Investigation and characterisation of the genetic variation in the coding region of the glycine N-acyltransferase gene

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It always seems impossible until it's done.
- Nelson Mandela 1918 - 2013

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ABSTRACT

Thorough investigation of the glycine conjugation pathway has been neglected over the last 30 years. Environmental factors, nutrition, and the chronic use of medications are increasing the exposure of humans to benzoate and drugs that are metabolized to acyl-CoA intermediates. Glycine conjugation of mitochondrial acyl-CoAs, catalysed by glycine N-acyltransferase (GLYAT, E.C. 2.3.1.13), is an important metabolic pathway responsible for maintaining adequate levels of free coenzyme A (CoASH). However, because of the small number of pharmaceutical drugs that are conjugated to glycine, the pathway has not yet been characterised in detail. Therefore, one of the objectives of this thesis was to develop a better understanding of glycine conjugation and its role in metabolism. In humans and animals a number of endogenous and xenobiotic organic acids are conjugated to glycine. Glycine conjugation has generally been assumed to be a detoxification mechanism, increasing the water solubility of organic acids in order to facilitate urinary excretion. However, recently it was proposed that the role of the amino acid conjugations, including glycine conjugation, is to regulate systemic levels of amino acids that are also utilised as neurotransmitters in the central nervous systems of animals. The glycine deportation hypothesis was based on the observation that, compared to glucuronidation, glycine conjugation does not significantly increase the water solubility of aromatic acids. A thorough review of the literature for this thesis showed that the major role of glycine conjugation, however, is to dispose of the end products of phenylpropionate metabolism. The review also introduced the new perspective that mitochondrial glycine conjugation prevents the accumulation of benzoate in the mitochondrial matrix by forming hippuric acid a less lipophilic conjugate that can be more readily transported out of the mitochondria. Although organic anion transporters can export benzoate from the matrix, this process would likely be futile because benzoic acid can simply diffuse back into the matrix. Hippurate, however, is significantly less lipophilic and therefore less capable of diffusing into the matrix. It is therefore not the transport out of the mitochondrial matrix that is facilitated by glycine conjugation, but rather the ability of the glycine conjugates to re-enter the matrix that is decreased. Lastly, glycine conjugation of benzoate also exacerbates the dietary deficiency of glycine in humans. Because the resulting shortage of glycine can negatively influence brain neurochemistry and the synthesis of collagen, nucleic acids, porphyrins, and other important metabolites, the risks of using benzoate as a preservative should not be underestimated.

To date, no defect of the glycine conjugation pathway has been reported and this, together with the fact that GLYAT plays an important role in hepatic metabolism, suggests that this pathway is essential for survival. GLYAT activity affects mitochondrial ATP production, glycine availability, CoASH availability and the toxicity of various organic acids. Therefore, variation in the glycine conjugation pathway could influence liver cancer, musculoskeletal development and mitochondrial energy metabolism. Significant interindividual variation exists in glycine conjugation capacity. The molecular basis for this variability is not known. The main aim of this thesis was to investigate and characterise the genetic variation in the coding region of the *GLYAT* gene. This was accomplished by firstly, investigating the influence of non-synonymous single nucleotide polymorphisms (SNPs) on the enzyme activity of a recombinant human GLYAT and secondly, by analysing the level of genetic

variation in the coding region of the *GLYAT* gene using existing worldwide population data. To investigate the influence of non-synonymous SNPs in the *GLYAT* gene on the enzyme activity, a recombinant human GLYAT was prepared, and characterised. Site-directed mutagenesis was used to generate six variants of the enzyme (K16N; S17T; R131H; N156S; F168L; R199C). The variants were expressed, purified, and enzymatically characterised. The enzyme activities of the K16N, S17T and R131H variants were similar to that of the wild-type, whereas the N156S variant was more active, the F168L variant less active, and the R199C variant was inactive. The results showed that SNP variations in the human *GLYAT* gene can influence the kinetic properties of the enzyme. The genetic variation data of the human *GLYAT* open reading frame (ORF) available on public databases was investigated by formulating the hypothesis that due to the essential nature of the glycine conjugation pathway, the genetic variation in the ORF of the *GLYAT* gene should be low and that deleterious alleles will be found at low frequencies. Data from the i) 1000 Genome Project, ii) the HapMap Project, and iii) the Khoi-San/Bantu Sequencing Project was downloaded from available databases. Sequence data of the coding region of a small cohort of South African Afrikaner Caucasian individuals was also generated and included in the analyses. In the GLYAT ORF of the 1537 individuals analysed, only two haplotypes (S_{156} and $T_{17}S_{156}$) out of 14 haplotypes were identified in all populations as having the highest haplotype frequencies (70% and 20% respectively). The $S_{156}C_{199}$ and $S_{156}H_{131}$ haplotypes, which have a deleterious effect on the enzyme activity of a recombinant human GLYAT, were detected at very low frequencies. The results of this study indicated that the *GLYAT* ORF is remarkably conserved, which supports the hypothesis that the glycine conjugation pathway is an essential detoxification pathway. The findings presented in this thesis highlight the importance that future investigations should determine the *in vivo* capacity of the glycine conjugation pathway for the detoxification of benzoate and other xenobiotics.

Keywords: glycine N-acyltransferase; GLYAT; detoxification; xenobiotics; single nucleotide polymorphim; SNP; enzyme activity; conserved open reading frame

OPSOMMING

Deeglike ondersoek van die glisienkonjugeringsweg is oor die afgelope 30 jaar verwaarloos. Omgewingsfaktore, voeding en die kroniese gebruik van medikasie verhoog die blootstelling van die mens aan bensoaat en middels wat gemetaboliseer word tot asiel-KoA-tussengangers. Glisienkonjugering van mitochondriale asiel-KoA-verbindings, wat gekataliseer word deur glisien N-asieltransferase (GLIAT, EC 2.3.1.13), is 'n belangrike metaboliese weg wat verantwoordelik is vir die handhawing van voldoende vlakke van vrye koënsiem A (KoA). Die glisienkonjugeringsweg is nog nie goed gekarakteriseer nie as gevolg van die klein aantal farmaseutiese middels wat aan glisien konjugeer. Daarom is een van die doelwitte van hierdie studie om 'n beter begrip van glisienkonjugering en die rol van hierdie pad in die metabolisme te ontwikkel. In mense en diere word 'n aantal endogene en xenobiotiese organiese sure aan glisien gekonjugeer en was dit algemeen aanvaar dat die glisienkonjugeringweg `n detoksifiseringsmeganisme is wat die wateroplosbaarheid van organiese sure verhoog om die uitskeiding in urine te fasiliteer. Onlangs is daar egter voorgestel dat die rol van die aminosuurkonjugeringsreaksies, insluitend glisienkonjugering, is om sistemiese vlakke van aminosure wat ook gebruik word as neurotransmitters in die sentrale senuweestelsel van diere, te reguleer. Die glisiendeportasie hipotese is gebaseer op die waarneming dat, in vergelyking met glukuronidering, glisienkonjugering nie beduidend die wateroplosbaarheid van aromatiese sure verhoog nie. 'n Deeglike bestudering van die literatuur vir hierdie studie het getoon dat die primêre rol van glisienkonjugering eerder is om die eindprodukte van fenielpropionaatmetabolisme te verwyder. Hieruit het ook die nuwe perspektief na vore gekom dat mitokondriale glisienkonjugering die opbou van bensoaat in die mitokondriale matriks verhoed deur die vorming van hippuursuur `n minder lipofiele verbinding wat meer geredelik uit die mitokondria vervoer kan word. Alhoewel organiese anioon transporters bensoaat uit die matriks kan vervoer, sal hierdie proses waarskynlik nutteloos wees, aangesien bensoënsuur net eenvoudig weer terug in die matriks kan beweeg. Hippuursuur, is egter aansienlik minder lipofiel en dus minder daartoe in staat om weer terug in die matriks te diffundeer. Dit is dus nie die verwydering vanuit die mitokondriale matriks wat gefasiliteer word deur glisienkonjugering nie, maar eerder die vermoë van die glisienkonjugering verbindings om terug in die matriks in te beweeg. Laastens, glisienkonjugering van bensoaat vererger ook die dieët tekort van glisien in die mens. Die risiko van die gebruik van bensoaat as 'n preserveermiddel moenie onderskat word nie, omdat die gevolglike tekort aan glisien 'n negatiewe effek kan hê op brein neurochemie en die sintese van kollageen, nukleïensure, porferiene en ander belangrike metaboliete.

Geen defek van die glisienkonjugeringsweg is al beskryf nie en, dit tesame met die feit dat GLIAT 'n belangrike rol speel in lewermetabolisme, dui daarop dat hierdie metaboliese weg noodsaaklik is vir oorlewing. GLIAT aktiwiteit beïnvloed mitokondriale ATP-produksie, glisien beskikbaarheid, KoA beskikbaarheid en die toksisiteit van verskeie organiese sure. Daarom kan variasie in die glisienkonjugeringsweg lewerkanker, skeletale ontwikkeling en mitokondriale energie-metabolisme negatief beïnvloed. Beduidende interindividuele variasie bestaan in die kapasiteit van die glisienkonjugeringsweg. Die molekulêre basis vir hierdie variasie is nie bekend nie. Die hoofdoel van hierdie studie was om die genetiese variasie in die koderingsgebied van die *GLIAT* geen

te ondersoek en te karakteriseer. Om aan die hoofdoel te voldoen is `n tweeledige aanslag gevolg: i) die invloed van nie-sinonieme enkel nukleotied polimorfismes (ENPs) op die ensiemaktiwiteit van 'n rekombinante menslike GLIAT is bepaal; ii) die vlak van genetiese variasie in die koderingsgebied van die *GLIAT* geen is ontleed deur van bestaande wêreldwye bevolkingsdata gebruik te maak. Om die invloed van nie-sinonieme ENPs in die *GLIAT* geen op die ensiemaktiwiteit te ondersoek, is 'n rekombinante menslike GLIAT voorberei en gekarakteriseer. Puntmutasies is geïnduseer om ses weergawes van die ensiem te genereer (K16N; S17T; R131H; N156S; F168L; R199C). Hierdie variante is uitgedruk, gesuiwer en ensiematies gekarakteriseer. Die ensiem aktiwiteite van die K16N, S17T en R131H variante was soortgelyk aan dié van die wilde-tipe, terwyl die N156S variant meer aktief was, die F168L variant minder aktief, en die R199C variant was onaktief. Die resultate het getoon dat ENP verskille in die menslike *GLIAT* geen die kinetiese eienskappe van die ensiem kan beïnvloed. Die data ten opsigte van die genetiese variasie van die menslike GLIAT oop leesraam (OLR) wat beskikbaar is in openbare databasisse is ondersoek deur die volgende hipotese te formuleer: as gevolg van die noodsaaklike aard van die glisienkonjugeringsweg, sal die genetiese variasie in die OLR van die *GLIAT* geen laag wees en skadelike allele sal teen lae frekwensies gevind word. Data van die i) 1000 Genoomprojek, ii) die HapMapprojek, en iii) die Khoi-San / Bantoe Projek is afgelaai van beskikbare databasisse. Volgorde data van die kodering gebied van 'n klein groep van die Suid-Afrikaanse blanke Afrikaner individue is ook gegenereer en ingesluit in die ontleding. In die GLIAT OLR van die 1537 individue wat ontleed is, is slegs twee haplotipes (S_{156} en $T_{17}S_{156}$) uit 14 haplotipes in alle bevolkings geïdentifiseer met die hoogste haplotipe frekwensies (70% en 20% onderskeidelik). Die $S_{156}C_{199}$ en $S_{156}H_{131}$ haplotipes, wat 'n nadelige uitwerking op die ensiemaktiwiteit van 'n rekombinante menslike GLIAT het, is teen baie lae frekwensies teenwoordig. Die resultate van hierdie studie het aangedui dat die GLIAT OLR merkwaardig gekonserveerd is, wat die hipotese ondersteun dat die glisienkonjugeringsweg 'n noodsaaklike detoksifiseringsweg is. Die bevindinge van hierdie studie bevestig die belangrikheid dat toekomstige ondersoeke die *in vivo* kapasiteit van die glisienkonjugeringsweg moet bepaal vir die detoksifisering van bensoaat en ander xenobiotika.

Sleutelwoorde: glisien N-asieltransferase; GLIAT; detoksifisering; xenobiotika; enkel nukleotied polimorfismes; ENP; ensiem aktiwiteit; gekonserveerde oop leesraam

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CHAPTER 1: PREFACE

1.1 STUDY MOTIVATION AND RATIONALE

In humans, a diverse array of enzymes evolved in order to process endobiotics and to protect organisms from xenobiotics. These enzymes are distributed in many tissues, but are most prevalent within the liver, the major drug-metabolizing organ. In most cases the metabolites that form as a result of drug metabolism are more water soluble than the parent molecules, and more readily excreted in the bile or urine (Bock, 2014). The cytochrome P450 enzymes account for the biotransformation of the majority of existing pharmaceutical drugs. Other oxidative enzymes, though certainly important in the biotransformation of some drugs (e.g. xanthine oxidase contribute to the overall metabolism of substances such as caffeine and theophylline), are not responsible for the metabolism of many pharmaceutical drugs compared to the CYP enzymes (Dorne et al., 2004). Synthetic or Phase II biotransformation reactions consist of either conjugation or nucleophilic trapping in which moieties such as acetate, sulphate, glucuronic acid (conjugations), or glutathione (nucleophilic trapping) are added to either pre-existing functional groups or functional groups arising from oxidative biotransformation reactions. It is now also possible to evaluate the genotypes and to perform detoxification profiling for some drug metabolizing enzymes. New polymorphisms are being discovered continuously, and genotype alone does not always predict phenotype. Nevertheless, the emerging strategies for assessing both genotype of drug-metabolizing enzymes and the phenotypic expression of their activity is bringing the era of personalised medicine closer (Abul-Husn et al., 2014).

Even though the glycine conjugation pathway was one of the first metabolic pathways to be discovered, in 1841 (Ure, 1841), the biggest challenge regarding the investigation of the glycine conjugation pathway remains the fact that this pathway is still very poorly characterised. This becomes clearly evident when comparing the number of publications available on Pubmed (http://www.ncbi.nlm.nih.gov/pubmed September 2014) on the cytochrome P450 (CYP450) phase I detoxification enzymes (79 890) to those on N-acyltransferase (NAT) (18 387) and glycine N-acyltransferase (GLYAT) (47) both phase II detoxification enzymes. This may be because of the small number of pharmaceutical drugs that are metabolised to glycine conjugates and the difficulty in obtaining human liver samples and xenobiotic acyl-CoA substrates for research (for reviewe see Knights et al., 2007). There is now a growing realisation that glycine conjugation is one of the fundamentally important homeostatic mechanisms in animal physiology (Beloborodova et al., 2012; Beyoglu and Idle, 2012; Fedotcheva et al., 2008; Park et al., 2013; Wu et al., 2013).

A range of xenobiotic acylglycines are excreted in urine, indicating that either the parent xenobiotic or a carboxylate metabolite is a substrate for esterification to CoASH, and that the acyl-CoA is a substrate for glycine conjugation (Campbell et al., 1988; Duffy et al., 1995; Huang et al., 1994; Kasuya et al., 2000; for review see Knights and Miners, 2012; Rechner et al., 2002; Sakuma, 1991; Tanaka and Isselbacher, 1967). The toxicity of xenobiotic carboxylates is partially determined by the extent to which an acyl-CoA, that cannot be

conjugated to glycine or some other acceptor, is formed (Begriche et al., 2011; Fromenty and Pessayre, 1995; for reviewe see Knights and Miners, 2012; for reviewe see Knights et al., 2007). This leads to accumulation of the acyl-CoA, which can have several toxic effects in addition to disrupting mitochondrial energy production (Begriche et al., 2011; Pessayre et al., 2012). In severe cases, this can lead to hepatic steatosis and death (Knights et al., 2007; Mitchell et al., 2008). Since the glycine conjugation pathway is saturable, variation in the rate of glycine conjugation influences the clearance of xenobiotics and thus toxicity (Knights et al., 2007; Temellini et al., 1993). If the rate of conjugation by GLYAT is low, glycine conjugation may not prevent the toxicity of an acyl-CoA, even if it is a substrate for the enzyme (Bartlett and Gompertz, 1974; Fenton et al., 2013; Knights et al., 2007; Sweetman and Williams, 2013).

Glycine conjugation is a two-step process and the overall rate of glycine conjugation can be influenced by several factors, including the availability of ATP, CoASH, and glycine; genetic variation in the *ACSM2B* (encoding HXMA) and *GLYAT* genes as well as variable expression of HXMA and GLYAT (Gregus et al., 1993a, b; Knights and Miners, 2012; Knights et al., 2007; Krieger and Tanaka, 1976). Existing publications on interindividual variation in the glycine conjugation pathway do not discriminate between variation in acyl-CoA formation (HXMA) and variation in glycine conjugation (GLYAT). Previous investigators did not design experimental strategies that account for all or even some of these factors. An example of this is the study wherein Temellini and co-workers demonstrated significant interindividual variation in the ability of human liver homogenates to synthesise hippurate from benzoate, ATP, CoASH, and glycine (Temellini et al., 1993). This study did not, however, account for the genetic variation or for the possible variable expression of HXMA and GLYAT. It is also very difficult to compare studies presented in the literature due to differences in the quality of the enzyme preparations from liver samples studied as well as differences in the experimental conditions that were used. Therefore, the kinetic parameters reported in the literature for human GLYAT vary substantially, and cannot be compared (Bartlett and Gompertz, 1974; Gregersen et al., 1986; Kelley and Vessey, 1994; Kelley and Vessey, 1993; Kolvraa and Gregersen, 1986; Mawal and Qureshi, 1994; van der Westhuizen et al., 2000). The genetic variation in the *GLYAT* gene and its effect on enzyme activity was also not taken into account in these studies. Very little is currently known about the influence of genetic variation in the *ACSM2B* and *GLYAT* genes on the glycine conjugation pathway. Interindividual variation in responsiveness to administration of glycine and benzoate, respectively, was observed in both isovaleric acidemia and hyperammonemia (Dercksen et al., 2012; Itoh et al., 1996; Sweetman and Williams, 2013; Vest and Salzberg, 1965). It was suggested that interindividual variation in GLYAT activity may partly account for this. The *GLYAT* gene has also been linked to the development of liver cancer (Matsuo et al., 2012) and musculoskeletal development (Guo et al., 2013), although the mechanism behind this is still unclear at present.

Therefore, it is important to study the relationships between genetic variation in the *GLYAT* gene, GLYAT enzyme activity, the *in vivo* rate of glycine conjugation, and physiological consequences of variation in the glycine conjugation pathway. Very little is currently known regarding the genetic variation in the *GLYAT* gene as well as the effect of these polymorphisms on the activity of GLYAT. Expression and characterisation of a

recombinant human GLYAT enzyme and its variants can make an important contribution to our understanding of variation in glycine conjugation, given the difficulty in obtaining human liver tissue samples for research.

1.2 AIMS AND SPECIFIC OBJECTIVES OF THIS STUDY

1.2.1 Main aims

a. To generate a recombinant human GLYAT enzyme in order to investigate the influence of non-synonymous polymorphisms on enzyme activity.

b. To characterise the genetic variation in the coding region of the *GLYAT* gene using worldwide population data.

1.2.2 Specific Objectives

a. To investigate a variety of expression systems to find one that can express high levels of a biologically active recombinant human GLYAT. These will include bacterial systems that have fusion proteins to increase solubility, cold shock promoters and chaperones.

b. To purify the recombinant human GLYAT using purification systems applicable to each expression system.

c. To determine the enzyme kinetics of the recombinant human GLYAT *in vitro* and establish whether the recombinant enzyme kinetics is comparable to what is reported in the literature.

d. To establish if the catalytic residue (Glu^{227}) identified in bovine GLYAT is also the catalytic residue in human GLYAT through the construction of a site-directed mutant (E227Q).

e. To compare the relative enzyme activity of the variants (K16N; S17T; R131H; N156S; F168L; R199C) to that of the wild-type human GLYAT.

f. To investigate whether human GLYAT activity is regulated by reversible acetylation on Lys^{19} as was shown for the paralogue GLYATL2 (Waluk et al., 2012). The corresponding K20Q and K20R mutants of GLYAT will be expressed, purified, and assayed as for the other variants in this study.

g. To characterise the SNPs and haplotypes in the coding region of the *GLYAT* gene using worldwide population data. To generate sequence data for the coding region of the *GLYAT* gene of 61 healthy South African Afrikaner Caucasian individuals.

1.3 STRUCTURE OF THIS THESIS

This thesis is presented in five chapters that include three peer-reviewed articles.

Chapter 2: Literature review

A review of the literature on glycine conjugation and GLYAT was compiled by combining the information of the relevant parts in two peer reviewed articles published from this thesis (Paper I - Appendix A; Paper II – Appendix B) together with additional relevant information not covered in the two review papers.

- Paper I: **Glycine conjugation: Importance in metabolism, the role of glycine N-acyltransferase, and the factors that influence interindividual variation**

  Christoffel Petrus Stephanus Badenhorst, **Rencia van der Sluis**, Elardus Erasmus, and Alberdina Aike van Dijk

  Published in: ***Expert Opinion on Drug Metabolism and Toxicology*** (2013) 9: 1139-1153

- Paper II: **A new perspective on the importance of glycine conjugation in the metabolism of aromatic acids.**

  Badenhorst CP, Erasmus E, **van der Sluis R**, Nortje C, van Dijk AA.

  Published in: ***Drug Metabolism Reviews***. 2014 46 (3): 343-361

Chapter 3: Generation of a recombinant human GLYAT and the analyses of the influence of genetic variations on the enzyme activity of a recombinant GLYAT

This chapter consists of a paper describing the effect of genetic variations on the enzyme activity of a recombinant human GLYAT.

- Paper III: **Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase**

  Rencia van der Sluis, Christoffel Petrus Stephanus Badenhorst, Francois Hendrikus van der Westhuizen, and Alberdina Aike van Dijk

  Published in: ***Gene*** (2013) 515: 447-453

Chapter 4: Characterisation and haplotype analyses of non-synonymous polymorphisms in the glycine N-acyltransferase gene using data from worldwide populations

This chapter consists of a submitted manuscript in which the allele and haplotype frequency of the non-synonymous SNP in the human *GLYAT* gene were analysed using worldwide population data. Sequencing data of the coding region of the *GLYAT* gene was also generated for a co-hort of 61 South African individuals.

- Submitted manuscript to *Pharmacogenetics and Genomics* (Manuscript number: PGEN-2014-114): **Conservation of the coding regions of the glycine N-acyltransferase gene and its implications for the detoxification of xenobiotics in humans**

  Rencia van der Sluis, Christoffel P. Badenhorst, Elardus Erasmus, Etresia van Dyk, Francois H. van der Westhuizen, Alberdina A. van Dijk.

Chapter 5: Summary and conclusion

This chapter includes the general discussion, recommendations and conclusions of this study.

References

References are provided at the end of the thesis. The references used in Chapter two, four and five are listed according to the requirement stipulated in the manual for post-graduate studies of the NWU. The references used in Chapter three are provided as specified by each journal in which the articles were published.

Materials and Methods

The materials and methods used in this study are described in the *Materials and Methods* sections of the published paper III and submitted manuscript IV.

1.4 APPENDIXES

Appendix A: Paper I

Appendix B: Paper II

Appendix C: An example of the consent form used for the submitted manuscript (Chapter 4)

Appendix D: The recombinant therapeutic GLYAT patent application, PCT/IB2011/053721

Appendix E: List of publications and scientific posters

1.5 AUTHOR CONTRIBUTIONS

Papers I and II presented in Chapter 2: C.P.S. Badenhorst, E. Erasmus and C. Nortje were involved in the review of the literature and manuscript writing of the sections that focus on the glycine conjugation pathway as a whole as well as its role in metabolism. R. van der Sluis was involved in the review of the literature and manuscript writing of the sections that focus on the *GLYAT* and *ACSM2B* genes and their roles in interindividual variation. A.A. van Dijk was involved in manuscript writing and supervision.

Paper III presented in Chapter 3: R. van der Sluis was involved in the design of the study, laboratory assays (specifically the development of a suitable expression system), data analyses and manuscript writing. C.P.S. Badenhorst was involved in laboratory assays (specifically the enzyme assays), data analyses and manuscript writing. A.A van Dijk and F.H. van der Westhuizen were involved in study design, manuscript writing and supervision.

Submitted manuscript in Chapter 4: R. van der Sluis was involved in the design of the study, laboratory assays, data analyses and manuscript writing. E. van Dyk was involved in the next generation sequencing of the South African samples on the Ion PGM. C.P.S. Badenhorst and E. Erasmus were involved in manuscript writing. F.H. van der Westhuizen and A.A. van Dijk were involved in manuscript writing and supervision.

All authors signed the declarations on this page:

As a co-author, I hereby approve and give consent that the mentioned articles can be used for the PhD thesis of R. van der Sluis. I declare that my role in the study, as indicated above, is a representation of my actual contributions.

Signature:	*Mrs R. van der Sluis*	Signature:	*Dr. E. van Dyk*
Signature:	*Dr. C.P.S. Badenhorst*	Signature:	*Prof. F.H. van der Westhuizen*
Signature:	*Mr. E. Erasmus*	Signature:	*Prof. A.A. van Dijk*
Signature:	*Ms. C. Nortje*		

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

During the 20th century great progress was made regarding therapies against all major diseases. However, drug therapy often fails to be curative and may in fact cause substantial adverse effects. Moreover, worldwide use of these drugs has revealed substantial interindividual differences in therapeutic response. Recognition of interindividual differences in drug response is an essential step towards optimizing therapy. A substantial portion of the variability in drug response is genetically determined, with age, nutrition, health status, environmental exposure, and concurrent therapy playing important contributory roles. These observations of highly variable drug response, which began in the early 1950s, have ushered in the era of personalised medicine and have led to the birth of a new scientific discipline called pharmacogenetics. Pharmacogenetics focuses on drug response as a function of genetic differences among individuals. Applied to non-therapeutic foreign substances (xenobiotics), the equivalent term "toxicogenetics" is used (Mancinelli et al., 2000).

Lipophilic drugs, dietary phytochemicals, environmental pollutants and many endobiotics have to be detoxified, eliminated or homeostatically controlled by drug-metabolizing enzymes (DMEs) (Chawla et al., 2001; Nebert, 1991; Omiecinski et al., 2011). Drug-metabolizing Phase I and II enzyme families, drug transporters (Phase III) and their ligand activated transcription factors probably evolved as a system necessary for homeostatic control of lipophilic endobiotics and detoxification of xenobiotics (Bock, 2014). DMEs have been found to be well developed very early in evolution, for example, in the echinoderm sea urchin (Goldstone et al., 2006). In contrast to other enzyme systems, DMEs exhibit broad substrate specificity for lipophilic compounds, which is why they are able to metabolise developed drugs.

2.2. BIOTRANSFORMATION REACTIONS

The study of drug metabolism (also referred to as detoxification or biotransformation) is important in the understanding of the clearance kinetics of pharmaceutical drugs, the structuring of dosage regimens, the pharmacology and toxicology of drug metabolites, and the interactions of multivalent drug combinations. Drugs are eliminated from the body through the biotransformation of hydrophobic drug molecules to more hydrophilic molecules (Bachmann, 2009). The first human study of drug metabolism occurred in 1841 when Ure noted that hippuric acid could be isolated from the urine after the ingestion of benzoic acid (Ure, 1841). Hoffman reported the first metabolic interaction between drugs in 1877 when he found that quinine could decrease the formation of hippuric acid from benzoic acid (Gibson and Skett, 2001). In the mid-nineteenth century the role of biotransformation on drug action was recognized after the discovery and characterisation of a red liver pigment as a cytochrome (Gibson and Skett, 2001; Klingenberg, 1958; Omura et al., 1965). Although the enzymes that mediate drug metabolism are found in many tissues, it is within the liver and the epithelial cells of the upper portion of the intestines where most drug metabolism occurs. In mammals, the

liver is the principal organ responsible for xenobiotic biotransformation. A xenobiotic, such as a drug, is a molecule that is foreign to the body, whereas an endobiotic is a molecule that is normally found in the body.

Xenobiotic elimination requires the combined processes of metabolism and transport and is divided into four phases (Phase 0, I, II and III) (Doring and Petzinger, 2014) (

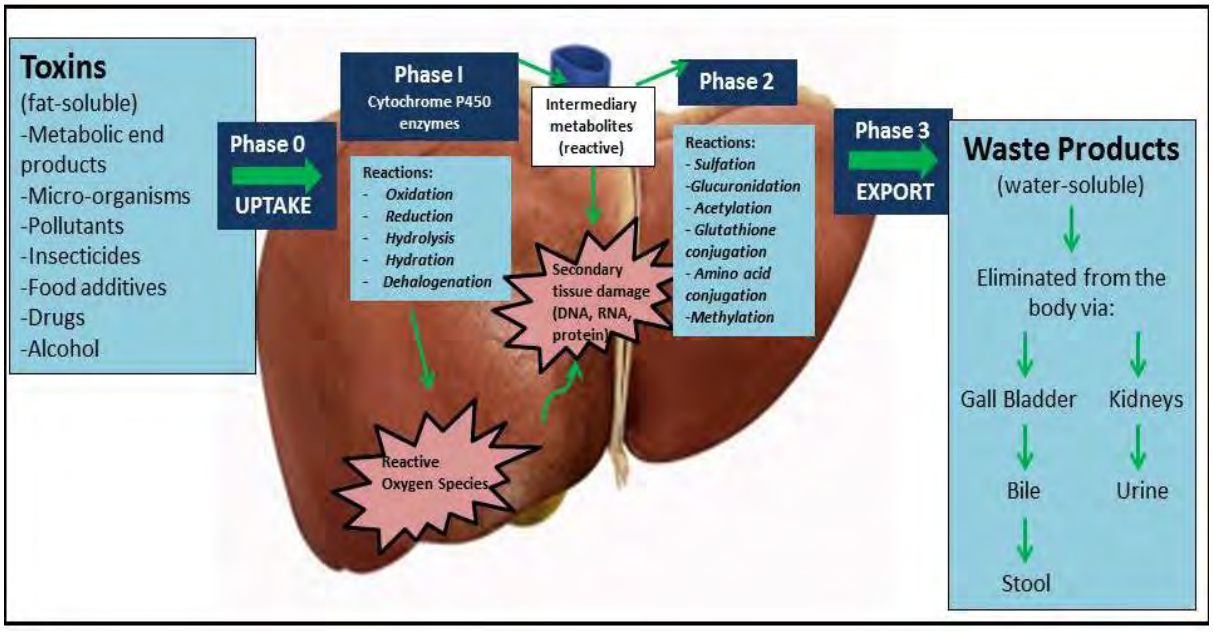

Figure 2.1). Phase 0 carrier-mediated uptake occurs at the blood-facing basolateral membrane and is histologically separated from the bile-facing canalicular membrane and the urine-facing tubule brush border membrane (Petzinger and Geyer, 2006). Phase 0 transporters mainly belong to the solute carrier (SLC)21/SLCO family of organic anion transporting polypeptides (OATPs) (Doring and Petzinger, 2014). In the middle of the twentieth century R. T. Williams proposed the dichotomous scheme of drug metabolism consisting of an initial phase (Phase I) followed by a second (Phase II) (Williams, 1959). In Phase I, a drug is either activated or inactivated by one of three types of irreversible chemical modifications or biotransformation reactions, namely oxidation, reduction or hydrolysis. Williams characterized Phase II as a synthetic phase, which is an additional inactivation step. Josephy et al. (2005) stated that Williams's dichotomous scheme of drug metabolism is dated and should be supplemented by terminology that groups metabolic reactions into four simple categories. Fundamentally all drug metabolism falls into one of the following four categories: i) Oxidative reactions that are catalysed by cytochromes P450 (CYPs), monamine oxidases (MAOs), peroxidases, xanthine oxidase (XO), or alcohol dehydrogenase; ii) Reductive reactions that typically involve either azo or nitro-reductions; iii) Conjugation reactions in which electrophilic adenosine-containing cofactors such as ATP, phosphoadenosine phosphosulfate (PAPS), Acetyl coenzymeA (acetyl-CoA), uridine diphosphateglucuronic acid (UDPGA), or S-adenosylmethionine(SAM) react with nucleophilic groups such as –OH or $–NH_2$; and iv) Nucleophilic trapping in which water, glutathione or other nucleophiles react with electrophiles (Josephy et al., 2005). Ishikawa et al. (1992) introduced the term Phase III, which is the next sequential step of drug metabolites awaiting elimination and having passed Phase I and Phase II metabolism (Ishikawa, 1992). Phase III refers to substrate

transport across the plasma membranes of mammalian cells without any chemical modification. Phase III drug elimination pumps belong to the class of ABC carriers, including the GS-X pump, named multidrug resistance-associated protein 1 (MRP1) and P-gp (Doring and Petzinger, 2014). Only Phase I and Phase II biotransformation will be discussed in detail in Sections 2.3. and 2.4.

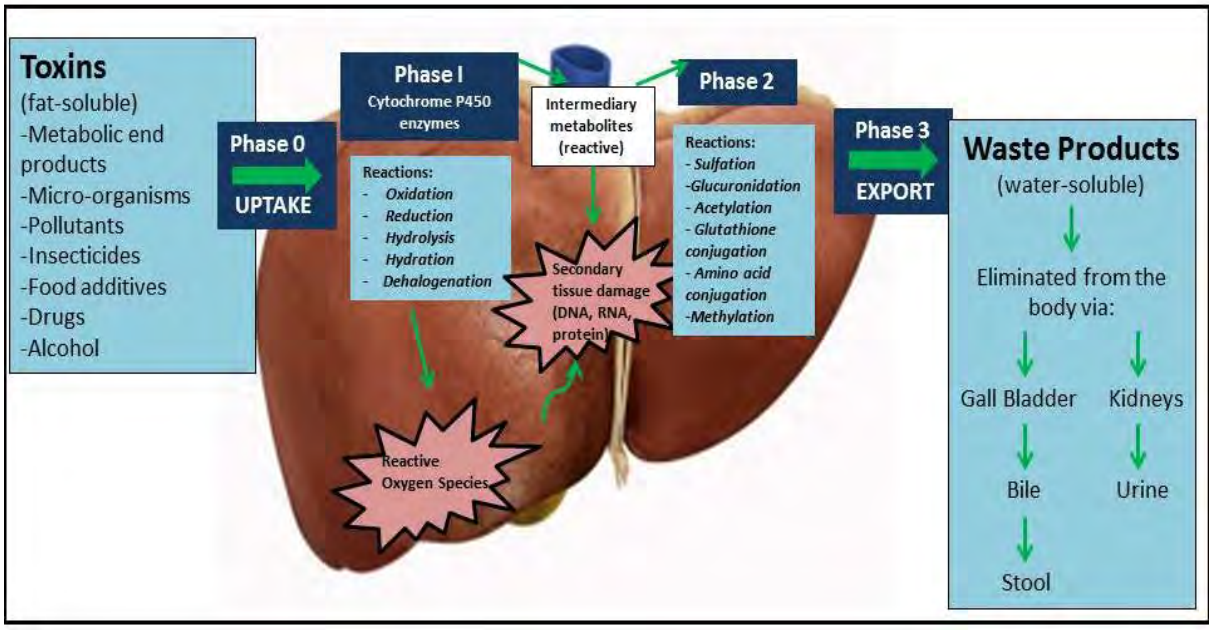

Figure 2.1: Sequential phases of drug elimination by metabolism and membrane transport. The diagram is a representation of the situation in the liver. Phase 0 delivers drugs by carrier-mediated uptake from the blood into a liver cell. In Phase I, drugs are either activated or inactivated by oxidation, reduction or hydrolysis. Phase II is a synthetic phase. The excretion phase III of metabolite conjugates is achieved by transporter pumps such as MRP2, MDR1/P-gp and BCRP at the canalicular hepatocyte membrane. Adapted from (Doring and Petzinger, 2014; Liska, 1998).

In Phase I, drugs with ester and amide bonds are subject to hydrolysis by esterase or amidase enzymes, respectively. A few drugs are subject to reductive reactions. Drug oxidations are among the most prominent type of biotransformation reactions, and can encompass aliphatic or aromatic hydroxylations, N-oxidations, N-dealkylations, O-dealkylations, S-dealkylations, sulfoxidations, deaminations, or desulfurations (Gibson and Skett, 2001) (Table 2.1).

The Phase II conjugation reactions include glucuronidation of hydroxyl, carboxyl, and amino groups; sulfonation of aromatic phenolic groups, alcohols, and amino groups; acetylation of aliphaticamines, aromatic amines, and hydrazino groups; glycine conjugation with carboxylic acids; glutathione conjugation with

electrophiles; and methylation of aliphatic amines, N-containing heterocycles, phenols, and thiols (Gibson and Skett, 2001) (Table 2.2).

Table 2.1: Phase I Biotransformation reactions

Reaction Type	Type of Enzyme	Representative Substrates
Hydrolysis		
Ester	Esterase	Cocaine, esmolol, aspirin, enalapril, capecitabine, succinylcholine
Amide	Amidase	Procainamide, loperamide, pyrazinamide, indapamide
Reduction		
Nitro reduction	Cytochrome P450, cytochrome P450 reductase	Chloramphenicol
Hydration	Epoxide hydrolase	Benzo[a]pyrene 4,5-epoxide
Oxidation		
Aromatic hydroxylation	Mixed function oxidase (cytochromes P450), Xanthine oxidase	Phenytoin, warfarin, midazolam, carvedilol Caffeine, theophylline
Aliphatic hydroxylation	Mixed function oxidase (cytochromes P450)	Midazolam, cyclosporine, ibuprofen, valproic acid
O-dealkylation	Mixed function oxidase (cytochromes P450)	Dextromethorphan, codeine, fluvoxamine
N-dealkylation	Mixed function oxidase (cytochromes P450)	Fluoxetine, imipramine, diazepam, flurazepam, olanzapine
N-oxidation	Mixed function oxidase (cytochromes P450)	Acetaminophen, quinidine
S-oxidation	Mixed function oxidase (cytochromes P450)	S-omeprazole, cimetidine, thioridazine
Deamination	Mixed function oxidase (monoamine oxidase)	Amphetamine, epinephrine

Adapted from (Bachmann, 2009).

Table 2.2: Phase II Biotransformation reactions

Reaction Type	Type of Enzyme	Representative Substrates
Glucuronidation	Glucuronosyl transferase	Acetaminophen, lorazepam, morphine, chloramphenicol
Acetylation	N-acetyltransferases	Isoniazid, procainamide
Sulfation	Sulfotransferases	Acetaminophen
Glutathione conjugation	Glutathione-S-transferases	Acetaminophen

Adapted from (Bachmann, 2009).

2.3. PHASE I BIOTRANSFORMATION REACTIONS

2.3.1. Introduction

Mixed function oxidases catalyse the oxidation of a diverse array of lipophilic drug substrates as well as endobiotics such as steroid and thyroid hormones, fatty acids, and arachidonic acid metabolites. The most widely studied gene family of mixed-function oxidases and the one that participates more than any other in the oxidation of pharmaceutical drugs is the cytochrome P450 family (Bachmann, 2009). It has been estimated that about 75% of all currently marketed drugs are processed by the cytochrome P450 enzymes. The cytochrome P450 (CYP) enzymes are incredibly important in terms of the number of existing drugs that they process, their substrate specificity, polymorphisms, and propensity to be determinants in drug–drug interactions (Ioannides, 1996).

2.3.2. Evolution and cytochrome P450 classification

After the recognition of the important role of the cytochrome P450s in drug oxidations dating back to the 1950s and 1960s, there was a relatively sharp scientific interest in these enzymes. It is thought that the original ancestral P450 gene arose approximately 3 billion years ago followed by rounds of expansion associated with gene and genome duplication. Although the cytochrome P450 family is mainly seen as xenobiotic processing enzymes, this gene superfamily likely originated to process endobiotics. There are hundreds of P450 gene families among animals, plants and bacteria. Only 15 enzymes from three gene families are involved extensively in human drug metabolism. The enzyme families are identified by Arabic numbers after the prefix CYP, a shorthand reference to cytochrome P450. The three key cytochrome P450 or CYP families responsible for pharmaceutical drug metabolism in humans are CYP1, CYP2 and CYP3. Subfamilies are denoted with uppercase letters (e.g., CYP1A). Individual enzymes are identified with the family designation, subfamily designation and another Arabic numeral (e.g., CYP1A2). CYP encoding genes are designated in italics (e.g. *CYP1A2*) (Ioannides, 1996).

2.3.3. Cytochrome P450 polymorphisms

Pharmacogenetics is a term that is used frequently with reference to sequence variations in the alleles of individual CYP enzymes. Although exposure of individuals to a wide array of substances (including drugs and environmental chemicals) and/or conditions (such as infection and liver dysfunction) can alter the expression or activity of their CYP enzymes, genetically based variation in the amino acid sequences of the CYP enzymes also has been shown to be responsible for significant interindividual variations in rates of drug metabolism. Often, individuals who express a variant form of the enzyme, metabolize drug substrates much more slowly than normal. Even a single nucleotide polymorphism (SNP) in a CYP gene may result in altered rates of drug metabolism. Some alleles may be duplicated or multiplied even more, giving rise to higher than normal levels of enzymatic activity and ultra-rapid metabolic phenotypes. A good example of this is CYP2D6*2XN, where the

N denotes the number of copies of the active gene. The frequency of occurrence of CYP allelic variants among various ethnic and other subgroups continue to be characterized. CYP2D6, CYP2C9, and CYP2C19 are among the most highly polymorphic of all CYP enzymes. Of these, the polymorphism of CYP2D6, which is responsible for the metabolism of 25% of known drugs, has been studied most extensively. In theory, knowledge of CYP genotypes should be useful in predicting individual phenotypes that will help in the selection of the most appropriate drug dosages for each person. Moreover, CYP genotyping is now commercialized for clinical application; that is, for predicting who might be candidates for high, normal or low doses of certain drugs, or who might not be candidates for certain drugs at all (Houston et al., 2003).

In 2006 the U.S. Food and Drug Administration approved Roche's AmpliChip genotyping device. This device, coupled with microarray instrumentation of Affymetrix, is currently available to assist physicians and other healthcare providers to genotype a patient's CYP2D6 or CYP2C19 using a sample of blood. The patient's CYP2D6 or CYP2C19 genotype is reported to the physician to aid in decisions about dosing drugs that are processed by CYP2D6 or CYP2C19. The simplest example of this can be shown for CYP2C19. Suppose the normal allele is designated as *1 (CYP2C19*1), and the two variant alleles that can be expressed are *2 and *3, respectively. An individual could express one of the following combinations of two of these alleles: *1*1; *1*2; *1*3; *2*2;*2*3; or *3*3. From a single blood sample, the Ampli-Chip technology would determine which allelic combination of CYP2C19 an individual has, and also whether or not a particular combination was likely to mean that the individual is a poor metabolizer of substrates for CYP2C19 or an extensive (i.e. normal) metabolizer of CYP2C19 substrates. As it turns out, as long as an individual has one *1 allele, regardless of whichever other allele (s)he possesses, then (s)he will be an extensive (normal) metabolizer of CYP2C19 substrates. So, for example, if a patient had the CYP2C19*2*3 allele, then the patient's physician would learn that the patient is a poor metabolizer of CYP2C19 substrates. If the patient required treatment with, for example phenytoin, it would be known from the outset that the safe and effective dose of phenytoin would likely be smaller than for most other patients (Bachmann, 2009).

However, in spite of the promise for individualizing drug therapy for substrates of CYP2C19 and CYP2D6, detecting correlations between single SNPs, diplotypes or haplotypes of CYPs and phenotypic variations in drug metabolism will likely remain an important challenge in clinical pharmacology and drug metabolism for some time to come. Several issues that hamper establishing predictive correlations include: i) The growing number of genetic variants for each CYP that are continually being discovered; ii) The growing recognition that genomics is currently not a particularly good predictor of proteomics; iii) Lack of uniform agreement on the methods for clinically phenotyping CYP activities; and iv) The relatively large sample sizes (i.e. populations) that must be simultaneously genotyped and phenotyped (Bachmann, 2009).

2.4. PHASE II BIOTRANSFORMATION REACTIONS

Phase II biotransformation reactions generally serve as a detoxification step in drug metabolism through conjugation reactions namely glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation. Phase II drug metabolising enzymes are mainly transferases including UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs) and methyltransferases (Jancova et al., 2010). Examples of Phase II reactions are shown in Table 2.2.

Phase II drug metabolising enzymes play an important role in the biotransformation of endogenous compounds and xenobiotics to more easily excretable forms as well as in the metabolic inactivation of pharmacologically active compounds. Phase II enzymes have attracted much less attention in clinical pharmacology than cytochrome P450 because drug interactions involving these enzymes are relatively rare. A decrease in the metabolising capacity of Phase II enzymes can lead to toxic effects of clinically used drugs (Jancova et al., 2010). Although Phase II reactions are generally seen as detoxifying reactions, the conjugates formed might mediate adverse effects. An example of this is conjugates acting as carriers for potentially carcinogenic compounds in the activation of benzylic alcohols, polycyclic aromatic hydrocarbons, aromatic hydroxylamines, hydroxamic acid and nitroalkanes by sulfotransferases (Glatt, 2000). Interindividual differences exist in the metabolic response of Phase II enzymes. External (smoking, medication, nutrition and environmental effects) and internal (age, sex, diseases and polymorphisms) factors can influence the activity of Phase II enzymes. For the purpose of this literature review only glycine conjugation will be discussed in detail.

> ***Note: i)*** *To prevent repetition of the contents of Paper I (Appendix A) and Paper II (Appendix B) parts relevant to the motivation of this thesis were rearranged and summarised for the purpose of this Chapter. Some of the paragraphs were taken verbatim from these two papers.*
>
> ***ii)*** *The literature review of the genetic variation of the ACSM2B and GLYAT genes and its effect on the enzyme activity of the respective proteins (HXMA and GLYAT) will be discussed in more detail in this Chapter as this was not extensively covered in Paper I and II.*

2.4.1. The history of glycine conjugation

The study of drug metabolism started with the discovery of glycine conjugation. The excretion of hippuric acid after ingestion of benzoic acid was discovered in 1841 by Alexander Ure (Ure, 1841). This was later confirmed by Wilhelm Keller in 1842 who ingested 32 grains of benzoic acid and isolated hippuric acid from his urine the next morning (Keller, 1842). In 1845 it was demonstrated by Dessaignes that hippuric acid was in fact an amide conjugate between glycine and benzoic acid, making this the first conjugation reaction to be discovered. Since this epic discovery, interest in glycine conjugation has faded significantly. The small range of substrates for

glycine conjugation, when compared with glucuronidation, may have contributed to the relatively little research that has been done on the glycine conjugation pathway (Knights et al., 2007).

2.4.2. The metabolic significance of glycine conjugation is not yet well understood

Collectively, conjugation reactions have traditionally been referred to as phase II biotransformation processes, indicating that the conjugates formed are less toxic or more readily excreted than the parent metabolites (Caldwell, 1982, 1984). Although the more recent discovery of reactive conjugates such as some acylglucuronides has led some researchers to abandon the concept of phase II biotransformation, it is still generally useful to think of conjugation reactions as mechanisms to accelerate excretion of a vast number of bioactive compounds (Caldwell, 1982; Ritter, 2000). What is unclear, however, is whether the glycine conjugation of benzoic acid should be viewed as a detoxification process. Recently an alternative to the "detoxification hypothesis" of glycine conjugation, the "glycine deportation hypothesis", was introduced. This hypothesis states that the primary purpose of amino acid conjugation reactions is to regulate levels of amino acids that are also utilised as neurotransmitters. This is because benzoate is usually conjugated to glycine, glutamine, glutamate, arginine, ornithine, or taurine, amino acids that are also utilised as neurotransmitters in the central nervous system (CNS) of animals. According to the glycine deportation hypothesis, accumulation of these neurotransmitters in the CNS, which would be neurotoxic, is avoided by the irreversible urinary excretion of these amino acids as conjugates to aromatic acids such as benzoic acid. The basis of this argument is that amino acid conjugation does not significantly increase the water solubility of aromatic acids, as has generally been assumed. For example, the solubility of benzoic acid in water is 3.4 g/l, and that of hippuric acid is only 3.75 g/l. In the case of phenylacetic acid, water solubility decreases from 16.6 g/l to 7.3 g/l or 2.12 g/l, when conjugated to glycine or glutamine, respectively. In contrast, the glucuronidation of benzoate increases water solubility to 263 g/l. According to the glycine deportation hypothesis, glucuronidation would thus be a better choice of conjugation reaction if the goal was to increase water solubility and facilitate urinary excretion (Beyoglu and Idle, 2012; Beyoglu et al., 2012).

2.4.3. The glycine conjugation of benzoic acid

Several xenobiotic organic acids are metabolised by conjugation to glycine in the mitochondrial matrix in mammalian liver and kidney, followed by urinary excretion (for review see Knights and Miners, 2012; Knights et al., 2007). The most abundant amino acid conjugate excreted in the urine of almost all mammals is hippurate, the glycine conjugate of benzoate (Lees et al., 2013). Hippurate is formed from benzoate in two steps (Figure 2.2). First, benzoate is activated to benzoyl-CoA by the action of a mitochondrial ATP dependent acid:CoA ligase, which in humans has been identified as HXM-A (E.C. 6.2.1.2), encoded by the *ACSM2B* gene (Knights, 1998; Knights and Drogemiller, 2000; Schachter and Taggart, 1953; Vessey et al., 1999; Vessey et al., 2003). Second, the enzyme glycine N-acyltransferase (GLYAT, E.C. 2.3.1.13) binds benzoyl-CoA and catalyses the acylation of glycine to form hippurate and CoASH (Schachter and Taggart, 1953, 1954a).

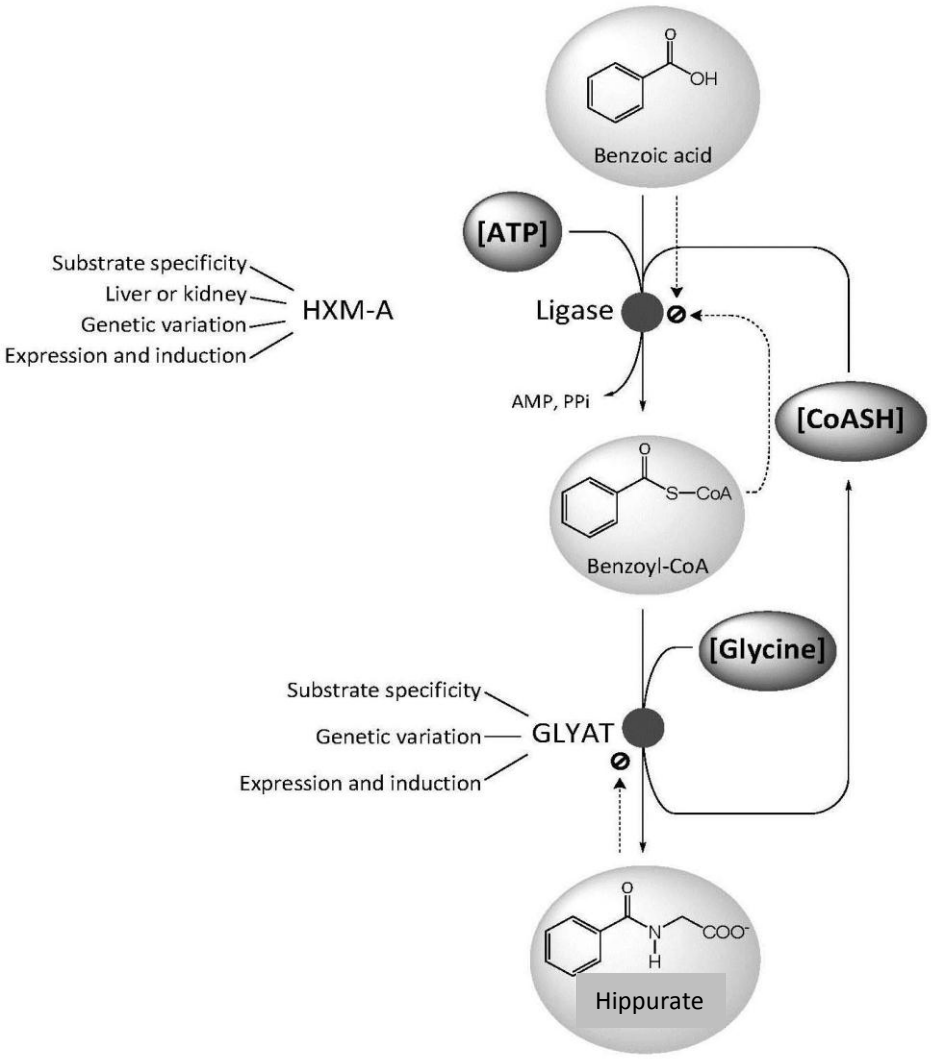

Figure 2.2: Glycine conjugation of benzoic acid. The glycine conjugation pathway consists of two steps. First benzoate is ligated to CoASH to form the high energy benzoyl-CoA thioester. This reaction is catalysed by the HXM-A medium chain acid:CoA ligase and requires energy in the form of ATP. Some acyl-CoA esters can competitively inhibit the ligase enzymes. The benzoyl-CoA is then conjugated to glycine by GLYAT to form hippurate, releasing CoASH. In addition to the factors listed in the boxes, the levels of ATP, CoASH, and glycine may influence the overall rate of the glycine conjugation pathway. The black circles indicate the ligase and GLYAT enzymes. Abbreviations: ATP, adenosine triphosphate; AMP, adenosine monophosphate; PPi, Pyrophosphate; GLYAT, glycine N-acyltransferase; CoASH, coenzyme A (Paper I, Appendix A, Figure 1).

2.4.4. Glycine conjugation is part of the phenylpropionate catabolism pathway

The natural substrates for glycine conjugation include benzoate, salicylate, 4-hydroxybenzoate, 3-hydroxybenzoate, 4-aminobenzoate, and 2-furoate. However, the major source of glycine conjugation

substrates seems to be the metabolites of dietary polyphenols produced by microorganisms in the gut (Figure 2.3) (for review see Knights and Miners, 2012; Rechner et al., 2002).

The major families of polyphenols in food are the flavan-3-ols, flavonols, flavanones, anthocyanins, and hydroxycinnamates. These are metabolised by the gut microbiota to simpler aromatic compounds such as phenylpropionate, 3-hydroxyphenylpropionate, and 4-hydroxyphenylpropionate (Bravo, 1998; Jenner et al., 2005; Rechner et al., 2002; Tsao, 2010). Phenylpropionate is transported to the liver where it is first activated to phenylpropionyl-CoA, although it is not clear which enzyme catalyses this reaction. Since phenylpropionyl-CoA is a good substrate for medium-chain acyl-CoA dehydrogenase (MCAD), it is rapidly converted to cinnamoyl-CoA (Mao et al., 1994). This is why phenylpropionyl-CoA is used in enzyme assays for the confirmation of MCAD deficiency (Rinaldo et al., 1990). Cinnamoyl-CoA continues through the β-oxidation cycle to yield acetyl-CoA and benzoyl-CoA (Dakin, 1908; Knoop, 1904; Saltzman and Caraway, 1953). Benzoyl-CoA cannot be further catabolised, and is conjugated to glycine instead. This results in hippurate being the major urinary metabolite of polyphenol metabolism and phenylpropionate catabolism (Dakin, 1908; Knoop, 1904; Rechner et al., 2002).

It is now known that the amount of glycine conjugates excreted in the urine largely depends on the dietary intake of polyphenolic compounds, and the extent to which the polyphenols are fermented in the colon (Lees et al., 2013; Phipps et al., 1998; Rechner et al., 2002). The polyphenol fermentation products produced in the colon depend on the type and amount of food consumed, its transit time through the digestive system, and the composition of the gut microbiome (Bravo, 1998; Fedotcheva et al., 2008; Jenner et al., 2005; Lees et al., 2013; Rechner et al., 2002; Smith and Macfarlane, 1996). Suppression of microbial activity, by antibiotic use for example, results in decreased production of compounds like phenylpropionate and consequently lower urinary hippurate excretion (Lees et al., 2013). This is further supported by the observation that germ free mice, bred under sterile conditions, excrete about one-seventeenth the amount of hippurate excreted by conventional mice fed the same diet. Exposure of germ free mice to a normal laboratory environment results in microbial colonisation of the colon and normalisation of urinary hippurate levels after about three weeks (Wikoff et al., 2009).

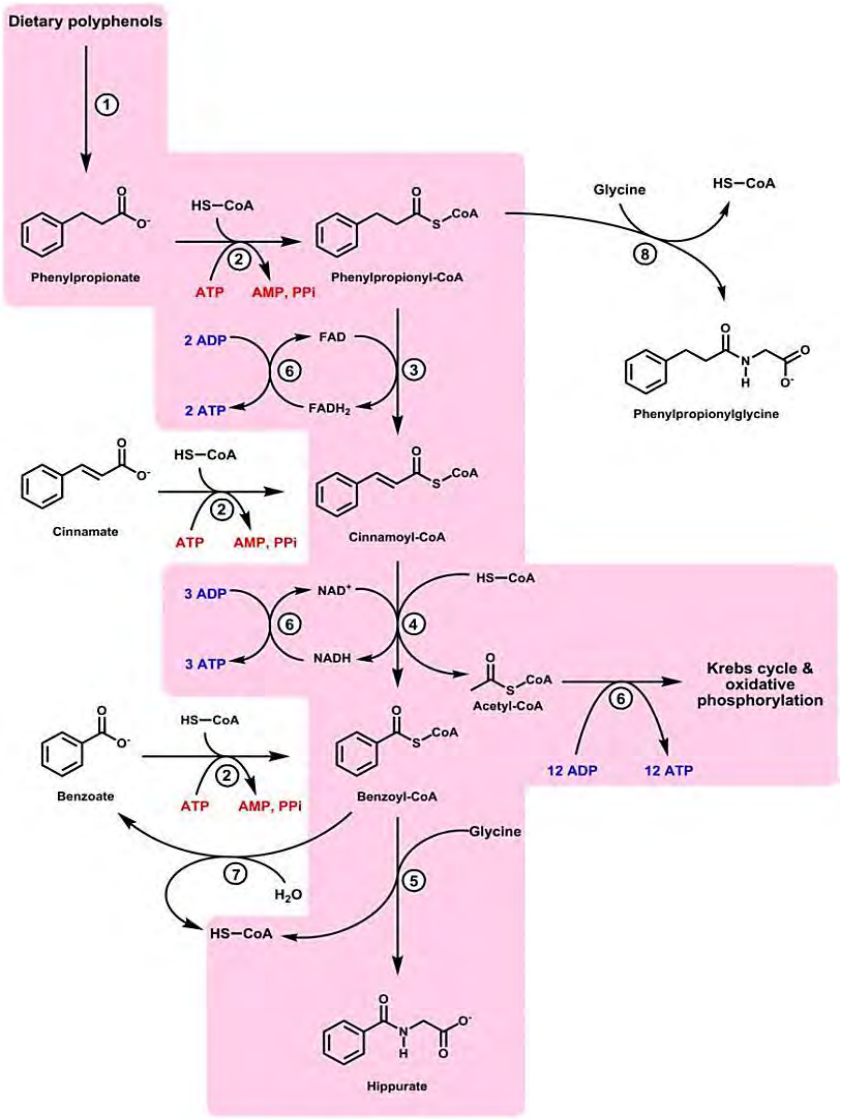

Figure 2.3: A schematic representation of the glycine conjugation pathway. The part of the pathway on the pink background depicts the natural glycine conjugation pathway, as it is described in this paper. 1) Dietary polyphenols are converted by the gut microbiota to simple aromatic acids such as phenylpropionate, cinnamate, and benzoate. 2) After absorption and transport to the liver, the aromatic acids are activated to acyl-CoA thioesters by ATP dependent acid:CoA ligases. The consumption of ATP is demonstrated by red. 3) Phenylpropionyl-CoA is converted to cinnamoyl-CoA by medium-chain acyl-CoA dehydrogenase, the first step of the β-oxidation cycle. 4) Cinnamoyl-CoA is further oxidised to benzoyl-CoA and acetyl-CoA. 5) Benzoyl-CoA is converted to hippurate by glycine N-acyltransferase. 6) The β-oxidation of phenylpropionate generates $FADH_2$, NADH, and acetyl-CoA, which are used by the Krebs cycle and the electron transport chain to produce ATP. The production of ATP is demonstrated by blue. 7) When the capacity of the glycine conjugation pathway is exceeded benzoyl-CoA accumulates, resulting in CoASH sequestration. Hydrolysis of benzoyl-CoA, by thioesterases with high affinity for benzoyl-CoA, releases benzoate and CoASH. 8) In individuals with a deficiency of medium-chain acyl-CoA dehydrogenase, phenylpropionyl-CoA accumulates and is converted by glycine N-acyltransferase to phenylpropionylglycine (Paper II, Appendix B, Figure 1).

2.4.5. The toxicity of benzoate and other aromatic acids

2.4.5.1. The toxicity of benzoate and phenylacetate to animals and cultured cells

Conjugation of large doses of benzoate or phenylacetate results in significant decreases in plasma glycine, which is associated with neurological symptoms in rats, dogs, and humans (Abdo et al., 1998; Beyoglu and Idle, 2012; Beyoglu et al., 2012; de Vries et al., 1948). High doses of benzyl acetate result in severe brain damage in rats as a result of glycine depletion, an effect cancelled out by the administration of glycine but not alanine (Abdo et al., 1998). When glycine is depleted, the detoxification of benzoate is significantly impaired, and significant damage occurs to the livers and kidneys of rats and dogs (Abdo et al., 1998; Griffith et al., 1989; Sherwin and Kennard, 1919; White, 1941). Plasma concentrations of aspartate aminotransferase and alkaline phosphatase, markers of liver damage, are increased in rats following benzoate administration. There is also evidence that benzoate and phenylacetate are neurotoxic (Batshaw and Brusilow, 1981; Praphanphoj et al., 2000). In addition to gut abnormalities, defects of pronephric tubes, cardiac oedema, and misalignment of muscle fibres, sodium benzoate also causes motor neuron innervations and abnormal expression of the acetylcholine receptor, tyrosine hydroxylase, and a dopamine transporter in zebrafish embryos and larvae (Bichara et al., 2014; Chen et al., 2009; Tsay et al., 2007). In human and mouse neurons and astrocytes, benzoate increases the expression of DJ-1, a protein with neuroprotective properties in Parkinson's disease (Khasnavis and Pahan, 2012). Benzoate can directly inhibit brain enzymes and can also increase the turnover of serotonin in the brains of mice and humans by competing with tryptophan for binding to albumin (Batshaw et al., 1988; Ross and Wootton, 1964). Phenylacetate causes permanent disruption of neurological development if administered to newborn rats (Wen et al., 1980). Similarly, abnormal neurological development of the foetus has been reported in cases of untreated maternal phenylketonuria, which is characterised by increased phenylacetate levels (Fulton et al., 1980). A potential mechanism of pathogenesis is the formation of phenylacetyl-CoA in the brain, where it strongly inhibits choline acetyltransferase and disrupts neuronal utilisation of acetyl-CoA (Loo et al., 1980; Loo et al., 1979; Loo et al., 1985).

Incubation of cultured primary rat hepatocytes with benzoate results in dose-dependent depression of protein and DNA synthesis and decreased activities of ornithine transcarbamylase and tyrosine aminotransferase (Oyanagi et al., 1987). Incubation of human leukocytes with benzyl acetate, benzyl alcohol, benzaldehyde, or benzoate results in significant DNA damage, reflecting the genotoxicity of these compounds (Abe and Sasaki, 1977; Demir et al., 2010; Yilmaz et al., 2009; Zengin et al., 2011). Benzoate influences the mitochondrial membrane potential and matrix calcium levels in cultured rat cortical neurons and human clonal epithelial cells, and induces cell death in a dose-dependent manner (Park et al., 2011; Park et al., 2013). The mechanisms of toxicity of aromatic acids like benzoate and cinnamate have not been clearly defined, but involves sequestration of CoASH, formation of toxic acyl-CoA thioesters, and disruption of mitochondrial integrity by interaction of the aromatic acids with the inner mitochondrial membrane.

2.4.5.2. Toxicity mediated by acyl-CoA formation

Comprehensive understanding of coenzyme A metabolism was advanced by the introduction of the concept of CASTOR disorders (Coenzyme A Sequestration, Toxicity, and Redistribution). In CASTOR disorders the synthesis of CoASH or the degradation of acyl-CoA thioesters is impaired, resulting in a combination of CoASH sequestration and accumulation of toxic acyl-CoAs (Mitchell et al., 2008). Therefore, it is very useful to keep in mind that the toxicity of an organic acid is largely determined by the extent to which an acyl-CoA is formed that cannot be further metabolised (Figure 2.4) (for reviewe see Knights and Miners, 2012; Knights et al., 2007; Mitchell et al., 2008).

2.4.5.2.1. The metabolic consequences of CoASH sequestration

The first step in the metabolism of several organic acid xenobiotics is the ATP dependent activation to an acyl-CoA thioester by hepatic acid:CoA ligases (Caldwell, 1982; for reviewe see Knights et al., 2007; Schachter and Taggart, 1953). In addition to the toxicity of the thioesters, acyl-CoA formation can cause sequestration of CoASH, which negatively impacts metabolism on many levels (Knights and Drogemiller, 2000; Knights et al., 2007; Mitchell et al., 2008; Zhang et al., 2007b). Coenzyme A is perhaps one of the most important molecules in biochemistry, participating in β-oxidation, ketogenesis, lipogenesis, glucose catabolism, gluconeogenesis, and several other metabolic processes such as amino acid metabolism and the Krebs cycle (Figure 2.4). Sequestration of CoASH can therefore inhibit these vital metabolic processes (Mitchell et al., 2008; Zhang et al., 2007b). Oxidation of propionate by bovine liver tissue is inhibited by phenylpropionate, cinnamate, and benzoate (Cremin Jr et al., 1994). Sequestration of CoASH by benzoate also inhibits β-oxidation, lipogenesis, ureagenesis, gluconeogenesis, ketogenesis, and the utilisation of ketone bodies in cultured rat hepatocytes and isolated mitochondria (Beynen and Geelen, 1982; Cyr et al., 1991; Griffith et al., 1989; Kalbag and Palekar, 1988; Palekar and Kalbag, 1991; Yao et al., 1994). The levels of ATP and acetyl-CoA in the brains of hyperammonemic rats treated with benzoate also decrease as a result of CoASH sequestration (Ratnakumari et al., 1993). The main routes for regeneration of free CoASH from xenobiotic acyl-CoAs are carnitine conjugation, hydrolysis, and glycine conjugation (Figure 2.4) (Knights and Drogemiller, 2000; Knights et al., 2007; Mitchell et al., 2008).

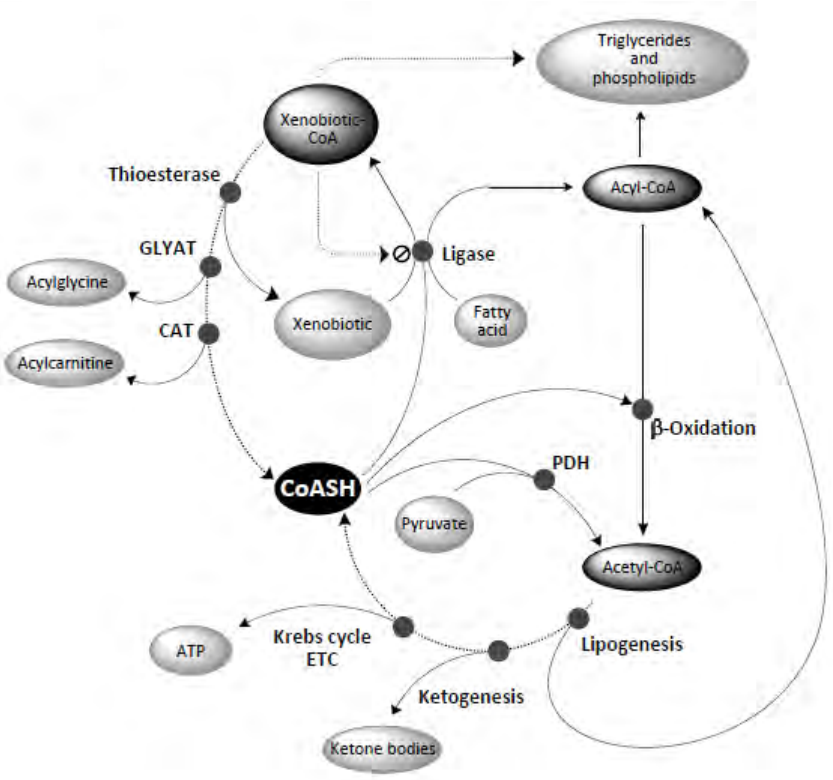

Figure 2.4: An overview of coenzyme A and acyl-CoA metabolism. The main pathways that produce and consume CoASH are demonstrated. It is extremely important that CoASH always be available in the cell because of its role in β-oxidation and the conversion of pyruvate to acetyl-CoA. Acetyl-CoA is the product of most catabolic reactions and provides the fuel for ketogenesis and mitochondrial ATP production. When xenobiotics are converted to xenobiotic-CoA esters, CoASH can be sequestered, disrupting ATP synthesis from pyruvate and fatty acids. As demonstrated by the fine broken arrows, accumulating xenobiotic-CoAs can inhibit the acid:CoA ligases or be incorporated into unnatural triglycerides and membrane phospholipids. The bold broken arrows indicate pathways that release bound CoASH. The black circles indicate important processes involved in the formation and degradation of acyl-CoAs. Abbreviations: GLYAT, glycine N-acyltransferase; CAT, carnitine acyltransferase; PDH, pyruvate dehydrogenase; CoASH, coenzyme A (Paper I and II, Appendix A and B, respectively, Figure 2).

2.4.5.2.2. The toxic effects of accumulated acyl-CoA thioesters

It is well known that formation of a more reactive acyl-CoA is an important mechanism mediating the toxicity of organic acids such as benzoate, phenylacetate, salicylate, valproate, and clofibrate. A major reason for the increased reactivity of acyl-CoA metabolites is the electrophilic nature of the thioester bond. This makes acyl-CoAs reactive to nucleophiles such as glutathione and the cysteine, serine, lysine, and histidine residues of proteins. For example, covalent modification of glutathione and cellular proteins by the acyl-CoA metabolites of phenylacetate, 2-phenylpropionate, and 2,4-dichlorophenoxyacetate has been demonstrated (Caldwell, 1984; Grillo and Benet, 2002; Grillo and Lohr, 2009; for review see Knights et al., 2007; Li et al., 2002; Li et al., 2003; Olsen et al., 2007; Sidenius et al., 2004).

Acyl-CoA metabolites can also inhibit several enzymes, causing complex effects that are difficult to predict (for review see Knights et al., 2007; Mitchell et al., 2008). Examples include the inhibition of choline acetyltransferase by phenylacetyl-CoA, inhibition of pyruvate carboxylase by propionyl-CoA and isovaleryl-CoA, inhibition of acid:CoA ligase enzymes by benzoyl-CoA and salicylyl-CoA, inhibition of the glycine cleavage system by valproyl-CoA and propionyl-CoA, and perturbation of protein kinase C function by clofibroyl-CoA (Erfle and Sauer, 1969; Knights et al., 2007; Kolvraa, 1979; Loo et al., 1980; Mortensen et al., 1980). In addition to acting as inhibitors, acyl-CoAs can also behave as alternative substrates for enzymes, resulting in the biosynthesis of abnormal metabolites. For example, when propionyl-CoA accumulates it can substitute for acetyl-CoA, resulting in the formation of methylcitrate by citrate synthase and *N*-propionylglutamate by *N*-acetylglutamate synthetase. The resulting shortage of *N*-acetylglutamate results in disruption of the urea cycle (Fenton et al., 2013). Accumulated propionyl-CoA and isovaleryl-CoA can substitute for acetyl-CoA in lipogenesis to produce odd-chain and branched-chain fatty acids (Fenton et al., 2013; Sweetman and Williams, 2013). Similarly, 2-arylpropionyl-CoAs, metabolites of nonsteroidal anti-inflammatory drugs, can be incorporated into adipocyte triglycerides (Knights et al., 2007). Very important cases involve the reaction of isovaleryl-CoA and benzoyl-CoA with carnitine, catalysed by carnitine acyltransferase enzymes (Itoh et al., 1996; Sakuma, 1991). This results in the excretion of acylcarnitines in the urine and can cause systemic carnitine depletion, which negatively impacts fatty acid oxidation and energy metabolism (Itoh et al., 1996; Lheureux and Hantson, 2009; Sakuma, 1991).

2.4.5.3. Benzoate and other aromatic acids stimulate ROS production and inhibit complex I

The aromatic acids phenylpropionate, cinnamate, and phenylacetate accumulate in the plasma of septic patients as a result of microbial metabolism in the blood. Sepsis is characterised by extremely high ROS production, oxidative stress, and mitochondrial damage (Beloborodova et al., 2012). The accumulating aromatic acids stimulate the production of ROS in isolated liver mitochondria and inhibits the activity of complex I of the electron transport chain (Beloborodova et al., 2012; Fedotcheva et al., 2008; Fedotcheva et al., 2012). Although the mechanisms of mitochondrial toxicity of the aromatic acids are not well understood, several recent advances in our understanding of this process will be briefly reviewed here.

Benzoate, salicylate, phenylpropionate, cinnamate, valproate, and isovalerate are all known to induce the mitochondrial permeability transition, an important mechanism in the pathogenesis of disorders related to Reye's syndrome (Battaglia et al., 2005; Beloborodova et al., 2012; Fedotcheva et al., 2008; Trost and Lemasters, 1996). Mitochondrial permeability transition is the common final pathway leading to acute cell death as a result of mitochondrial dysfunction, is induced by several mild oxidants such as *t*-butylhydroperoxide and diamide, and is inhibited by cyclosporin A (Fedotcheva et al., 2008; Kushnareva and Sokolove, 2000; Lemasters et al., 2002; Nakagawa and Moldeus, 1998; Nakagawa and Moore, 1999; Trost and Lemasters, 1996, 1997). It is characterised by the opening of a large non-selective pore which leads to uncoupling of oxidative phosphorylation, generation of ROS, mitochondrial swelling, release of cytochrome c, and apoptosis (Beloborodova et al., 2012; Bradshaw and Pfeiffer, 2006; Kushnareva and Sokolove, 2000; Trost

and Lemasters, 1997). An important first step leading to the permeability transition seems to be the formation of a small non-selective pore, distinct from the pore involved in mitochondrial permeability transition. The opening of this pore allows the passage of cations such as calcium, but not protons, and is thought to be stimulated by pro-oxidants and by salicylate in the inner mitochondrial membrane (Battaglia et al., 2005). Calcium efflux from this pore, coupled to calcium import by the calcium uniporter, results in rapid collapse of the membrane potential, which is stimulated by external calcium and inhibited by EGTA (Battaglia et al., 2005; Fedotcheva et al., 2008; Kushnareva and Sokolove, 2000; Trost and Lemasters, 1997). Collapse of the membrane potential precedes opening of the permeability transition pore and is not inhibited by cyclosporin A (Kushnareva and Sokolove, 2000).

Although it is not clear how aromatic acids induce ROS production and the mitochondrial permeability transition, it has been suggested that diffusion of these compounds into the inner mitochondrial membrane alters the properties of the membrane and stimulates opening of the initial small pore (Bernardi et al., 1994; Broekemeier and Pfeiffer, 1995; Trost and Lemasters, 1997). *N*-Ethylmaleimide can promote opening of the pore, which is inhibited by dithiothreitol, suggesting that oxidation of critical sulfhydryl groups on the inner mitochondrial membrane is important for opening the pore or for keeping it in an open state (Fedotcheva et al., 2008; Trost and Lemasters, 1997). In the mitochondria of cultured tobacco cells, benzoate and salicylate directly inhibit the transfer of electrons from complex I to ubiquinone, stimulating ROS generation (Norman et al., 2004). This could cause oxidation of sulfhydryl groups in the membrane, leading to inhibition of complex I and opening of the initial pore. This idea is supported by the observation that reducing agents like dithiothreitol can prevent the inhibition of complex I caused by *N*-Ethylmaleimide or aromatic acids (Fedotcheva et al., 2008). The inhibition of complex I results in ROS production upon oxidation of complex I and complex II substrates (Fedotcheva et al., 2008; Murphy, 2009). Oxidation of complex II substrates is not inhibited, since this complex is less sensitive to oxidative stress than complex I (Beloborodova et al., 2012; Fedotcheva et al., 2008; Fedotcheva et al., 2012; Murphy, 2009).

A related explanation for the damaging effects of aromatic acids on mitochondria is the ability of lipophilic compounds to disrupt the structural integrity of biological membranes by partitioning into the phospholipid bilayer, thereby increasing its permeability to small molecules and ions in a nonspecific manner (Politycka, 1997; Sikkema et al., 1995; Ye et al., 2006). For example, benzoate can increase the conductance of biological membranes, and causes leakage of lactate from intact mitochondria (Cremin Jr et al., 1994; Gutknecht, 1992). Increased membrane permeability can result in dissipation of membrane potential and uncoupling of oxidative phosphorylation, as demonstrated by the effects of cyclohexane on yeast mitochondria (Sikkema et al., 1995). This effect on membrane integrity also explains the observation that intracellular membrane trafficking in yeast is disturbed by benzoic acid, which also seems to negatively influence the fission and fusion of mitochondria (Hazan et al., 2004; Park et al., 2011; Park et al., 2013). The ability of a compound to diffuse into and accumulate in biological membranes is directly related to its lipophilicity (Lakeram et al., 2007; Sikkema et al., 1995). For example, the ability of the parabens, alkyl esters of 4-hydroxybenzoic acid, to induce mitochondrial damage depends on the alkyl-chain length and is linearly related to the octanol/water partition

coefficient, a reflection of lipophilicity (Lakeram et al., 2007; Nakagawa and Moldeus, 1998; Nakagawa and Moore, 1999). The ROS production and complex I inhibition in isolated mitochondria induced by aromatic acids also seem to be influenced by the lipophilicity of the compound (cinnamate > benzoate > phenylpropionate > phenylacetate) (Beloborodova et al., 2012; Fedotcheva et al., 2008; Fedotcheva et al., 2012; Politycka, 1997; Ye et al., 2006). The hydroxylated aromatic acids such as phenyllactate, 4-hydroxyphenylacetate, and 4-hydroxyphenyllactate are less lipophilic, do not stimulate ROS generation, and because of their hydroxyl groups, can act as free radical scavenging antioxidants (Fedotcheva et al., 2008).

2.4.6. The importance of glycine conjugation in the metabolism of aromatic acids

2.4.6.1. Glycine conjugation is important for the maintenance of CoASH levels

Several metabolic processes are dependent on CoASH and are inhibited by CoASH sequestration (Figure 2.4). Glycine conjugation is important for the maintenance of CoASH levels, by preventing its sequestration as benzoyl-CoA (for review see Knights and Miners, 2012; Knights et al., 2007). For example, the inhibition of gluconeogenesis in bovine liver mitochondria by benzoate-induced CoASH sequestration is reversed by the addition of glycine, which stimulates hippurate synthesis, decreases benzoyl-CoA levels, and increases CoASH levels. As a result acetyl-CoA levels are restored and the rate of gluconeogenesis normalises (Griffith et al., 1989). The accumulation of benzoyl-CoA and sequestration of CoASH caused by benzoate is similar to the situation in isovaleric acidemia, suggesting that isovaleric acidemia can shed some light on the importance of glycine conjugation (Trost and Lemasters, 1996). Isovaleric acidemia is a defect of leucine catabolism caused by mutations in the isovaleryl-CoA dehydrogenase gene, resulting in accumulation of isovaleryl-CoA in mitochondria (Tanaka et al., 1966). This accumulation results in sequestration of CoASH and formation of large amounts of isovaleric acid, which is toxic to mitochondria (Trost and Lemasters, 1996). Because isovaleryl-CoA is a good substrate for GLYAT, glycine conjugation restores CoASH levels and produces isovalerylglycine, a less toxic metabolite that is more readily excreted by the kidneys (Bartlett and Gompertz, 1974; Tanaka and Isselbacher, 1967). As a result of the excessive glycine conjugation, depletion of glycine is often observed in these patients. Glycine supplementation restores glycine levels, increases isovalerylglycine excretion, and generally results in normal mental and physical development of these patients (Dercksen et al., 2012; Itoh et al., 1996; Sweetman and Williams, 2013; Tanaka and Isselbacher, 1967). Alternatively, supplementation with carnitine leads to increased isovalerylcarnitine excretion, which also restores CoASH levels, detoxifies isovaleric acid, and results in effective treatment of the disease. Interestingly, carnitine supplementation does not necessarily decrease isovalerylglycine excretion, and may even stimulate it (Itoh et al., 1996; Sweetman and Williams, 2013). Deficiency of medium-chain acyl-CoA dehydrogenase also demonstrates the role of glycine conjugation in maintaining CoASH levels. Patients with defects of MCAD cannot metabolise phenylpropionyl-CoA to cinnamoyl-CoA, resulting in the accumulation of phenylpropionyl-CoA in mitochondria. As a result, high levels of phenylpropionylglycine are excreted in the urine (Figure 2.3) (Bennett et al., 1990).

An important mechanism by which glycine conjugation reduces the acyl-CoA mediated toxicity of organic acids is by converting the electrophilic thioester to the inert peptide bond of the glycine conjugate (Caldwell, 1982, 1984). Unlike thioesters, and even esters such as acylglucuronides, peptide conjugates are chemically stable and do not react with nucleophiles such as proteins and glutathione (Caldwell, 1982, 1984; Grillo and Benet, 2002; Grillo and Lohr, 2009; Li et al., 2002; Li et al., 2003; Olsen et al., 2007; Sidenius et al., 2004). Since covalent modification by acyl-CoAs is dependent on both time and the concentration of the acyl-CoA thioester, it seems reasonable to assume that the toxicities of these electrophiles are significantly decreased by glycine conjugation, although this is yet to be experimentally verified (Caldwell, 1984; Grillo and Lohr, 2009).

2.4.6.2. Glycine conjugation decreases the lipophilicity of benzoic acid

It has usually been assumed that glycine conjugation increases the water solubility of benzoate in order to facilitate urinary excretion. However, the water solubility of sodium benzoate (629 g/l) is so high that insolubility cannot possibly explain the toxicity of this compound. Furthermore, glycine conjugation only slightly increases the water solubility of benzoic acid from 3.4 g/l to 3.75 g/l for hippuric acid (Beyoglu and Idle, 2012; Beyoglu et al., 2012). This suggests that increasing water solubility is not the mechanism by which glycine conjugation decreases the toxicity of benzoate. We suggest that glycine conjugation decreases the toxicity of benzoate, not because hippuric acid is more water soluble than benzoic acid, but because hippuric acid is less lipophilic than benzoic acid. Despite having similar water solubilities, hippuric acid is significantly less lipophilic than benzoic acid, as reflected by their LogP values of 0.31 and 1.87, respectively (Hansch and Leo, 1995). This difference in LogP values reflects an almost 40-fold difference in octanol-water partition coefficients for these compounds. Based on the strong correlation between lipophilicity and ability to diffuse into biological membranes, this means that benzoic acid will be significantly more capable of partitioning into the hydrophobic interiors of phospholipid bilayers (Lakeram et al., 2007; Nakagawa and Moldeus, 1998; Nakagawa and Moore, 1999; Sikkema et al., 1995). This argument is supported by the observation that benzoic acid can be extracted from acidified urine by the non-polar solvent benzene, while hippuric acid remains in the aqueous phase after extraction with benzene and ether (Dakin, 1910).

2.4.6.3. The influence of decreased lipophilicity on the toxicity and mitochondrial export of benzoate

The mitochondrial toxicity of a compound is strongly correlated to its lipophilicity, because lipophilic compounds diffuse into and disrupt the structural integrity of biological membranes. It therefore seems reasonable to suggest that hippurate will be significantly less toxic to mitochondria than benzoic acid, despite the current lack of experimental evidence for this hypothesis (Figure 2.5, numbers 2, 7, and 10).

It can also be argued that glycine conjugation facilitates removal of benzoate from the mitochondrial matrix. It has been demonstrated that benzoate accumulates in the mitochondrial matrix to a concentration about 50-times higher than that in the external solution. This is the result of the pH gradient of about 2 units over the inner mitochondrial membrane (Gatley and Sherratt, 1977; Gatley and Sherratt, 1976). The low pH of about

6.0 in the intermembrane space favours the formation of benzoic acid, which diffuses into the matrix where the higher pH of about 7.8 favours dissociation to benzoate, which becomes trapped in the matrix (Figure 2.5, numbers 1 to 3) (Davies et al., 2011; Gatley and Sherratt, 1977; Gatley and Sherratt, 1976; Porcelli et al., 2005; Strauss et al., 2008). Both benzoate and hippurate can be exported from the mitochondrial matrix by organic anion transporters (Deguchi et al., 2005; Schwab et al., 2001; Yoshimura et al., 1998). However, it would be futile to simply export benzoate from the matrix, since it can reform benzoic acid in the more acidic intermembrane space, and diffuse back over the inner membrane into the matrix (Figure 2.5, numbers 2 and 3) (Piper et al., 2001; Piper, 1999). In contrast, unidirectional export of hippurate is possible, since the less lipophilic hippuric acid cannot diffuse back over the inner mitochondrial membrane at a significant rate (Figure 2.5, arrows 2, 5, and 7). The model described here is supported by the observation that benzoate clearance is limited by the rate of hippurate synthesis, which is about one-fifth of the maximum rate of hippurate excretion (Figure 2.5, arrows 2, 4, and 5) (Gregus et al., 1992, 1993a).

2.4.7. The impact of glycine conjugation on glycine metabolism

2.4.7.1. Glycine homeostasis is not the primary purpose of the glycine conjugation pathway

The major system involved in glycine homeostasis is the glycine cleavage system, which has a high metabolic capacity and is expressed in several tissues, including liver and brain (Kikuchi et al., 2008). Defects of the glycine cleavage system result in nonketotic hyperglycinemia (NKH), characterised by significant elevations of glycine concentrations in plasma and CSF (Conter et al., 2006; Hamosh and Johnston, 2013; Hiraga et al., 1981; Kure et al., 2004; Perry et al., 1975). The most useful diagnostic parameter is the CSF/plasma glycine ratio, which is normally below 0.02, with values of 0.09 or greater being diagnostic for NKH (Hamosh and Johnston, 2013; Krieger et al., 1977; Perry et al., 1975). Although there are no completely satisfactory treatments for this disorder, administration of sodium benzoate is used to decrease plasma and CSF glycine levels, as the excess glycine is excreted in the urine as hippurate. The effectiveness of this therapy is variable and glycine levels or CSF/plasma glycine ratios are rarely decreased to satisfactory levels (Applegarth and Toone, 2001; Barshop et al., 1989; Beyoglu and Idle, 2012; Beyoglu et al., 2012; Krieger et al., 1977). The failure of this therapy to completely normalise glycine levels and CSF/plasma glycine ratios, despite the large doses of benzoate used, suggests that the contribution of the glycine deportation system to glycine homeostasis is small, compared to that of the glycine cleavage system (Beyoglu and Idle, 2012; Beyoglu et al., 2012; Hamosh and Johnston, 2013; Krieger et al., 1977).

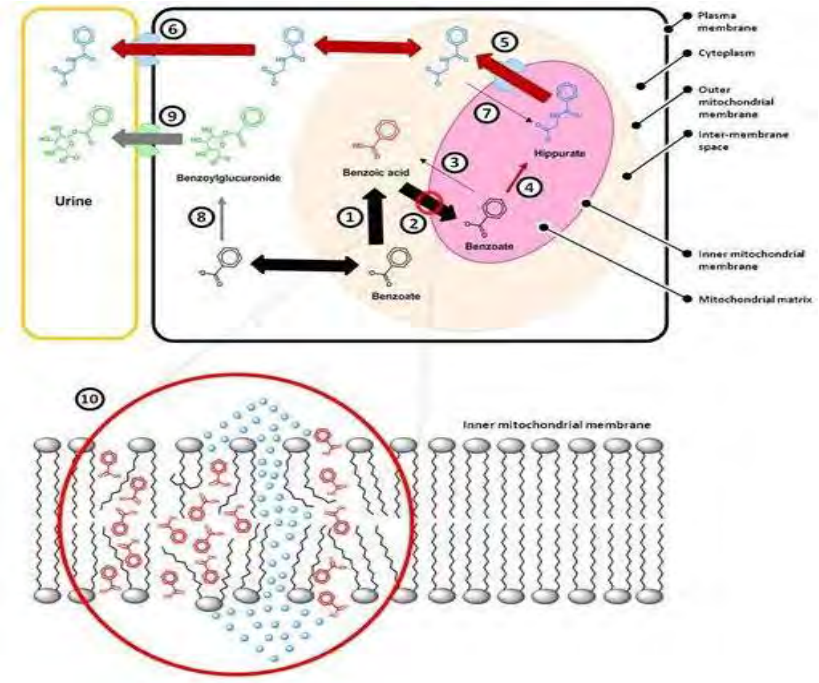

Figure 2.5: The detoxification of benzoate by means of glycine conjugation in hepatocytes. 1) Because the pH in the mitochondrial inter-membrane space is lower than that of the cytoplasm and the mitochondrial matrix, the conversion of benzoate (black) to benzoic acid (red) is favoured. 2) Benzoic acid can freely diffuse across the mitochondrial inner membrane into the mitochondrial matrix. In the matrix the higher pH favours the dissociation of benzoic acid to form benzoate. 3) Benzoate accumulates in the mitochondrial matrix to concentrations about 50 times higher than that in the surrounding solution. This is demonstrated by the width of the black arrows. Therefore, the thick black arrow 2, pointing to the matrix, is 50 times wider than arrow 3, pointing out of the matrix space. 4) In the mitochondrial matrix benzoate is converted to hippurate (blue). 5) Hippurate is transported out of the matrix by organic anion transporters. The rate at which hippurate can be excreted is about five times higher than that at which it can be synthesised. This is demonstrated by the red arrow 5 being five times wider than red arrow 4, which represents hippurate synthesis. 6) Finally, hippurate is irreversibly excreted into urine (yellow block) by organic anion transporters. 7) Hippuric acid is less lipophilic than benzoic acid and therefore diffuses into the matrix space at a significantly lower rate. This is indicated by black arrow 7 being 40 times thinner than black arrow 2, which represents the diffusion of benzoic acid into the matrix. 8) In the endoplastic riticulum, benzoate can also be converted to benzoyl-glucuronide by UDP-glucuronosyltransferases, as represented by the grey arrows. Because benzoate accumulates in the mitochondrial matrix, benzoylglucuronide synthesis, which is localised to the endoplastic reticulum, only occurs when the capacity of the mitochondrial glycine conjugation system is exceeded. 9) Benzoylglucuronide is then also excreted in the urine. 10) This section zooms in on a part of the inner mitochondrial membrane. It represents the diffusion of the lipophilic benzoic acid into the inner mitochondrial membrane, which causes disruption of the phospholipid bilayer and results in permeabilisation of the membrane. This is represented by the small blue spheres leaking across the membrane (Paper II, Appendix B, Figure 3).

After phenylketonuria, NKH is the second most prevalent defect of amino acid catabolism, affecting up to one in 64 000 individuals in some populations (Hamosh and Johnston, 2013). Therefore, being a carrier of NKH is not lethal or extremely deleterious for human health, despite the higher plasma and CSF glycine concentrations observed in carriers, compared to controls (Kure et al., 2004; Von Wendt and Similä, 1982). It is interesting to note, however, that no defect of the glycine conjugation system, which makes the smaller contribution to glycine homeostasis, has been reported in humans. This seems to suggest that glycine conjugation is a vital metabolic process, and that its primary purpose is not the regulation of glycine levels. However, as suggested by the glycine deportation hypothesis, consumption of large quantities of aromatic acids can result in significant excretion of glycine in the urine (as hippurate or phenylacetylglycine) (Beyoglu and Idle, 2012; Beyoglu et al., 2012; de Vries et al., 1948).

2.4.7.2. Benzoate and human glycine deficiency

Glycine is commonly thought to be a nonessential amino acid since it can be synthesised from serine by serine hydroxymethyltransferase (Wu et al., 2013). Serine itself is synthesised from 3-phosphoglycerate, an intermediate of glycolysis (Figure 2.6).

Serine hydroxymethyltransferase converts serine to glycine, 5,10-methylenetetrahydrofolate (THF-C1), and NADH. Additional THF-C1 and NADH are produced from glycine by the glycine cleavage system. In recent years it has become apparent that glycine is a conditionally essential amino acid, as the metabolic demand for glycine often exceeds the capacity for glycine synthesis (Melendez-Hevia et al., 2009; Wu, 2013; Wu et al., 2013). For example, glycine availability has been shown to be limiting in the developing human foetus, in young men, birds, and in milk-fed pigs (Jackson et al., 1997; Jackson et al., 1996; Wang et al., 2013; Wu, 2010, 2013; Wu et al., 2013). In fact, it has been estimated that human adults have a shortage of about 10 grams of glycine per day. This glycine deficiency, and the resulting deficiency of THF-C1 units, can influence the metabolism of collagen, glutathione, creatine, nucleic acids, and porphyrins (Brosnan et al., 2011; Melendez-Hevia et al., 2009; Wang et al., 2013). Glycine shortage can be exacerbated by benzoate and other substrates for glycine conjugation (Jackson et al., 1987; Xie et al., 2013). In addition to natural sources of benzoate, humans are nowadays exposed to ever increasing amounts of this compound, since it is used as a preservative in food and pharmaceuticals (Piper, 1999). In some populations where a lot of preserved food is consumed, benzoate intake can be as high as 280% of the recommended daily allowance, which is reflected by unusually high levels of urinary hippurate (Lees et al., 2013; Tfouni and Toledo, 2002).

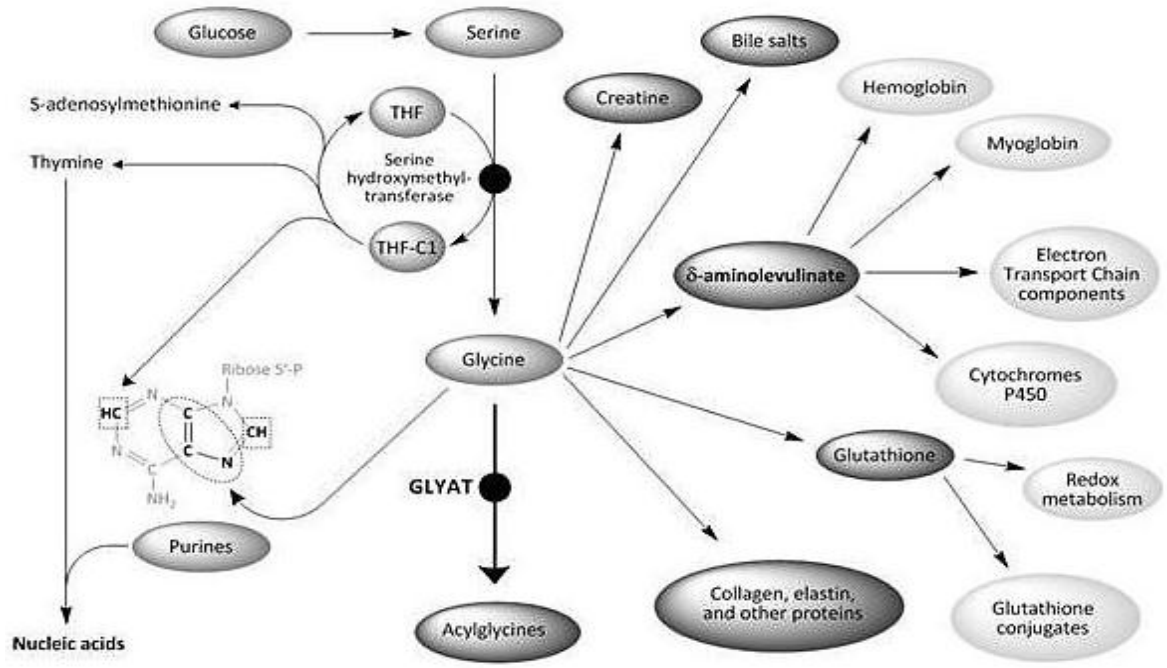

Figure 2.6: Biosynthesis and metabolic consumption of glycine. Glycine is biosynthesised from serine by serine hydroxymethyltransferase. The reaction converts THF (tetrahydrofolate) to THF-C1 (tetrahydrofolate-C1) for each molecule of glycine produced. The total amount of glycine synthesised can thus not exceed the amount of THF-C1 consumed through production of compounds such as S-adenosylmethionine, thymine, and purines. Glycine is consumed in the production of creatine, bile salts, porphyrins, collagen, elastin, other proteins, and glutathione. Black circles represent the serine hydroxymethyltransferase and GLYAT enzymes. The bold arrow indicates formation of xenobiotic acylglycines by GLYAT. The parts of purine rings derived from glycine and THF-C1 are indicated by the dashed ellipse and squares, respectively (Paper II, Appendix B, Figure 4).

2.4.8. Interindividual variation in glycine conjugation

Because the glycine conjugation system is readily saturable, the metabolism of benzoate is dose dependent (Amsel and Levy, 1969; for review see Knights and Miners, 2012; for review see Knights et al., 2007; Kubota and Ishizaki, 1991; Poon and Pang, 1995). The major metabolite of benzoate is hippurate, but when very large doses are administered benzoylcarnitine and benzoylglucuronide may also be formed (Bray et al., 1951; Sakuma, 1991). The formation of these secondary metabolites depends on both the dose of benzoate and the capacity of the individual glycine conjugation system in question (Bray et al., 1951; Saltzman and Caraway, 1953). For humans, significant interindividual variation in urinary hippurate excretion has been documented. One study determined the daily hippurate excretion as 6285 ± 4008 µmol for men and 4793 ± 3239 µmol for women. The Human Metabolome Database reports urinary hippurate concentrations ranging from 27.92 to 932.66 µmol (Lees et al., 2013; Wijeyesekera et al., 2012). It is important to distinguish between variation in the amount of hippurate excreted, and variation in the rate of glycine conjugation. These two concepts are usually not clearly distinguished in the literature, which can lead to confusion. For example, patients with

Crohn's disease are characterised by significantly lower urinary hippurate levels. This is associated with significant alterations of the gut microbiome, which result in decreased fermentation of dietary polyphenols and lower production of phenylpropionate. However, these individuals do not demonstrate a decreased rate of hepatic glycine conjugation, as demonstrated by normal conversion of an oral dose of benzoate to hippurate (Williams et al., 2010). In the next section the factors that influence the rate of glycine conjugation, and the capacity of the glycine conjugation pathway, are discussed.

2.4.9. Factors that influence interindividual variation in the rate of glycine conjugation

2.4.9.1. Availability of ATP, CoASH, and glycine

The first step in the glycine conjugation of benzoate requires CoASH for the synthesis of benzoyl-CoA (Figure 2.2). The availability of unacylated CoASH can therefore directly limit the maximum rate of glycine conjugation (Gregus et al., 1996a; Gregus et al., 1992). In one study pantothenate, a precursor for CoASH biosynthesis, was administered to a hyperglycinemia patient on benzoate therapy. This resulted in increased urinary hippurate excretion and attenuation of seizures, but there was no way to determine whether hepatic CoASH levels were actually increased. However, it was demonstrated that addition of pantothenate to the culture medium of HepG2 cells resulted in significant increases in cellular CoASH content and increased conversion of benzoate to hippurate (Palekar, 2000). The synthesis of benzoyl-CoA is also dependent on ATP (Figure 2.2). Since ATP is hydrolysed to AMP, two molecules of ATP are consumed per molecule of benzoate (Schachter and Taggart, 1953, 1954a). A shortage of ATP will, therefore, limit the rate of glycine conjugation by limiting the formation of benzoyl-CoA (Gregus et al., 1996a). The rates of glycine conjugation of cinnamate, benzoate, and 4-aminobenzoate have been measured extensively as a test of liver function, and correlate well with hepatic reserve (Duffy et al., 1995; Furuya et al., 1995; Krahenbuhl et al., 1997; Probstein and Londe, 1940; Saltzman and Caraway, 1953). It is thought that the mitochondrial dysfunction associated with liver disease, which leads to decreased ATP production, explains the lower rate of glycine conjugation in hepatitis patients (Gregus et al., 1996a; for review see Knights et al., 2007). The availability of glycine is another factor known to strongly influence the rate of hippurate synthesis (Figure 2.2) (de Vries et al., 1948; Gregus et al., 1992, 1993a; Levy, 1979). Therefore, although hippurate synthesis in humans is saturable with large doses of benzoate, the co-administration of glycine can significantly increase the rates of hippurate formation and benzoate clearance (for review see Knights and Miners, 2012; Levy, 1979). The availability of glycine also limits hippurate synthesis in rat liver. In one study the hepatic glycine content of rats was doubled by the administration of cysteamine, a potent inhibitor of the mitochondrial glycine cleavage system, without affecting the levels of ATP and CoASH. The result was a 50% increase in the rate of benzoate conjugation (Gregus et al., 1993a). Because the increase in the rate of hippurate formation was smaller than the increase in glycine concentration, other factors such as CoASH availability seem to limit hippurate synthesis when excess glycine is available (Gregus et al., 1996a, b; Gregus et al., 1992, 1993a).

Although glycine supplementation stimulates hippurate synthesis, the glycine conjugation of salicylic acid is not enhanced by co-administration of glycine (Amsel and Levy, 1969). This is because the limiting step in the pathway depends on the substrate: hippurate formation for benzoate, and salicylyl-CoA formation for salicylate. This is consistent with the observation that the rate of salicylyl-CoA formation by human liver HXM-A is less than 1% of that for benzoyl-CoA formation (for review see Knights et al., 2007; Vessey et al., 1999). There are three important differences when phenylpropionate, rather than benzoate, is the substrate for glycine conjugation (Figure 2.3). First, the conjugation of benzoate consumes two molecules of ATP, while the β-oxidation of phenylpropionate can theoretically yield 15 molecules of ATP. Second, an extra molecule of CoASH is needed for the oxidation of phenylpropionate (Houten and Wanders, 2010). The availability of ATP and CoASH for hippurate synthesis may thus depend on whether the substrate is phenylpropionate, cinnamate, or benzoate. Finally, the rates of mitochondrial uptake and activation to acyl-CoAs may be different for these substrates, because of differences in structure and lipophilicity (Hansch et al., 1968; Kasuya et al., 1990, 1991; Masuda et al., 1996).

2.4.9.2. Genetic variation in ACSM2B and GLYAT

Early investigations revealed a greater similarity in the metabolism of aspirin between identical twins than between fraternal twins, suggesting that genetic variation is partly responsible for interindividual variation in the glycine conjugation of salicylate (Furst et al., 1977). However, very little is known about the influence of genetic variation on the glycine conjugation pathway (Boomgaarden et al., 2009).

2.4.9.3. Expression of HXMA (encoded by ACSM2B) and GLYAT

Both the ligase and transferase enzymes are inducible, although the mechanisms involved are not well understood. Pre-treatment of rats with salicylate for six days increases the capacity of liver and kidney homogenates for synthesis of salicylylglycine, hippurate, and 4-aminohippurate (Irjala, 1972). Salicylate pre-treatment also increases the rate of salicylylglycine formation in humans (Furst et al., 1977). Recently it was demonstrated that dietary restriction results in increased urinary acylglycine excretion, which correlates with increased expression of GLYAT in the liver of rats (Wen et al., 2013). Also, investigation of hepatocellular carcinoma specimens further demonstrated that GLYAT expression is transcriptionally regulated (Matsuo et al., 2012).

2.4.10. Glycine conjugation in metabolic diseases

In several organic acidemias an acyl-CoA accumulates to toxic levels because of a defect of the enzyme acting on it (Bonafe et al., 2000; Tanaka and Isselbacher, 1967; Wanders et al., 1999). This result not only in CoASH sequestration, but because of thioesterase activity free organic acids are released, causing potentially deadly acidoses (Gompertz, 1974; Ogier and Saudubray, 2002; Tanaka et al., 1966). Because some of the acyl-CoAs that accumulate in organic acidemias are substrates for GLYAT, glycine conjugation impacts on the biochemical

profiles and clinical outcomes of some of these metabolic defects (Bartlett and Gompertz, 1974; Bonafe et al., 2000; Gompertz, 1974; Gron et al., 1978; Krieger and Tanaka, 1976). Glycine conjugation under these abnormal conditions sheds light on the important role GLYAT plays in maintaining CoASH levels.

In some cases the accumulating acyl-CoA can be conjugated to glycine by GLYAT, decreasing the severity of CoASH sequestration and avoiding acidosis, as a less toxic acylglycine is formed and excreted (Bartlett and Gompertz, 1974; Bonafe et al., 2000; Dercksen et al., 2012; Tanaka and Isselbacher, 1967). For example, in isovaleric acidemia, where isovaleryl-CoA accumulates, large amounts of isovalerylglycine is excreted in the urine because isovaleryl-CoA is a good substrate for GLYAT (Krieger and Tanaka, 1976; Sweetman and Williams, 2013; Tanaka and Isselbacher, 1967). However, in propionic acidemia, only relatively small amounts of propionate are excreted as propionylglycine (Fenton et al., 2013). Glycine conjugates are also excreted in several other organic acidemias, and include 3-methylcrotonylglycine, hexanoylglycine, butyrylglycine, and tiglylglycine (Bonafe et al., 2000; Gompertz, 1974; Ogier and Saudubray, 2002; Wanders et al., 1999). There is no simple relationship between GLYAT substrate selectivity, in terms of K_M and V_{max} parameters, and acyl group structure. This makes it difficult to predict the extent to which glycine conjugation will influence the outcome of any particular organic acidemia (Bartlett and Gompertz, 1974; for review see Knights and Miners, 2012).

Enzyme replacement therapy has been used effectively to treat several lysosomal storage diseases (for example Gaucher disease, Fabry disease and Pompe disease), adenosine deaminase deficiency, cystic fibrosis and other diseases (Brady et al., 1974; Cantz and Kresse, 1974; Chan et al., 2005; Munck et al., 2009). Instead of replacing a defective enzyme, the body can be supplemented with an enzyme to enhance detoxification by the liver (Nandi et al., 1979; Temellini et al., 1993). Currently, there is no enzyme enhancement therapy available to increase the detoxification of xenobiotics (Sears and Genuis, 2012). Enzyme replacement therapy was used to treat the deficiency of a mitochondrial protein, lipoamide dehydrogenase (LAD; also known as E3). This was done by recombinant expression of LAD fused to a signal peptide that directs the protein into hepatocytes and then targets the mitochondria for entry. This approach has been shown to correct the enzyme deficiency in cultured patient hepatocytes (Rapoport et al., 2008). Later it was shown that the defective enzyme could be replaced by using the TAT-LAD fusion protein in an *in vivo* mouse model using E3-deficient mice. A single administration of TAT-LAD resulted in a significant increase in the enzymatic activity of the mitochondrial multienzyme pyruvate dehydrogenase complex within the liver, heart and, the brain of TAT-LAD-treated E3-deficient mice (Rapoport et al., 2011). This is encouraging since it suggests that, at least in principle, it should be possible to direct a recombinant therapeutic GLYAT to liver mitochondria for the treatment of organic acidemias.

2.4.11. The increased demand for glycine conjugation in modern life

In modern times increasing exposure to benzoate, salicylate, solvents, and drugs that are metabolised to acyl-CoA intermediates places more pressure on the glycine conjugation pathway, possibly exacerbating

metabolic glycine shortage (Campbell et al., 1988; Huang et al., 1994; Jackson, 1991; for review see Knights and Miners, 2012; for review see Knights et al., 2007; Melendez-Hevia et al., 2009). Therefore the consequences of interindividual variation in the glycine conjugation pathway may become more significant as more xenobiotic organic acids are encountered in the future (Knights et al., 2007).

In addition to xenobiotics, short chain fatty acids (SCFAs) produced by intestinal microbes are another potential source of substrates for glycine conjugation and may contribute to glycine depletion under some conditions. Gut microbes produce large amounts of SCFAs which account for 5 to 10% of the total dietary energy intake in humans (Kimura et al., 2011). Indeed, the gut contains an active SCFA ligase for metabolising these organic acids (Vessey, 2001). Gut dysbiosis, caused by antibiotic use, for example, can result in increased SCFA production and this has been associated with obesity and diabetes (Holmes et al., 2011; Prakash et al., 2011). SCFAs are not usually conjugated to glycine, as this would be energetically wasteful (Bartlett and Gompertz, 1974; Bonafe et al., 2000; Wanders et al., 1999). However, if sufficiently large amounts of SCFAs are produced, hepatic metabolism of CoASH and glycine will be affected. A recent observation in our laboratory seems to support this idea. A patient with unusually high levels of urinary butyrate complained of bad body odour and was referred to our laboratory by a physician. The increased butyrate excretion was not the result of a short chain acyl-CoA dehydrogenase defect, and gut dysbiosis was suspected. Glycine supplementation was recommended and this resulted in significantly increased butyrylglycine excretion, decreased butyrate excretion and disappearance of the body odour (unpublished results).

2.4.12. Enzymes involved in the glycine conjugation pathway

2.4.12.1. ATP dependent acid:CoA ligases (HXMA encoded by ACSM2B) (Figure 2.2)

Several fatty acids, and xenobiotic carboxylic acids that are conjugated to amino acids, must first be activated to acyl-CoAs by ATP dependent acid:CoA ligases (Knights, 1998; for review see Knights and Miners, 2012; for review see Knights et al., 2007; Schachter and Taggart, 1953). These ligase enzymes exhibit selectivity for short, medium, long, or very long chain fatty acids (Knights, 1998). Most xenobiotics that undergo glycine conjugation are activated by the mitochondrial medium chain ligases, which also activate C4 to C12 acids for β-oxidation (Kasuya et al., 1996b; Killinberg et al., 1971; Knights, 1998; Knights and Drogemiller, 2000). The acyl-CoA species increase the reactivity of the carboxylic acid and therefore amino acid conjugation may be viewed as a mechanism of cell protection. In humans, however, formation of a xenobiotic-CoA does not necessarily result in subsequent amino acid conjugation (Knights, 1998). The xenobiotic-CoA can function as an alternative substrate in pathways of intermediary metabolism, such as lipid biosynthesis (Fears, 1985).

2.4.12.1.1. Nomenclature and substrate specificity of ACSM

Distinguishing substrate and inhibitor profiles for bovine medium chain xenobiotic-CoA ligases from information in the literature is complicated due to the fact that previous studies have used crude

mitochondrial preparations and an inconsistency in nomenclature of the enzymes (Knights and Drogemiller, 2000). The original medium chain CoA ligase (butyrate-CoA ligase, EC 6.2.1.2) identified in bovine liver by Mahler, could activate benzoic acid but not salicylic acid and *p*-aminosalicylic acid (Mahler et al., 1953). Subsequently, a medium-chain fatty acid:CoA ligase (AMP) which activates salicylate was purified from bovine liver. This enzyme was distinct from the butyrate medium-chain fatty acid:CoA ligase (AMP) (EC 6.2.1.2) described by Mahler et al. (1953).The enzyme with salicyl-CoA synthetase activity is more easily inactivated, migrates more slowly toward the anode during disc gel electrophoresis, and utilizes a wider spectrum of aromatic acid substrates than the enzyme described earlier. Evidence was obtained that both enzymes could catalyze the activation of hexanoate (preferred substrate), benzoate, *o*-methoxybenzoate, and anthranilate, but only the "salicylate" enzyme displayed activity with salicylate and *p*-aminosalicylate (Killenberg et al., 1971). Groot (1976) identified three soluble ATP-dependent acyl-CoA synthetases in guinea-pig liver mitochondria: (a) a medium-chain acyl-CoA synthetase, (b) a salicylate activating enzyme, and (c) a propionyl-CoA synthetase. A complete separation of these enzymes has been accomplished and the resulting preparation of propionyl-CoA synthetase accepted acetate, propionate and butyrate as substrates with a high preference for propionate (Groot, 1976).

Although much research has been done on several mammalian CoA-ligases (Fujino et al., 2001; Kasuya et al., 1996a; Killenberg et al., 1971; Knights and Drogemiller, 2000; Mahler et al., 1953; Schachter and Taggart, 1953), there is limited information in humans about the identity of individual xenobiotic CoA ligases responsible for the metabolism of a variety of drugs and chemicals (Knights and Drogemiller, 2000). The nomenclature of the enzymes is also very confusing and inconsistent. An attempt will be made here to clarify this situation.

Five medium chain xenobiotic activating enzymes have been described. These are *ACSM1*, *ACSM2A* and *ACSM2B*, *ACSM3*, *ACSM4* and *ACSM5* (Watkins et al., 2007). The ACSM gene family consists of four members located on chromosome 16p12 and 13 (ACSM1, 2, 3, 5) and the *ACSM4* gene on chromosome 12p3. Due to a chromosomal duplication event of approximately 80 kb the *ACSM2* gene exists in two loci, ACSM2A and ACSM2B (Loftus et al., 1999; Martin et al., 2004). The coding sequences of ACSM2A and ACSM2B are nearly identical with 98.8% nucleic acid identity. The biological function of ACSM2A and ASCM5 have not been characterised in any species (Watkins et al., 2007). Vessey and co-workers characterised two human liver medium chain ligases, HXMA and HXMB. HXMA showed preferential activity to benzoate while the preferred substrate for HXMB is hexanoate. There is evidence of activation of valproate by HXMA, while salicylate can be activated by both HXMA and HXMB. The substrate specificity for salicylate is, however, very low when compared to benzoate and hexanoate. Neither enzyme was effective at activating short-chain fatty acids as is shown by the very poor affinity of the enzymes for butyrate. A short-chain fatty acid:CoA ligase was also isolated, which had activity toward propionate and butyrate, but not acetate, hexanoate or benzoate (Vessey et al., 1999). ACSM2B is the predominant transcript identified in human liver when compared to ACSM1, 2A, 3 and 5 (Boomgaarden et al., 2009).

Vessey et al. (2003) incorrectly stated that a human (instead of a mouse) medium-chain acyl-CoA synthetase (MACS1) was previously identified and characterised (Fujino et al., 2001) which has kinetic properties similar to those of HXMB (Vessey et al., 2003). The murine MACS1 enzyme preferentially used octanoate as a substrate, while human HXM-B preferred hexanoate as substrate followed by octanoate. There is no substrate specificity information available in the literature on the human MACS1 (synonyms: ACSM1 and BUCS1). HXMA has been expressed in COS cells. Comparison of the HXMA sequence to the MACS1 sequence (supposedly similar to HXMB) reveals that they are distinct proteins, with 56.2% amino acid sequence homology (Vessey et al., 2003). This further indicates that HXMB is not ACSM2A because ACSM2A has 98% similarity to ACSM2B (Watkins et al., 2007).

According to BRENDA (www.brenda-enzymes.org) ACSM1 (synonyms: MACS1, BUCS1 and possible HXMB), ACSM2A (synonyms: MACS2, ACSM2) (Lindner et al., 2006) and ACSM2B (synonyms: HXMA, ACSM2, HYST1046) (Vessey et al., 2003) are all classified as human butyrate-CoA ligase (EC 6.2.1.2) enzymes. This results in some confusion as short-chain acyl-coenzyme A synthetases (ACSs) typically activate acetate, propionate or butyrate. Although there is overlap in chain length specificity between the short and medium chain ACS (Watkins et al., 2007), neither HXMA or HXMB is effective at activating short-chain fatty acids such as butyrate. ACSM2B (HXMA) has a clear substrate preference for benzoate (Vessey et al., 1999; Vessey et al., 2003), while it seems that ACSM1 (HXMB) preferably activates hexonate (Vessey et al., 1999). ACSM2B (HXMA) and ACSM1 (HXMB) can both activate salicylate but very poorly. This means that salicylate is clearly not the preferred substrate for either of these enzymes. The substrate specificity heterogeneity of the medium-chain fatty acid:CoA ligases complicates the understanding of pathways in which these enzymes participate. Therefore, care should be taken in future studies to clearly state which ACSM is studied, especially in the case of ACSM2A and 2B.

2.4.12.1.2. Interindividual variation and genetic polymorphisms of the *ACSM2B* gene

HXMA is encoded by the *ACSM2B* gene (Vessey et al., 2003; Watkins et al., 2007). ACSM2B is located on chromosome 16p12 on the reverse strand. It consists of just over 40 000 base pairs and contains 15 exons of which 13 are coding exons. There is a total of 1651 SNPs located in the *ACSM2B* gene of which 59 are synonymous and 168 are missense variants (www.ensembl.org, Sept 2014, ENST00000567001). No information is available on the influence of genetic variation on the enzyme activity of ACSM2B. A whole exome sequencing study on melanoma samples identified two missense SNPs (rs144594944 and rs148136861) within the *ACSM2B* gene that were not present in normal tissues. These two SNPs were, however, not recurrent mutations identified in melanoma (Wei et al., 2011).

2.4.12.2. Glycine N-acyltransferase (GLYAT) (Figure 2.2)

2.4.12.2.1. Biochemical and enzymatic characteristics of GLYAT

GLYAT is a monomeric detoxification enzyme found in the mitochondrial matrix of mammalian liver and kidney (Gregersen et al., 1986; Kelley and Vessey, 1990, 1994; Kelley and Vessey, 1993; Kolvraa and Gregersen, 1986; Lau et al., 1977; Matsuo et al., 2012; Mawal and Qureshi, 1994; Nandi et al., 1979; Schachter and Taggart, 1954a; van der Westhuizen et al., 2000; Vessey and Lau, 1998). GLYAT was first identified in bovine liver mitochondria in 1953 and subsequently isolated and characterised from human liver mitochondria in 1976 (Schachter and Taggart, 1953; Webster et al., 1976). GLYAT catalyses the transfer of an acyl group from an acyl-CoA to the amino group of glycine, forming an acylglycine and CoASH. Both products of the reaction are powerful inhibitors, and product inhibition is readily observed in enzyme assays (Kelley and Vessey, 1990; Schachter and Taggart, 1954b). Human GLYAT can use several endogenous and xenobiotic acyl-CoAs as substrates, as is evidenced by excretion of corresponding acylglycines in urine (Bartlett and Gompertz, 1974; Bonafe et al., 2000; Campbell et al., 1988; Gron et al., 1978; Huang et al., 1994; for review see Knights and Miners, 2012; Sakuma, 1991; Tanaka and Isselbacher, 1967).

Very little information is available on the kinetic parameters of human GLYAT (Gregersen et al., 1986; Kelley and Vessey, 1994; Mawal and Qureshi, 1994; van der Westhuizen et al., 2000; Webster et al., 1976). The apparent K_M (benzoyl-CoA) value is reported to range from 13 µM to 57.9 mM, and the V_{max} value using benzoyl-CoA and glycine is reported to range from 543 nmol/min/mg to 17.1 µmol/min/mg . This large variation in reported values is difficult to explain, but differences in the method of kinetic analysis, substrate quality, enzyme quality, experimental technique, and perhaps genetic heterogeneity of the *GLYAT* gene may be responsible (Webster et al., 1976). The molecular mass of human GLYAT has been reported as 24, 27, 30, and 30.5 kDa (Kelley and Vessey, 1994; Mawal and Qureshi, 1994; van der Westhuizen et al., 2000; Webster et al., 1976). This variation in reported values may be partly explained by the different techniques used in the different studies. For example, bovine GLYAT bound to the gel filtration matrix, which resulted in erroneous molecular mass estimates (Kelley and Vessey, 1990).

No structure has been reported for GLYAT, thus little is known about structure-function relationships. However, GLYAT is a member of the GNAT (Gcn5-related N-acetyltransferase) superfamily. Because of the remarkable structural conservation in the GNAT superfamily, a molecular model of bovine GLYAT could be generated by homology modelling. The model was used to propose that Glu^{227}, a highly conserved residue, is catalytically important (Figure 2.7). Kinetic characterisation and pH profiling of an E227Q mutant demonstrated that Glu^{227} acts as a general base that deprotonates glycine before nucleophilic attack on the carbonyl of the acyl-CoA thioester (Badenhorst et al., 2012).[1]

[1]Erratum: In Badenhorst et al., (2012) it is reported that Glu^{226} of bovine GLYAT is the catalytic residue. However, this residue was incorrectly numbered and should be Glu^{227} of the bovine GLYAT reference sequence (NP_803479).

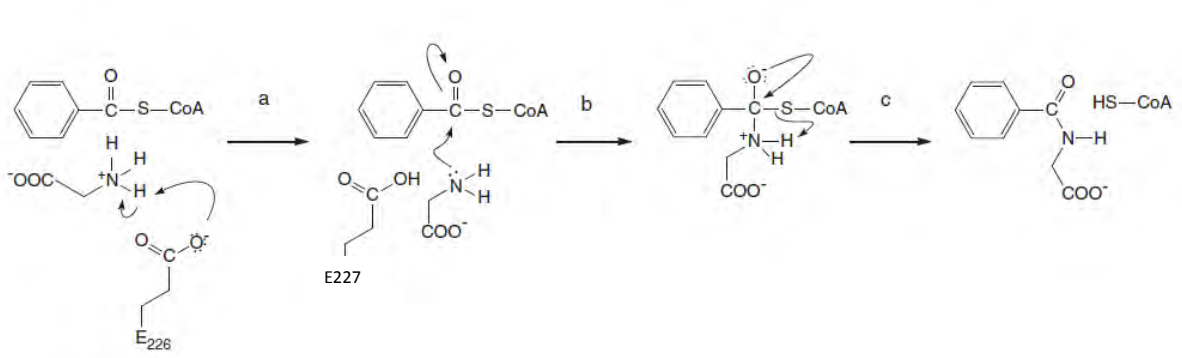

Figure 2.7: A schematic representation of the general base-catalyzed, ternary-complex mechanism proposed for bovine GLYAT. A, for nucleophilic attack to occur, the glycine amino group must be deprotonated by Glu227. B, a tetrahedral intermediate is formed after nucleophilic attack by the amino group of glycine on the thioester carbonyl group. C, finally, the tetrahedral intermediate collapses, forming the peptide product and CoA. ChemDraw 10.0 (CambridgeSoft, Cambridge, MA) was used to produce this schematic (Badenhorst., et al 2012 with permission).

2.4.12.2.2. Substrate specificity

Human GLYAT can use several endogenous and xenobiotic acyl-CoAs as substrates, as is evidenced by excretion of corresponding acylglycines in urine (Bartlett and Gompertz, 1974; Bonafe et al., 2000; Campbell et al., 1988; Gron et al., 1978; Huang et al., 1994; for review see Knights and Miners, 2012; Sakuma, 1991; Tanaka and Isselbacher, 1967). Benzoyl-CoA is the preferred substrate for the orthologs of GLYAT that have been isolated from the livers and kidneys of several mammals, including rats, rabbits, sheep, cows, rhesus monkeys, and humans (Gregersen et al., 1986; Kelley and Vessey, 1990, 1994; Kelley and Vessey, 1993; Kolvraa and Gregersen, 1986; Mawal and Qureshi, 1994; Nandi et al., 1979; Schachter and Taggart, 1954a; Webster et al., 1976). Several other acyl-CoAs, including salicylyl-CoA, 4-aminobenzoyl-CoA, hexanoyl-CoA, and isovaleryl-CoA, can act as acyl donor substrates, but much less efficiently (Badenhorst et al., 2012; Bartlett and Gompertz, 1974; Kolvraa and Gregersen, 1986; Mawal and Qureshi, 1994; Nandi et al., 1979). While the preferred acyl-acceptor substrate for GLYAT is always glycine, the amino acids asparagine, glutamine, glutamate, alanine, serine, and threonine can also be used, but at low rates that seem to be irrelevant under normal physiological conditions (van der Westhuizen et al., 2000; Webster et al., 1976). This variation in substrate specificity might be explained by genetic variations present in GLYAT.

2.4.12.2.3. The *GLYAT* gene and genetic variation

The human *GLYAT* gene is located on chromosome 11 at position 11q12, spans 23 200 base pairs, and contains 6 exons. Two splice variants of human GLYAT mRNA exist, coding for isoforms a (296 residues) and b (162 residues). The transcript for isoform b does not contain exon 6, and there is no protein level evidence for the existence of isoform b. Within the *GLYAT* gene there are 815 known SNPs (www.ensembl.org, Sept 2014,

ENST00000344743), of which 20 are synonymous and 76 are non-synonymous. Only two studies, on relatively small groups of Japanese and French Caucasian individuals, have reported on novel genetic polymorphisms and allele frequencies of SNPs in human GLYAT (Lino Cardenas et al., 2010; Yamamoto et al., 2009). The N156S variant had allele frequencies of 97% and 85% in the French Caucasian and Japanese populations, respectively. Because of this high frequency, it was suggested that the N156S allele, rather than the reference sequence (NM_201648.2), should be considered the wild-type allele (Lino Cardenas et al., 2010; Yamamoto et al., 2009). The 1000 genome sequencing project (Abecasis et al., 2012) and HapMap project (Smith, 2008) have also characterised genetic variations within the *GLYAT* gene. It is not yet known whether genetic variation in the *GLYAT* gene influences the overall rate of glycine conjugation *in vivo*.

Very little is known about the influence of genetic variation on the glycine conjugation pathway. Interindividual variation in responsiveness to administration of glycine and benzoate, respectively, was observed in both isovaleric acidemia and hyperammonemia (Dercksen et al., 2012; Itoh et al., 1996; Sweetman and Williams, 2013; Vest and Salzberg, 1965). For example, variation in the clinical outcome and responsiveness to glycine supplementation was observed in a group of South African isovaleric acidemia patients, all homozygous for the same isovaleryl-CoA dehydrogenase mutation (Dercksen et al., 2012). It was suggested that interindividual variation in GLYAT activity may partly account for this.

2.4.12.2.4. Paralogs of the human *GLYAT* gene

GLYAT is one of four putative glycine conjugating enzymes. Two GLYAT-like genes, *GLYAT-L1* and *GLYAT-L2*, are located with the *GLYAT* gene on chromosome 11q12.1, while the *GLYAT-L3* gene is located on chromosome 6p12.3 (Matsuo et al., 2012; Waluk et al., 2010; Zhang et al., 2007a). In addition to GLYAT, primates have another transferase that conjugates arylacetyl-CoAs to glutamine, forming phenylacetylglutamine and indoleacetylglutamine (Kelley and Vessey, 1994; Webster et al., 1976). The *GLYAT-L1* gene codes for the glutamine conjugating enzyme in humans (Matsuo et al., 2012). Both mitochondrial and cytoplasmic localisation of GLYAT-L1 have been reported, and this could be explained by the two alternative splice variants of GLYAT-L1 mRNA, which code for two isoforms (333 and 302 amino acid residues respectively) with distinct N-termini and possibly different subcellular localisation (Webster et al., 1976; Zhang et al., 2007a). The 302 residue isoform of GLYAT-L1 is located in the cytoplasm and transcriptionally activates the heat shock factor pathway in HEK293T cells (Zhang et al., 2007a). The two isoforms might thus have different functions in the mitochondria and cytoplasm (Matsuo et al., 2012; Webster et al., 1976; Zhang et al., 2007a). It has not been investigated why primates, unlike other mammals, conjugate arylacetates to glutamine instead of glycine.

GLYAT-L2 mRNA is expressed in salivary gland, trachea, spinal cord, and skin fibroblasts. The enzyme is localised to the endoplasmic reticulum, and a recombinant GLYAT-L2 catalyses the formation of long chain acylglycines such as N-arachidonoylglycine and N-oleoylglycine (Waluk et al., 2010; Waluk et al., 2012). These are members of a class of cannabinoid-like signalling hormones that activate G-protein-coupled receptors and have anti-nociceptive, anti-inflammatory, and anti-proliferative effects (Bradshaw and Walker, 2005). GLYAT-

L2 activity is regulated by acetylation on Lys^{19}, and mutation of Lys^{19} of a recombinant GLYAT-L2 to arginine or glutamine resulted in a 70 to 80% decrease in enzyme activity (Waluk et al., 2012). No enzyme activity has been reported for GLYAT-L3, which does not seem to have the catalytic glutamate residue proposed for the GLYAT reaction mechanism, but the significance of this is unclear (Badenhorst et al., 2012; Waluk et al., 2010).

2.4.12.2.5. GLYAT, liver cancer and hepatitis

Investigation of hepatocellular carcinoma specimens demonstrated that GLYAT expression is transcriptionally regulated. Complete down regulation of transcription of the *GLYAT* gene was observed in 32 of 41 hepatocellular carcinoma specimens investigated, with significant down regulation in the other nine specimens (Matsuo et al., 2012). This was confirmed by immunohistochemistry using a GLYAT specific antibody, which revealed that GLYAT is not expressed in cancerous cells, but is expressed in neighbouring healthy hepatocytes. Interestingly, GLYAT expression was found to be significant and similar in all noncancerous liver specimens studied, including sixty samples from patients with chronic hepatitis of various aetiologies (Matsuo et al., 2012). This may be explained by the dependence of rapidly proliferating cancer cells on the availability of glycine, which is needed for nucleic acid synthesis (Jain et al., 2012). The down-regulation of GLYAT expression in hepatocellular carcinoma could therefore be a strategy to conserve glycine, which is needed for DNA synthesis. Hippurate can significantly inhibit tumour growth, which might be another reason why GLYAT expression is down-regulated in cancer cells (Spustova and Oravec, 1989). Furthermore, hippurate has recently been identified in metabolomics studies as important for the prediction of cancer recurrence, with decreased hippurate levels correlating to recurrence of oral cancer (Xie et al., 2012; Ye et al., 2012).

It has been reported that the fraction of 4-aminobenzoate excreted as glycine conjugates correlates well to functional hepatic reserves in patients with hepatitis. Therefore, the measurement of glycine conjugation of 4-aminobenzoate has been proposed as a liver function test (Duffy et al., 1995; Furuya et al., 1995; Lebel et al., 2003). GLYAT expression is normal in hepatitis specimens and it has been suggested that the lower glycine conjugation observed for hepatitis patients could be explained by impaired hepatic β-oxidation and lower availability of ATP for ligation of benzoate to CoASH (for review see Knights et al., 2007; Matsuo et al., 2012).

2.4.12.2.6. GLYAT, glycine and musculoskeletal development

A well-known consequence of glycine shortage is decreased turnover of collagen, an important structural protein. Although decreased collagen turnover is not lethal, it leads to accumulation of damage to connective tissues such as the skin, thereby accelerating the ageing process, especially in long-lived animals such as humans (Melendez-Hevia et al., 2009; Wu et al., 2013). In addition to influencing collagen turnover, glycine shortage seems to be related to the growth and development of the musculoskeletal system in several complicated ways. It was recently proposed that the *GLYAT* gene may be involved in determining lean muscle mass and bone size in humans (Guo et al., 2013). Approximately 690 000 SNPs were analysed in large groups of unrelated Han Chinese (1627) and American Caucasian (2286) individuals, to search for variations in the

genome that correlate to variation in lean muscle mass and bone size. Fourteen SNPs with significant correlation were identified, three of which are located in or near the *GLYAT* gene (rs2507838, rs7116722, and rs11826261). Unfortunately, the effect of these genetic variations are not known, but it has been suggested that the influence of GLYAT on glycine metabolism could explain this observation (Guo et al., 2013).

Glycine is commonly considered a non-essential amino acid because it can be synthesised from serine (Lewis et al., 2005; Melendez-Hevia et al., 2009). However, studies over the past two decades have shown that glycine is in fact a semi-essential amino acid, that humans may have a daily shortage of about 10 grams of glycine and that this may impact on collagen turnover and the synthesis of bile acids, creatine, glutathione, and haem (Jackson, 1991; Lewis et al., 2005; Melendez-Hevia et al., 2009; Oates and West, 2006). Under certain conditions, GLYAT can conjugate sufficient amounts of glycine to limit its availability for other metabolic processes (Loots et al., 2005; Piper et al., 1973; van der Westhuizen et al., 1999). For example, it has been shown that administration of benzoate to rats can reverse chemically induced porphyria by diverting glycine away from haem biosynthesis. This results in normalisation of urinary δ-aminolevulinate, porphobilinogen, and porphyrin levels, an effect cancelled out by co-administration of glycine (Oates and West, 2006; Piper et al., 1973). The recently reported correlation of SNPs in and near the *GLYAT* gene to variation in lean muscle mass and bone size could be explained, in part, by the impact of GLYAT on the availability of glycine for the synthesis of creatine, collagen, and elastin (Guo et al., 2013; Lewis et al., 2005; Melendez-Hevia et al., 2009). It is interesting that apart from the normal expression of GLYAT in liver and kidney, low levels of GLYAT expression has also been observed in skeletal muscle, but the significance of this observation is unclear (Matsuo et al., 2012).

CHAPTER 3:

GENERATION OF A RECOMBINANT HUMAN GLYAT AND THE ANALYSES OF THE INFLUENCE OF GENETIC VARIATIONS ON THE ENZYME ACTIVITY OF A RECOMBINANT GLYAT

PREFACE

Interindividual variation in the glycine conjugation pathway needs further investigation because previous studies do not discriminate between variation in acyl-CoA formation and variation in glycine conjugation. It is important to remember that glycine conjugation is a two-step process and that the overall rate of glycine conjugation can be influenced by several factors (Reviewed in Chapter 2). Given the difficulty in obtaining human liver tissues for research, the expression and characterisation of a recombinant human GLYAT enzyme and its variants can make an important contribution to our understanding of variation in glycine conjugation.

A previous study in our laboratory generated a recombinant bovine GLYAT, which was subsequently used to characterise the enzymatic activity and catalytic mechanism of the recombinant bovine GLYAT enzyme (Badenhorst et al., 2012). However, the pColdIII bacterial expression system and purification methods used in this study were not effective in generating a soluble active recombinant human GLYAT. This necessitated the identification and optimisation of an expression system and purification method that would result in adequate quantities of soluble active recombinant human GLYAT in order to characterise the influence of genetic variants on the enzyme activity of human GLYAT *in vitro*.

Several expression systems were investigated and included: i) the mammalian expression vector pcDNA3.1(-) (Invitrogen, LifeTechnologies, Carlsbad, California); ii) the bacterial expression vector pColdIII (TAKARA, Shiga, Japan) together with several different chaperones (Takara, Madison, WI); iii) the bacterial expression vector pMAL-c5X (New England Biolabs) and iv) the bacterial expression vector pET32a(+) (Novagen, Darmstadt, Germany). For the mammalian expression system, no expression of human GLYAT could be detected with western blot analyses in the CHO-K1 cells, using an anti-GLYAT polyclonal antibody. This mammalian expression system was then abandoned in favour of a bacterial expression system. With the pColdIII bacterial expression system together with two different chaperones, namely pG-Tf2 (which expresses groES-groEL-tig) and pTf16 (which expresses tig) insoluble GLYAT protein was obtained. Expression of human GLYAT as a maltose binding protein (MBP) fusion protein also did not yield soluble GLYAT protein. Finally, co-expression of MBP/hGLYAT with the chaperones groEL and groES resulted in a soluble enzymatically active human GLYAT. The soluble human GLYAT protein was purified using amylose magnetic beads, but the purification step could not successfully be optimised to exclude all of the contaminating proteins. For the pET32a(+) expression system several factors were then optimised which included: the expression medium, the Isopropyl β-D-1-

thiogalactopyranoside (IPTG) concentration, culture temperature, lysis buffer, lysis method, co-expression of chaperones and purification methods used. The optimised expression protocol (discussed in Paper III) resulted in an optimal protein yield of approximately 900 µg soluble enzymatically active human GLYAT protein per 50 ml culture. The level of expressed soluble human GLYAT was affected by the freshness of the glycerol stock. Increased expression was observed when starting a culture from a newly transformed colony. The trx-tag fused to the human GLYAT protein could not be removed as this resulted in precipitation of the human GLYAT protein. Soluble enzymatically active human GLYAT could be generated using both the pMal and pET bacterial expression systems. There was no difference in the expression level of both recombinant human GLYAT fusion proteins: i) MBP-hGLYAT and ii) trx-His-hGLYAT. The purification of the MBP-hGLYAT fusion protein with the amylose beads resulted in co-purification of the chaperones GroEL and GroES as well as other contaminating proteins. The Ni-affinity purification of the trx-His-hGLYAT fusion protein resulted in a single purified protein. It was therefore difficult to compare the level of relative enzyme activity between MBP-hGLYAT and trx-His-hGLYAT as equal amounts of protein would not contain equal amounts of the two different recombinant human GLYAT fusion protein molecules.

The influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase was investigated and the results are presented and discussed in Paper III below.

> *It should be noted that, although there are currently 76 known non-synonymous polymorphisms within GLYAT, there were only six known polymorphisms when this study commenced in 2011. So at that time all known non-synonymous SNPs were included in the study.*

Paper III

Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase

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Short Communication

Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase

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ABSTRACT

Human glycine N-acyltransferase (human GLYAT) detoxifies a wide range of endogenous and xenobiotic metabolites, including benzoate and salicylate. Significant inter-individual variation exists in glycine conjugation capacity. The molecular basis for this variability is not known. To investigate the influence of single nucleotide polymorphisms (SNPs) in the GLYAT coding sequence on enzyme activity, we expressed and characterised a recombinant human GLYAT. Site-directed mutagenesis was used to generate six non-synonymous SNP variants of the enzyme (K16N; S17T; R131H; N156S; F168L; R199C). The variants were expressed, purified, and enzymatically characterised. The enzyme activities of the K16N, S17T and R131H variants were similar to that of the wild-type, whereas the N156S variant was more active, the F168L variant less active, and the R199C variant was inactive. We also generated an E227Q mutant, which lacks the catalytic residue proposed by Badenhorst et al. (2012). This mutant was inactive compared to the wild-type recombinant human GLYAT. A molecular model of human GLYAT containing coenzyme A (CoA) was generated which revealed that the inactivity of the R199C variant could be due to the substitution of the highly conserved Arg^{199} and destabilisation of an α-loop-α motif which is important for substrate binding in the GNAT superfamily. The finding that SNP variations in the human GLYAT gene influence the kinetic properties of the enzyme may explain some of the inter-individual variation in glycine conjugation capacity, which is relevant to the metabolism of xenobiotics such as aspirin and the industrial solvent xylene, and to the treatment of some metabolic disorders.

1. Introduction

Detoxification is an essential physiological process as it serves to decrease the toxicity of compounds that are not catabolised (Liska, 1998). These compounds include several endogenous metabolites such as steroid hormones and exogenous toxins such as compounds in food or industrial chemicals (Campbell et al., 1988). Detoxification is generally divided into two phases of which phase I detoxification activates metabolites by adding functional groups to them. The activated compounds are often more toxic than the original compounds and are rapidly acted on by phase II detoxification systems (Kinzig-Schippers et al., 2005). These reactions include methylation and conjugation to sulfate, glucorunide and glycine. The purpose of these conjugation reactions is to make the compounds less toxic and more soluble for excretion in the urine and bile (Jakoby and Ziegler, 1990; Liska, 1998; Swinney et al., 2006).

Impaired phase II detoxification has been associated with adverse reactions to pharmaceutical drugs and may be involved in the pathogenesis of complex multifactorial diseases like cancer (Nebert et al., 1996; Wallig, 2004). Glycine N-acyltransferase (EC 2.3.1.13, GLYAT) is a phase II detoxification enzyme found in mitochondria of mammalian liver and kidney (Nandi et al., 1979; Schachter and Taggart, 1954). GLYAT is a member of the Gcn5-related N-acetyltransferase (GNAT) superfamily of N-acyltransferases which uses an acyl-CoA and glycine as substrates (Schachter and Taggart, 1954; Vetting et al., 2005). The products of the reaction are free CoA and an acylglycine that is less toxic and more readily excreted by the kidneys than the original compound (Bartlett and Gompertz, 1974). Little is currently understood about the physiological significance of glycine conjugation and the implications of its impairment (Bartlett and Gompertz, 1974; Campbell et al., 1988; Gregersen et al., 1986; Kolvraa and Gregersen, 1986; Tanaka and Isselbacher, 1967).

A large number of metabolic disorders, collectively named CASTOR disorders (coenzyme A sequestration, toxicity, and redistribution), influence the metabolism of CoA (Mitchell et al., 2008; Ogier and Saudubray, 2002). One of the primary mechanisms of pathogenesis in CASTOR disorders is depletion of free CoA. This depletion is caused by the accumulation of acyl-CoAs, which in turn leads to a depletion of free carnitine, as acylcarnitines are excreted in the urine. Conjugation of accumulating acyl-CoAs to glycine normalises cellular metabolism as levels of free CoA and carnitine are restored (Bartlett and Gompertz, 1974; Mitchell et al.,

Abbreviations: CASTOR, coenzyme A sequestration, toxicity, and redistribution; CoA, coenzyme A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); human GLYAT, human glycine N-acyltransferase; GNAT, Gcn5-related N-acetyltransferase; SNP, single nucleotide polymorphism.

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Table 1
Parameters for GLYAT isolated from human liver.

Parameters	Values	Reference
Protein size (kDa)	27	van der Westhuizen et al. (2000)
	30	Mawal and Qureshi (1994)
	30.5	Kelley and Vessey (1994)
	33.9	Based on sequence NM_201648.2
K_M (benzoyl-CoA) (μM)	13	van der Westhuizen et al. (2000)
	67 ± 5	Kelley and Vessey (1994)
	209	Matsuo et al. (2012)
	57900	Mawal and Qureshi (1994)
V_{max} (nmol/min/mg)	543 ± 21	van der Westhuizen et al. (2000)
	807	Matsuo et al. (2012)
	17100	Mawal and Qureshi (1994)

2008; Sakuma, 1991; Tanaka and Isselbacher, 1967). For example, in isovaleric acidemia, isovaleryl-CoA accumulates to toxic levels. Since isovaleryl-CoA is a good substrate for GLYAT, glycine supplementation enhances excretion of isovalerylglycine, making it a very effective therapy (Dercksen et al., 2012; Tanaka and Isselbacher, 1967; Tanaka et al., 1966).

Xenobiotics which are detoxified by conjugation to glycine include benzoate, salicylate, and methyl-benzoate, a metabolite of the industrial solvent xylene (Levy, 1965; Tremblay and Quereshi, 1993). Competition between different substrates for glycine conjugation is demonstrated by the reduced excretion of salicylurate and methyl-hippurate when aspirin and xylene are co-administered to healthy individuals (Campbell et al., 1988). Significant inter-individual variability in glycine conjugation capacity has been demonstrated using human liver samples (Temellini et al., 1993). There is also large variation in the amount of glycine conjugates excreted after administering benzoate to different individuals (Campbell et al., 1988). No work to date has explained this inter-individual variation in glycine conjugation capacity, but substrate availability and levels of GLYAT expression may be important factors (Matsuo et al., 2012; Tanaka and Isselbacher, 1967). It is not known at present whether known non-synonymous variations in the GLYAT gene may account for some of this variability (Lino Cardenas et al., 2010; Yamamoto et al., 2009).

The enzymatic characteristics of GLYAT have been studied using enzymes isolated from the liver and kidney of several mammals (Bartlett and Gompertz, 1974; Gregersen et al., 1986; Kelley and Vessey, 1993, 1994; Kolvraa and Gregersen, 1986; Mawal and Qureshi, 1994). The values reported for the molecular mass, apparent Michaelis constant (K_{Mapp}), and maximum velocity of human liver GLYAT, are highly variable (Table 1). This variation in values is unexplained, but factors like substrate quality and/or concentration, experimental technique, and genetic heterogeneity of the GLYAT gene may be responsible (Kolvraa and Gregersen, 1986; Lino Cardenas et al., 2010; Yamamoto et al., 2009).

Recently, there have been several reports on the expression, purification, and partial enzymatic characterisation of recombinant GLYATs. A recombinant human GLYATL2 (GLYAT-like 2) was shown to be an acyltransferase that produces long-chain acylglycines, precursors to cannabinoid-like signalling hormones. It was also demonstrated that human GLYATL2 is regulated by reversible acetylation of Lys^{19} (Waluk et al., 2010, 2012). Site-directed mutagenesis revealed that Glu^{226} is the catalytically important residue of a recombinant bovine GLYAT (Badenhorst et al., 2012). Most recently, a recombinant human GLYAT was expressed, purified, and partially characterised (Matsuo et al., 2012). In the work we present here, we used site-directed mutagenesis to investigate the influence of SNPs in the human GLYAT gene on the enzymatic properties of a bacterially expressed recombinant human GLYAT.

2. Methods

2.1. Generation of human GLYAT variants

The human GLYAT reference sequence (GenBank ID: NM_201648.2), cloned into a pET32a(+) expression vector, was purchased from GeneArt (Piscataway, NJ). Mutations of the GLYAT coding sequence, to generate the desired GLYAT variants (Table 2), were introduced using the Phusion Site-Directed Mutagenesis kit (Finnzymes, Vantaa, Finland). The following mutagenic oligonucleotides were employed: **K16N**, CTG CAG ATG CTG GAG AAT TCC TTG AGG AAG; **S17T**, CAG ATG CTG GAG AAA ACC TTG AGG AAG AGC; **K20R**, TCC TTG AGG CGG AGC CTC CCA; **K20Q**, TCC TTG AGG CAG AGC CTC CCA; **R131H**, GTC AAA CAA ACA CAA CAC ATT CTC TAT ATG GCA; **N156S**, AAG ATT TTA TCT CCC AGT GGT GGC AAA CCC AAG; **F168L**, ATC AAC CAA GAG ATG TTA AAA CTC TCA TCC ATG; **R199C**, CAG AGA TTC ATT GAG TGC TGC ATT CAG ACC TTT; and **E227Q**, ATG GAC CAG ACT GGA CAG ATG AGA ATG GCA. All oligonucleotides are in the standard 5′→3′ orientation, and were purchased from IDT (Coralville, Iowa). All constructs were Sanger sequenced to confirm introduction of the various mutations.

2.2. Expression and nickel-affinity purification of recombinant human GLYAT and selected variants

All proteins were expressed with an N-terminal His-tag to facilitate purification. Expression of the recombinant human GLYAT variants was carried out as follows. Purified plasmid DNA was used to transform Origami™ cells (Novagen, Madison, WI) already containing the pGro7 chaperone expression plasmid (Takara, Madison, WI). An expression medium containing 2% bacto-tryptone, 1.25% yeast extract, 0.625% NaCl, 0.5% $Na_2HPO_4 \cdot 12H_2O$, 0.1% KH_2PO_4, and 0.2% glucose was used. Colonies of co-transformed Origami™ cells were used to inoculate 10 ml cultures in expression medium containing 50 μg/ml ampicillin, 20 μg/ml chloramphenicol, and 2 mg/ml L-arabinose. The cultures were incubated overnight at 37 °C. Cells were harvested by centrifugation at 4000 ×*g* for 5 min and resuspended in 100 ml of the same antibiotic containing medium. All centrifugation steps were performed at 4 °C. The cultures were further incubated at 37 °C until they reached an optical density of 0.4 at 600 nm. The cultures were then transferred to 28 °C for 24 h. Cells were harvested by centrifugation at 4000 ×*g* for 15 min. Cells were resuspended in 10 ml of a buffer, pH 8.0, containing 50 mM NaH_2PO_4, 300 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and

Table 2
Variants of human GLYAT investigated in this study.

Recombinant human GLYAT variants		Allele frequencies (%)	
Amino acid change from wild-type	Accession number or RS number	95 Japanese subjects (Yamamoto et al., 2009)	55 Caucasian subjects (Lino Cardenas et al., 2010)
Wild-type	NM_201648.2		
K16N	rs17850556	Not detected	Not detected
S17T	rs10896818	27.4	16.4
R131H	rs117149346	0.5	Not detected
N156S	rs675815	85.3	97.3
F168L	rs1045359	Not detected	Not detected
R199C	rs138125182	Not detected	0.9
E227Q	Novel variant	n/a	n/a

10 μl of Lysonase Bioprocessing Reagent (Novagen, Madison, WI). The resuspended cells were incubated at room temperature for 10 min before being disrupted by five passes through a 22 G needle. The lysates were cleared by centrifugation at 14 000 ×*g* for 25 min and passed through Protino Ni-TED 2000 columns equilibrated with buffer LEW (Macherey-Nagel Inc., Bethlehem, PA). Columns were washed with 10 ml of buffer LEW before eluting the bound proteins with 3 ml of buffer EB (Macherey-Nagel Inc., Bethlehem, PA). Eluates were immediately desalted on 10 ml columns of Sephadex G25 (Sigma, St. Louis, MO), pre-equilibrated with 25 mM Tris–HCl, pH 8.0. Glycerol was added to 12.5% (v/v), followed by concentration to approximately 1 ml using Vivaspin 6 ultra-filtration devices (GE Healthcare, Björkgatan, Uppsala, Sweden). Expression and purification procedures were monitored by means of SDS-PAGE and Coomassie brilliant blue staining (Laemmli, 1970). Protein concentrations were determined based on the bicinchoninic acid method (Smith et al., 1985) using a kit from Sigma-Aldrich (Sigma, St. Louis, MO). To confirm that the purified proteins contained equal amounts of human GLYAT, western blot analyses were performed using an anti-GLYAT polyclonal antibody (Abcam, Cambridge, MA).

2.3. Enzyme assays for determination of relative activities and K_{Mapp} values

The relative activities of the recombinant human GLYAT variants were determined using a colourimetric assay that measures glycine dependent release of CoA at 412 nm, in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Kolvraa and Gregersen, 1986). Enzyme assays were generally 100 μl in volume, and contained 50 mM Tris-acetate, pH 8.0, 200 mM glycine, 100 μM DTNB, 200 μM benzoyl-CoA, and 0.5 μg of a particular recombinant human GLYAT variant. Reactions were carried out at 37 °C in 96-well plates and monitored for 10 min using a BioTek plate reader and accompanying Gen5 software (BioTek, Winooski, VT). Activities from triplicate assays were calculated from the linear change at 412 nm over 10 min, and expressed as nmol/min/mg. For estimation of V_{max} and apparent K_M (benzoyl-CoA) values, similar conditions were used, except that the concentration of benzoyl-CoA was varied from 10 μM to 100 μM in a 200 μl reaction, containing 1.5 μg of a particular recombinant human GLYAT variant. Initial velocities were calculated over the first 2.5 min, where the reaction was linear even for the assays where a low concentration of benzoyl-CoA was used. Data of six replicate assays were used to estimate kinetic parameters by nonlinear regression, using SigmaPlot 12.0 (Systat software, Inc., San Jose, CA).

2.4. Molecular modelling and sequence analyses

To investigate the possible structural significance of the amino acid variations investigated in this study, molecular modelling was used. A molecular model of human GLYAT, containing a molecule of CoA, and a multiple alignment of sequences with high homology to human GLYAT, were generated as described previously (Badenhorst et al., 2012). The unusually high degree of structural homology in the GNAT superfamily of enzymes (Dyda et al., 2000; Vetting et al., 2005) allowed an approximate model of human GLYAT to be generated, using template structure 1SQH. The use of this model is discussed by Badenhorst (Badenhorst et al., 2012). UCSF Chimera (Pettersen et al., 2004) was used to generate molecular graphics and to investigate positions on the model of residues significant to this study.

3. Results and discussion

3.1. Wild-type human GLYAT and selected variants: constructs and expression

In this study the catalytic effects of selected amino acid substitutions on the activity of a recombinant human GLYAT were investigated. The variants studied included six known polymorphisms K16N, S17T, R131H, N156S, F168L, and R199C. An E227Q mutant lacking the proposed GLYAT catalytic residue (Badenhorst et al., 2012), was also investigated. Wild-type recombinant human GLYAT and the selected variants (Table 2) were expressed from pET32a(+) in a soluble form as Trx–His-fusions of approximately 56 kDa. The thioredoxin (Trx) fusion protein is a highly soluble polypeptide (Sachdev and Chirgwin, 1998) used to enhance the production of soluble, enzymatically active human GLYAT. The expression of soluble, enzymatically active recombinant human GLYAT could be improved by lowering the temperature of expression to 28 °C and by not using isopropyl-1-thio-β-D-galactopyranoside to induce expression. The recombinant enzymes were highly purified by means of nickel-affinity chromatography (Fig. 1) and could be stored at 4 °C for at least one week without loss of enzyme activity.

3.2. Relative activities

To investigate the relative activities of the wild-type human GLYAT and selected variants, 0.5 μg of each enzyme was assayed using substrate concentrations where V_{max} could be measured (200 μM benzoyl-CoA and 200 mM glycine) (Nandi et al., 1979). Relative activities of the variants, in nmol/min/mg, were calculated from the 10 min period where the reaction proceeded linearly (Fig. 2). The wild-type recombinant human GLYAT had activity comparable to the K16N, S17T, and R131H variants. The N156S variant showed an increase in enzyme activity when compared to the wild-type GLYAT, while the F168L variant showed a decreased enzyme activity. Both the R199C and E227Q variants of recombinant human GLYAT were virtually inactive, having less than 5% of the activity of the wild-type. To verify that each variant was assayed using the same amount of recombinant GLYAT protein, SDS-PAGE and western blot analyses were performed on samples diluted to equal protein concentration (Fig. 1). The relative enzyme activity shown in Fig. 2 was consistently observed using independently expressed and purified batches of enzymes.

3.3. Kinetic parameters

The recombinant human GLYAT variants were further compared by determining V_{max} values and K_{Mapp} (benzoyl-CoA) values. Lineweaver–Burk plots are shown in Fig. 3, and the results are summarised in Table 3. The V_{max} values range from approximately 500 nmol/min/mg to 1200 nmol/min/mg, and the K_{Mapp} (benzoyl-CoA) values range from approximately 20 μM to 70 μM (Table 3).

The kinetic parameters reported in the literature for human GLYAT vary substantially. The K_{Mapp} (benzoyl-CoA) is reported to be between 13 μM and 57.9 mM, and the V_{max} value between 543 nmol/min/mg and 17100 nmol/min/mg (Table 1) (Bartlett and Gompertz, 1974; Gregersen et al., 1986; Kelley and Vessey, 1993, 1994; Kolvraa and Gregersen, 1986; Mawal and Qureshi, 1994; van der Westhuizen et al., 2000). The V_{max} values we determined for the recombinant human GLYAT variants fall within the range reported in the literature. The K_{Mapp} (benzoyl-CoA) values we determined for the recombinant human GLYAT variants fall on the lower end of the range in the literature, being comparable to the values of 13 μM (van der Westhuizen et al., 2000) and 67 μM (Kelley and Vessey, 1994). It is difficult to explain the large variation in literature values, but differences in the quality of enzyme preparations studied may be partly responsible. The exact experimental conditions used in previous reports are unclear, making interpretation difficult.

The recombinant enzyme characterised by Matsuo et al. (2012) is the N156S variant. They report a K_M (benzoyl-CoA) of 209 μM (Matsuo et al., 2012). This value is much higher than the value of 38 μM we determined for the N156S variant (Table 3). This difference in K_{Mapp} value obtained for the N156S variant could be due to significant differences in the reaction composition, substrate concentrations used, quality of purified protein preparations used, and data analysis.

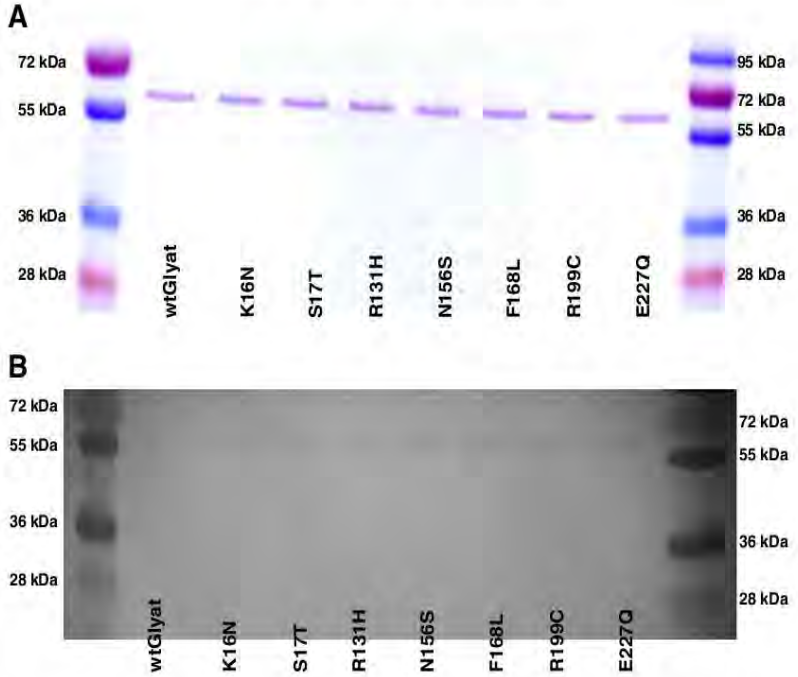

Fig. 1. 10% SDS-PAGE (A) and western blot analyses (B) of the purified protein samples of the wild-type human GLYAT and the selected variants. Lane 1: protein ladder – 28 kDa, 36 kDa, 55 kDa, and 72 kDa; Lane 2: wild type human GLYAT; Lane 3: K16N; Lane 4: S17T; Lane 5: R131H; Lane 6: N156S; Lane 7: F168L; Lane 8: R199C; Lane 9: E227Q and Lane 10: protein ladder – 28 kDa, 36 kDa, 55 kDa, 72 kDa, and 95 kDa. The purified recombinant proteins are visible as a band of approximately 56 kDa.

When comparing the kinetic parameters of the wild-type to that of the variants, the wild-type, K16N, and S17T variants had comparable K_{Mapp} (benzoyl-CoA) values (24 μM, 21 μM, and 28 μM, respectively). The R131H variant had an increased K_{Mapp} (benzoyl-CoA) value of about 71 μM. Interestingly, the N156S variant had a V_{max} value (1230 nmol/min/mg) greater than that of the wild-type enzyme (730 nmol/min/mg), despite having a higher K_{Mapp} (benzoyl-CoA) value of 38 μM. The F168L variant also had an increased K_{Mapp} value of about 53 μM, but a decreased V_{max} value of 500 nmol/min/mg. The R199C substitution resulted in less than 5% of wild-type activity, and its K_M (benzoyl-CoA) could not be determined.

The differences in V_{max} and K_{Mapp} (benzoyl-CoA) values reported in this study, for variants of recombinant human GLYAT, may partly explain the significant variation in glycine conjugation capacity that has been observed between human liver samples (Temellini et al., 1993). However, since Temellini and co-workers investigated the formation of hippurate from benzoate, ATP, and glycine, using hepatocyte homogenates, there may also have been other factors, such as variation in the rate of benzoyl-CoA formation, that influenced the rates of glycine conjugation. The inter-individual variation in urinary acylglycine excretion may similarly be accounted for, in part, by the different properties we report here for human GLYAT variants (Campbell et al., 1988; Sakuma, 1991).

The clinical variability of certain inborn errors of organic acid metabolism may be influenced by inter-individual differences in glycine conjugation capacity. This is demonstrated by the variation in clinical presentation of a large group of South African isovaleric acidemia patients, all having the same disease-causing mutation (Dercksen et al., 2012). Because of its critical importance in the management of

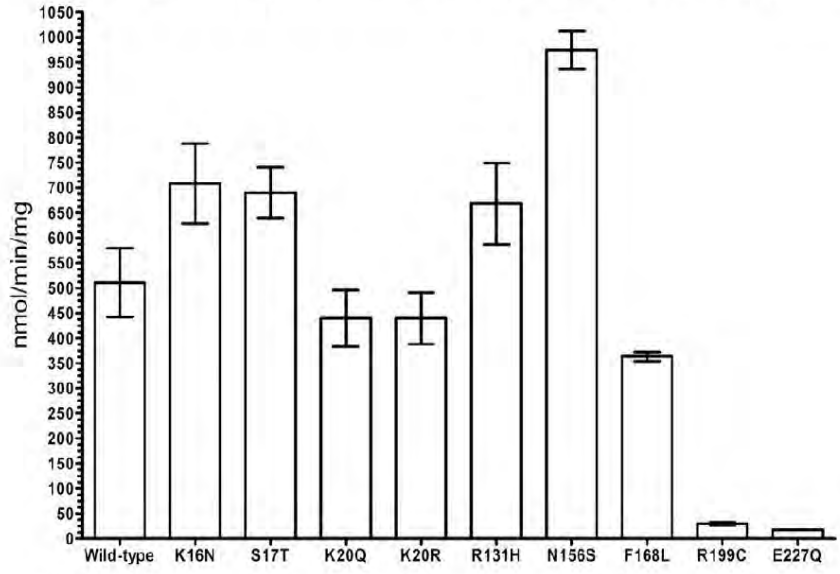

Fig. 2. Relative enzyme activities of recombinant human GLYAT and selected variants. Enzymes (0.5 μg) were assayed at 37 °C using 200 μM benzoyl-CoA and 200 mM glycine. Error bars indicate the mean ± standard deviation for triplicate assays.

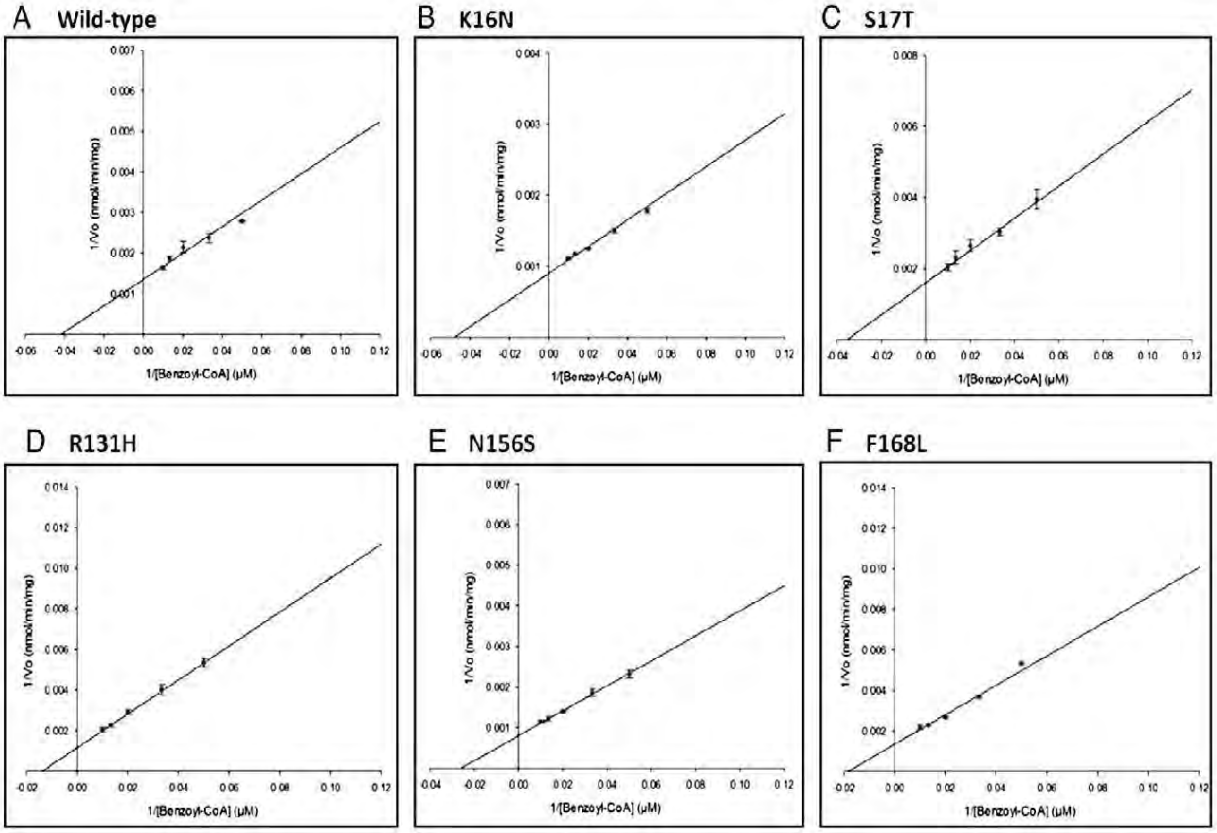

Fig. 3. Lineweaver–Burk plots for determination of K_{Mapp} (benzoyl-CoA) and V_{max} values at 200 mM glycine. Error bars indicate the mean ± standard deviation for six replicate assays. Benzoyl-CoA concentrations used were 20 uM, 30 uM, 50 uM, 75 uM, and 100 uM. A) Wild-type recombinant human GLYAT; B–F) K16N, S17T, R131H, N156S, and F168L recombinant human GLYAT variants.

isovaleric acidemia, variation in glycine conjugation capacity is thought to impact on clinical presentation of the disease, and this needs to be further investigated (Bartlett and Gompertz, 1974; Dercksen et al., 2012; Tanaka and Isselbacher, 1967). Another question that needs to be addressed is whether variants of human GLYAT have different substrate specificities, which would also help explain why some patients with isovaleric acidemia excrete more glycine conjugates than others (Dercksen et al., 2012).

It has been proposed that the fraction of a dose of para-aminobenzoate, excreted as glycine conjugates (hippurates), be used as a convenient test of liver function. It was demonstrated that there exists a correlation between this hippurate ratio and the extent of liver damage, with a lower hippurate ratio indicating more severe damage (Duffy et al., 1995). Based on the known inter-individual variation in glycine conjugation capacity, and the different kinetic parameters of the GLYAT variants reported in this study, we suggest that tests of liver function based on the hippurate ratio be interpreted with caution.

Table 3
Kinetic parameters obtained for recombinant human GLYAT variants.

Variant	K_{Mapp} (benzoyl-CoA) μM[a]	V_{max} (nmol/min/mg)[a]
Wild-type	24 ± 3	730 ± 30
K16N	21 ± 1	1030 ± 20
S17T	28 ± 5	665 ± 40
R131H	71 ± 11	1040 ± 85
N156S	38 ± 4	1230 ± 60
F168L	53 ± 6	500 ± 30

[a] Values are reported as mean ± standard deviation for six replicates.

3.4. Molecular model and sequence analyses

Previously, a molecular model of bovine GLYAT was generated, which suggested that the highly conserved Glu^{226} residue was catalytically important, and this was supported by investigation of an E226Q mutant (Badenhorst et al., 2012). We constructed, expressed, and purified the equivalent E227Q recombinant human GLYAT. This variant had no enzyme activity (Fig. 2). A molecular model of human GLYAT, based on the model reported for bovine GLYAT (Badenhorst et al., 2012), was constructed to help explain some of the differences between the human GLYAT variants investigated in this study. Locations of the Arg^{131}, Asn^{156}, Phe^{168}, and Arg^{199} residues of human GLYAT are indicated on the model (Fig. 4A). Residues in contact with these residues (Asp^{48}, Leu^{242} and Thr^{244}) are also indicated. Conservation of selected residues is also indicated on part of a multiple sequence alignment (Fig. 4B).

The R131H substitution removes a positively charged residue from a binding cavity on the model, perhaps explaining its increased K_{Mapp} value for the negatively charged benzoyl-CoA (Fig. 3 and Table 3). The F168L substitution alters the highly conserved Phe^{168}, which on the model is in contact with the well conserved Leu^{242}, found with Thr^{244} on the first turn of an alpha helix important for acyl-CoA binding in the GNAT superfamily. The Thr^{244} residue is in contact with the CoA pyrophosphate moiety, a very common feature of GNAT enzymes (Dyda et al., 2000; Vetting et al., 2005). This may explain the high K_{Mapp} (benzoyl-CoA) and low V_{max} values obtained for the F168L variant (Fig. 3 and Table 3). The inactivity of the R199C variant may be explained by the presence of the highly conserved Arg^{199} in an α-loop-α motif, where it is in contact with the highly conserved, negatively charged Asp^{48}. Substitution of Arg^{199} may destabilise this motif, which is important for substrate binding in the GNAT superfamily

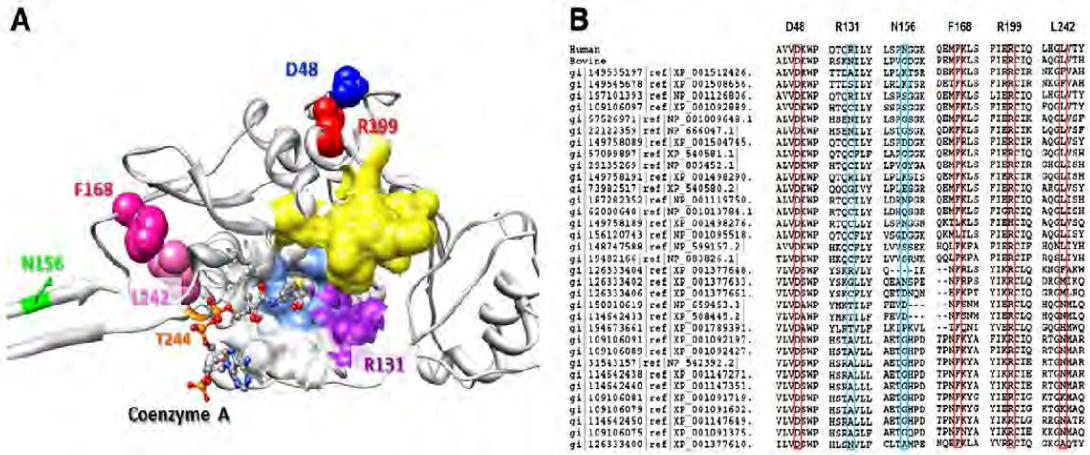

Fig. 4. A molecular model and multiple sequence alignment of human GLYAT to visualise selected residues mutated in this study. A) The molecular model of human GLYAT, with bound CoA. Arg^{131} (in purple) forms part of a putative substrate binding cavity (light blue). Asn^{156} (green) is located on a poorly modelled loop. Phe^{168} (dark pink) is in contact with Leu^{242} (lighter pink), on the same helix as Thr^{244} (orange), which is in contact with the pyrophosphate of CoA. Arg^{199} (red) is in contact with Asp^{48} (blue). Arg^{199} is part of an α-loop-α motif important for substrate binding (yellow loop). UCSF Chimera was used to generate this image. B) A multiple alignment of sequences homologous to human GLYAT. Accession numbers are shown to the left of the alignment. The Arg^{131} and Asn^{156} residues are not well conserved. The Phe^{168} residue and the Leu^{242} residue are well conserved. The Arg^{199} and Asp^{48} residues are also well conserved. ClustalX 2.0.12 was used to align sequences.

(Dyda et al., 2000; Vetting et al., 2005). On the model, Asn^{156} is on a poorly predicted loop from Lys^{159} to Met^{167}, making interpretation of its role in enzyme activity difficult (Fig. 4A). However, it has been suggested that residues 130 to 180 of human GLYAT may be important in substrate binding (Matsuo et al., 2012), which could perhaps explain why the N156S variant had altered K_{Mapp} (benzoyl-CoA) and V_{max} values.

3.5. Allele frequencies of selected variants

The allele frequencies of the SNPs for S17T, R131H, N156S, and R199C human GLYAT have been investigated in small Japanese and French Caucasian populations (Lino Cardenas et al., 2010; Yamamoto et al., 2009). There seems to be some interesting correlations between the allele frequencies reported for these variants and the kinetic parameters we determined. The altered kinetic parameters of R131H, F168L and R199C (Fig. 3 and Table 3) may explain the low frequencies of these alleles (Table 2). In contrast, the allele frequencies for the N156S variant were 85% and 97% (Table 2). Based on the higher activity (Fig. 2) and higher allele frequency of the N156S variant, it appears that choosing the GLYAT sequence from GenBank (NM_201648.2, Table 2) as a reference sequence for a typical wild-type human GLYAT, may be erroneous. It was also suggested by Lino Cardenas et al. (2010) that the N156S variant is the wild-type allele in the French Caucasian population.

3.6. Regulation of human GLYAT activity

Amino acid substitutions in the GLYAT sequence can influence mitochondrial import and perhaps regulation of enzyme activity (Stadtler et al., 2005; Waluk et al., 2012). The activity of human GLYATL2, a paralogue of human GLYAT, is regulated by reversible acetylation on Lys^{19}. Enzyme activities of K19Q and K19R mutants of GLYATL2 were decreased by about 80%, demonstrating the importance of this residue (Waluk et al., 2012). To investigate whether human GLYAT activity is regulated similarly, the corresponding K20Q and K20R mutants of GLYAT were expressed, purified, and assayed as for the other variants in this study. These mutants had activity comparable to the wild-type GLYAT, indicating that human GLYAT is not similarly subject to regulation by modification of this residue (Fig. 2).

3.7. Conclusion

By using a recombinant human GLYAT we demonstrated that SNP variations found in the human GLYAT gene may result in altered kinetic properties of the enzyme. These differences may help explain some of the observed inter-individual variation in glycine conjugation capacity, which is relevant to the treatment of some metabolic disorders and to the metabolism of xenobiotics such as aspirin and the industrial solvent xylene.

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References

Badenhorst, C.P.S., Jooste, M., van Dijk, A.A., 2012. Enzymatic characterization and elucidation of the catalytic mechanism of a recombinant bovine glycine N-acyltransferase. Drug Metab. Dispos. 40, 346–352.
Bartlett, K., Gompertz, D., 1974. The specificity of glycine-N-acylase and acylglycine excretion in the organicacidaemias. Biochem. Med. 10, 15–23.
Campbell, L., Wilson, H.K., Samuel, A.M., Gompertz, D., 1988. Interactions of m-xylene and aspirin metabolism in man. Br. J. Ind. Med. 45, 127–132.
Dercksen, M., et al., 2012. Clinical variability of isovaleric acidemia in a genetically homogeneous population. J. Inherit. Metab. Dis. 2012, 17.
Duffy, L.F., Kerzner, B., Seeff, L., Barr, S.B., Soldin, S.J., 1995. Preliminary assessment of glycine conjugation of para-aminobenzoic acid as a quantitative test of liver function. Clin. Biochem. 28, 527–530.
Dyda, F., Klein, D.C., Hickman, A.B., 2000. GCN5-related N-acetyltransferases: a structural overview. Annu. Rev. Biophys. Biomol. Struct. 29, 81–103.
Gregersen, N., Kolvraa, S., Mortensen, P.B., 1986. Acyl-CoA: glycine N-acyltransferase: in vitro studies on the glycine conjugation of straight- and branched-chained acyl-CoA esters in human liver. Biochem. Med. Metab. Biol. 35, 210–218.
Jakoby, W., Ziegler, D., 1990. The enzymes of detoxification. J. Biol. Chem. 265, 20715–20718.
Kelley, M., Vessey, D.A., 1993. Isolation and characterization of mitochondrial acyl-CoA: glycine N-acyltransferases from kidney. J. Biochem. Toxicol. 8, 63–69.
Kelley, M., Vessey, D., 1994. Characterization of the acyl-CoA: amino acid N-acyltransferases from primate liver mitochondria. J. Biochem. Toxicol. 9, 153–158.
Kinzig-Schippers, M., et al., 2005. Should we use N-acetyltransferase type 2 genotyping to personalize isoniazid doses? Antimicrob. Agents Chemother. 1733–1738.
Kolvraa, S., Gregersen, N., 1986. Acyl-CoA:glycine N-acyltransferase: organelle localization and affinity toward straight- and branched-chained acyl-CoA esters in rat liver. Biochem. Med. Metab. Biol. 36, 98–105.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Levy, G., 1965. Pharmacokinetics of salicylate elimination in man. J. Pharm. Sci. 54, 959–967.
Lino Cardenas, C.L., et al., 2010. Genetic polymorphisms of glycine N-acyltransferase (GLYAT) in a French Caucasian population. Xenobiotica 40, 853–861.
Liska, D.J., 1998. The detoxification enzyme systems. Altern. Med. Rev. 3, 187–198.

Matsuo, M., et al., 2012. Designation of enzyme activity of glycine-N-acyltransferase family genes and depression of glycine-N-acyltransferase in human hepatocellular carcinoma. Biochem. Biophys. Res. Commun. 420, 901–906.
Mawal, Y.R., Qureshi, I.A., 1994. Purification to homogeneity of mitochondrial acyl CoA: glycine n-acyltransferase from human liver. Biochem. Biophys. Res. Commun. 205, 1373–1379.
Mitchell, G.A., Gauthier, N., Lesimple, A., Wang, S.P., Mamer, O., Qureshi, I., 2008. Hereditary and acquired diseases of acyl-coenzyme A metabolism. Mol. Genet. Metab. 94, 4–15.
Nandi, D.L., Lucas, S.V., Webster Jr., L.T., 1979. Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine N-acyltransferase from bovine liver mitochondria. Purification and characterization. J. Biol. Chem. 254, 7230–7237.
Nebert, D., McKinnon, R., Puga, A., 1996. Human drug-metabolizing enzyme polymorphisms: effects on risk of toxicity and cancer. DNA Cell Biol. 15, 273–280.
Ogier, D.B., Saudubray, J.M., 2002. Branched-chain organic acidurias. Semin. Neonatol. 7, 65–74.
Pettersen, E.F., et al., 2004. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612.
Sachdev, D., Chirgwin, J.M., 1998. Solubility of proteins isolated from inclusion bodies is enhanced by fusion to maltose-binding protein or thioredoxin. Protein Expr. Purif. 12, 122–132.
Sakuma, T., 1991. Alteration of urinary carnitine profile induced by benzoate administration. Arch. Dis. Child. 66, 873–875.
Schachter, D., Taggart, J.V., 1954. Glycine N-acylase: purification and properties. J. Biol. Chem. 208, 263–275.
Smith, P.K., et al., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.
Stadtler, S.C., et al., 2005. Mitochondrial targeting signals and mature peptides of 3-methylcrotonyl-CoA carboxylase. Biochem. Biophys. Res. Commun. 939–946.
Swinney, R., Hsu, S., Tomlinson, G., 2006. Phase I and phase II enzyme polymorphisms and childhood cancer. J. Investig. Med. 54, 303–320.
Tanaka, K., Isselbacher, K.J., 1967. The isolation and identification of N-isovalerylglycine from urine of patients with isovaleric acidemia. J. Biol. Chem. 242, 2966–2972.
Tanaka, K., Budd, M.A., Efron, M.L., Isselbacher, K.J., 1966. Isovaleric acidemia: a new genetic defect of leucine metabolism. Proc. Natl. Acad. Sci. U. S. A. 56, 236–242.
Temellini, A., Mogavero, S., Giulianotti, P.C., Pietrabissa, A., Mosca, F., Pacifici, G.M., 1993. Conjugation of benzoic acid with glycine in human liver and kidney: a study on the interindividual variability. Xenobiotica 23, 1427–1433.
Tremblay, G., Quereshi, I., 1993. The biochemistry and toxicology of benzoic acid metabolism and its relationship to the elimination of waste nitrogen. Pharmacol. Ther. 60, 63–90.
van der Westhuizen, F.H., Pretorius, P.J., Erasmus, E., 2000. The utilization of alanine, glutamic acid, and serine as amino acid substrates for glycine N-acyltransferase. J. Biochem. Mol. Toxicol. 14, 102–109.
Vetting, M.W., et al., 2005. Structure and functions of the GNAT superfamily of acetyltransferases. Arch. Biochem. Biophys. 433, 212–226.
Wallig, M.A., 2004. Glucuronidation and susceptibility to chemical carcinogenesis. Toxicol. Sci. 1–2.
Waluk, D.P., Schultz, N., Hunt, M.C., 2010. Identification of glycine N-acyltransferase-like 2 (GLYATL2) as a transferase that produces N-acyl glycines in humans. FASEB J. 24, 2795–2803.
Waluk, D.P., Sucharski, F., Sipos, L., Silberring, J., Hunt, M.C., 2012. Reversible lysine acetylation regulates the activity of human glycine n-acyltransferase-like 2 (hGLYATL2): implications for production of glycine-conjugated signalling molecules. J. Biol. Chem. 287 (20) (May 11 2012), 16158–16167.
Yamamoto, A., Nonen, S., Fukuda, T., Yamazaki, H., Azuma, J., 2009. Genetic polymorphisms of glycine N-acyltransferase in Japanese individuals. Drug Metab. Pharmacokinet. 24, 114–117.

CHAPTER 4:

CHARACTERISATION AND HAPLOTYPE ANALYSES OF NON-SYNONYMOUS POLYMORPHISMS IN THE GLYCINE N-ACYLTRANSFERASE GENE USING DATA FROM WORLDWIDE POPULATIONS

Paper IV – Submitted manuscript

Conservation of the coding regions of the glycine N-acyltransferase gene and its implications for the detoxification of xenobiotics in humans

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Title Page

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Short title: Conservation of glycine N-acyltransferase

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Abstract

Thorough investigation of the glycine conjugation pathway has been neglected over the last 30 years. Environmental factors, nutrition, and the chronic use of medications are increasing the exposure of humans to benzoate and drugs that are metabolized to acyl-CoA intermediates. To date no defect of the glycine conjugation pathway has been reported and this, together with the fact that glycine N-acyltransferase (GLYAT) plays an important role in hepatic metabolism, suggests that this pathway is essential for survival. We hypothesised that the genetic variation in the open reading frame (ORF) of the *GLYAT* gene should be low and that deleterious alleles will be found at low frequencies. This hypothesis was investigated by analysing the genetic variation data of the human *GLYAT* ORF available on public databases. We also sequenced the coding region of a small cohort of South African Afrikaner Caucasian individuals. In the GLYAT ORF of the 1537 individuals analysed, only two haplotypes, S_{156} and $T_{17}S_{156}$, were identified in all populations as having the highest haplotype frequencies (70% and 20% respectively). The $S_{156}C_{199}$ and $S_{156}H_{131}$ haplotypes, which have a deleterious effect on the enzyme activity of a recombinant human GLYAT, were detected at very low frequencies. The results of this study indicated that the *GLYAT* ORF is conserved and supports the hypothesis that the glycine conjugation pathway is an essential detoxification pathway. These findings underline the importance of future investigations to determine the *in vivo* capacity of the glycine conjugation pathway for the detoxification of benzoate and other xenobiotics.

Key Words

Glycine N-acyltransferase; conserved open reading frame; detoxification; xenobiotics; salicylic acid

1. Introduction

It is becoming increasingly evident that an impaired ability to excrete xenobiotics is an important factor contributing to multi-factorial diseases such as systemic lupus erythematosus, chronic fatigue syndrome and Parkinson's disease (Bandmann et al., 1997; Daly et al., 1993; Haiser & Turnbaugh, 2013; Kawajiri et al., 1990; Meyer, 1990; Nebert et al., 1991; Nebert et al., 2013; Ritchie et al., 1980; Tang & Hazen, 2014). Several detoxification systems exist, which convert xenobiotics to more hydrophilic conjugates that can be excreted in the urine (for review see Badenhorst et al., 2014; for review see Knights et al., 2007; Wallig, 2004). Hippurate, the glycine conjugate of benzoate, is a hydrophilic metabolite excreted in the urine of humans and other mammals. After urea and creatinine, hippurate is often the most abundant metabolite found in urine, at concentrations of up to 932.66 µmol/mmol creatinine (Lees et al., 2013). Hippurate is the product of metabolic interaction between a mammalian host and the microorganisms inhabiting its gastrointestinal tract (Lees et al., 2013; Phipps et al., 1998; Wikoff et al., 2009). In humans, abnormalities of the gut microbiota are associated with disorders such as Crohn's disease (Williams et al., 2010), autism (MacFabe et al., 2007), diabetes (Schooneman et al., 2013), obesity (Caricilli and Saad, 2014), and cancer (Rabot et al., 2010). Interestingly, these disorders are also associated with abnormal urinary excretion of hippurate (reviewed in (Lees et al., 2013)). Therefore, the microbial and mammalian metabolic pathways resulting in hippurate biosynthesis are of considerable medical interest (Manach et al., 2004). Recently interest in glycine conjugation has increased, due to the emerging clinical relevance of glycine conjugation after research in this field had been neglected for several decades (Badenhorst et al., 2014; Badenhorst et al., 2013; Beyoglu and Idle, 2012; Lees et al., 2013).

Glycine N-acyltransferase (GLYAT, EC 2.3.1.13) is responsible for the glycine conjugation of xenobiotics (Nandi et al., 1979; Schachter and Taggart, 1954; Tanaka and Isselbacher, 1967) such as benzoic acid, salicylic acid and several endogenous metabolites such as isovaleric acid (Bartlett and Gompertz, 1974; Campbell et al., 1988). Xenobiotics that undergo glycine conjugation are activated to an acyl-CoA by the mitochondrial medium chain ligases (Killinberg et al., 1971; Knights, 1998). The microorganisms in the mammalian gut metabolise dietary polyphenols to simple aromatic acids such as phenylpropionate and benzoate. These lipophilic compounds are absorbed by the gut and transported to the liver, where they are metabolised to benzoyl-CoA. The constant exposure of the liver to aromatic acids can lead to the sequestration of hepatic coenzyme A (CoASH) as xenobiotic acyl-CoAs. GLYAT prevents CoASH sequestration by catalysing the conversion of benzoyl-CoA to hippurate and CoASH. Since CoASH sequestration would negatively impact hepatic metabolism by inhibiting processes such as β-oxidation, lipogenesis, and other important metabolic pathways, it is clear that GLYAT plays a central role in maintaining hepatic homeostasis (Badenhorst et al., 2014).

In addition to natural sources of benzoate, the wide use of benzoate as a preservative in food and pharmaceuticals is increasing the exposure of humans to benzoate (Piper, 1999). In some populations where the consumption of preserved food is high, benzoate intake can be as high as 280% of the recommended daily allowance. This is reflected by unusually high levels of urinary hippurate (Lees et al., 2013; Tfouni and Toledo, 2002). Benzoic acid and sodium benzoate are generally recognized as safe in foods according to the U.S. Food

and Drug Administration (Nair, 2001). However, several studies have demonstrated the genotoxicity of benzoate (Abe and Sasaki, 1977; Demir et al., 2010; Fabisch and Fellner, 1957; Yilmaz et al., 2009; Zengin et al., 2011). Interindividual variation in glycine conjugation can result in individuals experiencing different toxic effects of the same compound. Very little is currently known regarding the effect of genetic variation in GLYAT on the *in vivo* glycine conjugation rate.

The human *GLYAT* gene is located on chromosome 11 at position 11q12. It spans 23 200 base pairs and contains six exons. Within the *GLYAT* gene there are 815 known single nucleotide polymorphisms (SNPs) (www.ensembl.org, September 2014, ENST00000344743), of which 76 are non-synonymous. Only two studies, on small cohorts of Japanese and French Caucasian individuals, have reported on novel genetic polymorphisms and allele frequencies of SNPs in the *GLYAT* gene (Lino Cardenas et al., 2010; Yamamoto et al., 2009). In both these studies the N156S variant was the dominant allele, with frequencies of 85% and 97% in the Japanese and French Caucasian populations, respectively. Because of the high allele frequency of the N156S allele, Lino Cardenas *et al.* suggested that the N156S variant, rather than the reference sequence (NM_201648.2), should be regarded as the wild-type. We subsequently demonstrated that an N156S variant of a recombinant human GLYAT had higher enzyme activity than a recombinant human GLYAT encoded by the reference sequence (NM_201648.2) (van der Sluis et al., 2013), supporting the suggestion that the N156S variant is the wild-type human GLYAT. We also demonstrated that some variants negatively influence GLYAT enzyme activity by decreasing substrate affinity (R131H) or decreasing catalytic rate (F168L and R199C) (van der Sluis et al., 2013).

Interestingly, no defect of the glycine conjugation pathway in humans has been reported to date. Due to the important role of GLYAT in hepatic metabolism, we postulated that the *GLYAT* gene is essential for survival and must therefore be well conserved. Genetic variation in the human *GLYAT* gene and its consequences are thus of considerable interest. Here, we investigated this hypothesis by analysing the data on genetic variation in the human *GLYAT* ORF available in public databases. The data from the 1000 Genomes Project, the HapMap Project, and the Khoi-San/Bantu sequencing project was used. Since very few studies have focused on the analyses of genetic variation of a particular gene within the Afrikaner population (Heathfield et al., 2013; Kruse et al., 2009; Rodriguez-Murillo et al., 2014; Xu et al., 2012; Xu et al., 2011; Xu et al., 2009), we also started to investigate the genetic variation in the *GLYAT* gene of a small group of Afrikaner Caucasian individuals by sequencing the coding regions of their *GLYAT* genes. There is no data available for the *GLYAT* gene in the Afrikaner Caucasian population and this group is of particular interest as several founder mutations have been identified in the Afrikaner Caucasian population due to the dramatic population growth of the European settlers at the Cape since 1672 (Abecasis et al., 2004; Reeves et al., 2004; Roos et al., 2009; Tipping et al., 2001).

This is the first worldwide population analysis of the non-synonymous SNPs within GLYAT as well as the first study to report on the genetic variation found within the *GLYAT* gene of a South African Afrikaner Caucasian population.

2. Materials & Methods

2.1. GLYAT sequences obtained from public databases

Nucleic acid sequences for the coding region of GLYAT were obtained from the following public databases: i) the 1000 Genome Project (Abecasis et al., 2012), ii) the HapMap Project (Smith, 2008), iii) the Khoi-San/Bantu Sequencing Project (May et al., 2013) and primate sequences (Table 1).

The complete genome sequence for four Khoi-San individuals and one Bantu individual from southern Africa (May et al., 2013) is available on the Sequence Read Archive (SRA) database (www.ncbi.nlm.nih.gov/sra). The exome sequencing reads for the *GLYAT* gene were downloaded and analysed using CLC Genomic Workbench 7 to identify non-synonymous SNPs.

Non-human primate GLYAT sequence data for *Pan troglodytes*, *Pan paniscus*, *Gorillagi* and *Macaca mulatta* were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/). These sequences represented the out-group in the phylogenetic analyses.

Table 1: Population data used for the present study

Source (Reference)	Population	Description	n[1]
Present study	SA	South African Caucasian Afrikaner population	61
1000 Genomes Project [50]	AFR	African	246
	EUR	European	379
	ASN	East Asian	286
	AMR	Ad Mixed American	181
HapMap project [51]	CEU	Utah residents with Northern and Western European ancestry	180
	CHB	Han Chinese in Beijing China	90
	JPT	Japanese in Tokyo, Japan	91
	YRI	Yoruba in Ibadan, Nigeria	18
Khoi-San/Bantu sequencing project [52]	KB1, NB1, TK1 and MD8	Indigenous Namibian hunter-gatherers	4
	ABT	Bantu	1
Primate NCBI database (http://www.ncbi.nlm.nih.gov/)	*Pan troglodyte, Pan paniscus, Gorillagi* and *Macaca mulatta*	Common chimpanzee, Pygmy chimpanzee, Gorilla and Rhesus Macaque	5

[1] *Number of individuals in the test sample*

2.2. South African Afrikaner Caucasian population samples

Ethical approval (NWU-0096-08-A1) was obtained from the Ethics Review Board of the North-West University, South Africa prior to sample collection. Sequencing data from 61 healthy unrelated South African Afrikaner Caucasian volunteers from a pilot study was included, as this group is not represented on any of the public databases. The volunteers were from the Potchefstroom area in the North-West province of South-Africa. Written informed consent was obtained from all participants. An example of the consent form is given in Appendix C.

2.3. PCR amplification and Ion Torrent semiconductor sequencing of the coding region of the human *GLYAT* gene

An 8 ml sample of blood was drawn from each individual. Genomic DNA was isolated from these whole blood samples using the Flexigene DNA isolation kit (QIAGEN, Valencia, CA, USA). Primer sets for PCR used to amplify the 5' flanking and coding regions of the human *GLYAT* gene were used as previously described by Yamamoto et al. (2009). These amplification reactions were performed in a final volume of 50 µL using 100 ng template DNA, 1x HF Phusion buffer (pH 8), 2.5 mM of each deoxynucleotide (dNTP), 5% (v/v) DMSO, 0.5 µM of each primer and 1 U of Phusion high-fidelity polymerase (Finnzymes). For all PCR reactions an initial denaturation step at 95°C for 10 min was performed followed by denaturation at 95°C for 30s and annealing of the primers at the respective T_m values for 30s. Annealing was followed by an extension step at 72°C for 1 min and amplicons were generated for 35 cycles, followed by a final elongation step of 5 min at 72°C. The amplicons were prepared for sequencing by compiling a sample for each individual containing equimolar amounts of each DNA fragment (150 femtomole).

Sequencing libraries were prepared using the Ion Plus Fragment Library kit and Ion Express™ Barcode adapters. Libraries were combined and a sequencing template was prepared using the Ion PGM™ Template OT2 kit and the Ion OneTouch system. Sequencing was performed on an Ion PGM™ sequencer using the Ion PGM™ Sequencing 200 Kit and Ion 314 chip with a flow number of 520. The sequencing data obtained was analysed using VariantCaller version 3.0, which is part of the Torrent Suite™ software, together with CLC Genomic Workbench 7 to identify polymorphisms. The SNPs identified with Ion PGM™ sequencing were validated using Sanger sequencing.

2.4. Identification and phasing of haplotypes

The 1000 Genomes Project used the SHAPEIT2 (Delaneau et al., 2012; Delaneau et al., 2013) software programme for haplotype phasing of the variant genotypes. In this study, the Arlequin ver 3.5 programme (Excoffier et al., 2005) was used to perform haplotype phasing of the 1000 Genomes data, the South African Afrikaner data and the Bantu and Khoisan data. Since the *GLYAT* gene is located on the reverse strand, all variations and sequence data reflect the negative ('-' or "reverse") strand. Currently, no method to estimate gametic phase appears to be uniformly superior in all scenarios (Excoffier et al., 2003).

2.5. Phylogenetic analyses

MEGA version 6.0 (Hall, 2013; Tamura et al., 2013) was used to perform the phylogenetic analyses. Representative amino acid sequences for each haplotype within each population were included in the dataset to reduce the phylogenetic complexity. Before analyses, the amino acid sequences were aligned using MUSCLE (Edgar, 2004) and maximum likelihood fits for 48 different amino acid substitution models were performed to find the best protein model (in this case the Jones Taylor Thornton model (Jones et al., 1992)) to use for the construction of the phylogenetic tree. The robustness of the tree topology was evaluated by bootstrap analysis using a resampling size of 1000 replicates. The tree was rooted using the GLYAT sequence of the non-human primate *Macaca mulatta*.

2.6. Tajima's Neutrality Test

Tajima's D test is a statistical test to distinguish between a DNA sequence evolving randomly and one evolving under a non-random process such as directional selection (Fu and Li, 1993; Simonsen et al., 1995; Tajima, 1989). MEGA version 6.0 (Hall, 2013; Tamura et al., 2013) was used to calculate the Tajima's D value on the amino acid alignment of all the human GLYAT sequences from all the populations. The analyses involved 2317 amino acid sequences. All positions containing gaps and missing data were eliminated.

3. Results and Discussion

The nucleotide variation of xenobiotic metabolising enzymes needs to be characterised to identify variants that play a role in xenobiotic response (Mortensen et al., 2011). The glycine conjugation pathway is an essential detoxification pathway that removes toxic acyl-CoA intermediates and plays an important role in maintaining adequate levels of free CoASH (Badenhorst et al., 2014; Badenhorst et al., 2013). The aim of this study was to identify the non-synonymous SNPs in the *GLYAT* gene using public available datasets as well as a new South African Afrikaner Caucasian cohort. The genetic variation was characterised within populations and haplotypes were determined in order to facilitate future genotype-phenotype association studies.

3.1. Inter-population data analyses

3.1.1. The 1000 Genomes Project data

The 1000 Genomes Project sequenced the complete genomes of 1092 individuals of African, Ad Mixed American, East Asian and European decent (Table 1) (Abecasis et al., 2012). In this study we used the data generated by the 1000 Genomes Project to analyse the allele frequencies of the 13 non-synonymous SNPs identified in the human *GLYAT* gene (Table 2). The N156S polymorphism had the highest homozygous genotype frequency of 89.9%, followed by S17T (4.6%) and R131H (0.1%). The remaining 10 non-synonymous SNPs were only detected as heterozygotes. Inspection of the genotype frequencies within the different population groups (Table 2) revealed that most of the variants were found in only one of the four population

groups. The N36S polymorphism was only present in the African population; M65T, N85K and N85T in the European population; T73I, R199C and T244M in the East Asian population; and A251T and P295L in the American population.The exception was M272T that was found in both the American and East Asian population. We previously showed that the R131H and R199C variants negatively impact on the enzyme kinetics of a recombinant human GLYAT (van der Sluis et al., 2013). The R131H variant was identified as a homozygote in one East Asian individual and previously (Yamamoto et al., 2009) as a heterozygote in one individual out of 95 Japanese individuals. The R199C variant was detected once in a heterozygous East Asian individual's genome and was also previously observed in one heterozygote out of 55 French Caucasian individuals (Lino Cardenas et al., 2010).

3.1.2. The HapMap Project dataset

The allele frequencies of the S17T, N156S, and F168L polymorphisms, genotyped by the HapMap project for 379 individuals, are summarised in Table 3. In all the populations the N156S variant had the highest allele frequency ranging between 81% and 100% (Table 3). The S17T variant had the second highest allele frequency in all populations, ranging from 13% to 22%. These allele frequencies are similar to those determined using the 1000 Genomes Project data (Table 2). The F168L SNP was submitted to the dbSNP database (www.ncbi.nlm.nih.gov/SNP) on the 13 September 2000 but has not been validated or reported since. Here, the F168L variant was not identified in any of the other populations studied, but was one of the non-synonymous SNPs genotyped in the HapMap project (Thorisson et al., 2005). All individuals were genotyped to be 100% homozygous for the wild-type allele (Table 3).

3.1.3. The Khoisan and Bantu dataset

Individuals from the southern African region have been under represented with regard to whole genome sequencing projects, while northern and central Africans have been well studied in both the HapMap and 1000 Genomes Projects. Limited reference data is available for the south-eastern Bantu speaking individuals (May et al., 2013). Schuster et al. (2010) sequenced the genomes of four indigenous Namibian hunter-gatherers referred to as KB1, NB1, TK1 and MD8 respectively, as well as the Bantu individual, Archbishop Desmund Tutu (ABT) (Schuster et al., 2010).

Table 2: Non-synonymous SNPs and genotype frequencies identified in the 1000 Genome sequencing data of 1092 individuals within the coding region of GLYAT

SNP	Genotype frequency (%) of all 1092 individuals			Genotype frequency (%) of the different population groups											
				African (AFR)			American (AMR)			East Asian (ASN)			European (EUR)		
	hmz (wt)[1]	htz[1]	hmz (snp)[1]	hmz (wt)	htz	hmz (snp)	hmz (wt)	htz	hmz (snp)	hmz (wt)	htz	hmz (snp)	hmz (wt)	htz	hmz (snp)
S17T	63.2 (T/T)	32.2 (T/A)	4.6 (T/T)	67.8	30.5	1.6	61.3	32.6	6.1	57.3	41.3	4.5	65.4	31.1	3.4
N36S	99.9 (A/A)	0.1 (A/G)	0 (A/A)	99.6	0.4	0	100	0	0	100	0	0	0	0	0
M65T	99.9 (T/T)	0.1 (T/C)	0 (C/C)	100	0	0	100	0	0	100	0	0	99.7	0.26	0
T73I	99.5 (C/C)	0.5 (C/T)	0 (T/T)	100	0	0	100	0	0	98.3	1.7	0	100	0	0
N85K	99.8 (C/C)	0.2 (C/A)	0 (A/A)	100	0	0	100	0	0	100	0	0	99.3	0.7	0
N85T	99.8 (A/A)	0.2 (A/C)	0 (C/C)	100	0	0	100	0	0	100	0	0	99.5	0.5	0
R131H	98.2 (G/G)	1.7 (G/A)	0.1 (A/A)	100	0	0	100	0	0	93	6.6	0.4	100	0	0
N156S	0.7 (A/A)	10.3 (A/G)	89.9 (G/G)	0	0	100	0	5	95	2.8	29	68.2	0	5.3	94.7
R199C	99.9 (C/C)	0.1 (C/T)	0 (T/T)	100	0	0	100	0	0	99.7	0.3	0	100	0	0
T244M	99.9 (C/C)	0.1 (C/T)	0 (T/T)	100	0	0	100	0	0	99.7	0.3	0	100	0	0
A251T	99.9 (G/G)	0.1 (G/A)	0 (A/A)	100	0	0	99.4	0.6	0	100	0	0	100	0	0
M272T	96.6 (T/T)	0.4 (T/C)	0 (C/C)	100	0	0	99.4	0.6	0	100	0	0	99.2	0.8	0
P295L	99.9 (C/C)	0.1 (C/T)	0 (T/T)	100	0	0	99.4	0.6	0	100	0	0	100	0	0

[1] *hmz (wt) - Homozygote (wild-type); htz – Heterozygote; hmz (SNP) - Homozygote (SNP)*

Table 3: Non-synonymous SNPs and allele frequencies, within the coding region of GLYAT, genotyped by the HapMap project in 379 individuals.

Population	S17T		N156S		F168L	
	WT	SNP	WT	SNP	WT	SNP
CEU	0.86	0.14	0.03	0.97	1	0
CHB	0.81	0.18	0.12	0.81	1	0
JPT	0.78	0.22	0.16	0.84	1	0
YRI	0.87	0.13	0	1	1	0

CEU – Utah residents with Northern and Western ancestry; CHB – Han Chinese in Beijing China; JPT – Japanese in Tokyo, Japan; YRI – Yoruba in Ibadan, Nigeria; WT – wild-type allele; SNP – single nucleotide polymorphism allele

Three novel (P40L, V46L and V47M) and two known SNPs (S17T and N156S) were identified in the Bantu individual (ABT). Several studies have shown that the discovery rate of novel sequence variants is higher in African populations (Altshuler et al., 2010; Buchanan et al., 2012; Ramsay, 2012). The four Khoisan individuals analysed in this study were all homozygous for the N156S SNP, while TK1 and MD8 were heterozygous for the S17T SNP. Previous studies have shown that two Khoisan individuals were genetically more different from one another than a European and Asian individual would be (Henn et al., 2011; Schuster et al., 2010; Tishkoff et al., 2009). Interestingly, this high degree of genetic variation reported in previous studies was not observed in the GLYAT sequences analysed in this study, as the four Khoisan individuals were highly similar to each other. This might indicate that the *GLYAT* gene is conserved even in individuals where a high degree of genetic diversity is expected.

3.1.4. South African Afrikaner Caucasian population

To investigate whether the founder effect has influenced the allele frequencies of polymorphisms in the South African Afrikaner Caucasian population, the coding region of the *GLYAT* gene of a small group of 61 Afrikaner Caucasian individuals was also sequenced. One novel (Q61L) and 11 known polymorphisms of the *GLYAT* gene were identified. Of the 11 known polymorphisms, two (S17T and N156S) were non-synonymous and nine were located in the intron regions (Table 4). The N156S variant was homozygous in all individuals resulting in the non-synonymous SNP with the highest allele frequency (100%), followed by Q61L (12.3%) and S17T (2.5%) (Table 4). Interestingly, one in four of the Afrikaner individuals were heterozygous for the novel Q61L polymorphism. No homozygotes for the Q61L polymorphism were identified in this small cohort. The high allele frequency of the Q61L polymorphism suggests that this variant has become prevalent in the Afrikaner Caucasian population due to a founder effect.

Table 4: Summary of the polymorphisms identified in the nucleotide sequence of the *GLYAT* gene in 61 South African Caucasian Afrikaners.

Polymorphism (rs)	Location (gene)	Effect (protein)	Allele frequency		Genotype frequency		
			Allele	%	Genotype	n[1]	%
rs615701 (5' Flanking)	g.58500426C>G		C G	3.3 96.7	CC CG GG	0 4 57	0.0 6.5 93.4
rs507224 (5' Flanking)	g.58499979A>G		A G	3.3 96.7	AA AG GG	0 27 34	0.0 6.6 93.4
rs75425382 (5' Flanking)	g.58499707C>T		C T	13.1 86.9	CC TC TT	7 2 52	11.5 3.3 85.2
rs1938722 (Intron 1)	g.58498835T>A		T A	75.4 24.6	TT TA AA	32 28 1	52.5 45.9 1.6
rs539085 (Intron 1)	g.58498825C>G		C G	3.3 96.7	CC CG GG	0 4 57	0.0 6.6 93.4
rs10896818 (Exon 2)	g.58491921T>A	p.Ser17Thr	T A	97.5 2.5	TT TA AA	58 3 0	95.1 4.9 0.0
Novel (Exon 3)	g.58482762A>T	p.Gln61Leu	A T	87.7 12.3	AA AT TT	46 15 0	75.4 24.6 0.0
rs610165 (Intron 3)	g.58480583T>A		T A	3.3 96.7	TT TA AA	0 4 57	0.0 6.6 93.4
rs2509908 (Intron 4)	g.58480155C>T		C T	98.4 1.6	CC CT TT	59 2 0	96.7 3.3 0.0
rs675815 (Exon 5)	g.58478084A>G	p.Asn156Ser	A G	0.0 100	AA AG GG	0 0 61	0.0 0.0 100
rs675757 (Intron 5)	g.58478039A>G		A G	22.1 77.9	AA AG GG	4 19 38	6.5 31.2 62.3
rs675423 (Intron 5)	g.58478026C>T		C T	46.7 53.3	CC CT TT	2 53 6	3.3 86.9 9.8

[1]*Number of individuals*

3.1.5. Haplotype phasing of the non-synonymous SNPs identified in the *GLYAT* gene of different population groups

Haplotype distributions between populations can provide insights in the diversity, the history and the migrations of human populations. The 1000 Genomes Project used the SHAPEIT2 programme to phase the variant genotypes. The phased haplotype data is available on the UCSC Genome Browser (genome.ucsc.edu/) (Karolchik et al., 2014). When only taking into account the non-synonymous variations, SHAPEIT2 analyses identified 13 haplotypes. However, one haplotype ($T_{85}T_{85}S_{156}$) on this database does not correspond to the non-synonymous SNP data that is available for any individual on the Ensembl database. To clarify this issue, the original data input was scrutinised. Two individuals (NA20756 and NA20810) have two non-synonymous SNPs at amino acid 85, namely N85K and N85T. This would result in two distinct haplotypes for these

individuals, which means that a total of fourteen distinct haplotypes exist for the 1000 Genomes sequencing data project (Table 5). In this study, the phasing of the 1000 Genomes data was repeated using the Arlequin version 3.5 programme (Excoffier et al., 2005). The Arlequin programme also called the $T_{85}T_{85}S_{156}$ haplotype instead of two separate haplotypes, namely $T_{85}S_{156}$ and $K_{85}S_{156}$. When this was corrected manually, the Arlequin analyses resulted in 14 haplotypes for the 1000 Genomes data (Table 5). For both the SHAPEIT2 and Arlequin analyses, nucleic acid sequences are used as input data. The problem with the incorrect identification of the $T_{85}T_{85}S_{156}$ haplotype is due to the genetic code. The $T_{85}S_{156}$ haplotype results in an AAC (Asn) $\rightarrow$ A<u>C</u>C (Thr) change, while the $K_{85}S_{156}$ haplotype results in an AAC (Asn) $\rightarrow$ AA<u>A</u> (Lys) change. The algorithms used by SHAPEIT2 and Arlequin result in a nucleic acid sequence with an AAC (Asn) $\rightarrow$ A<u>CA</u> (Thr) change explaining why the $K_{85}S_{156}$ haplotype is not called even though it is present in individuals NA20756 and NA20810 on the Ensembl database. Although the SHAPEIT2 and Arlequin analyses identified slightly different haplotypes, the haplotypes with the highest allele frequencies namely S_{156} (~70%), $T_{17}S_{156}$ (~20%), wild-type (~5%) and $H_{131}S_{156}$ (0.9%) were identified by both programmes (Table 5). Except for the T73I variant, all other SNPs were always found in combination with the N156S SNP.

Allele defined haplotypes were assigned for the South African Caucasian Afrikaner population and the Khoisan/Bantu population using the Arlequin version 3.5 programme (Excoffier et al., 2005) (Table 5). Haplotype analyses of the 61 South African Caucasian Afrikaner cohort data identified four distinct haplotypes [S_{156} (86.9%), $L_{61}S_{156}$ (10.7%), $T_{17}L_{61}S_{156}$ (1.6%) and T_{17} S_{156} (0.8%)], while three distinct haplotypes [S_{156} (60%), $T_{17}S_{156}$ (30%) and $L_{40}L_{46}M_{47}S_{156}$ (10%)] were identified in the Bantu and Khoisan individuals.

Table 5: Phased haplotypes for the coding regions of the *GLYAT* gene for all the populations analysed in this study.

Haplotypes	Amino acid residue of variant	rs number	Haplotype frequency (%) for different population groups						
			SA (Total)	Khoisan/ Bantu (Total)	1000 genomes (Total)	AFR[1]	AMR[1]	ASN[1]	EUR[1]
S_{156}	AA156 N/S	rs675815	86.9	60	S:71.1;A: 72.3	S:25.1;A:25.9	S:17.3;A:17.3	S:19.7; A:19.5	S:37.9;A:37.3
$T_{17}S_{156}$	AA17 S/T; AA156 N/S	rs10896818;rs675815	0.8	30	S:20.6;A:20.4	S:18.4;A:18.4	S:18.0;A:17.5	S:31.6;A:32.1	S:32.0; A:31.9
$L_{61}S_{156}$	AA61 Q/L; AA156 N/S	novel; rs675815	10.7	-	-	-	-	-	-
$L_{40}L_{46}M_{47}S_{156}$	novel; novel; novel; AA156 N/S	novel; novel; novel; rs675815	-	10	-	-	-	-	-
wild-type	Reference sequence	(NM_201648.2)	-	-	S:5.59;A:5.7	S:0;A:0	S:7.38;A:7.3	S:76.2;A:75.8	S:16.4;A:16.9
$T_{17}L_{61}S_{156}$	AA17 S/T; AA61 Q/L; AA156 N/S	rs10896818; novel; rs675815	1.6	-	-	-	-	-	-
$H_{131}S_{156}$	AA131 R/H; AA156 N/S	rs117149346; rs675815	-	-	S:0.96;A:0.92	S:0;A:0	S:0;A:0	S:100;A:100	S:0;A:0
I_{73}	AA73 T/I	rs144761620	-	-	S:0.23;A:0.2	S:0;A:0	S:0;A:0	S:100;A:100	S:0;A:0
$S_{156}T_{272}$	AA156 N/S; AA272 M/T	rs675815; rs149668211	-	-	S:0.18;A:0.09	S:0;A:0	S:25.0;A:0	S:0;A:0	S:75.0;A:100
$T_{85}S_{156}$	AA85 N/T; AA156 N/S	rs142339923; rs675815	-	-	S:0.05;A:0.09	S:0;A:0	S:0;A:0	S:0;A:0	S:100;A:100
$K_{85}S_{156}$	AA85 N/K; AA156 N/S	rs137908036; rs675815	-	-	S:0.05;A:0.09	S:0;A:0	S:0;A:0	S:0;A:0	S:100;A: 100
$S_{36}S_{156}$	AA36 N/S; AA156 N/S	rs201306322; rs675815	-	-	S:0.05;A:-	S:100; A:-	S:0;A:-	S:0;A:-	S:0; A:-
$T_{17}S_{36}S_{156}$	AA17 S/T; AA36 N/S; AA156 N/S	rs201306322; rs675815			S:-;A: 0.05	S: -;A: 100	S: -;A: 0	S: -;A: 0	S: -;A: 0
$T_{65}S_{156}$	AA65 M/T; AA156 N/S	rs145971997; rs675815	-	-	S:0.05;A:0.05	S:0;A 0	S:0;A:0	S:0;A:0	S:100;A:100
$S_{156}C_{199}$	AA156 N/S; AA199 R/C	rs675815; rs138125182	-	-	S:0.05;A:0.05	S:0;A:0	S:0;A:0	S:100;A:100	S:0;A:0
$S_{156}M_{244}$	AA156 N/S; AA244 T/M	rs675815; rs142279294	-	-	S:0.05;A: -	S:0;A: -	S:0;A: -	S:100;A: -	S:0;A: -
$H_{131}S_{156}M_{244}$	AA131 R/H; AA156 N/S; AA244 T/M	rs117149346; rs675815; rs142279294			S: -;A: 0.05	S: -;A: 0	S: -;A: 0	S: -;A: 100	S: -;A: 0
$S_{156}T_{251}$	AA156 N/S; AA251 A/T	rs675815; rs200165614	-	-	S:0.05;A: 0.05	S:0;A: 0	S:100;A: 100	S:0;A: 0	S:0;A: 0
$S_{156}L_{295}$	AA156 N/S; AA295 P/L	rs675815; rs200812874	-	-	S:0.05;A:-	S:0;A:-	S:100;A:-	S:0;A:-	S:0;A:-
$T_{17}S_{156}L_{295}$	AA17 S/T; AA156 N/S; AA295 P/L	rs675815; rs200812874	-	-	S: -;A: 0.05	S: -;A: 0	S: -;A: 100	S: -;A: 0	S: -;A: 0

SA – South African Caucasian Afrikaner cohort; AFR – African; AMR – Ad Mixed American; ASN – East Asian; EUR – European; S -SHAPEIT2 analyses; A – Arlequin analyses. [1]Each population group is a column in the table which contains the percentage of that haplotype that is found in each group. This is not the same as the percentage of each group that has the haplotype. For example, 19.7% for a particular haplotype in the ASN column where N=286 of 1092 means that of the 1092 occurrences of the haplotype, 215 or 19.7% are found in the ASN group.

3.1.6. Phylogenetic analyses and evolutionary conservation of the coding region of the *GLYAT* gene

Genetic data suggests that chimpanzees and humans may have diverged from a common ancestor 7-6 million years ago (Lovejoy, 1981; McGrew, 2010; Sibley and Ahlquist, 1984). Therefore, comparative studies between apes and humans help to understand the patterns of evolutionary change that characterise the human lineage. To determine which haplotype represents the ancestral allele and which haplotypes arose since the chimpanzee and human split, phylogenetic analyses were performed (Figure 1). The robustness of the tree was assessed using a 1000 bootstrap replicates. The human/chimpanzee clade had good bootstrap support of 78%, while ancestral nodes within this clade had bootstrap support below 60% (Figure 1) (Taylor and Piel, 2004). The phylogenetic analyses suggest that the S_{156} haplotype is the ancestral allele as it occurs in all the primate sequences. All the other haplotypes arose after the split of the human and chimpanzee lineages. It seems that, within each population group (EUR, AFR, AMR, ASN, SA and Khoisan), the S_{156} haplotype was the ancestral haplotype. The S_{156} haplotype is also the non-synonymous SNP with the highest allele frequency and highest relative enzyme activity (Lino Cardenas et al., 2010; van der Sluis et al., 2013). The haplotype diversity is however not population specific as sequences from different populations did not group together. This further indicates that the *GLYAT* gene is conserved across all population groups. From the phylogenetic analyses, it is clear that relatively few changes have occurred within the *GLYAT* ORF since the chimpanzee and human split as the *Pan Paniscus* and *Pan Troglodyte* haplotypes group within the human clade. The phylogenetic analyses therefore support our hypothesis that the coding regions of the *GLYAT* gene are well conserved in evolution.

It has been demonstrated that genetic polymorphisms in some human genes have been influenced by the different human sustenance modes of human cultures (pastoralists, agriculturalists, and hunter-gatherers). An example of this is polymorphisms of the *NAT2* gene, which results in a range of slow, intermediate, and fast acetylator phenotypes. Interestingly, some modern human diets have created a selective pressure favouring slow and intermediate acetylator phenotypes (Dorne et al., 2005; Sabbagh et al., 2011). The results of the present study did not reveal a similar degree of polymorphism in the human *GLYAT* gene. A low level of genetic diversity in the *GLYAT* ORF was observed in all worldwide populations investigated. Only two haplotypes (S_{156} and $T_{17}S_{156}$) were consistently found in all populations at relatively high frequencies (approximately 70% and 20% respectively) with the exception of the South African Afrikaner Caucasian population (S_{156} - 86.9%; $L_{61}S_{156}$ - 10.7%). The $S_{156}C_{199}$ haplotype, which has been shown to have less than 5% residual GLYAT enzyme activity, has a very low frequency (0.05%) and is always found in combination with the S_{156} allele, suggesting that homozygotes of the $S_{156}C_{199}$ haplotype might not be viable. The $S_{156}H_{131}$ haplotype also has a low allele frequency and it has been suggested that this may be a result of the decreased substrate affinity of the R131H variant of human GLYAT (van der Sluis et al., 2013).

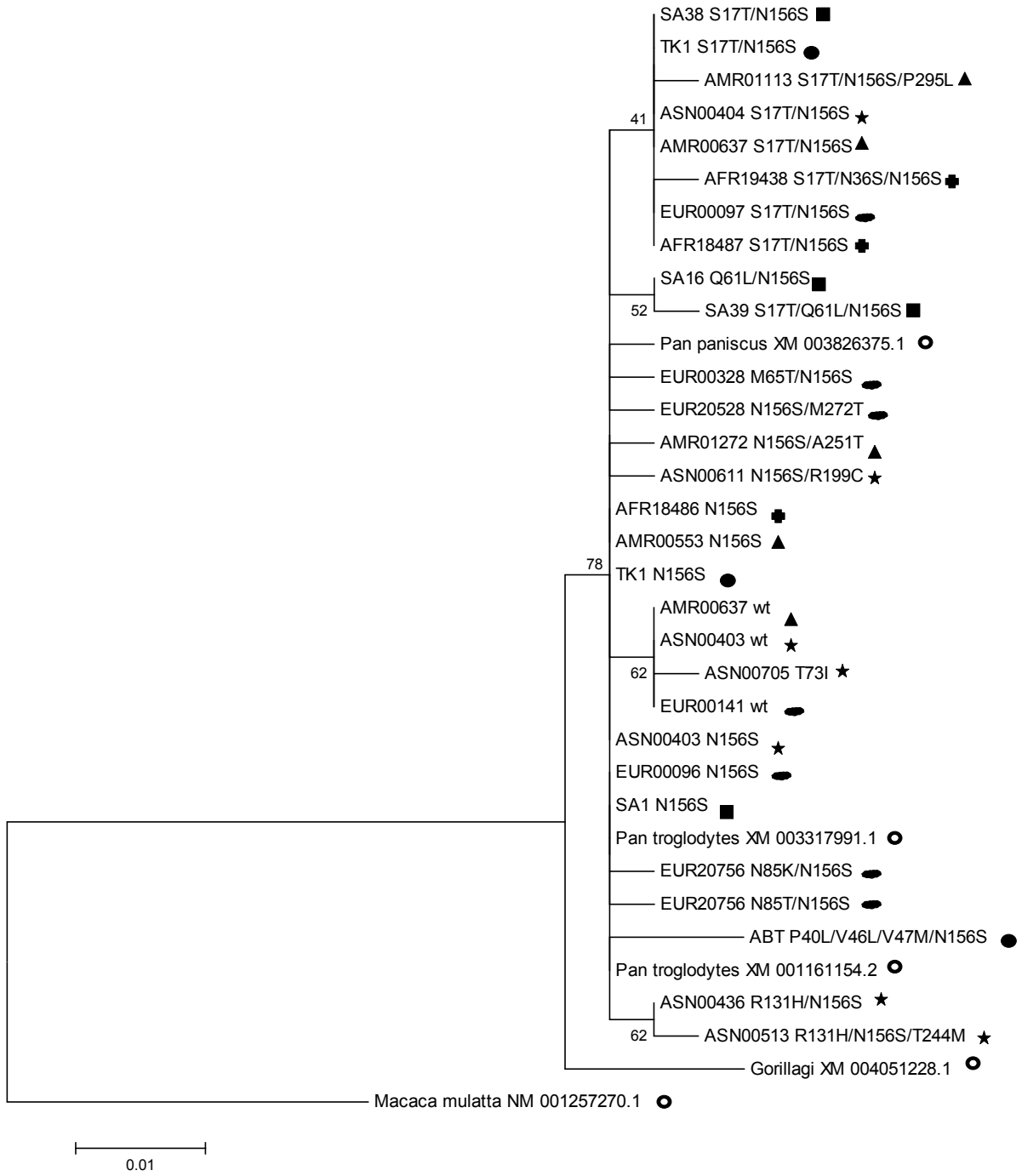

Figure 1: A maximum likelihood phylogenetic tree representing the haplotypes of the *GLYAT* gene within each population. Branch lengths are indicated (bar = 0.01 amino acid substitutions per site). The reliability of the phylogenetic tree was estimated using the bootstrap method (1000 replicates). The tree was rooted using the Macaca mulatta GLYAT sequence. The different populations are indicated by colours: South African Caucasian Afrikaner cohort (SA) – ■; Khoisan/Bantu (TK1, ABT) – ●; Ad Mixed American (AMR) – ▲; East Asian (ASN) – ★; African (AFR) – ✚ ; European (EUR) – ⬬ and primate – ⭘.

3.1.7. Tajima's Neutrality Test

To assess whether the human *GLYAT* gene is evolving neutrally, the Tajima's D value was determined. A gene evolves neutrally when genetic polymorphisms do not have a significant effect on the reproductive fitness of an organism, usually because the gene is not essential for survival. Therefore, frequencies of neutral mutations can fluctuate randomly from one generation to the next. However, if a gene is essential for survival, natural selection maintains deleterious mutations at very low frequencies, a process known as purifying selection. A negative Tajima's D value indicates that a large number of genetic variants occur at low frequency, which suggests that the gene is under selective pressure. Based on coalescent simulations conducted under a model of neutral evolution, 95% of the time the Tajima's D value falls between -1.74 and 1.64 (Fu and Li, 1993; Simonsen et al., 1995; Tajima, 1989) . In this study a Tajima's D value of -1.87 was calculated for the human *GLYAT* ORF. This suggests that the *GLYAT* gene is under selective pressure, and deleterious alleles, are maintained at low frequencies in the population. This is supported by the large number of low frequency alleles observed in this study as well as the low frequency of the R199C variant (Table 5), which has only 5% of the wild-type activity (van der Sluis et al., 2013).

3.2. Implications of a conserved GLYAT ORF for xenobiotic metabolism

3.2.1. Gut microbial metabolism and the vital role of glycine conjugation

As discussed in the introduction, GLYAT plays a vital role in hepatic homeostasis by conjugating benzoyl-CoA to glycine, to form hippurate and regenerate CoASH (reviewed in (Badenhorst et al., 2014)). The gut microbiome contributes a great deal to this detoxification load. Because of this important metabolic function, and because no genetic defect of glycine conjugation in humans has been reported in the literature, we hypothesised that the *GLYAT* gene is essential for human survival. This hypothesis led to the prediction that the *GLYAT* gene should be well conserved, with deleterious mutations occurring at low frequencies. The results obtained in this study seem to support this hypothesis. Rare genetic polymorphisms that have previously been shown to negatively influence GLYAT enzyme activity (van der Sluis et al., 2013) were detected at very low frequencies and most occur only as heterozygotes and in a single population group (Table 5). The present study also supports the previous suggestion that the S_{156} haplotype should be considered to be the "wild-type" human GLYAT, as this haplotype has the highest allele frequency in all populations studied as well as the highest relative enzyme activity (Lino Cardenas et al., 2010; van der Sluis et al., 2013).

3.2.2. Glycine conjugation of salicylate

Since the glycine conjugation pathway can be saturated, the rate of glycine conjugation influences the clearance of xenobiotics and therefore also toxicity (for review see Knights et al., 2007; Temellini et al., 1993). Glycine conjugation may not prevent the toxicity of an acyl-CoA if the rate of conjugation by GLYAT is low (e.g. $S_{156}C_{199}$ variant), even if the acyl-CoA is a substrate for the enzyme (Bartlett and Gompertz, 1974; Fenton et al., 2013; for review see Knights et al., 2007; Sweetman and Williams, 2013). Sodium salicylate, the active

component of aspirin, is one of the most widely used drugs worldwide (Cheng, 2007; Levine et al., 2005; Lewis et al., 1983), but is not recommended for children because it may cause Reye's syndrome (Larsen, 1997; Trost and Lemasters, 1996). Salicylate is eliminated in humans mainly by conjugation with glycine to form salicylurate followed by glucuronidation (Levy and Procknal, 1968). The appearance of salicyluric acid in the urine can be significantly inhibited by the administration of benzoic acid. This effect is not prevented by the co-administration of glycine (Amsel and Levy, 1969). It has also been shown that repeated administration of small doses of benzoate (0.5 g every 30 min) can inhibit salicylurate formation for extended periods of time (Levy and Amsel, 1966), which means that salicylate can readily accumulate in the body so that chronic administration may result in toxicity and liver damage, especially in young children (Levy and Yaffe, 1975; Zimmerman, 1981). On average, dietary intake and gut microbial metabolism result in exposure to the equivalent of 0.6 g to 1.3 g of benzoic acid per day, which is higher if foods preserved with benzoate are consumed frequently (Beyoglu and Idle, 2012). Aspirin doses as low as 0.6 g inhibit glucuronidation of salicylate (Levy and Procknal, 1968). This can result in a situation where salicylate accumulates for long periods because it can neither be conjugated to glycine (due to inhibition by benzoate) or to glucuronic acid (due to inhibition by high levels of aspirin).

To our knowledge, the most recent study on the kinetic elimination of salicylate was performed in 1986 (Greenblatt et al., 1986) while the glycine conjugation pathway is still rather poorly understood (for review see Badenhorst et al., 2013; Knights and Miners, 2012; for review see Knights et al., 2007; Lees et al., 2013). The work of Levy and co-workers needs to be repeated in light of the increased levels of benzoate and aspirin humans are exposed to nowadays. A number of important drug biotransformation processes in humans have a relatively limited capacity (Levy, 1965; Levy and Amsel, 1966; Levy and Matsuzawa, 1967). Environmental factors, nutrition, and the chronic use of medications are increasing the exposure of humans to benzoate, salicylate, solvents, and drugs that are metabolized to acyl-CoA intermediates. As we encounter more xenobiotics in the future, the consequences of interindividual variation (deleterious polymorphisms and differences in GLYAT expression levels) in the glycine conjugation pathway may have greater consequences (Badenhorst et al., 2013). It is, therefore, important to determine the *in vivo* capacity of the glycine conjugation pathway for the detoxification of benzoate.

3.2.3. Factors that influence interindividual variation in glycine conjugation rates

Several factors influence the rate of glycine conjugation such as the concentrations of ATP, CoASH, and glycine, genetic polymorphisms in the *HXM-A* and *GLYAT* genes, and the level of transcription of the *HXM-A* and *GLYAT* genes (Badenhorst et al., 2014). Using human liver samples, it was demonstrated that there is interindividual variation in the capacity for hippurate synthesis from benzoate, ATP, CoASH, and glycine (Temellini et al., 1993). In another study it was found that some isovaleric acidemia patients do not respond very well to glycine supplementation therapy, even in South Africa, where all known patients are homozygous for the same disease-causing mutation (Dercksen et al., 2012). We previously suggested that variation in the human *GLYAT* gene could contribute to the interindividual variation in the glycine conjugation rates observed in humans as well as help explain why some South African isovaleric acidemia patients do not respond well to glycine

supplementation therapy (Badenhorst et al., 2014; Badenhorst et al., 2013; van der Sluis et al., 2013). However, because of the small number and low allele frequencies of non-synonymous variants of human GLYAT reported here, it seems unlikely that non-synonymous SNPs in the human *GLYAT* gene contribute significantly to interindividual variation in glycine conjugation rates. Transcriptional regulation may thus have a much greater influence on hepatic glycine conjugation rates. GLYAT expression is underdeveloped in newborns, with children under seven months of age having 5% to 40% of normal GLYAT activity. GLYAT enzyme activity reaches its peak at about 18 months of age, stays at this level until approximately the age of 40, and then declines slightly in the elderly (Mawal et al., 1997; Temellini et al., 1993). It has also recently been demonstrated that GLYAT expression is transcriptionally down-regulated in hepatocellular carcinoma specimens, and that diet influences the expression of GLYAT in the livers of rats (Matsuo et al., 2012; Wen et al., 2013). In the case of the South African isovaleric acidemia patients, factors other than polymorphisms of the *GLYAT* gene may therefore better explain the interindividual variation in responsiveness to glycine supplementation.

4. Conclusion

There is a growing realisation that glycine conjugation and its effect on metabolism as a whole is far more complex than previously thought and that current mechanistic and genetic knowledge on this key detoxification pathway is lacking (Badenhorst et al., 2014; Beyoglu and Idle, 2012). Several pathological conditions such as diabetes, obesity, gut dysbiosis, autism, schizophrenia, depression, hepatitis, and cancer (Beyoglu and Idle, 2012; Beyoglu et al., 2012; Lees et al., 2013) have been correlated to the levels of hippurate excreted in urine. These diseases are also associated with alterations of gut microbial populations, but the relationships between gut microbial metabolism, glycine conjugation in the liver, urinary hippurate excretion, and disease are poorly understood (Holmes et al., 2011). This means that a thorough characterisation of all factors involved in the glycine conjugation pathway is necessary in order to successfully interpret the effect of xenobiotics on this pathway (Clayton et al., 2009).

This study aimed to characterise the genetic variation in the *GLYAT* ORF in order to facilitate future studies to correlate *in vitro / in vivo* rates of glycine conjugation to the genetic variations in GLYAT. Only two haplotypes, namely S_{156} and $T_{17}S_{156}$, were identified in all populations as having the highest haplotype frequencies (70% and 20% respectively). This supports a previous suggestion (Lino Cardenas et al., 2010) that the S_{156} haplotype should be considered as the wild-type sequence. The results in this study indicate that the *GLYAT* ORF is highly conserved. This is perhaps because the glycine conjugation pathway is an essential detoxification pathway and it would be important to confirm this experimentally *in vivo* using a GLYAT knockdown model. The essential nature of the glycine conjugation pathway can also explain why no metabolic defect of this pathway has been described to date. Future pharmacological studies should take note of the possibility that the glycine conjugation pathway in the modern human nutritional and environmental milieu might be stressed and that the capacity of this important detoxification pathway should be properly evaluated in *in vivo* studies.

5. References

Abe, S., Sasaki, M., 1977. Chromosome aberrations and sister chromatid exchanges in Chinese hamster cells exposed to various chemicals. Journal of the National Cancer Institute 58, 1635-1641.

Abecasis, G.R., Auton, A., Brooks, L.D., DePristo, M.A., Durbin, R.M., Handsaker, R.E., Kang, H.M., Marth, G.T., McVean, G.A., 2012. An integrated map of genetic variation from 1,092 human genomes. Nature 491, 56-65.

Abecasis, G.R., Burt, R.A., Hall, D., Bochum, S., Doheny, K.F., Lundy, S.L., Torrington, M., Roos, J.L., Gogos, J.A., Karayiorgou, M., 2004. Genomewide scan in families with schizophrenia from the founder population of Afrikaners reveals evidence for linkage and uniparental disomy on chromosome 1. American journal of human genetics 74, 403-417.

Altshuler, D.M., Gibbs, R.A., Peltonen, L., Altshuler, D.M., Gibbs, R.A., Peltonen, L., Dermitzakis, E., Schaffner, S.F., Yu, F., Peltonen, L., Dermitzakis, E., Bonnen, P.E., Altshuler, D.M., Gibbs, R.A., de Bakker, P.I., Deloukas, P., Gabriel, S.B., Gwilliam, R., Hunt, S., Inouye, M., Jia, X., Palotie, A., Parkin, M., Whittaker, P., Yu, F., Chang, K., Hawes, A., Lewis, L.R., Ren, Y., Wheeler, D., Gibbs, R.A., Muzny, D.M., Barnes, C., Darvishi, K., Hurles, M., Korn, J.M., Kristiansson, K., Lee, C., McCarrol, S.A., Nemesh, J., Dermitzakis, E., Keinan, A., Montgomery, S.B., Pollack, S., Price, A.L., Soranzo, N., Bonnen, P.E., Gibbs, R.A., Gonzaga-Jauregui, C., Keinan, A., Price, A.L., Yu, F., Anttila, V., Brodeur, W., Daly, M.J., Leslie, S., McVean, G., Moutsianas, L., Nguyen, H., Schaffner, S.F., Zhang, Q., Ghori, M.J., McGinnis, R., McLaren, W., Pollack, S., Price, A.L., Schaffner, S.F., Takeuchi, F., Grossman, S.R., Shlyakhter, I., Hostetter, E.B., Sabeti, P.C., Adebamowo, C.A., Foster, M.W., Gordon, D.R., Licinio, J., Manca, M.C., Marshall, P.A., Matsuda, I., Ngare, D., Wang, V.O., Reddy, D., Rotimi, C.N., Royal, C.D., Sharp, R.R., Zeng, C., Brooks, L.D., McEwen, J.E., 2010. Integrating common and rare genetic variation in diverse human populations. Nature 467, 52-58.

Amsel, L.P., Levy, G., 1969. Drug biotransformation interactions in man II: A pharmacokinetic study of the simultaneous conjugation of Benzoic and Salicylic Acids with Glycine. Journal of Pharmaceutical Sciences 58, 321-326.

Badenhorst, C.P., Erasmus, E., van der Sluis, R., Nortje, C., van Dijk, A.A., 2014. A new perspective on the importance of glycine conjugation in the metabolism of aromatic acids. Drug Metabolism Review 46 (3): 343-361

Badenhorst, C.P., van der Sluis, R., Erasmus, E., van Dijk, A.A., 2013. Glycine conjugation: importance in metabolism, the role of glycine N-acyltransferase, and factors that influence interindividual variation. Expert opinion on drug metabolism & toxicology 9 (9): 1139-1153.

Bandmann, O., Vaughan, J., Holmans, P., Marsden, C.D., Wood, N.W., 1997. Association of slow acetylator genotype for N-acetyltransferase 2 with familial Parkinson's disease. . Lancet 350, 1136-1139.

Bartlett, K., Gompertz, D., 1974. The specificity of glycine-N-acylase and acylglycine excretion in the organicacidaemias. Biochem.Med. 10, 15-23.

Beyoglu, D., Idle, J.R., 2012. The glycine deportation system and its pharmacological consequences. Pharmacology & therapeutics 135, 151-167.

Beyoglu, D., Smith, R.L., Idle, J.R., 2012. Dog bites man or man bites dog? The enigma of the amino acid conjugations. Biochemical pharmacology 83, 1331-1339.

Buchanan, C.C., Torstenson, E.S., Bush, W.S., Ritchie, M.D., 2012. A comparison of cataloged variation between International HapMap Consortium and 1000 Genomes Project data. Journal of the American Medical Informatics Association : JAMIA 19, 289-294.

Campbell, L., Wilson, H.K., Samuel, A.M., Gompertz, D., 1988. Interactions of m-xylene and aspirin metabolism in man. Br J Ind Med 45, 127-132.

Caricilli, A.M., Saad, M.J., 2014. Gut microbiota composition and its effects on obesity and insulin resistance. Current opinion in clinical nutrition and metabolic care 17, 312-318.

Cheng, T.O., 2007. The history of aspirin. Texas Heart Institute journal / from the Texas Heart Institute of St. Luke's Episcopal Hospital, Texas Children's Hospital 34, 392-393.

Clayton, T.A., Baker, D., Lindon, J.C., Everett, J.R., Nicholson, J.K., 2009. Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. Proceedings of the National Academy of Sciences of the United States of America 106, 14728-14733.

Daly, A.K., Cholerton, S., Gregory, W., Idle, J., 1993. Metabolic polymorphisms. Pharmacol.Ther. 57, 129-160.

Delaneau, O., Marchini, J., Zagury, J.F., 2012. A linear complexity phasing method for thousands of genomes. Nature methods 9, 179-181.

Delaneau, O., Zagury, J.F., Marchini, J., 2013. Improved whole-chromosome phasing for disease and population genetic studies. Nature methods 10, 5-6.

Demir, E., Kocaoglu, S., Kaya, B., 2010. Assessment of genotoxic effects of benzyl derivatives by the comet assay. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 48, 1239-1242.

Dercksen, M., Duran, M., Ijlst, L., Mienie, L.J., Reinecke, C.J., Ruiter, J.P., Waterham, H.R., Wanders, R.J., 2012. Clinical variability of isovaleric acidemia in a genetically homogeneous population. Journal of inherited metabolic disease 2012, 17.

Dorne, J.L., Walton, K., Renwick, A.G., 2005. Human variability in xenobiotic metabolism and pathway-related uncertainty factors for chemical risk assessment: a review. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 43, 203-216.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research 32, 1792-1797.

Excoffier, L., Laval, G., Balding, D., 2003. Gametic phase estimation over large genomic regions using an adaptive window approach. Human genomics 1, 7-19.

Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evolutionary bioinformatics online 1, 47-50.

Fabisch, W., Fellner, C., 1957. Excretion of hippuric acid in schizophrenic and depressive patients after E.C.T. Psychosomatic medicine 19, 320-325.

Fenton, W.A., Gravel, R.A., Rosenblatt, D.S., 2013. Part 9: Organic acids. Chapter 94: Disorders of Propionate and Methylmalonate Metabolism, The Online Metabolic and Molecular Bases of Inherited Diseases. McGraw Hill.

Fu, Y.X., Li, W.H., 1993. Statistical tests of neutrality of mutations. Genetics 133, 693-709.

Greenblatt, D.J., Abernethy, D.R., Boxenbaum, H.G., Matlis, R., Ochs, H.R., Harmatz, J.S., Shader, R.I., 1986. Influence of age, gender, and obesity on salicylate kinetics following single doses of aspirin. Arthritis and rheumatism 29, 971-980.

Haiser, H. J., and Turnbaugh, P. J. 2013. Developing a metagenomic view of xenobiotic metabolism. Pharmacological research 69, 21-31.

Hall, B.G., 2013. Building phylogenetic trees from molecular data with MEGA. Molecular biology and evolution 30, 1229-1235.

Heathfield, L., Lacerda, M., Nossek, C., Roberts, L., Ramesar, R.S., 2013. Stargardt disease: towards developing a model to predict phenotype. European journal of human genetics : EJHG 21, 1173-1176.

Henn, B.M., Gignoux, C.R., Jobin, M., Granka, J.M., Macpherson, J.M., Kidd, J.M., Rodriguez-Botigue, L., Ramachandran, S., Hon, L., Brisbin, A., Lin, A.A., Underhill, P.A., Comas, D., Kidd, K.K., Norman, P.J., Parham, P., Bustamante, C.D., Mountain, J.L., Feldman, M.W., 2011. Hunter-gatherer genomic diversity suggests a southern African origin for modern humans. Proceedings of the National Academy of Sciences of the United States of America 108, 5154-5162.

Holmes, E., Li, J.V., Athanasiou, T., Ashrafian, H., Nicholson, J.K., 2011. Understanding the role of gut microbiome-host metabolic signal disruption in health and disease. Trends in microbiology 19, 349-359.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Computer applications in the biosciences : CABIOS 8, 275-282.

Karolchik, D., Barber, G.P., Casper, J., Clawson, H., Cline, M.S., Diekhans, M., Dreszer, T.R., Fujita, P.A., Guruvadoo, L., Haeussler, M., Harte, R.A., Heitner, S., Hinrichs, A.S., Learned, K., Lee, B.T., Li, C.H., Raney, B.J., Rhead, B., Rosenbloom, K.R., Sloan, C.A., Speir, M.L., Zweig, A.S., Haussler, D., Kuhn, R.M., Kent, W.J., 2014. The UCSC Genome Browser database: 2014 update. Nucleic acids research 42, D764-770.

Kawajiri, K., Nakachi, K., Imai, K., Yoshii, A., Shinodaa, N., Watanabe, J., 1990. Identification of genetically high risk individuals to lung cancer by DNA polymorphisms of the cytochrome P45 0IA1 gene. FEBS Lett. 263, 131-133.

Killinberg, P.G., Davidson, E.D., Webster, L.T., 1971. Evidence for a medium-chain fatty acid: coenzyme A ligase (adenosine monophosphate) that activates salicylate. Mol. Pharmacol. 7, 260-268.

Knights, K.M., 1998. Role of hepatic fatty acid:coenzyme A ligases in the metabolism of xenobiotic carboxylic acids. Clininal and Experimental Pharmacology and Physiology 25, 776-782.

Knights, K.M., Miners, J.O., 2012. Amino Acid Conjugation: A Novel Route of Xenobiotic Carboxylic Acid Metabolism in Man, 1st ed. John Wiley & Sons, Inc. .

Knights, K.M., Sykes, M.J., Miners, J.O., 2007. Amino acid conjugation: contribution to the metabolism and toxicity of xenobiotic carboxylic acids. Expert opinion in drug metabolism and toxicology. 3, 159-168.

Kruse, M., Schulze-Bahr, E., Corfield, V., Beckmann, A., Stallmeyer, B., Kurtbay, G., Ohmert, I., Schulze-Bahr, E., Brink, P., Pongs, O., 2009. Impaired endocytosis of the ion channel TRPM4 is associated with human progressive familial heart block type I. The Journal of clinical investigation 119, 2737-2744.

Larsen, S.U., 1997. Reye's syndrome. Medicine, science, and the law 37, 235-241.

Lees, H.J., Swann, J.R., Wilson, I.D., Nicholson, J.K., Holmes, E., 2013. Hippurate: The Natural History of a Mammalian-Microbial Cometabolite. Journal of proteome research.

Levine, M., Wong, D., Brown, D.F., Nadel, E.S., 2005. Chest pain and arthritis. The Journal of emergency medicine 29, 91-95.

Levy, G., 1965. Pharmacokinetics of salicylate elimination in man. J Pharm Sci 54, 959-967.

Levy, G., Amsel, L.P., 1966. Kinetics of competitive inhibition of salicylic acid conjugation with glycine in man. Biochemical pharmacology 15, 1033-1038.

Levy, G., Matsuzawa, T., 1967. Pharmacokinetics of salicylamide elimination in man. The Journal of pharmacology and experimental therapeutics 156, 285-293.

Levy, G., Procknal, J.A., 1968. Drug biotransformation interactions in man. I. Mutual inhibition in glucuronide formation of salicylic acid and salicylamide in man. J Pharm Sci 57, 1330-1335.

Levy, G., Yaffe, S.J., 1975. Clinical implications of salicylate-induced liver damage. American journal of diseases of children (1960) 129, 1385-1386.

Lewis, H.D., Jr., Davis, J.W., Archibald, D.G., Steinke, W.E., Smitherman, T.C., Doherty, J.E., 3rd, Schnaper, H.W., LeWinter, M.M., Linares, E., Pouget, J.M., Sabharwal, S.C., Chesler, E., DeMots, H., 1983. Protective effects of aspirin against acute myocardial infarction and death in men with unstable angina. Results of a Veterans Administration Cooperative Study. The New England journal of medicine 309, 396-403.

Lino Cardenas, C.L., Bourgine, J., Cauffiez, C., Allorge, D., Lo-Guidice, J.M., Broly, F., Chevalier, D., 2010. Genetic polymorphisms of Glycine N-acyltransferase (GLYAT) in a French Caucasian population. Xenobiotica; the fate of foreign compounds in biological systems 40, 853-861.

Lovejoy, C.O., 1981. The origin of man. Science (New York, N.Y.) 211, 341-350.

MacFabe, D.F., Cain, D.P., Rodriguez-Capote, K., Franklin, A.E., Hoffman, J.E., Boon, F., Taylor, A.R., Kavaliers, M., Ossenkopp, K.P., 2007. Neurobiological effects of intraventricular propionic acid in rats: possible role of short chain fatty acids on the pathogenesis and characteristics of autism spectrum disorders. Behavioural brain research 176, 149-169.

Manach, C., Scalbert, A., Morand, C., Remesy, C., Jimenez, L., 2004. Polyphenols: food sources and bioavailability. The American journal of clinical nutrition 79, 727-747.

Matsuo, M., Terai, K., Kameda, N., Matsumoto, A., Kurokawa, Y., Funase, Y., Nishikawa, K., Sugaya, N., Hiruta, N., Kishimoto, T., 2012. Designation of enzyme activity of glycine-N-acyltransferase family genes and depression of glycine-N-acyltransferase in human hepatocellular carcinoma. Biochem.Biophys.Res.Commun. 420, 901-906.

Mawal, Y., Paradis, K., Qureshi, I.A., 1997. Developmental profile of mitochondrial glycine N-acyltransferase in human liver. J.Pediatr. 130, 1003-1007.

May, A., Hazelhurst, S., Li, Y., Norris, S.A., Govind, N., Tikly, M., Hon, C., Johnson, K.J., Hartmann, N., Staedtler, F., Ramsay, M., 2013. Genetic diversity in black South Africans from Soweto. BMC genomics 14, 644.

McGrew, W.C., 2010. In search of the last common ancestor: new findings on wild chimpanzees. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 365, 3267-3276.

Meyer, U.A., 1990. Genetic polymorphisms of drug metabolism. Fundamental and Clinical Pharmacology 4, 595-615.

Mortensen, H.M., Froment, A., Lema, G., Bodo, J.M., Ibrahim, M., Nyambo, T.B., Omar, S.A., Tishkoff, S.A., 2011. Characterization of genetic variation and natural selection at the arylamine N-acetyltransferase genes in global human populations. Pharmacogenomics 12, 1545-1558.

Nair, B., 2001. Final report on the safety assessment of Benzyl Alcohol, Benzoic Acid, and Sodium Benzoate. Int J Toxicol 20, 23-50.

Nandi, D.L., Lucas, S.V., Webster, L.T., Jr., 1979. Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine N-acyltransferase from bovine liver mitochondria. Purification and characterization. J.Biol.Chem. 254, 7230-7237.

Nebert, D.W., Petersen, D.D., Puga, A., 1991. Human AH locus polymorphism and cancer: inducibility of CYP1A1 and other genes by combustion products and dioxin. Pharmacogenetics 1, 68-78.

Nebert, D. W., Zhang, G., Vesell, E. S., 2013. Genetic risk prediction: individualized variability in susceptibility to toxicants. Pharmacology and Toxicology 53, 355-375.

Phipps, A.N., Stewart, J., Wright, B., Wilson, I.D., 1998. Effect of diet on the urinary excretion of hippuric acid and other dietary-derived aromatics in rat. A complex interaction between diet, gut microflora and substrate specificity. Xenobiotica; the fate of foreign compounds in biological systems 28, 527-537.

Piper, P.W., 1999. Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. Free radical biology & medicine 27, 1219-1227.

Rabot, S., Rafter, J., Rijkers, G.T., Watzl, B., Antoine, J.M., 2010. Guidance for substantiating the evidence for beneficial effects of probiotics: impact of probiotics on digestive system metabolism. The Journal of nutrition 140, 677S-689S.

Ramsay, M., 2012. Africa: continent of genome contrasts with implications for biomedical research and health. FEBS letters 586, 2813-2819.

Reeves, M.D., Yawitch, T.M., van der Merwe, N.C., van den Berg, H.J., Dreyer, G., van Rensburg, E.J., 2004. BRCA1 mutations in South African breast and/or ovarian cancer families: evidence of a novel founder mutation in Afrikaner families. International journal of cancer. Journal international du cancer 110, 677-682.

Ritchie, J.C., Cloan, T.P., Idle, J.R., Smith, R.L., 1980. Ciba Foundation Symposium 76: Toxicological implications of polymorphic drug metabolism. Excerpta Medica, 219-244.

Rodriguez-Murillo, L., Xu, B., Roos, J.L., Abecasis, G.R., Gogos, J.A., Karayiorgou, M., 2014. Fine mapping on chromosome 13q32-34 and brain expression analysis implicates MYO16 in schizophrenia. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 39, 934-943.

Roos, J.L., Pretorius, H.W., Karayiorgou, M., 2009. Clinical characteristics of an Afrikaner founder population recruited for a schizophrenia genetic study. Annals of the New York Academy of Sciences 1151, 85-101.

Sabbagh, A., Darlu, P., Crouau-Roy, B., Poloni, E.S., 2011. Arylamine N-acetyltransferase 2 (NAT2) genetic diversity and traditional subsistence: a worldwide population survey. PloS one 6, e18507.

Schachter, D., Taggart, J.V., 1954. Glycine N-acylase: purification and properties. J.Biol.Chem. 208, 263-275.

Schooneman, M.G., Vaz, F.M., Houten, S.M., Soeters, M.R., 2013. Acylcarnitines: reflecting or inflicting insulin resistance? Diabetes 62, 1-8.

Schuster, S.C., Miller, W., Ratan, A., Tomsho, L.P., Giardine, B., Kasson, L.R., Harris, R.S., Petersen, D.C., Zhao, F., Qi, J., Alkan, C., Kidd, J.M., Sun, Y., Drautz, D.I., Bouffard, P., Muzny, D.M., Reid, J.G., Nazareth, L.V., Wang, Q., Burhans, R., Riemer, C., Wittekindt, N.E., Moorjani, P., Tindall, E.A., Danko, C.G., Teo, W.S., Buboltz, A.M., Zhang, Z., Ma, Q., Oosthuysen, A., Steenkamp, A.W., Oostuisen, H., Venter, P., Gajewski, J., Zhang, Y., Pugh, B.F., Makova, K.D., Nekrutenko, A., Mardis, E.R., Patterson, N., Pringle, T.H., Chiaromonte, F., Mullikin, J.C., Eichler, E.E., Hardison, R.C., Gibbs, R.A., Harkins, T.T., Hayes, V.M., 2010. Complete Khoisan and Bantu genomes from southern Africa. Nature 463, 943-947.

Sibley, C.G., Ahlquist, J.E., 1984. The phylogeny of the hominoid primates, as indicated by DNA-DNA hybridization. Journal of molecular evolution 20, 2-15.

Simonsen, K.L., Churchill, G.A., Aquadro, C.F., 1995. Properties of statistical tests of neutrality for DNA polymorphism data. Genetics 141, 413-429.

Smith, A.V., 2008. Browsing HapMap Data Using the Genome Browser. CSH protocols 2008, pdb prot5023.

Sweetman, L., Williams, J.C., 2013. Part 9: Organic acids Chapter 93: Branched Chain Organic Acidurias, The Online Metabolic and Molecular Bases of Inherited Diseases. McGraw Hill.

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585-595.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular biology and evolution 30, 2725-2729.

Tanaka, K., Isselbacher, K.J., 1967. The isolation and identification of N-isovalerylglycine from urine of patients with isovaleric acidemia. Journal of biological chemistry. 242, 2966-2972.

Tang, W. H., and Hazen, S. L., 2014. The contributory role of gut microbiota in cardiovascular disease. The journal of clinical investigation 124 (10), 4204-4211.

Taylor, D.J., Piel, W.H., 2004. An assessment of accuracy, error, and conflict with support values from genome-scale phylogenetic data. Molecular biology and evolution 21, 1534-1537.

Temellini, A., Mogavero, S., Giulianotti, P.C., Pietrabissa, A., Mosca, F., Pacifici, G.M., 1993. Conjugation of benzoic acid with glycine in human liver and kidney: a study on the interindividual variability. Xenobiotica; the fate of foreign compounds in biological systems 23, 1427-1433.

Tfouni, S.A., Toledo, M.C., 2002. Estimates of the mean per capita daily intake of benzoic and sorbic acids in Brazil. Food additives and contaminants 19, 647-654.

Thorisson, G.A., Smith, A.V., Krishnan, L., Stein, L.D., 2005. The International HapMap Project Web site. Genome research 15, 1592-1593.

Tipping, A.J., Pearson, T., Morgan, N.V., Gibson, R.A., Kuyt, L.P., Havenga, C., Gluckman, E., Joenje, H., de Ravel, T., Jansen, S., Mathew, C.G., 2001. Molecular and genealogical evidence for a founder effect in Fanconi anemia families of the Afrikaner population of South Africa. Proceedings of the National Academy of Sciences of the United States of America 98, 5734-5739.

Tishkoff, S.A., Reed, F.A., Friedlaender, F.R., Ehret, C., Ranciaro, A., Froment, A., Hirbo, J.B., Awomoyi, A.A., Bodo, J.M., Doumbo, O., Ibrahim, M., Juma, A.T., Kotze, M.J., Lema, G., Moore, J.H., Mortensen, H., Nyambo, T.B., Omar, S.A., Powell, K., Pretorius, G.S., Smith, M.W., Thera, M.A., Wambebe, C., Weber, J.L., Williams, S.M., 2009. The genetic structure and history of Africans and African Americans. Science (New York, N.Y.) 324, 1035-1044.

Trost, L.C., Lemasters, J.J., 1996. The mitochondrial permeability transition: a new pathophysiological mechanism for Reye's syndrome and toxic liver injury. The Journal of pharmacology and experimental therapeutics 278, 1000-1005.

van der Sluis, R., Badenhorst, C.P.S., Van der Westhuizen, F.H., van Dijk, A.A., 2013. Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase. Gene 515, 447-453.

Wallig, M.A., 2004. Glucuronidation and susceptibility to chemical carcinogenesis. Toxicological sciences, 1-2.

Wen, H., Yang, H.J., An, Y.J., Kim, J.M., Lee, D.H., Jin, X., Park, S.W., Min, K.J., Park, S., 2013. Enhanced phase II detoxification contributes to beneficial effects of dietary restriction as revealed by multi-platform metabolomics studies. Molecular & cellular proteomics : MCP 12, 575-586.

Wikoff, W.R., Anfora, A.T., Liu, J., Schultz, P.G., Lesley, S.A., Peters, E.C., Siuzdak, G., 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proceedings of the National Academy of Sciences of the United States of America 106, 3698-3703.

Williams, H.R., Cox, I.J., Walker, D.G., Cobbold, J.F., Taylor-Robinson, S.D., Marshall, S.E., Orchard, T.R., 2010. Differences in gut microbial metabolism are responsible for reduced hippurate synthesis in Crohn's disease. BMC Gastroenterol 10, 108.

Xu, B., Ionita-Laza, I., Roos, J.L., Boone, B., Woodrick, S., Sun, Y., Levy, S., Gogos, J.A., Karayiorgou, M., 2012. De novo gene mutations highlight patterns of genetic and neural complexity in schizophrenia. Nature genetics 44, 1365-1369.

Xu, B., Roos, J.L., Dexheimer, P., Boone, B., Plummer, B., Levy, S., Gogos, J.A., Karayiorgou, M., 2011. Exome sequencing supports a de novo mutational paradigm for schizophrenia. Nature genetics 43, 864-868.

Xu, B., Woodroffe, A., Rodriguez-Murillo, L., Roos, J.L., van Rensburg, E.J., Abecasis, G.R., Gogos, J.A., Karayiorgou, M., 2009. Elucidating the genetic architecture of familial schizophrenia using rare copy number variant and linkage scans. Proceedings of the National Academy of Sciences of the United States of America 106, 16746-16751.

Yamamoto, A., Nonen, S., Fukuda, T., Yamazaki, H., Azuma, J., 2009. Genetic polymorphisms of glycine N-acyltransferase in Japanese individuals. Drug Metab Pharmacokinet 24, 114-117.

Yilmaz, S., Unal, F., Yuzbasioglu, D., 2009. The in vitro genotoxicity of benzoic acid in human peripheral blood lymphocytes. Cytotechnology.

Zengin, N., Yuzbasioglu, D., Unal, F., Yilmaz, S., Aksoy, H., 2011. The evaluation of the genotoxicity of two food preservatives: sodium benzoate and potassium benzoate. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 49, 763-769.

Zhang, H., Lang, Q., Li, J., Zhong, Z., Xie, F., Ye, G., Wan, B., Yu, L., 2007. Molecular cloning and characterization of a novel human glycine N-acyltransferase gene GLYAT1, which activates transcriptional activity of HSE pathway. Int. J. Mol. Sci. 8, 433-444.

Zimmerman, H.J., 1981. Effects of aspirin and acetaminophen on the liver. Archives of internal medicine 141, 333-342.

CHAPTER 5: CLOSING REMARKS

In this final chapter, conclusions of the main findings and results of this study (presented in Chapters 2-4) and their implications for the understanding of the glycine conjugation pathway will be presented. Some aspects that still need to be investigated in order to further elucidate the understanding of this essential pathway will also be highlighted.

5.1 Summary and conclusion

5.1.1 The purpose of the glycine conjugation pathway is to detoxify dietary polyphenols

Research on the glycine conjugation pathway has been neglected for the last 30 years. Therefore, the first aim of this study was to develop a better understanding of the role of glycine conjugation in metabolism. Two review articles were published after conducting a thorough investigation of the literature (Papers I, Appendix A and Paper II, Appendix B). Paper I focused on the elucidation of the primary role of GLYAT in the detoxification of polyphenols and related aromatic acids encountered in the diet. The factors that influence interindividual variation in the rate of glycine conjugation were also reviewed, with a focus on the significance of genetic variation in the human *GLYAT* gene. This review highlighted the importance of glycine conjugation in preventing CoASH sequestration, which would result from the accumulation of acyl-CoA metabolites such as benzoyl-CoA.

Recently, it was argued that glycine conjugation should be viewed as a neuroregulatory process, important for the regulation of cerebrospinal fluid (CSF) glycine levels, rather than as a detoxification mechanism (Beyoglu and Idle, 2012). The glycine deportation hypothesis stated that the irreversible urinary excretion of glycine as a conjugate to benzoate prevents the accumulation of glycine, which is a neurotransmitter, to toxic levels. In Paper II the glycine deportation hypothesis was critically analysed and compared to the traditional view that glycine conjugation is a detoxification mechanism. As evidence that glycine conjugation should be viewed as a neuroregulatory process instead of a detoxification process Beyoglu et al. 2012 argued that hippuric acid is not significantly more water soluble than benzoic acid. The most important contribution of Paper II was the demonstration that glycine conjugation decreases the toxicity of benzoate and other aromatic acids by forming less lipophilic conjugates rather than more water soluble conjugates. Because of its lipophilic nature, benzoic acid is capable of passively diffusing across the mitochondrial inner membrane into the matrix space, where it accumulates due to the pH gradient over the inner membrane. Although benzoate can be exported from the matrix by organic anion transporters, this process would likely be futile because benzoic acid can simply diffuse back into the matrix. Hippurate, however, is significantly less lipophilic and therefore less capable of diffusing into the matrix. It was concluded that, while providing a valuable new perspective, the glycine deportation system does not provide a suitable alternative to the view of glycine conjugation as a detoxification mechanism.

In summary, Papers I and II demonstrated that: i) the major source of glycine conjugation substrates seems to be the metabolites of dietary polyphenols produced by microorganisms in the gut; ii) the glycine conjugation pathway evolved as a detoxification pathway and iii) the glycine conjugation pathway is essential for the maintenance of homeostatic CoASH levels.

5.1.2 Polymorphisms in the coding region of the human *GLYAT* gene may influence the catalytic properties of a recombinant human GLYAT

The main aim of this thesis was to investigate and characterise the genetic variation in the coding region of the *GLYAT* gene. This was accomplished by firstly, investigating the influence of non-synonymous SNPs on the enzyme activity of a recombinant GLYAT (as discussed in this section) and secondly, by analysing the level of genetic variation in the coding region of the *GLYAT* gene using worldwide population data (discussed in Section 5.1.3.).

To investigate the influence of genetic variations on the enzyme activity of GLYAT, the wild-type sequence (GenBank ID: NM_201648.2), of human GLYAT was purchased and cloned into a pET32a(+) expression vector. Using site-directed mutagenesis, six recombinant human GLYAT variants were generated containing known SNPs in the human *GLYAT* gene. These variants were expressed and purified by means of nickel-affinity chromatography. For each variant, the relative enzyme activities (using benzoyl-CoA and glycine as substrates), and the K_M (benzoyl-CoA) kinetic parameters were then determined. These results were reported in Paper III. The main conclusions of this paper were that: i) Known SNP variations in the human *GLYAT* gene can influence the enzyme activity and K_M (benzoyl-CoA) of a recombinant human GLYAT; ii) As previously suggested (Lino Cardenas et al., 2010), the N156S variant should be regarded as the wild-type allele due to this variant's relative high enzyme activity and allele frequency; iii) There seemed to be a correlation between the kinetic parameters of the recombinant human GLYAT variants investigated and the allele frequencies of these variants reported in previous studies (Lino Cardenas et al., 2010; Yamamoto et al., 2009); iv) These differences in relative enzyme activity and kinetic parameters may help explain some of the observed interindividual variation in glycine conjugation capacity observed in the literature and v) human GLYAT is not subject to regulation by reversible acetylation on Lys[19].

5.1.3 Evidence for the conservation of the coding region of the *GLYAT* gene

To date, no defect of the glycine conjugation pathway has been reported and this, together with the fact that GLYAT plays an important role in hepatic metabolism, indicates that this pathway might be essential for survival. We hypothesised that the genetic variation in the open reading frame (ORF) of the *GLYAT* gene should be low and that deleterious alleles will be found at low frequencies. This hypothesis was investigated by analysing the genetic variation data of the human *GLYAT* ORF of 1476 individuals available on public databases. We also sequenced the coding region of 61 South African Afrikaner Caucasian individuals. The main findings of Paper IV (submitted manuscript) were: i) The N156S variant should be considered the wild-type

allele as it has the highest allele and haplotype frequency in all populations studied; ii) Deleterious alleles were consistently found at very low allele frequencies, which indicates that the glycine conjugation pathway is essential for survival; iii) The high allele frequency of the novel Q61L polymorphism suggests that this variant has become prevalent in the Afrikaner Caucasian population due to a putative founder effect; iv) Both the haplotype analyses and the phylogenetic analyses supported our hypothesis that the coding regions of the *GLYAT* gene are well conserved during evolution; v) Due to the small number and low allele frequencies of non-synonymous variants of human GLYAT, it seems unlikely that non-synonymous SNPs in the human *GLYAT* gene contribute significantly to interindividual variation in glycine conjugation rates.

5.1.4 Conclusion

In summary, there were three major and contributing findings made by the investigations presented in this thesis. These findings resulted from both an extensive review of available literature on GLYAT and its role in human metabolism, as well as subsequent empirical scientific research on the variants found in the *GLYAT* gene and their consequence on enzyme function. Firstly, it is proposed that glycine conjugation should be viewed as a detoxification mechanism of dietary polyphenols that results in decreasing the lipophilic nature of benzoic acid by conjugation to glycine and the formation of hippurate that is significantly less lipophilic. This prevents benzoate from continuously diffusing back across the mitochondrial inner membrane. Secondly, experimental investigations in this thesis showed that the six non-synonymous SNPs (K16N; S17T; R131H; N156S; F168L; R199C) investigated have an influence on the enzyme activity of a wild-type recombinant GLYAT (NM_201648.2). The relative enzyme activities of the variants ranged from 5% - 190% of that of the wild-type enzyme while the K_M (benzoyl-CoA) ranged from 21 µM to 71 µM. Thirdly, the *GLYAT* gene is remarkably conserved and alleles with a negative effect on the enzyme activity (R199C) or substrate specificity (R131H) are found at very low frequencies. These findings support our hypothesis that the glycine conjugation pathway is possibly essential for survival. Therefore, because this pathway evolved for the efficient detoxification of dietary polyphenols, the modern diet with the increased use of benzoate as a preservative could place a great deal of strain on this pathway. We previously suggested that variation in the human *GLYAT* gene is one of the factors that could contribute to the interindividual variation in the glycine conjugation rates observed in humans as well as help explain why some South African isovaleric acidemia patients do not respond well to glycine supplementation therapy. This study clearly showed that the variety of haplotypes found in the different populations studied is very low, which might indicate that variation in the *GLYAT* gene itself might be a very small contributing factor to the overall variation seen in the glycine conjugation pathway.

5.2 Suggestions for future research

5.2.1 Investigation of the influence of the SNPs and haplotypes on the enzyme activity and substrate specificity of a recombinant GLYAT

In this study the effect of six non-synonymous SNPs on the enzyme activity of a recombinant GLYAT were investigated. Currently there are 76 known SNPs in the *GLYAT* gene reported on the Ensembl database. This study identified 14 probable haplotypes for the *GLYAT* gene using available prediction software. It would be valuable to investigate what the influence of the haplotypes identified is on the catalytic properties of a recombinant human GLYAT. Another question that needs to be addressed is whether variants of human GLYAT have different substrate specificities, which might help explain why some patients with isovaleric acidemia excrete more glycine conjugates than others (Dercksen et al., 2012) and why several other glycine conjugates have been identified in urine (Loots et al., 2007). Recently, the concept of dual-use codons ("duons") was introduced. These are highly conserved codons that simultaneously specify both amino acid and transcription factor recognition sites. Transcription factor binding is directly affected by 17% of SNPs within duons (Stergachis et al., 2013). Therefore, future studies should also investigate the possible influence of duons on the expression levels of GLYAT as well as the influence of variation in the non-coding parts of the *GLYAT* gene. This is especially important as it was speculated in Chapter 4 that variation in the expression levels of GLYAT might have a large effect on the overall variation in observed glycine conjugation ability. It has also been reported that some of the variations in the introns of the *GLYAT* gene may influence development of the musculoskeletal system (Guo et al., 2013).

5.2.2 Correlation of polymorphisms in the *GLYAT* gene with the *in vivo* glycine conjugation rate

Previous *in vivo* studies of glycine conjugation are difficult to compare because of differences in the substrates used, substrate dosages, sample collection protocols, metabolite analyses, and test animals used. At present, nothing is known regarding the relationship between polymorphisms in the *GLYAT* gene and the *in vivo* glycine conjugation rate. Since environmental factors, nutrition, and the chronic use of medications are increasing the exposure of modern humans to benzoate, salicylate, solvents and drugs that are metabolised to acyl-CoA intermediates, the findings in this study imply that it will, in future, also become important to determine the *in vivo* capacity of the glycine conjugation pathway for the detoxification of benzoate and other xenobiotics such as aspirin.

5.2.3 Enzyme augmentation therapy

Since the late 1970s, glycine conjugation has been used in the treatment of hyperammonemia in urea cycle disorders. The treatment is based on the synthesis and excretion of hippurate as an alternative pathway to divert nitrogen from the urea cycle (Bursilow et al., 1979; Feoli-Fonseca et al., 1996). In isovaleric acidemia

there is a toxic build-up of isovaleric acid. Supplementation with glycine leads to increased synthesis and excretion of isovaleric acid as isovalerylglycine. Therefore, GLYAT plays an important role by effectively detoxifying substantial quantities of the toxic organic acids (Sweetman and Williams, 2013; Tanaka and Isselbacher, 1967). Glycine conjugates are also excreted in several other organic acidemias, and include 3-methylcrotonylglycine, hexanoylglycine, butyrylglycine, tiglylglycine (Bonafe et al., 2000; Gompertz, 1974; Ogier and Saudubray, 2002; Wanders et al., 1999).

Enzyme replacement therapy has been used effectively to treat several lysosomal storage diseases (for example Gaucher disease, Fabry disease and Pompe disease), adenosine deaminase deficiency and cystic fibrosis (Brady et al., 1974; Cantz and Kresse, 1974; Chan et al., 2005; Munck et al., 2009). Enzyme replacement therapy was also used to treat the deficiency of a mitochondrial protein, lipoamide dehydrogenase (LAD; also known as E3). This was done by recombinant expression of LAD fused to a signal peptide that directs the protein into hepatocytes and then targets the mitochondria for entry. This approach has been shown to correct the enzyme deficiency in cultured patient hepatocytes (Rapoport et al., 2008). Later it was shown that the defective enzyme could be replaced by using the TAT-LAD fusion protein in an *in vivo* mouse model using E3-deficient mice. A single administration of TAT-LAD resulted in a significant increase in the enzymatic activity of the mitochondrial multi-enzyme pyruvate dehydrogenase complex within the liver, heart and the brain of TAT-LAD-treated E3-deficient mice (Rapoport et al., 2011). Recently, enzyme augmentation therapy has been introduced with the treatment of Alpha-1 antitrypsin deficiency with the intravenous infusion of purified human plasma-derived alpha-1 antitrypsin (Mohanka et al., 2012). Currently, there is no enzyme augmentation therapy available to increase the detoxification of xenobiotics (Sears and Genuis, 2012).

It is important to realise that glycine conjugation may not prevent the toxicity of an acyl-CoA, even if it is a good substrate for the enzyme, if the rate of conjugation by GLYAT is low (e.g. R199C heterozygous individual) (Bartlett and Gompertz, 1974; Fenton et al., 2013; reviewed in Knights et al., 2007; Sweetman and Williams, 2013). It is also possible that the glycine conjugation pathway is placed under stress with the modern diet and increased use of preservatives resulting in deficient detoxification of benzoate. The detoxification load of this pathway might be decreased through enzyme augmentation therapy, although one should then be careful not to deplete important cofactors and substrates. A GLYAT variant that preferentially forms isovaleryl or propionyl glycine conjugates could potentially also be used in enzyme augmentation therapy. The study of Rapoport et al. (2008) showed that it should be possible to direct a recombinant therapeutic GLYAT to liver mitochondria for the treatment of organic acidemias. A patent application (PCT/IB2011/053721) for the therapeutic use of a recombinant GLYAT has been filed and is included as Appendix D.

5.2.4 Further characterisation of the remaining factors that can influence the variation in the glycine conjugation rate

The levels of hippurate excreted in urine seem to correlate with a wide range of pathological conditions such as diabetes, obesity, gut dysbiosis, autism, schizophrenia, depression, hepatitis, and cancer (Beyoglu and Idle,

2012; Beyoglu et al., 2012; Lees et al., 2013). However, it is currently impossible to accurately interpret the relationships between hippurate excretion and these disease conditions, since the glycine conjugation pathway is still rather poorly understood (for review see Knights and Miners, 2012; Lees et al., 2013). The limiting step in the glycine conjugation pathway depends on the xenobiotic used (Kasuya et al., 2000; Temellini et al., 1993). Future studies should use at least salicylate and benzoate as probe compounds, on separate occasions, to enable differentiation between variation in acid:CoA ligase and GLYAT activities, respectively. The major precursor for hippurate synthesis is phenylpropionic acid, produced by the gut microbiota from dietary polyphenols (Fedotcheva et al., 2008; Jenner et al., 2005; Rechner et al., 2002). This means that the rates of glycine conjugation measured using benzoate as a probe compound may not always be physiologically relevant. Therefore, it would be valuable to compare the *in vivo* rates of hippurate formation from phenylpropionate, cinnamate, and benzoate, and to compare the stimulatory effect of glycine supplementation in each case.

In conclusion, there is a growing realisation that glycine conjugation and its effect on metabolism as a whole is far more complex than previously thought and that current mechanistic and genetic knowledge on this key detoxification pathway is lacking to a great extent. Finally, because of the glycine deportation and ROS production induced by benzoate, the health risks of using this preservative should not be underestimated any longer.

REFERENCES

Abdo, K.M., Wenk, M.L., Harry, G.J., Mahler, J., Goehl, T.J., Irwin, R.D., 1998. Glycine modulates the toxicity of benzyl acetate in F344 rats. Toxicologic pathology 26, 395-402.

Abe, S., Sasaki, M., 1977. Chromosome aberrations and sister chromatid exchanges in Chinese hamster cells exposed to various chemicals. Journal of the national cancer institute 58, 1635-1641.

Abecasis, G.R., Auton, A., Brooks, L.D., DePristo, M.A., Durbin, R.M., Handsaker, R.E., Kang, H.M., Marth, G.T., McVean, G.A., 2012. An integrated map of genetic variation from 1,092 human genomes. Nature 491, 56-65.

Abul-Husn, N.S., Owusu Obeng, A., Sanderson, S.C., Gottesman, O., Scott, S.A., 2014. Implementation and utilization of genetic testing in personalized medicine. Pharmacogenomics and personalized medicine 7, 227-240.

Amsel, L.P., Levy, G., 1969. Drug biotransformation interactions in man II: A pharmacokinetic study of the simultaneous conjugation of Benzoic and Salicylic Acids with Glycine. Journal of pharmaceutical sciences 58, 321-326.

Applegarth, D.A., Toone, J.R., 2001. Nonketotic hyperglycinemia (glycine encephalopathy): laboratory diagnosis. Molecular genetics and metabolism 74, 139-146.

Bachmann, K., 2009. Drug metabolism. Elsevier, USA.

Badenhorst, C.P.S., Jooste, M., van Dijk, A.A., 2012. Enzymatic characterization and elucidation of the catalytic mechanism of a recombinant bovine glycine N-acyltransferase. Drug metabolism and disposition: the biological fate of chemicals 40, 346-352.

Barshop, B.A., Breuer, J., Holm, J., Leslie, J., Nyhan, W.L., 1989. Excretion of hippuric acid during sodium benzoate therapy in patients with hyperglycinaemia or hyperammonaemia. Journal of inherited metabolic disease 12, 72-79.

Bartlett, K., Gompertz, D., 1974. The specificity of glycine-N-acylase and acylglycine excretion in the organicacidaemias. Biochemical medicine. 10, 15-23.

Batshaw, M.L., Brusilow, S.W., 1981. Evidence of lack of toxicity of sodium phenylacetate and sodium benzoate in treating urea cycle enzymopathies. Journal of inherited metabolic disease 4, 231.

Batshaw, M.L., Hyman, S.L., Coyle, J.T., Robinson, M.B., Qureshi, I.A., Mellits, E.D., Quaskey, S., 1988. Effect of sodium benzoate and sodium phenylacetate on brain serotonin turnover in the ornithine transcarbamylase-deficient sparse-fur mouse. Pediatric research 23, 368-374.

Battaglia, V., Salvi, M., Toninello, A., 2005. Oxidative stress is responsible for mitochondrial permeability transition induction by salicylate in liver mitochondria. The journal of biological chemistry 280, 33864-33872.

Begriche, K., Massart, J., Robin, M.A., Borgne-Sanchez, A., Fromenty, B., 2011. Drug-induced toxicity on mitochondria and lipid metabolism: Mechanistic diversity and deleterious consequences for the liver. Journal of hepatology 54, 773-794.

Beloborodova, N., Bairamov, I., Olenin, A., Shubina, V., Teplova, V., Fedotcheva, N., 2012. Effect of phenolic acids of microbial origin on production of reactive oxygen species in mitochondria and neutrophils. Journal of biomedical science 19, 89.

Bennett, M.J., Pollitt, R.J., Taitz, L.S., Variend, S., 1990. Medium-chain acyl-CoA dehydrogenase deficiency: a useful diagnosis five years after death. Clinical chemistry 36, 1695-1697.

Bernardi, P., Broekemeier, K.M., Pfeiffer, D.R., 1994. Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. Journal of bioenergetics and biomembranes 26, 509-517.

Beynen, A.C., Geelen, M.J., 1982. Short-term inhibition of fatty acid biosynthesis in isolated hepatocytes by mono-aromatic compounds. Toxicology 24, 183-197.

Beyoglu, D., Idle, J.R., 2012. The glycine deportation system and its pharmacological consequences. Pharmacology and therapeutics 135, 151-167.

Beyoglu, D., Smith, R.L., Idle, J.R., 2012. Dog bites man or man bites dog? The enigma of the amino acid conjugations. Biochemical pharmacology 83, 1331-1339.

Bichara, D., Calcaterra, N.B., Arranz, S., Armas, P., Simonetta, S.H., 2014. Set-up of an infrared fast behavioral assay using zebrafish (Danio rerio) larvae, and its application in compound biotoxicity screening. Journal of applied toxicology 34 (2), 214-9.

Bock, K.W., 2014. Homeostatic control of xeno- and endobiotics in the drug-metabolizing enzyme system. Biochemical pharmacology 90, 1-6.

Bonafe, L., Troxler, H., Kuster, T., Heizmann, C.W., Chamoles, N.A., Burlina, A.B., Blau, N., 2000. Evaluation of Urinary Acylglycines by Electrospray Tandem Mass Spectrometry in Mitochondrial Energy Metabolism Defects and Organic Acidurias. Molecular genetics and metabolism 69, 302-311.

Boomgaarden, I., Vock, C., Klapper, M., Doring, F., 2009. Comparative analyses of disease risk genes belonging to the acyl-CoA synthetase medium-chain (ACSM) family in human liver and cell lines. Biochemical genetics 47, 739-748.

Bradshaw, H.B., Walker, J.M., 2005. The expanding field of cannabimimetic and related lipid mediators. British journal of pharmacology 144, 459-465.

Bradshaw, P.C., Pfeiffer, D.R., 2006. Loss of NAD(H) from swollen yeast mitochondria. BMC biochemistry 7, 3.

Brady, R.O., Pentchev, P.G., Gal, A.E., Hibbert, S.R., Dekaban, A.S., 1974. Replacement therapy for inherited enzyme deficiency: use of purified glucocerebrosidase in Gaucher's disease. New England journal of medicine 291, 989-993.

Bravo, L., 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutrition reviews 56, 317-333.

Bray, H.G., Thorpe, W.V., White, K., 1951. Kinetic studies of the metabolism of foreign organic compounds; the formation of benzoic acid from benzamide toluene, benzyl alcohol and benzaldehyde and its conjugation with glycine and glucuronic acid in the rabbit. The Biochemical journal 48, 88-96.

Broekemeier, K.M., Pfeiffer, D.R., 1995. Inhibition of the mitochondrial permeability transition by cyclosporin A during long time frame experiments: relationship between pore opening and the activity of mitochondrial phospholipases. Biochemistry 34, 16440-16449.

Brosnan, J.T., da Silva, R.P., Brosnan, M.E., 2011. The metabolic burden of creatine synthesis. Amino acids 40, 1325-1331.

Bursilow, S.W., Valle, D.L., Batshaw, M.L., 1979. New pathways of nitrogen excretion in inborn errors of urea synthesis. Lancet II, 452-454.

Caldwell, J., 1982. Conjugation reactions in foreign-compound metabolism: definition, consequences, and species variations. Drug metabolism reviews 13, 745-777.

Caldwell, J., 1984. Xenobiotic acyl-coenzymes A: critical intermediates in the biochemical pharmacology and toxicology of carboxylic acids. Biochemical society transactions 12, 9-11.

Campbell, L., Wilson, H.K., Samuel, A.M., Gompertz, D., 1988. Interactions of m-xylene and aspirin metabolism in man. British journal of industrial medicine 45, 127-132.

Cantz, M., Kresse, H., 1974. Sandhoff disease: defective glycosaminoglycan catabolism in cultured fibroblasts and its correction by b-N-acetylhexosaminidase. European journal of biochemistry 47, 581-590.

Chan, B., Wara, D., Bastian, J., Hershfield, M.S., Bohnsack, J., Azen, C.G., Parkman, R., Weinberg, K., Kohn, D.B., 2005. Long-term efficacy of enzyme replacement therapy for Adenosine deaminase (ADA)-deficient Severe Combined Immunodeficiency (SCID). Clinical immunology 117, 133-143.

Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J., 2001. Nuclear receptors and lipid physiology: opening the X-files. Science 294, 1866-1870.

Chen, Q., Huang, N.N., Huang, J.T., Chen, S., Fan, J., Li, C., Xie, F.K., 2009. Sodium benzoate exposure downregulates the expression of tyrosine hydroxylase and dopamine transporter in dopaminergic neurons in developing zebrafish. Birth defects research. Part B, Developmental and reproductive toxicology 86, 85-91.

Conter, C., Rolland, M.O., Cheillan, D., Bonnet, V., Maire, I., Froissart, R., 2006. Genetic heterogeneity of the GLDC gene in 28 unrelated patients with glycine encephalopathy. Journal of inherited metabolic disease 29, 135-142.

Cremin Jr, J.D., Drackley, J.K., Grum, D.E., Hansen, L.R., Fahey Jr, G.C., 1994. Effects of reduced phenolic acids on metabolism of propionate and palmitate in bovine liver tissue in vitro. Journal of dairy science 77, 3608-3617.

Cyr, D.M., Egan, S.G., Brini, C.M., Tremblay, G.C., 1991. On the mechanism of inhibition of gluconeogenesis and ureagenesis by sodium benzoate. Biochemical pharmacology 42, 645-654.

Dakin, H.D., 1908. Comparative studies of the mode of oxidation of phenyl derivatives of fatty acids by the animal organism and by hydrogen peroxide. Journal of biological chemistry 4, 419-435.

Dakin, H.D., 1910. The fate of sodium benzoate in the human organism. Journal of biological chemistry 7, 103-108.

Davies, K.M., Strauss, M., Daum, B., Kief, J.H., Osiewacz, H.D., Rycovska, A., Zickermann, V., Kuhlbrandt, W., 2011. Macromolecular organization of ATP synthase and complex I in whole mitochondria. Proceedings of the national academy of sciences of the United States of America 108, 14121-14126.

de Vries, A., Alexander, B., Quamo, Y., 1948. Studies on amino acid metabolism. Iii. Plasma glycine concentration and hippuric acid formation following the ingestion of benzoate 1. The journal of clinical investigation 27, 665-668.

Deguchi, T., Takemoto, M., Uehara, N., Lindup, W.E., Suenaga, A., Otagiri, M., 2005. Renal clearance of endogenous hippurate correlates with expression levels of renal organic anion transporters in uremic rats. The journal of pharmacology and experimental therapeutics 314, 932-938.

Demir, E., Kocaoglu, S., Kaya, B., 2010. Assessment of genotoxic effects of benzyl derivatives by the comet assay. Food and chemical toxicology : an international journal published for the British industrial biological research association 48, 1239-1242.

Dercksen, M., Duran, M., Ijlst, L., Mienie, L.J., Reinecke, C.J., Ruiter, J.P., Waterham, H.R., Wanders, R.J., 2012. Clinical variability of isovaleric acidemia in a genetically homogeneous population. Journal of inherited metabolic disease 2012, 17.

Doring, B., Petzinger, E., 2014. Phase 0 and phase III transport in various organs: Combined concept of phases in xenobiotic transport and metabolism. Drug metabolism reviews 46 (3), 261-82.

Dorne, J.L.C.M., Walton, K., Renwick, A.G., 2004. Human variability for metabolic pathways with limited data (CYP2A6, CYP2C9, CYP2E1, ADH, esterases, glycine and sulphate conjugation). Food and chemical toxicology 42, 397-421.

Duffy, L.F., Kerzner, B., Seeff, L., Barr, S.B., Soldin, S.J., 1995. Preliminary assessment of glycine conjugation of para-aminobenzoic acid as a quantitative test of liver function. Clinical biochemistry 28, 527-530.

Erfle, J.D., Sauer, F., 1969. The inhibitory effects of acyl-coenzyme a esters on the pyruvate and α-oxoglutarate dehydrogenase complexes. Biochimica et biophysica acta (BBA) - Enzymology 178, 441-452.

Fears, R., 1985. Lipophilic xenobiotic conjugates: the pharmacological and toxicological consequences of the participation of drugs and other foreign compounds as substrates in lipid biosynthesis. Progress in lipid research 24, 177-195.

Fedotcheva, N.I., Kazakov, R.E., Kondrashova, M.N., Beloborodova, N.V., 2008. Toxic effects of microbial phenolic acids on the functions of mitochondria. Toxicology letters 180, 182-188.

Fedotcheva, T.A., Shimanovskii, N.L., Kruglov, A.G., Teplova, V.V., Fedotcheva, N.I., 2012. Role of mitochondrial thiols of different localization in the generation of reactive oxygen species. Biochemistry (Moscow) supplement series b biomedical chemistry A 6, 92-99.

Fenton, W.A., Gravel, R.A., Rosenblatt, D.S., 2013. Part 9: Organic acids. Chapter 94: Disorders of Propionate and Methylmalonate Metabolism, The online metabolic and molecular bases of inherited diseases. McGraw Hill.

Feoli-Fonseca, J.C., Lambert, M., Mitchell, G., Melancon, S.B., Dallaire, L., Millington, D.S., Qureshi, I.A., 1996. Chronic Sodium Benzoate Therapy in Children with Inborn Errors of Urea Synthesis: Effect on Carnitine Metabolism and Ammonia Nitrogen Removal. Biochemical and molecular medicine 57, 31-36.

Fromenty, B., Pessayre, D., 1995. Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. Pharmacology and therapeutics 67, 101-154.

Fujino, T., Takei, Y.A., Sone, H., Ioka, R.X., Kamataki, A., Magoori, K., Takahashi, S., Sakai, J., Yamamoto, T.T., 2001. Molecular identification and characterization of two medium-chain acyl-CoA synthetases, MACS1 and the Sa gene product. The journal of biological chemistry 276, 35961-35966.

Fulton, T.R., Triano, T., Rabe, A., Loo, Y.H., 1980. Phenylacetate and the enduring behavioral deficit in experimental phenylketonuria. Life sciences 27, 1271-1281.

Furst, D.E., Gupta, N., Paulus, H.E., 1977. Salicylate Metabolism in Twins. Evidence suggesting a generic influence and induction of salicylurate formation. The journal of clinical investigation 60, 32-42.

Furuya, K.N., Durie, P.R., Roberts, E.A., Soldin, S.J., Verjee, Z., Yung-Jato, L., Giesbrecht, E., Ellis, L., 1995. Glycine Conjugation of Para-Aminobenzoic Acid (PABA): A Quantitative Test of Liver Function. Clinical biochemistry 28, 531-540.

Gatley, S.J., Sherratt, H.S., 1977. The synthesis of hippurate from benzoate and glycine by rat liver mitochondria. Submitochondrial localization and kinetics. The biochemical journal 166, 39-47.

Gatley, S.J., Sherratt, S.A., 1976. The localization of hippurate synthesis in the matrix of rat liver mitochondria. Biochemical society transactions 4, 525-526.

Gibson, G., Skett, P., 2001. Introduction to drug metabolism, 3 ed, Cheltenham, UK.

Glatt, H., 2000. Sulfotransferases in the bioactivation of xenobiotics. Chemico-biological interactions 129, 141-170.

Goldstone, J.V., Hamdoun, A., Cole, B.J., Howard-Ashby, M., Nebert, D.W., Scally, M., Dean, M., Epel, D., Hahn, M.E., Stegeman, J.J., 2006. The chemical defensome: environmental sensing and response genes in the Strongylocentrotus purpuratus genome. Developmental biology 300, 366-384.

Gompertz, D., 1974. Inborn errors of organic acid metabolism. Clinics in endocrinology and metabolism 3.

Gregersen, N., Kolvraa, S., Mortensen, P.B., 1986. Acyl-CoA: glycine N-acyltransferase: in vitro studies on the glycine conjugation of straight- and branched-chained acyl-CoA esters in human liver. Biochemical medicine and metabolic biology 35, 210-218.

Gregus, Z., Fekete, T., Halaszi, E., Klaassen, C.D., 1996a. Does hepatic ATP depletion impair glycine conjugation in vivo? Drug metabolism and disposition: the biological fate of chemicals 24, 1347-1354.

Gregus, Z., Fekete, T., Halaszi, E., Klaassen, C.D., 1996b. Lipoic acid impairs glycine conjugation of benzoic acid and renal excretion of benzoylglycine. Drug metabolism and disposition: the biological fate of chemicals 24, 682-688.

Gregus, Z., Fekete, T., Varga, F., Klaassen, C.D., 1992. Availability of glycine and coenzyme A limits glycine conjugation in vivo. Drug metabolism and disposition: the biological fate of chemicals 20, 234-240.

Gregus, Z., Fekete, T., Varga, F., Klaassen, C.D., 1993a. Dependence of glycine conjugation on availability of glycine: role of the glycine cleavage system. Xenobiotica; the fate of foreign compounds in biological systems 23, 141-153.

Gregus, Z., Fekete, T., Varga, F., Klaassen, C.D., 1993b. Effect of valproic acid on glycine conjugation of benzoic acid. Journal of pharmacology and experimental therapeutics 267, 1068-1075.

Griffith, A.D., Cyr, D.M., Egan, S.G., Tremblay, G.C., 1989. Inhibition of pyruvate carboxylase by sequestration of coenzyme A with sodium benzoate. Archives of biochemistry and biophysics 269, 201-207.

Grillo, M.P., Benet, L.Z., 2002. Studies on the reactivity of clofibryl-S-acyl-CoA thioester with glutathione in vitro. Drug metabolism and disposition: the biological fate of chemicals 30, 55-62.

Grillo, M.P., Lohr, M.T., 2009. Covalent binding of phenylacetic acid to protein in incubations with freshly isolated rat hepatocytes. Drug metabolism and disposition: the biological fate of chemicals 37, 1073-1082.

Gron, I., Gregersen, N., Kolvraa, S., Rasmussen, K., 1978. Formation of *N*-dicarboxyl-mono-glycines catalyzed bt glycine-*N*-Acylase. Biochemical medicine 19, 133-140.

Groot, P.H., 1976. Acyl-CoA synthetases in guinea-pig liver mitochondria. Purification and characterization of a distinct propionyl-CoA synthetase. Biochim biophys acta 441, 260-267.

Guo, Y., Zhang, L., Liu, Y., Hu, H., Li, J., Tian, Q., Yu, P., Zhang, F., Yang, T., Guo, Y., Peng, X., Dai, M., Chen, W., Deng, H., 2013. Suggestion of *GLYAT* gene underlying variation of bone size and body lean mass as revealed by a bivariate genome-wide association study. Human genetics 132, 189-199.

Gutknecht, J., 1992. Aspirin, acetaminophen and proton transport through phospholipid bilayers and mitochondrial membranes. Molecular and cellular biochemistry 114, 3-8.

Hamosh, A., Johnston, M.V., 2013. Part 8: Amino acids. Chapter 90: Nonketotic hyperglycinemia, The online metabolic and molecular bases of inherited diseases. McGraw Hill.

Hansch, C., Leo, A., 1995. Exploring QSAR. American chemical society, Washington.

Hansch, C., Lien, E.J., Helmer, F., 1968. Structure-activity correlations in the metabolism of drugs. Archives of biochemistry and biophysics 128, 319-330.

Hazan, R., Levine, A., Abeliovich, H., 2004. Benzoic acid, a weak organic acid food preservative, exerts specific effects on intracellular membrane trafficking pathways in Saccharomyces cerevisiae. Applied and environmental microbiology 70, 4449-4457.

Hiraga, K., Kochi, H., Hayasaka, K., Kikuchi, G., Nyhan, W.L., 1981. Defective glycine cleavage system in nonketotic hyperglycinemia. Occurrence of a less active glycine decarboxylase and an abnormal aminomethyl carrier protein. The journal of clinical investigation 68, 525-534.

Holmes, E., Li, J.V., Athanasiou, T., Ashrafian, H., Nicholson, J.K., 2011. Microbes and Metabolism. Understanding the role of gut microbiome–host metabolic signal disruption in health and disease. Trends in microbiology 19, 349-359.

Houston, J., Kenworthy, K., Galetin, A., 2003. Typical and atypical enzyme kinetics. Fontis Media, Lausanne, Switzerland.

Houten, S.M., Wanders, R.J., 2010. A general introduction to the biochemistry of mitochondrial fatty acid beta-oxidation. Journal of inherited metabolic disease 33, 469-477.

Huang, M., Jin, C., Liu, Y., Li, B., Qu, Q., Uchida, Y., Inoue, O., Nakatsuka, H., Watanabe, T., Ikeda, M., 1994. Exposure of workers to a mixture of toluene and xylenes. I metabolism. Occupational and environmental medicine 51, 42-46.

Ioannides, C., 1996. Cytochromes P450: Metabolic and toxicological aspects. CRC Press.

Irjala, K., 1972. Synthesis of p-aminohippuric, hippuric, and salicyluric acids in experimental animals and man. Annales Academiae Scientiarum Fennicae. Ser. A.5, Medica 154, 1-40.

Ishikawa, T., 1992. The ATP-dependent glutathione S-conjugate export pump. Trends in biochemical sciences 17, 463-468.

Itoh, T., Ito, T., Ohba, S., Sugiyama, N., Mizuguchi, K., Yamaguchi, S., Kidouchi, K., 1996. Effect of carnitine administration on glycine metabolism in patients with isovaleric acidemia: significance of acetylcarnitine determination to estimate the proper carnitine dose. The Tohoku journal of experimental medicine 179, 101-109.

Jackson, A.A., 1991. The glycine story. European journal of clinical nutrition 45, 59-65.

Jackson, A.A., Badaloo, A.V., Forrester, T., Hibbert, J.M., Persaud, C., 1987. Urinary excretion of 5-oxoproline (pyroglutamic aciduria) as an index of glycine insufficiency in normal man. The British journal of nutrition 58, 207-214.

Jackson, A.A., Persaud, C., Hall, M., Smith, S., Evans, N., Rutter, N., 1997. Urinary excretion of 5-L-oxoproline (pyroglutamic acid) during early life in term and preterm infants. Archives of disease in childhood. Fetal and neonatal edition 76, F152-157.

Jackson, A.A., Persaud, C., Meakins, T.S., Bundy, R., 1996. Urinary excretion of 5-L-oxoproline (pyroglutamic acid) is increased in normal adults consuming vegetarian or low protein diets. The journal of nutrition 126, 2813-2822.

Jain, M., Nilsson, R., Sharma, S., Madhusudhan, N., Kitami, T., Souza, A.L., Kafri, R., Kirschner, M.W., Clish, C.B., Mootha, V.K., 2012. Metabolite Profiling Identifies a Key Role for Glycine in Rapid Cancer Cell Proliferation. Science. 336, 1040-1044.

Jancova, P., Anzenbacher, P., Anzenbacherova, E., 2010. Phase II drug metabolizing enzymes. Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia 154, 103-116.

Jenner, A.M., Rafter, J., Halliwell, B., 2005. Human fecal water content of phenolics: the extent of colonic exposure to aromatic compounds. Free radical biology & medicine 38, 763-772.

Josephy, P., Peter Guengerich, F., Miners, J.O., 2005. "Phase I and Phase II" drug metabolism: terminology that we should phase out? Drug metabolism reviews 37, 575-580.

Kalbag, S.S., Palekar, A.G., 1988. Sodium benzoate inhibits fatty acid oxidation in rat liver: Effect on ammonia levels. Biochemical medicine and metabolic biology 40, 133-142.

Kasuya, F., Igarashi, K., Fukui, M., 1990. Glycine conjugation of the substituted benzoic acids in vitro: structure-metabolism relationship study. Journal of pharmacobio-dynamics 13, 432-440.

Kasuya, F., Igarashi, K., Fukui, M., 1991. Glycine conjugation of the substituted benzoic acids in mice: structure-metabolism relationship study II. Journal of pharmacobio-dynamics 14, 671-677.

Kasuya, F., Igarashi, K., Fukui, M., 1996a. Inhibition of a medium chain acyl-CoA synthetase involved in glycine conjugation by carboxylic acids. Biochemical pharmacology 52, 1643-1646.

Kasuya, F., Igarashi, K., Fukui, M., Nokihara, K., 1996b. Purification and characterization of a medium chain acyl-coenzyme A synthetase. Drug metabolism disposition. 24, 879-883.

Kasuya, F., Yamaoka, Y., Osawa, E., Igarashi, K., Fukui, M., 2000. Difference of the liver and kidney in glycine conjugation of ortho-substituted benzoic acids. Chemico-Biological Interactions 125, 39-50.

Keller, W., 1842. On the Conversion of Benzoic Acid into Hippuric Acid. Provincial Medical Journal and Retrospect of the Medical sciences 4, 256-257.

Kelley, M., Vessey, D., 1990. The effects of ions on the conjugation of xenobiotics by the aralkyl-CoA and arylacetyl-CoA N-acyltransferases from bovine liver mitochondria. Journal of biochemical toxicolology 5, 125-135.

Kelley, M., Vessey, D., 1994. Characterization of the acyl-CoA: Amino acid N-acyltransferases from primate liver mitochondria. Journal of biochemical toxicology 9, 153-158.

Kelley, M., Vessey, D.A., 1993. Isolation and characterization of mitochondrial acyl-CoA: glycine N-acyltransferases from kidney. Journal of biochemical toxicology 8, 63-69.

Khasnavis, S., Pahan, K., 2012. Sodium benzoate, a metabolite of cinnamon and a food additive, upregulates neuroprotective Parkinson disease protein DJ-1 in astrocytes and neurons. Journal of neuroimmune pharmacology : the official journal of the society on neuroImmune pharmacology 7, 424-435.

Kikuchi, G., Motokawa, Y., Yoshida, T., Hiraga, K., 2008. Glycine cleavage system: reaction mechanism, physiological significance, and hyperglycinemia. Proceedings of the Japan Academy. Series B, Physical and biological sciences 84, 246-263.

Killenberg, P.G., Davidson, E.D., Webster, L.T., 1971. Evidence for a medium-chain fatty acid: coenzyme A ligase (adenosine monophosphate) that activates salicylate. Molecular pharmacology 7, 260-268.

Kimura, I., Inoue, D., Maeda, T., Hara, T., Ichimura, A., Miyauchi, S., Kobayashi, M., Hirasawa, A., Tsujimoto, G., 2011. Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). Proceedings of the national academy of sciences 108, 8030-8035.

Klingenberg, M., 1958. Pigments of rat liver microsomes. Archives of biochemistry and biophysics 75, 376-386.

Knights, K.M., 1998. Role of hepatic fatty acid:coenzyme A ligases in the metabolism of xenobiotic carboxylic acids. Clininal and experimental pharmacology and physiology 25, 776-782.

Knights, K.M., Drogemiller, C.J., 2000. Xenobiotic-CoA Ligases: Kinetic and Molecular Characterization. Current drug metabolism 1, 49-66.

Knights, K.M., Miners, J.O., 2012. Amino Acid Conjugation: A Novel Route of Xenobiotic Carboxylic Acid Metabolism in Man, 1st ed. John Wiley & Sons, Inc.

Knights, K.M., Sykes, M.J., Miners, J.O., 2007. Amino acid conjugation: contribution to the metabolism and toxicity of xenobiotic carboxylic acids. Expert opinion in drug metabolism and toxicology. 3, 159-168.

Knoop, F., 1904. Der Abbau aromatischer Fettsäuren im Tierkörper.

Kolvraa, S., 1979. Inhibition of the glycine cleavage system by branched-chain amino acid metabolites. Pediatric research 13, 889-893.

Kolvraa, S., Gregersen, N., 1986. Acyl-CoA:glycine N-acyltransferase: organelle localization and affinity toward straight- and branched-chained acyl-CoA esters in rat liver. Biochemical medicine and metabolic biology 36, 98-105.

Krahenbuhl, L., Reichen, J., Talos, C., Krahenbuhl, S., 1997. Benzoic acid metabolism reflects hepatic mitochondrial function in rats with long-term extrahepatic cholestasis. Hepatology (Baltimore, Md.) 25, 278-283.

Krieger, I., Tanaka, K., 1976. Therapeutic Effects of Glycine in Isovaleric Acidemia. Pediatric research 10, 25-29.

Krieger, I., Winbaum, E.S., Eisenbrey, A.B., 1977. Cerebrospinal fluid glycine in nonketotic hyperglycinemic: effect of treatment with sodium benzoate and a ventricular shunt. Metabolism: clinical and experimental 26, 517-524.

Kubota, K., Ishizaki, T., 1991. Dose-dependent pharmacokinetics of benzoic acid following oral administration of sodium benzoate to humans. European journal of clinical pharmacology 41, 363-368.

Kure, S., Ichinohe, A., Kojima, K., Sato, K., Kizaki, Z., Inoue, F., Yamanaka, C., Matsubara, Y., 2004. Mild variant of nonketotic hyperglycinemia with typical neonatal presentations: mutational and in vitro expression analyses in two patients. The journal of pediatrics 144, 827-829.

Kushnareva, Y.E., Sokolove, P.M., 2000. Prooxidants open both the mitochondrial permeability transition pore and a low-conductance channel in the inner mitochondrial membrane. Archives of biochemistry and biophysics 376, 377-388.

Lakeram, M., Lockley, D.J., Sanders, D.J., Pendlington, R., Forbes, B., 2007. Paraben transport and metabolism in the biomimetic artificial membrane permeability assay (BAMPA) and 3-day and 21-day Caco-2 cell systems. Journal of biomolecular screening 12, 84-91.

Lau, E.P., Haley, B.E., Barden, R.E., 1977. Photoaffinity labeling of acyl-coenzyme A:glycine N-acyltransferase with p-azidobenzoyl-coenzyme A. Biochemistry 16, 2581-2585.

Lebel, S., Nakamachi, Y., Hemming, A., Verjee, Z., Phillips, M.J., Furuya, K.N., 2003. Glycine conjugation of para-aminobenzoic acid (PABA): a pilot study of a novel prognostic test in acute liver failure in children. Journal of pediatric gastroenterology and nutrition 36, 62-71.

Lees, H.J., Swann, J.R., Wilson, I.D., Nicholson, J.K., Holmes, E., 2013. Hippurate: The Natural History of a Mammalian-Microbial Cometabolite. Journal of proteome research 12 (4), 1527-46.

Lemasters, J.J., Qian, T., He, L., Kim, J.S., Elmore, S.P., Cascio, W.E., Brenner, D.A., 2002. Role of mitochondrial inner membrane permeabilization in necrotic cell death, apoptosis, and autophagy. Antioxidants and redox signaling 4, 769-781.

Levy, G., 1979. Pharmacokinetics of salicylate in man. Drug metabolism reviews 9, 3-19.

Lewis, R.M., Godfrey, K.M., Jackson, A.A., Cameron, I.T., Hanson, M.A., 2005. Low Serine Hydroxymethyltransferase Activity in the Human Placenta Has Important Implications for Fetal Glycine Supply. The journal of clinical endocrinology and metabolism 90, 1594-1598.

Lheureux, P.E., Hantson, P., 2009. Carnitine in the treatment of valproic acid-induced toxicity. Clinical toxicology 47, 101-111.

Li, C., Benet, L.Z., Grillo, M.P., 2002. Studies on the chemical reactivity of 2-phenylpropionic acid 1-O-acyl glucuronide and S-acyl-CoA thioester metabolites. Chemical research in toxicology 15, 1309-1317.

Li, C., Grillo, M.P., Benet, L.Z., 2003. In vitro studies on the chemical reactivity of 2,4-dichlorophenoxyacetyl-S-acyl-CoA thioester. Toxicology and applied pharmacology 187, 101-109.

Lindner, I., Rubin, D., Helwig, U., Nitz, I., Hampe, J., Schreiber, S., Schrezenmeir, J., Doring, F., 2006. The L513S polymorphism in medium-chain acyl-CoA synthetase 2 (MACS2) is associated with risk factors of the metabolic syndrome in a Caucasian study population. Molecular nutrition & food research 50, 270-274.

Lino Cardenas, C.L., Bourgine, J., Cauffiez, C., Allorge, D., Lo-Guidice, J.M., Broly, F., Chevalier, D., 2010. Genetic polymorphisms of Glycine N-acyltransferase (GLYAT) in a French Caucasian population. Xenobiotica; the fate of foreign compounds in biological systems 40, 853-861.

Liska, D.J., 1998. The detoxification enzyme systems. Alternative medicine review : a journal of clinical therapeutic 3, 187-198.

Loftus, B.J., Kim, U.J., Sneddon, V.P., Kalush, F., Brandon, R., Fuhrmann, J., Mason, T., Crosby, M.L., Barnstead, M., Cronin, L., Deslattes Mays, A., Cao, Y., Xu, R.X., Kang, H.L., Mitchell, S., Eichler, E.E., Harris, P.C., Venter, J.C., Adams, M.D., 1999. Genome duplications and other features in 12 Mb of DNA sequence from human chromosome 16p and 16q. Genomics 60, 295-308.

Loo, Y.H., Fulton, T., Miller, K., Wisniewski, H.M., 1980. Phenylacetate and brain dysfunction in experimental phenylketonuria: synaptic development. Life sciences 27, 1283-1290.

Loo, Y.H., Fulton, T., Miller, K.A., Wisniewski, H.M., 1979. Vulnerability of the immature rat brain to phenylacetate intoxication: postnatal development of the detoxication mechanism. Journal of neurochemistry 32, 1699-1705.

Loo, Y.H., Potempska, A., Wisniewski, H.M., 1985. A biochemical explanation of phenyl acetate neurotoxicity in experimental phenylketonuria. Journal of neurochemistry 45, 1596-1600.

Loots, D.T., Mienie, L.J., Erasmus, E., 2007. Amino-acid depletion induced by abnormal amino-acid conjugation and protein restriction in isovaleric acidemia. European journal of clinical nutrition 61, 1323-1327.

Loots, D., Erasmus, E., Mienie, L.J., 2005. Identification of 19 New Metabolites Induced by Abnormal Amino Acid Conjugation in Isovaleric Acidemia,. Clinical chemistry 51, 1510-1512.

Mahler, H.R., Wakil, S.J., Bock, R.M., 1953. Studies on fatty acid oxidation. I. Enzymatic activation of fatty acids. The Journal of biological chemistry 204, 453-468.

Mancinelli, L., Cronin, M., Sadee, W., 2000. Pharmacogenomics: the promise of personalized medicine. AAPS pharmSci 2, E4.

Mao, L.-F., Chu, C., Schulz, H., 1994. Hepatic .beta.-oxidation of 3-phenylpropionic acid and the stereospecific dehydration of (R)- and (S)-3-hydroxy-3-phenylpropionyl-CoA by different enoyl-CoA hydratases. Biochemistry 33, 3320-3326.

Martin, J., Han, C., Gordon, L.A., Terry, A., Prabhakar, S., She, X., Xie, G., Hellsten, U., Chan, Y.M., Altherr, M., Couronne, O., Aerts, A., Bajorek, E., Black, S., Blumer, H., Branscomb, E., Brown, N.C., Bruno, W.J., Buckingham, J.M., Callen, D.F., Campbell, C.S., Campbell, M.L., Campbell, E.W., Caoile, C., Challacombe, J.F., Chasteen, L.A., Chertkov, O., Chi, H.C., Christensen, M., Clark, L.M., Cohn, J.D., Denys, M., Detter, J.C., Dickson, M., Dimitrijevic-Bussod, M., Escobar, J., Fawcett, J.J., Flowers, D., Fotopulos, D., Glavina, T., Gomez, M., Gonzales, E., Goodstein, D., Goodwin, L.A., Grady, D.L., Grigoriev, I., Groza, M., Hammon, N., Hawkins, T., Haydu, L., Hildebrand, C.E., Huang, W., Israni, S., Jett, J., Jewett, P.B., Kadner, K., Kimball, H., Kobayashi, A., Krawczyk, M.C., Leyba, T., Longmire, J.L., Lopez, F., Lou, Y., Lowry, S., Ludeman, T., Manohar, C.F., Mark, G.A., McMurray, K.L., Meincke, L.J., Morgan, J., Moyzis, R.K., Mundt, M.O., Munk, A.C., Nandkeshwar, R.D., Pitluck, S., Pollard, M., Predki, P., Parson-Quintana, B., Ramirez, L., Rash, S., Retterer, J., Ricke, D.O., Robinson, D.L., Rodriguez, A., Salamov, A., Saunders, E.H., Scott, D., Shough, T., Stallings, R.L., Stalvey, M., Sutherland, R.D., Tapia, R., Tesmer, J.G., Thayer, N., Thompson, L.S., Tice, H., Torney, D.C., Tran-Gyamfi, M., Tsai, M., Ulanovsky, L.E., Ustaszewska, A., Vo, N., White, P.S., Williams, A.L., Wills, P.L., Wu, J.R., Wu, K., Yang, J., Dejong, P., Bruce, D., Doggett, N.A., Deaven, L., Schmutz, J., Grimwood, J., Richardson, P., Rokhsar, D.S., Eichler, E.E., Gilna, P., Lucas, S.M., Myers, R.M., Rubin, E.M., Pennacchio, L.A., 2004. The sequence and analysis of duplication-rich human chromosome 16. Nature 432, 988-994.

Masuda, T., Nakamura, K., Jikihara, T., Kasuya, F., Igarashi, K., Fukui, M., Takagi, T., Fujiwara, H., 1996. 3D-Quantitative Structure-Activity Relationships for Hydrophobic Interactions: Comparative Molecular Field Analysis (CoMFA) including Molecular Lipophilicity Potentials as Applied to the Glycine Conjugation of Aromatic as well as Aliphatic Carboxylic Acids. Quantitative structure-activity relationships 15, 194-200.

Matsuo, M., Terai, K., Kameda, N., Matsumoto, A., Kurokawa, Y., Funase, Y., Nishikawa, K., Sugaya, N., Hiruta, N., Kishimoto, T., 2012. Designation of enzyme activity of glycine-N-acyltransferase family genes and depression of glycine-N-acyltransferase in human hepatocellular carcinoma. Biochemical and biophysical research communications 420, 901-906.

Mawal, Y.R., Qureshi, I.A., 1994. Purification to homogeneity of mitochondrial acyl coa:glycine n-acyltransferase from human liver. Biochemical and biophysical research communications 205, 1373-1379.

Melendez-Hevia, E., De Paz-Lugo, P., Cornish-Bowden, A., Cardenas, M.L., 2009. A weak link in metabolism: the metabolic capacity for glycine biosynthesis does not satisfy the need for collagen synthesis. Journal of biosciences 34, 853-872.

Mitchell, G.A., Gauthier, N., Lesimple, A., Wang, S.P., Mamer, O., Qureshi, I., 2008. Hereditary and acquired diseases of acyl-coenzyme A metabolism. Molecular genetics and metabolism 94, 4-15.

Mohanka, M., Khemasuwan, D., Stoller, J.K., 2012. A review of augmentation therapy for alpha-1 antitrypsin deficiency. Expert opinion on biological therapy 12, 685-700.

Mortensen, P.B., Kolvraa, S., Christensen, E., 1980. Inhibition of the glycine cleavage system: hyperglycinemia and hyperglycinuria caused by valproic acid. Epilepsia 21, 563-569.

Munck, A., Duhamel, J.F., Lamireau, T., Le Luyer, B., Le Tallec, C., Bellon, G., Roussey, M., Foucaud, P., Ginies, J.L., Houzel, A., Marguet, C., Guillot, M., David, V., Kapel, N., Dyard, F., Henniges, F., 2009. Pancreatic enzyme replacement therapy for young cystic fibrosis patients. Journal of cystic fibrosis 8, 14-18.

Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. The biochemical journal 417, 1-13.

Nakagawa, Y., Moldeus, P., 1998. Mechanism of p-hydroxybenzoate ester-induced mitochondrial dysfunction and cytotoxicity in isolated rat hepatocytes. Biochemical pharmacology 55, 1907-1914.

Nakagawa, Y., Moore, G., 1999. Role of mitochondrial membrane permeability transition in p-hydroxybenzoate ester-induced cytotoxicity in rat hepatocytes. Biochemical pharmacology 58, 811-816.

Nandi, D.L., Lucas, S.V., Webster, L.T., Jr., 1979. Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine N-acyltransferase from bovine liver mitochondria. Purification and characterization. Journal of biological chemistry. 254, 7230-7237.

Nebert, D.W., 1991. Proposed role of drug-metabolizing enzymes: regulation of steady state levels of the ligands that effect growth, homeostasis, differentiation, and neuroendocrine functions. Molecular endocrinology 5, 1203-1214.

Norman, C., Howell, K.A., Millar, A.H., Whelan, J.M., Day, D.A., 2004. Salicylic acid is an uncoupler and inhibitor of mitochondrial electron transport. Plant physiology 134, 492-501.

Oates, P.S., West, A.R., 2006. Heme in intestinal epithelial cell turnover, differentiation, detoxification, inflammation, carcinogenesis, absorption and motility. World journal of gastroenterology 12, 4281-4295.

Ogier, d.B., Saudubray, J.M., 2002. Branched-chain organic acidurias. Seminars in neonatology 7, 65-74.

Olsen, J., Li, C., Skonberg, C., Bjornsdottir, I., Sidenius, U., Benet, L.Z., Hansen, S.H., 2007. Studies on the metabolism of tolmetin to the chemically reactive acyl-coenzyme A thioester intermediate in rats. Drug metabolism and disposition: the biological fate of chemicals 35, 758-764.

Omiecinski, C.J., Vanden Heuvel, J.P., Perdew, G.H., Peters, J.M., 2011. Xenobiotic metabolism, disposition, and regulation by receptors: from biochemical phenomenon to predictors of major toxicities. Toxicological sciences : an official journal of the society of toxicology 120 Suppl 1, S49-75.

Omura, T., Sato, R., Cooper, D.Y., Rosenthal, O., Estabrook, R.W., 1965. Function of cytochrome P-450 of microsomes. Federation proceedings 24, 1181-1189.

Oyanagi, K., Kuniya, Y., Nagao, M., Tsuchiyama, A., Nakao, T., 1987. Cytotoxicities of sodium benzoate in primary culture of hepatocytes from adult rat liver. The Tohoku journal of experimental medicine 152, 47-51.

Palekar, A., 2000. Effect of pantothenic acid on hippurate formation in sodium benzoate-treated HepG2 cells. Pediatric research 48, 357-359.

Palekar, A.G., Kalbag, S.S., 1991. Amino acids in the rat liver and plasma and some metabolites in the liver after sodium benzoate treatment. Biochemical medicine and metabolic biology 46, 52-58.

Park, H.-W., Park, E.H., Yun, H.-M., Rhim, H., 2011. Sodium benzoate-mediated cytotoxicity in mammalian cells. Journal of food biochemistry 35, 1034-1046.

Park, W.H., Kang, Y.C., Piao, Y., Pak, D.H., Pak, Y.K., 2013. Causal effects of synthetic chemicals on mitochondrial deficits and diabetes pandemic. Archives of pharmacalogical research 36, 178-188.

Perry, T.L., Urquhart, N., MacLean, J., Evans, M.E., Hansen, S., Davidson, A.G.F., Applegarth, D.A., MacLeod, P.J., Lock, J.E., 1975. Nonketotic Hyperglycinemia. New England journal of medicine 292, 1269-1273.

Pessayre, D., Fromenty, B., Berson, A., Robin, M.A., Lettéron, P., Moreau, R., Mansouri, A., 2012. Central role of mitochondria in drug-induced liver injury. Drug metabolism reviews. 44, 34-87.

Petzinger, E., Geyer, J., 2006. Drug transporters in pharmacokinetics. Naunyn-Schmiedeberg's archives of pharmacology 372, 465-475.

Phipps, A.N., Stewart, J., Wright, B., Wilson, I.D., 1998. Effect of diet on the urinary excretion of hippuric acid and other dietary-derived aromatics in rat. A complex interaction between diet, gut microflora and substrate specificity. Xenobiotica; the fate of foreign compounds in biological systems 28, 527-537.

Piper, P., Calderon, C.O., Hatzixanthis, K., Mollapour, M., 2001. Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiology 147, 2635-2642.

Piper, P.W., 1999. Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. Free radical biology & medicine 27, 1219-1227.

Piper, W.N., Condie, L.W., Tephly, T.R., 1973. The role of substrates for glycine acyltransferase in the reversal of chemically induced porphyria in the rat. Archives of biochemistry and biophysics 159, 671-677.

Politycka, B., 1997. Free and glucosylated phenolics, phenol β-glucosyltransferase activity and membrane permeability in cucumber roots affected by derivatives of cinnamic and benzoic acids. Acta physiologiae plantarum 19, 311-317.

Poon, K., Pang, K.S., 1995. Benzoic acid glycine conjugation in the isolated perfused rat kidney. Drug metabolism and disposition: the biological fate of chemicals 23, 255-260.

Porcelli, A.M., Ghelli, A., Zanna, C., Pinton, P., Rizzuto, R., Rugolo, M., 2005. pH difference across the outer mitochondrial membrane measured with a green fluorescent protein mutant. Biochemical and biophysical research communications 326, 799-804.

Prakash, S., Rodes, L., Coussa-Charley, M., Tomaro-Duchesneau, C., 2011. Gut microbiota: next frontier in understanding human health and development of biotherapeutics. Biologics: targets and therapy 5, 71-86.

Praphanphoj, V., Boyadjiev, S.A., Waber, L.J., Brusilow, S.W., Geraghty, M.T., 2000. Three cases of intravenous sodium benzoate and sodium phenylacetate toxicity occurring in the treatment of acute hyperammonaemia. Journal of inherited metabolic disease 23, 129-136.

Probstein, J.G., Londe, S., 1940. Studies of liver function by means of quick's hippuric acid test. Annals of surgery 111, 230-245.

Rapoport, M., Saada, A., Elpeleg, O., Lorberboum-Galski, H., 2008. TAT-mediated delivery of LAD restores pyruvate dehydrogenase complex activity in the mitochondria of patients with LAD deficiency. Molecular therapy 16, 691-697.

Rapoport, M., Salman, L., Sabag, O., Patel, M.S., Lorberboum-Galski, H., 2011. Successful TAT-mediated enzyme replacement therapy in a mouse model of mitochondrial E3 deficiency. Journal of molecular medicine 89, 161-170.

Ratnakumari, L., Qureshi, I.A., Butterworth, R.F., 1993. Effect of sodium benzoate on cerebral and hepatic energy metabolites in spf mice with congenital hyperammonemia. Biochemical pharmacology 45, 137-146.

Rechner, A.R., Kuhnle, G., Bremner, P., Hubbard, G.P., Moore, K.P., Rice-Evans, C.A., 2002. The metabolic fate of dietary polyphenols in humans. Free radical biology & medicine 33, 220-235.

Rinaldo, P., O'Shea, J.J., Welch, R.D., Tanaka, K., 1990. The enzymatic basis for the dehydrogenation of 3-phenylpropionic acid: in vitro reaction of 3-phenylpropionyl-CoA with various acyl-CoA dehydrogenases. Pediatric research 27, 501-507.

Ritter, J.K., 2000. Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. Chemico-biological interactions 129, 171-193.

Ross, P., Wootton, I.D.P., 1964. Glycine conjugation and toxicity of phenolic acids. Clinica chimica acta 9, 434-440.

Sakuma, T., 1991. Alteration of urinary carnitine profile induced by benzoate administration. Archives of disease in childhood 66, 873-875.

Saltzman, A., Caraway, W.T., 1953. Cinnamic acid as a test substance in the evaluation of liver function. The journal of clinical investigation 32, 711-719.

Schachter, D., Taggart, J.V., 1953. Benzoyl coenzyme A and hippurate synthesis. The journal of biological chemistry 203, 925-934.

Schachter, D., Taggart, J.V., 1954a. Glycine N-acylase: purification and properties. Journal of biological chemistry. 208, 263-275.

Schachter, D., Taggart, J.V., 1954b. Product inhibition in the glygine N-acylase reaction. Journal of biological chemistry. 211, 271-278.

Schwab, A.J., Tao, L., Yoshimura, T., Simard, A., Barker, F., Pang, K.S., 2001. Hepatic uptake and metabolism of benzoate: a multiple indicator dilution, perfused rat liver study. American journal of physiology. Gastrointestinal and liver physiology 280, G1124-1136.

Sears, M.E., Genuis, S.J., 2012. Environmental determinants of chronic disease and medical approaches: Recognition, avoidance, supportive, therapy, and detoxification. Journal of environmental and public health 2012, 1-15.

Sherwin, C.P., Kennard, K.S., 1919. Toxicity of phenylacetic acid. Journal of biological chemistry 40, 259-264.

Sidenius, U., Skonberg, C., Olsen, J., Hansen, S.H., 2004. In vitro reactivity of carboxylic acid-CoA thioesters with glutathione. Chemical research in toxicology 17, 75-81.

Sikkema, J., de Bont, J.A., Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological reviews 59, 201-222.

Smith, A.V., 2008. Browsing HapMap Data Using the Genome Browser. CSH protocols 2008, pdb prot 5023.

Smith, E.A., Macfarlane, G.T., 1996. Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. The journal of applied bacteriology 81, 288-302.

Spustova, V., Oravec, C., 1989. Antitumor effect of hippurate. An experimental study using various mouse tumor strains. Neoplasma 36, 317-320.

Stergachis, A.B., Haugen, E., Shafer, A., Fu, W., Vernot, B., Reynolds, A., Raubitschek, A., Ziegler, S., LeProust, E.M., Akey, J.M., Stamatoyannopoulos, J.A., 2013. Exonic transcription factor binding directs codon choice and affects protein evolution. Science 342, 1367-1372.

Strauss, M., Hofhaus, G., Schroder, R.R., Kuhlbrandt, W., 2008. Dimer ribbons of ATP synthase shape the inner mitochondrial membrane. The EMBO journal 27, 1154-1160.

Sweetman, L., Williams, J.C., 2013. Part 9: Organic acids Chapter 93: Branched Chain Organic Acidurias, The online metabolic and molecular bases of inherited diseases. McGraw Hill.

Tanaka, K., Budd, M.A., Efron, M.L., Isselbacher, K.J., 1966. Isovaleric acidemia: a new genetic defect of leucine metabolism. Proceedings of the national academy of sciences of the United States of America 56, 236-242.

Tanaka, K., Isselbacher, K.J., 1967. The isolation and identification of N-isovalerylglycine from urine of patients with isovaleric acidemia. Journal of biological chemistry. 242, 2966-2972.

Temellini, A., Mogavero, S., Giulianotti, P.C., Pietrabissa, A., Mosca, F., Pacifici, G.M., 1993. Conjugation of benzoic acid with glycine in human liver and kidney: a study on the interindividual variability. Xenobiotica; the fate of foreign compounds in biological systems 23, 1427-1433.

Tfouni, S.A., Toledo, M.C., 2002. Estimates of the mean per capita daily intake of benzoic and sorbic acids in Brazil. Food additives and contaminants 19, 647-654.

Trost, L.C., Lemasters, J.J., 1996. The mitochondrial permeability transition: a new pathophysiological mechanism for Reye's syndrome and toxic liver injury. The Journal of pharmacology and experimental therapeutics 278, 1000-1005.

Trost, L.C., Lemasters, J.J., 1997. Role of the mitochondrial permeability transition in salicylate toxicity to cultured rat hepatocytes: implications for the pathogenesis of Reye's syndrome. Toxicology and applied pharmacology 147, 431-441.

Tsao, R., 2010. Chemistry and biochemistry of dietary polyphenols. Nutrients 2, 1231-1246.

Tsay, H.J., Wang, Y.H., Chen, W.L., Huang, M.Y., Chen, Y.H., 2007. Treatment with sodium benzoate leads to malformation of zebrafish larvae. Neurotoxicology and teratology 29, 562-569.

Ure, A., 1841. On gouty concretions; with a new method of treatment. Pharm J Trans 1, 24.

van der Sluis, R., Badenhorst, C.P.S., Van der Westhuizen, F.H., van Dijk, A.A., 2013. Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase. Gene 515, 447-453.

Van der Westhuizen, B.M., Terblanche, E., Mienie, L.J., 1999. Benzoylserine and benzoylthreonine in propionic acidaemia treated with sodium benzoate. South African medical journal. 89, 125-127.

van der Westhuizen, F.H., Pretorius, P.J., Erasmus, E., 2000. The utilization of alanine, glutamic acid, and serine as amino acid substrates for glycine N-acyltransferase. Journal of biochemical and molecular toxicology. 14, 102-109.

Vessey, D.A., 2001. Isolation and preliminary characterization of the medium-chain fatty acid:CoA ligase responsible for activation of short- and medium-chain fatty acids in colonic mucosa from swine. Gigestive diseases and sciences. 46, 438-442.

Vessey, D.A., Kelley, M., Warren, R.S., 1999. Characterization of the CoA ligases of human liver mitochondria catalyzing the activation of short- and medium-chain fatty acids and xenobiotic carboxylic acids. Biochimica et biophysica acta 1428, 455-462.

Vessey, D.A., Lau, E., Kelley, M., Warren, R.S., 2003. Isolation, Sequencing, and Expression of a cDNA for the HXM-A Form of Xenobiotic/Medium-Chain Fatty Acid:CoA Ligase from Human Liver Mitochondria. Journal of biochemical and molecular toxicology 17, 1-6.

Vessey, D.A., Lau, E.P., 1998. Determination of the Sequence of the Arylacetyl Acyl-CoA:Amino Acid N-Acyltransferase from Bovine Liver Mitochondria and Its Homology to the Aralkyl Acyl-CoA:Amino Acid N-Acyltransferase. Journal of biochemical and molecular toxicology 12, 275-279.

Vest, M.F., Salzberg, R., 1965. Conjugation reactions in the newborn infant: The metabolism of para-aminobenzoic acid Archives of disease in childhood., 1965, 40, 97. 40, 97-105.

Von Wendt, L., Similä, S., 1982. Experience with non-ketotic hyperglycinaemia in Finland. Journal of inherited metabolic disease 5, 111-112.

Waluk, D.P., Schultz, N., Hunt, M.C., 2010. Identification of glycine N-acyltransferase-like 2 (GLYATL2) as a transferase that produces N-acyl glycines in humans. FASEB journal 24, 2795-2803.

Waluk, D.P., Sucharski, F., Sipos, L., Silberring, J., Hunt, M.C., 2012. Reversible lysine acetylation regulates the activity of human glycine n-acyltransferase-like 2 (hGLYATL2):Implications for production of glycine-conjugated signalling molecules. Journal of biological chemistry. 287 (20),16158-67.

Wanders, R.J.A., Vreken, P., den Boer, M.E.J., Wijberg, F.A., van Gennip, A.H., Ijlst, L., 1999. Disorders of mitochondrial fatty acyl-CoA b-oxidation. Journal of inherited metabolic diseases. 22, 442-487.

Wang, W., Wu, Z., Dai, Z., Yang, Y., Wang, J., Wu, G., 2013. Glycine metabolism in animals and humans: implications for nutrition and health. Amino acids. 45 (3), 463-77.

Watkins, P.A., Maiguel, D., Jia, Z., Pevsner, J., 2007. Evidence for 26 distinct acyl-coenzyme A synthetase genes in the human genome. Journal of lipid research 48, 2736-2750.

Webster, L.T., Siddiqui, U.A., Lucas, S.V., Strong, J.M., Mieyal, J.J., 1976. Identification of separate acyl- CoA:glycine and acyl-CoA:L-glutamine N-acyltransferase activities in mitochondrial fractions from liver of rhesus monkey and man. Journal of biological chemistry. 251, 3352-3358.

Wei, X., Walia, V., Lin, J.C., Teer, J.K., Prickett, T.D., Gartner, J., Davis, S., Stemke-Hale, K., Davies, M.A., Gershenwald, J.E., Robinson, W., Robinson, S., Rosenberg, S.A., Samuels, Y., 2011. Exome sequencing identifies GRIN2A as frequently mutated in melanoma. Nature genetics 43, 442-446.

Wen, G.Y., Wisniewski, H.M., Shek, J.W., Loo, Y.H., Fulton, T.R., 1980. Neuropathology of phenylacetate poisoning in rats: an experimental model of phenylketonuria. Annals of neurology 7, 557-566.

Wen, H., Yang, H.J., An, Y.J., Kim, J.M., Lee, D.H., Jin, X., Park, S.W., Min, K.J., Park, S., 2013. Enhanced phase II detoxification contributes to beneficial effects of dietary restriction as revealed by multi-platform metabolomics studies. Molecular and cellular proteomics 12, 575-586.

White, A., 1941. Growth-Inhibition Produced in Rats by the Oral Administration of Sodium Benzoate: Effects of Various Dietary Supplements. The Yale journal of biology and medicine 13, 759-768.

Wijeyesekera, A., Clarke, P.A., Bictash, M., Brown, I.J., Fidock, M., Ryckmans, T., Yap, I.K.S., Chan, Q., Stamler, J., Elliott, P., Holmes, E., Nicholson, J.K., 2012. Quantitative UPLC-MS/MS analysis of the gut microbial co-metabolites phenylacetylglutamine, 4-cresyl sulphate and hippurate in human urine: INTERMAP Study. Analytical methods 4, 65-72.

Wikoff, W.R., Anfora, A.T., Liu, J., Schultz, P.G., Lesley, S.A., Peters, E.C., Siuzdak, G., 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proceedings of the national academy of sciences of the United States of America 106, 3698-3703.

Williams, H.R.T., Cox, I.J., D.G., W., Cobbold, J.F.L., Taylor-Robinson, S.D., Marshall, S.E., Orchard, T.R., 2010. Differences in gut microbial metabolism are responsible for reduced hippurate synthesis in Chron's disease. BMC Gastroenterology 10, 108.

Williams, R., 1959. Detoxification mechanisms: The metabolism and detoxification of drugs, toxic substances and other organic compounds, 2nd ed ed. Chapman and Hall Ltd, London.

Wu, G., 2010. Functional amino acids in growth, reproduction, and health. Advances in nutrition 1, 31-37.

Wu, G., 2013. Functional amino acids in nutrition and health. Amino acids. 45 (3), 407-11.

Wu, G., Wu, Z., Dai, Z., Yang, Y., Wang, W., Liu, C., Wang, B., Wang, J., Yin, Y., 2013. Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. Amino acids 44, 1107-1113.

Xie, G., Chen, T., Qiu, Y., Shi, P., Zheng, X., Su, M., Zhao, A., Zhou, Z., Jia, W., 2012. Urine metabolite profiling offers potential early diagnosis of oral cancer. Metabolomics 8, 220-231.

Xie, W., Wood, A.R., Lyssenko, V., Weedon, M.N., Knowles, J.W., Alkayyali, S., Assimes, T.L., Quertermous, T., Abbasi, F., Paananen, J., Häring, H., Hansen, T., Pedersen, O., Smith, U., Laakso, M., Dekker, J.M., Nolan, J.J., Groop, L., Ferrannini, E., Adam, K.-P., Gall, W.E., Frayling, T.M., Walker, M., 2013. Genetic variants associated with glycine metabolism and their role in insulin sensitivity and type 2 diabetes. Diabetes. 62 (6), 2141-50.

Yamamoto, A., Nonen, S., Fukuda, T., Yamazaki, H., Azuma, J., 2009. Genetic polymorphisms of glycine N-acyltransferase in Japanese individuals. Drug metabolism and pharmacokinetics 24, 114-117.

Yao, K.-W., Mao, L.-F., Luo, M.J., Schulz, H., 1994. The relationship between mitochondrial activation and toxicity of some substituted carboxylic acids. Chemico-biological interactions 90, 225-234.

Ye, G., Zhu, B., Yao, Z., Yin, P., Lu, X., Kong, H., Fan, F., Jiao, B., Xu, G., 2012. Analysis of urinary metabolic signatures of early hepatocellular carcinoma recurrence after surgical removal using gas chromatography-mass spectrometry. Journal of proteome research 11, 4361-4372.

Ye, S.F., Zhou, Y.H., Sun, Y., Zou, L.Y., Yu, J.Q., 2006. Cinnamic acid causes oxidative stress in cucumber roots, and promotes incidence of Fusarium wilt. Environmental and experimental botany 56, 255-262.

Yilmaz, S., Unal, F., Yuzbasioglu, D., 2009. The in vitro genotoxicity of benzoic acid in human peripheral blood lymphocytes. Cytotechnology 60, 55-61.

Yoshimura, T., Schwab, A.J., Tao, L., Barker, F., Pang, K.S., 1998. Hepatic uptake of hippurate: a multiple-indicator dilution, perfused rat liver study. The American journal of physiology 274, G10-20.

Zengin, N., Yuzbasioglu, D., Unal, F., Yilmaz, S., Aksoy, H., 2011. The evaluation of the genotoxicity of two food preservatives: sodium benzoate and potassium benzoate. Food and chemical toxicology : an international journal published for the British industrial biological research association 49, 763-769.

Zhang, H., Lang, Q., Li, J., Zhong, Z., Xie, F., Ye, G., Wan, B., Yu, L., 2007a. Molecular cloning and characterization of a novel human glycine N-acyltransferase gene GLYAT1, which activates transcriptional activity of HSE pathway. International journal of molecular sciences 8, 433-444.

Zhang, Y., Chohnan, S., Virga, K.G., Stevens, R.D., Ilkayeva, O.R., Wenner, B.R., Bain, J.R., Newgard, C.B., Lee, R.E., Rock, C.O., Jackowski, S., 2007b. Chemical Knockout of Pantothenate Kinase Reveals the Metabolic and Genetic Program Responsible for Hepatic Coenzyme A Homeostasis. Chemistry and biology, 291-302.

APPENDIX A: PAPER I

Glycine conjugation: Importance in metabolism, the role of glycine N-acyltransferase, and the factors that influence interindividual variation

Christoffel Petrus Stephanus Badenhorst, **Rencia van der Sluis**, Elardus Erasmus, and Alberdina Aike van Dijk

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Review

EXPERT OPINION

Glycine conjugation: importance in metabolism, the role of glycine *N*-acyltransferase, and factors that influence interindividual variation

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Introduction: Glycine conjugation of mitochondrial acyl-CoAs, catalyzed by glycine *N*-acyltransferase (GLYAT, E.C. 2.3.1.13), is an important metabolic pathway responsible for maintaining adequate levels of free coenzyme A (CoASH). However, because of the small number of pharmaceutical drugs that are conjugated to glycine, the pathway has not yet been characterized in detail. Here, we review the causes and possible consequences of interindividual variation in the glycine conjugation pathway.

Areas covered: The authors review the importance of CoASH in metabolism, formation and toxicity of xenobiotic acyl-CoAs, and mechanisms for restoring levels of CoASH. They focus on GLYAT, glycine conjugation, how genetic variation in the GLYAT gene could influence glycine conjugation, and the emerging roles of glycine metabolism in cancer and musculoskeletal development.

Expert opinion: The substrate selectivity of GLYAT and its variants needs to be further characterized, as organic acids can be toxic if the corresponding acyl-CoA is not a substrate for glycine conjugation. GLYAT activity affects mitochondrial ATP production, glycine availability, CoASH availability, and the toxicity of various organic acids. Therefore, variation in the glycine conjugation pathway could influence liver cancer, musculoskeletal development, and mitochondrial energy metabolism.

Keywords: acyl-coenzyme A, benzoate, CASTOR disorder, coenzyme A, coenzyme A sequestration, GLYAT, glycine conjugation, glycine *N*-acyltransferase, hepatocellular carcinoma, xenobiotics

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1. Introduction

The study of drug metabolism started with the discovery of glycine conjugation. The excretion of hippuric acid after ingestion of benzoic acid was discovered in 1841 by Alexander Ure [1]. This was later confirmed by Wilhelm Keller in 1842 who ingested 32 grains of benzoic acid and isolated hippuric acid from his urine the next morning [2]. Later, in 1845, it was demonstrated by Dessaignes that hippuric acid was in fact an amide conjugate between glycine and benzoic acid, making this the first conjugation reaction to be discovered [3]. Since this epic discovery, interest in glycine conjugation has faded significantly, and only sporadically has anything on the subject been published in the last 168 years. In this review, we wish to re-emphasize the importance of glycine conjugation and clarify its influence on the metabolism of CoASH and glycine. We also point out some

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Article highlights.

- GLYAT is the enzyme responsible for glycine conjugation of the acyl-CoA esters of several xenobiotic organic acids.
- Glycine conjugation is important for the detoxification of benzoate and hydroxybenzoates that are conjugated to coenzyme A in the liver and kidneys.
- Interindividual variation in glycine conjugate excretion has been observed but the mechanisms underlying this variation are not understood.
- SNPs in human GLYAT have been shown to influence the enzymatic activity, but it is not clear how this influences variation in the glycine conjugation pathway.
- The high exposure to xenobiotics in modern times may exacerbate dietary glycine deficiency.
- Because of its influence on glycine availability, GLYAT may play a role in the development of hepatocellular carcinoma and may be involved in musculoskeletal development.

This box summarizes key points contained in the article.

serious deficiencies, of paramount importance, in our understanding of the glycine conjugation pathway.

Humans have several biotransformation systems, including conjugation to sulfate, glucuronate, and glycine, that convert various endogenous and xenobiotic metabolites to more hydrophilic conjugates that can be excreted in the urine [3-8]. The resulting conjugates are often less toxic than the parent compound, with some exceptions such as reactive acyl-glucuronides [9]. Glycine *N*-acyltransferase (GLYAT) is responsible for the glycine conjugation of xenobiotics such as benzoic acid (**Figure 1**). Although the small range of substrates for glycine conjugation, when compared with glucuronidation, may have contributed to the relatively little research that has been done on GLYAT [3], we will argue that the enzyme plays a central role in maintaining CoASH homeostasis in the liver. We briefly review and discuss acyl-CoA metabolism, glycine conjugation, interindividual variations, and some factors that may influence glycine conjugation. Finally, we review the literature on the GLYAT gene and enzyme, and what is known about genetic variation in the GLYAT gene and its consequences.

2. Acyl-CoA metabolism and toxicity

2.1 The importance of coenzyme A in metabolism

Coenzyme A is an extremely important molecule that can be seen as a central hub around which much of metabolism revolves [10-13]. Acyl-CoA esters are important intermediates in many anabolic and catabolic reactions. Almost all catabolic reactions result in the formation of acetyl-CoA, the fuel for both oxidative phosphorylation and lipogenesis (**Figure 2**) [10,14]. It is thus clear that disturbances of coenzyme A metabolism, and changes in the relationships between CoASH and acyl-CoAs, can have severe and far-reaching consequences for metabolism as a whole [12,13]. Therefore, coenzyme A metabolism is tightly regulated, and even under ischemic conditions, levels of free and acylated CoASH in the liver stay the same, despite a doubling in acetyl-CoA levels [12].

An interesting study in which rats were fed with the pantothenate analog hopantenate demonstrates the tight regulation of hepatic coenzyme A metabolism. Hopantenate inhibits CoASH biosynthesis, and the rats died of hypoglycemia within 2 weeks with fatty liver and mitochondrial dysmorphology [13]. It was shown that hopantenate initiates a transcriptional reprogramming of the liver, which leads to an increase in expression of acyl-CoA thioesterases, and pyruvate dehydrogenase kinase isoform 1, which decreases pyruvate dehydrogenase activity. The result is increased liberation and decreased consumption of CoASH [11-13]. These observations emphasize the importance of tight regulation of hepatic CoASH metabolism and the consequences of disruption of CoASH homeostasis.

2.2 Formation of xenobiotic acyl-CoAs

Several fatty acids and xenobiotic carboxylic acids that are conjugated to amino acids must first be activated to acyl-CoAs by ATP-dependent acid:CoA ligases [3,4,15,16]. These ligase enzymes exhibit selectivity for short-, medium-, long-, or very long-chain fatty acids [15]. Several long-chain forms have been identified, which have different activities and tissue localization, and enable site-specific activation of fatty acids for specific metabolic requirements [12,17]. Most xenobiotics that undergo glycine conjugation are activated by the mitochondrial medium-chain ligases, which also activate C4–C12 acids for β-oxidation [15,18-20]. This dual role of the medium-chain ligases for fatty acid oxidation and xenobiotic activation is one of the reasons why mitochondrial accumulation of xenobiotic acyl-CoA esters may interfere with β-oxidation and disturb mitochondrial metabolism [3,21-23].

Four distinct medium-chain ligases, XL-I, XL-II, XL-III, and XL-J, have been identified in bovine liver, and have overlapping substrate specificities [18,19,22,24,25]. XL-I, XL-II, and XL-III all activate C3–C10 fatty acids and a range of arylacetic and aromatic carboxylic acids, including benzoate, 4-aminobenzoate, 4-chlorobenzoate, 4-nitrobenzoate, napthylacetate, and salicylate [15,18,20]. In humans, there are five medium-chain xenobiotic-activating enzymes. These are ACSM1, ACSM2A and ACSM2B, ACSM3, and ACSM5 [26]. Vessey *et al.* characterized two human liver medium-chain ligases, HXM-A and HXM-B, with activity toward a range of xenobiotics [3,18,27,28]. HXM-A is encoded by the ACSM2A gene [26,28]. These enzymes are less well characterized than the corresponding bovine enzymes, but have been shown to activate benzoate, hexanoate, octanoate, and decanoate. There is evidence of activation of valproate by HXM-A and salicylate by HXM-B [3,27,28]. Xenobiotics that involve activation to an acyl-CoA ester include pivalate,

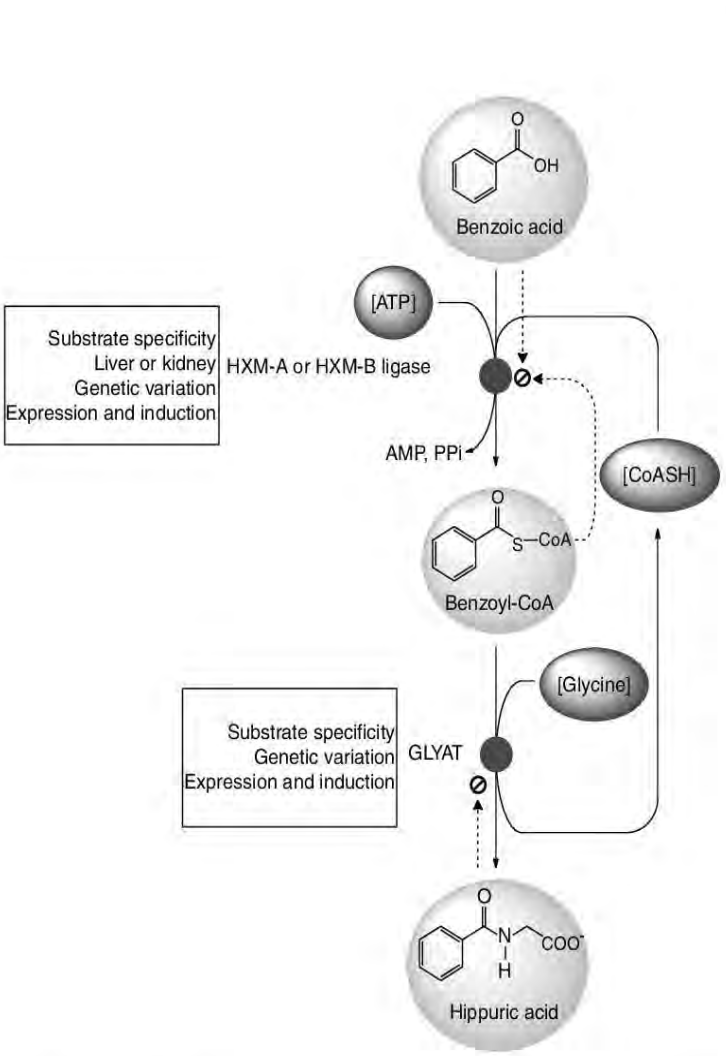

Figure 1. Glycine conjugation of benzoic acid. The glycine conjugation pathway consists of two steps. First benzoate is ligated to CoASH to form the high-energy benzoyl-CoA thioester. This reaction is catalyzed by the HXM-A and HXM-B medium-chain acid:CoA ligases and requires energy in the form of ATP. Some acyl-CoA esters can competitively inhibit the ligase enzymes. The benzoyl-CoA is then conjugated to glycine by GLYAT to form hippuric acid, releasing CoASH. In addition to the factors listed in the boxes, the levels of ATP, CoASH, and glycine may influence the overall rate of the glycine conjugation pathway. The black circles indicate the ligase and GLYAT enzymes.

AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; CoASH: Coenzyme A; GLYAT: Glycine *N*-acyltransferase; PPi: Pyrophosphate.

valproate, benzoate, salicylate, phenylbutyrate, and several others, summarized in **Table 1**. Depending on the xenobiotic, glycine conjugation may occur primarily in either the liver or kidney, reflecting differences between hepatic and renal acyl-CoA formation, but this is not discussed here [29]. If a xenobiotic acyl-CoA is formed that cannot be metabolized further, it will accumulate, resulting in toxicity [4,23,30]. The mechanisms of acyl-CoA toxicity are briefly described in the following section, before looking at pathways that can restore CoASH levels and homeostasis.

2.3 Mechanisms of acyl-CoA toxicity and pathogenesis

All disorders, acquired or inherited, that involve coenzyme A sequestration, toxicity, or redistribution were conceptually united into a group called CASTOR disorders by Mitchell *et al.* [12]. In CASTOR disorders, the degradation of acyl-CoA esters is impaired [10,14]. Grouping of the CASTOR disorders enables a clearer grasp of the underlying pathophysiology and enables better understanding of potential therapeutic strategies. The mechanisms of pathogenesis can be divided broadly into effects caused by depletion of CoASH, and effects caused by the accumulated acyl-CoA itself [4,12,30].

2.3.1 *Depletion of CoASH*

Depletion of CoASH is often one of the most severe consequences of acyl-CoA accumulation [4,12,13,31,32]. As described in Section 2.1, coenzyme A is a central metabolic hub and depletion can, indirectly, have far-reaching implications for both intermediary and energy metabolism. When CoASH

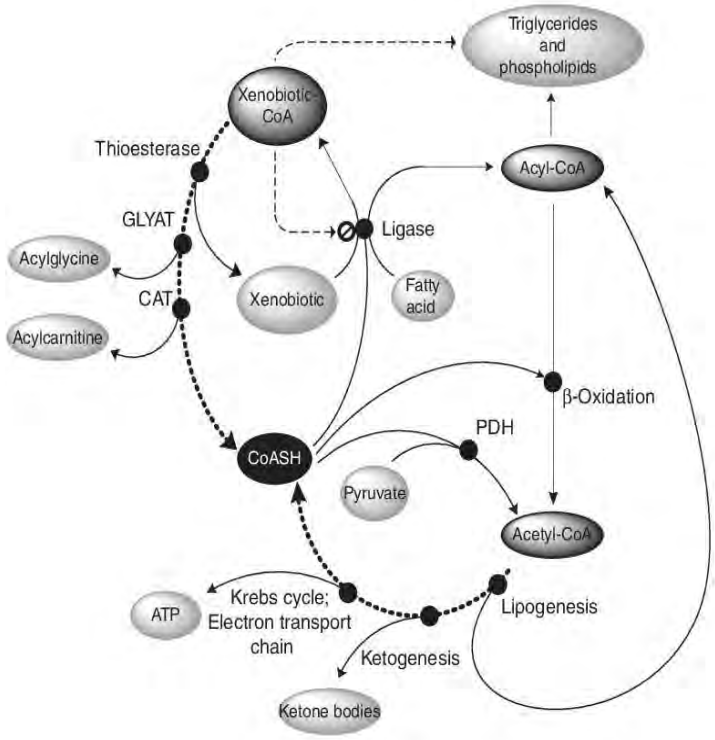

Figure 2. An overview of coenzyme A and acyl-CoA metabolism. The main pathways that produce and consume CoASH are demonstrated. It is extremely important that CoASH always be available in the cell because of its role in β-oxidation and the conversion of pyruvate to acetyl-CoA. Acetyl-CoA is the product of most catabolic reactions and provides the fuel for ketogenesis and mitochondrial ATP production. When xenobiotics are converted to xenobiotic-CoA esters, CoASH can be sequestered, disrupting the ATP synthesis from pyruvate and fatty acids. As demonstrated by the fine broken arrows, accumulating xenobiotic-CoAs can inhibit the acid:CoA ligases or be incorporated into unnatural triglycerides and membrane phospholipids. The bold broken arrows indicate pathways that release bound CoASH. The black circles indicate important processes involved in the formation and degradation of acyl-CoAs.

CAT: Carnitine acyltransferase; CoASH: Coenzyme A; GLYAT: Glycine *N*-acyltransferase; PDH: Pyruvate dehydrogenase.

becomes limiting, energy metabolism is impacted on several levels [13,14,33]. The consumption of glucose, a primary metabolic fuel, results in the formation of pyruvate, which requires CoASH in order to be converted to acetyl-CoA by pyruvate dehydrogenase [13]. CoASH is also needed for β-oxidation of fatty acids, which are broken down to two-carbon units in the form of acetyl-CoA [10,13,14,31,33]. Thus, if CoASH is depleted, glucose and lipids cannot be efficiently utilized for the production of energy by oxidative phosphorylation. The result is diminished capacity for mitochondrial ATP production, increased dependence on glycolysis, and altered ratios of cellular NAD^+ and NADH [12,13,31,32]. NAD^+ is required for activity of the sirtuins, a family of NAD^+-dependent deacetylases and ADP-ribosyltransferases [34]. These proteins play important roles in energy metabolism by regulating the activities of enzymes involved in gluconeogenesis, β-oxidation, and the electron transport chain [34]. Disturbances of NAD^+ levels can thus negatively impact the regulation of energy metabolism, but this falls outside the scope of this review.

The effects of CoASH sequestration are demonstrated by the metabolism of valproate, an anti-epileptic drug that is metabolized to valproyl-CoA. As valproyl-CoA is not a substrate for glycine conjugation, it can accumulate in the liver, deplete CoASH, and may eventually cause hepatic steatosis [3,30,31,33]. This effect is not caused by an α-fluorinated derivative of valproate, which is either not a good substrate for ligation to CoASH, or because of its increased acidity forms a less stable thioester that spontaneously hydrolyses. As a result, this α-fluorinated derivative does not cause CoASH sequestration and hepatic steatosis [3].

2.3.2 *Toxic effects of accumulating acyl-CoAs*

Accumulation of acyl-CoAs can also negatively influence energy metabolism by causing a depletion of carnitine, which is the transporter of fatty acids over mitochondrial membranes [10,14]. When an acyl-CoA accumulates to high enough amounts, it may become a substrate for carnitine acyltransferases, resulting in the formation of an acyl-carnitine that can be

Table 1. Xenobiotics that are metabolized to acyl-CoA and glycine conjugates.

Xenobiotic	Glycine conjugate formed in humans	CoA sequestration or toxicity	Notes
2,4,5-Trichlorophenoxyacetate	No	Unknown	Glycine conjugate formed by bovine GLYAT [3]
2,4-Dichlorophenoxyacetate	No	Unknown	Glycine conjugate formed by bovine GLYAT [3]
3-Hydroxybenzoate	Yes	No	Product of dietary polyphenol fermentation by gut microorganisms [44]
4-Aminobenzoate (PABA)	Yes	No	Seems to be well tolerated; slow glycine conjugation of PABA has been correlated to liver failure and hepatitis probably because of decreased formation of aminobenzoyl-CoA [3,47,65,66]
4-Hydroxybenzoate	Yes	No	Product of dietary polyphenol fermentation by gut microorganisms [44]
Astemizole	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Benzoate	Yes	No	Good substrate for glycine conjugation; large doses of benzoate are tolerated; we believe that benzoate would cause severe CoASH sequestration in the absence of GLYAT activity [2,27,45,59,78]
Brompheniramine	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Ferulic acid	Yes	No	From metabolism of ferulate-containing plant material by gut microorganisms [100]
Hypoglycine	Yes	Sequestration and toxicity	Glycine conjugation is not fast enough to detoxify the acyl-CoA metabolite, which is an irreversible inhibitor of dehydrogenase enzymes [3,4]
Ibuprofen	No	Sequestration	Taurine conjugate is formed; interaction between salicylate and ibuprofen was observed for the bovine medium-chain ligase enzymes; causes CoASH sequestration in rat liver [3,4,22]
Indoleacetic acid	Yes	No	Usually conjugated to glutamine; associated with gut microbe dysbiosis; not activated to acyl-CoA by HXM-A or HXM-B [27,81,100]
Naphthylacetic acid	No	Unknown	Weak activation by HXM-A and HXM-B; unlikely to cause CoASH sequestration [27]
Nicotinic acid	Unknown	No	Very weak activation by HXM-B [27]
Permethrin	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Phenylacetic acid	Yes	Unknown	Usually conjugated to glutamine; associated with gut microbe dysbiosis [81,100]
Pivalic acid	No	Sequestration	Not a substrate for human GLYAT [3,32]
Salicylate	Yes	Uncertain, toxic	Activation of salicylate to salicylyl-CoA is slow, making CoASH sequestration unlikely; toxicity and associated Rye-like syndrome are possibly caused by inhibition of carnitine acyltransferases by salicylyl-CoA; salicylic acid at therapeutic doses can also inhibit bovine ligase enzymes, suggesting another mechanism of toxicity [3,22,27,39,78]
Toluene	Yes	No	Metabolized to hippuric acid; no interaction was observed between toluene and the xylenes at the doses used in the investigation [83]
Triflusal	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Valproate	No	Sequestration and toxicity	Can cause hepatic steatosis because of CoASH sequestration [3,18,67]
Xylenes	Yes	No	These solvents are metabolized to methylhippurates; *m*-xylene has been shown to interact with salicylate conjugation [63,83]

PABA: *Para*-aminobenzoic acid.

excreted in the urine [21,32,35,36]. For example, benzoic acid administration results in benzoyl-carnitine excretion and a decrease in plasma-free carnitine levels [35].

Xenobiotic acyl-CoAs can substitute for acetyl-CoA in lipogenesis, resulting in odd-chain, branched-chain, aromatic, and other unnatural fatty acids, which cannot be properly catabolized and may be incorporated into cell membranes [3,30,31]. For example, propionyl-CoA, which accumulates in propionic acidemia, can be the substrate for synthesis of odd- and branched-chain fatty acids [37]. It has also been shown that 2-arylpropionyl-CoA esters, metabolites of the nonsteroidal anti-inflammatory drugs (NSAIDS), can be incorporated into adipocyte triglycerides [15,31]. Enzymes may be competitively or allosterically inhibited by acyl-CoAs, with effects that are difficult to predict [12,21-23,30,38,39]. For example, protein kinase C activity, important in signal transduction, is perturbed by ciprofibroyl-CoA, a metabolite of the hypolipidaemic drug ciprofibrate [15]. Propionyl-CoA, at high concentrations, inhibits formation of N-acetylglutamate by N-acetylglutamate synthetase, resulting in urea cycle dysfunction and hyperammonemia [37].

2.4 Restoration of CoASH levels

There are a few basic mechanisms that can restore depleted CoASH reserves, including conjugation to amino acids or to carnitine, and hydrolysis of acyl-CoAs by thioesterases (**Figure 2**) [3,12,40]. Acyl-CoA thioesterases hydrolyze acyl-CoA esters to free organic acids and CoASH. This is an indispensable metabolic necessity, because CoASH must always be available to maintain a proper metabolic milieu [10,14]. Thioesterases may have selectivity for short-, medium-, long-, and very long-chain acyl-CoAs, and are found in almost every compartment of the cell, including the cytoplasm, peroxisomes, microsomes, and mitochondria [3,12,41]. There is a direct relationship between cellular levels of CoASH, long-chain acyl-CoAs, peroxisomal β-oxidation, and cellular thioesterase activity, with thioesterases playing a role in regulation of peroxisomal and intracellular lipid metabolism [10,41]. It has been suggested that the accumulation of a particular xenobiotic acyl-CoA will, in part, reflect the relative activity and substrate selectivity of the various thioesterases [15]. In the following section, the main focus is on glycine conjugation, a primary mechanism for the restoration of CoASH levels.

3. Glycine conjugation and interindividual variation

3.1 The metabolic role of glycine conjugation

Although GLYAT can conjugate a variety of endogenous and xenobiotic acyl-CoAs to glycine, the normal metabolic role of GLYAT seems to be the detoxification of dietary benzoates [4,42-45]. On a daily basis, humans consume varying quantities of benzoate, a metabolite found in plant material [45]. In addition, plant material contains complex polyphenols, which are fermented by the colonic flora to benzoate, 3- and 4-hydroxybenzoates, and the corresponding hydroxyphenyl-propionates [44]. After intestinal absorption, these compounds are transported to the liver, where they are conjugated to CoASH by the medium-chain xenobiotic acid:CoA ligases [3,15,18,45]. Because these benzoates are ubiquitous in plant-containing diets, it is clear that hepatic CoASH would be rapidly sequestered if the xenobiotic acyl-CoAs could not be further metabolized. GLYAT plays a major role in restoring CoASH levels by conjugating these xenobiotic acyl-CoAs to glycine. Therefore, excretion as the corresponding glycine conjugates is the major metabolic fate of ingested polyphenols [44,45]. Other natural substrates for conjugation to glycine include salicylate, a common plant metabolite, and 4-aminobenzoate [39,46-48]. About 83 – 90% of ingested benzoate and about 75 – 84% of ingested salicylate are excreted as glycine conjugates [49]. Decreased benzoate production by the gut microorganisms in patients with Crohn's disease is correlated with decreased hippurate excretion in the urine [45].

3.2 Glycine conjugation in metabolic diseases

In several organic acidemias, an acyl-CoA accumulates to toxic levels because of a defect of the enzyme acting on it [14,43,50]. This results not only in CoASH sequestration, but because of thioesterase activity, free organic acids are released, causing potentially deadly acidoses [51-53]. Because some of the acyl-CoAs that accumulate in organic acidemias are substrates for GLYAT, glycine conjugation impacts on the biochemical profiles and clinical outcomes of some of these metabolic defects [42,43,53-55]. Glycine conjugation under these abnormal conditions sheds light on the important role GLYAT plays in maintaining CoASH levels.

In some cases, the accumulating acyl-CoA can be conjugated to glycine by GLYAT, decreasing the severity of CoASH sequestration and avoiding acidosis, as a less toxic acylglycine is formed and excreted [42,43,50,56]. It was demonstrated that a relationship exists between the kinetics and substrate selectivity of a bovine liver GLYAT, and the acylglycines excreted in the urine of patients with organic acidemias [42]. For example, in isovaleric acidemia, where isovaleryl-CoA accumulates, large amounts of isovalerylglycine are excreted in the urine because isovaleryl-CoA is a good substrate for GLYAT [50,55,57]. However, in propionic acidemia, only relatively small amounts of propionate are excreted as propionylglycine [37]. This is because, despite having similar K_M values for isovaleryl-CoA and propionyl-CoA, bovine GLYAT conjugates propionyl-CoA at a much lower rate [42,58]. Unfortunately, a similar comparison cannot yet be made for the human enzyme, as its kinetic parameters are not as well characterized. Glycine conjugates are also excreted in several other organic acidemias, and include 3-methylcrotonylglycine, hexanoylglycine, butyrylglycine, and tiglylglycine [14,43,52,53]. There is no simple relationship between GLYAT substrate selectivity, in terms of K_M and V_{max} parameters, and acyl

group structure. This makes it difficult to predict the extent to which glycine conjugation will influence the outcome of any particular organic acidemia [4,42].

3.3 Glycine availability can be a limiting factor in glycine conjugation

The presence of isovalerylglycine in the urine of patients with isovaleric acidemia suggested that glycine supplementation may help to detoxify the accumulating isovaleryl-CoA [50,55]. In fact, glycine supplementation has been shown to increase the levels of isovalerylglycine excreted, and usually decreases the severity of the disease to allow normal physical and mental development [50,55,57]. This suggests that availability of glycine may be a limiting factor in glycine conjugation under some conditions [50,59,60]. When glycine conjugation is maximal, glycine may be depleted, and other amino acids may be used, as suggested by the detection of isovaleryl-conjugates of 19 other amino acids in the urine of isovaleric acidemia patients [61]. It was proposed, although not experimentally verified, that these conjugates are also formed by GLYAT. Glycine availability also influences benzoate conjugation, as demonstrated by the dose-dependent increase in hippurate formation after administration of glycine [59]. Cysteamine, which decreases the activity of the glycine cleavage system and doubles hepatic glycine content, increases the benzoate clearance by 50% in rats [59].

3.4 Interindividual variation in glycine conjugation

Glycine conjugation is also manipulated for the treatment of hyperammonemia in urea cycle disorders, by administration of benzoate, which is conjugated to form hippurate, allowing excess nitrogen to be excreted from the body [36,60]. Interindividual variation in responsiveness to administration of glycine and benzoate, respectively, was observed in both isovaleric acidemia and hyperammonemia [56,57,62]. For example, variation in clinical outcome and responsiveness to glycine supplementation was observed in a group of South African isovaleric acidemia patients, all homozygous for the same isovaleryl-CoA dehydrogenase mutation [56]. It was suggested that interindividual variation in GLYAT activity may partly account for this, but further investigation is needed.

It has also been shown that there is significant interindividual variation in the rate of glycine conjugation of xenobiotics [35,48,49,62,63]. Greater similarity between identical twins than between fraternal twins in the glycine conjugation of salicylate suggested that there is a genetic component to this variation [48]. Using human liver samples, it was demonstrated that there is interindividual variation in the capacity for hippurate synthesis from benzoate, and that the elderly seem to have a slightly decreased capacity [64]. In a large group of subjects, a coefficient of variation of approximately 15 – 24% was found for the formation of hippurate and salicylurate from benzoate and salicylate, respectively [49]. However, no significant difference was found between the mean values for children, adults, the elderly, or patients with liver disease. There was, however, higher variation between the individuals with liver disease, suggesting that the rate of glycine conjugation is influenced by liver disease (Section 4.1) [47,49,65,66]. Glycine conjugation is a saturable process, and this has consequences for co-administration of different substrates for this pathway [3,4,39,45,48,49,64]. For example, although there is interindividual variation in the total amount of glycine conjugates excreted by healthy adults, the total amount of glycine conjugates excreted is the same for each individual whether aspirin and *m*-xylene (excreted as 3-methylhippurate) are administered separately or simultaneously [63].

It is important to note that all these studies on glycine conjugation of xenobiotics report variation for the whole pathway, including ligation to CoASH and conjugation to glycine [3,35,48,49,62-64]. Several factors can influence the overall rate of glycine conjugation, including the availability of ATP, CoASH, and glycine, variation in acid:CoA ligase activity, and GLYAT enzyme activity (**Figure 1**) [3,4,55,59,67]. The limiting step is substrate dependent and can be either ligation to CoASH, as with salicylate, or conjugation to glycine, as with benzoate [3,29,64].

4. GLYAT, liver cancer, hepatitis, and musculoskeletal development

4.1 GLYAT, liver cancer, and hepatitis

Recently, a complete downregulation of transcription of the GLYAT gene was observed in 32 of 41 hepatocellular carcinoma specimens investigated, with significant downregulation in the other nine specimens [68]. This was confirmed by immunohistochemistry using a GLYAT-specific antibody, which revealed that GLYAT is not expressed in cancerous cells, but is expressed in neighboring healthy hepatocytes. Interestingly, GLYAT expression was found to be significant and similar in all noncancerous liver specimens studied, including 60 samples from patients with chronic hepatitis of various etiologies [68]. This observation may be explained by the expression of GLYAT in differentiated hepatocytes, but not in dedifferentiated cancerous cells. On the basis of these findings, Matsuo *et al.* proposed that suppression of GLYAT transcription may be a novel marker of hepatocellular carcinoma and is a key event in the development of liver cancer. There could also be a relationship between GLYAT activity, glycine availability, and cancer cell proliferation [69,70]. This is further elaborated on in Section 7.

It has been reported that the fraction of 4-aminobenzoate excreted as glycine conjugates correlates well to functional hepatic reserves in patients with hepatitis. Therefore, the measurement of glycine conjugation of 4-aminobenzoate has been proposed as a liver function test [47,65,66]. GLYAT expression is normal in hepatitis specimens and it has been suggested that the lower glycine conjugation observed for hepatitis patients could be explained by impaired hepatic β-oxidation and lower availability of ATP for ligation of benzoate to CoASH [3,68].

4.2 GLYAT, glycine, and musculoskeletal development

It was recently proposed that the GLYAT gene may be involved in determining lean muscle mass and bone size in humans [71]. About 690 000 single nucleotide polymorphisms (SNPs) were analyzed in large groups of unrelated Han Chinese (1627) and American Caucasian (2286) individuals to search for variations in the genome that correlate to variation in lean muscle mass and bone size. Fourteen SNPs with significant correlation were identified, three of which are located in or near the GLYAT gene (rs2507838, rs7116722, and rs11826261). Guo *et al.* explained this correlation by stating that GLYAT is important in the metabolism of glucose, but we are not aware of a direct relationship between GLYAT and glucose metabolism. The correlation they report is significant, however, suggesting that GLYAT may play an as yet unknown role in musculoskeletal development [71]. The significance of this observation is further elaborated on in Section 7.

5. Glycine *N*-acyltransferase

5.1 Biochemical and enzymatic characteristics of GLYAT

GLYAT is a monomeric detoxification enzyme found in the mitochondrial matrix of mammalian liver and kidney [58,60,68,72-80]. GLYAT was first identified in bovine liver mitochondria in 1953 and subsequently isolated and characterized from human liver mitochondria in 1976 [16,81]. GLYAT catalyses the transfer of an acyl group from an acyl-CoA to the amino group of glycine, forming an acylglycine and CoASH. Both products of the reaction are powerful inhibitors, and product inhibition is readily observed in enzyme assays [73,82]. Human GLYAT can use several endogenous and xenobiotic acyl-CoAs as substrates, as is evidenced by excretion of corresponding acylglycines in urine (**Table 1**) [4,35,42,43,50,54,63,83]. However, very little information is available on the kinetic parameters of human GLYAT [60,72,74,78,81]. The apparent K_M (benzoyl-CoA) value is reported to range from 13 µM to 57.9 mM, and the V_{max} value using benzoyl-CoA and glycine is reported as 700 nmol/min/mg and 17.1 µmol/min/mg (**Table 2**). This large variation in reported values is difficult to explain, but differences in the method of kinetic analysis, substrate quality, enzyme quality, experimental technique, and perhaps genetic heterogeneity of the GLYAT gene may be responsible [81,84]. The molecular mass of human GLYAT has been reported as 24, 27, 30, and 30.5 kDa [60,74,78,81]. This variation in reported values may be partly explained by the different techniques used in the different studies. For example, Kelley and Vessey [73] found that bovine GLYAT bound to their gel filtration matrix, resulting in erroneous molecular mass estimates.

No structure has been reported for GLYAT; thus, little is known about structure–function relationships. However, GLYAT is a member of the GNAT (Gcn5-related N-acetyltransferase) superfamily. Because of the remarkable structural conservation in the GNAT superfamily, a molecular model of bovine GLYAT could be generated by homology modeling [85]. The model was used to propose that Glu^{226}, a highly conserved residue, is catalytically important. Kinetic characterization and pH profiling of an E226Q mutant demonstrated that Glu^{226} acts as a general base that deprotonates glycine before nucleophilic attack on the carbonyl of the acyl-CoA thioester (**Figure 3**) [85].

5.2 The GLYAT gene and genetic variation

The human GLYAT gene is located on chromosome 11 at position 11q12, spans over 23 000 base pairs, and contains six exons [40]. Two splice variants of human GLYAT mRNA exist, coding for isoforms a (296 residues) and b (162 residues). The transcript for isoform b does not contain exon 6, and there is no protein level evidence for the existence of isoform b [86]. Within the GLYAT gene, there are approximately 668 known SNPs (www.ensembl.org, February 2013), of which 12 are synonymous and 39 are nonsynonymous. Only two studies on relatively small groups of Japanese and French Caucasian individuals have reported novel genetic polymorphisms and allele frequencies of SNPs in human GLYAT [86,87]. The N156S variant had allele frequencies of 97 and 85% in the French Caucasian and Japanese populations, respectively. Because of this high frequency, it was suggested that the N156S allele, rather than the reference sequence (NM_201648.2), should be considered as the wild-type allele [86,87].

In a recent study, the relative enzyme activities of six known polymorphisms (K16N, S17T, R131H, N156S, F168L, and R199C) of a recombinant human GLYAT were compared to the enzyme encoded by the reference sequence (NM_201648.2) [84]. The N156S variant had a greater relative activity than the reference sequence, and this might further support the suggestion that the N156S allele represents the wild-type enzyme. It is interesting to note that the variants with low allelic frequencies (R131H, F168L and R199C) had higher apparent K_M (benzoyl-CoA) values or lower relative enzyme activity when compared to the reference sequence [84,86,87]. The V_{max} values of the variants investigated range from approximately 500 to 1200 nmol/min/mg, and the apparent K_M (benzoyl-CoA) values range from approximately 20 to 70 µM (**Table 3**). Compared to the reference sequence, the K16N, S17T, and R131H variants had similar activities, the F168L variant had decreased activity and an increased K_M (benzoyl-CoA) value, while the R199C variant had < 5% activity. These results indicate that SNP variations found in the human GLYAT gene may result in altered properties of the enzyme, and could perhaps explain some of the differences in kinetic parameters reported in the literature [84]. A molecular model of human GLYAT was used to help explain the altered kinetic properties of the R131H, F168L, and R199C variants of human GLYAT [84].

5.3 Paralogs of the human GLYAT gene

GLYAT is one of four putative glycine-conjugating enzymes. Two GLYAT-like genes, GLYAT-L1 and GLYAT-L2, are

Table 2. Kinetic parameters of human GLYAT.

Parameters	Values
K_M (benzoyl-CoA) (μM)	13 [60] 67 ± 5 [74] 57900 [78]
V_{max} (nmol/min/mg)	700 [60] 17100 [78]

located with the GLYAT gene on chromosome 11q12.1, while the GLYAT-L3 gene is located on chromosome 6p12.3 [40,68,88]. In addition to GLYAT, primates have another transferase that conjugates arylacetyl-CoAs to glutamine, forming phenylacetylglutamine and indoleacetylglutamine [74,81]. The GLYAT-L1 gene codes for the glutamine-conjugating enzyme in humans [68]. Both mitochondrial and cytoplasmic localizations of GLYAT-L1 have been reported, and this could be explained by the two alternative splice variants of GLYAT-L1 mRNA, which code for two isoforms (333 and 302 residues) with distinct N-termini and possibly different subcellular localization [40,81]. The 302-residue isoform of GLYAT-L1 is located in the cytoplasm and transcriptionally activates the heat shock factor pathway in HEK293T cells [40]. The two isoforms might thus have different functions in the mitochondria and cytoplasm [40,68,81]. It has not been investigated why primates, unlike other mammals, conjugate arylacetates to glutamine instead of glycine.

GLYAT-L2 mRNA is expressed in salivary gland, trachea, spinal cord, and skin fibroblasts. The enzyme is localized to the endoplasmic reticulum, and a recombinant GLYAT-L2 catalyses the formation of long-chain acylglycines such as N-arachidonoylglycine and N-oleoylglycine [88,89]. These are members of a class of cannabinoid-like signaling hormones that activate G-protein-coupled receptors and have antinociceptive, anti-inflammatory, and antiproliferative effects [90]. GLYAT-L2 activity is regulated by acetylation on Lys^{19} and mutation of Lys^{19} of a recombinant GLYAT-L2 to arginine or glutamine resulted in a 70 – 80% decrease in enzyme activity [89]. Mutation of the equivalent Lys^{20} residue of a recombinant human GLYAT to arginine or glutamine did not cause a similar reduction in enzyme activity, suggesting that acetylation of this lysine residue is not important in regulation of GLYAT activity [84,89]. No enzyme activity has been reported for GLYAT-L3, which does not seem to have the catalytic glutamate residue proposed for the GLYAT reaction mechanism, but the significance of this is unclear [85,88].

6. Summary

Compared to the cytochrome P450 and UDP-glucuronosyltransferase superfamilies of biotransformation enzymes, GLYAT is not very well characterized [3,4]. This may be because of the small number of pharmaceutical drugs that are metabolized to glycine conjugates and the difficulty in obtaining human material and xenobiotic acyl-CoA substrates for research [3]. In this review, we have demonstrated that glycine conjugation is an important metabolic pathway that plays a role in the metabolism of CoASH and glycine and can influence mitochondrial energy production (**Figures 2** and **4**) [4,84]. Recent studies suggest that GLYAT may also be an important factor in the development of hepatocellular carcinoma and could influence musculoskeletal development and growth [68,71].

A range of xenobiotic acylglycines are excreted in urine, indicating that either the parent xenobiotic or a carboxylate metabolite is a substrate for ligation to CoASH, and that the acyl-CoA is a substrate for glycine conjugation (**Table 1**) [4,29,35,44,50,63,65,83]. The toxicity of xenobiotic carboxylates is partially determined by the extent to which an acyl-CoA, that cannot be conjugated to glycine or some other acceptor, is formed [3,4,31,33]. This leads to accumulation of the acyl-CoA, which can have several toxic effects in addition to disrupting mitochondrial energy production [23,33]. In severe cases, this can lead to hepatic steatosis and death [3,12]. As the glycine conjugation pathway is saturable, variation in the rate of glycine conjugation influences the clearance of xenobiotics and thus toxicity [3,64]. If the rate of conjugation by GLYAT is low, glycine conjugation may not prevent the toxicity of an acyl-CoA, even if it is a substrate for the enzyme (**Table 1**) [3,37,42,57].

It is often unclear whether variation in the rate of glycine conjugation results from differences in acid:CoA ligase activity or GLYAT activity (**Figure 1**) [4,55,59,67]. This is complicated by the observation that the limiting step depends on the xenobiotic, making it difficult to compare the results of different studies [29,64]. The recent expression and characterization of recombinant human GLYAT enzymes made an important contribution to our understanding of variation in glycine conjugation by demonstrating that genetic variation in the GLYAT gene can influence GLYAT enzyme activity [68,84,88,89].

7. Expert opinion

7.1 GLYAT and its relationship to liver cancer and musculoskeletal development

It was recently proposed that the GLYAT gene may play important roles in development of both hepatocellular carcinoma and the musculoskeletal system [68,71]. We suggest that these relationships may be explained by the role of GLYAT in glycine metabolism. Glycine is commonly considered as a nonessential amino acid because it can be synthesized from serine (**Figure 4**) [91,92]. However, studies over the past two decades have shown that glycine is in fact a semi-essential amino acid, that humans may have a daily shortage of about 10 g of glycine and that this may impact on collagen turnover and the synthesis of bile acids, creatine, glutathione, and heme [8,91-93].

Under certain conditions, GLYAT can conjugate sufficient amounts of glycine to limit its availability for other metabolic

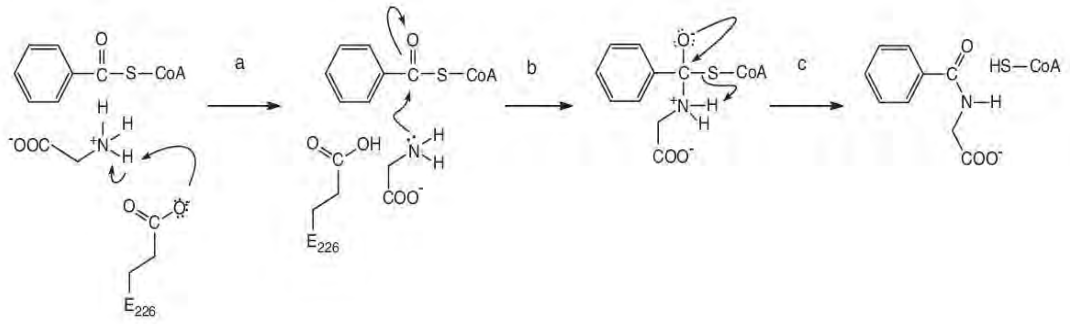

Figure 3. The catalytic mechanism proposed for bovine GLYAT. Bovine GLYAT employs a ternary complex mechanism, where Glu^{226} serves as a general base catalyst. (a) For nucleophilic attack to take place, the glycine amino group must be deprotonated by Glu^{226}; (b) A tetrahedral intermediate is formed, following the nucleophilic attack by the amino group of glycine on the thioester carbonyl group. (c) Finally, the tetrahedral intermediate collapses, forming benzoylglycine and CoASH. ChemDraw 10.0 (CambridgeSoft, Cambridge, MA) was used to produce this schematic.
Reproduced with permission [85] from the American Society for Pharmacology and Experimental Therapeutics.

Table 3. Kinetic parameters of recombinant human GLYAT enzymes.

Recombinant GLYAT	K_M (benzoyl-CoA) (μM)	V_{max} (nmol/min/mg)
Flag-His_6-hGLYAT (N156S)	209	807 [68]
Trx-His_6-hGLYAT (NM_201648.2)	24 ± 3	730 ± 30 [84]
Trx-His_6-hGLYAT (K16N)	21 ± 1	1030 ± 20 [84]
Trx-His_6-hGLYAT (S17T)	28 ± 5	665 ± 40 [84]
Trx-His_6-hGLYAT (R131H)	71 ± 11	1040 ± 85 [84]
Trx-His_6-hGLYAT (N156S)	38 ± 4	1230 ± 60 [84]
Trx-His_6-hGLYAT (F168L)	53 ± 6	500 ± 30 [84]
Trx-His_6-hGLYAT (R199C)	Not determined	Not determined [84]

processes (**Figure 4**) [61,94,95]. For example, it has been shown that administration of benzoate to rats can reverse chemically induced porphyria by diverting glycine away from heme biosynthesis. This results in normalization of urinary δ-aminolevulinate, porphobilinogen, and porphyrin levels, an effect cancelled out by co-administration of glycine [8,94]. We suggest that the recently reported correlation of SNPs in and near the GLYAT gene to variation in lean muscle mass and bone size could be explained, in part, by the impact of GLYAT on the availability of glycine for the synthesis of creatine, collagen, and elastin [71,91,92]. It is interesting that apart from the normal expression of GLYAT in liver and kidney, low levels of GLYAT expression have also been observed in skeletal muscle, but the significance of this observation is unclear [68].

It was recently demonstrated that glycine is a metabolite crucial for rapid division of cancer cells and that inhibition of glycine uptake or biosynthesis impaired the cancer cell growth, probably by slowing the synthesis of nucleic acids (**Figure 4**) [69]. We suggest that this helps to explain why GLYAT is not expressed in hepatocellular carcinoma, as depletion of hepatic glycine by GLYAT would inhibit rapid proliferation of cancer cells [68-70,96,97]. This could have significant implications for both the diagnosis and treatment of liver cancer.

7.2 The increased demand for glycine conjugation in modern life

In modern times increasing exposure to benzoate, salicylate, solvents, and drugs that are metabolized to acyl-CoA intermediates places more pressure on the glycine conjugation pathway, possibly exacerbating metabolic glycine shortage discussed previously [3,4,63,83,84,91,93]. Therefore, the consequences of interindividual variation in the glycine conjugation pathway may become more significant as more xenobiotic organic acids are encountered in the future [3].

In addition to xenobiotics, SCFAs produced by intestinal microbes are another potential source of substrates for glycine conjugation and may contribute to glycine depletion under some conditions [98]. Gut microbes produce large amounts of SCFAs that account for 5 – 10% of the total dietary energy intake in humans [98]. Indeed, the gut contains an active SCFA ligase for metabolizing these organic acids [99]. Gut dysbiosis, caused by antibiotic use, for example, can result in increased SCFA production and this has been associated with obesity and diabetes [100,101]. SCFAs are not usually

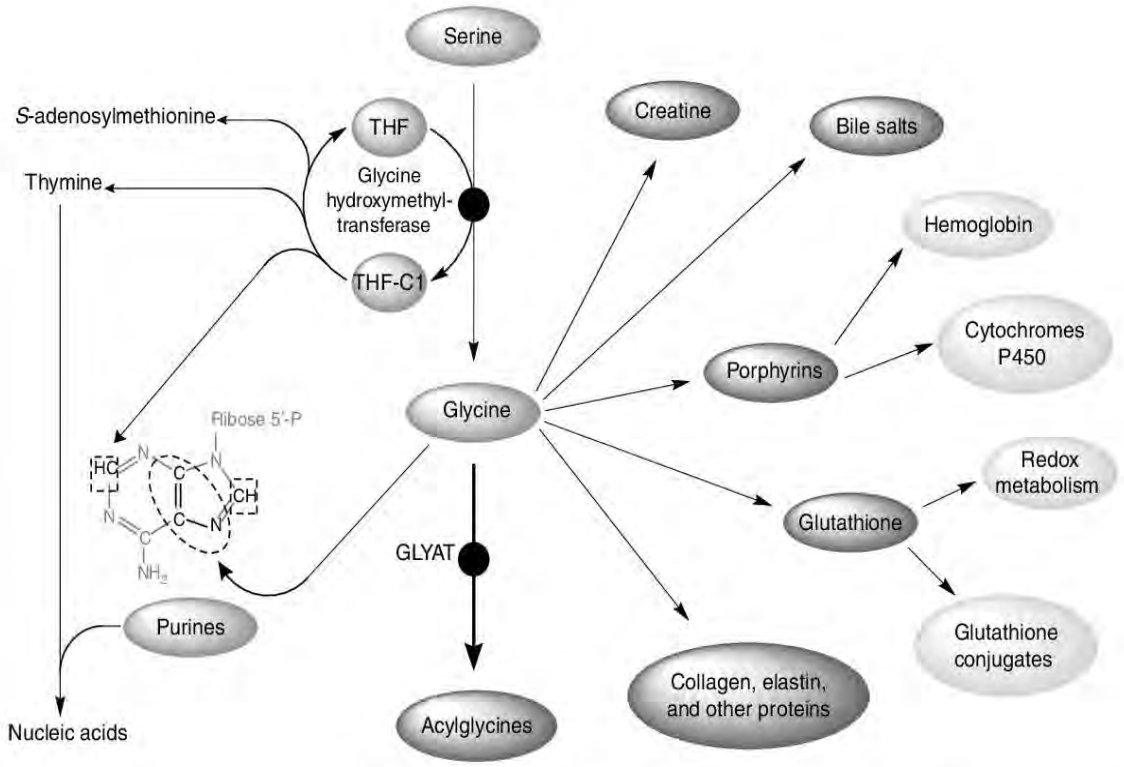

Figure 4. Biosynthesis and metabolic consumption of glycine. Glycine is biosynthesized from serine by glycine hydroxymethyltransferase. The reaction converts tetrahydrofolate (THF) to tetrahydrofolate-C1 (THF-C1) for each molecule of glycine produced. The total amount of glycine synthesized can thus not exceed the amount of THF-C1 consumed through the production of *S*-adenosylmethionine, thymine, and purines. Glycine is used in the production of glutathione, creatinine, bile salts, porphyrins, collagen, elastin, and other proteins. The bold arrow indicates the formation of xenobiotic acylglycines by GLYAT. The parts of purine rings derived from glycine and THF-C1 are indicated by the dashed ellipse and squares, respectively. The black circles indicate the glycine hydroxymethyltransferase and GLYAT enzymes.

conjugated to glycine, as this would be energetically wasteful [14,42,43]. However, it is our opinion that if sufficiently large amounts of SCFAs are produced, hepatic metabolism of CoASH and glycine will be affected. A recent observation in our laboratory seems to support this idea. A patient with unusually high levels of urinary butyrate complained of bad body odor and was referred to our laboratory by a physician. The increased butyrate excretion was not the result of a short-chain acyl-CoA dehydrogenase defect, and gut dysbiosis was suspected. Glycine supplementation was recommended and this resulted in significantly increased butyrylglycine excretion, decreased butyrate excretion, and disappearance of the body odor (unpublished results).

7.3 Future investigations of interindividual variation in glycine conjugation

In conclusion, we believe that it is important to study the relationships between genetic variation in the GLYAT gene, GLYAT enzyme activity, the *in vivo* rate of glycine conjugation, and physiological consequences of variation in the glycine conjugation pathway.

Existing publications on interindividual variation in the glycine conjugation pathway do not discriminate between variation in acyl-CoA formation and variation in glycine conjugation. It is important to remember that glycine conjugation is a two-step process and that the overall rate of glycine conjugation can be influenced by several factors (**Figure 1**) [3,4,55,59,67]. Most importantly, the limiting step in the glycine conjugation pathway depends on the xenobiotic used [29,64]. We suggest that future studies employ at least salicylate and benzoate as probe compounds, on separate occasions, to enable differentiation between variation in acid:CoA ligase and GLYAT activities, respectively.

The use of benzoate as a probe compound is, however, complicated by the metabolism of gut microorganisms. It was mentioned in Section 3.1 that gut dysbiosis in Crohn's disease results in decreased microbial benzoate production and lower levels of hippurate in urine [45]. Gut metabolism will thus influence relative increases in urinary hippurate levels after benzoate ingestion without necessarily affecting the rate of hepatic glycine conjugation [44,45]. Therefore, it is important to determine increases in hippurate excretion rather

than ratios to baseline levels [45]. An alternative probe compound is 4-aminobenzoate [65]. However, substituted benzoates are generally activated to acyl-CoAs more slowly than benzoate [24,27,29]. This suggests that glycine conjugation of 4-aminobenzoate, as with salicylate, is limited at the acid: CoA ligase step. This is consistent with the suggestion that the decreased glycine conjugation of 4-aminobenzoate by hepatitis patients is because of decreased mitochondrial ATP production [3]. Determination of hippurate formed from an oral dose of stable isotope labelled benzoate is one suggestion to simplify the interpretation of benzoate conjugation data, which we believe cannot be substituted for by salicylate or 4-aminobenzoate.

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Declaration of interest

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Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Ure A. On gouty concretions; with a new method of treatment. Pharm J Trans 1841;1:24
2. Keller W. On the conversion of benzoic acid into hippuric acid. Prov Med J Retrosp Med Sci 1842;4(13):256-7
3. Knights KM, Sykes MJ, Miners JO. Amino acid conjugation: contribution to the metabolism and toxicity of xenobiotic carboxylic acids. Expert Opin Drug Metab Toxicol 2007;3(2):159-68

•• **A very good review of amino acid conjugation of xenobiotics.**

4. Knights KM, Miners JO. Amino acid conjugation: a novel route of xenobiotic carboxylic acid metabolism in man. 1st edition. John Wiley & Sons, Inc; 2012
5. Wallig MA. Glucuronidation and susceptibility to chemical carcinogenesis. Toxicol Sci 2004;78(1):1-2
6. Grillo MP. Drug-S-acyl-glutathione thioesters: synthesis, bioanalytical properties, chemical reactivity, biological formation and degradation. Curr Drug Metab 2011;12(3):229-44
7. Sidenius U, Skonberg C, Olsen J, Hansen SH. In vitro reactivity of carboxylic acid-CoA thioesters with glutathione. Chem Res Toxicol 2004;17:75-81
8. Oates PS, West AR. Heme in intestinal epithelial cell turnover, differentiation, detoxifi cation, infl ammation, carcinogenesis, absorption and motility. World J Gastroenterol 2006;12(27):4281-95
9. Potter T, Lewis R, Luker T, et al. In silico prediction of acyl glucuronide reactivity. J Comput Aided Mol Des 2011;25:997-1005
10. Eaton S, Bartlett K, Pourfarzam M. Mammalian mitochondrial b-oxidation. Biochem J 1996;320:345-57
11. Leonardi R, Zhang Y, Rock CO, et al. Back in action. Prog Lip Res 2005;44:125-53
12. Mitchell GA, Gauthier N, Lesimple A, et al. Hereditary and acquired diseases of acyl-coenzyme A metabolism. Mol Genet Metab 2008;94(1):4-15

•• **Describes the CASTOR disorders and provides a very good introduction to coenzyme A metabolism.**

13. Zhang Y, Chohnan S, Virga KG, et al. Chemical knockout of pantothenate kinase reveals the metabolic and genetic program responsible for hepatic coenzyme a homeostasis. Chem Biol 2007;14(3):291-302

•• **Describes the effects of hopantenate on CoASH metabolism. It is an excellent demonstration of the importance of CoASH in energy metabolism and describes the transcriptional reprogramming of cells that results from CoASH depletion.**

14. Wanders RJA, Vreken P, den Boer MEJ, et al. Disorders of mitochondrial fatty acyl-CoA b-oxidation. J Inher Metab Dis 1999;22:442-87
15. Knights KM. Role of hepatic fatty acid: coenzyme A ligases in the metabolism of xenobiotic carboxylic acids. Clin Exp Pharmacol Physiol 1998;25:776-82
16. Schachter D, Taggart JV. Benzoyl coenzyme A and hippurate synthesis. J Biol Chem 1953;203:925-34
17. Mashek DG, Bornfeldt KE, Coleman RA, et al. Revised nomenclature for the mammalian long-chain acyl-CoA synthetase gene family. J Lipid Res 2004;45:1958-61
18. Knights KM, Drogemiller CJ. Xenobiotic-CoA Ligases: kinetic and Molecular Characterization. Curr Drug Metab 2000;1:49-66

• **A very useful review of the medium-chain acid:CoA ligases that are important in xenobiotic metabolism and amino acid conjugation.**

19. Kasuya F, Igarashi K, Fukui M, Nokihara K. Purification and characterization of a medium chain acyl-coenzyme A synthetase. Drug Metab Dispos 1996;24(8):879-83
20. Killinberg PG, Davidson ED, Webster LT. Evidence for a medium-chain fatty acid: coenzyme A ligase (adenosine monophosphate) that activates salicylate. Mol Pharmacol 1971;7:260-8
21. Vessey DA, Chen W, Ramsay RR. Effect of carboxylic acid xenobiotics and their metabolites on the activity of carnitine acyltransferases. Biochem J 1991;279:895-7
22. Vessey DA, Hu J, Kelley M. Interaction of salicylate and ibuprofen with the carboxylic acid: coA ligases from bovine liver mitochondria. J Biochem Toxicol 1996;11(2):73-8
23. Pessayre D, Fromenty B, Berson A, et al. Central role of mitochondria in drug-induced liver injury. Drug Metab Rev 2012;44(1):34-87

• **An excellent recent review on the role of mitochondria in drug induced liver injury explaining how drugs can cause CoASH sequestration, inhibit β-oxidation and ATP synthesis, and**

interfere with mitochondrial DNA replication and integrity.

24. Vessey DA, Hu J. Isolation from bovine liver mitochondria and characterization of three distinct carboxylic acid: coA ligases with activity toward xenobiotics. J Biochem Toxicol 1995;10(6):329-37

25. Vessey DA, Kelley M. Characterization of the reaction mechanism for the XL-I form of bovine liver xenobiotic/medium-chain fatty acid:coA ligase. Biochem J 2001;357:283-8

26. Watkins PA, Maiguel D, Jia Z, Pevsner J. Evidence for 26 distinct acyl-coenzyme A synthetase genes in the human genome. J Lipid Res 2007;48:2736-50

27. Vessey DA, Kelley M, Warren RS. Characterization of the CoA ligases of human liver mitochondria catalyzing the activation of short- and medium-chain fatty acids and xenobiotic carboxylic acids. Biochim Biophys Acta 1999;1428:455-62

28. Vessey DA, Lau E, Kelley M, Warren RS. Isolation, Sequencing, and Expression of a cDNA for the HXM-A Form of Xenobiotic/Medium-Chain Fatty Acid:coA Ligase from Human Liver Mitochondria. J Biochem Mol Toxicol 2003;17(1):1-6

29. Kasuya F, Yamaoka Y, Osawa E, et al. Difference of the liver and kidney in glycine conjugation of ortho-substituted benzoic acids. Chem Biol Interact 2000;125:39-50

30. Yao KW, Mao LF, Luo MJ, Schulz H. The relationship between mitochondrial activation and toxicity of some substituted carboxylic acids. Chem Biol Interact 1994;90(3):225-34

31. Fromenty B, Pessayre D. Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. Pharmacol Ther 1995;67(1):101-54

•• **A very good introduction to hepatotoxicity resulting from inhibition of mitochondrial β-oxidation. Explains how some pharmaceuticals cause CoASH sequestration and consequences thereof.**

32. Ruff LJ, Brass EP. Metabolic effects of pivalate in isolated rat hepatocytes. Toxicol Appl Pharmacol 1991;110(2):295-302

33. Begriche K, Massart J, Robin MA, et al. Drug-induced toxicity on mitochondria and lipid metabolism: mechanistic diversity and deleterious consequences for the liver. J Hepatol 2011;54:773-94

• **A good overview of drug induced mitochondrial toxicity and effects on lipid metabolism.**

34. Guarente L. Sirtuins, aging, and medicine. N Engl J Med 2011;364:2235-44

35. Sakuma T. Alteration of urinary carnitine profile induced by benzoate administration. Arch Dis Child 1991;66:873-5

36. Feoli-Fonseca JC, Lambert M, Mitchell G, et al. Chronic sodium benzoate therapy in children with inborn errors of urea synthesis: effect on carnitine metabolism and ammonia nitrogen removal. Biochem Mol Med 1996;57:31-6

37. Fenton WA, Gravel RA, Rosenblatt DS. Part 9: organic acids. Chapter 94: disorders of propionate and methylmalonate metabolism. In: Vale D, Beaudet AL, Vogelstein B, et al., editors, The online metabolic and molecular bases of inherited diseases. McGraw Hill, New York; 2013

38. Geelen MJH. Medium-chain fatty acids as short-term regulators of hepatic lipogenesis. Biochem J 1994;302:141-6

39. Glasgow JFT, Middleton B. Reye syndrome—insights on causation and prognosis. Arch Dis Child 2001;85:351-3

40. Zhang H, Lang Q, Li J, et al. Molecular cloning and characterization of a novel human glycine N-acyltransferase gene GLYAT1, which activates transcriptional activity of HSE pathway. Int J Mol Sci 2007;8:433-44

41. Hunt MC, Siponen MI, Alexson SEH. The emerging role of acyl-CoA thioesterases and acyltransferases in regulating peroxisomal lipid metabolism. Biochim Biophys Acta 2012;1822:1397-410

42. Bartlett K, Gompertz D. The specificity of glycine-N-acylase and acylglycine excretion in the organicacidaemias. Biochem Med 1974;10(1):15-23

• **Of great interest to the study of inborn errors of organic acid metabolism. Describes a relationship between the acylglycines excreted by organic acidemia patients and the substrate specificity of a bovine GLYAT.**

43. Bonafe L, Troxler H, Kuster T, et al. Evaluation of urinary acylglycines by electrospray tandem mass spectrometry in mitochondrial energy metabolism defects and organic acidurias. Mol Genet Metab 2000;69:302-11

44. Rechner AR, Kuhnle G, Bremner P, et al. The metabolic fate of dietary polyphenols in humans. Free Radic Biol Med 2002;33(2):220-35

45. Williams HRT, Cox IJ, Walker DG, et al. Differences in gut microbial metabolism are responsible for reduced hippurate synthesis in Chron's disease. BMC Gastroenterol 2010;10:108

46. Paterson JR, Lawrence JR. Salicylic acid: a link between aspirin, diet and the prevention of colorectal cancer. Q J Med 2001;94:445-8

47. Furuya KN, Durie PR, Roberts EA, et al. Glycine conjugation of Para-Aminobenzoic Acid (PABA): a quantitative test of liver function. Clin Biochem 1995;28(5):531-40

48. Furst DE, Gupta N, Paulus HE. Salicylate metabolism in twins. Evidence suggesting a generic influence and induction of salicylurate formation. J Clin Invest 1977;60:32-42

49. Dorne JLCM, Walton K, Renwick AG. Human variability for metabolic pathways with limited data (CYP2A6, CYP2C9, CYP2E1, ADH, esterases, glycine and sulphate conjugation). Food Chem Toxicol 2004;42:397-421

50. Tanaka K, Isselbacher KJ. The isolation and identification of N-isovalerylglycine from urine of patients with isovaleric acidemia. J Biol Chem 1967;242(12):2966-72

51. Tanaka K, Budd WA, Efron ML, Isselbacher KJ. Isovaleric acidemia: a new genetic defect of leucine metabolism. Biochemistry 1966;56:236-42

52. Ogier DB, Saudubray JM. Branched-chain organic acidurias. Semin Neonatol 2002;7(1):65-74

53. Gompertz D. Inborn errors of organic acid metabolism. Clin Endocrinol Metab 1974;3:107-130

54. Gron I, Gregersen N, Kolvraa S, Rasmussen K. Formation of N-dicarboxyl-mono-glycines catalyzed bt glycine-N-Acylase. Biochem Med 1978;19:133-40

55. Krieger I, Tanaka K. Therapeutic effects of glycine in isovaleric acidemia. Pediatr Res 1976;10:25-9

56. Dercksen M, Duran M, Ijlst L, et al. Clinical variability of isovaleric acidemia in a genetically homogeneous population. J Inherit Metab Dis 2012;2012:17

57. Sweetman L, Williams JC. Part 9: organic acids Chapter 93: branched chain organic acidurias. In: Vale D, Beauder AL, Vogelstein B, et al., editors, The online metabolic and molecular bases of inherited diseases; McGraw Hill, New York; 2013

58. Nandi DL, Lucas SV, Webster LT Jr. Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine N-acyltransferase from bovine liver mitochondria. Purification and characterization. J Biol Chem 1979;254(15):7230-7

59. Gregus Z, Fekete T, Varga F, Klaassen CD. Dependence of glycine conjugation on availability of glycine: role of the glycine cleavage system. Xenobiotica 1993;23(2):141-53

60. van der Westhuizen FH, Pretorius PJ, Erasmus E. The utilization of alanine, glutamic acid, and serine as amino acid substrates for glycine N-acyltransferase. J Biochem Mol Toxicol 2000;14(2):102-9

• **Demonstrates that GLYAT can also conjugate benzoate to other amino acids, including serine, alanine, asparagine, glutamine, and glutamate.**

61. Loots D, Erasmus E, Mienie LJ. Identification of 19 new metabolites induced by abnormal amino acid conjugation in isovaleric acidemia. Clin Chem 2005;51(8):1510-12

62. Vest MF, Salzberg R. Conjugation reactions in the newborn infant: the metabolism of para-aminobenzoic acid. Arch Dis Childh 1965;40:97-105

63. Campbell L, Wilson HK, Samuel AM, Gompertz D. Interactions of m-xylene and aspirin metabolism in man. Br J Ind Med 1988;45(2):127-32

64. Temellini A, Mogavero S, Giulianotti PC, et al. Conjugation of benzoic acid with glycine in human liver and kidney: a study on the interindividual variability. Xenobiotica 1993;23(12):1427-33

65. Duffy LF, Kerzner B, Seeff L, et al. Preliminary assessment of glycine conjugation of para-aminobenzoic acid as a quantitative test of liver function. Clin Biochem 1995;28(5):527-30

66. Lebel S, Nakamachi Y, Hemming A, et al. Glycine conjugation of para-aminobenzoic acid (PABA): a pilot study of a novel prognostic test in acute liver failure in children. J Pediatr Gastroenterol Nutr 2003;36(1):62-71

67. Gregus Z, Fekete T, Varga F, Klaassen CD. Effect of valproic acid on glycine conjugation of benzoic acid. J Pharmacol Exp Ther 1993;267(3):1068-75

68. Matsuo M, Terai K, Kameda N, et al. Designation of enzyme activity of glycine-N-acyltransferase family genes and depression of glycine-N-acyltransferase in human hepatocellular carcinoma. Biochem Biophys Res Commun 2012;420(4):901-6

•• **First report on the properties of bacterially expressed recombinant human GLYAT and GLYAT-L1 enzymes. Demonstrates that GLYAT-L1 is the glutamine transferase in humans, and that GLYAT is down regulated in hepatocellular carcinoma.**

69. Jain M, Nilsson R, Sharma S, et al. Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. Science 2012;336(6084):1040-4

• **Reports the results of CORE (consumption and release) analyses on the NCI-60 panel of cancer cell lines. Glycine was found to be a metabolite crucial to rapid proliferation of cancerous cells.**

70. Renwick SB, Snell K, Baumann U. The crystal structure of human cytosolic serine hydroxymethyltransferase: a target for cancer chemotherapy. Structure 1998;6(9):1105-16

71. Guo Y, Zhang L, Liu Y, et al. Suggestion of GLYAT gene underlying variation of bone size and body lean mass as revealed by a bivariate genome-wide association study. Hum Genet 2013;132(2):189-99

•• **Reports that SNPs in and near the human GLYAT gene correlate to variation in bone size and lean muscle mass, suggesting that GLYAT plays a role in musculoskeletal development.**

72. Gregersen N, Kolvraa S, Mortensen PB. Acyl-CoA: glycine N-acyltransferase: in vitro studies on the glycine conjugation of straight- and branched-chained acyl-CoA esters in human liver. Biochem Med Metab Biol 1986;35(2):210-18

73. Kelley M, Vessey D. The effects of ions on the conjugation of xenobiotics by the aralkyl-CoA and arylacetyl-CoA N-acyltransferases from bovine liver mitochondria. J Biochem Toxicol 1990;5:125-35

74. Kelley M, Vessey D. Characterization of the acyl-CoA: amino acid N-acyltransferases from primate liver mitochondria. J Biochem Toxicol 1994;9:153-8

75. Kelley M, Vessey DA. Isolation and characterization of mitochondrial acyl-CoA: glycine N-acyltransferases from kidney. J Biochem Toxicol 1993;8(2):63-9

76. Kolvraa S, Gregersen N. Acyl-CoA: glycine N-acyltransferase: organelle localization and affinity toward straight- and branched-chained acyl-CoA esters in rat liver. Biochem Med Metab Biol 1986;36(1):98-105

77. Lau EP, Haley BE, Barden RE. Photoaffinity labeling of acyl-coenzyme A:glycine N-acyltransferase with p-azidobenzoyl-coenzyme A. Biochemistry 1977;16(12):2581-5

78. Mawal YR, Qureshi IA. Purification to homogeneity of mitochondrial acyl coa: glycine n-acyltransferase from human liver. Biochem Biophys Res Commun 1994;205(2):1373-9

79. Schachter D, Taggart JV. Glycine N-acylase: purification and properties. J Biol Chem 1954;208(1):263-75

• **Of historical interest. The first report of glycine N-acyltransferase activity from bovine liver.**

80. Vessey DA, Lau EP. Determination of the Sequence of the arylacetyl acyl-CoA: amino acid N-acyltransferase from bovine liver mitochondria and its homology to the aralkyl acyl-CoA:amino acid N-acyltransferase. J Biochem Mol Toxicol 1998;12(5):275-9

81. Webster LT, Siddiqui UA, Lucas SV, et al. Identification of separate acyl-CoA:glycine and acyl-CoA:L-glutamine N-acyltransferase activities in mitochondrial fractions from liver of

rhesus monkey and man. J Biol Chem 1976;251(11):3352-8

• **Of historical interest. The first report of glycine N-acyltransferase isolated from human liver. Demonstrates that primates have separate benzoylglycine and phenylacetylglutamine synthesizing enzymes.**

82. Schachter D, Taggart JV. Product inhibition in the clygine N-acylase reaction. J Biol Chem 1954;211(1):271-8

83. Huang M, Jin C, Liu Y, et al. Exposure of workers to a mixture of toluene and xylenes. I metabolism. Occup Environ Med 1994;51:42-6

84. van der Sluis R, Badenhorst CPS, van der Westhuizen FH, van Dijk AA. Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase. Gene 2013;515:447-53

•• **This paper describes the enzymatic consequences of SNPs in the GLYAT gene on a recombinant human GLYAT enzyme.**

85. Badenhorst CPS, Jooste M, van Dijk AA. Enzymatic characterization and elucidation of the catalytic mechanism of a recombinant bovine glycine N-acyltransferase. Drug Metab Dispos 2012;40(2):346-52

• **A molecular model of bovine GLYAT is described and is used to propose a catalytic mechanism for the GLYAT enzymes.**

86. Lino Cardenas CL, Bourgine J, Cauffiez C, et al. Genetic polymorphisms of Glycine N-acyltransferase (GLYAT) in a French Caucasian population. Xenobiotica 2010;40(12):853-61

• **Describes novel genetic variations in a small group of French-Caucasian individuals and suggests that the N156S variant of human GLYAT be considered the wild-type.**

87. Yamamoto A, Nonen S, Fukuda T, et al. Genetic polymorphisms of glycine N-acyltransferase in Japanese individuals. Drug Metab Pharmacokinet 2009;24(1):114-17

• **The first publication on genetic variations in the human GLYAT gene in a small group of Japanese individuals.**

88. Waluk DP, Schultz N, Hunt MC. Identification of glycine N-acyltransferase-like 2 (GLYATL2) as a transferase that produces N-acyl glycines in humans. FASEB J 2010;24(8):2795-803

•• **Reports bacterial expression and characterisation of a recombinant human GLYAT-L2, demonstrating that the enzyme catalyses glycine conjugation of long-chain acyl-CoAs.**

89. Waluk DP, Sucharski F, Sipos L, et al. Reversible lysine acetylation regulates activity of human glycine N-acyltransferase-like 2 (hGLYATL2): implications for production of glycine-conjugated signaling molecules. J Biol Chem 2012;287(20):16158-67

90. Bradshaw HB, Walker JM. The expanding field of cannabimimetic and related lipid mediators. Br J Pharmacol 2005;144:459-65

91. Melendez-Hevia E, De Paz-Lugo P, Cornish-Bowden A, Cardenas ML. A weak link in metabolism: the metabolic capacity for glycine biosynthesis does not satisfy the need for collagen synthesis. J Biosci 2009;34(6):853-72

•• **An excellent review of glycine metabolism, arguing that there is a significant deficiency of glycine in the average adult diet and that this may negatively impact on the rate of collagen turnover.**

92. Lewis RM, Godfrey KM, Jackson AA, et al. Low serine hydroxymethyltransferase activity in the human placenta has important implications for fetal glycine supply. J Clin Endocrinol Metab 2005;90(3):1594-8

93. Jackson AA. The glycine story. Eur J Clin Nutr 1991;45(2):59-65

94. Piper WN, Condie LW, Tephly TR. The role pf substrates for glycine acyltransferase in the reversal of chemically induced porphyria in the rat. Arch Biochem Biophy 1973;159:671-7

95. van der Westhuizen FH, Terblanche E, Mienie LJ. Benzoylserine and benzoylthreonine in propionic acidaemia treated with sodium benzoate. SAMJ 1999;89(12):125-7

96. Kastanos EK, Woldman YY, Appling DR. Role of mitochondrial and cytoplasmic serine hydroxymethyltransferase isozymes in de novo purine synthesis in saccharomyces cerevisiae. Biochemistry 1997;36:14956-64

97. Fulda S, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. Nat Rev Drug Discov 2010;9:447-64

98. Kimura I, Inoue D, Maeda T, et al. Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). PNAS 2011;108(19):8030-5

99. Vessey DA. Isolation and preliminary characterization of the medium-chain fatty acid:coA ligase responsible for activation of short- and medium-chain fatty acids in colonic mucosa from swine. Dig Dis Sci 2001;46:438-42

100. Holmes E, Li JV, Athanasiou T, et al. Microbes and Metabolism. Understanding the role of gut microbiome–host metabolic signal disruption in health and disease. Trends Microbiol 2011;19(7):349-59

101. Prakash S, Rodes L, Coussa-Charley M, Tomaro-Duchesneau C. Gut microbiota: next frontier in understanding human health and development of biotherapeutics. Biologics 2011;5:71-86

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APPENDIX B: PAPER II

A new perspective on the importance of glycine conjugation in the metabolism of aromatic acids.

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REVIEW ARTICLE

A new perspective on the importance of glycine conjugation in the metabolism of aromatic acids

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Abstract

A number of endogenous and xenobiotic organic acids are conjugated to glycine, in animals ranging from mosquitoes to humans. Glycine conjugation has generally been assumed to be a detoxification mechanism, increasing the water solubility of organic acids in order to facilitate urinary excretion. However, the recently proposed glycine deportation hypothesis states that the role of the amino acid conjugations, including glycine conjugation, is to regulate systemic levels of amino acids that are also utilized as neurotransmitters in the central nervous systems of animals. This hypothesis is based on the observation that, compared to glucuronidation, glycine conjugation does not significantly increase the water solubility of aromatic acids. In this review it will be argued that the major role of glycine conjugation is to dispose of the end products of phenylpropionate metabolism. Furthermore, glucuronidation, which occurs in the endoplasmic reticulum, would not be ideal for the detoxification of free benzoate, which has been shown to accumulate in the mitochondrial matrix. Glycine conjugation, however, prevents accumulation of benzoic acid in the mitochondrial matrix by forming hippurate, a less lipophilic conjugate that can be more readily transported out of the mitochondria. Finally, it will be explained that the glycine conjugation of benzoate, a commonly used preservative, exacerbates the dietary deficiency of glycine in humans. Because the resulting shortage of glycine can negatively influence brain neurochemistry and the synthesis of collagen, nucleic acids, porphyrins, and other important metabolites, the risks of using benzoate as a preservative should not be underestimated.

Keywords

Coenzyme A, CASTOR disorders, glycine N-acyltransferase, glycine conjugation, glycine deportation, benzoate, hippurate

History

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The history of glycine conjugation

Benzoic acid was the first compound investigated with regard to its fate in the body. As early as the eighteenth century, benzoate was used ''therapeutically'' as a tonic to stimulate respiration. In 1801 it was suggested that urine contained a compound similar to benzoate, rather than benzoate itself, and in 1829 Liebig discovered hippuric acid in the urine of horses (reviewed in Conti & Bickel, 1977). In 1841 Ure discovered that ingestion of benzoic acid resulted in urinary excretion of hippurate, a finding confirmed in 1842 by Keller (Keller, 1842; Ure, 1841). In 1845, Dessaignes demonstrated that hippurate is a peptide conjugate of benzoic acid and glycine (reviewed in Conti & Bickel, 1977). These discoveries marked the start of the new era of biotransformation research, and by the end of the nineteenth century most of the major pathways of drug metabolism had been discovered. These include oxidation and reduction, and conjugation to glucuronic acid, sulfate, and acetate (Conti & Bickel, 1977). Subsequently, very large numbers of enzymes and substrates were characterized for the oxidation, reduction, hydrolysis, glucuronidation, and sulfonation pathways (Conti & Bickel, 1977; Coon, 2005; Gamage et al., 2006; Guillemette, 2003; Oates & West, 2006; Ritter, 2000; Rodriguez-Antona et al., 2010; Weinshilboum et al., 1997). These biotransformation reactions are still the subject of much research in pharmacology and toxicology because of their impact on metabolism and predisposition to adverse drug responses (Gamage et al., 2006; Guillemette, 2003; Ritter, 2000; Rodriguez-Antona et al., 2010). Interest in glycine conjugation, however, significantly faded shortly after its discovery. It has been argued that this may be because of the small number of pharmaceutical drugs that are conjugated to glycine (Badenhorst et al., 2013; Knights & Miners, 2012; Knights et al., 2007). More recently, interest in the importance of glycine conjugation has increased again, and a recent review concluded that glycine conjugation, the ''poor cousin'' of the drug metabolism family, may now have ''inherited a fortune'' (Beyoglu & Idle, 2012; Beyoglu et al., 2012). This is because it is becoming clear that glycine conjugation is a very important metabolic pathway that can influence the metabolism of glycine, ATP, and CoASH, versatile metabolites

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that participate in a large number of metabolic pathways (Sections ''A brief introduction to glycine conjugation'', ''Factors that influence interindividual variation in the rate of glycine conjugation'', and ''The impact of glycine conjugation on glycine metabolism'').

The metabolic significance of glycine conjugation is not yet well understood

Collectively, conjugation reactions have traditionally been referred to as phase II biotransformation processes, indicating that the conjugates formed are less toxic or more readily excreted than the parent metabolites (Caldwell, 1982, 1984). Although the more recent discovery of reactive conjugates such as some acylglucuronides has led some researchers to abandon the concept of phase II biotransformation, it is still generally useful to think of conjugation reactions as mechanisms to accelerate excretion of a vast number of bioactive compounds (Caldwell, 1982; Ritter, 2000). What is unclear, however, is whether the glycine conjugation of benzoic acid should be viewed as a detoxification process. Recently an alternative to the ''detoxification hypothesis'' of glycine conjugation, the ''glycine deportation hypothesis'', was introduced. This hypothesis states that the primary purpose of amino acid conjugation reactions is to regulate levels of amino acids that are also utilized as neurotransmitters (Beyoglu & Idle, 2012; Beyoglu et al., 2012). This is because benzoate is usually conjugated to glycine, glutamine, glutamate, arginine, ornithine, or taurine, amino acids that are also utilized as neurotransmitters in the central nervous system (CNS) of animals. According to the glycine deportation hypothesis, accumulation of these neurotransmitters in the CNS, which would be neurotoxic, is avoided by the irreversible urinary excretion of these amino acids as conjugates to aromatic acids such as benzoic acid. The basis of this argument is that amino acid conjugation does not significantly increase the water solubility of aromatic acids, as has generally been assumed (Beyoglu & Idle, 2012; Beyoglu et al., 2012). For example, the solubility of benzoic acid in water is 3.4 g/l, and that of hippuric acid is only 3.75 g/l. In the case of phenylacetic acid, water solubility decreases from 16.6 g/l to 7.3 g/l or 2.12 g/l, when conjugated to glycine or glutamine, respectively. In contrast, the glucuronidation of benzoate increases water solubility to 263 g/l. According to the glycine deportation hypothesis, glucuronidation would thus be a better choice of conjugation reaction if the goal was to increase water solubility and facilitate urinary excretion (Beyoglu & Idle, 2012; Beyoglu et al., 2012).

Developing a more comprehensive understanding of the glycine conjugation pathway

The purpose of this article is to review the literature on glycine conjugation, in an attempt to clarify the role of the glycine conjugation pathway in metabolism. To achieve this goal of clarification, several important arguments will be made. First, that it may be misleading to view benzoic acid as the typical substrate for the glycine conjugation pathway, and that the glycine conjugation reaction should rather be viewed as a part of the phenylpropionate catabolism pathway (Section ''A brief introduction to glycine conjugation'' and Figure 1). Second, that aromatic acids are toxic to mitochondria and that glycine conjugation serves to decrease this toxicity (Sections ''The toxicity of benzoate and other aromatic acids'' and ''The importance of glycine conjugation in the metabolism of aromatic acids''). Finally, it will be argued that glycine deportation does not seem to be the primary purpose of the glycine conjugation pathway, but is rather a phenomenon that becomes important when large amounts of substrates for glycine conjugation are consumed. It will thus be suggested that the glycine deportation phenomenon forms part of the neurological mechanisms that enable animals to limit their consumption of foods rich in aromatic compounds such as benzoic acid (Section ''The impact of glycine conjugation on glycine metabolism'').

A brief introduction to glycine conjugation

The glycine conjugation of benzoic acid

Several xenobiotic organic acids are metabolized by conjugation to glycine in the mitochondrial matrix in mammalian liver and kidney, followed by urinary excretion (Knights & Miners, 2012; Knights et al., 2007). The most abundant amino acid conjugate excreted in the urine of almost all mammals is hippurate, the glycine conjugate of benzoate (Lees et al., 2013). Hippurate is formed from benzoate in two steps (Figure 1, reactions 2 and 5). First, benzoate is activated to benzoyl-CoA by the action of a mitochondrial ATP dependent acid:CoA ligase, which in humans has been identified as HXM-A (E.C. 6.2.1.2), encoded by the ACSM2A gene (Knights, 1998; Knights & Drogemiller, 2000; Schachter & Taggart, 1953; Vessey et al., 1999; Vessey 2003). Second, the enzyme glycine N-acyltransferase (GLYAT, E.C. 2.3.1.13) binds benzoyl-CoA and catalyses the acylation of glycine to form hippurate and CoASH (Schachter & Taggart, 1953, 1954). Benzoyl-CoA is the preferred substrate for the orthologs of GLYAT that have been isolated from the livers and kidneys of several mammals, including rats, rabbits, sheep, cows, rhesus monkeys, and humans (Gregersen et al., 1986; Kelley & Vessey, 1990, 1993, 1994; Kolvraa & Gregersen, 1986; Mawal & Qureshi, 1994; Nandi et al., 1979; Schachter & Taggart, 1954; Webster et al., 1976). Several other acyl-CoAs, including salicylyl-CoA, 4-aminobenzoyl-CoA, hexanoyl-CoA, and isovaleryl-CoA, can act as acyl donor substrates, but much less efficiently (Badenhorst et al., 2012; Bartlett & Gompertz, 1974; Kolvraa & Gregersen, 1986; Mawal & Qureshi, 1994; Nandi et al., 1979). While the preferred acyl-acceptor substrate for GLYAT is always glycine, the amino acids asparagine, glutamine, glutamate, alanine, serine, and threonine can also be used, but at low rates that seem to be irrelevant under normal physiological conditions (van der Westhuizen et al., 2000; Webster et al., 1976). A second N-acyltransferase, glycine phenylacetyltransferase (E.C. 2.3.1.192), is responsible for the glycine conjugation of phenylacetate (Kelley & Vessey, 1990; Nandi et al., 1979; Vessey & Lau, 1998). In primates, including rhesus monkeys and humans, phenylacetate is conjugated to glutamine, instead of glycine (Knights & Miners, 2012; Webster et al., 1976). In humans the glutamine phenylacetyltransferase (E.C. 2.3.1.14) is encoded by the GLYAT-L1 gene

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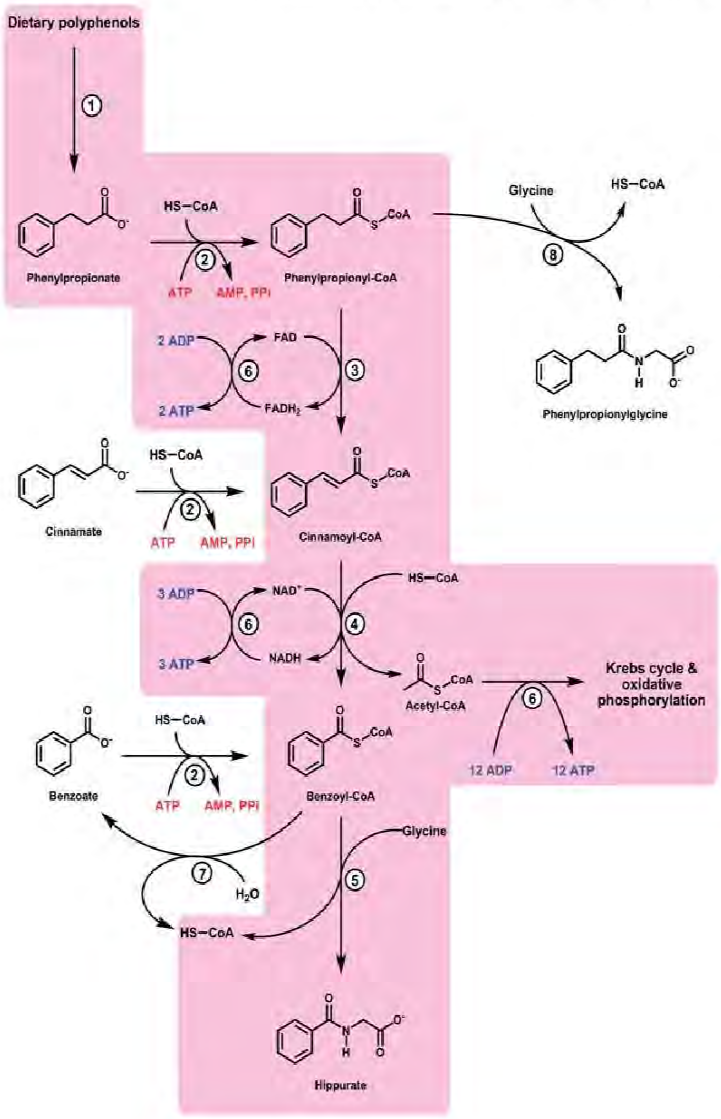

Figure 1. A schematic representation of the glycine conjugation pathway. The part of the pathway on the pink background depicts the natural glycine conjugation pathway, as it is described in this paper. (1) Dietary polyphenols are converted by the gut microbiota to simple aromatic acids such as phenylpropionate, cinnamate, and benzoate. (2) After absorption and transport to the liver, the aromatic acids are activated to acyl-CoA thioesters by ATP dependent acid:CoA ligases. The consumption of ATP is demonstrated by red. (3) Phenylpropionyl-CoA is converted to cinnamoyl-CoA by medium-chain acyl-CoA dehydrogenase, the first step of the β-oxidation cycle. (4) Cinnamoyl-CoA is further oxidized to benzoyl-CoA and acetyl-CoA. (5) Benzoyl-CoA is converted to hippurate by glycine N-acyltransferase. (6) The β-oxidation of phenylpropionate generates $FADH_2$, NADH, and acetyl-CoA, which are used by the Krebs cycle and the electron transport chain to produce ATP. The production of ATP is demonstrated by blue. (7) When the capacity of the glycine conjugation pathway is exceeded benzoyl-CoA accumulates, resulting in CoASH sequestration. Hydrolysis of benzoyl-CoA, by thioesterases with high affinity for benzoyl-CoA, releases benzoate and CoASH. (8) In individuals with a deficiency of medium-chain acyl-CoA dehydrogenase, phenylpropionyl-CoA accumulates and is converted by glycine N-acyltransferase to phenylpropionylglycine.

(Matsuo et al., 2012). It is currently not understood why separate acyltransferases exist for benzoyl-CoA and phenylacetyl-CoA, or why the primate enzymes use glutamine instead of glycine for the conjugation of phenylacetyl-CoA (Badenhorst et al., 2013; Knights & Miners, 2012).

Glycine conjugation is part of the phenylpropionate catabolism pathway

The natural substrates for glycine conjugation include benzoate, salicylate, 4-hydroxybenzoate, 3-hydroxybenzoate, 4-aminobenzoate, and 2-furoate. However, the major source of glycine conjugation substrates seems to be the metabolites of dietary polyphenols produced by microorganisms in the gut (Figure 1, reactions 1 to 5) (Knights & Miners, 2012; Rechner et al., 2002). The major families of polyphenols in food are the flavan-3-ols, flavonols, flavanones, anthocyanins, and hydroxycinnamates. These are metabolized by the gut microbiota to simpler aromatic compounds such as phenylpropionate, 3-hydroxyphenylpropionate, and 4-hydroxyphenylpropionate (Bravo, 1998; Jenner et al., 2005; Rechner et al., 2002; Tsao, 2010). Phenylpropionate is transported to the liver where it is first activated to phenylpropionyl-CoA, although it is not clear which enzyme catalyses this reaction. Since phenylpropionyl-CoA is a good substrate for medium-chain acyl-CoA dehydrogenase (MCAD), it is rapidly converted to cinnamoyl-CoA (Mao et al., 1994). This is why phenylpropionyl-CoA is used in enzyme assays for the confirmation of MCAD deficiency (Rinaldo et al., 1990). Cinnamoyl-CoA continues through the β-oxidation cycle to yield acetyl-CoA and benzoyl-CoA (Saltzman & Caraway, 1953; Dakin, 1908; Knoop, 1904). Benzoyl-CoA cannot be further catabolized, and is conjugated to glycine instead. This results in hippurate being the major urinary metabolite of polyphenol metabolism and phenylpropionate catabolism (Dakin, 1908; Knoop, 1904; Rechner et al., 2002).

It is now known that the amount of glycine conjugates excreted in the urine largely depends on the dietary intake of polyphenolic compounds, and the extent to which the polyphenols are fermented in the colon (Lees et al., 2013; Phipps et al., 1998; Rechner et al., 2002). The polyphenol fermentation products produced in the colon depend on the

type and amount of food consumed, its transit time through the digestive system, and the composition of the gut microbiome (Bravo, 1998; Fedotcheva et al., 2008; Rechner et al., 2002; Smith & Macfarlane, 1996; Jenner et al., 2005; Lees et al., 2013). Suppression of microbial activity, by antibiotic use for example, results in decreased production of compounds like phenylpropionate and consequently lower urinary hippurate excretion (Lees et al., 2013). This is further supported by the observation that germ free mice, bred under sterile conditions, excrete about one-seventeenth the amount of hippurate excreted by conventional mice fed the same diet (Wikoff et al., 2009). Exposure of germ free mice to a normal laboratory environment results in microbial colonization of the colon and normalization of urinary hippurate levels after about three weeks (Wikoff et al., 2009).

Interindividual variation in glycine conjugation

Because the glycine conjugation system is readily saturable, the metabolism of benzoate is dose dependent (Amsel & Levy, 1969; Knights & Miners, 2012; Knights et al., 2007; Kubota & Ishizaki, 1991; Poon & Pang, 1995). The major metabolite of benzoate is hippurate, but when very large doses are administered benzoylcarnitine and benzoylglucuronide may also be formed (Bray et al., 1951; Sakuma, 1991). The formation of these secondary metabolites depends on both the dose of benzoate and the capacity of the individual glycine conjugation system in question (Bray et al., 1951; Saltzman & Caraway, 1953). For humans, significant interindividual variation in urinary hippurate excretion has been documented. One study determined the daily hippurate excretion as 6285 ± 4008 μmol for men and 4793 ± 3239 μmol for women. The Human Metabolome Database reports urinary hippurate concentrations ranging from 27.92 to 932.66 μmol/mmol creatinine (Lees et al., 2013; Wijeyesekera et al., 2012). It is important to distinguish between variation in the amount of hippurate excreted, and variation in the rate of glycine conjugation. These two concepts are usually not clearly distinguished in the literature, which can lead to confusion. For example, patients with Crohn's disease are characterized by significantly lower urinary hippurate levels. This is associated with significant alterations of the gut microbiome, which result in decreased fermentation of dietary polyphenols and lower production of phenylpropionate (Section ''Glycine conjugation is part of the phenylpropionate catabolism pathway''). However, these individuals do not demonstrate a decreased rate of hepatic glycine conjugation, as demonstrated by normal conversion of an oral dose of benzoate to hippurate (Williams et al., 2010). In the next section the factors that influence the rate of glycine conjugation, and the capacity of the glycine conjugation pathway, are discussed.

Factors that influence interindividual variation in the rate of glycine conjugation

Availability of ATP, CoASH, and glycine

The first step in the glycine conjugation of benzoate requires CoASH for the synthesis of benzoyl-CoA (Figure 1, reaction 2). The availability of unacylated CoASH can therefore directly limit the maximum rate of glycine conjugation (Gregus et al., 1996a, 1992). In one study pantothenate, a precursor for CoASH biosynthesis, was administered to a hyperglycinemia patient on benzoate therapy. This resulted in increased urinary hippurate excretion and attenuation of seizures, but there was no way to determine whether hepatic CoASH levels were actually increased. However, it was demonstrated that addition of pantothenate to the culture medium of HepG2 cells resulted in significant increases in cellular CoASH content and increased conversion of benzoate to hippurate (Palekar, 2000). The synthesis of benzoyl-CoA is also dependent on ATP (Figure 1). Since ATP is hydrolysed to AMP, two molecules of ATP are consumed per molecule of benzoate (Schachter & Taggart, 1953, 1954). A shortage of ATP will, therefore, limit the rate of glycine conjugation by limiting the formation of benzoyl-CoA (Gregus et al., 1996a). The rates of glycine conjugation of cinnamate, benzoate, and 4-aminobenzoate have been measured extensively as a test of liver function, and correlate well with hepatic reserve (Duffy et al., 1995; Furuya et al., 1995; Krahenbuhl et al., 1997; Probstein & Londe, 1940; Saltzman & Caraway, 1953). It is thought that the mitochondrial dysfunction associated with liver disease, which leads to decreased ATP production, explains the lower rate of glycine conjugation in hepatitis patients (Badenhorst et al., 2013; Gregus et al., 1996a; Knights et al., 2007). The availability of glycine is another factor known to strongly influence the rate of hippurate synthesis (Figure 1) (de Vries et al., 1948; Gregus et al., 1992, 1993; Levy, 1979). Therefore, although hippurate synthesis in humans is saturable with large doses of benzoate, the co-administration of glycine can significantly increase the rates of hippurate formation and benzoate clearance (Knights & Miners, 2012; Levy, 1979). The availability of glycine also limits hippurate synthesis in rat liver. In one study the hepatic glycine content of rats was doubled by the administration of cysteamine, a potent inhibitor of the mitochondrial glycine cleavage system, without affecting the levels of ATP and CoASH. The result was a 50% increase in the rate of benzoate conjugation (Gregus et al., 1993). Because the increase in the rate of hippurate formation was smaller than the increase in glycine concentration, other factors such as CoASH availability seem to limit hippurate synthesis when excess glycine is available (Gregus et al., 1992, 1993, 1996a, b).

Although glycine supplementation stimulates hippurate synthesis, the glycine conjugation of salicylic acid is not enhanced by co-administration of glycine (Amsel & Levy, 1969). This is because the limiting step in the pathway depends on the substrate: hippurate formation for benzoate, and salicylyl-CoA formation for salicylate. This is consistent with the observation that the rate of salicylyl-CoA formation by human liver HXM-A is less than 1% of that for benzoyl-CoA formation (Knights et al., 2007; Vessey et al., 1999). There are three important differences when phenylpropionate, rather than benzoate, is the substrate for glycine conjugation (Figure 1). First, the conjugation of benzoate consumes two molecules of ATP, while the β-oxidation of phenylpropionate can theoretically yield 15 molecules of ATP. Second, an extra molecule of CoASH is needed for the oxidation of phenylpropionate (Houten & Wanders, 2010). The availability of ATP and CoASH for hippurate synthesis

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may thus depend on whether the substrate is phenylpropionate, cinnamate, or benzoate. Finally, the rates of mitochondrial uptake and activation to acyl-CoAs may be different for these substrates, because of differences in structure and lipophilicity (Masuda et al., 1996; Hansch et al., 1968; Kasuya et al., 1990, 1991).

Genetic variation in HXM-A and GLYAT

Early investigations revealed a greater similarity in the metabolism of aspirin between identical twins than between fraternal twins, suggesting that genetic variation is partly responsible for interindividual variation in the glycine conjugation of salicylate (Furst et al., 1977). However, very little is known about the influence of genetic variation on the glycine conjugation pathway. One study described an association between aspirin intolerance and an SNP (rs1133607) in the ACSM2A gene, which encodes HXM-A, the salicylate activating enzyme (Agundez et al., 2009; Vessey et al., 1999). This suggests that genetic variation may influence the rate of activation of aromatic acids by HXM-A, although very little is known about the actual influence of genetic variation on the enzyme activity of this ligase (Boomgaarden et al., 2009).

Genetic variation in the human GLYAT gene can influence the enzyme activity of a bacterially expressed recombinant human GLYAT (van der Sluis et al., 2013). Although the effects of only six SNPs on enzyme activity were determined, it is interesting to note that the variant with the highest enzyme activity had the highest allele frequency, and that those with negative effects on enzyme activity occur very rarely (van der Sluis et al., 2013; McVean, 2012). For example, it was suggested that the N156S variant should be considered the ''wild-type'' allele because it has a much higher allele frequency and higher enzyme activity than that encoded by the reference sequence (NM_201648.2). In contrast, only a single case of each of the R131H, F168L, and R199C variants, with negative impacts on GLYAT enzyme activity, have been reported (Lino Cardenas et al., 2010; McVean, 2012; van der Sluis et al., 2013; Yamamoto et al., 2009). It is not yet known whether genetic variation in the GLYAT gene influences the overall rate of glycine conjugation *in vivo* (Badenhorst et al., 2013; van der Sluis et al., 2013).

Expression of HXM-A and GLYAT

Both the ligase and transferase enzymes are inducible, although the mechanisms involved are not well understood. Pre-treatment of rats with salicylate for six days increases the capacity of liver and kidney homogenates for the synthesis of salicylylglycine, hippurate, and 4-aminohippurate (Irjala, 1972). Salicylate pre-treatment also increases the rate of salicylylglycine formation in humans (Furst et al., 1977). Recently it was demonstrated that dietary restriction results in increased urinary acylglycine excretion, which correlates with increased expression of GLYAT in the liver of rats (Wen et al., 2013). Also, investigation of hepatocellular carcinoma specimens further demonstrated that GLYAT expression is transcriptionally regulated (Matsuo et al., 2012).

The toxicity of benzoate and other aromatic acids

The toxicity of benzoate and phenylacetate to animals and cultured cells

Conjugation of large doses of benzoate or phenylacetate results in significant decreases in plasma glycine, which is associated with neurological symptoms in rats, dogs, and humans (Abdo et al., 1998; Beyoglu & Idle, 2012; Beyoglu et al., 2012; de Vries et al., 1948). High doses of benzyl acetate result in severe brain damage in rats as a result of glycine depletion, an effect cancelled out by the administration of glycine but not alanine (Abdo et al., 1998). When glycine is depleted, the detoxification of benzoate is significantly impaired, and significant damage occurs to the livers and kidneys of rats and dogs (Abdo et al., 1998; Griffith et al., 1989; Sherwin & Kennard, 1919; White, 1941). Plasma concentrations of aspartate amino transferase and alkaline phosphatase, markers of liver damage, are increased in rats following benzoate administration. There is also evidence that benzoate and phenylacetate are neurotoxic (Batshaw & Brusilow, 1981; Praphanphoj et al., 2000). In addition to gut abnormalities, defects of pronephric tubes, cardiac oedema, and misalignment of muscle fibres, sodium benzoate causes motor neuron defects and abnormal expression of the acetylcholine receptor, tyrosine hydroxylase, and a dopamine transporter in zebrafish embryos and larvae (Bichara et al., 2014; Chen et al., 2009; Tsay et al., 2007). In human and mouse neurons and astrocytes, benzoate increases the expression of DJ-1, a protein with neuroprotective properties in Parkinson's disease (Khasnavis & Pahan, 2012). Benzoate can directly inhibit brain enzymes and can also increase the turnover of serotonin in the brains of mice and humans by competing with tryptophan for binding to albumin (Batshaw et al., 1988; Ross & Wootton, 1964). Phenylacetate causes permanent disruption of neurological development if administered to newborn rats (Wen et al., 1980). Similarly, abnormal neurological development of the foetus has been reported in cases of untreated maternal phenylketonuria, which is characterized by increased phenylacetate levels (Fulton et al., 1980). A potential mechanism of pathogenesis is the formation of phenylacetyl-CoA in the brain, where it strongly inhibits choline acetyltransferase and disrupts neuronal utilization of acetyl-CoA (Loo et al., 1979, 1980, 1985).

Incubation of cultured primary rat hepatocytes with benzoate results in dose-dependent depression of protein and DNA synthesis and decreased activities of ornithine transcarbamylase and tyrosine aminotransferase (Oyanagi et al., 1987). Incubation of human leukocytes with benzyl acetate, benzyl alcohol, benzaldehyde, or benzoate results in significant DNA damage, reflecting the genotoxicity of these compounds (Abe & Sasaki, 1977; Demir et al., 2010; Yilmaz et al., 2009; Zengin et al., 2011). Benzoate influences the mitochondrial membrane potential and matrix calcium levels in cultured rat cortical neurons and human clonal epithelial cells, and induces cell death in a dose-dependent manner (Park et al., 2011, 2013). The mechanisms of toxicity of aromatic acids like benzoate and cinnamate have not been clearly defined, but involves sequestration of CoASH, formation of toxic acyl-CoA thioesters, and disruption of mitochondrial integrity by interaction of the aromatic acids

with the inner mitochondrial membrane. These mechanisms are briefly reviewed in Sections ''Toxicity mediated by acyl-CoA formation'' and ''Benzoate and other aromatic acids stimulate ROS production and inhibit complex I''.

Toxicity mediated by acyl-CoA formation

Comprehensive understanding of coenzyme A metabolism was advanced by the recent introduction of the concept of CASTOR disorders (Coenzyme A Sequestration, Toxicity, and Redistribution). In CASTOR disorders the synthesis of CoASH or the degradation of acyl-CoA thioesters is impaired, resulting in a combination of CoASH sequestration and accumulation of toxic acyl-CoAs (Mitchell et al., 2008). Therefore, it is very useful to keep in mind that the toxicity of an organic acid is largely determined by the extent to which an acyl-CoA is formed that cannot be further metabolized (Figure 2) (Badenhorst et al., 2013; Knights & Miners, 2012; Knights et al., 2007; Mitchell et al., 2008).

The metabolic consequences of CoASH sequestration

The first step in the metabolism of several organic acid xenobiotics is the ATP dependent activation to an acyl-CoA thioester by hepatic acid:CoA ligases (Badenhorst et al., 2013; Caldwell, 1982; Knights et al., 2007; Schachter & Taggart, 1953). In addition to the toxicity of the thioesters, acyl-CoA formation can cause sequestration of CoASH, which negatively impacts metabolism on many levels (Knights & Drogemiller, 2000; Knights et al., 2007; Mitchell et al., 2008; Zhang et al., 2007). Coenzyme A is perhaps one of the most important molecules in biochemistry, participating in β-oxidation, ketogenesis, lipogenesis, glucose catabolism, gluconeogenesis, and several other metabolic processes such as amino acid metabolism and the Krebs cycle (Figure 2). Sequestration of CoASH can therefore inhibit these vital metabolic processes (Mitchell et al., 2008; Zhang et al., 2007). Oxidation of propionate by bovine liver tissue is inhibited by phenylpropionate, cinnamate, and benzoate (Cremin Jr et al., 1994). Sequestration of CoASH by benzoate also inhibits β-oxidation, lipogenesis, ureagenesis, gluconeogenesis, ketogenesis, and the utilization of ketone bodies in cultured rat hepatocytes and isolated mitochondria (Beynen & Geelen, 1982; Cyr et al., 1991; Griffith et al., 1989; Kalbag & Palekar, 1988; Palekar & Kalbag, 1991; Yao et al., 1994). The levels of ATP and acetyl-CoA in the brains of hyperammonemic rats treated with benzoate also decrease as a result of CoASH sequestration (Ratnakumari et al., 1993). The main routes for regeneration of free CoASH from xenobiotic acyl-CoAs are carnitine conjugation, hydrolysis, and glycine conjugation (Figure 2) (Knights & Drogemiller, 2000; Knights et al., 2007; Mitchell et al., 2008). The importance of glycine conjugation in the maintenance of hepatic CoASH levels and normal metabolic function is discussed in Section ''The importance of glycine conjugation in the metabolism of aromatic acids''.

The toxic effects of accumulated acyl-CoA thioesters

It is well known that formation of a more reactive acyl-CoA is an important mechanism mediating the toxicity of organic acids such as benzoate, phenylacetate, salicylate, valproate, and clofibrate. A major reason for the increased reactivity of acyl-CoA metabolites is the electrophilic nature of the thioester bond (Caldwell, 1984; Grillo & Benet, 2002; Grillo & Lohr, 2009; Knights et al., 2007; Li et al., 2002, 2003;

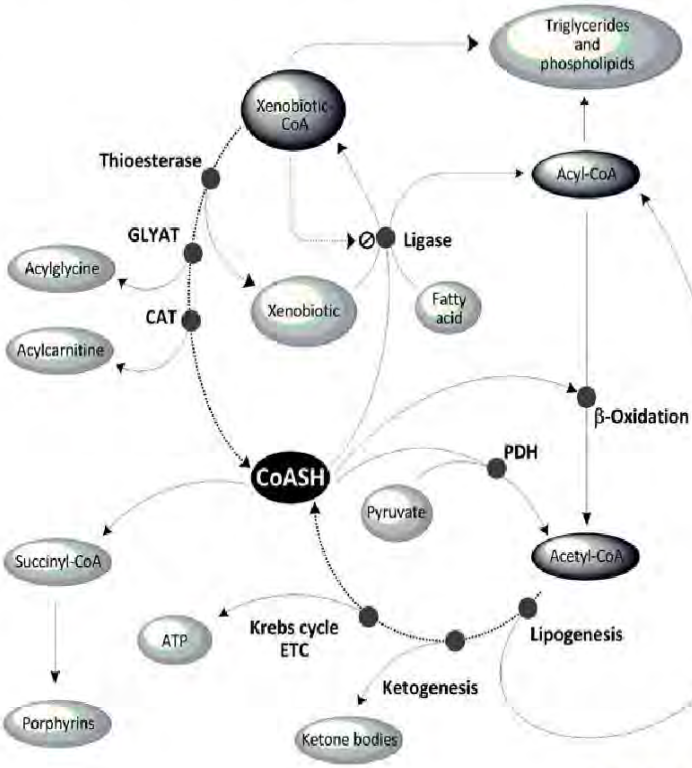

Figure 2. An overview of coenzyme A and acyl-CoA metabolism. The main pathways that produce and consume CoASH are demonstrated. It is very important that CoASH always be available in the cell because of its role in β-oxidation and the conversion of pyruvate to acetyl-CoA. Acetyl-CoA is the product of most catabolic reactions and provides the fuel for ketogenesis and mitochondrial ATP production. When xenobiotics are converted to xenobiotic-CoA esters, CoASH can be sequestered, disrupting ATP synthesis from pyruvate and fatty acids. As demonstrated by the fine broken arrows, accumulating xenobiotic-CoAs can inhibit the acid:CoA ligases or be incorporated into unnatural triglycerides and membrane phospholipids. The bold broken arrows indicate pathways that release bound CoASH. Black circles represent processes or enzymes involved in the metabolism of CoASH. Abbreviations: GLYAT, glycine N-acyltransferase; CAT, carnitine acyltransferase; PDH, pyruvate dehydrogenase; ETC, electron transport chain; CoASH, coenzyme A. Reproduced with minor modifications (Badenhorst et al., 2013) with permission from Informa Healthcare.

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Olsen et al., 2007; Sidenius et al., 2004). This makes acyl-CoAs reactive to nucleophiles such as glutathione and the cysteine, serine, lysine, and histidine residues of proteins. For example, covalent modification of glutathione and cellular proteins by the acyl-CoA metabolites of phenylacetate, 2-phenylpropionate, and 2,4-dichlorophenoxyacetate has been demonstrated (Caldwell, 1984; Grillo & Benet, 2002; Grillo & Lohr, 2009; Knights et al., 2007; Li et al., 2002, 2003; Olsen et al., 2007; Sidenius et al., 2004).

Acyl-CoA metabolites can also inhibit several enzymes, causing complex effects that are difficult to predict (Knights et al., 2007; Mitchell et al., 2008). Examples include the inhibition of choline acetyltransferase by phenylacetyl-CoA, inhibition of pyruvate carboxylase by propionyl-CoA and isovaleryl-CoA, inhibition of acid:CoA ligase enzymes by benzoyl-CoA and salicylyl-CoA, inhibition of the glycine cleavage system by valproyl-CoA and propionyl-CoA, and perturbation of protein kinase C function by clofibroyl-CoA (Erfle & Sauer, 1969; Knights et al., 2007; Kolvraa, 1979; Loo et al., 1980; Mortensen et al., 1980). In addition to acting as inhibitors, acyl-CoAs can also behave as alternative substrates for enzymes, resulting in the biosynthesis of abnormal metabolites. For example, when propionyl-CoA accumulates it can substitute for acetyl-CoA, resulting in the formation of methylcitrate by citrate synthase and *N*-propionylglutamate by *N*-acetylglutamate synthetase. The resulting shortage of *N*-acetylglutamate results in disruption of the urea cycle (Fenton et al., 2013). Accumulated propionyl-CoA and isovaleryl-CoA can substitute for acetyl-CoA in lipogenesis to produce odd-chain and branched-chain fatty acids (Fenton et al., 2013; Sweetman & Williams, 2013). Similarly, 2-arylpropionyl-CoAs, metabolites of nonsteroidal anti-inflammatory drugs, can be incorporated into adipocyte triglycerides (Knights et al., 2007). Very important cases involve the reaction of isovaleryl-CoA and benzoyl-CoA with carnitine, catalysed by carnitine acyltransferase enzymes (Itoh et al., 1996; Sakuma, 1991). This results in the excretion of acylcarnitines in the urine and can cause systemic carnitine depletion, which negatively impacts fatty acid oxidation and energy metabolism (Itoh et al., 1996; Lheureux & Hantson, 2009; Sakuma, 1991).

Benzoate and other aromatic acids stimulate ROS production and inhibit complex I

The aromatic acids phenylpropionate, cinnamate, and phenylacetate accumulate in the plasma of septic patients as a result of microbial metabolism in the blood. Sepsis is characterized by extremely high ROS production, oxidative stress, and mitochondrial damage (Beloborodova et al., 2012). The accumulating aromatic acids stimulate the production of ROS in isolated liver mitochondria and inhibits the activity of complex I of the electron transport chain (Beloborodova et al., 2012; Fedotcheva et al., 2008, 2012). Although the mechanisms of mitochondrial toxicity of the aromatic acids are not well understood, several recent advances in our understanding of this process will be briefly reviewed here.

Benzoate, salicylate, phenylpropionate, cinnamate, valproate, and isovalerate are all known to induce the mitochondrial permeability transition, an important mechanism in the pathogenesis of disorders related to Reye's syndrome (Battaglia et al., 2005; Beloborodova et al., 2012; Fedotcheva et al., 2008; Trost & Lemasters, 1996). Mitochondrial permeability transition is the common final pathway leading to acute cell death as a result of mitochondrial dysfunction, is induced by several mild oxidants such as *t*-butylhydroperoxide and diamide, and is inhibited by cyclosporin A (Fedotcheva et al., 2008; Kushnareva & Sokolove, 2000; Lemasters et al., 2002; Nakagawa & Moldeus, 1998; Nakagawa & Moore, 1999; Trost & Lemasters, 1996, 1997). It is characterized by the opening of a large non-selective pore which leads to uncoupling of oxidative phosphorylation, generation of ROS, mitochondrial swelling, release of cytochrome c, and apoptosis (Beloborodova et al., 2012; Bradshaw & Pfeiffer, 2006; Kushnareva & Sokolove, 2000; Trost & Lemasters, 1997). An important first step leading to the permeability transition seems to be the formation of a small non-selective pore, distinct from the pore involved in mitochondrial permeability transition. The opening of this pore allows the passage of cations such as calcium, but not protons, and is thought to be stimulated by pro-oxidants and by salicylate in the inner mitochondrial membrane (Battaglia et al., 2005). Calcium efflux from this pore, coupled to calcium import by the calcium uniporter, results in rapid collapse of the membrane potential, which is stimulated by external calcium and inhibited by EGTA (Battaglia et al., 2005; Fedotcheva et al., 2008; Kushnareva & Sokolove, 2000; Trost & Lemasters, 1997). Collapse of the membrane potential precedes opening of the permeability transition pore and is not inhibited by cyclosporin A (Kushnareva & Sokolove, 2000).

Although it is not clear how aromatic acids induce ROS production and the mitochondrial permeability transition, it has been suggested that diffusion of these compounds into the inner mitochondrial membrane alters the properties of the membrane and stimulates opening of the initial small pore (Bernardi et al., 1994; Broekemeier & Pfeiffer, 1995; Trost & Lemasters, 1997). *N*-Ethylmaleimide can promote opening of the pore, which is inhibited by dithiothreitol, suggesting that oxidation of critical sulfhydryl groups on the inner mitochondrial membrane is important for opening the pore or for keeping it in an open state (Fedotcheva et al., 2008; Trost & Lemasters, 1997). In the mitochondria of cultured tobacco cells, benzoate and salicylate directly inhibit the transfer of electrons from complex I to ubiquinone, stimulating ROS generation (Norman et al., 2004). This could cause oxidation of sulfhydryl groups in the membrane, leading to inhibition of complex I and opening of the initial pore. This idea is supported by the observation that reducing agents like dithiothreitol can prevent the inhibition of complex I caused by *N*-Ethylmaleimide or aromatic acids (Fedotcheva et al., 2008). The inhibition of complex I results in ROS production upon oxidation of complex I and complex II substrates (Fedotcheva et al., 2008; Murphy, 2009). Oxidation of complex II substrates is not inhibited, since this complex is less sensitive to oxidative stress than complex I (Beloborodova et al., 2012; Fedotcheva et al., 2008, 2012; Murphy, 2009).

A related explanation for the damaging effects of aromatic acids on mitochondria is the ability of lipophilic compounds

to disrupt the structural integrity of biological membranes by partitioning into the phospholipid bilayer, thereby increasing its permeability to small molecules and ions in a nonspecific manner (Politycka, 1997; Sikkema et al., 1995; Ye et al., 2006). For example, benzoate can increase the conductance of biological membranes, and causes leakage of lactate from intact mitochondria (Cremin Jr et al., 1994; Gutknecht, 1992). Increased membrane permeability can result in dissipation of membrane potential and uncoupling of oxidative phosphorylation, as demonstrated by the effects of cyclohexane on yeast mitochondria (Sikkema et al., 1995). This effect on membrane integrity also explains the observation that intracellular membrane trafficking in yeast is disturbed by benzoic acid, which also seems to negatively influence the fission and fusion of mitochondria (Hazan et al., 2004; Park et al., 2011, 2013). The ability of a compound to diffuse into and accumulate in biological membranes is directly related to its lipophilicity (Lakeram et al., 2007; Sikkema et al., 1995). For example, the ability of the parabens, alkyl esters of 4-hydroxybenzoic acid, to induce mitochondrial damage depends on the alkyl-chain length and is linearly related to the octanol/water partition coefficient, a reflection of lipophilicity (Lakeram et al., 2007; Nakagawa & Moldeus, 1998; Nakagawa & Moore, 1999). The ROS production and complex I inhibition in isolated mitochondria induced by aromatic acids also seem to be influenced by the lipophilicity of the compound (cinnamate > benzoate > phenylpropionate > phenylacetate) (Beloborodova et al., 2012; Fedotcheva et al., 2008, 2012; Politycka, 1997; Ye et al., 2006). The hydroxylated aromatic acids such as phenyllactate, 4-hydroxyphenylacetate, and 4-hydroxyphenyllactate are less lipophilic, do not stimulate ROS generation, and because of their hydroxyl groups, can act as free radical scavenging antioxidants (Fedotcheva et al., 2008).

The importance of glycine conjugation in the metabolism of aromatic acids

Glycine conjugation is important for the maintenance of CoASH levels

As discussed in Section ''Toxicity mediated by acyl-CoA formation'', several metabolic processes are dependent on CoASH and are inhibited by CoASH sequestration (Figure 2). Glycine conjugation is important for the maintenance of CoASH levels, by preventing its sequestration as benzoyl-CoA (Knights & Miners, 2012; Knights et al., 2007). For example, the inhibition of gluconeogenesis in bovine liver mitochondria by benzoate-induced CoASH sequestration is reversed by the addition of glycine, which stimulates hippurate synthesis, decreases benzoyl-CoA levels, and increases CoASH levels. As a result acetyl-CoA levels are restored and the rate of gluconeogenesis normalizes (Griffith et al., 1989). The accumulation of benzoyl-CoA and sequestration of CoASH caused by benzoate is similar to the situation in isovaleric acidemia, suggesting that isovaleric acidemia can shed some light on the importance of glycine conjugation (Badenhorst et al., 2013; Trost & Lemasters, 1996). Isovaleric acidemia is a defect of leucine catabolism caused by mutations in the isovaleryl-CoA dehydrogenase gene, resulting in accumulation of isovaleryl-CoA in mitochondria (Tanaka et al., 1966). This accumulation results in sequestration of CoASH and formation of large amounts of isovaleric acid, which is toxic to mitochondria (Trost & Lemasters, 1996). Because isovaleryl-CoA is a good substrate for GLYAT, glycine conjugation restores CoASH levels and produces isovalerylglycine, a less toxic metabolite that is more readily excreted by the kidneys (Bartlett & Gompertz, 1974; Tanaka & Isselbacher, 1967). As a result of the excessive glycine conjugation, depletion of glycine is often observed in these patients. Glycine supplementation restores glycine levels, increases isovalerylglycine excretion, and generally results in normal mental and physical development of these patients (Dercksen et al., 2012; Itoh et al., 1996; Tanaka & Isselbacher, 1967; Sweetman & Williams, 2013). Alternatively, supplementation with carnitine leads to increased isovalerylcarnitine excretion, which also restores CoASH levels, detoxifies isovaleric acid, and results in effective treatment of the disease. Interestingly, carnitine supplementation does not necessarily decrease isovalerylglycine excretion, and may even stimulate it (Itoh et al., 1996; Sweetman & Williams, 2013). Deficiency of medium-chain acyl-CoA dehydrogenase also demonstrates the role of glycine conjugation in maintaining CoASH levels. Patients with defects of MCAD cannot metabolize phenylpropionyl-CoA to cinnamoyl-CoA, resulting in the accumulation of phenylpropionyl-CoA in mitochondria. As a result, high levels of phenylpropionylglycine are excreted in the urine (Figure 1, reaction 8) (Bennett et al., 1990).

An important mechanism by which glycine conjugation reduces the acyl-CoA mediated toxicity of organic acids is by converting the electrophilic thioester to the inert peptide bond of the glycine conjugate (Caldwell, 1982, 1984). Unlike thioesters, and even esters such as acylglucuronides, peptide conjugates are chemically stable and do not react with nucleophiles such as proteins and glutathione (Caldwell, 1982, 1984; Grillo & Benet, 2002; Grillo & Lohr, 2009; Li et al., 2002, 2003; Olsen et al., 2007; Sidenius et al., 2004). Since covalent modification by acyl-CoAs is dependent on both time and the concentration of the acyl-CoA thioester, it seems reasonable to assume that the toxicities of these electrophiles are significantly decreased by glycine conjugation, although this is yet to be experimentally verified (Caldwell, 1984; Grillo & Lohr, 2009).

Glycine conjugation decreases the lipophilicity of benzoic acid

As mentioned in the introduction, it has usually been assumed that glycine conjugation increases the water solubility of benzoate in order to facilitate urinary excretion. However, the water solubility of sodium benzoate (629 g/l) is so high that insolubility cannot possibly explain the toxicity of this compound. Furthermore, glycine conjugation only slightly increases the water solubility of benzoic acid from 3.4 g/l to 3.75 g/l for hippuric acid (Beyoglu & Idle, 2012; Beyoglu et al., 2012). This suggests that increasing water solubility is not the mechanism by which glycine conjugation decreases the toxicity of benzoate. We suggest that glycine conjugation decreases the toxicity of benzoate, not because

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hippuric acid is more water soluble than benzoic acid, but because hippuric acid is less lipophilic than benzoic acid. Despite having similar water solubilities, hippuric acid is significantly less lipophilic than benzoic acid, as reflected by their LogP values of 0.31 and 1.87, respectively (Hansch & Leo, 1995). This difference in LogP values reflects an almost 40-fold difference in octanol-water partition coefficients for these compounds. Based on the strong correlation between lipophilicity and ability to diffuse into biological membranes, this means that benzoic acid will be significantly more capable of partitioning into the hydrophobic interiors of phospholipid bilayers (Section ''Benzoate and other aromatic acids stimulate ROS production and inhibit complex I'') (Lakeram et al., 2007; Sikkema et al., 1995; Nakagawa & Moldeus, 1998; Nakagawa & Moore, 1999). This argument is supported by the observation that benzoic acid can be extracted from acidified urine by the non-polar solvent benzene, while hippuric acid remains in the aqueous phase after extraction with benzene and ether (Dakin, 1910).

The influence of decreased lipophilicity on the toxicity and mitochondrial export of benzoate

As discussed in Section ''Benzoate and other aromatic acids stimulate ROS production and inhibit complex I'', the mitochondrial toxicity of a compound is strongly correlated to its lipophilicity, because lipophilic compounds diffuse into and disrupt the structural integrity of biological membranes. It therefore seems reasonable to suggest that hippurate will be significantly less toxic to mitochondria than benzoic acid, despite the current lack of experimental evidence for this hypothesis (Figure 3, numbers 2, 7, and 10). It can also be

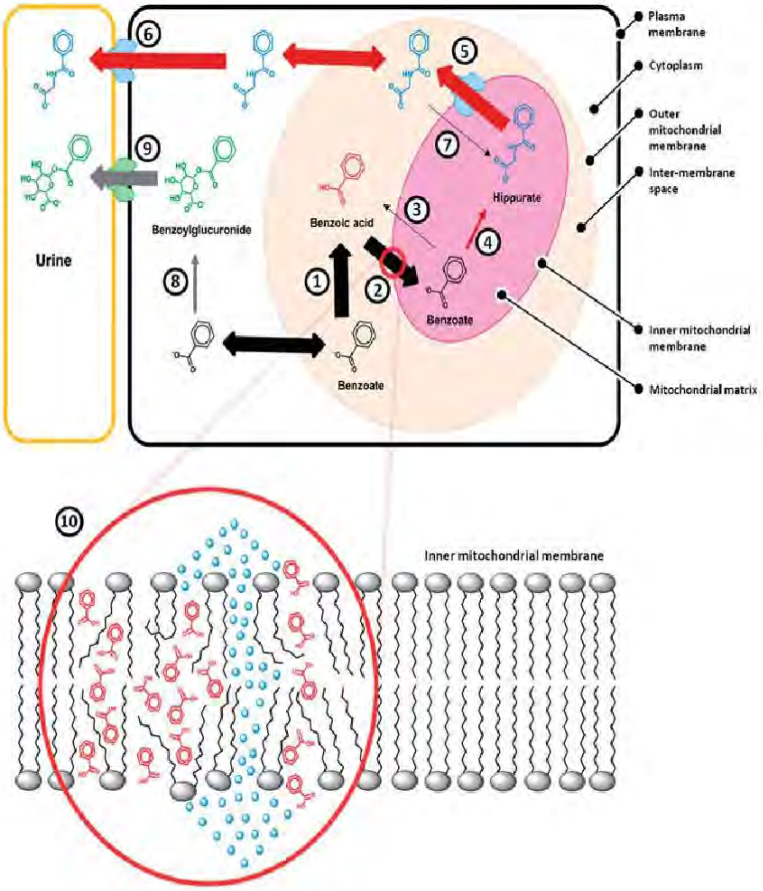

Figure 3. The detoxification of benzoate by means of glycine conjugation in hepatocytes. (1) Because the pH in the mitochondrial inter-membrane space is lower than that of the cytoplasm and the mitochondrial matrix, the conversion of benzoate (black) to benzoic acid (red) is favoured. (2) Benzoic acid can freely diffuse across the mitochondrial inner membrane into the mitochondrial matrix. In the matrix the higher pH favours the dissociation of benzoic acid to form benzoate. (3) Benzoate accumulates in the mitochondrial matrix to concentrations about 50 times higher than that in the surrounding solution. This is demonstrated by the width of the black arrows. Therefore, the thick black arrow 2, pointing to the matrix, is 50 times wider than arrow 3, pointing out of the matrix space. (4) In the mitochondrial matrix benzoate is converted to hippurate (blue). (5) Hippurate is transported out of the matrix by organic anion transporters. The rate at which hippurate can be excreted is about five times higher than that at which it can be synthesized. This is demonstrated by the red arrow 5 being five times wider than red arrow 4, which represents hippurate synthesis. (6) Finally, hippurate is irreversibly excreted into urine (yellow block) by organic anion transporters. (7) Hippuric acid is less lipophilic than benzoic acid and therefore diffuses into the matrix space at a significantly lower rate. This is indicated by black arrow 7 being 40 times thinner than black arrow 2, which represents the diffusion of benzoic acid into the matrix. (8) In the cytoplasm, benzoate can also be converted to benzoylglucuronide by UDP-glucuronosyltransferases, as represented by the grey arrows. Because benzoate accumulates in the mitochondrial matrix, benzoylglucuronide synthesis, which is localized to the cytoplasm, only occurs when the capacity of the mitochondrial glycine conjugation system is exceeded. (9) Benzoylglucuronide is then also excreted in the urine. (10) This section zooms in on a part of the inner mitochondrial membrane. It represents the diffusion of the lipophilic benzoic acid into the inner mitochondrial membrane, which causes disruption of the phospholipid bilayer and results in permeabilization of the membrane. This is represented by the small blue spheres leaking across the membrane.

argued that glycine conjugation facilitates removal of benzoate from the mitochondrial matrix. It has been demonstrated that benzoate accumulates in the mitochondrial matrix to a concentration about 50-times higher than that in the external solution. This is the result of the pH gradient of about 2 units over the inner mitochondrial membrane (Gatley & Sherratt, 1977; Gatley & Sherratt, 1976). The low pH of about 6.0 in the intermembrane space favours the formation of benzoic acid, which diffuses into the matrix where the higher pH of about 7.8 favours dissociation to benzoate, which becomes trapped in the matrix (Figure 3, numbers 1 to 3) (Davies et al., 2011; Strauss et al., 2008; Porcelli et al., 2005; Gatley & Sherratt, 1976, 1977). Both benzoate and hippurate can be exported from the mitochondrial matrix by organic anion transporters (Deguchi et al., 2005; Schwab et al., 2001; Yoshimura et al., 1998). However, it would be futile to simply export benzoate from the matrix, since it can reform benzoic acid in the more acidic intermembrane space, and diffuse back over the inner membrane into the matrix (Figure 3, numbers 2 and 3) (Piper, 1999; Piper et al., 2001). In contrast, unidirectional export of hippurate is possible, since the less lipophilic hippuric acid cannot diffuse back over the inner mitochondrial membrane at a significant rate (Figure 3, arrows 2, 5, and 7). The model described here is supported by the observation that benzoate clearance is limited by the rate of hippurate synthesis, which is about one-fifth of the maximum rate of hippurate excretion (Figure 3, arrows 2, 4, and 5) (Gregus et al., 1992, 1993).

The impact of glycine conjugation on glycine metabolism

Glycine homeostasis is not the primary purpose of the glycine conjugation pathway

The major system involved in glycine homeostasis is the glycine cleavage system, which has a high metabolic capacity and is expressed in several tissues, including liver and brain (Kikuchi et al., 2008). Defects of the glycine cleavage system result in nonketotic hyperglycinemia (NKH), characterized by significant elevations of glycine concentrations in plasma and CSF (Conter et al., 2006; Hiraga et al., 1981; Kure et al., 2004; Perry et al., 1975; Hamosh & Johnston, 2013). The most useful diagnostic parameter is the CSF/plasma glycine ratio, which is normally below 0.02, with values of 0.09 or greater being diagnostic for NKH (Krieger et al., 1977; Perry et al., 1975; Hamosh & Johnston, 2013). Although there are no completely satisfactory treatments for this disorder, administration of sodium benzoate is used to decrease plasma and CSF glycine levels, as the excess glycine is excreted in the urine as hippurate. The effectiveness of this therapy is variable and glycine levels or CSF/plasma glycine ratios are rarely decreased to satisfactory levels (Applegarth & Toone, 2001; Barshop et al., 1989; Krieger et al., 1977; Beyoglu & Idle, 2012; Beyoglu et al., 2012). The failure of this therapy to completely normalize glycine levels and CSF/plasma glycine ratios, despite the large doses of benzoate used, suggests that the contribution of the glycine deportation system to glycine homeostasis is small, compared to that of the glycine cleavage system (Krieger et al., 1977; Hamosh & Johnston, 2013; Beyoglu & Idle, 2012; Beyoglu et al., 2012).

After phenylketonuria, NKH is the second most prevalent defect of amino acid catabolism, affecting up to one in 64 000 individuals in some populations (Hamosh & Johnston, 2013). Therefore, being a carrier of NKH is not lethal or extremely deleterious for human health, despite the higher plasma and CSF glycine concentrations observed in carriers, compared to controls (Kure et al., 2004; Von Wendt and Similä, 1982). It is interesting to note, however, that no defect of the glycine conjugation system, which makes the smaller contribution to glycine homeostasis, has been reported in humans. This seems to suggest that glycine conjugation is a vital metabolic process, and that its primary purpose is not the regulation of glycine levels. However, as suggested by the glycine deportation hypothesis, consumption of large quantities of aromatic acids can result in significant excretion of glycine in the urine (as hippurate or phenylacetylglycine) (Beyoglu & Idle, 2012; Beyoglu et al., 2012; de Vries et al., 1948). The rest of this section discusses the impact of this glycine deportation phenomenon on metabolism as a whole.

Benzoate and human glycine deficiency

Glycine is commonly thought to be a nonessential amino acid since it can be synthesized from serine by serine hydroxymethyltransferase (Wu et al., 2013). Serine itself is synthesized from 3-phosphoglycerate, an intermediate of glycolysis (Figure 4). Serine hydroxymethyltransferase converts serine to glycine, 5,10-methylenetetrahydrofolate (THF-C1), and NADH. Additional THF-C1 and NADH are produced from glycine by the glycine cleavage system. In recent years it has become apparent that glycine is a conditionally essential amino acid, as the metabolic demand for glycine often exceeds the capacity for glycine synthesis (Melendez-Hevia et al., 2009; Wu, 2013; Wu et al., 2013). For example, glycine availability has been shown to be limiting in the developing human foetus, in young men, birds, and in milk-fed pigs (Jackson et al., 1996, 1997; Wang et al., 2013; Wu, 2010, 2013; Wu et al., 2013). In fact, it has been estimated that human adults have a shortage of about 10 grams of glycine per day. This glycine deficiency, and the resulting deficiency of THF-C1 units, can influence the metabolism of collagen, glutathione, creatine, nucleic acids, and porphyrins (Brosnan et al., 2011; Melendez-Hevia et al., 2009; Wang et al., 2013). It has been argued that glycine shortage can be exacerbated by benzoate and other substrates for glycine conjugation (Au et al., 2013; Badenhorst et al., 2013; Jackson et al., 1987). In addition to natural sources of benzoate (Section "A brief introduction to glycine conjugation"), humans are nowadays exposed to ever increasing amounts of this compound, since it is used as a preservative in food and pharmaceuticals (Piper, 1999). In some populations where a lot of preserved food is consumed, benzoate intake can be as high as 280% of the recommended daily allowance, which is reflected by unusually high levels of urinary hippurate (Lees et al., 2013; Tfouni & Toledo, 2002).

Glycine conjugation, glutathione, and diabetes

A useful marker of glycine depletion is the urinary excretion of pyroglutamate, which is spontaneously formed when insufficient glycine is available for glutathione synthesis

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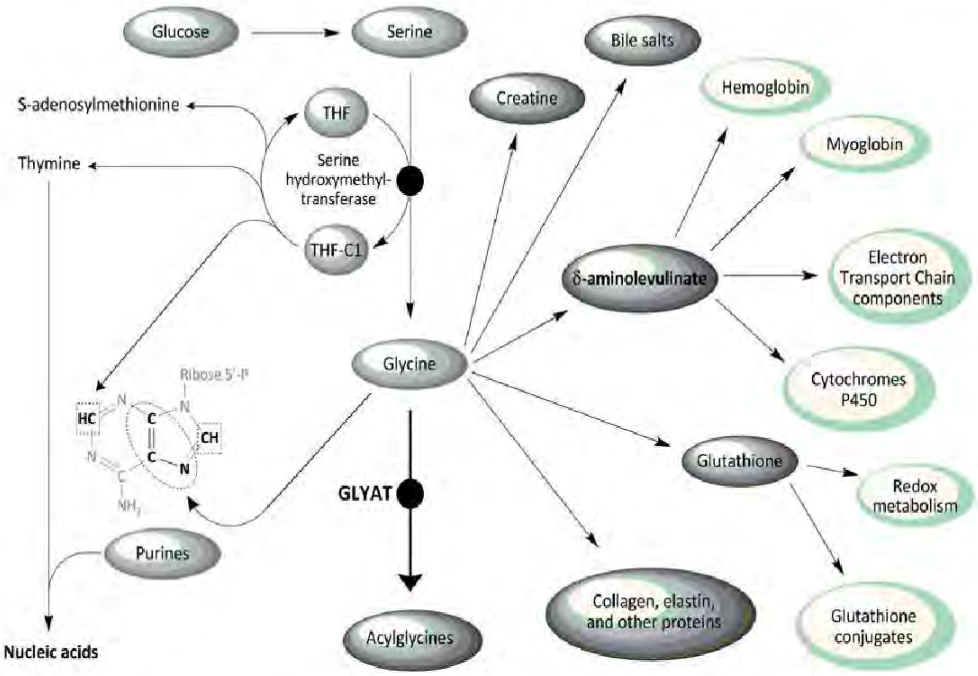

Figure 4. Biosynthesis and metabolic consumption of glycine. Glycine is biosynthesized from serine by serine hydroxymethyltransferase. The reaction converts THF (tetrahydrofolate) to THF-C1 (tetrahydrofolate-C1) for each molecule of glycine produced. The total amount of glycine synthesized can thus not exceed the amount of THF-C1 consumed through production of compounds such as S-adenosylmethionine, thymine, and purines. Glycine is consumed in the production of creatine, bile salts, porphyrins, collagen, elastin, other proteins, and glutathione. Black circles represent the serine hydroxymethyltransferase and GLYAT enzymes. The bold arrow indicates formation of xenobiotic acylglycines by GLYAT. The parts of purine rings derived from glycine and THF-C1 are indicated by the dashed ellipse and squares, respectively. Reproduced with minor modifications (Badenhorst et al., 2013) with permission from Informa Healthcare.

(Jackson et al., 1996, 1997, 2004; Jackson et al., 1997, 1996). Vegetarians, who consume large amounts of substrates for glycine conjugation, and often insufficient protein, excrete larger amounts of pyroglutamate than omnivorous humans (Metges et al., 2000; Persaud et al., 1996). Benzoate administration to humans significantly increases pyroglutamate excretion, indicating glycine shortage and impaired glutathione synthesis (Jackson et al., 1987). Glutathione is crucial in all cells of the body for maintaining a reducing environment and for neutralizing the harmful ROS constantly produced by mitochondria (Wu et al., 2004). A deficiency of glutathione is associated with inhibition of mitochondrial complex I, increased levels of ROS, and oxidative damage such as lipid peroxidation (Jha et al., 2000; Sekhar et al., 2011a, b).

Metabolites associated with glycine and glutathione metabolism are the most important metabolites differentiating diabetics from controls in metabolomics studies. However, this is not clearly understood at present, and warrants further investigation (Xie et al., 2013). Diabetes is characterized by a depletion of glutathione and very high levels of ROS (Bandeira et al., 2013; Pitocco et al., 2010; Sekhar et al., 2011a). The shortage of glutathione is due to impaired synthesis, resulting from a shortage of glycine and cysteine. Dietary supplementation with glycine and cysteine results in increased glutathione synthesis and decreases the levels of ROS and lipid peroxidation to normal (Sekhar et al., 2011a). It is interesting to note that diabetes is associated with an abnormal gut microbiome and significantly elevated urinary hippurate, indicating increased glycine conjugation of microbial metabolites (Lees et al., 2013; Zuppi et al., 2002). Although it is not known whether the increased synthesis of hippurate is responsible for the glycine shortage in these individuals, they may be more sensitive to the negative consequences of consuming benzoate as a preservative, which can exacerbate the glycine deficiency (Badenhorst et al., 2013; Lees et al., 2013; Sekhar et al., 2011a; Zuppi et al., 2002). Glycine shortage could also decrease the rate of benzoate clearance, and therefore increase the levels of benzoate circulating in plasma. This can stimulate ROS production and damage mitochondria, important factors in the development of insulin insensitivity (Beloborodova et al., 2012; Park et al., 2013; Trost & Lemasters, 1996, 1997; Wang et al., 2010). Interestingly, administration of benzoate to sheep rapidly increases plasma levels of glucose, insulin, and glucagon, but the significance of this observation is unclear at present (Mineo et al., 1995).

Glycine N-acyltransferase and the musculoskeletal system

A well-known consequence of glycine shortage is decreased turnover of collagen, an important structural protein. Although decreased collagen turnover is not lethal, it leads to accumulation of damage to connective tissues such as the skin, thereby accelerating the ageing process, especially in long-lived animals such as humans (Melendez-Hevia et al., 2009; Wu et al., 2013). In addition to influencing collagen

turnover, glycine shortage seems to be related to the growth and development of the musculoskeletal system in several complicated ways. A recent study revealed a statistically significant correlation between lean muscle mass and bone size, and three SNPs in the human GLYAT gene. Unfortunately, the effect of these genetic variations are not known, but it has been suggested that the influence of GLYAT on glycine metabolism could explain this observation (Badenhorst et al., 2013; Guo et al., 2013). Depletion of glycine by conjugation to benzoate, for example, has been shown to result in reduced weight gain and weight loss in rats, an effect cancelled out by co-administration of glycine (White, 1941). The amount of hippurate synthesized, however, depends mostly on the extent of exposure to hippurate precursors like benzoate (Section ''A brief introduction to glycine conjugation''). Genetic variation in GLYAT is therefore not likely to significantly influence the amount of glycine consumed in the synthesis of hippuric acid, and other factors must explain the relationship between genetic variation in GLYAT and development of the musculoskeletal system. The effects of increased levels of plasma benzoate on muscle function are not yet clearly understood, but its negative effect on mitochondrial energy production may be a contributing factor (Section ''The toxicity of benzoate and other aromatic acids''). Other factors that could influence muscle growth and function are plasma levels of glucose, insulin, and glucagon, which may be influenced by benzoate (Mineo et al., 1995). It is known that hippurate can inhibit the utilization of glucose by human muscle and kidney cells, but this effect is only observed at the high concentrations of hippurate resulting from chronic renal failure (Spustova et al., 1987, 1989; Spustova & Dzurik, 1991; Spustova & Oravec, 1989). Interestingly, GLYAT is expressed weakly in skeletal muscle and the pancreas, but glycine *N*-acyltransferase activity has not been reported in these tissues and the significance of this observation remains unclear (Matsuo et al., 2012).

Glycine conjugation and cancer

In hepatocellular carcinoma specimens the expression of GLYAT is significantly or completely downregulated at the transcriptional level (Matsuo et al., 2012). This may be explained by the dependence of rapidly proliferating cancer cells on the availability of glycine, which is needed for nucleic acid synthesis (Figure 4) (Jain et al., 2012). Two molecules of glycine and one THF-C1 are needed for the synthesis of purines, and *de novo* thymidine synthesis requires THF-C1 produced by the glycine cleavage system (Melendez-Hevia et al., 2009; Wu, 2010, 2013; Wu et al., 2013). The downregulation of GLYAT expression in hepatocellular carcinoma could therefore be a strategy to conserve glycine, which is needed for DNA synthesis (Badenhorst et al., 2013). Benzoate has been shown to inhibit DNA synthesis in cultured rat hepatocytes, but it is not clear whether this is because of glycine depletion (Oyanagi et al., 1987). The impact of benzoate conjugation on the availability of glycine for purine synthesis is indirectly demonstrated by the observation that uricotelic birds and reptiles, which excrete waste nitrogen as uric acid, do not synthesize hippurate (Bridges et al., 1970; Smith, 1958). Two molecules of glycine are needed for uric acid synthesis, perhaps explaining why benzoate is conjugated to ornithine instead (Figure 4) (Smith, 1958). Further indirect evidence for decreased purine synthesis, as a result of increased hippurate synthesis, is the reduced urinary excretion of uridine by diabetic patients, who also excrete significantly more hippurate than controls (Lees et al., 2013; van Doorn et al., 2007).

In addition to glycine, glutamine is a crucial nutrient for cancer cells (Wise & Thompson, 2010). Utilization of glutamine is initiated by its conversion to glutamate by glutaminases, followed by transamination of glutamate to α-ketoglutarate, which enters the Krebs cycle. The rate of growth of cancer cells is proportional to glutaminase activities (Knox et al., 1969; Linder-Horowitz et al., 1969). Phosphate-dependent and phosphate-independent glutaminases, with different metabolic functions, are found in humans and other animals. Phosphate-independent glutaminase activity, thought to be important in the regulation of renal ammoniagenesis and pH balance, is stimulated by hippurate. In contrast, the mitochondrial phosphate-dependent glutaminases are inhibited by hippurate under acidic conditions (Dzurik et al., 2001; Krivosikova et al., 1998). Hippurate could thus inhibit the utilization of glutamine by tumours, which prefer acidic environments. This is supported by the observation that hippurate can significantly inhibit tumour growth (Spustova & Oravec, 1989). Furthermore, hippurate has recently been identified in metabolomics studies as important for the prediction of cancer recurrence, with decreased hippurate levels correlating to recurrence of oral cancer (Xie et al., 2012; Ye et al., 2012).

Benzoate and glycine conjugation may influence porphyrin metabolism

Porphyrins are crucial components of haemoglobin, myoglobin, electron transport chain complexes, and cytochrome P450 enzymes (Figures 2 and 4). Porphyrin synthesis starts with the mitochondrial condensation of glycine and succinyl-CoA to form δ-aminolevulinate (Piper et al., 1973). To date, there is no direct evidence that glycine availability limits porphyrin synthesis. However, benzoate decreases the faecal excretion of porphyrin metabolites by rats, an effect cancelled out by administration of glycine, which by itself increases the excretion of these metabolites (Lucas & Orten, 1954). Benzoate can also be used to treat chemically induced porphyria in rats, by decreasing glycine availability and inhibiting porphyrin synthesis, an effect cancelled out by the co-administration of glycine (Piper et al., 1973). In a recently published hypothesis it was argued that the glycine dependence of cancer cells cannot be completely explained by the increased demand for nucleic acid synthesis. The authors point out that cancer cells are specifically dependent on mitochondrial glycine synthesis, while purine biosynthesis occurs in the cytoplasm (di Salvo et al., 2013). Furthermore, Chinese hamster ovary cell lines with defects of mitochondrial serine hydroxymethyltransferase are glycine auxotrophs

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and have reduced activities of mitochondrial complex IV, which assembles incorrectly if haem is deficient (Atamna et al., 2001; Ye et al., 2010).

An issue that has not been addressed in the literature is whether succinyl-CoA availability can limit the synthesis of δ-aminolevulinate, and therefore porphyrins (Figures 2 and 4). Synthesis of succinyl-CoA from either succinate or α-ketoglutarate requires CoASH. It is therefore possible that sequestration of CoASH by benzoate would limit the synthesis of succinyl-CoA and therefore decrease porphyrin synthesis. This is indirectly supported by the observation that ketone body oxidation in rat heart causes CoASH sequestration, which results in decreased synthesis of succinyl-CoA by α-ketoglutarate dehydrogenase (Russell & Taegtmeyer, 1992). If benzoate influences the availability of succinyl-CoA and glycine it is possible that the synthesis of the cytochromes P450 may be affected, which could result in abnormal phase I detoxification. This topic must be urgently investigated, as abnormal succinyl-CoA ligase activity has been linked to deleterious effects on mitochondrial DNA synthesis (Elpeleg et al., 2005; Furuyama & Sassa, 2000).

Benzoate consumption may influence appetite and feeding behavior

In ecology, the ''detoxification limitation hypothesis'', based on observations of animal feeding behavior over the past four decades, states that animals can sense the toxic load of food and either reduce food intake, or switch diets, in response to a high intake of toxic plant secondary metabolites. This prevents the animal from ingesting an amount of toxins exceeding its detoxification capacity (Marsh et al., 2006). The emetic system is one mechanism mediating an aversion to the diet, explaining why anti-emetic drugs such as metoclopramide and ondansetron can suppress this response. However, the decreased consumption of diets containing benzoate by possums is not suppressed by anti-emetics, but supplementing the benzoate containing diet with glycine restores food intake to normal (Marsh et al., 2005). This suggests that plasma or CSF glycine levels may be important in mediating the response to benzoate. Although it has been argued in this review that regulation of CNS glycine concentration is not the primary function of the glycine conjugation system, short-term fluctuations of plasma and CNS glycine do result from ingestion of benzoate and other substrates for glycine conjugation (Beyoglu & Idle, 2012; Beyoglu et al., 2012; de Vries et al., 1948). Imbalanced diets rapidly influence the levels of amino acids in rat brain, which is sensed in the anterior piriform cortex by a GCN2 kinase mediated signal transduction pathway that results in an aversive response to the food source (Maurin et al., 2005). We hypothesize that this mechanism could partly explain the decreased appetite induced in humans, monkeys, dogs, rats, possums, and hens by the administration of benzoate or phenylacetate (Batshaw et al., 1988; Marsh et al., 2005; Sherwin & Kennard, 1919). From this perspective, the glycine deportation hypothesis seems to support, rather than contradict, the view that glycine conjugation is important for the elimination of the toxic end products of aromatic acid metabolism (Badenhorst et al., 2013; Beyoglu & Idle, 2012; Beyoglu et al., 2012).

Summary and conclusions

The primary purpose of glycine conjugation is the detoxification of benzoate and benzoyl-CoA

The main objective of this review has been to develop a deeper understanding of the role of glycine conjugation in metabolism. It was demonstrated that glycine conjugation, far from being an uninteresting and unimportant metabolic process, is one of the fundamentally important homeostatic mechanisms in animal physiology. It was demonstrated that glycine conjugation is crucial to the maintenance of adequate levels of CoASH in the liver and kidney, and for decreasing the intrinsic toxicity of aromatic acids by increasing their hydrophilicity (Section ''The importance of glycine conjugation in the metabolism of aromatic acids'' and Figure 3). It was also argued that the recently proposed glycine deportation hypothesis (Beyoglu & Idle, 2012; Beyoglu et al., 2012) could help explain the aversion animals develop to diets containing high levels of benzoate (Caldwell, 1982; Caldwell, 1984; Au et al., 2013; Marsh et al., 2006; Marsh et al., 2005). Finally, because of the glycine deportation and ROS production induced by benzoate, the health risks of using this preservative should not be underestimated (Park et al., 2013; Beloborodova et al., 2012; Beyoglu & Idle, 2012; Fedotcheva et al., 2008; Wu et al., 2013).

The glycine conjugation pathway is still very poorly characterized

The levels of hippurate excreted in urine seem to correlate with a wide range of pathological conditions such as diabetes, obesity, gut dysbiosis, autism, schizophrenia, depression, hepatitis, and cancer (Lees et al., 2013; Beyoglu & Idle, 2012; Beyoglu et al., 2012). However, it is currently impossible to accurately interpret the relationships between hippurate excretion and these disease conditions, since the glycine conjugation pathway is still rather poorly understood (Badenhorst et al., 2013; Knights & Miners, 2012; Knights et al., 2007; Lees et al., 2013). Therefore, the influence of genetic variation in the ACSM2A and GLYAT genes on the activities of the HXM-A and GLYAT enzymes, as well as the relationship between these factors and the *in vivo* rate of glycine conjugation, must be investigated in more detail. Here some studies, that could significantly advance our understanding of the glycine conjugation pathway, are suggested.

Determination of in vivo *and* in vitro *glycine conjugation rates*

Previous *in vivo* studies of glycine conjugation are difficult to compare because of differences in the substrates, substrate dosages, sample collection protocols, metabolite analyses, and test animals used. Most studies have made no attempt to identify the limiting step of the glycine conjugation pathway, making it difficult to estimate the relative importance of variation in HXM-A and GLYAT enzyme activities to this

process (Badenhorst et al., 2013; Knights & Miners, 2012). As discussed in Section ''A brief introduction to glycine conjugation'', the commonly accepted view of benzoate as the primary substrate for glycine conjugation may be inaccurate. The major precursor for hippurate synthesis is phenylpropionic acid, produced by the gut microbiota from dietary polyphenols (Fedotcheva et al., 2008; Jenner et al., 2005; Rechner et al., 2002). This means that the rates of glycine conjugation measured using benzoate as a probe compound may not always be physiologically relevant. Phenylpropionate, cinnamate, and benzoate differ in their ability to stimulate ROS generation and inhibit respiration (Beloborodova et al., 2012; Fedotcheva et al., 2008, 2012). It would therefore be valuable to compare the *in vivo* rates of hippurate formation from phenylpropionate, cinnamate, and benzoate, and to compare the stimulatory effect of glycine supplementation in each case. It is also important to compare the *in vitro* rates of activation of these three hippurate precursors to acyl-CoA esters by the human liver HXM-A and HXM-B (E.C. 6.2.1.2) ligases, as well as the rate of benzoyl-CoA formation through β-oxidation. This information is crucial to developing an understanding of the factors that influence variation in the rate of glycine conjugation *in vivo*. It would also be valuable to compare glycine cleavage system activities to glycine availability, which is a major factor influencing the rate of glycine conjugation *in vivo* (de Vries et al., 1948).

Investigation of the influence of genetic variation in the ligase and transferase genes

As discussed in Section ''Genetic variation in HXM-A and GLYAT'', it is now known that genetic variation in the human GLYAT gene influences the enzyme activity of a recombinant human GLYAT. However, of the 55 nonsynonymous SNPs in the human GLYAT gene that are currently known, only six have been investigated (van der Sluis et al., 2013). It would therefore be valuable if more of these variations were characterized in terms of enzyme activity, substrate selectivity, stability, and mitochondrial import. The influence of variation in the non-coding parts of the gene also need to be investigated, since it has been reported that some of these variations may influence development of the musculoskeletal system (Guo et al., 2013). It is possible that these genetic variations influence expression of the gene, but at present this is not understood at all. It is known that hepatic expression of GLYAT is influenced by diet in rats, and that decreased transcription of GLYAT results in decreased GLYAT protein content of hepatocellular carcinoma specimens (Matsuo et al., 2012; Wen et al., 2013). However, it is not yet known how variation in hepatic GLYAT activity influences the *in vivo* rate of glycine conjugation, since we do not yet understand the conditions under which GLYAT activity is the limiting factor in this pathway.

The study of Temellini and co-workers demonstrated significant interindividual variation in the ability of human liver homogenates to synthesize hippurate from benzoate, ATP, CoASH, and glycine. This suggests that the amounts and activities of the ligase and transferase enzymes are responsible for the variation, but these factors were not determined separately (Temellini et al., 1993). As far as we know, the association between aspirin intolerance and an SNP (rs1133607) in the ACSM2A gene, which encodes HXM-A, is the only information currently available to suggest that genetic variation in the acid:CoA ligase enzymes can influence the metabolism of salicylate and other benzoate derivatives (Agundez et al., 2009; Boomgaarden et al., 2009; Vessey et al., 1999). The use of standardized protocols for the characterization of recombinant enzymes and their variants seems to hold great promise for this field, given the difficulty in obtaining human liver tissues for research (Knights et al., 2007; van der Sluis et al., 2013; Vessey et al., 1999).

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References

Abdo KM, Wenk ML, Harry GJ, et al. (1998). Glycine modulates the toxicity of benzyl acetate in F344 rats. Toxicol Pathol 26:395–402.

Abe, S, Sasaki M. (1977). Chromosome aberrations and sister chromatid exchanges in Chinese hamster cells exposed to various chemicals. J Natl Cancer Inst 58:1635–1641.

Agundez JA, Martinez C, Perez-Sala D, et al. (2009). Pharmacogenomics in aspirin intolerance. Curr Drug Metab 10: 998–1008.

Amsel LP, Levy G. (1969). Drug biotransformation interactions in man II: a pharmacokinetic study of the simultaneous conjugation of benzoic and salicylic acids with glycine. J Pharm Sci 58:321–326.

Applegarth DA, Toone JR. (2001). Nonketotic hyperglycinemia (glycine encephalopathy): laboratory diagnosis. Mol Genet Metab 74:139–146.

Atamna H, Liu, J, Ames BN. (2001). Heme deficiency selectively interrupts assembly of mitochondrial complex IV in human fibroblasts: revelance to aging. J Biol Chem 276:48410–48416.

Au J, Marsh KJ, Wallis IR, Foley WJ. (2013). Whole-body protein turnover reveals the cost of detoxification of secondary metabolites in a vertebrate browser. J Comp Physiol B 183:993–1003.

Badenhorst CP, Van Der Sluis R, Erasmus, E, Van Dijk AA. (2013). Glycine conjugation: importance in metabolism, the role of glycine N-acyltransferase, and factors that influence interindividual variation. Expert Opin Drug Metab Toxicol 9:1139–1153.

Badenhorst CPS, Jooste, M, Van Dijk AA. (2012). Enzymatic characterization and elucidation of the catalytic mechanism of a recombinant bovine glycine N-acyltransferase. Drug Metab Dispos 40:346–352.

Bandeira S, Fonseca LJ, Guedes G, et al. (2013). Oxidative stress as an underlying contributor in the development of chronic complications in diabetes mellitus. Int J Mol Sci 14:3265–3284.

Barshop BA, Breuer J, Holm J, et al. (1989). Excretion of hippuric acid during sodium benzoate therapy in patients with hyperglycinaemia or hyperammonaemia. J Inherit Metab Dis 12:72–79.

Bartlett K, Gompertz D. (1974). The specificity of glycine-N-acylase and acylglycine excretion in the organicacidaemias. Biochem Med 10: 15–23.

Batshaw ML, Brusilow SW. (1981). Evidence of lack of toxicity of sodium phenylacetate and sodium benzoate in treating urea cycle enzymopathies. J Inherit Metab Dis 4:231.

DOI: 10.3109/03602532.2014.908903

Batshaw ML, Hyman SL, Coyle JT, et al. (1988). Effect of sodium benzoate and sodium phenylacetate on brain serotonin turnover in the ornithine transcarbamylase-deficient sparse-fur mouse. Pediatr Res 23:368–374.

Battaglia V, Salvi, M, Toninello A. (2005). Oxidative stress is responsible for mitochondrial permeability transition induction by salicylate in liver mitochondria. J Biol Chem 280:33864–33872.

Beloborodova N, Bairamov I, Olenin A, et al. (2012). Effect of phenolic acids of microbial origin on production of reactive oxygen species in mitochondria and neutrophils. J Biomed Sci 19:89–97.

Bennett MJ, Pollitt RJ, Taitz LS, Variend S. (1990). Medium-chain acyl-CoA dehydrogenase deficiency: A useful diagnosis five years after death. Clin Chem 36:1695–1697.

Bernardi P, Broekemeier KM, Pfeiffer DR. (1994). Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. J Bioenerg Biomembr 26:509–517.

Beynen AC, Geelen MJ. (1982). Short-term inhibition of fatty acid biosynthesis in isolated hepatocytes by mono-aromatic compounds. Toxicology 24:183–197.

Beyoglu, D, Idle JR. (2012). The glycine deportation system and its pharmacological consequences. Pharmacol Ther 135:151–167.

Beyoglu D, Smith RL, Idle JR. (2012). Dog bites man or man bites dog? The enigma of the amino acid conjugations. Biochem Pharmacol 83: 1331–1339.

Bichara D, Calcaterra NB, Arranz S, et al. (2014). Set-up of an infrared fast behavioral assay using zebrafish (*Danio rerio*) larvae, and its application in compound biotoxicity screening. J Appl Toxicol 34:214–219.

Boomgaarden I, Vock C, Klapper, M, Doring F. (2009). Comparative analyses of disease risk genes belonging to the acyl-CoA synthetase medium-chain (ACSM) family in human liver and cell lines. Biochem Genet 47:739–748.

Bradshaw PC, Pfeiffer DR. (2006). Loss of NAD(H) from swollen yeast mitochondria. BMC Biochem 7:3.

Bravo L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev 56:317–333.

Bray HG, Thorpe WV, White K. (1951). Kinetic studies of the metabolism of foreign organic compounds; the formation of benzoic acid from benzamide toluene, benzyl alcohol and benzaldehyde and its conjugation with glycine and glucuronic acid in the rabbit. Biochem J 48:88–96.

Bridges JW, French MR, Smith RL, Williams RT. (1970). The fate of benzoic acid in various species. Biochem J 118:47–51.

Broekemeier KM, Pfeiffer DR. (1995). Inhibition of the mitochondrial permeability transition by cyclosporin A during long time frame experiments: relationship between pore opening and the activity of mitochondrial phospholipases. Biochemistry 34:16440–16449.

Brosnan JT, Da Silva RP, Brosnan ME. (2011). The metabolic burden of creatine synthesis. Amino Acids 40:1325–1331.

Caldwell J. (1982). Conjugation reactions in foreign-compound metabolism: definition, consequences, and species variations. Drug Metab Rev 13:745–777.

Caldwell J. (1984). Xenobiotic acyl-coenzymes A: critical intermediates in the biochemical pharmacology and toxicology of carboxylic acids. Biochem Soc Trans 12:9–11.

Chen Q, Huang NN, Huang JT, et al. (2009). Sodium benzoate exposure downregulates the expression of tyrosine hydroxylase and dopamine transporter in dopaminergic neurons in developing zebrafish. Birth Defects Res B Dev Reprod Toxicol 86:85–91.

Conter C, Rolland MO, Cheillan D, et al. (2006). Genetic heterogeneity of the GLDC gene in 28 unrelated patients with glycine encephalopathy. J Inherit Metab Dis 29:135–42.

Conti A, Bickel MH. (1977). History of drug metabolism: discoveries of the major pathways in the 19th century. Drug Metab Rev 6:1–50.

Coon MJ. (2005). Cytochrome P450: nature's most versatile biological catalyst. Annu Rev Pharmacol Toxicol 45:1–25.

Cremin Jr JD, Drackley JK, Grum DE, et al. (1994). Effects of reduced phenolic acids on metabolism of propionate and palmitate in bovine liver tissue in vitro. J Dairy Sci 77:3608–3617.

Cyr DM, Egan SG, Brini CM, Tremblay GC. (1991). On the mechanism of inhibition of gluconeogenesis and ureagenesis by sodium benzoate. Biochem Pharmacol 42:645–654.

Dakin HD. (1908). Comparative studies of the mode of oxidation of phenyl derivatives of fatty acids by the animal organism and by hydrogen peroxide. J Biol Chem 4:419–435.

Dakin HD. (1910). The fate of sodium benzoate in the human organism. J Biol Chem 7:103–108.

Davies KM, Strauss M, Daum B, et al. (2011). Macromolecular organization of ATP synthase and complex I in whole mitochondria. Proc Natl Acad Sci USA 108:14121–14126.

De Vries A, Alexander, B, Quamo Y. (1948). Studies on amino acid metabolism. III. Plasma glycine concentration and hippuric acid formation following the ingestion of benzoate 1. J Clin Invest 27: 665–668.

Deguchi T, Takemoto M, Uehara N, et al. (2005). Renal clearance of endogenous hippurate correlates with expression levels of renal organic anion transporters in uremic rats. J Pharmacol Exp Ther 314: 932–938.

Demir E, Kocaoglu S, Kaya B. (2010). Assessment of genotoxic effects of benzyl derivatives by the comet assay. Food Chem Toxicol 48: 1239–1242.

Dercksen M, Duran M, Ijlst L, et al. (2012). Clinical variability of isovaleric acidemia in a genetically homogeneous population. J Inherit Metab Dis 35:1021–1029.

Di Salvo ML, Contestabile R, Paiardini, A, Maras B. (2013). Glycine consumption and mitochondrial serine hydroxymethyltransferase in cancer cells: the heme connection. Med Hypotheses 80:633–636.

Duffy LF, Kerzner B, Seeff L, et al. (1995). Preliminary assessment of glycine conjugation of para-aminobenzoic acid as a quantitative test of liver function. Clin Biochem 28:527–530.

Dzurik R, Spustova V, Krivosikova, Z, Gazdikova K. (2001). Hippurate participates in the correction of metabolic acidosis. Kidney Int Suppl 78:S278–S281.

Elpeleg O, Miller C, Hershkovitz E, et al. (2005). Deficiency of the ADP-forming succinyl-CoA synthase activity is associated with encephalomyopathy and mitochondrial DNA depletion. Am J Hum Genet 76:1081–1086.

Erfle JD, Sauer F. (1969). The inhibitory effects of acyl-coenzyme a esters on the pyruvate and α-oxoglutarate dehydrogenase complexes. Biochim Biophys Acta 178:441–452.

Fedotcheva NI, Kazakov RE, Kondrashova MN, Beloborodova NV. (2008). Toxic effects of microbial phenolic acids on the functions of mitochondria. Toxicol Lett 180:182–188.

Fedotcheva TA, Shimanovskii NL, Kruglov AG, et al. (2012). Role of mitochondrial thiols of different localization in the generation of reactive oxygen species. Biochemistry (Moscow) 6:92–99.

Fenton WA, Gravel RA, Rosenblatt DS. (2013). Part 9: Organic acids. Chapter 94: Disorders of propionate and methylmalonate metabolism. In: Vale D, Beaudet AL, Vogelstein B, et al., eds. The Online Metabolic and Molecular Bases of Inherited Diseases. New York: McGraw Hill.

Fulton TR, Triano T, Rabe, A, Loo YH. (1980). Phenylacetate and the enduring behavioral deficit in experimental phenylketonuria. Life Sci 27:1271–1281.

Furst DE, Gupta, N, Paulus HE. (1977). Salicylate metabolism in twins. Evidence suggesting a generic influence and induction of salicylurate formation. J Clin Invest 60:32–42.

Furuya KN, Durie PR, Roberts EA, et al. (1995). Glycine Conjugation of para-aminobenzoic acid (PABA): A quantitative test of liver function. Clin Biochem 28:531–540.

Furuyama K, Sassa S. (2000). Interaction between succinyl CoA synthetase and the heme-biosynthetic enzyme ALAS-E is disrupted in sideroblastic anemia. J Clin Invest 105:757–764.

Gamage N, Barnett A, Hempel N, et al. (2006). Human sulfotransferases and their role in chemical metabolism. Toxicol Sci 90:5–22.

Gatley SJ, Sherratt SA. (1976). The localization of hippurate synthesis in the matrix of rat liver mitochondria. Biochem Soc Trans 4: 525–526.

Gatley SJ, Sherratt HS. (1977). The synthesis of hippurate from benzoate and glycine by rat liver mitochondria. Submitochondrial localization and kinetics. Biochem J 166:39–47.

Gregersen N, Kolvraa S, Mortensen PB. (1986). Acyl-CoA: glycine N-acyltransferase: in vitro studies on the glycine conjugation of straight- and branched-chained acyl-CoA esters in human liver. Biochem Med Metab Biol 35:210–218.

Gregus Z, Fekete T, Halaszi, E, Klaassen CD. (1996a). Does hepatic ATP depletion impair glycine conjugation in vivo? Drug Metab Dispos 24:1347–1354.

Gregus Z, Fekete T, Halaszi E, Klaassen CD. (1996b). Lipoic acid impairs glycine conjugation of benzoic acid and renal excretion of benzoylglycine. Drug Metab Dispos 24:682–688.

Gregus Z, Fekete T, Varga, F, Klaassen CD. (1992). Availability of glycine and coenzyme A limits glycine conjugation in vivo. Drug Metab Dispos 20:234–240.

Gregus Z, Fekete T, Varga, F, Klaassen CD. (1993). Dependence of glycine conjugation on availability of glycine: Role of the glycine cleavage system. Xenobiotica 23:141–153.

Griffith AD, Cyr DM, Egan SG, Tremblay GC. (1989). Inhibition of pyruvate carboxylase by sequestration of coenzyme A with sodium benzoate. Arch Biochem Biophys 269:201–207.

Grillo MP, Benet LZ. (2002). Studies on the reactivity of clofibryl-S-acyl-CoA thioester with glutathione in vitro. Drug Metab Dispos 30: 55–62.

Grillo MP, Lohr MT. (2009). Covalent binding of phenylacetic acid to protein in incubations with freshly isolated rat hepatocytes. Drug Metab Dispos 37:1073–1082.

Guillemette C. (2003). Pharmacogenomics of human UDP-glucuronosyltransferase enzymes. Pharmacogenomics J 3:136–158.

Guo Y, Zhang L, Liu Y, et al. (2013). Suggestion of GLYAT gene underlying variation of bone size and body lean mass as revealed by a bivariate genome-wide association study. Hum Genet 132:189–199.

Gutknecht J. (1992). Aspirin, acetaminophen and proton transport through phospholipid bilayers and mitochondrial membranes. Mol Cell Biochem 114:3–8.

Hamosh A, Johnston MV. (2013). Part 8: Amino acids. Chapter 90: Nonketotic hyperglycinemia. In: Vale D, Beaudet AL, Vogelstein B, et al., eds. The Online Metabolic and Molecular Bases of Inherited Diseases. New York: McGraw Hill.

Hansch C, Leo A. (1995). Exploring QSAR. Washington: American Chemical Society.

Hansch C, Lien EJ, Helmer F. (1968). Structure-activity correlations in the metabolism of drugs. Arch Biochem Biophys 128:319–330.

Hazan R, Levine, A, Abeliovich H. (2004). Benzoic acid, a weak organic acid food preservative, exerts specific effects on intracellular membrane trafficking pathways in *Saccharomyces cerevisiae*. Appl Environ Microbiol 70:4449–4457.

Hiraga K, Kochi H, Hayasaka K, et al. (1981). Defective glycine cleavage system in nonketotic hyperglycinemia. Occurrence of a less active glycine decarboxylase and an abnormal aminomethyl carrier protein. J Clin Invest 68:525–534.

Houten SM, Wanders RJ. (2010). A general introduction to the biochemistry of mitochondrial fatty acid beta-oxidation. J Inherit Metab Dis 33:469–477.

Irjala K. (1972). Synthesis of p-aminohippuric, hippuric, and salicyluric acids in experimental animals and man. Ann Acad Sci Fenn A 154: 1–40.

Itoh T, Ito T, Ohba S, et al. (1996). Effect of carnitine administration on glycine metabolism in patients with isovaleric acidemia: Significance of acetylcarnitine determination to estimate the proper carnitine dose. Tohoku J Exp Med 179:101–109.

Jackson AA, Badaloo AV, Forrester T, et al. (1987). Urinary excretion of 5-oxoproline (pyroglutamic aciduria) as an index of glycine insufficiency in normal man. Br J Nutr 58:207–214.

Jackson AA, Gibson NR, Lu Y, Jahoor F. (2004). Synthesis of erythrocyte glutathione in healthy adults consuming the safe amount of dietary protein. Am J Clin Nutr 80:101–107.

Jackson AA, Persaud C, Hall M, et al. (1997). Urinary excretion of 5-L-oxoproline (pyroglutamic acid) during early life in term and preterm infants. Arch Dis Child Fetal Neonatal Ed 76:F152–F157.

Jackson AA, Persaud C, Meakins TS, Bundy R. (1996). Urinary excretion of 5-L-oxoproline (pyroglutamic acid) is increased in normal adults consuming vegetarian or low protein diets. J Nutr 126: 2813–2822.

Jain M, Nilsson R, Sharma S, et al. (2012). Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. Science 336:1040–1044.

Jenner AM, Rafter, J, Halliwell B. (2005). Human fecal water content of phenolics: the extent of colonic exposure to aromatic compounds. Free Radic Biol Med 38:763–772.

Jha N, Jurma O, Lalli G, Liu Y, et al. (2000). Glutathione depletion in PC12 results in selective inhibition of mitochondrial complex I activity. Implications for Parkinson's disease. J Biol Chem 275: 26096–26101.

Kalbag SS, Palekar AG. (1988). Sodium benzoate inhibits fatty acid oxidation in rat liver: effect on ammonia levels. Biochem Med Metab Biol 40:133–142.

Kasuya F, Igarashi, K, Fukui M. (1990). Glycine conjugation of the substituted benzoic acids in vitro: Structure-metabolism relationship study. J Pharmacobiodyn 13:432–440.

Kasuya F, Igarashi, K, Fukui M. (1991). Glycine conjugation of the substituted benzoic acids in mice: Structure-metabolism relationship study II. J Pharmacobiodyn 14:671–677.

Keller W. (1842). On the conversion of benzoic acid into hippuric acid. Provincial Med J Retrospect Med Sci 4:256–257.

Kelley M, Vessey D. (1990). The effects of ions on the conjugation of xenobiotics by the aralkyl-CoA and arylacetyl-CoA N-acyltransferases from bovine liver mitochondria. J Biochem Toxicol 5:125–135.

Kelley M, Vessey D. (1994). Characterization of the acyl-CoA: Amino acid N-acyltransferases from primate liver mitochondria. J Biochem Toxicol 9:153–158.

Kelley M, Vessey DA. (1993). Isolation and characterization of mitochondrial acyl-CoA: Glycine N-acyltransferases from kidney. J BiochemToxicol 8:63–69.

Khasnavis S, Pahan K. (2012). Sodium benzoate, a metabolite of cinnamon and a food additive, upregulates neuroprotective Parkinson disease protein DJ-1 in astrocytes and neurons. J Neuroimmune Pharmacol 7:424–435.

Kikuchi G, Motokawa Y, Yoshida, T, Hiraga K. (2008). Glycine cleavage system: reaction mechanism, physiological significance, and hyperglycinemia. Proc Jpn Acad Ser B Phys Biol Sci 84:246–263.

Knights KM. (1998). Role of hepatic fatty acid:coenzyme A ligases in the metabolism of xenobiotic carboxylic acids. Clin Exper Pharmacol Physiol 25:776–782.

Knights KM, Drogemiller CJ. (2000). Xenobiotic-CoA ligases: Kinetic and molecular characterization. Current Drug Metab 1:49–66.

Knights KM, Miners JO. (2012). Amino acid conjugation: A novel route of xenobiotic carboxylic acid metabolism in man. In: AV Lyubimov, ed. Encyclopedia of Drug Metabolism and Interactions. Somerset, USA: John Wiley and Sons, 595–610.

Knights KM, Sykes MJ, Miners JO. (2007). Amino acid conjugation: Contribution to the metabolism and toxicity of xenobiotic carboxylic acids. Expert Opin Drug Metab Toxicol 3:159–168.

Knoop F. (1904). Der Abbau aromatischer Fettsäuren im Tierkörper.

Knox WE, Horowitz ML, Friedell GH. (1969). The proportionality of glutaminase content to growth rate and morphology of rat neoplasms. Cancer Res 29:669–680.

Kolvraa S. (1979). Inhibition of the glycine cleavage system by branched-chain amino acid metabolites. Pediatr Res 13:889–893.

Kolvraa S, Gregersen N. (1986). Acyl-CoA:glycine N-acyltransferase: organelle localization and affinity toward straight- and branched-chained acyl-CoA esters in rat liver. Biochem Med Metab Biol 36: 98–105.

Krahenbuhl L, Reichen J, Talos, C, Krahenbuhl S. (1997). Benzoic acid metabolism reflects hepatic mitochondrial function in rats with long-term extrahepatic cholestasis. Hepatology 25:278–283.

Krieger I, Winbaum ES, Eisenbrey AB. (1977). Cerebrospinal fluid glycine in nonketotic hyperglycinemic: Effect of treatment with sodium benzoate and a ventricular shunt. Metabolism 26:517–524.

Krivosikova Z, Spustova, V, Dzurik R. (1998). Participation of P-dependent and P-independent glutaminases in rat kidney ammoniagenesis and their modulation by metabolic acidosis, hippurate and insulin. Physiol Res 47:177–183.

Kubota K, Ishizaki T. (1991). Dose-dependent pharmacokinetics of benzoic acid following oral administration of sodium benzoate to humans. Eur J Clin Pharmacol 41:363–368.

Kure S, Ichinohe A, Kojima K, et al. (2004). Mild variant of nonketotic hyperglycinemia with typical neonatal presentations: mutational and in vitro expression analyses in two patients. J Pediatr 144:827–829.

Kushnareva YE, Sokolove PM. (2000). Prooxidants open both the mitochondrial permeability transition pore and a low-conductance channel in the inner mitochondrial membrane. Arch Biochem Biophys 376:377–388.

DOI: 10.3109/03602532.2014.908903

Lakeram M, Lockley DJ, Sanders DJ, et al. (2007). Paraben transport and metabolism in the biomimetic artificial membrane permeability assay (BAMPA) and 3-day and 21-day Caco-2 cell systems. J Biomol Screen 12:84–91.

Lees HJ, Swann JR, Wilson ID, et al. (2013). Hippurate: the natural history of a mammalian-microbial cometabolite. J Proteome Res 12: 1527–1546.

Lemasters JJ, Qian T, He L, et al. (2002). Role of mitochondrial inner membrane permeabilization in necrotic cell death, apoptosis, and autophagy. Antioxid Redox Signal 4:769–781.

Levy G. (1979). Pharmacokinetics of salicylate in man. Drug Metab Rev 9:3–19.

Lheureux PE, Hantson P. (2009). Carnitine in the treatment of valproic acid-induced toxicity. Clin Toxicol (Phila) 47:101–111.

Li C, Benet LZ, Grillo MP. (2002). Studies on the chemical reactivity of 2-phenylpropionic acid 1-O-acyl glucuronide and S-acyl-CoA thioester metabolites. Chem Res Toxicol 15:1309–1317.

Li C, Grillo MP, Benet LZ. (2003). In vitro studies on the chemical reactivity of 2,4-dichlorophenoxyacetyl-S-acyl-CoA thioester. Toxicol Appl Pharmacol 187:101–109.

Linder-Horowitz M, Knox WE, MORRIS HP. (1969). Glutaminase activities and growth rates of rat hepatomas. Cancer Res 29: 1195–1199.

Lino Cardenas CL, Bourgine J, Cauffiez C, et al. (2010). Genetic polymorphisms of Glycine N-acyltransferase (GLYAT) in a French Caucasian population. Xenobiotica 40:853–861.

Loo YH, Fulton T, Miller, K, Wisniewski HM. (1980). Phenylacetate and brain dysfunction in experimental phenylketonuria: synaptic development. Life Sci 27:1283–1290.

Loo YH, Fulton T, Miller KA, Wisniewski HM. (1979). Vulnerability of the immature rat brain to phenylacetate intoxication: Postnatal development of the detoxication mechanism. J Neurochem 32: 1699–1705.

Loo YH, Potempska, A, Wisniewski HM. (1985). A biochemical explanation of phenyl acetate neurotoxicity in experimental phenylketonuria. J Neurochem 45:1596–1600.

Lucas, J, Orten JM. (1954). Dietary protein and glycine as precursors of porphyrins in the rat. J Nutr 52:89–97.

Mao L-F, Chu C, Schulz H. (1994). Hepatic.beta.-oxidation of 3-phenylpropionic acid and the stereospecific dehydration of (R)- and (S)-3-hydroxy-3-phenylpropionyl-CoA by different enoyl-CoA hydratases. Biochemistry 33:3320–3326.

Marsh KJ, Wallis IR, Andrew RL, Foley WJ. (2006). The detoxification limitation hypothesis: where did it come from and where is it going? J Chem Ecol 32:1247–1266.

Marsh KJ, Wallis IR, Foley WJ. (2005). Detoxification rates constrain feeding in common brushtail possums (*Trichosurus vulpecula*). Ecology 86:2946–2954.

Masuda T, Nakamura K, Jikihara T, et al. (1996). 3D-quantitative structure-activity relationships for hydrophobic interactions: Comparative molecular field analysis (CoMFA) including molecular lipophilicity potentials as applied to the glycine conjugation of aromatic as well as aliphatic carboxylic acids. Quantitative Struct-Activity Relationships 15:194–200.

Matsuo M, Terai K, Kameda N, et al. (2012). Designation of enzyme activity of glycine-N-acyltransferase family genes and depression of glycine-N-acyltransferase in human hepatocellular carcinoma. Biochem Biophys Res Commun 420:901–906.

Maurin AC, Jousse C, Averous J, et al. (2005). The GCN2 kinase biases feeding behavior to maintain amino acid homeostasis in omnivores. Cell Metab 1:273–277.

Mawal YR, Qureshi IA. (1994). Purification to homogeneity of mitochondrial acyl coa:glycine n-acyltransferase from human liver. Biochem Biophys Res Commun 205:1373–1379.

McVean GA. (2012). An integrated map of genetic variation from 1,092 human genomes. Nature 491:56–65.

Melendez-Hevia E, De Paz-Lugo P, Cornish-Bowden A, Cardenas ML. (2009). A weak link in metabolism: the metabolic capacity for glycine biosynthesis does not satisfy the need for collagen synthesis. J Biosci 34:853–872.

Metges CC, Yu YM, Cai W, et al. (2000). Oxoproline kinetics and oxoproline urinary excretion during glycine- or sulfur amino acid-free diets in humans. Am J Physiol Endocrinol Metab 278: E868–E876.

Mineo H, Ohdate T, Fukumura K, et al. (1995). Effects of benzoic acid and its analogues on insulin and glucagon secretion in sheep. Eur J Pharmacol 280:149–154.

Mitchell GA, Gauthier N, Lesimple A, et al. (2008). Hereditary and acquired diseases of acyl-coenzyme A metabolism. Mol Genet Metab 94:4–15.

Mortensen PB, Kolvraa, S, Christensen E. (1980). Inhibition of the glycine cleavage system: Hyperglycinemia and hyperglycinuria caused by valproic acid. Epilepsia 21:563–569.

Murphy MP. (2009). How mitochondria produce reactive oxygen species. Biochem J 417:1–13.

Nakagawa Y, Moldeus P. (1998). Mechanism of p-hydroxybenzoate ester-induced mitochondrial dysfunction and cytotoxicity in isolated rat hepatocytes. Biochem Pharmacol 55:1907–1914.

Nakagawa Y, Moore G. (1999). Role of mitochondrial membrane permeability transition in p-hydroxybenzoate ester-induced cytotoxicity in rat hepatocytes. Biochem Pharmacol 58:811–816.

Nandi DL, Lucas SV, Webster Jr LT. (1979). Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine N-acyltransferase from bovine liver mitochondria. Purification and characterization. J Biol Chem 254:7230–7237.

Norman C, Howell KA, Millar AH, et al. (2004). Salicylic acid is an uncoupler and inhibitor of mitochondrial electron transport. Plant Physiol 134:492–501.

Oates PS, West AR. (2006). Heme in intestinal epithelial cell turnover, differentiation, detoxification, inflammation, carcinogenesis, absorption and motility. World J Gastroenterol 12:4281–4295.

Olsen J, Li C, Skonberg C, et al. (2007). Studies on the metabolism of tolmetin to the chemically reactive acyl-coenzyme A thioester intermediate in rats. Drug Metab Dispos 35:758–764.

Oyanagi K, Kuniya Y, Nagao M, et al. (1987). Cytotoxicities of sodium benzoate in primary culture of hepatocytes from adult rat liver. Tohoku J Exp Med 152:47–51.

Palekar A. (2000). Effect of pantothenic acid on hippurate formation in sodium benzoate-treated HepG2 cells. Pediatr Res 48:357–359.

Palekar AG, Kalbag SS. (1991). Amino acids in the rat liver and plasma and some metabolites in the liver after sodium benzoate treatment. Biochem Med Metab Biol 46:52–58.

Park H-W, Park EH, Yun H-M, Rhim H. (2011). Sodium benzoate-mediated cytotoxicity in mammalian cells. J Food Biochem 35: 1034–1046.

Park WH, Kang YC, Piao Y, et al. (2013). Causal effects of synthetic chemicals on mitochondrial deficits and diabetes pandemic. Arch Pharm Res 36:178–188.

Perry TL, Urquhart N, Maclean J, et al. (1975). Nonketotic hyperglycinemia. N Eng J Med 292:1269–1273.

Persaud C, Forrester T, Jackson AA. (1996). Urinary excretion of 5-L-oxoproline (pyroglutamic acid) is increased during recovery from severe childhood malnutrition and responds to supplemental glycine. J Nutr 126:2823–2830.

Phipps AN, Stewart J, Wright, B, Wilson ID. (1998). Effect of diet on the urinary excretion of hippuric acid and other dietary-derived aromatics in rat. A complex interaction between diet, gut microflora and substrate specificity. Xenobiotica 28:527–537.

Piper P, Calderon CO, Hatzixanthis K, Mollapour M. (2001). Weak acid adaptation: The stress response that confers yeasts with resistance to organic acid food preservatives. Microbiology 147:2635–2642.

Piper PW. (1999). Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. Free Radic Biol Med 27:1219–1227.

Piper WN, Condie LW, Tephly TR. (1973). The role pf substrates for glycine acyltransferase in the reversal of chemically induced porphyria in the rat. Arch Biochem Biophys 159:671–677.

Pitocco D, Zaccardi F, Di Stasio E, et al. (2010). Oxidative stress, nitric oxide, and diabetes. Rev Diabet Stud 7:15–25.

Politycka B. (1997). Free and glucosylated phenolics, phenol β-glucosyltransferase activity and membrane permeability in cucumber roots affected by derivatives of cinnamic and benzoic acids. Acta Physiologiae Plantarum 19:311–317.

Poon K, Pang KS. (1995). Benzoic acid glycine conjugation in the isolated perfused rat kidney. Drug Metab Dispos 23:255–260.

Porcelli AM, Ghelli A, Zanna C, et al. (2005). pH difference across the outer mitochondrial membrane measured with a green fluorescent protein mutant. Biochem Biophys Res Commun 326:799–804.

Praphanphoj V, Boyadjiev SA, Waber LJ, et al. (2000). Three cases of intravenous sodium benzoate and sodium phenylacetate toxicity occurring in the treatment of acute hyperammonaemia. J Inherit Metab Dis 23:129–136.

Probstein JG, Londe S. (1940). Studies of liver function by means of quick's hippuric acid test. Ann Surg 111:230–245.

Ratnakumari L, Qureshi IA, Butterworth RF. (1993). Effect of sodium benzoate on cerebral and hepatic energy metabolites in spf mice with congenital hyperammonemia. Biochem Pharmacol 45:137–146.

Rechner AR, Kuhnle G, Bremner P, et al. (2002). The metabolic fate of dietary polyphenols in humans. Free Radical Biol Med 33: 220–235.

Rinaldo P, O'shea JJ, Welch RD, Tanaka K. (1990). The enzymatic basis for the dehydrogenation of 3-phenylpropionic acid: In vitro reaction of 3-phenylpropionyl-CoA with various acyl-CoA dehydrogenases. Pediatr Res 27:501–507.

Ritter JK. (2000). Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. Chem Biol Interact 129:171–193.

Rodriguez-Antona C, Gomez A, Karlgren M, et al. (2010). Molecular genetics and epigenetics of the cytochrome P450 gene family and its relevance for cancer risk and treatment. Hum Genet 127:1–17.

Ross P, Wootton IDP. (1964). Glycine conjugation and toxicity of phenolic acids. Clin Chim Acta 9:434–440.

Russell 3rd RR,, Taegtmeyer H. (1992). Coenzyme A sequestration in rat hearts oxidizing ketone bodies. J Clin Invest 89:968–973.

Sakuma T. (1991). Alteration of urinary carnitine profile induced by benzoate administration. Arch Dis Child 66:873–875.

Saltzman A, Caraway WT. (1953). Cinnamic acid as a test substance in the evaluation of liver function. J Clin Invest 32:711–719.

Schachter, D, Taggart JV. (1953). Benzoyl coenzyme A and hippurate synthesis. J Biol Chem 203:925–934.

Schachter, D, Taggart JV. (1954). Glycine N-acylase: Purification and properties. J Biol Chem 208:263–275.

Schwab AJ, Tao L, Yoshimura T, et al. (2001). Hepatic uptake and metabolism of benzoate: A multiple indicator dilution, perfused rat liver study. Am J Physiol Gastrointest Liver Physiol 280: G1124–G1136.

Sekhar RV, Mckay SV, Patel SG, et al. (2011a). Glutathione synthesis is diminished in patients with uncontrolled diabetes and restored by dietary supplementation with cysteine and glycine. Diabetes Care 34: 162–167.

Sekhar RV, Patel SG, Guthikonda AP, et al. (2011b). Deficient synthesis of glutathione underlies oxidative stress in aging and can be corrected by dietary cysteine and glycine supplementation. Am J Clin Nutr 94: 847–853.

Sherwin CP, Kennard KS. (1919). Toxicity of phenylacetic acid. J Biol Chem 40:259–264.

Sidenius U, Skonberg C, Olsen J, Hansen SH. (2004). In vitro reactivity of carboxylic acid-CoA thioesters with glutathione. Chem Res Toxicol 17:75–81.

Sikkema J, De Bont JA, Poolman B. (1995). Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201–222.

Smith EA, MacFarlane GT. (1996). Enumeration of human colonic bacteria producing phenolic and indolic compounds: Effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. J Appl Bacteriol 81:288–302.

Smith JN. (1958). Comparative detoxication. 5. Conjugation of aromatic acids in reptiles: formation of ornithuric acid, hippuric acid and glucuronides. Biochem J 69:509–516.

Spustova V, Cernay, P, Golier I. (1989). Inhibition of glucose utilization in uremia by hippurate: liquid chromatographic isolation and mass spectrometric and nuclear magnetic resonance spectroscopic identification. J Chromatogr 490:186–192.

Spustova V, Dzurik R. (1991). Effect of hippurate on glucose utilization in rat kidney cortex slices. Ren Physiol Biochem 14:42–47.

Spustova V, Dzurik R, Gerykova M. (1987). Hippurate participation in the inhibition of glucose utilization in renal failure. Czech Med 10: 79–89.

Spustova V, Oravec C. (1989). Antitumor effect of hippurate. An experimental study using various mouse tumor strains. Neoplasma 36: 317–320.

Strauss M, Hofhaus G, Schroder RR, Kuhlbrandt W. (2008). Dimer ribbons of ATP synthase shape the inner mitochondrial membrane. EMBO J 27:1154–1160.

Sweetman L, Williams JC. (2013). Part 9: Organic acids. Chapter 93: Branched chain organic acidurias. In: Vale D, Beaudet AL, Vogelstein B, et al., eds. The Online Metabolic and Molecular Bases of Inherited Diseases. New York: McGraw Hill.

Tanaka K, Budd MA, Efron ML, Isselbacher KJ. (1966). Isovaleric acidemia: a new genetic defect of leucine metabolism. Proc Natl Acad Sci USA 56:236–242.

Tanaka, K, Isselbacher KJ. (1967). The isolation and identification of N-isovalerylglycine from urine of patients with isovaleric acidemia. J Biol Chem 242:2966–2972.

Temellini A, Mogavero S, Giulianotti PC, et al. (1993). Conjugation of benzoic acid with glycine in human liver and kidney: A study on the interindividual variability. Xenobiotica 23:1427–1433.

Tfouni SA, Toledo MC. (2002). Estimates of the mean per capita daily intake of benzoic and sorbic acids in Brazil. Food Addit Contam 19: 647–654.

Trost LC, Lemasters JJ. (1996). The mitochondrial permeability transition: a new pathophysiological mechanism for Reye's syndrome and toxic liver injury. J Pharmacol Exp Ther 278:1000–1005.

Trost LC, Lemasters JJ. (1997). Role of the mitochondrial permeability transition in salicylate toxicity to cultured rat hepatocytes: implications for the pathogenesis of Reye's syndrome. Toxicol Appl Pharmacol 147:431–441.

Tsao R. (2010). Chemistry and biochemistry of dietary polyphenols. Nutrients 2:1231–1246.

Tsay HJ, Wang YH, Chen WL, et al. (2007). Treatment with sodium benzoate leads to malformation of zebrafish larvae. Neurotoxicol Teratol 29:562–569.

Ure A. (1841). On hippuric acid and its tests. Pharmac Transactions I: 317–318.

van der Sluis R, Badenhorst CPS, Van Der Westhuizen FH, Van Dijk AA. (2013). Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase. Gene 515:447–453.

van der Westhuizen FH, Pretorius PJ, Erasmus E. (2000). The utilization of alanine, glutamic acid, and serine as amino acid substrates for glycine N-acyltransferase. J Biochem Mol Toxicol 14:102–109.

van Doorn M, Vogels J, Tas A, et al. (2007). Evaluation of metabolite profiles as biomarkers for the pharmacological effects of thiazolidinediones in Type 2 diabetes mellitus patients and healthy volunteers. Br J Clin Pharmacol 63:562–574.

Vessey DA, Kelley M, Warren RS. (1999). Characterization of the CoA ligases of human liver mitochondria catalyzing the activation of short- and medium-chain fatty acids and xenobiotic carboxylic acids. Biochim Biophys Acta 1428:455–462.

Vessey DA, Lau E, Kelley M, Warren RS. (2003). Isolation, sequencing, and expression of a cDNA for the HXM-A form of xenobiotic/medium-chain fatty acid:CoA ligase from human liver mitochondria. J Biochem Mol Toxicol 17:1–6.

Vessey DA, Lau EP. (1998). Determination of the sequence of the arylacetyl acyl-CoA:amino acid n-acyltransferase from bovine liver mitochondria and its homology to the aralkyl acyl-CoA:amino acid n-acyltransferase. J Biochem Mol Toxic 12:275–279.

von Wendt L, Similä, S. (1982). Experience with non-ketotic hyperglycinaemia in Finland. J Inherited Metab Dis 5:111–112.

Wang CH, Wang CC, Wei YH. (2010). Mitochondrial dysfunction in insulin insensitivity: implication of mitochondrial role in type 2 diabetes. Ann N Y Acad Sci 1201:157–165.

Wang W, Wu Z, Dai Z, et al. (2013). Glycine metabolism in animals and humans: Implications for nutrition and health. Amino Acids 45: 463–477.

Webster LT, Siddiqui UA, Lucas SV, et al. (1976). Identification of separate acyl-CoA:glycine and acyl-CoA:L-glutamine N-acyltransferase activities in mitochondrial fractions from liver of rhesus monkey and man. J Biol Chem 251:3352–3358.

Weinshilboum RM, Otterness DM, Aksoy IA, et al. (1997). Sulfation and sulfotransferases 1: Sulfotransferase molecular biology: cDNAs and genes. FASEB J 11:3–14.

Wen GY, Wisniewski HM, Shek JW, et al. (1980). Neuropathology of phenylacetate poisoning in rats: an experimental model of phenylketonuria. Ann Neurol 7:557–566.

Wen H, Yang HJ, An YJ, et al. (2013). Enhanced phase II detoxification contributes to beneficial effects of dietary restriction as revealed by multi-platform metabolomics studies. Mol Cell Proteomics 12: 575–586.

DOI: 10.3109/03602532.2014.908903

White A. (1941). Growth-inhibition produced in rats by the oral administration of sodium benzoate: Effects of various dietary supplements. Yale J Biol Med 13:759–768.

Wijeyesekera A, Clarke PA, Bictash M, et al. (2012). Quantitative UPLC-MS/MS analysis of the gut microbial co-metabolites phenylacetylglutamine, 4-cresyl sulphate and hippurate in human urine: INTERMAP Study. Analyt Methods 4:65–72.

Wikoff WR, Anfora AT, Liu J, et al. (2009). Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci USA 106:3698–3703.

Williams HRT, Cox IJ, Cobbold JFL, et al. (2010). Differences in gut microbial metabolism are responsible for reduced hippurate synthesis in Chron's disease. BMC Gastroenterol 10:108.

Wise DR, Thompson CB. (2010). Glutamine addiction: A new therapeutic target in cancer. Trends Biochem Sci 35:427–433.

Wu G. (2010). Functional amino acids in growth, reproduction, and health. Adv Nutr 1:31–37.

Wu G. (2013). Functional amino acids in nutrition and health. Amino Acids 45:407–411.

Wu G, Fang YZ, Yang S, et al. (2004). Glutathione metabolism and its implications for health. J Nutr 134:489–492.

Wu G, Wu Z, Dai Z, et al. (2013). Dietary requirements of ''nutritionally non-essential amino acids'' by animals and humans. Amino Acids 44: 1107–1113.

Xie G, Chen T, Qiu Y, et al. (2012). Urine metabolite profiling offers potential early diagnosis of oral cancer. Metabolomics 8:220–231.

Xie W, Wood AR, Lyssenko V, et al. (2013). Genetic variants associated with glycine metabolism and their role in insulin sensitivity and type 2 diabetes. Diabetes 62:2141–2150.

Yamamoto A, Nonen S, Fukuda T, Yamazaki, H, Azuma J. (2009). Genetic polymorphisms of glycine N-acyltransferase in Japanese individuals. Drug Metab Pharmacokinet 24:114–117.

Yao K-W, Mao L-F, Luo MJ, Schulz H. (1994). The relationship between mitochondrial activation and toxicity of some substituted carboxylic acids. Chem-Biol Interact 90:225–234.

Ye G, Zhu B, Yao Z, et al. (2012). Analysis of urinary metabolic signatures of early hepatocellular carcinoma recurrence after surgical removal using gas chromatography-mass spectrometry. J Proteome Res 11:4361–4372.

Ye SF, Zhou YH, Sun Y, et al. (2006). Cinnamic acid causes oxidative stress in cucumber roots, and promotes incidence of Fusarium wilt. Environ Exper Botany 56:255–262.

Ye YL, Chan YT, Liu HC, et al. (2010). Depleted folate pool and dysfunctional mitochondria associated with defective mitochondrial folate proteins sensitize Chinese ovary cell mutants to tert-butylhydroperoxide-induced oxidative stress and apoptosis. J Nutr Biochem 21:793–800.

Yilmaz S, Unal F, Yuzbasioglu D. (2009). The in vitro genotoxicity of benzoic acid in human peripheral blood lymphocytes. Cytotechnology 60:55–61.

Yoshimura T, Schwab AJ, Tao L, et al. (1998). Hepatic uptake of hippurate: A multiple-indicator dilution, perfused rat liver study. Am J Physiol 274:G10–G20.

Zengin N, Yuzbasioglu D, Unal F, et al. (2011). The evaluation of the genotoxicity of two food preservatives: Sodium benzoate and potassium benzoate. Food Chem Toxicol 49:763–769.

Zhang Y, Chohnan S, Virga KG, et al. (2007). Chemical knockout of pantothenate kinase reveals the metabolic and genetic program responsible for hepatic coenzyme a homeostasis. Chem Biol 14: 291–302.

Zuppi C, Messana I, Tapanainen P, et al. (2002). Proton nuclear magnetic resonance spectral profiles of urine from children and adolescents with type 1 diabetes. Clin Chem 48:660–662.

APPENDIX C: EXAMPLE OF CONSENT FORM USED IN CHAPTER 4

Consent

Title of project: Enzymatic characterisation of the influence of genetic variations on a recombinant human glycine N-acyltransferase

Please read carefully and feel free to ask any questions you may have.

I, ...hereby consent to the removal of blood from myself.

The purpose of the investigation is to:

- isolate genomic DNA for sequencing to identify single nucleotide polymorphisms of GLYAT (glycine-N-acyltransferase)

The result(s) of the analysis carried out on the sample(s) will be made known to me, if all the protocols necessary for the experiment could be performed successfully.

I understand that:

1. Conventional procedures and techniques are employed by trained staff to obtain and process the samples and that consequently the risk to my health is minimal.
2. Confidentiality and anonymity of the sample(s) is (are) respected: the samples are coded by numbers. My written permission is required for release of identifiable information to any other party.
3. The research may be of no direct benefit to me but the samples may provide general insight into disease and health.
4. No commercial claims can be made on developments from these investigations by myself.
5. Permission to investigate may be withdrawn at any time by me, without prejudice to my future *medical* care.

Signed ..... Date.....

Personnell number .....

Witnessed by (Signatures) (1)..... (2).....

Printed Names & Dates ....

Toestemming

Titel van projek: Enzymatic characterisation of the influence of genetic variations on a recombinant human glycine N-acyltransferase

Lees asseblief versigtig en voel vry om enige vrae te vra.

Ek, ...gee hiermee toestemming vir die verwydering van bloed van myself.

Die doel van die studie is om:

- Genomiese DNA te isoleer, waarvan die geenvolgorde bepaal sal word om sodoende enkel nukleotied polimorfismes te identifiseer van GLIAT (glisien asieltransferase)

Die resultate van die studie sal beskikbaar gestel word aan u indien alle stappe in die eksperiment suksesvol uitgevoer kon word.

Ek verstaan dat:

1. Standaard prosedures en tegnieke gebruik word deur opgeleide personeel om monsters te verkry en te verwerk en dat daar sodoende `n minimale gesonheidsrisiko bestaan.
2. Alle monsters sal vertroulik gehou word en word gekodeer met nommers. My skriftelike toestemming is nodig om persoonlike inligting vry te stel.
3. Die navorsing mag dalk nie `n onmiddelike voordeel vir my inhou nie, maar die resultate sal lei tot insig in algemene gesondheid.
4. Geen kommersielle aanspraak mag deur myself gemaak word op ontwikkelings vanuit die studie nie.
5. Toestemming om deel te neem aan die studie mag ter enige tyd onttrek word deur myself.

Handtekening (proefpersoon) ..... Datum.....

Personeelnmmer .....

Getuienis by (handtekening) (1) ..... (2) .....

Naam (drukskrif) & Datum ....

APPENDIX D: GLYAT AUGMENTATION THERAPY PATENT PCT/IB2011/053721

Recombinant therapeutic GLYAT patent application

Inventors:

Christoffel Petrus Stephanus Badenhorst, Lodewyk Jacobus Mienie, **Rencia van der Sluis**, and Alberdina Aike van Dijk

International application number:

PCT/IB2011/053721

The patent application was filed by:

At van Rooy, DM Kisch Inc., Pretoria, South Africa

US 20130224175A1

(19) **United States**

(12) **Patent Application Publication**

Mienie et al.

(10) **Pub. No.: US 2013/0224175 A1**

(43) **Pub. Date: Aug. 29, 2013**

(54) **RECOMBINANT THERAPEUTIC GLYCINE N-ACYLTRANSFERASE**

(75) Inventors: **Lodewyk Jacobus Mienie**, Potchefstroom (ZA); **Alberdina Aike Van Dijk**, Potchefstroom (ZA); **Christoffel Petrus Stephanus Badenhorst**, Potchefstroom (ZA); **Rencia Van Der Sluis**, Potchefstroom (ZA)

(73) Assignee: **North-West University**, Potchefstroom (ZA)

(21) Appl. No.: **13/818,481**

(22) PCT Filed: **Aug. 24, 2011**

(86) PCT No.: **PCT/IB2011/053721**

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C12N 9/10 (2006.01)

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CPC ***C12N 9/1029*** (2013.01)
USPC **424/94.5**; 435/193

(57) **ABSTRACT**

This invention relates to a method of producing a recombinant enzyme, more particularly, this invention relates to a method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT (E.G. 2.1.3.13)). including the steps of providing a suitable expression host; preparing a vector including a gene for expressing GLYAT in the expression host to form an expression plasmid; transforming the host with the expression plasmid to form an expression system; expressing the GLYAT gene in the expression system; and separating the expressed GLYAT from the expression system.

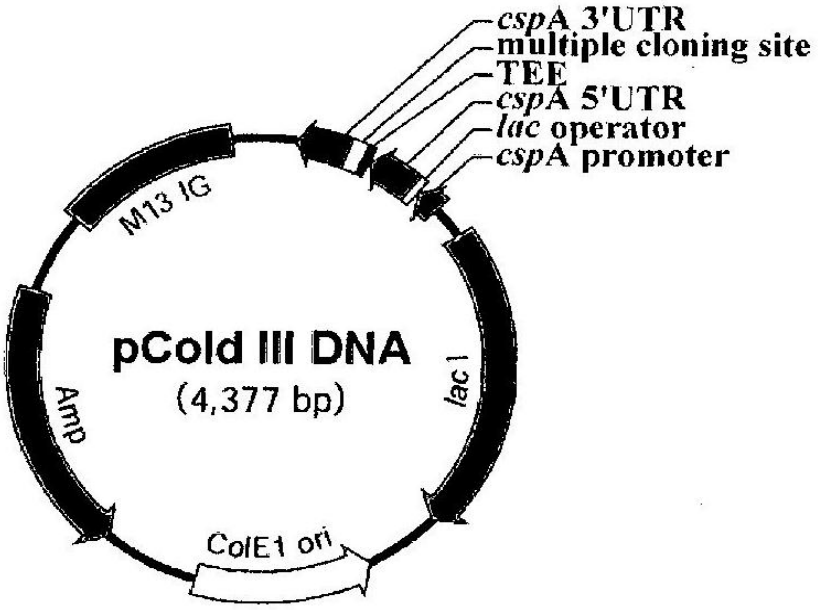


FIGURE 1

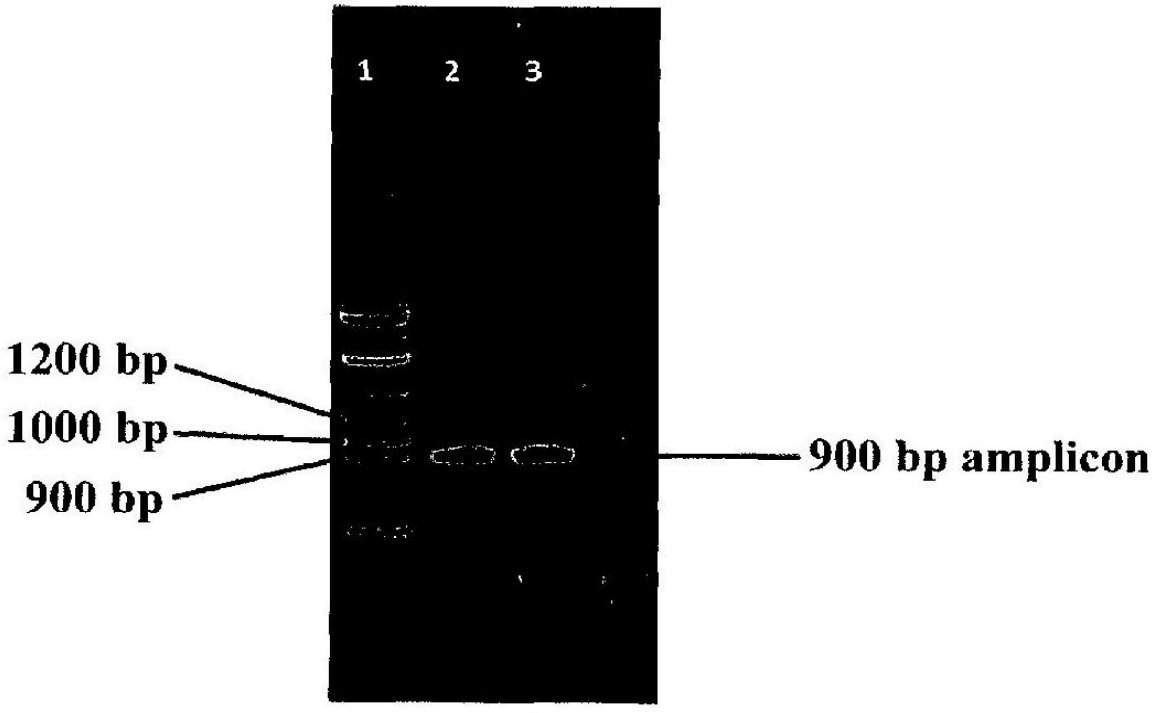


FIGURE 2

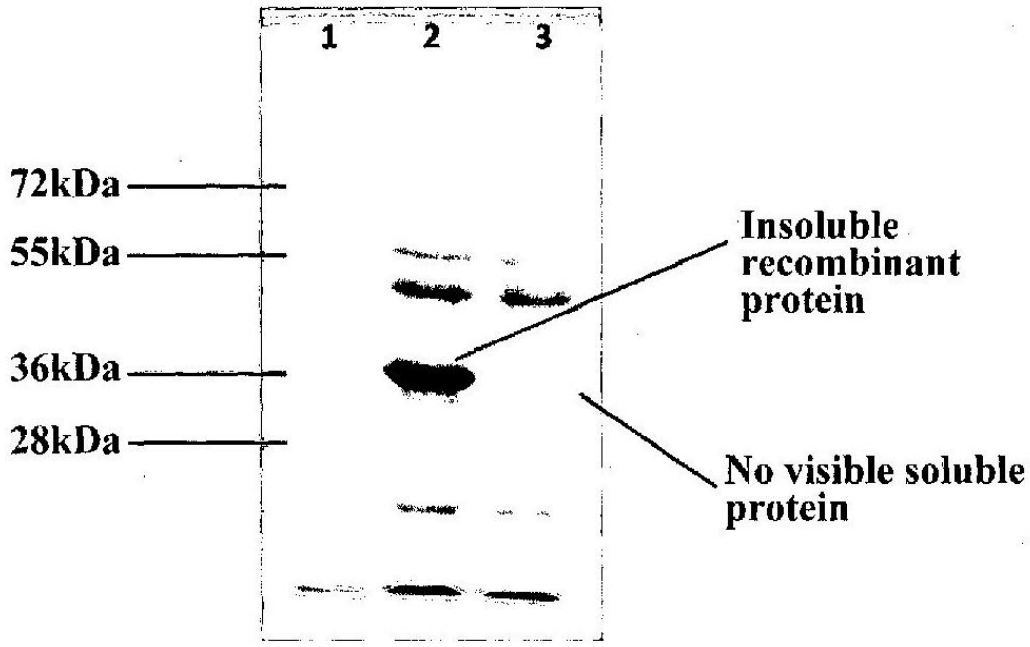
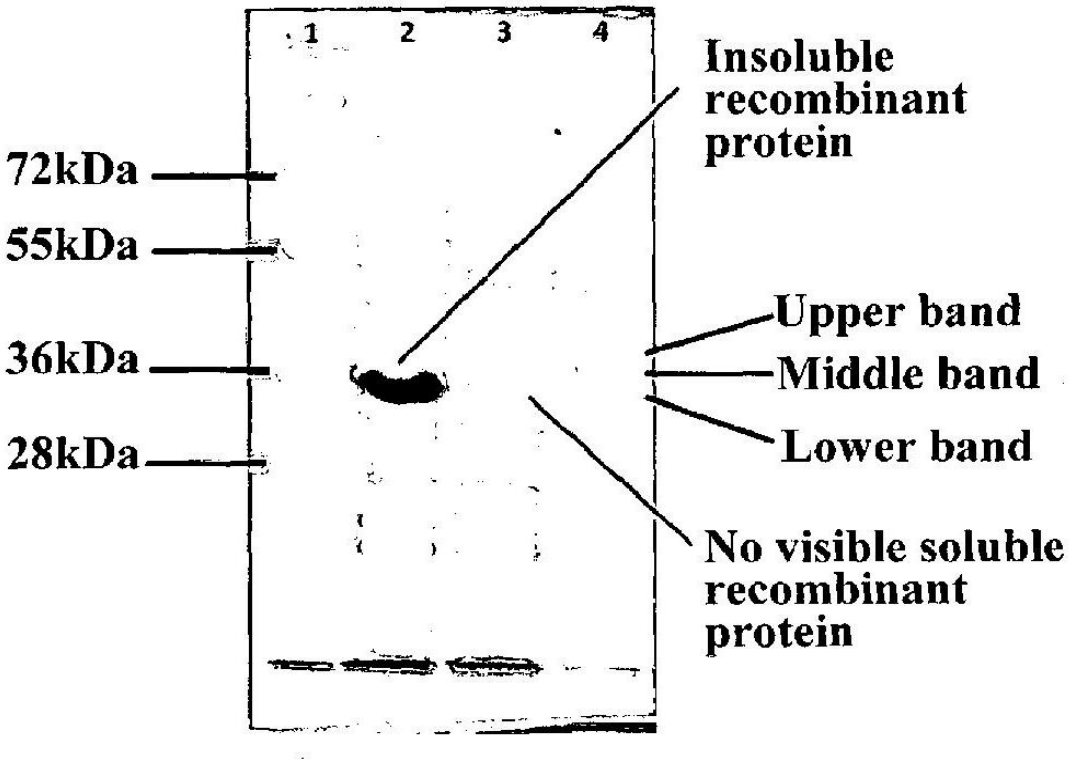

1
2
3
72kDa
55kDa
36kDa
28kDa
Insoluble recombinant protein
No visible soluble protein

FIGURE 3


1
2
3
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55kDa
36kDa
28kDa
Insoluble recombinant protein
Upper band
Middle band
Lower band
No visible soluble recombinant protein

FIGURE 4

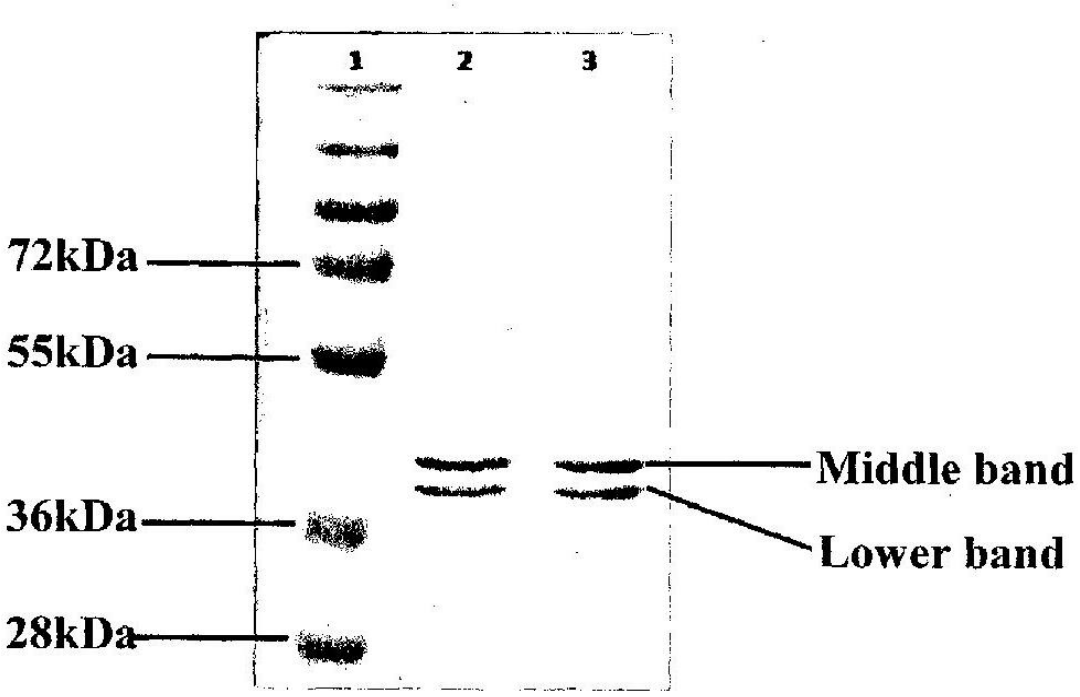
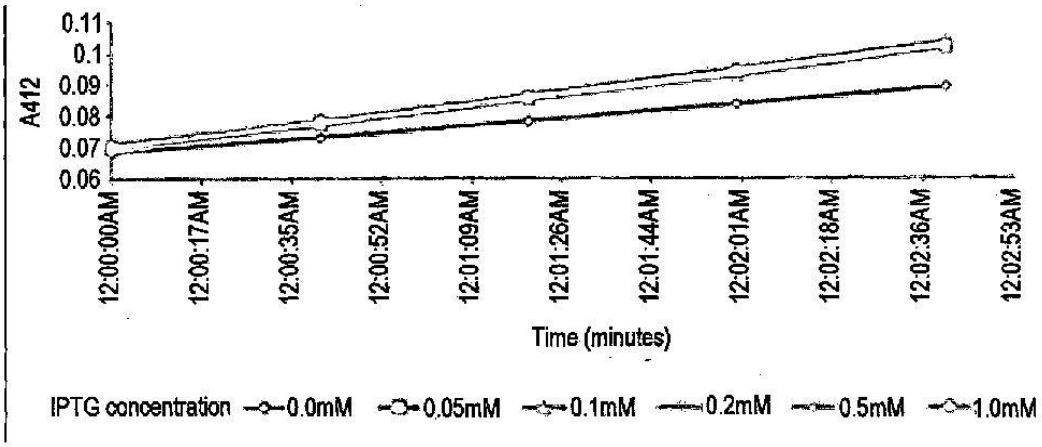


FIGURE 5

FIGURE 9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12:01:26AM
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Time (minutes)
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0.2mM
0.5mM
1.0mM

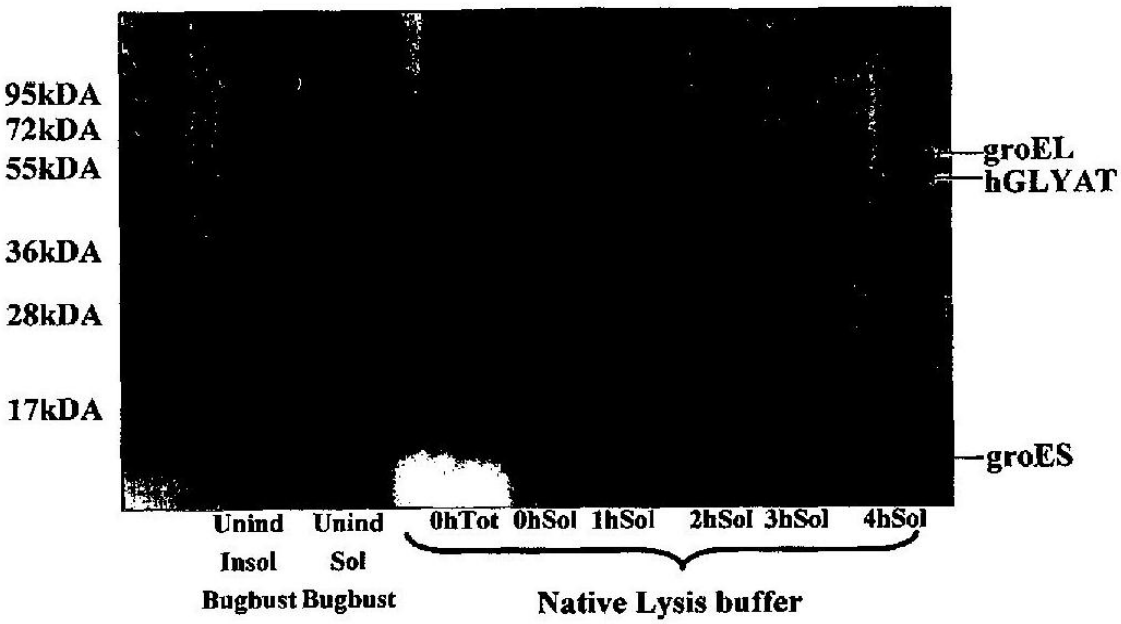

FIGURE 6

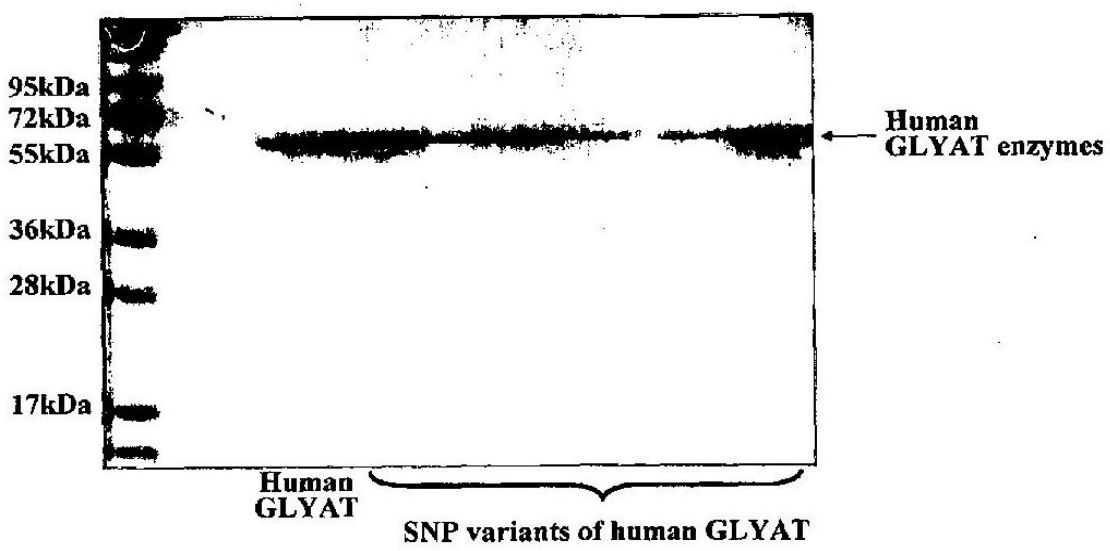

FIGURE 7

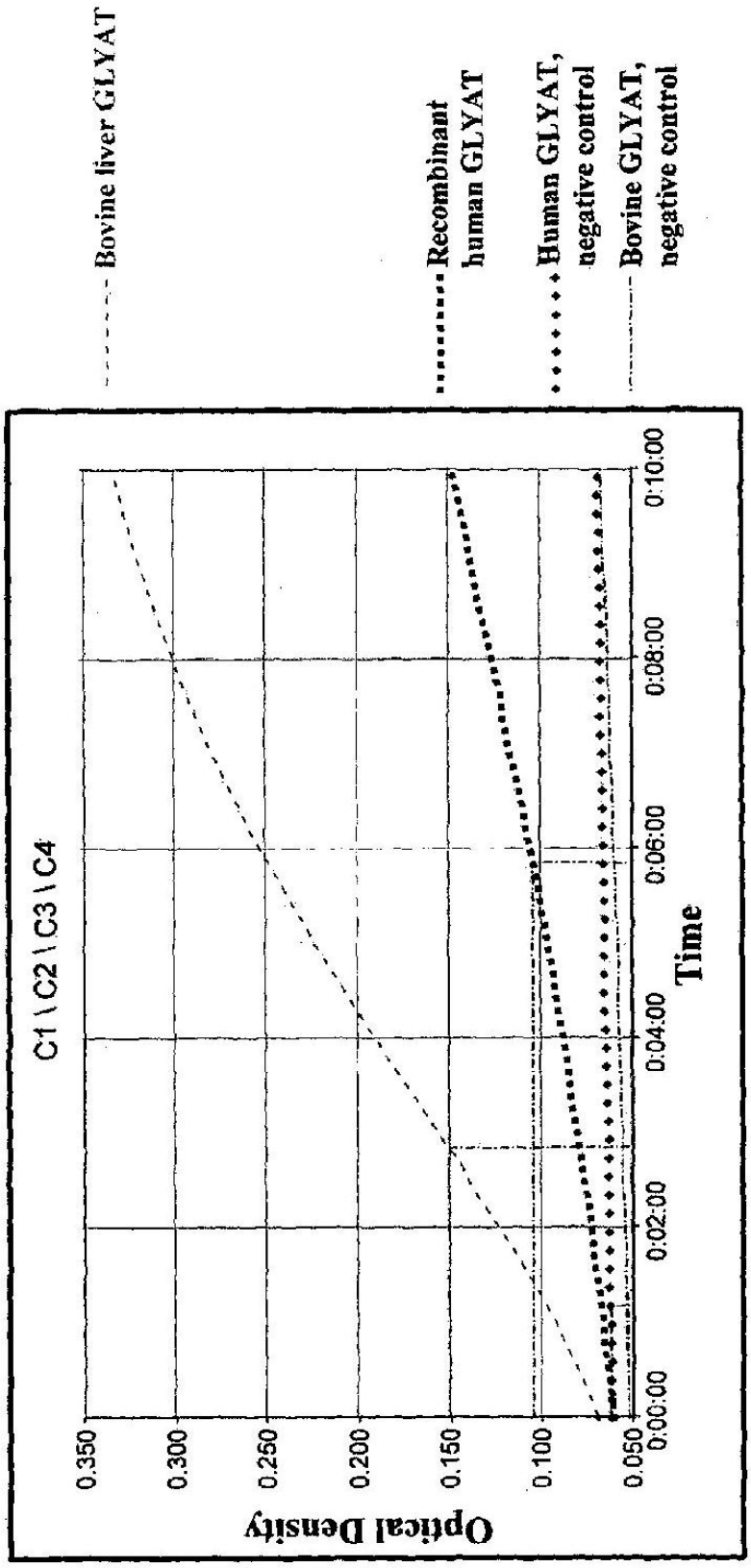
Bovine liver GLYAT
Recombinant human GLYAT
Human GLYAT, negative control
Bovine GLYAT, negative control
C1 \ C2 \ C3 \ C4
Optical Density
0.350
0.300
0.250
0.200
0.150
0.100
0.050
Time
0:00:00
0:02:00
0:04:00
0:06:00
0:08:00
0:10:00

FIGURE 8

US 2013/0224175 A1 Aug. 29, 2013

RECOMBINANT THERAPEUTIC GLYCINE N-ACYLTRANSFERASE

INTRODUCTION AND BACKGROUND TO THE INVENTION

[0001] This invention relates to a method of producing a recombinant enzyme. More particularly, this invention relates to a method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT (E.C. 2.3.1.13)).

[0002] Detoxification of toxic metabolites by the human body is an essential physiological process. The detoxification process decreases the toxicity of several endogenous metabolites, such as steroid hormones, and exogenous toxins, which could include compounds in food or industrial chemicals.

[0003] The detoxification process is divided into three main phases. Phase I detoxification activates metabolites by adding functional groups. The activated compounds generated by phase I detoxification are often more reactive and toxic than the original metabolites, and are further processed by phase II detoxification systems. In phase II detoxification, a range of conjugation reactions serve to make the activated compounds less toxic and more soluble, for excretion in the urine and bile. Phase III detoxification involves the elimination of toxins from cells.

[0004] Organic acidemias are a group of metabolic disorders caused by dysfunctional organic acid metabolism. The deficiency of certain metabolic enzymes causes the accumulation of acids which are not normally present in high levels in the human body. There are several known organic acidemias, with methylmalonic acidemia, propionic acidemia, isovaleric acidemia, glutaric aciduria, and maple syrup urine disease being some common examples.

[0005] Isovaleric acidemia is an autosomal recessive disorder. It is caused by a deficiency of isovaleryl coenzyme A dehydrogenase. A deficiency of this enzyme results in accumulation of intermediates of leucine catabolism, including isovaleric acid, 3- and 4-hydroxyisovaleric acid, isovalerylcarnitine and isovalerylglycine.

[0006] Isovalerylglycine is formed when isovaleric acid conjugates to glycine by glycine N-acyltransferase (GLYAT). The isovalerylglycine is less toxic than isovaleric acid, indicating that glycine conjugation is of critical importance in the treatment of isovaleric acidemia.

[0007] Urea cycle disorder is a genetic disorder caused by an enzyme deficiency in the urea cycle responsible for eliminating ammonia from the blood stream. In urea cycle disorders, nitrogen accumulates in the form of ammonia resulting in hyperammonemia which ultimately causes irreversible brain damage, coma and/or death.

[0008] A known method for enhancing glycine conjugation capacity in individuals suffering from organic acidemias is the administration of glycine supplements. Assays on liver samples have however shown that there is great variability in the glycine conjugation capacity in humans.

[0009] It is therefore evident that a means of augmenting the natural capacity for glycine conjugation would not only be beneficial to the general health of humans but may further present as an alternative therapeutic strategy for individuals affected by organic acidemias, urea cycle disorders, aminoaciduria, and exposure to some xenobiotic chemicals.

[0010] GLYAT is an enzyme responsible for the phase II detoxification of several toxic organic acids by means of conjugation to glycine. Several toxic compounds, both xenobiotic and endogenously derived metabolites, are detoxified by conjugation to glycine. In addition to GLYAT's role in the detoxification of benzoic acid, the enzyme is also important in the management of certain inborn errors of metabolism.

[0011] To date, no system for the bacterial expression and purification of an enzymatically active recombinant GLYAT has been reported.

[0012] A disadvantage associated with the lack of a system for expression of an enzymatically active recombinant GLYAT is that there is no commercially viable product currently available for directly improving the capacity of the glycine-conjugation detoxification system, particularly in the case of patients with metabolic disorders.

OBJECTS OF THE INVENTION

[0013] It is accordingly an object of the present invention to provide a novel method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT) enzyme and to provide GLYAT produced with such a method.

[0014] It is a further object of the invention to provide use of a pharmaceutically effective amount of GLYAT in a method of enhancing detoxification and for treating and/or preventing metabolic disorders in mammals.

[0015] It is yet another object of the invention to provide a method of enhancing detoxification in mammals and for treating and/or preventing metabolic disorders with which the aforesaid disadvantage may be overcome or at least minimised.

SUMMARY OF THE INVENTION

[0016] According to a first aspect of the invention there is provided a method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT) including the steps of:

[0017] providing a suitable expression host;

[0018] preparing a vector including a gene for expressing GLYAT in the expression host to form an expression plasmid;

[0019] transforming the host with the expression plasmid to form an expression system;

[0020] expressing the GLYAT gene in the expression system; and

[0021] separating the expressed GLYAT from the expression system.

[0022] Further according to the invention the step of separating the expressed GLYAT from the expression system may include the steps of separating the water soluble fraction of the expression system from the insoluble material and concentrating or lyophilising the separated GLYAT.

[0023] Further according to the invention the expression host may be selected from the group consisting of eukaryotic systems, including yeast cell expression-, insect cell expression- and mammalian cell expression systems; prokaryotic systems, including *Escherichia coli* and *Bacillus subtilis* and archaeon systems.

[0024] Further according to the invention the method includes a further step of combining the separated expressed GLYAT with glycine.

[0025] According to a second aspect of the invention there is provided water soluble enzymatically active recombinant GLYAT prepared in accordance with the first aspect of the invention.

[0026] According to a third aspect of the invention there is provided a medicament comprising water soluble enzymatically active recombinant GLYAT prepared in accordance with the first aspect of the invention.

[0027] According to a fourth aspect of the invention there is provided use of a pharmaceutically effective amount of water soluble enzymatically active recombinant GLYAT prepared in accordance with the first aspect of the invention in a method of:

[0028] improving the capacity of a glycine-conjugation detoxification system;

[0029] enhancing detoxification; or

[0030] treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin in mammals.

[0031] According to a fifth aspect of the invention water soluble enzymatically active recombinant GLYAT may be used in a method of:

[0032] improving the capacity of a glycine-conjugation detoxification system;

[0033] enhancing detoxification; or

[0034] treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin,

in mammals by administering to a mammal in need thereof a biologically effective amount of between 0.1 mg and 160 mg of water soluble enzymatically active recombinant GLYAT per kilogram of body mass depending on the demand for increased glycine conjugation.

[0035] According to a sixth aspect of the invention there is provided use of a pharmaceutically effective amount of water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of the first aspect of the invention in a method of manufacturing a medicament for use in:

[0036] improving the capacity of a glycine-conjugation detoxification system;

[0037] enhancing detoxification; or

[0038] treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin in mammals.

[0039] Further according to the invention the metabolic disorders may be any one or more of the conditions selected from the group consisting of organic acidemias selected from propionic acidemia, isovaleric acidemia and glutaric aciduria, aminoacidurias selected from maple syrup urine disease and hyperglycinemia; and urea cycle disorder.

[0040] According to the seventh aspect of the invention there is provided a medicament prepared from water soluble enzymatically active recombinant GLYAT in accordance with the first aspect of the invention together with at least one inert pharmaceutically acceptable carrier or diluents in a dosage form selected from the group consisting of tablets; capsules; suspension; syrup; intradermal-; intramuscular-; intravenous-; and subcutaneous injection.

[0041] The water soluble enzymatically active recombinant GLYAT may be administered by intravenous injection (IV) with a preparation of the enzyme in a form that is targeted to the desired sub-cellular compartments. Alternatively, water soluble enzymatically active recombinant GLYAT may be administered by using a GLYAT enzyme fused to the membrane permeating TAT (transactivator of transcription) peptide, allowing the recombinant enzyme to effectively cross cell membranes to reach the desired mitochondrial matrix. Further alternatively, water soluble enzymatically active recombinant GLYAT may be administered by using a colloidal system that contains unique and stable lipid-based submicron- and micron-sized structures.

[0042] Further according to the invention the step of administering the biologically effective amount of water soluble enzymatically active recombinant GLYAT may include the further step of administering the water soluble enzymatically active recombinant GLYAT in combination with glycine to further stimulate glycine conjugation capacity.

BRIEF DESCRIPTION OF THE FIGURES

[0043] The invention will now be described further, by way of example only, with reference to the accompanying figures wherein:

[0044] FIG. **1**: is a diagram illustrating the pColdIII expression vector used for expression of bovine GLYAT in accordance with a preferred embodiment of the invention;

[0045] FIG. **2**: is a polymerase chain reaction (PCR) amplification of an open reading frame (ORF) encoding bovine GLYAT from a plasmid into which the ORF encoding bovine GLYAT had already been cloned (the original PCR amplification and cloning were performed using cDNA from bovine liver);

[0046] FIG. **3**: is a sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretogram (PAGE) illustrating the total and soluble fractions of the expression of recombinant bovine GLYAT (lanes **2** and **3**, respectively);

[0047] FIG. **4**: is an SDS-PAGE analysis illustrating the total and soluble fractions of the expression of recombinant bovine GLYAT (lanes **2** and **3**, respectively) as well as the partially purified enzyme (nickel affinity chromatography) in lane **4**;

[0048] FIG. **5**: is an SDS-PAGE analysis illustrating the enzyme after partial purification using nickel affinity chromatography (in this purification 20 mM imidazole was added to the wash purification buffers);

[0049] FIG. **6**: is an SDS-PAGE analysis illustrating the expression of a soluble recombinant human GLYAT gene (lanes **4** to **9**), with an N-terminal fusion of the hexahistidine tag and Trx-tag;

[0050] FIG. **7**: is an SDS-PAGE analysis illustrating the nickel-affinity purification of wild-type recombinant human GLYAT (lane **3**) and single nucleotide polymorphism (SNP) variants of human GLYAT (lanes **4** to **9**); and

[0051] FIG. **8**: is a spectrophotometric assay illustrating enzyme activity of recombinant human GLYAT and bovine liver GLYAT in the presence and absence of glycine.

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

[0052] According to a preferred embodiment of the invention there is provided a method for producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT).

[0053] The method includes the steps of providing a suitable expression host providing a GLYAT expressing gene; preparing a vector including a gene for expressing GLYAT in the expression host to form an expression plasmid; transforming the host with the expression plasmid to form an expression system; expressing the GLYAT in the expression system; separating the expressed GLYAT from the expression system; and combining the separated expressed GLYAT with glycine.

[0054] The expression host is selected from the group consisting of eukaryotic systems, including yeast cell expression, insect cell expression and mammalian cell expression, prokaryotic systems, including *Escherichia coli* and *Bacillus subtilis* and archaeon systems. It was found that *Escherichia coli* (*E. coli*) provided a particularly suitable host.

[0055] The gene encoding bovine GLYAT was isolated from bovine liver RNA and cloned, by means of reverse transcription and polymerase chain reaction (PCR) amplification, into a pColdIII expression vector (as illustrated in FIG. **1**). The pColdIII expression vector allows for the expression of a protein in *E. coli* at 15 degrees Celsius, which enhances the expression of soluble, enzymatically active recombinant proteins.

[0056] Various other vectors could also be used for the expression of recombinant human and bovine GLYAT, or other GLYAT variants, in eukaryotic, prokaryotic and archaeon expression hosts.

[0057] In order to obtain a suitable vector, a histidine-tag (His-tag) is attached to the C-terminus of the gene. In the alternative to C-terminal histidine tags, tags are selected from the group consisting of N-terminal hexahistidine tags, maltose binding protein (MBP), glutathione S-transferase, GST tags and Strep-Tag II.

[0058] GLYAT is alternatively expressed without any purification tags, and separated from the proteins of the expression host by utilising known protein purification strategies. Owing to the fact that GLYAT is a nucleotide-cofactor binding enzyme, it may further alternatively be purified by affinity chromatography.

EXAMPLE 1

Recombinant Bovine GLYAT

[0059] Recombinant bovine GLYAT was cloned into a set of three modified pColdIII (pColdIII-E, pColdIII-A and pColdIII-EH) expression vectors encoding C-terminal histidine tags.

[0060] In order to clone the coding sequence into the expression vectors, the sequence is amplified through polymerase chain reaction (PCR) using primers containing NdeI and XhoI restriction enzyme sites to facilitate directional cloning. The PCR reaction mixtures contained 1X Takara ExTaq buffer, 10 nmol of each dNTP, 25 pmol of each primer, approximately 50 ng of template DNA and 2 units of Takara ExTaq polymerase, in a final volume of 50 μl. Thermal cycling conditions were 94 degrees Celsius for 1 min, then 30 cycles of 94 degrees Celsius for 30 seconds, 70 degrees Celsius for 30 seconds, and 72 degrees Celsius for 1 minute, followed by a final extension at 72 degrees Celsius for 10 minutes.

[0061] After transforming *E. coli* with an expression plasmid containing a recombinant GLYAT coding sequence, colonies were screened for desired recombinant plasmids using either restriction analysis or PCR amplification. A colony was considered to be positive if an excised fragment of approximately 900 bp could be seen on an agarose gel, as illustrated in FIG. **2**.

[0062] The recombinant protein was purified using a nickel affinity purification process. Upon passage through a resin with nickel ions immobilised onto it, the histidine tags fused to the recombinant GLYAT binds tightly to the column matrix, by forming coordinate bonds with the nickel ions immobilised on its surface. This enables most other proteins to be washed from the column, while the histidine tagged GLYAT remains bound. The tagged protein was eluted with a buffer containing a high concentration of imidazole, which displaced the coordinate bonds between the histidine residues and nickel ions, resulting in a partially purified recombinant protein.

[0063] Referring to FIG. **3**, a sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretogram (PAGE) was used to analyse the expression of bovine

[0064] GLYAT from the pColdIII vector. The proteins were visualised by staining with Coomassie brilliant blue. Lane **2** illustrates the total fraction of expressed protein and lane **3** illustrates the soluble fraction of bacterial lysate; with the soluble recombinant GLYAT expressed not being clearly visible on the background of bacterial proteins.

[0065] Referring to FIG. **4**, recombinant bovine GLYAT was expressed from pColdIII with a C-terminal histidine tag. The soluble fraction was passed through a nickel affinity purification column, to purify the tagged recombinant GLYAT enzyme. The levels of soluble recombinant bovine GLYAT expressed were low, therefore, the final eluate of the purification was significantly concentrated. SDS-PAGE analysis revealed the total fraction of expressed protein in lane **2**. Lane **3** represents the soluble fraction of the recombinant GLYAT with no significant amount of soluble recombinant GLYAT being visible against the background of bacterial proteins. Lane **4** illustrates the partially purified enzyme as a result of the nickel-affinity purification. The lower band indicates the active form of the GLYAT enzyme.

[0066] Referring to FIG. **5**, 20 mM imidazole was added to the column wash buffers of the purification kit. The use of imidazole in the buffers resulted in the majority of the previously co-purifying proteins being lost. The lower bands, in FIG. **5**, represent the enzymatically active bovine GLYAT and an unknown protein.

[0067] It was found that the recombinant bovine GLYAT enzyme, prepared in accordance with the invention, has similar biochemical characteristics to the GLYAT enzyme purified from bovine liver.

EXAMPLE 2

Recombinant Human GLYAT

[0068] The nucleotide sequence encoding the human GLYAT sequence was synthesised and cloned into the pET32 expression vector.

[0069] The pET32 expression vector enables the expression of human GLYAT with an N-terminal hexahistidine tag and an N-terminal Trx-tag, which respectively facilitates the purification and correct folding of the enzyme.

[0070] The expression vector encoding human GLYAT was transformed into Origami expression cells. The cells were also transformed with the pGro7 vector from Takara, which resulted in co-expression of the GroES and GroEL chaperone proteins. Chaperone proteins aid in the correct folding of proteins and increase the yield of soluble recombinant enzymes.

[0071] The Origami cells containing the plasmids for expression of recombinant human GLYAT and the chaperone proteins were grown in liquid culture. It was found that the optimal expression of soluble GLYAT occurs in the absence of IPTG (Isopropyl (β-D-1-thiogalactopyranoside), thus

allowing GLYAT to be expressed at slow basal rate as oppose to the known method of inducing the fusion protein with IPTG to express.

[0072] After expression, cells were harvested by means of centrifugation, and lysed using an optimised native lysis buffer containing 300 mM NaCl, 50 mM phosphate buffer, pH 8.0, 10% glycerol, 1% Triton-X, lysozyme, and protease inhibitors. The cell lysates were clarified, using centrifugation at 10,000 g for 30 minutes to remove the insoluble material and passed through Protino nickel affinity purification columns to selectively bind the hexahistidine tagged enzymes. The columns were washed, and the purified protein eluted in a final volume of 3 ml.

[0073] Referring to FIG. **6**, soluble recombinant human GLYAT was expressed with an N-terminal hexahistidine-Trx-fusion tag. Lane **1** contains molecular size markers. Lanes **2** and **3** contain the insoluble and soluble fractions, respectively, of a culture, of which the cells were lysed using the BugBuster protein extraction reagent. It was found that this lysis reagent was not suitable for the extraction of recombinant human GLYAT, as no soluble recombinant human GLYAT was visible.

[0074] As an alternative, the optimised native lysis buffer was used to isolate the protein from cultures expressing from 0 hours to 4 hours, and the soluble fractions were loaded in lanes 5 to 9. The hexahistidine-Trx-GLYAT fusion protein is indicated by the arrow, in the 55 kDa range.

[0075] Referring to FIG. **7**, the soluble recombinant human GLYAT fusion proteins are purified by means of nickel-affinity chromatography, using Protino Ni-TED columns. Lane **1** contains molecular weight markers, and lane **2** is empty. Lane **3** contains the wild-type recombinant human GLYAT fusion protein, after purification. Lanes **4** to **9** contain purified recombinant human GLYAT, as prepared in accordance with the invention, fusion proteins, of the known single nucleotide polymorphism (SNP) variations of the gene.

[0076] Referring to FIG. **8**, the resultant enzyme preparation was assayed for enzyme activity using the spectrophotometric assay for GLYAT. In the assay, bovine GLYAT is used as a positive control. Reactions without glycine were run as negative controls illustrating that the enzyme activity observed is glycine dependent. The recombinant human GLYAT illustrated an increase in optical density (OD) at 412 nm confirming the enzyme activity of recombinant human GLYAT, prepared in accordance with the invention, in the presence of glycine.

[0077] In addition to using a recombinant therapeutic GLYAT enzyme in the above described manner, it is possible that novel forms of the GLYAT enzyme may be obtained by rational and semi-rational enzyme engineering strategies, and these may alternatively be used for their specialised functions. Qualities of the GLYAT enzyme that may be subjected to modification by enzyme engineering strategies include catalytic rate, substrate specificity, stability, immunological aspects, and optimal substrate concentration.

[0078] There are six known natural SNP (single nucleotide polymorphism) variants of human GLYAT and site-directed mutagenesis was used to generate these variant coding sequences from the wild-type sequence. It was found that of the six SNP variants, two have higher enzyme activity than the wild-type GLYAT, and the rest have much lower activity than the wild-type GLYAT. It is to be expected that there would be clear benefits associated with the use of variants with increased catalytic rate, for example.

Further Findings and Analysis

[0079] In use, a pharmaceutically effective amount of 0.1 mg to 160 mg of the recombinant GLYAT enzyme per kilogram of body weight, depending on the nature and extent of the metabolic disorder, is administered to a patient in need thereof by way of intravenous injection (IV) with a preparation of the enzyme in a form targeting the desired sub-cellular compartments. Alternatively, the prepared recombinant GLYAT enzyme is administered by using a TAT (transactivator of transcription) peptide to act as a membrane permeating agent, which will allow the recombinant enzyme to effectively cross cell membranes to reach the desired mitochondrial matrix. Further alternatively, the prepared recombinant GLYAT enzyme is administered using a colloidal system that contains unique and stable lipid-based submicron- and micron-sized structures to enhance detoxification and to treat and/or prevent metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin in mammals.

[0080] The metabolic disorders may be any one or more of the conditions selected from the group consisting of organic acidemias selected from propionic acidemia, isovaleric acidemia and glutaric aciduria; aminoacidurias selected from maple syrup urine disease, and hyperglycinemia, and urea cycle disorder.

[0081] The recombinant GLYAT is further alternatively formulated into any one of the following dosage forms comprising tablet; capsule; suspension; syrup; intradermal-; intramuscular-; intravenous-; and subcutaneous injection.

[0082] A medicament prepared from the recombinant GLYAT in combination with glycine is used to directly improve the capacity of the glycine-conjugation detoxification system in the treatment of patients exposed to chemical and industrial solvents and in the emergency treatment of acute aspirin poisoning. Glycine conjugation of several organic acids is enhanced by the use of a recombinant therapeutic GLYAT enzyme.

[0083] It will be appreciated that in terms of the invention, variations in details in providing a novel method of producing a recombinant enzyme and more particularly relating a novel method of producing a water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT) enzyme, are possible without departing from the scope of the appended claims.

1. A method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT) including the steps of:

providing a suitable expression host;

preparing a vector including a gene for expressing GLYAT in the expression host to form an expression plasmid;

transforming the host with the expression plasmid to form an expression system;

expressing the GLYAT gene in the expression system; and

separating the expressed GLYAT from the expression system.

2. A method according to claim **1** wherein the step of separating the expressed GLYAT from the expression system includes the steps of separating the water soluble fraction of the expression system from the insoluble material and concentrating or lyophilising the separated GLYAT.

3. A method according to claim **1** wherein the expression host is selected from the group consisting of eukaryotic systems, including yeast cell expression-, insect cell expression-,

and mammalian cell expression systems: prokaryotic systems, including *Escherichia coli*, and *Bacillus subtilis*; and archaeon systems.

4. A method according to claim **1** including the further step of combining the separated expressed GLYAT with glycine.

5. Water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of claim **1**.

6. A medicament comprising water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of claim **1** **4**.

7. Use of a pharmaceutically effective amount of water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of claim **1** in a method of:

improving the capacity of a glycine-conjugation detoxification system;

enhancing detoxification; or

treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin in mammals.

8. Water soluble enzymatically active recombinant GLYAT for use in a method of:

improving the capacity of a glycine-conjugation detoxification system;

enhancing detoxification; or

treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin,

in mammals by administering to a mammal in need thereof a biologically effective amount of between 0.1 mg and 160 mg of water soluble enzymatically active recombinant GLYAT per kilogram of body weight.

9. Use of a pharmaceutically effective amount of water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of claim **1** in a method of manufacturing a medicament for use in:

improving the capacity of a glycine-conjugation detoxification system;

enhancing detoxification; or

treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin in mammals.

10. Use according to claim **7** wherein the metabolic disorders are any one or more of the conditions selected from the group consisting of organic acidemias selected from propionic acidemia, isovaleric acidemia and glutaric aciduria; aminoacidurias selected from maple syrup urine disease and hyperglycinemia, and urea cycle disorder.

11. Use according to claim **7**, wherein the water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of claim **1** is administered in combination with glycine in order to further stimulate glycine conjugation capacity.

12. A medicament comprising water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of claim **1** **4** in combination with glycine, together with at least one inert pharmaceutically acceptable carrier or diluents in a dosage form selected from a group comprising tablet; capsule; suspension; syrup; TAT (transactivator of transcription) peptide; a colloidal system that contains unique and stable lipid-based submicron- and micron-sized structures; intradermal-; intramuscular-; intravenous-; and subcutaneous injection.

13. A method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT) as herein described and exemplified, with reference to the accompanying figures.

* * * * *

APPENDIX E: LIST OF PUBLICATIONS AND SCIENTIFIC POSTERS

Peer Reviewed Publications:

1. **R. van der Sluis**, C.P.S. Badenhorst, F.H. van der Westhuizen, and A.A. van Dijk. 2013. Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase. *Gene (2013) 515: 447-453.* (Paper III)

2. C.P.S. Badenhorst, **R. van der Sluis**, E. Erasmus, and A.A. van Dijk. 2013. Glycine conjugation: Importance in metabolism, the role of glycine N-acyltransferase, and the factors that influence interindividual variation. *Expert Opinion on Drug Metabolism and Toxicology 9: 1139-1153.* (Paper I)

3. C.P.S. Badenhorst, E Erasmus, **R. van der Sluis**, C. Nortje, A.A. van Dijk. 2014. A new perspective on the importance of glycine conjugation in the metabolism of aromatic acids. *Drug Metabolism Reviews 46 (3): 343-361* (Paper II)

Submitted Manuscript:

4. **R. van der Sluis**, C.P.S. Badenhorst, E. Erasmus, E. van Dyk, F.H. van der Westhuizen, A.A. van Dijk. Conservation of the coding regions of the glycine N-acyltransferase gene and its implications for the detoxification of xenobiotics in humans. (Manuscript submitted to *Pharmacogenetics and Genomics*). (Paper IV).

Scientific Posters Presented at Conferences

2012

- Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase

  Van der Sluis, R., Badenhorst, C.P.S., Erasmus, E. and van Dijk, A.A.

  Presented at the 2012 joint South African Genetics and Bioinformatics Society Conference in Stellenbosch, South Africa.

2013

- Genetic polymorphisms of glycine N-acyltransferase in a South African cohort identified with Ion Torrent semiconductor sequencing

  Van der Sluis, R., van Dyk, E. and van Dijk, A.A.

  Presented at the 2013 Southern African Society for Human Genetics (SASHG) conference in Sandton, South Africa.

2014

- Characterisation and haplotype analyses of the non-synonymous polymorphisms in the glycine N-acyltransferase gene using worldwide populations

  Rencia van der Sluis, Christoffel P.S. Badenhorst, Elardus Erasmus, Etresia van Dyk, Francois H. van der Westhuizen and Alberdina A. van Dijk.

  Presented at the 2014 joint South African Genetics and Bioinformatics Society Conference in Rawesonville, South Africa