

**Tyrosinemia type I as a model for studying
epigenetic events in the aetiology of metabolic
disease associated hepatocarcinoma**

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**Thesis submitted for the degree Doctor of Philosophy in
Biochemistry at the Potchefstroom Campus of the
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Potchefstroom

2011

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Vir my ouers en Rupert

“But I call to God, and the Lord saves me.

*I will present my thank offering to you.
For you have delivered me...”*

Psalm 55:16 & 56:12

ABSTRACT

A number of inherited metabolic defects result in the initiation and progression of various cancers, although the aetiology of these cancers is poorly understood. One such inborn error of metabolism (IEM) is hereditary tyrosinemia type 1 (HT1), a clinically severe disease with characteristic development of hepatocellular carcinoma (HCC). HT1 results from a deficient fumarylacetoacetate hydrolase enzyme (FAH) in the tyrosine catabolic pathway, and damaging upstream tyrosine metabolites can accumulate. The mechanism underlying the pathophysiology of HT1 remains unclear, and some patients develop HCC in spite of treatment. HT1 was accordingly chosen as a model for the study of metabolic defect associated cancers.

Since HCC is localized in the liver, a limited number of models are available for its study. Although several HT1 models have been described in the literature, these models all lack some elements of a true human HT1 intracellular environment. Developing a model for HT1 in cultured cells was therefore deemed necessary to mimic the intracellular environment that is present in patients with this disease, also allowing the long-term evaluation of cellular changes. RNA interference technology (RNAi) is proposed as an effective method to establish such a human cell model, and a shRNA knock-down system was chosen to target the *FAH* gene. Several double-stable *FAH* knock-down cell lines were subsequently established and evaluated to assess their suitability for studies of this kind.

The aetiology of HCC in HT1 remains largely unknown, but it has been proposed that epigenetic factors may be involved in its pathophysiology, although it has not been studied thus far. Although the roles the accumulated metabolites play in the initiation of IEM-associated cancers remain vague, there is ample indication that an epigenetically regulated mechanism may be involved in HT1. Aberrant DNA methylation profiles can result in genomic instability and altered gene expression patterns, and these changes are often associated with cancer development. A semi-targeted analysis of the global DNA methylation status of HepG2 cells following direct exposure to the accumulating HT1 metabolites was therefore performed. Global DNA methylation was measured with the cytosine extension assay. This thesis reports that exposure of hepatic cells to succinylacetone (SA) and *p*-hydroxyphenylpyruvate (pHPPA) does not appear to change the levels of global DNA methylation in HepG2 cells, and the results suggest that this is not a directly affected factor in the development of HT1-associated liver disease. However, it cannot entirely be excluded that these mechanisms may be affected over a longer period of accumulating metabolite exposure. This study also identified long-term cyclic patterns in DNA methylation, and a shift in these patterns was observed as a result of exposure to the metabolites SA and pHPPA.

The accumulating HT1 metabolites have been proposed to be alkylating agents, and are thought to contribute to the frequent HCC development. O⁶-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme responsible for the alkyl adduct removal, has been shown to be involved in the development of HCC (not of HT1 origin). *MGMT* has frequently been found to have altered promoter methylation patterns and/or expression patterns in these cancers, and this compelled the investigation into the effect of accumulating HT1 metabolites on the promoter methylation and

expression of this enzyme. *MGMT* methylation and subsequent expression did not seem to be directly affected by the HT1 metabolites used in this study.

Although oxidative stress responses are known to be activated in HT1, the direct effect of the accumulating metabolites on the production of reactive oxygen species (ROS) and their subsequent damage on a protein level have not been investigated. ROS can interfere with normal DNA methylation mechanisms, thereby changing the DNA methylation patterns of cells. This potential interaction compelled the investigation as to whether the HT1 metabolites affect intracellular ROS production in any way. HepG2 cells were treated with both SA and pHPPA, and subsequently ROS levels were determined with a flow cytometric analysis. Protein carbonyl concentrations were also determined spectrophotometrically as a generic marker of oxidative protein damage. The results clearly indicated a marked increase in intracellular ROS production, which possibly led to oxidative protein damage. The results indicate that oxidative stress response activation in HT1 is probably a direct result of SA and pHPPA induced ROS production, and these metabolites may therefore play a more significant role in the development of HT1 associated liver disease than thought previously.

The research tested the hypothesis that DNA methylation alterations could play a role in HT1-associated HCC development, and the conclusion was that changes in the levels of DNA methylation is probably not a significant contributing factor. This study did, however, identify long-term cyclic patterns to be present in the DNA methylation, as well as a shift in these patterns as a result of the metabolites SA and pHPPA. Direct evidence of ROS production in HT1 is presented, and SA and pHPPA was identified to be, at least in part, responsible for the oxidative stress present in HT1 patients.

Keywords:

Hereditary tyrosinemia type 1; Hepatocellular carcinoma, DNA methylation; DNA alkylation; Reactive oxygen species; Oxidative stress.

SAMEVATTING

Die onderwerp van hierdie studie was “Tirosinemie tipe I as ’n model vir die bestudering van epigenetiese gebeure in die etiologie van hepatokarsinoom geassosieer met ’n metaboliese siekte”. ’n Aantal aangebore metaboliese defekte veroorsaak die ontstaan en ontwikkeling van verskillende kankers waarvan die etiologie nog onbekend is. Een van hierdie aangebore metaboliese defekte (IEM) is oorerflike tirosinemie tipe 1 (HT1), ’n klinies ernstige siekte met karakteristieke ontwikkeling van hepatosellulêre karsinoom (HCC). HT1 ontstaan a.g.v. ’n defektiewe fumarielasetoasetaat-hidrolase ensiem (FAH) in die tirosien kataboliese weg, en skadelike voorafgaande tirosien-metaboliete akkumuleer. Die onderliggende meganisme van die patofisiologie van HT1 bly onduidelik, en sommige pasiënte ontwikkel HCC ten spyte van behandeling. HT1 is gevolglik gekies as ’n model vir die studie van metaboliese defek geassosieerde kankers.

Aangesien HCC in die lewer geleë is, is slegs ’n beperkte aantal modelle beskikbaar vir die studie daarvan. Alhoewel ’n verskeidenheid HT1 modelle al beskryf is, kort al hierdie modelle sommige aspekte van die intrasellulêre omgewing van ware menslike HT1. Die ontwikkeling van ’n model vir HT1 in gekweekte selle was daarom nodig geag om die intrasellulêre omgewing wat in HT1 pasiënte teenwoordig is na te boots, asook om die langtermyn bestudering van sellulêre veranderinge moontlik te maak. RNS-tussenkoms tegnologie (RNAi) is voorgestel as ’n effektiewe metode om so ’n menslikesel model te vestig, en ’n shRNA klopsisteem is gekies om die *FAH*-geen te teiken. Verskeie dubbel-stabiele *FAH* klopsisteem sellyne is gevolglik gevestig en geëvalueer om hul geskiktheid vir studies van hierdie aard te beoordeel.

Die etiologie van HCC in HT1 is grootliks onbekend, maar dit is voorgestel dat epigenetiese faktore in die patofisiologie betrokke mag wees, maar is tot nog toe nie bestudeer nie. Alhoewel die rol wat die akkumulerende metaboliete speel in die inisiasie van IEM-geassosieerde kankers onduidelik bly, is daar aanduidings dat ’n epigeneties-gereguleerde meganisme in HT1 betrokke mag wees. Foutiewe DNS-metileringsprofiel kan genomiese onstabieliteit en veranderde geenuitdrukingspatrone veroorsaak, en hierdie veranderinge word dikwels met die ontwikkeling van kanker geassosieer. ’n Semi-gerigte analise van die globale DNS-metileringsstatus van HepG2-selle na die direkte blootstelling aan die akkumulerende HT1-metaboliete is gevolglik uitgevoer. Globale DNS-metileringsstatus is gemeet met die sitosien uitbreiding metode. In hierdie studie is waargeneem dat die blootstelling van hepatiese selle aan suksinielasetoon (SA) en *p*-hidroksiefenilpiruvaat (pHPPA) nie die vlakke van globale DNS-metileringsstatus in HepG2-selle beïnvloed nie, en dit kan uit die resultate afgelei word dat hierdie faktor nie betrokke is in die ontwikkeling van HT1-geassosieerde lewersiekte nie, alhoewel dit nie algeheel uitgesluit kan word dat hierdie meganismes oor ’n langer tydperk van akkumulerende metabolietblootstelling geaffekteer mag word nie. In hierdie studie is ook langtermyn sikliese patrone in DNS-metileringsstatus waargeneem, asook ’n verskuiwing in die patroon na blootstelling van die selle aan die metaboliete SA en pHPPA.

Die akkumulerende HT1-metaboliete word beskou as alkilerings agente, en dra vermoedelik by tot die HCC-ontwikkeling. O⁶-metielguanien-DNS metieltransferase (MGMT), ’n DNS-herstel ensiem

verantwoordelik vir die verwydering van alkiel-addukte, is betrokke in die ontwikkeling van HCC (nie van HT1 nie), omdat daar waargeneem is dat *MGMT* veranderde promotor metileringspatrone en/of uitdrukkingspatrone in hierdie kankers het. Dit was gerade geag om die effek van die akkumulerende HT1-metaboliete op die promotor en uitdrukking van hierdie ensiem te bestudeer. Uit hierdie studie blyk dit dat *MGMT* metilering en die gevolglike uitdrukking nie geaffekteer is deur die HT1 metaboliete nie.

Alhoewel dit bekend is dat 'n oksidatiewe stresrespons in HT1 geaktiveer word, is die direkte effek van die akkumulerende metaboliete op die vorming van reaktiewe suurstof spesies (ROS) en hulle gevolglike effek op proteïenvlak, nog nie in HT1 bestudeer nie. ROS kan met normale DNS-metilering meganismes inmeng, en daardeur die DNS-metileringpatrone van selle verander. Hierdie potensiële interaksie het die ondersoek na die effek van die HT1 metaboliete of die intrasellulêre ROS-produksie genoodsaak. HepG2-selle is met beide SA en pHPPA behandel, waarna die ROS-vlakke met vloeisitometrie bepaal is. Proteïen karbonielkonsentrasies is ook spektrofotometries bepaal as 'n generiese merker vir oksidatiewe proteïenskade. Die resultate het duidelik getoon dat daar 'n merkbare toename in die intrasellulêre ROS-produksie was, wat moontlik tot die oksidatiewe skade van proteïene gelei het. Die resultate toon aan dat die aktivering van oksidatiewe stres respons in HT1 waarskynlik 'n direkte gevolg is van SA- en pHPPA-geïnduseerde ROS-produksie, en dat hierdie metaboliete gevolglik 'n baie belangriker rol mag speel in die ontwikkeling van HT1-geassosieerde lewersiekte as wat vroeër gedink is.

Die navorsing het die hipotese dat DNS-metileringsveranderinge 'n rol mag speel in HT1-geassosieerde HCC ontwikkeling, getoets. Die gevolgtrekking waartoe gekom is, is dat veranderinge in die vlakke van DNS-metilering waarskynlik nie 'n baie belangrike bydraende faktor is nie. Die studie het wel langtermyn sikliese veranderinge in DNS metilering geïdentifiseer, sowel as 'n skuif in hierdie patrone a.g.v. die metaboliete SA en pHPPA. Direkte bewyse van ROS-produksie in HT1 is verkry, en SA en pHPPA is aangetoon om, ten minste gedeeltelik, verantwoordelik te wees vir die oksidatiewe stres wat in HT1-pasiënte gesien is.

Sleutelwoorde:

Oorerflike tirosinemie tipe 1; Hepatosellulêre karsinoom, DNS-metilering; DNS-alkilering; Reaktiewe suurstof spesies; Oksidatiewe stres.

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LIST OF ABBREVIATIONS

I. Abbreviations

[³ H]dCTP:	[³ H]deoxycytidine triphosphate
8-OHdG:	8-hydroxy-2-deoxyguanosine
18S rRNA:	Ribosomal protein 18S gene
A:	Adenine
ACTB:	Human β-actin gene
AGT:	O ⁶ -alkylguanine-DNA methyltransferase enzyme
Amp ^r :	Ampicillin resistant gene
BER:	Base excision repair
BSA:	Bovine serum albumin
C:	Cytosine
cDNA:	Reverse transcribed DNA
CEA:	Cytosine extension assay
CO ₂ :	Carbon dioxide
CpG:	CG dinucleotide
DCF:	2',7'-dichlorofluorescein
DCFH-DA:	2',7'-dichlorodihydrofluorescein diacetate
ddH ₂ O:	Double distilled water
DMEM:	Dulbecco's Modified Eagles Medium
DNA:	Deoxyribonucleic acid
DNMT/DMT:	DNA methyltransferase
DNPH:	2,4-dinitrophenylhydrazine
dox:	Doxycycline
dpm:	Disintegrations per minute count
DSB:	Double strand break
dsRNA:	Double-stranded RNA
<i>E. coli</i> :	Escherichia coli
EDTA:	Ethylenediaminetetra acetic acid
EGTA:	Ethylene glycol tetraacetic acid
ER:	Endoplasmic reticulum
ERK:	Extracellular signal-regulated protein kinase
<i>et al</i> :	Latin: and others
FAA:	Fumarylacetoacetate
<i>fah</i> :	Bacterial fumarylacetoacetate hydrolase gene

<i>Fah</i> :	Mouse fumarylacetoacetate hydrolase gene
<i>FAH</i> :	Human fumarylacetoacetate hydrolase gene
FAH:	Fumarylacetoacetate hydrolase enzyme
FBS:	Foetal bovine serum
FL:	Fluorescence channel
FRW:	Forward primer
FSC:	Forward scatter channel
<i>g</i> :	Gravitational force
g:	Gram
G:	Guanine
G418:	Neomycin
<i>GAPDH</i> :	Human glyceraldehyde-3-phosphate dehydrogenase gene
GSH:	Cellular glutathione
H ₂ O ₂ :	Hydrogen peroxide
HCC:	Hepatocellular carcinoma
HCl:	Hydrochloric acid
HGA:	Homogentisic acid
<i>Hpd</i> :	Mouse 4-hydroxyphenylpyruvate dioxygenase
HT1:	Hereditary tyrosinemia type 1
IEM:	Inborn error of metabolism
kb:	Kilo base pairs
kDa:	Kilo Dalton
L or l:	Litre
M:	Molar
MAA:	Maleylacetoacetate
MAPK:	Mitogen-activated protein kinase
MBD:	Methyl-binding Domain
MFI:	Mean fluorescence intensity
mg:	Milligram
MgCl ₂ :	Magnesium chloride
<i>MGMT</i> :	Human O ⁶ -methylguanine-DNA methyltransferase gene
MGMT:	O ⁶ -methylguanine-DNA methyltransferase enzyme
miRNA:	Micro RNA
ml:	Millilitre
mM:	Millimolar
MMR:	Mismatch repair
Mr:	Molecular weight
mRNA:	Messenger RNA
MSP:	Methylation-specific PCR

n.a.:	Not available
Na ₂ HPO ₄ :	di-Sodium hydrogen orthophosphate
NaCl:	Sodium chloride
NaOH:	Sodium hydroxide
NER:	Nucleotide excision repair
ng:	Nanogram
nm:	Nanometres
nmol:	Nanomole
NTBC:	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione
O ⁶ alkylG:	O ⁶ -alkylguanine
O ⁶ meG:	O ⁶ -methylguanine
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PEI:	Apyrogenic polyethylenimine
pen:	Penicillin
pH:	Potential of hydrogen
pHPPA:	<i>p</i> -hydroxyphenylpyruvate
Prxs:	Peroxiredoxin
qMSP:	Real-time methylation-specific PCR
qPCR:	Quantitative real-time PCR
RISC:	RNA-induced silencing complex
REV:	Reverse primer
RNA:	Ribonucleic acid
RNAi:	RNA interference
ROS:	Reactive oxygen species
rRNA:	Ribosomal RNA
rtTA:	Reverse tetracycline controlled transactivator
SA:	Succinylacetone
SAA:	Succinylacetoacetate
SAM:	S-adenosylmethionine
SCE:	Sister chromatid exchange
shRNA:	Small hairpin RNA
siRNA:	Small interfering RNA
SSC:	Side scatter channel
std. dev.:	Standard deviation
strep:	Streptomycin
T:	Thymine
TA:	Transcriptional activating domain
TCA:	Trichloroacetic acid
tet:	Tetracycline

<i>tet</i> O:	<i>Tet</i> operator
<i>tet</i> R:	Tetracycline repressor
TRE:	Tet-Regulatory Element
Tris-Cl:	2-Amino-2-(hydroxymethyl)-1,3-propandiol-hydrochloride
Trx:	Thioredoxins
tTA:	Transcription factor
tTS:	Tetracycline-controlled transcriptional suppressor
u:	Units
U:	Uracil
UV:	Ultra violet
V:	Volt
v/v:	Volume per volume (ml per 100 ml)
w/v:	Weight per volume (g per 100 ml)

II. Symbols

α	Alpha
β	Beta
Δ	Delta
$\Delta\Delta C_t$	Comparative cycle threshold method
κ	Kappa
%	Percentage
°C	Degrees Celsius
μ g	Microgram
μ l	Microlitre
μ M	Micromolar
#	Catalogue number
[]	Concentration
-	Minus
+	Plus
=	Equals
/	Division

CHAPTER 1

INTRODUCTION

1.1. Introduction

Inherited metabolic defects are abnormalities of specific catabolic enzymes, and have been studied extensively for many years. The severity of these abnormalities differs greatly, but in a number of them the resulting pathology includes the initiation and progression of various cancers. The aetiology of these associated cancers, however, remains poorly understood.

One such inborn error of metabolism (IEM) is hereditary tyrosinemia type 1 (HT1), a very well-described, clinically severe disease with characteristic development of hepatocellular carcinoma (HCC). In fact, it is the metabolic defect with the highest incidence of primary liver cancer (Russo *et al.*, 2001). HT1 results from a deficient fumarylacetoacetate hydrolase enzyme (FAH) in the tyrosine catabolic pathway, and upstream metabolites then accumulate (Scott, 2006). The mechanism underlying the pathophysiology of HT1 remains unclear, and although treatment is available, some patients still develop HCC (Mitchell *et al.*, 2001). HT1 is therefore an excellent model for the study of metabolic defect associated cancers.

This study explores the effect of HT1 and its ensuing metabolite accumulation on underlying cellular changes, specifically on an epigenetic level. Literature relevant to the subjects of initiation and development of HCC, epigenetic involvement and HT1-associated cellular changes is discussed in an effort to compile a hypothesis on the involvement of each factor in the eventual pathophysiology of the disease. The hypothesis is then tested experimentally, and the results are considered in the context of the literature. This study also describes the need for a new HT1 model, enabling long-term evaluation of cellular changes in a human HT1 intracellular environment. RNA interference (RNAi) is proposed as an effective method to establish such a human cell model, and several double-stable *FAH* knock-down cell lines are subsequently established and evaluated to assess their suitability for this study.

The outcome of this thesis can be of significance in several research fields, including epigenetics, inborn errors of metabolism, and molecular biology in general, and can be influential to different aspects of patient care. It will lead to further research and will provide a knowledge base in the fields of IEMs, their associated pathophysiology and epigenetic alterations in these diseases.

This chapter will give a brief overview of different models available for the study of IEM-associated liver pathologies and the significance of epigenetic alterations with regard to carcinogenesis. A brief problem statement, aim and objectives are then provided, followed by the research outline and thesis structure. The chapter ends with the publication status of the research and collaborations which contributed to the study.

1.1.1. Models for liver-specific studies

In order to study the aetiology of IEM-associated cancers, it is necessary to study the genetic and epigenetic alterations which occur during the development of these pathologies. A limited number of models are available for the study of HCC, since it is localized in the liver. Liver biopsies of diagnosed patients are rarely available due to the trauma associated with the procedure and the fragile state of the patients. These primary hepatocytes also divide poorly and rarely survive (Vogel *et al.*, 2004). Several knockout mouse-models have been described in the literature, where the *Fah* gene is disrupted. These mice are subject to neonatal lethality, unless treated with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) to prevent the full onset of the IEM, and once treatment is removed the pathology progresses. These mouse-models were not available for this study, and would not necessarily reflect the events in human patients since disease progression is much faster than in humans (Mitchell *et al.*, 2001). The “rescue” of the mice with NTBC treatment is also incomplete, since HCC and kidney damage are still observed.

A third model entails the imitation of the events in the liver of the affected patients by directly exposing hepatocytes to the accumulating metabolites present in the IEM, and subsequently studying the effects. This is an ideal short-term model, suitable to determine the immediate effects of various concentrations of the accumulating metabolites. It does, however, not allow for long-term studies into these effects, and since the hepatocytes of patients are exposed to continuous levels of these metabolites, the model only provides limited answers. Nevertheless, it is a good initial model for such studies.

The fourth option entails establishing a human cell model of HT1. This is possible through the knock-down of the *FAH* gene in an established cell line. This is the preferred model, since it allows long-term, controlled analysis of the effects of the accumulating metabolites with a base line of no previous exposure. A more detailed description of current HT1 models is given in Section 3.2.

1.1.2. Epigenetic alterations

Epigenetic alterations are heritable modifications in gene expression and chromatin organisation with no alterations in the primary DNA sequence (Das and Singal, 2004, Esteller, 2008, Sawan *et al.*, 2008). These epigenetic traits can possibly explain the phenotypic and disease susceptibility variations within a population, which cannot be explained by genetics alone (Esteller, 2008). Poudrier and colleagues suggested that epigenetic and other factors could modify the phenotype in HT1 (Poudrier *et al.*, 1998), and although the role the accumulated metabolites play in the initiation of IEM-associated cancers remain vague, there is ample indication that an epigenetically regulated mechanism may be involved in HT1 (Mitchell *et al.*, 2001).

DNA methylation is one of the most common epigenetic events in the human genome, and entails the covalent addition of a methyl group to the 5-carbon of a cytosine pyrimidine ring in a CpG dinucleotide in mammals (Das and Singal, 2004). Alterations of normal DNA methylation patterns are frequently associated with malignancies and carcinogenesis (Watson *et al.*, 2003, Castanotto *et al.*, 2005). It has also been suggested that aberrant epigenetic gene expression regulation plays an equally important role in disease development as mutations, deletions, and

other gene expression disregulations (Ho and Tang, 2007). Epigenetic mechanisms, alterations and their significance are discussed in more detail in Section 2.4.

1.2. Problem statement

The aetiology of HCC development in HT1 remains poorly understood. Although epigenetic involvement in the pathophysiology of HT1 has been suggested, it has not been investigated. The possible role of such epigenetic aberrations in the associated HCC development is, therefore, still unclear. Since no reports on the involvement of epigenetic events in HT1 could be found in the literature, it therefore needs to be explored and developed. Research pertaining to the genetic basis of HT1 was the focus of a parallel study and was not included in this study (see Section 1.6).

To date, most research pertaining to HT1 was performed using non-human models. In the studies where human cell cultures were used, these cells did not contain the enzyme defect in its genome, and the cells were exposed to estimated concentrations of metabolites which accumulate in HT1. This approach, however, does not enable long-term exposure and lacks the complete intracellular HT1 environment. Therefore, it was necessary to establish a HT1 model in cultured human hepatic cells which would enable long-term, controlled evaluation of cellular changes during the development of the associated pathophysiology.

1.3. Research aims and objectives

The main objective of this study is to investigate the role of epigenetic alterations in the aetiology of hereditary tyrosinemia type 1 associated hepatocarcinoma, as a model for inborn error of metabolism associated cancers.

The study has two main approaches. Firstly, a human cell culture model of HT1 will be developed using RNAi technology, enabling long-term study of epigenetic alterations and their consequences. Secondly, cultured hepatocytes will be exposed directly to metabolites which accumulate in HT1 to establish the various assays to be performed with the cell culture model, and to determine their usefulness for such studies.

1.4. Study outline

This section provides a brief outline of the study. The project has three experimental parts, illustrated in Figure 1.1. These parts consist of:

- Establishing a knock-down model of HT1 in cultured human hepatic cells (HepG2).
- Mimicking HT1 conditions in HepG2 cells through direct exposure to accumulating metabolites.
- Examining various epigenetic parameters in each of these models, and evaluating the possible effect of the process to create the model on each parameter measured.

It is necessary to perform all analyses at each step in the creation of the knock-down model to ensure that each individual alteration of the cells will not influence the eventual results.

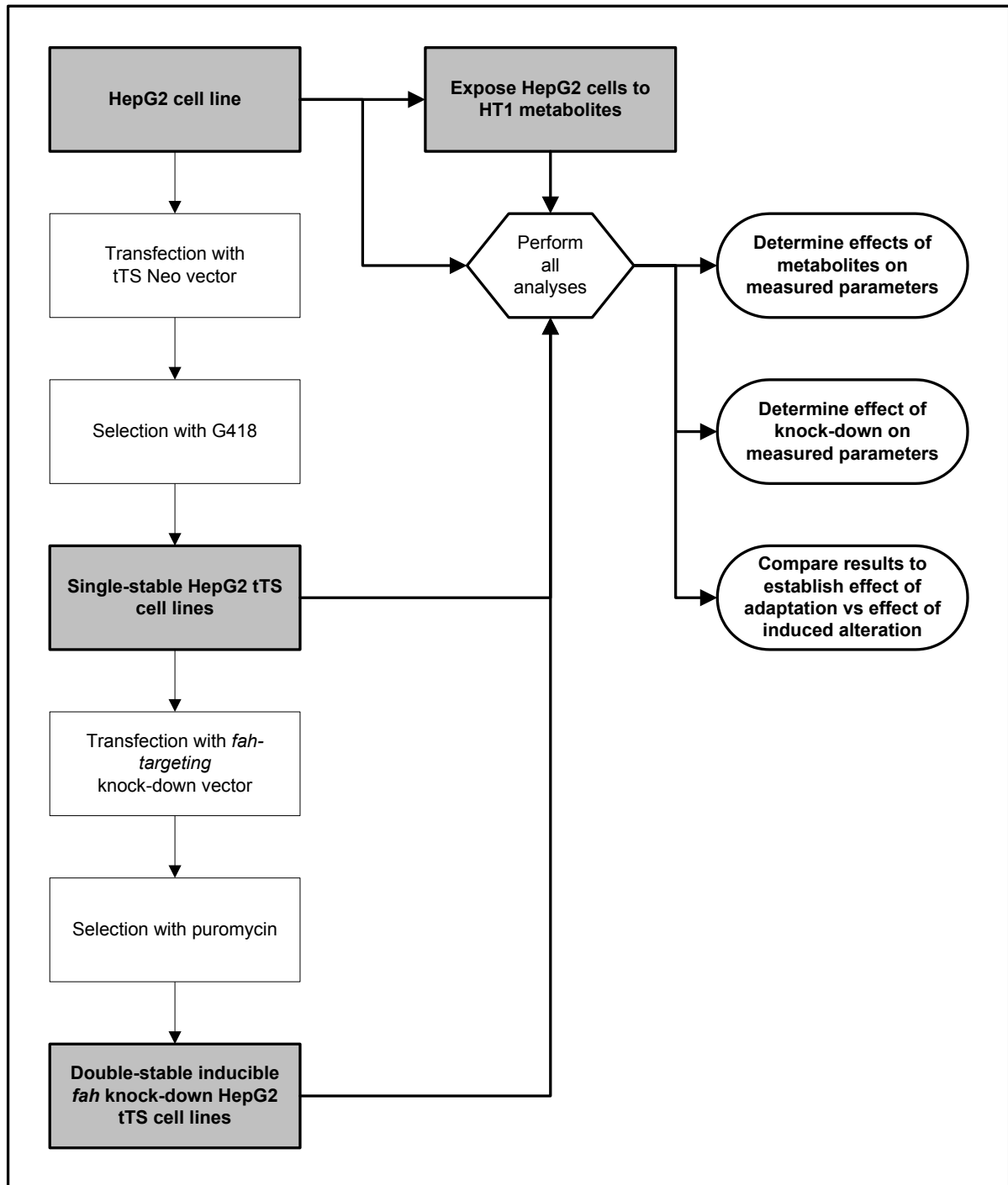


Figure 1.1. A diagram depicting the basic layout of the study. The three main experimental parts consisting of establishing a knock-down model of HT1 in cultured cells, mimicking HT1 conditions in HepG2 cells through exposure to accumulated metabolites, and examining various epigenetic parameters in each of these models are shown. The grey blocks indicate all points at which the epigenetic parameters will be measured, including points in the development of the model.

The basic outline of the thesis consists of the following: in Chapter 2 a brief literature overview of all related subjects will be given, including a discussion of HT1, HCC, epigenetic mechanisms, DNA methylation and DNA alkylation in carcinogenesis and oxidative stress. This chapter ends with a hypothesis on the involvement of all these factors in the development of HCC in HT1.

Chapter 3 outlines the process to establish the knock-down model for HT1. The various known HT1 models are described, and their advantages and shortcomings are highlighted. The principles of RNAi are presented, followed by a discussion of the RNAi system chosen for this study and its implementation. This chapter ends with the evaluation of the established cell lines.

Chapter 4 is dedicated to the design of the various epigenetic analyses. A brief background or basis of each method is given, followed by a concise description of the techniques used. Where necessary, preliminary results obtained during establishing of the assays will be provided.

Chapter 5 provides all the results obtained following the direct exposure of HepG2 cells to the HT1 metabolites, as well as the discussion of these results and how they relate to each other.

Final conclusions and future prospects are presented in Chapter 6. The significance of the results in light of current knowledge is evaluated, and aspects for further research are discussed.

In the appendices, technical information and data sets from the study are provided. Appendices A, B and C contain vector maps of all plasmids used in this study, while sequencing results of the *FAH*-targeting shRNA_pSIREN constructs are shown in Appendix D. All primer sequences and information are presented in Appendix E. In Appendix F, all original data sets obtained with the various assays are shown, with all buffer and solution compositions presented in Appendix G. In Appendix H, the reagent suppliers and catalogue numbers are listed.

1.5. Publication status of the research

Several manuscripts conveying the results and significance of this study were prepared and submitted to peer-reviewed journals for publication. These manuscripts were included in the thesis where appropriate. The publication status of these articles together with the abstracts is provided:

- Gouws, C. and Pretorius, P.J. 2011. **O⁶-methylguanine-DNA methyltransferase (MGMT): can function explain a suicidal mechanism?** *Medical Hypotheses*, 77:857-860.

Abstract:

Why does O⁶-methylguanine-DNA methyltransferase (MGMT), an indispensable DNA repair enzyme, have a mechanism which seems to run counter to its importance? This enzyme is key to the removal of detrimental alkyl adducts from guanine bases. Although the mechanism is well known, an unusual feature surrounds its mode of action, which is its so-called suicidal endpoint. In addition, induction of MGMT is highly variable and its kinetics is atypical. These features raise some questions on the seemingly paradoxical mechanism. In this manuscript we point out that, although there is ample literature regarding the “how” of the MGMT enzyme, we found a lack of information on “why” this specific mechanism is in place. We then ask whether we know all there is to know about MGMT, or if perhaps there is a further as yet unknown function for MGMT, or if the suicidal mechanism may play some kind of protective role in the cell.

This article is included in Chapter 2 as part of the literature study.

- Gouws, C. and Pretorius, P.J. 2011. **O⁶-methylguanine-DNA methyltransferase in the aetiology of hereditary tyrosinemia type 1 associated hepatocellular carcinoma.** *Journal of Inherited Metabolic Disease*. Status: manuscript to be submitted.

Abstract:

In the metabolic disorder hereditary tyrosinemia type 1 (HT1) there is an accumulation of damaging tyrosine metabolites, such as succinylacetone (SA) and p-hydroxyphenylpyruvate (pHPPA). Hepatocellular carcinoma (HCC) is characteristic of the disease, although its molecular mechanism remains largely unknown. The HT1 metabolites have been proposed to be alkylating agents, which can lead to a plethora of adverse events and are thought to contribute to the HCC development. O⁶-methylguanine-DNA methyltransferase (MGMT), a DNA alkylation repair enzyme, has been found to have altered promoter methylation patterns and/or expression patterns in non-HT1 HCC. Defective MGMT alkylation repair is thought to be a fundamental event in multistep HCC, but this enzyme has not been studied in HT1-associated HCC. In this study, we investigated the effect of the accumulating HT1 metabolites SA and pHPPA on the promoter methylation and mRNA expression of the MGMT gene to elucidate its role in the associated liver pathophysiology. Although the loss of MGMT expression is usually attributed to MGMT promoter CpG island hypermethylation, the results obtained in this study suggest that other factors control MGMT expression in HepG2 cells, since no correlation could be observed. The results also indicated no significant change in either the promoter methylation or the expression of MGMT as a result of treatment with the accumulating metabolites. It is suggested MGMT should be investigated on a protein level to identify possible depletion or protein damage, in which case the ensuing alkylation damage accumulation could play a role in the associated liver pathophysiology.

This manuscript is included in Chapter 5 as part of the evaluation of the results.

- Gouws, C., du Plessis, L.^a, and Pretorius, P.J. 2011. **Accumulating tyrosinemia type 1 metabolites induce ROS production and protein oxidation.** *Journal of Inherited Metabolic Disease*. Status: manuscript to be submitted.

Abstract:

Damaging tyrosine metabolites accumulate in the metabolic disorder hereditary tyrosinemia type 1 (HT1), which is caused by a defective fumarylacetoacetate hydrolase enzyme. Characteristic of the untreated chronic form of this disease is the development of hepatocellular carcinoma, but its molecular mechanism remains largely unknown. Although oxidative stress responses are known to be activated in HT1, the direct effect of the accumulating metabolites succinylacetone (SA) and para-hydroxyphenylpyruvate (pHPPA) on the production of radical oxygen species (ROS) and their subsequent damage on a protein level have not been investigated. In this study, HepG2 cells were treated with both SA and pHPPA, and subsequently ROS levels were determined with a flow cytometric analysis. Protein carbonyl concentrations were also determined spectrophotometrically as a generic marker of oxidative protein damage. Our results clearly indicate that SA and pHPPA markedly increased intracellular ROS production, which possibly led to oxidative protein damage.

^a Flow cytometric analyses were performed with the help of Dr. L. du Plessis (Unit for Drug Research and Development, North-West University).

Our results indicate that oxidative stress response activation in HT1 is probably a direct result of SA and pHPPA induced ROS production, and these metabolites may therefore play a more significant role in the development of HT1-associated liver disease than thought previously.

This manuscript is included in Chapter 5 as part of the evaluation of the results.

1.6. Collaborations

The knock-down model for HT1, as described in Chapter 3, was developed in a collaborative study between two PhD studies^b, as part of a larger study. However, both researchers contributed equally to the development and evaluation of the model, and each presented the process and results independent from the other. It was envisaged that this model, once properly established, would be used by various investigators to study different aspects of the aetiology of HT1.

The researcher also contributed intellectually to the conception and execution of the study from which the following paper (Wentzel *et al.*, 2010) and dissertation (Wentzel, 2009) emanated. This contribution included the standardization of the cytosine extension assay used in the study.

- Wentzel, J. F., Gouws, C., Huysamen, C., Van Dyk, E., Koekemoer, G. and Pretorius, P. J. 2010. **Assessing the DNA methylation status of single cells with the comet assay.** *Analytical Biochemistry*, 400:190-194.

Abstract:

The comet assay (single cell gel electrophoresis) is a cost-effective, sensitive, and simple technique that is traditionally used for analyzing and quantifying DNA damage in individual cells. The aim of this study was to determine whether the comet assay could be modified to detect changes in the levels of DNA methylation in single cells. We used the difference in methylation sensitivity of the isoschizomeric restriction endonucleases HpaII and MspI to demonstrate the feasibility of the comet assay to measure the global DNA methylation level of individual cells. The results were verified with the well-established cytosine extension assay. We were able to show variations in DNA methylation after treatment of cultured cells with 5-azacytidine and succinylacetone, an accumulating metabolite in human tyrosinemia type I.

^b The parallel study: VAN DYK, E. (2011) The molecular basis of the genetic mosaicism in hereditary tyrosinemia (HT1). *Centre for Human Metabonomics*. Potchefstroom, North-West University.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Inherited metabolic defects have been studied extensively for many years, and although the incidence of each specific defect is relatively small, they are quite abundant as a group (Saudubray *et al.*, 2006). In a typical inborn error of metabolism (IEM) there is an enzyme or protein with aberrant function or complete absence (Pollitt, 2008). These defects can often be linked to a specific genetic defect (Pollitt, 2008). The abnormalities range from having very severe to completely normal phenotypes, and the life expectancy of these individuals differ greatly (Pollitt, 2008). In numerous metabolic defects the resulting pathology includes the initiation and progression of various cancers, but the aetiology of these associated cancers remains unclear in most cases (Mitchell *et al.*, 2001). In recent years, epigenetic alterations of DNA have received increasing attention, specifically with regard to their association with cancer pathogenesis. The role of these epigenetic alterations in IEM-associated cancers, however, remains to be defined.

2.2. Inborn errors of metabolism

2.2.1. Introduction

Inherited abnormalities of specific enzymes involved in the catabolic pathways of amino acids, carbohydrates or lipids, often result in metabolic blockages with proximal toxic compounds accumulating in tissues (Wajner *et al.*, 2004, Saudubray *et al.*, 2006). IEMs may or may not be associated with liver manifestations, and if the liver is involved various histopathologic changes may be present (Arroyo and Crawford, 2006). Hepatitis, cirrhosis and hepatocellular carcinoma (HCC) have been associated with a number of germline mutation-caused IEMs, e.g. neonatal hemochromatosis, alpha-1-antitrypsin deficiency and tyrosinemia (Cha and DeMatteo, 2005, Arroyo and Crawford, 2006).

One of these inherited metabolic diseases, namely hereditary tyrosinemia type 1, involves the catabolic pathway of the amino acid tyrosine, as shown in Figure 2.1. This metabolic defect is frequently associated with the development of HCC and is, therefore, an excellent model for the study of metabolic defect associated cancers.

2.2.2. Hereditary tyrosinemia type 1

Hereditary tyrosinemia type 1 (HT1; OMIM 276700), also known as hepatorenal tyrosinemia, is the most clinically severe defect in the tyrosine catabolic pathway (see Figure 2.1) (Tanguay *et al.*, 1996, Russo *et al.*, 2001). It is an autosomal recessive inherited genetic defect of fumarylacetoacetate hydrolase (FAH; E.C.3.7.1.2), the last enzyme in the pathway (Lindblad *et al.*, 1977, Mitchell *et al.*, 2001). This defect is also one of the best described IEMs in the tyrosine

pathway (Russo *et al.*, 2001). The *FAH* gene is located on chromosome 15q23-q25 and spans 30 to 35 kb (Mitchell *et al.*, 2001, Scott, 2006). It forms fumarate and acetoacetate through hydrolysis, without the use of any known cofactors (Mitchell *et al.*, 2001, Scott, 2006). FAH is a 419 amino acid, 43 kDa soluble homodimer enzyme and has a basal level of expression in most tissues, but it is mainly expressed in the liver and kidneys (Tanguay *et al.*, 1996, St-Louis and Tanguay, 1997, Poudrier *et al.*, 1998, Russo *et al.*, 2001). Kvittingen and colleagues have observed residual FAH activity in patients of less than 2%, but a complete loss of FAH activity is thought to be fatal (Kvittingen *et al.*, 1993, Fernández-Cañón and Peñalva, 1995).

Although HT1 has a worldwide incidence of 1:100 000 to 1:200 000, there are regions with higher frequencies, including Scandinavia and Eastern Quebec, where as a result of a founder effect HT1 has an incidence of 1:16 000 and a carrier rate of 1:20 to 1:25 (Russo *et al.*, 2001, Langlois *et al.*, 2006, Scott, 2006). It was also in these patients where the first causal mutation was observed, namely the N16I missense mutation (Phaneuf *et al.*, 1992). Currently, there are 51 known *FAH* mutations, and since the genetic basis of this disease has been well-described, it will not be discussed in detail (See (St-Louis and Tanguay, 1997, Mitchell *et al.*, 2001, Cassiman *et al.*, 2009, Park *et al.*, 2009) and the Human Genome Mutation Database). An important observation in HT1 is the apparent lack of genotype-phenotype correlations, since similar genotypes do not result in the same phenotypes (Phaneuf *et al.*, 1992, Mitchell *et al.*, 2001, Russo *et al.*, 2001). HT1 is also known to have considerable molecular and biochemical heterogeneity (Phaneuf *et al.*, 1992). HT1 pathology is also characteristic, but not diagnostic (Nakamura *et al.*, 2007).

Two clinical phenotypic extremes have been described for HT1, the first of which is the acute form which has an early onset, shows rapid and severe liver and renal failure and is life threatening before 6-12 months (Tanguay *et al.*, 1996, Poudrier *et al.*, 1998, Langlois *et al.*, 2006, Scott, 2006). The chronic form has a slower onset and a less aggressive progress, but progressive liver dysfunction with nodular cirrhosis is characteristic of the disease, and almost 40% of patients will develop HCC (Poudrier *et al.*, 1998, Cha and DeMatteo, 2005). This classification has become outdated, however, due to increased genetic screening and early commencing of treatment (Russo *et al.*, 2001). Tissues affected, other than the liver, include the kidneys and peripheral nerves with painful neurologic crisis and rickets, but the phenotype and symptom severity are highly variable (Kvittingen *et al.*, 1993, Holme and Lindstedt, 1998, Mitchell *et al.*, 2001, Russo *et al.*, 2001, Scott, 2006).

Treatment of HT1 consists primarily of NTBC therapy (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione) (Russo *et al.*, 2001). This treatment diminishes or abolishes hepatic or neurologic decompensation within hours, although availability of the drug remains to be an issue (Mitchell *et al.*, 2001). This compound was developed as a herbicide, but was found to inhibit *p*-hydroxyphenylpyruvic acid dioxygenase (pHPPD; E.C.1.13.11.7), the second step in the tyrosine catabolic pathway (Mitchell *et al.*, 2001, Russo *et al.*, 2001). This inhibition results in decreased accumulation of the toxic metabolites discussed below. Although some researchers suggested that early treatment with NTBC can prevent HCC (Holme and Lindstedt, 1998), HCC has been observed as early as 15 months in spite of treatment since 5 months with NTBC (Mitchell *et al.*, 2001). Animal models also presented with HCC in spite of NTBC treatment, and it was proposed that NTBC does not block pHPPD completely (Grompe *et al.*, 1998, Al-Dhalimy *et al.*, 2002, Nakamura *et al.*, 2007). Other treatments include dietary restriction of tyrosine and phenylalanine

and liver transplantation, although both have complications (Langlois *et al.*, 2006, Scott, 2006). Langlois and associates showed a combination of diet restriction and NTBC treatment to be optimal for HT1 phenotype prevention, since NTBC treatment can result in elevated blood tyrosine levels if the diet is not restricted (Grompe *et al.*, 1998, Langlois *et al.*, 2006).

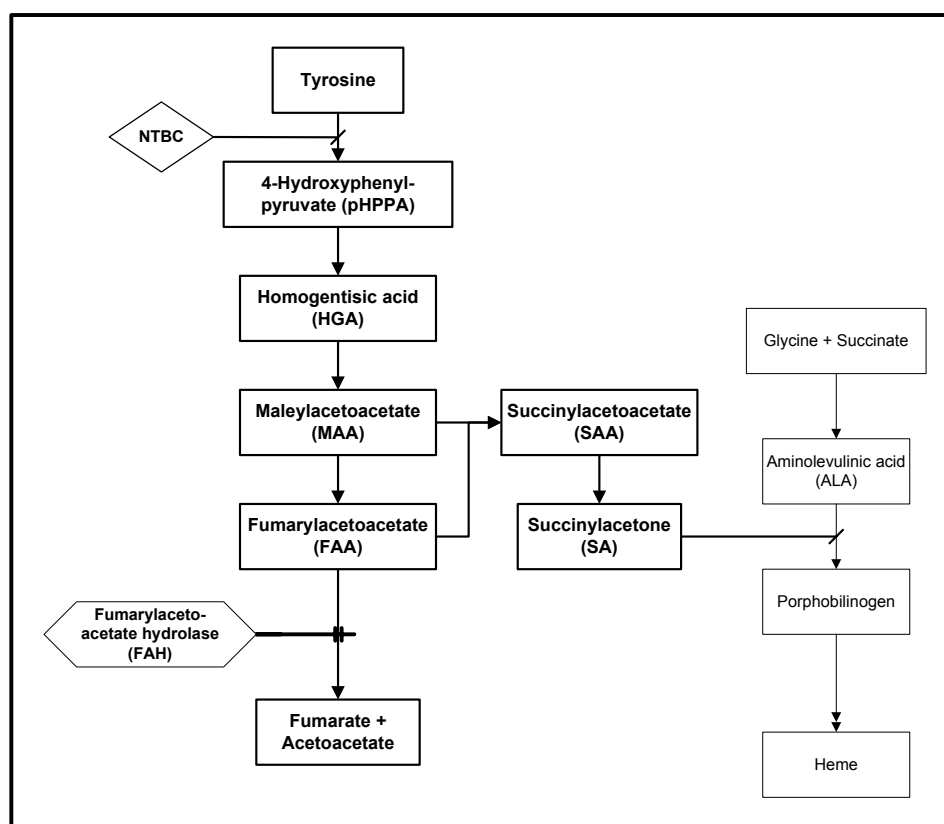


Figure 2.1. The Tyrosine catabolic pathway and its interrelationship with the heme metabolism. Deficient FAH inhibits the conversion of FAA to fumarate and acetoacetate, resulting in the accumulation of MAA and FAA. These are spontaneously converted to SA which inhibits aminolevulinic acid dehydratase. This results in the accumulation of ALA. (Adapted from (Mitchell *et al.*, 2001))

The aetiology of the frequent HCC development in HT1 is not clear yet, although the preferred hypothesis is that the accumulating metabolites are toxic and mutagenic, and possibly act as alkylating agents and/or disrupt sulfhydryl metabolism (Jorquera and Tanguay, 2001, Mitchell *et al.*, 2001, Russo *et al.*, 2001). As shown in Figure 2.1, maleylacetoacetate (MAA) and fumarylacetoacetate (FAA) are the compounds directly upstream from the FAH-mediated reaction and their derivatives, succinylacetoacetate (SAA) and succinylacetone (SA), are the result of the spontaneous conversion of FAA and MAA (Kvittingen *et al.*, 1993, Fernández-Cañón and Peñalva, 1995, Mitchell *et al.*, 2001). *p*-hydroxyphenylpyruvate, *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylacetic acid can also accumulate, although in much lower quantities (Mitchell *et al.*, 2001, Nakamura *et al.*, 2007).

FAA, and possibly MAA, is highly mutagenic and both have been proposed to be potent alkylating agents, since they have α,β -unsaturated carbonyl compound structures (Jorquera and Tanguay, 1997, Jorquera and Tanguay, 1999, Mitchell *et al.*, 2001). Both can also react with cellular glutathione (GSH) and protein sulfhydryl groups, possibly resulting in oxidative damage which apparently potentiates its mutagenicity (Mitchell *et al.*, 2001, Vogel *et al.*, 2004, Langlois *et al.*, 2006). They have been shown to be toxic to the cells, and this toxicity has been proposed to

induce necrosis and increased hepatocyte regeneration which can also be limited by GSH (Kvittingen *et al.*, 1994, Tanguay *et al.*, 1996, Langlois *et al.*, 2006). However, the GSH concentration measured in one tyrosinemic liver was reduced to about half of normal levels, suggesting its depletion in HT1 cells (Mitchell *et al.*, 2001).

FAA is thought to be the main effector in HT1 pathophysiology, and it has been shown to induce cell cycle arrest and apoptosis (Jorquera and Tanguay, 1999, Langlois *et al.*, 2006). Kubo and associates showed that FAA resulted in the release of cytochrome c from mitochondria, which could be the source of the apoptosis and eventually may lead to DNA damage (Kubo *et al.*, 1998, Nakamura *et al.*, 2007). In addition, FAA has been shown to cause genetic instability, activate the Ras/ERK pathway and disrupt Golgi apparatus. Bergeron and colleagues also showed that it launches an endoplasmic reticulum stress response in cells (Jorquera and Tanguay, 2001, Bergeron *et al.*, 2006). Jorquera and Tanguay indicated that FAA depleted GSH in cells, and that its cytotoxicity was dose- and GSH-dependent, but it did not induce ROS generation (Jorquera and Tanguay, 1997, Jorquera and Tanguay, 1999). It was also proposed that FAA could possibly impair DNA repair and cause mutations through alkylation damage of DNA (Jorquera and Tanguay, 1997, Jorquera and Tanguay, 1999).

SA is the diagnostic compound for HT1 in urine, since it is not detected in healthy subjects or in patients with other phenylalanine/tyrosine metabolism diseases (Fernández-Cañón and Peñalva, 1995, Kvittingen, 1995). SA has been shown to react nonenzymatically with proteins and free amino acids, especially with lysine, and through Schiff base formation results in adducts to these amino acids (Manabe *et al.*, 1985, Prieto-Alamo and Laval, 1998). SA can affect cell growth, immune function and renal tubular transport, and can cause acute porphyria-like neurological crisis by inhibiting aminolevulinic acid dehydratase (see Figure 2.1) (Kvittingen, 1995, Mitchell *et al.*, 2001). This inhibition is thought to be competitive and not due to protein destabilization (Mitchell *et al.*, 2001). The accumulating ALA is neurotoxic, and it is proposed to be the underlying cause of the neuropathy in HT1 (Mitchell *et al.*, 2001). SA also inhibits DNA ligase activity, and this leads to slow rejoining of Okazaki fragments and, possibly, genomic instability (Prieto-Alamo and Laval, 1998). Fisher and associates also showed that exposure of cells to SA resulted in cellular toxicity (Fisher *et al.*, 2008).

p-hydroxyphenylpyruvate (pHPPA) has been thought to be an unlikely cause for the symptoms observed in HT1, since it is present in other disorders which do not show the same phenotype as HT1 (Jorquera and Tanguay, 1997, Mitchell *et al.*, 2001). Van Dyk and Pretorius, however, showed that pHPPA can impair DNA repair in liver cells and suggested its involvement in HT1 pathophysiology (Van Dyk and Pretorius, 2005). They then showed that both SA and pHPPA impaired base-excision repair (BER), and to a lesser extent nucleotide-excision repair (NER), by decreasing the recognition and incision efficiency of the initiating proteins (Van Dyk *et al.*, 2010). The specific mechanism of the protein impairment, however, remains unclear.

HT1 is the metabolic disease with the highest risk for primary liver cancer, since hepatic damage is prominent in HT1, and in the chronic form more specifically HCC (Russo *et al.*, 2001, Vogel *et al.*, 2004).

2.3. Hepatocellular carcinoma

2.3.1. Introduction

HCC is one of the most common and deadly cancers worldwide, and it is prevalent in various underlying diseases including alcohol abuse, chronic viral hepatitis, aflatoxin exposure and hereditary metabolic disorders (Major and Collier, 1998, Feitelson *et al.*, 2002, Cha and DeMatteo, 2005, Sasaki, 2006, Van Thiel and Ramadori, 2010). It is an aggressive cancer with a poor prognosis and multifactorial pathogenesis (Elmore and Harris, 2001). In most of these cases, the aetiology of the disease is known, but the HCC risk differs between these aetiologies and the underlying hepatocarcinogenesis is still, for the most part, unclear (Kaneto *et al.*, 2001, Macheiner *et al.*, 2006, Harder *et al.*, 2008).

HCC is generally thought to result from a multi-step mechanism, rather than a single causative agent (Feitelson *et al.*, 2002, Leong and Leong, 2005, Harder *et al.*, 2008). It probably involves multiple etiological factors and complex interactions between them (Leong and Leong, 2005). HCC is a genetically heterogeneous tumour and is thought to result from multiple molecular pathways, including the gathering of genetic alterations during damaged liver tissue propagation (Cha and DeMatteo, 2005, Leong and Leong, 2005) (Reviewed in (Feitelson *et al.*, 2002, Feo *et al.*, 2009)). Phenotypically, HCC mostly occur in a setting of liver inflammation and chronic hepatitis, leading to fibrosis which progresses to cirrhosis (Leong and Leong, 2005, Harder *et al.*, 2008). In cirrhosis, regenerative hepatocytes in focal lesions often change to dysplastic nodules and finally progress to HCC lesions (Leong and Leong, 2005, Harder *et al.*, 2008). Between 60% and 90% of HCC patients present with cirrhosis, and it is the most general association of HCC (Major and Collier, 1998, Leong and Leong, 2005).

In the literature, several mechanisms of tumorigenesis have been associated with HCC, including oncogene activation, direct viral DNA insertion, loss of heterozygosity, tumour suppressor inactivation, DNA methylation and angiogenesis (Cha and DeMatteo, 2005, Kanai and Hirohashi, 2007, Harder *et al.*, 2008). However, the significance of these events in general, and specifically for HT1, is not clear. Genetic alterations observed in HCC include chromosomal deletions, mutations, gene amplifications, chromosomal re-arrangements and epigenetic alterations (Leong and Leong, 2005). Epigenetic alterations such as satellite DNA hypomethylation has been proposed to alter genetic stability and change gene expression patterns involved in tumour progression, and Van Thiel and associates suggested an epigenetic event to be the underlying mechanism of non-viral HCC (Feitelson *et al.*, 2002, Van Thiel and Ramadori, 2010). They proposed that the epigenetic event disturbs the cell cycle and this enhance the HCC risk through resulting in cell proliferation, differentiation, and senescence or a genetic polymorphism (Kaneto *et al.*, 2001, Van Thiel and Ramadori, 2010). They also suggested that the pathophysiologic mechanism included oncogene activation and oxidative stress (Van Thiel and Ramadori, 2010).

2.3.2. HCC in HT1

Prior to NTBC treatment, HCC was reported in 37% of HT1 patients older than two years (Weinberg *et al.*, 1976, Langlois *et al.*, 2006). This is the highest prevalence of liver malignancy in IEMs (Leong and Leong, 2005). HT1 patients present a sequence of morphologic changes, with a

rapid micro- to macro-nodular cirrhosis progression in only a few months (Dehner *et al.*, 1989, Leong and Leong, 2005). This develops into dysplasia and then HCC (Leong and Leong, 2005). Vogel and associates have shown that this liver damage is not characterised by apoptosis, but rather by necro-inflammation (Russo *et al.*, 2001, Vogel *et al.*, 2004). A phenomenon known as mosaicism or mutation reversion often occurs in HT1 patients, resulting in nodules with normal FAH being expressed and these patients often present lower clinical severity (Kvittingen *et al.*, 1994, Mitchell *et al.*, 2001, Demers *et al.*, 2003). These nodules also have a selective growth advantage and may explain the phenotypic variations between patients (Mitchell *et al.*, 2001). The mechanism for this high rate of reversion is not clear yet, although it has been proposed that the accumulating metabolites could be directly or indirectly responsible (Kvittingen *et al.*, 1994). It could also be a result of induced cell regeneration (Kvittingen *et al.*, 1993).

Although the aetiology of HT1-associated HCC remains unclear, various mechanisms have been investigated, and FAA has been proposed to initiate the carcinogenic process (Jorquera and Tanguay, 2001). In HT1, a high level of chromosome breakage was observed, suggesting genetic instability and hypersensitivity of the cells to DNA damaging agents, such as the accumulating metabolites (Gilbert-Barness *et al.*, 1990, Prieto-Alamo and Laval, 1998). Oxidative stress response pathways have also been shown to be activated in HT1 (Fisher *et al.*, 2008), and Orejuela and associates also proposed the involvement of the AKT survival pathway, leading to cell death resistance (Orejuela *et al.*, 2008). Chronic cellular damage and increased mitotic rates can predispose the DNA for a higher mutation frequency, and this combined with the observed cell death resistance may result in the high incidence of HCC in HT1 (Kvittingen *et al.*, 1994, Vogel *et al.*, 2004).

The role of the accumulating metabolites in the initiation of HT1-associated HCC remains vague, however, there are some suggestions indicating that an epigenetically regulated mechanism may be involved (Mitchell *et al.*, 2001). One such indication is the diverse phenotypes in patients with the same genotype (Phaneuf *et al.*, 1992, Mitchell *et al.*, 2001, Russo *et al.*, 2001), while altered gene expression patterns in the liver of HT1 patients is another (Haber *et al.*, 1996, Luijterink *et al.*, 2003). This altered expression has also been shown not to be completely normalized by NTBC treatment (Luijterink *et al.*, 2003). Wentzel and associates also observed that short-term exposure of HepG2 cells to SA resulted in a decrease in global non-CpG island DNA methylation, while CpG island methylation seemed to increase slightly (Wentzel *et al.*, 2010).

In spite of the suggested involvement of epigenetic factors in HT1, no literature pertaining to the study of such factors in HT1, or any other amino aciduria, could be found^c.

2.4. Epigenetic events

2.4.1. Introduction

Epigenetic alterations are heritable modifications in gene expression and chromatin organisation with no associated alterations in the primary DNA sequence (Das and Singal, 2004, Esteller, 2008,

^c As per Pubmed search, using the keywords “inborn error of metabolism”, “metabolic defect”, “amino acid metabolism”, “tyrosinemia”, “epigenetic”, “DNA methylation”.

Sawan *et al.*, 2008, Taby and Issa, 2010). These epigenetic traits allow for extremely stable transmission of gene expression conditions from one cell generation to the next (Fazzari and Greally, 2004, Laird, 2005, Vaissière *et al.*, 2008), and can possibly explain the phenotypic and disease susceptibility variations within a population, which cannot be explained by genetics alone (Peaston and Whitelaw, 2006, Esteller, 2008). Increasing attention to the field of epigenetics has developed as the knowledge of the critical role it plays in development, gene transcription, cell differentiation and viral genome defence increased (Sawan *et al.*, 2008, Taby and Issa, 2010). Sawan and associates have stated that “Epigenetic mechanisms are versatile and adapted for specific cellular memory function not only during development but also during life-time”, and since these epigenetic mechanisms are so important, its deregulation has been associated with several human diseases, particularly cancer (Sawan *et al.*, 2008). There are two main chromosomal structures, namely heterochromatin and euchromatin, and epigenetic mechanisms are implicated in the arrangement, maintenance of and changing between these states (Peaston and Whitelaw, 2006).

There are three main epigenetic mechanisms, namely DNA methylation, histone modification and non-coding RNAs (Esteller, 2008, Sawan *et al.*, 2008, Vaissière *et al.*, 2008, LeBaron *et al.*, 2010). These various markers, however, appear to act in concert to regulate cellular processes and adaptations to endogenous and exogenous signals (Feinberg *et al.*, 2002, Sawan *et al.*, 2008, Vaissière *et al.*, 2008). Mammalian DNA methylation is the covalent addition of a methyl group to the 5-carbon of a cytosine pyrimidine ring in a CpG dinucleotide, and it is one of the most common epigenetic events in the human genome (Singal and Ginder, 1999, Prokhortchouk and Defossez, 2008, Sawan *et al.*, 2008). This epigenetic mechanism is the focus of this study, and will be discussed in more detail in Section 2.4.2.

Histones are the main protein components of chromatin which is involved in gene expression regulation (Esteller, 2008). Epigenetic information is stored in these molecular structures, and alterations include covalent post-translational modifications of the histone proteins, e.g. lysine acetylation, arginine and lysine methylation, and serine phosphorylation (Esteller, 2008, Sawan *et al.*, 2008, Hitchler and Domann, 2009). Histone modifications can influence DNA repair, DNA transcription and gene transcription (Esteller, 2008, Sawan *et al.*, 2008). Non-coding RNA is the most recent epigenetic mechanism, and it can maintain gene expression through microRNAs (miRNAs) (Ambros, 2004, Sawan *et al.*, 2008). miRNAs are 22-nucleotide non-coding RNAs with complementary sequences to the 3' untranslated regions of its target mRNAs (Ambros, 2004, Esteller, 2008). They have regulatory functions and are expressed in a tissue-specific manner (Ambros, 2004, Shi *et al.*, 2010).

2.4.2. DNA methylation

As mentioned in Section 2.4.1, DNA methylation entails the addition of a methyl group to cytosines in CpG dinucleotides, the so-called “fifth base” of DNA, and approximately 70% of these sites are methylated throughout the human genome (Singal and Ginder, 1999, Robertson and Jones, 2000, Prokhortchouk and Defossez, 2008, Sawan *et al.*, 2008, Illingworth and Bird, 2009). These groups protrude into the major groove of the double helix, and alter the biophysical features of the DNA resulting in enhanced sequence recognition by some proteins, and inhibited recognition by others (Prokhortchouk and Defossez, 2008, LeBaron *et al.*, 2010). CpG sites are widespread in the

genome, in regions such as gene bodies, endogenous repeats and transposable elements (Esteller, 2008, Illingworth and Bird, 2009, LeBaron *et al.*, 2010). These CpG sites are, however, underrepresented in the genome since methylated cytosines are spontaneously deaminated to thymine (Singal and Ginder, 1999, Frühwald and Plass, 2002, Das and Singal, 2004). This results in a CpG representation of only 21% of what would be expected in the human genome (Hitchler and Domann, 2009, Illingworth and Bird, 2009). If not repaired, these thymines can result in C:G to T:A mutations, and such repair represents a problem since thymine is not promptly recognized as foreign (Fazzari and Grealley, 2004, LeBaron *et al.*, 2010).

A number of these CpG sites are concentrated in specific CpG-rich, non-methylation regions or so-called CpG islands (CGIs) (Esteller, 2008, Illingworth and Bird, 2009, LeBaron *et al.*, 2010). CpG islands are regions of between 0.5 and 5 kb with a high G/C content, typically occurring every 100 kb, but predominantly in the upstream gene promoter regions of approximately 60-70% of all human genes (Singal and Ginder, 1999, Das and Singal, 2004, Fazzari and Grealley, 2004, Esteller, 2008, Illingworth and Bird, 2009). CpG sites are not uniformly methylated, resulting in both methylated and unmethylated regions and although CpG islands are usually hypomethylated, exceptions include imprinted genes and the inactive X-chromosome (Singal and Ginder, 1999, Dean *et al.*, 2005, LeBaron *et al.*, 2010).

The normal functions of DNA methylation are essential, and include gene expression regulation, transposon and repeat sequence silencing to promote chromosomal stability, control of cell differentiation, genomic imprinting and X-chromosome inactivation (Jones and Takai, 2001, Feinberg *et al.*, 2002, Pradhan and Esteve, 2003, Dean *et al.*, 2005, Esteller, 2008). This regulation by DNA methylation is very important in the maintenance of normal gene expression, since alterations of the normal methylation patterns are frequently associated with disease states and carcinogenesis (Watson *et al.*, 2003, Sawan *et al.*, 2008, LeBaron *et al.*, 2010). It has also been suggested that aberrant epigenetic gene expression regulation plays an equally important role in disease development as mutations, deletions, and other gene expression disregulations (Frühwald and Plass, 2002, Ho and Tang, 2007). This is supported by reports indicating that alterations in the epigenetic status of a gene can cause significant gene expression changes, such as the role hypermethylation, and subsequent silencing, of tumour suppressor genes is believed to play in the initiation and progression of many cancers (reviewed in (Frühwald and Plass, 2002, Franco *et al.*, 2008, Sawan *et al.*, 2008)).

Normal DNA methylation patterns are established very early in embryogenesis and is controlled and maintained by several co-dependent factors. These factors include maintenance and *de novo* methylation, methyl-group donor availability and cellular proliferation and differentiation (Goodman and Watson, 2002, Das and Singal, 2004). Should one or more of these factors change, it could result in hyper- and/or hypomethylation, and both have been implicated in carcinogenesis (Goodman and Watson, 2002, Watson *et al.*, 2003). A group of very important enzymes, known as DNA methyltransferases (DNMTs), are responsible for DNA methylation via the transfer of a methyl-group obtained from the methyl-donor, S-adenosyl-L-methionine (SAM) (Das and Singal, 2004, Franco *et al.*, 2008, Sawan *et al.*, 2008). To date, DNMT1 (isoforms DNMT1b, DNMT1o, DNMT1p), DNMT2, DNMT3a, DNMT3b and DNMT3L are known, although not all of them are involved in active DNA methylation, and cells with defective DNMTs present with major nuclear anomalies (Esteller, 2008) (Reviewed in (Jaenisch and Bird, 2003, Pradhan and Esteve, 2003,

Dean *et al.*, 2005, Franco *et al.*, 2008)). DNMT1, DNMT3a and DNMT3b are responsible for *de novo* methylation, which occurs when both DNA strands are unmethylated, although DNMT1 is mainly responsible for methylation when CpGs are methylated on only one DNA strand (hemi-methylated), also known as maintenance methylation (Okano *et al.*, 1999, Das and Singal, 2004, Franco *et al.*, 2008, Prokhortchouk and Defossez, 2008). This maintenance methylation usually occurs after DNA replication, and DNMT1 is highly conserved in eukaryotes (Singal and Ginder, 1999, Pradhan and Esteve, 2003, Prokhortchouk and Defossez, 2008). DNMT3L aids DNMT3a and DNMT3b as a cofactor during *de novo* methylation (Prokhortchouk and Defossez, 2008). There are other factors involved in methylation also, such as demethylases, methylation centers (which triggers DNA methylation) and methylation protection centers (Das and Singal, 2004). Two of these demethylating enzymes are 5-methylcytosine glycosylase and MBD2b which removes the methyl-group, while leaving the cytosine base intact (Das and Singal, 2004).

DNA sequences act as the substrate for transcription factors in the complex, controlled process of gene expression regulation (Das and Singal, 2004). This expression of the DNA is, however, influenced by the presence of DNA methylation, and the position of the methylation determines the effect on the expression (Das and Singal, 2004). For instance, if the methylation of the promoter region of a gene increases, the level of expression will usually decrease, while increased or decreased transcribed region methylation can have various effects on the expression levels (Boyes and Bird, 1992). This inverse relationship is, however, not universal for all genes (Singal and Ginder, 1999). There are three possible mechanisms for the repression of gene expression by DNA methylation. The first of these involve the direct interference of methylation with binding of specific transcription factors, such as AP-2, NF- κ B and E2F, with their recognition sites in the gene promoters (Singal and Ginder, 1999, Das and Singal, 2004, Hitchler and Domann, 2009). The second mechanism involves the direct binding of transcriptional repressor proteins (e.g. MeCP1, MeCP2, MBD1, MBD2 and MBD4) through a Methyl-binding Domain (MBD) which recognizes methylated DNA (Das and Singal, 2004, Prokhortchouk and Defossez, 2008, LeBaron *et al.*, 2010). Thirdly, gene expression can also be altered by histone modifications and chromatin structure, both of which are affected by DNA methylation, since methylation patterns direct histone deacetylation to specific residues (Das and Singal, 2004, Franco *et al.*, 2008, LeBaron *et al.*, 2010) (reviewed in (Taby and Issa, 2010)).

2.4.3. DNA methylation alterations and cancer

Originally, it was thought that progressive transformation of normal to malignant cells resulted from classical genetic mechanisms, which could include deletion or mutation of the genetic information (Das and Singal, 2004, Lehmann *et al.*, 2007). These events cause progressive alteration or loss of gene activity, and thereby initiate a series of events which ultimately leads to cancer (Das and Singal, 2004, Cha and DeMatteo, 2005). However, epigenetic alterations have increasingly been implicated in early tumorigenesis and in the progression of human cancers, including multistage carcinogenesis (Jones and Takai, 2001, Laird, 2003, Dean *et al.*, 2005, Kanai and Hirohashi, 2007, Taby and Issa, 2010).

Two DNA methylation alterations have been proposed to be involved in human cancer development. The first, global hypomethylation, is thought to occur early in neoplasia, causing genomic instability and loss of heterozygosity and the second, aberrant regional hypermethylation,

both of which can silence tumour suppressor genes or activate oncogenes (Singal and Ginder, 1999, Das and Singal, 2004, Franco *et al.*, 2008, Prokhortchouk and Defossez, 2008). Frequent observations of genome-wide DNA demethylation and chromatin hypoacetylation in carcinomas have been made, while specific genes show hypo- or hypermethylation which induce gene silencing or activation (Watson *et al.*, 2003, Dean *et al.*, 2005). It has been indicated that such gene inactivation can target tumour-suppressor genes, becoming an important event in the progression of tumours, including HCC (Pradhan and Esteve, 2003, Lehmann *et al.*, 2007, Esteller, 2008, Harder *et al.*, 2008). Genes responsible for the regulation of growth, the cell cycle, DNA repair, apoptosis, drug resistance, detoxification and metastasis have been found to be inactivated through promoter methylation even in pre-malignant lesions (Watson *et al.*, 2003, Das and Singal, 2004, Lehmann *et al.*, 2007). Some of these genes have been shown to be hypermethylated in several cancers, while others seem to be associated with specific cancers, and this hypermethylation also occurs at different cancer development stages (Frühwald and Plass, 2002, Das and Singal, 2004, Esteller, 2008). DNA hypomethylation, albeit thought to be a relatively late event in carcinogenesis, have been proposed to contribute to cancer development through transposon reactivation, loss of imprinting and induction of chromosomal instability (Frühwald and Plass, 2002, Eden *et al.*, 2003, Watson *et al.*, 2003, Esteller, 2008). It can also promote deletions, translocations and chromosomal rearrangements (Eden *et al.*, 2003, Esteller, 2008, Taby and Issa, 2010).

In addition, methylated cytosine have been shown to be unstable and highly mutable, and this in itself can cause mutations and threaten the genome (Wachsman, 1997, Sawan *et al.*, 2008). This higher mutability, compared to unmethylated cytosines, is due to altered repair capacity, spontaneous de-amination and cell division rate differences (Robertson and Jones, 2000, Fryxell and Moon, 2005). It seems that transition mutations of these bases frequently occur in tumour suppressor genes, possibly contributing to cancer development (Fryxell and Moon, 2005).

2.4.4. Epigenetic alterations in HT1 and associated HCC

Poudrier and colleagues have stated that “genotypic heterogeneity is not a sufficient explanation for clinical heterogeneity” and suggested that epigenetic and other factors could modify the phenotype in HT1 (Poudrier *et al.*, 1998). Whitelaw and Martin also proposed that epigenetic variations could explain different gene expression profiles in isogenic individuals, as well as mosaic patterns in an individual (Whitelaw and Martin, 2001, Peaston and Whitelaw, 2006).

Epigenetic alterations have been observed in several non-HT1 HCCs, including genome-wide hypomethylation (Lin *et al.*, 2001, Wilson *et al.*, 2006). Researchers have suggested that promoter methylation could be one of the founding aberrations in hepatocarcinogenesis, and they even proposed that it occurs before gene deletion or loss of heterozygosity (Kaneto *et al.*, 2001, Roncalli *et al.*, 2002). Roncalli and colleagues also found hypermethylation in cirrhosis to be frequently associated with hypermethylation in HCC (Roncalli *et al.*, 2002). Lin and associates, however, did not find any “serial of sequential increases of DNA hypomethylation from normal and noncirrhotic liver to cirrhosis” (Lin *et al.*, 2001). They subsequently suggested that DNA hypomethylation was not simply a historical incident during the start of the cancer, but rather an unceasing development during its progression (Lin *et al.*, 2001). Kanai and Hirohashi also proposed that DNA hypomethylation could increase during the initiation and progression of HCC (Kanai and Hirohashi,

2007). Epigenetic alterations could theoretically also be a secondary event, following genetic instability (Peaston and Whitelaw, 2006), in turn promoting further development of the disease or cancer. This is possible in HT1, since FAA has been shown to cause genetic instability (Jorquera and Tanguay, 2001). However, Kanai and Hirohashi showed that DNA methylation alterations preceded loss of heterozygosity and chromosomal instability in hepatocarcinogenesis (Kanai and Hirohashi, 2007).

Several genes have been indicated as potential targets for altered methylation in HCC (not of HT1 origin). *p16^{INK4A}*, a tumour suppressor gene, has been shown to have promoter methylation in 62-83% of HCC patients (See references in (Kaneto *et al.*, 2001, Roncalli *et al.*, 2002, Harder *et al.*, 2008)), while *E-cadherin* has been shown to present with increasing promoter methylation during HCC development, with associated decreasing expression of the gene (Kanai and Hirohashi, 2007). MGMT, a DNA repair enzyme, has also frequently been found to have altered promoter methylation patterns and/or expression patterns in HCC (not of HT1 origin) (Zhang *et al.*, 2003). Harder and associates, however, found MGMT promoter methylation to be an unlikely factor in HCC (Harder *et al.*, 2008). They did find a strong association between hepatocarcinogenesis and promoter methylation of the glutathione S-transferase gene (Harder *et al.*, 2008). It has also been suggested that *RASSF1A* suppression due to methylation is a common aberration in cancer which promotes tumour development (Di Gioia *et al.*, 2006). Various reports suggest that *RASSF1A* promoter methylation is a potential HCC marker, since the promoter is methylated in 67-100% of HCCs, while closely situated non-neoplastic liver tissue is not methylated (Di Gioia *et al.*, 2006). Di Gioia and colleagues also suggested that *RASSF1A* methylation occurs early in cirrhosis-associated HCC, but is not related to a specific aetiology (Di Gioia *et al.*, 2006). *NORE1B* promoter methylation has also been found in a large number of HCCs, and it was proposed that this inactivation could be influential in the escape of malignant liver cells from growth control (Macheiner *et al.*, 2006).

The GSH concentration measured in one HT1 liver was reduced to about half of normal levels (Mitchell *et al.*, 2001), and it has been shown that FAA, and possibly MAA, can react with GSH, or even deplete it (Jorquera and Tanguay, 1997, Mitchell *et al.*, 2001, Vogel *et al.*, 2004, Langlois *et al.*, 2006). GSH depletion and cell toxicity are frequently associated, and it is thought that this is a result of limited GSH conjugation (Lertratanangkoon *et al.*, 1997). Lertratanangkoon and colleagues, however, proposed that it could be a less direct mechanism, involving a cascade of events (Lertratanangkoon *et al.*, 1997). They subsequently showed that hepatic GSH depletion results in methionine depletion, which in turn can alter or impair DNA methylation (Lertratanangkoon *et al.*, 1997, Hitchler and Domann, 2009).

HCC is also associated with inflammation of the liver (Feo *et al.*, 2009), and chronic inflammation has been shown to be associated with hypermethylation, DNA mutation and genetic instability (Ushijima and Okochi-Takada, 2005, Schetter *et al.*, 2010).

2.5. DNA alkylation

2.5.1. Introduction

The mammalian genome is continuously subjected to both endogenous and exogenous hazards. Fortunately, cells are well equipped to detect and react to these insults against the integrity of the genome by means of a variety of DNA repair mechanisms, each with its own unique target(s) and mechanism (Doak *et al.*, 2008, Zhong *et al.*, 2010). However, aberrations in these processes are not uncommon and may ultimately lead to pathological conditions, including the accumulation of genetic mutations (Doak *et al.*, 2008).

One of the most important DNA repair mechanisms involves the enzyme O⁶-alkylguanine-DNA methyltransferase (AGT; also known as ATase, AGAT and MGMT). The human counterpart is most often referred to as MGMT (O⁶-methylguanine-DNA methyltransferase; E.C.2.1.1.63), its main function being the removal of highly promutagenic and cytotoxic O⁶-alkyl adducts on guanine bases (O⁶-alkylG) in DNA, thereby protecting cells against the adverse biological effects induced by alkylating agents (Liu and Gerson, 2006, Jacinto and Esteller, 2007, Kaina *et al.*, 2007, Zhong *et al.*, 2010). These adducts result from the action of alkylating agents such as *N*-nitroso compounds, which are commonly dispersed environmental carcinogens as well as endogenous metabolic products (Kaina, 1998, Major and Collier, 1998, Kaina *et al.*, 2007). One of these lesions, O⁶-methylguanine (O⁶-MeG), is very stable and it is crucial for cell viability to remove these adducts, as they result in C:T mismatches, which in turn lead to a plethora of adverse events, such as point mutations, sister chromatid exchanges (SCEs), chromosomal aberrations, cancer and cell death (Major and Collier, 1998, Jacinto and Esteller, 2007, Kaina *et al.*, 2007, Doak *et al.*, 2008). One MGMT molecule can repair only one alkyl adduct (Jacinto and Esteller, 2007, Kaina *et al.*, 2007). Consequently, the number of MGMT molecules in a cell can drop abruptly and be temporarily depleted if an excess of DNA adducts are present, and even permanently should the alkylation of the DNA persist (Lindahl *et al.*, 1982, Pieper, 1997, Liu and Gerson, 2006). Cells deficient in MGMT were also shown to be more susceptible to the damage caused by alkylating agents (Grady and Ulrich, 2007, Kaina *et al.*, 2007).

There are many excellent reviews explaining in great detail the mechanism and function of MGMT (Pieper, 1997, Pegg, 2000, Margison *et al.*, 2003, Kaina *et al.*, 2007), but an apparent dearth of clear explanations for the seemingly paradoxical nature of its mechanism, a suicidal enzyme with unsustainable induction. In the manuscript included at the end of this chapter, MGMT is discussed with regard to its properties and function, and some peculiarities surrounding the function and mechanism of MGMT is highlighted in the context of its great importance in maintaining the integrity of the genome. This information will, therefore, not be discussed in detail in this section.

2.5.2. DNA alkylation, MGMT and HT1-associated HCC

The accumulating HT1 metabolites have been proposed to be alkylating agents, and are thought to contribute to the frequent HCC development (Jorquera and Tanguay, 2001, Mitchell *et al.*, 2001, Russo *et al.*, 2001). This is important, since MGMT is known to be involved in the development of HCC (not of HT1 origin), and O⁶-meG appears to be central to the carcinogenesis initiation (Zhang *et al.*, 2003). The liver is an important site for precarcinogenic agent metabolic activation, thereby

possibly raising carcinogenic susceptibility (Major and Collier, 1998). The high levels of MGMT in the liver supports this, but some HCCs have been shown to completely lack MGMT activity (Major and Collier, 1998). Higher endogenous nitrosation rates, but decreased MGMT levels, have also been observed in cirrhosis patients (Collier *et al.*, 1993, Major and Collier, 1998). Whether these lowered levels persist in HCC is not clear yet, although some studies have indicated cases where this was true (Collier *et al.*, 1996, Major and Collier, 1998).

Major and Collier proposed defective MGMT alkylation repair to be “an early, possibly pivotal, event in the multistep process of hepatocellular carcinogenesis” (Major and Collier, 1998). They did, however, suggest that these MGMT level alterations were determined by genetic, as well as acquired factors (Major and Collier, 1998).

2.5.3. MGMT and DNA methylation

It is important to clarify DNA alkylation and DNA methylation, before the association between these events can be discussed. DNA alkylation entails the formation of alkyl adducts to specific bases, and this alkyl group can be a methyl group (Grady and Ulrich, 2007). This, for example, results in O⁶-methylation of guanine (O⁶-meG), which consistently cause DNA damage and mutations and could be related to the risk of cancer (Major and Collier, 1998, Jacinto and Esteller, 2007, Kaina *et al.*, 2007, Doak *et al.*, 2008). On the other hand, the general use of the term DNA methylation refers to the epigenetic mechanism which adds a methyl-group to cytosine in a CpG site in a DNA sequence. This mechanism is a normal process and is driven by DNA methyltransferases (Grady and Ulrich, 2007).

Loss of *MGMT* expression can be attributed mainly to *MGMT* promoter CpG island hypermethylation, as opposed to conventional deletions, mutations and rearrangements (Jacinto and Esteller, 2007, Kaina *et al.*, 2007, Sedgwick *et al.*, 2007, Hermes *et al.*, 2008). The methylation status of the *MGMT* promoter has been the focus of several studies, and hypermethylation was associated with colorectal cancer, brain cancer and oesophageal adenocarcinoma (Esteller *et al.*, 2001b, Yin *et al.*, 2003, Baumann *et al.*, 2006). *MGMT* has also been found to have altered promoter methylation patterns and/or expression patterns in HCC (not of HT1 origin) (Zhang *et al.*, 2003). Bearzatto and colleagues observed that in some cells, however, hypermethylation of its promoter did not result in decreased expression of *MGMT*, making this relationship between promoter methylation and transcription even more complex (Bearzatto *et al.*, 2000). Hermes and associates have shown the *MGMT* promoter region to be partially hypermethylated in HepG2 cells (Hermes *et al.*, 2008).

On the contrary, it has been indicated that alkylation damage preferably occur in GC-rich regions (Mattes *et al.*, 1988), and Hepburn and associates subsequently proposed that this could interfere with the enzymatic methylation of these regions (Hepburn *et al.*, 1991, Goodman and Watson, 2002). O⁶-meG has also been shown to inhibit binding of DNMTs to the DNA region, possibly leading to hypomethylation of adjacent cytosines in that region (Hepburn *et al.*, 1991, Wachsman, 1997). Through the spontaneous mispairing of O⁶-meG, it also decrease potential methylation sites, also potentially causing hypomethylation (Tan and Li, 1990, Hepburn *et al.*, 1991, Franco *et al.*, 2008).

2.6. Oxidative stress

2.6.1. Introduction

Reactive oxidant species (ROS) are normal by-products of aerobic respiration, p450 metabolism, nitric oxide synthesis and inflammation, but it can be derived from various exogenous sources as well (Sasaki, 2006, Cecarini *et al.*, 2007, Franco *et al.*, 2008). ROS consists of various partially reduced oxygen metabolites, such as superoxide anions and hydroxyl radicals (Halliwell and Aruoma, 1991, Sasaki, 2006). These highly reactive and unstable molecules can damage all biological components, including DNA, RNA, proteins and lipids, and may contribute to malignant transformation (Cerdeira and Weitzman, 1997, Mary *et al.*, 2004, Cecarini *et al.*, 2007, Hawkins *et al.*, 2009, Jung *et al.*, 2009). ROS accumulation is contested by various complex mechanisms, such as enzymatic antioxidants (e.g. superoxide dismutase, catalase) and nonenzymatic antioxidants (e.g. GSH, vitamin C) (Halliwell and Aruoma, 1991, Sasaki, 2006, Reuter *et al.*, 2010). Of these, the glutathione redox cycle is most important and tightly regulated (Sasaki, 2006). Normally, the bulk of ROS produced is detoxified into H₂O (Sasaki, 2006). Some ROS remains, however, and some is converted into H₂O₂ (Sasaki, 2006). In abnormal circumstances the production of ROS can increase, and once the ROS production exceeds the cellular antioxidant defences, oxidative stress ensues, compromising cellular homeostasis and function (Sasaki, 2006, Cecarini *et al.*, 2007, Franco *et al.*, 2008, Dunlop *et al.*, 2009). Various DNA repair enzymes are also present in cells to protect against ensuing DNA damage in the event of oxidative stress, but antioxidants protect cells more efficiently (Reuter *et al.*, 2010).

Since proteins are so abundant in the cells, it is a likely target and oxidative protein damage can cause amino acid structure alterations and enzymatic activity changes, affecting cell signalling, cellular structure and metabolism (Cecarini *et al.*, 2007, Hawkins *et al.*, 2009). These oxidized proteins have to be eliminated, but there are very few enzymatic repair systems for these proteins and its degradation is the main mechanism of removal (Mary *et al.*, 2004, Dunlop *et al.*, 2009). Intracellular proteases recognize this damage and remove it, but it appears that these proteolytic complexes can be directly affected by the oxidants, and in doing so compromise their function (Cecarini *et al.*, 2007, Dunlop *et al.*, 2009). Protein carbonyls are principal oxidation products, and therefore generic markers of protein oxidation (Cecarini *et al.*, 2007, Hawkins *et al.*, 2009). These carbonyls can react with lysine residues, causing protein aggregate formation which cannot be degraded through the normal mechanisms (Cecarini *et al.*, 2007). Should this lack of degradation continue, proteolytic processes can be inhibited and cellular dysfunction enhanced (Cecarini *et al.*, 2007). Accumulation of oxidized proteins has been indicated in several human diseases (see references in (Dalle-Donne *et al.*, 2003)), and in several of these the increase in the disease development corresponded with carbonyl concentrations.

ROS has been shown to damage DNA, and this damage can contribute to carcinogenesis by initiating tumours by causing mutations, cytotoxicity and forming genotoxic adducts (Cerdeira and Weitzman, 1997, Franco *et al.*, 2008). Other DNA lesions include deletions, single strand breaks, frameshifts and chromosomal rearrangements (Halliwell and Aruoma, 1991, Franco *et al.*, 2008, Klaunig *et al.*, 2010). These lesions can give rise to a permanently damaged cell population which can overcome cytotoxicity and continue proliferating, while normal cells are depleted (Cerdeira and

Weitzman, 1997). This can give rise to the clonal expansion of the damaged population (Cerdeira and Weitzman, 1997, Goodman and Watson, 2002, Feo *et al.*, 2009, Klaunig *et al.*, 2010).

2.6.2. Oxidative stress and DNA methylation

Intracellular ROS can cause various DNA lesions, including the mutagenic 8-hydroxy-2-deoxyguanosine (8-OHdG) and O⁶-methylguanine (O⁶-meG) (Franco *et al.*, 2008). These lesions are not only mutagenic, but they can modulate cell cycle regulation and alter epigenetic mechanisms, thereby changing the normal DNA methylation patterns of cells (Sasaki, 2006, Franco *et al.*, 2008). Weitzman and colleagues observed that when guanines are replaced with 8-OHdG, binding of DNMTs are inhibited, preventing the methylation of neighbouring cytosines and it may, therefore, cause DNA hypomethylation (Weitzman *et al.*, 1994, Sasaki, 2006). It has also been shown that some of these oxidation lesions in DNA can inhibit binding of MBPs (Valinluck *et al.*, 2004), while ROS has also been shown to increase DNA methyltransferase expression, which can result in global hypermethylation (Schetter *et al.*, 2010). It has been suggested that *de novo* methylation can be signalled by single-stranded DNA and, therefore, ROS induced single-strand breaks could alter DNA methylation patterns (Cerdeira and Weitzman, 1997, Wachsmann, 1997). It has, therefore, been suggested that oxidative DNA damage can alter DNA methylation patterns, and that these changes may be involved in disease development (Cerdeira and Weitzman, 1997, Valinluck *et al.*, 2004, Franco *et al.*, 2008).

2.6.3. Oxidative stress in HT1 and associated HCC

Cancer development is a frequent event in a number of common liver diseases with related oxidative stress, which suggests that ROS can cause mutations, changing the function of regulating proteins in DNA repair, cell cycle or apoptosis (Vogel *et al.*, 2004). This may be a factor in the pathophysiology of these liver diseases and their risk of HCC (Wang *et al.*, 2002, Vogel *et al.*, 2004). Substantial evidence implicates oxidative stress in several stages of carcinogenesis, including initiation and promotion (Sasaki, 2006, Franco *et al.*, 2008, Reuter *et al.*, 2010). It is also widely accepted that early stage hepatocarcinogenesis is associated with ROS production as a result of carcinogen metabolism and inflammation, which can cause genomic instability (Feo *et al.*, 2009, Schetter *et al.*, 2010).

GSH is a key molecule for the detoxification of H₂O₂, and its depletion in the mitochondria, for instance, can result in mitochondrial dysfunction and cell death (Sasaki, 2006). FAA, and possibly MAA, can react with or even deplete GSH, resulting in oxidative damage which potentiates the mutagenicity of the metabolites (Jorquera and Tanguay, 1997, Mitchell *et al.*, 2001, Vogel *et al.*, 2004, Langlois *et al.*, 2006). Dieter and colleagues therefore suggested that various tyrosine intermediates are capable of oxidative stress induction, and that this was possibly causally linked to the ensuing liver pathology (Dieter *et al.*, 2003). They also proposed that HT1 patients on NTBC treatment are chronically exposed to oxidative stress, since the NTBC-rescued 14CoS/14CoS mouse model had a significant oxidative stress response in the liver and kidneys (Dieter *et al.*, 2003). Several research groups have since shown that oxidative stress response pathways are activated in HT1 models, but few of them directly assayed ROS production and its ensuing cellular damage (Dieter *et al.*, 2003, Vogel *et al.*, 2004, Bergeron *et al.*, 2006, Fisher *et al.*, 2008). Vogel and colleagues did indicate an increase in oxidative DNA damage in *Fah*^{-/-} mice (Vogel *et al.*,

2004), but Jorquera and Tanguay measured ROS production following direct exposure experiments with FAA, and did not detect any increase in ROS production (Jorquera and Tanguay, 1997). Bergeron and associates showed the launch of an endoplasmic reticulum (ER) stress response in HT1, and subsequently stated that it was unclear whether the previously observed induction of the stress-related proteins, HO-1 and Hsps, was a result of oxidative or ER stress (Bergeron *et al.*, 2006). As such, the source of the oxidative stress response in HT1 remains unclear, and needs further exploration.

Approximately 90% of oxidized nuclear DNA bases are repaired by NER, and the remaining 10% by BER (Sasaki, 2006). As discussed in Section 2.2.2, however, both SA and pHPA can impair BER and NER by decreasing the recognition and incision efficiency of the initiating proteins (Van Dyk *et al.*, 2010). Increased oxidative DNA damage in *Fah*^{-/-} mice has been indicated (Vogel *et al.*, 2004), and therefore, these lesions will probably persist and it can therefore influence normal DNA methylation patterns.

2.7. Senescence in HCC

Terminal growth arrest is known as cellular senescence (Ozturk *et al.*, 2009). It is a response of cells to both exogenous and endogenous stress and damage (Ozturk *et al.*, 2009). Cells in this state present permanent cell cycle arrest, characteristic morphologic changes and altered gene expression patterns (Ozturk *et al.*, 2009). Senescence functions in the process of aging, but can also act as a protection mechanism against cancer (Ozturk *et al.*, 2009). Senescence has been indicated in patients with cirrhosis and HCC, but its specific role in these diseases remains unclear (Paradis *et al.*, 2001). Senescence can result from telomere shortening, ROS damage and oncogene activation, leading to DNA damage, which in turn then leads to the activation of the p53 pathway (for review, see (Ozturk *et al.*, 2009)). *In vivo* replicative senescence has been detected in 60% of HCCs (Paradis *et al.*, 2001).

2.8. Hypothesis^d

In inborn errors of metabolism (IEMs), an enzyme defect in a metabolic pathway results in the accumulation of various metabolites. The effect of these metabolites, if not removed/detoxified, can be extremely harmful to the cells and set in motion several cascades of events in these cells. In numerous IEMs this results in cancer. The IEM, hereditary tyrosinemia type 1 (HT1) is frequently associated with hepatocellular carcinoma (HCC) development, and it is therefore an excellent model for the study of IEM-associated cancers. HCC is a complex, multi-step, multi-factorial cancer, and it usually progress from hepatitis and inflammation to fibrosis. Cirrhosis then develop from which dysplastic nodules can progress, ending in HCC (See (1) in Figure 2.2). The aetiology of this HCC in HT1 remains unclear as yet.

^d For the sake of brevity and flow of the text, no references were included in the proposal of the hypothesis. All the relevant references were indicated in the text of the literature study.

In HT1, several metabolites accumulate including FAA, MAA, SA and pHPPA ((2) in Figure 2.2). These metabolites have been shown or suggested to induce multiple aberrations in the cells, and it is thought that these metabolites somehow initiate the carcinogenic process in the liver. Some of these effects they could have include impairing DNA repair, inducing cellular toxicity, decreasing or depleting cellular glutathione (GSH), inducing oxidative stress – possibly through increasing reactive oxygen species (ROS) production, alkylating DNA and altering epigenetic mechanisms such as DNA methylation. It is unclear which of these aberrations are responsible for the carcinogenesis initiation, or if a combination of several factors are at play. From the literature, several probable or possible interactions between these aberrations have been investigated, as well as some resulting cellular changes they could induce. Many of these interactions have been indicated in Figure 2.2, although it would become extremely complex if all possible changes and interactions should be added. Therefore, the most relevant elements of the hypothesis are shown.

Epigenetic mechanisms regulate gene expression and function, and aberrant DNA methylation has been associated with several cancers in the literature. However, the role of these DNA methylation alterations in IEM-associated cancers has not been studied to date. The involvement of epigenetic alterations in HT1 has been suggested, and there are several factors indicating its possible involvement, but to my knowledge it has not been studied thus far. Therefore, one of the aims of this study is to identify whether epigenetic alterations, specifically in DNA methylation, do occur due to the action of the accumulating metabolites or their effects.

Aberrant DNA methylation has been associated with changes in gene expression, tumour suppressor gene inactivation and oncogene activation (see (3) in Figure 2.2). Although these changes can directly be involved in cancer initiation and progression, they are also involved in several other mechanisms. For instance, the changes in gene expression can impair DNA repair, including the enzyme O⁶-methylguanine methyltransferase (MGMT) (see (4) in Figure 2.2). This enzyme is involved in the removal of alkyl-adducts from DNA bases. Since some of the metabolites have been proposed to be alkylating agents, their accumulation can lead to increased DNA alkylation damage, which can result in mutations and genetic instability. This can be an initiating factor in carcinogenesis. If MGMT is fully functional, it can counteract the increase in DNA alkylation (see (5) in Figure 2.2), but it has been shown that increased methylation of its promoter region can result in a decrease in its expression. Therefore, should alkylation damage increase as a result of the accumulating metabolites, and repair via MGMT decrease, ensuing deleterious effects may occur. It is proposed that this could be a significant contributing factor to HCC development in HT1.

This increased alkylation damage as a result of DNA methylation changes can also result in a positive feedback effect, since it has been shown that DNA alkylation itself can alter DNA methylation patterns and mechanisms (see (8) in Figure 2.2). The methylation alterations can possibly induce further alkylation damage and it can, therefore, result in a futile loop of DNA damage.

Another point of interest is the observation of increased oxidative stress responses in HT1. Several researchers have reported this, as discussed in Section 2.6.3., although it remains unclear if this response was initiated by oxidative or endoplasmic reticulum stress. The increased production of intracellular ROS has also not been indicated in HT1. Also, it has been shown that

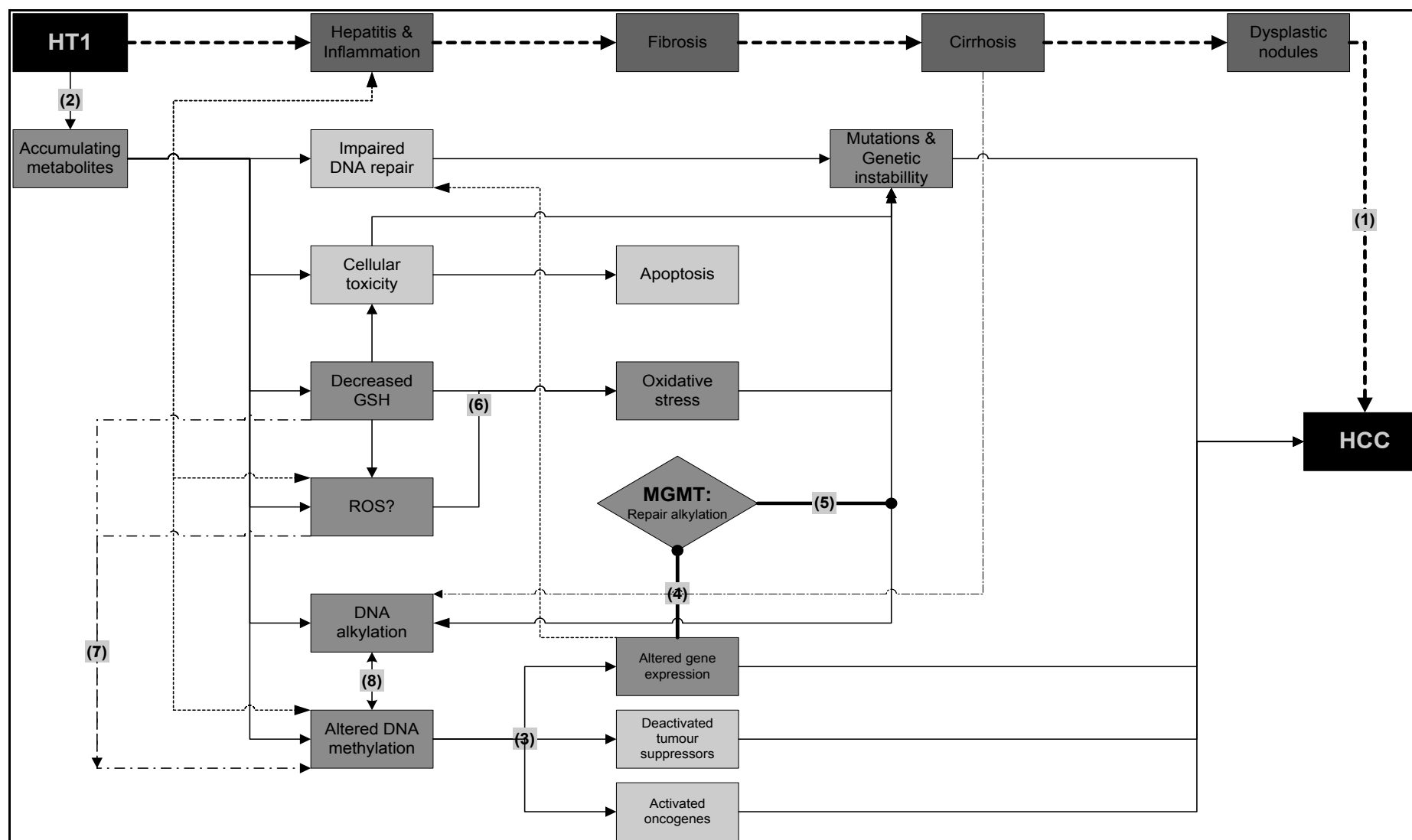


Figure 2.2. Flow diagram of the proposed mechanism for the initiation of HCC in HT1. In HT1, various metabolites accumulate (2). These compounds may have alkylating properties, resulting in alkylation damage, unless it is repaired by the MGMT enzyme (5). The accumulating metabolites may, however, also affect normal DNA methylation patterns, resulting in the aberrant regulation of various genes (3), including MGMT (4). These alterations can induce accumulation of alkylation damage (8), tumour initiation and progression and genomic instability. Other mechanisms affected and potentially involved in the initiation and progression of HCC (1) includes the presence of oxidative stress (6 and 7), chronic inflammation and mutation accumulation.

some of the metabolites can decrease GSH in the cells, which would also contribute to oxidative stress (see (6) in Figure 2.2). This is important in the study of the abovementioned aim, since it has been shown that both ROS and decreased GSH can influence normal DNA methylation (see (7) in Figure 2.2).

Ultimately, the mechanism proposed could be responsible for the initiation of cancer in inborn errors of metabolism.

2.9. In Summary

The hypothesis posits that the metabolites which accumulate in HT1 may alter normal DNA methylation patterns, which may lead to altered gene expression profiles in the cells. The metabolites also reduce GSH, and probably induce ROS, both of which can also influence normal DNA methylation. Since the metabolites probably induce alkylation damage to the DNA, the repair enzyme MGMT is crucial for the removal of such damage. The altered DNA methylation may affect MGMT, thereby reducing its expression and impairing the cells' capacity to deal with the continued damage. This ultimately leads to mutations and genomic instability, which can contribute to HCC development.

2.9.1. Aims

The specific aims for this study are:

- Establish an inducible knock-down model for HT1 in cultured human cells
- Investigate the role of epigenetic alterations in the aetiology of HT1-associated HCC, specifically pertaining to:
 - Global DNA methylation
 - The *MGMT* gene and the associated enzyme
 - Oxidative stress

2.9.2. Approach

The specific approach entails:

- Applying RNAi technology to establish an inducible knock-down HT1 model in cultured HepG2 cells
- Testing the hypothesis in a direct exposure model with the accumulating metabolites, SA and pHPPA
 - Measuring global DNA methylation
 - Measuring DNA methylation of the *MGMT* gene promoter and its expression
 - Measuring intracellular ROS production and protein oxidation

2.10. Published article

The following paper is a discussion of MGMT and some peculiarities surrounding its function and mechanism, as referred to in Section 2.5.1.

The manuscript was published in Medical Hypotheses, as mentioned in Section 1.5.



O⁶-methylguanine-DNA methyltransferase (MGMT): Can function explain a suicidal mechanism?

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ARTICLE INFO

Article history:

Received 28 June 2011

Accepted 27 July 2011

ABSTRACT

Why does O⁶-methylguanine-DNA methyltransferase (MGMT), an indispensable DNA repair enzyme, have a mechanism which seems to run counter to its importance? This enzyme is key to the removal of detrimental alkyl adducts from guanine bases. Although the mechanism is well known, an unusual feature surrounds its mode of action, which is its so-called suicidal endpoint. In addition, induction of MGMT is highly variable and its kinetics is atypical. These features raise some questions on the seemingly paradoxical mechanism. In this manuscript we point out that, although there is ample literature regarding the “how” of the MGMT enzyme, we found a lack of information on “why” this specific mechanism is in place. We then ask whether we know all there is to know about MGMT, or if perhaps there is a further as yet unknown function for MGMT, or if the suicidal mechanism may play some kind of protective role in the cell.

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Introduction

The mammalian genome is continuously subjected to both endogenous and exogenous hazards. Fortunately, cells are well equipped to detect and react to these insults against the integrity of the genome by means of a variety of DNA repair mechanisms, each with its own unique target(s) and mechanism [1,2]. However, aberrations in these processes are not uncommon and may ultimately lead to pathological conditions, including the accumulation of genetic mutations [1].

One of the most important DNA repair mechanisms involves the enzyme O⁶-alkylguanine-DNA methyltransferase (AGT; also known as ATase, AGAT and MGMT). The human counterpart is most often referred to as MGMT (O⁶-methylguanine-DNA methyltransferase; E.C.2.1.1.63), its main function being the removal of highly promutagenic and cytotoxic O⁶-alkyl adducts on guanine bases (O⁶-alkylG) in DNA, thereby protecting cells against the adverse biological effects induced by alkylating agents [2–5]. These adducts result from the action of alkylating agents such as *N*-nitroso compounds, which are commonly dispersed environmental carcinogens as well as endogenous metabolic products [5–7]. One of these lesions, O⁶-MeG (O⁶-methylguanine), is very stable and it is crucial for cell viability to remove these adducts, as they result in C:T mismatches, which in turn lead to a plethora of adverse events, such as point

mutations, sister chromatid exchanges (SCEs), chromosomal aberrations, cancer and cell death [1,3,5,7]. Cells deficient in MGMT were shown to be more susceptible to the damage caused by alkylating agents [5,8].

There are many excellent reviews explaining in great detail the mechanism and function of MGMT [5,9–11], but an apparent dearth of clear explanations for the seemingly paradoxical nature of its mechanism, a suicidal enzyme with unsustainable induction. In this paper we highlight some peculiarities surrounding the function and mechanism of MGMT, in the context of its great importance in maintaining the integrity of the genome. An important question is, therefore: why does an enzyme with such an important function act in such an apparently curious manner, and how efficiently can one use this enzyme if it may have characteristics still not understood?

Properties of MGMT

MGMT has been highly conserved in organisms from bacteria to mammalian species, and acts by transferring the alkyl adduct in a direct damage-reversal pathway from the oxygen in the O⁶-alkylG base, to a cysteine residue in its catalytic pocket [3,5,7,9,11]. This unconventional mechanism results in repaired DNA but in an irreversibly inactivated MGMT, thus being described as “suicidal” [1,5,12]. Following the transfer of the alkyl moiety, it seems that the alkylated MGMT protein attains an altered conformation, rendering it susceptible to be rapidly ubiquitinated and degraded by the proteasome, instead of being demethylated to reactivate the enzyme [3,5,7,9,11,13,14]. In other words, one MGMT molecule can repair only one alkyl adduct [3,5]. Consequently, the number

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of MGMT molecules in a cell can drop abruptly and be temporarily depleted if an excess of DNA adducts are present, and even permanently should the alkylation of the DNA persist [4,11,12].

O⁶-MeG is exclusively repaired by MGMT, while the larger adducts are also recognized by the nucleotide excision repair (NER) pathway [5,11,15]. The translation of O⁶-MeG to chromosomal aberrations and cell death is dependent on both mismatch repair (MMR) and the cell cycle [5,6]. Some opinions are that O⁶-MeG/thymine mismatch lesions are not processed correctly by MMR, because of the futile cycles of excision and reinsertion of thymine due to the mispairing properties of the O⁶-MeG base [1,5,6,11]. This futile MMR cycle results in these O⁶-MeG/thymine mismatches becoming toxic by producing tertiary lesions that in turn give rise to double strand breaks (DSBs) during DNA replication and ultimately cause genetic instability or cell death [4,5]. O⁶-MeG/thymine mismatches can also result in proto-oncogene activating G > A transition mutations [7,15].

MGMT has distinct tissue-specific variation in expression, with the highest levels of expression detected in the liver and colon [5,7]. The MGMT levels also vary remarkably between individuals [16], differing up to 8-fold, although the levels of expression in an individual appears to remain relatively constant over time [5]. Major and Collier, however, speculated that variations in the amount of MGMT between cells and individuals may be the result of variations in the protein degradation abilities via the ubiquitin pathway [7]. In tumours the expression of MGMT appears to be more variable, and some tumours have been reported to contain lower amounts of MGMT proteins than that detected in the surrounding tissue, while other tumours totally lack MGMT activity [3,5]. Loss of MGMT expression can be attributed mainly to *mgmt* promoter CpG island hypermethylation, as opposed to conventional deletions, mutations and rearrangements [3,5,13]. This epigenetic transcriptional regulation is suggested since mRNA levels usually parallel enzyme expression [7]. The methylation status of the *mgmt* promoter has been the focus of numerous studies, and hypermethylation was associated with colorectal cancer, brain cancer and oesophageal adenocarcinoma [17–19]. Bearzatto and colleagues [20] observed that in some cells, however, hypermethylation of its promoter did not result in decreased expression of MGMT, making this relationship between promoter methylation and transcription even more complex.

Ambiguity of MGMT induction

Reports in the literature are not consistent with reference to the inducibility of *mgmt* in mammalian cells. On the one hand it is seen as one of the best-studied inducible DNA repair enzymes in mammalian cells [5], and on the other hand there is doubt whether it is inducible or not, and to what extent it is inducible [9,21,22]. For instance, Pieper stated that in human cells, *mgmt* seemed to be “neither highly inducible nor repressible” [11], while Shrivastav and colleagues stated it to be “not inducible” [22].

Mammalian *mgmt* has been shown to be induced moderately (up to 6-fold) in rodents with parallel modulation in MGMT mRNA and protein [7,23]. Expression of the *mgmt* gene has been shown to be up-regulated in some models by a number of factors, including alkylating agents, ionizing radiation, DNA damage, partial hepatectomy and some genotoxins [5,9,24], however, the effectiveness of these agents show no universality [21]. This increased expression is the result of transcriptional stimulation, most probably by *mgmt* promoter activation, although the level of induction is modest and the exact mechanism is not clear yet [9,23]. The level of induction usually peaks between 24 and 48 h post-exposure, depending on the model, which suggests that the response is mediated through a cascade of reactions [9,23].

The level of modulation and the effectiveness of the inducing agents are highly variable and are influenced by, amongst others, the state of differentiation of the cells, species of organism and cell type [9,21]. For instance, it seems that some cultured human cells may be incapable of *mgmt* induction, even if the protein is expressed [9,23].

Why so peculiar?

The importance of MGMT in maintaining the genetic integrity of a cell, as described above, puts forward the anomaly of it having a mechanism of action which appears to run counter to its importance. The following discussion is an effort to elaborate on and, if only partially, contribute in unravelling these peculiarities to stimulate conversation on this enzyme.

Non-essential gene or crucial enzyme?

Whether MGMT can be described as a “true” enzyme at all remains controversial. The classic definition of an enzyme describes it as biological catalyst, increasing chemical reaction rates without any change in themselves [25]. Therefore, since MGMT simultaneously acts as the alkyltransferase and the alkyl acceptor, forming the chemically stable S-methyl-L-cystein derivative, the reaction cannot be described as strictly enzymatic [4,7,26]. Despite this, MGMT is considered to be a DNA repair enzyme, and its mechanism is therefore only viewed as such.

Since *mgmt* knockout mice appear to be viable, fertile, having a normal appearance and a normal lifespan, it is commonly stated that *mgmt* is a non-essential gene for viability [5,9]. However, since the persistent presence of O⁶-MeG in DNA results in at least six different negative endpoints, including cell death and tumour initiation [26], such knockout mice are more susceptible to toxicity, alkylation damage and its harmful effects [5,9,13]. This once again emphasizes the crucial role of this enzyme as a defence against the detrimental effects of DNA alkylation damage. In addition, MGMT is the sole defence mechanism to successfully rid a cell from O⁶-MeG lesions [5,27]. The mere existence of such a specific DNA repair system implies that endogenous and environmental exposures must be a permanent and significant burden in the biosphere [28], also highlighting the vital importance of sustained levels of this enzyme in the face of a sustained threat.

Suicide in the face of the enemy

Why such an important enzyme would have a gene expression and catalytic mechanism which apparently infringe on its primary function remains one of the most perplexing questions surrounding MGMT. This mechanism, that can indeed be called suicidal, is a mechanism of action which under normal circumstances can maintain its functionality, but under continuous pressure of the cell results in its own depletion and the subsequent persistence of the damage-causing adducts which it was supposed to remove. One can rightly ask whether this “deficiency” makes alkylating agents more dangerous than other similar chemicals targeting DNA.

It is still not clear whether *mgmt* is inducible in human cells, and to what extent. This in itself is an intriguing question which remains as yet unanswered. The next query presenting itself would then be if *mgmt* is in fact not inducible in human cells, why was this capability lost? Since the enzyme has been shown to be inducible in lower mammals, and the *Escherichia coli* homologue Ada is well-known to be highly inducible [12,22], it would be expected that the same is possible in human cells. Should *mgmt* be proven to be inducible in human cells to the same extent as in other species,

the question would remain why not all alkylating agents can induce *mgmt* to the same extent. The damage caused remains detrimental and needs to be removed with the same readiness, regardless of its origin?

Why so different from its associates?

The genome is not only under constant attack from alkylating agents, but also from oxidative sources. The lesions caused by the oxidative damage, including 8-oxo-7,8-dihydroguanine, 5-(hydroxymethyl)uracil and 5,6-dihydroxy-5,6-dihydrothymine, are similar to O⁶-MeG lesions given that they may not be directly lethal to the cell, but eventually could be highly mutagenic [29].

Unsurprisingly, there are repair mechanisms in place to deal with oxidative damage, such as the peroxiredoxin (Prxs) antioxidant enzymes, and oxidized Cys residues are specifically reduced by nuclear thioredoxins (Trx) [30]. The mechanism of action of MGMT becomes even more perplexing when the bifunctional nature of these Prx enzymes is taken into the arena of cell protection. Not only is the oxidation-induced Prx inactivation reversible, but under stress conditions the inactivated peroxidase can possibly act as a molecular chaperone in the cell [30].

One can therefore again ask why alkylated MGMT molecules are not re-activated in a similar manner if the analogous function is considered. Also, the suggested function of the inactivated Prx highlights the possibility of the altered MGMT molecule having unknown functionalities, as discussed later.

Calling in re-enforcements or protecting against the unknown – another perspective

A few explanations have been proposed to elucidate this perplexing behaviour of MGMT, though none is entirely satisfactory. These proposals include the notion of whether it may be energetically too expensive to remove the methyl group from the enzyme [3,13]. Another idea pertains to the removal of inactivated MGMT. Major and Collier proposed that the methylated MGMT protein is too rapidly ubiquitinated and degraded by the proteasome, prohibiting the reactivation by demethylation [7]. They also hypothesized that the conformational change of alkylated MGMT may be important for decreasing the amount of enzyme in the nucleus in order to recruit additional active MGMT molecules [7]. Also, Pegg and colleagues postulated that “The rapid removal of this inactive protein may be necessary to allow continued repair by other molecules of MGMT or the replacement of the molecules used up in the repair reaction” [31]. These thoughts therefore suggest that each MGMT molecule used must be removed to recruit a replacement molecule to continue the repair process.

We propose a different line of thought; that one possible “justification” for the rapid removal would be the establishing of an even more detrimental effect resulting from the sustained presence of alkylated MGMT. It has been reported that methylated MGMT can bind to oestrogen receptors, subsequently inhibiting its function as a transcription factor, although the physiological role of this is not clear [32]. Also, Lindahl and colleagues considered that the alkylated MGMT may have an undefined regulatory function which still needs to be elucidated [12]. We ask whether alkylated MGMT is degraded promptly to prevent such “unspecific” binding which could be detrimental to the cell by interfering with normal regulation of cellular processes. Also, can the limited upregulation of MGMT therefore be a preventative “defensive mechanism” against an overload of the ubiquitination pathway during the degradation of the inactivated MGMT; should the balance between synthesis and degradation be overextended?

Conclusions and prospects

In the literature there are reviews and publications regarding the properties and mode of action of the MGMT enzyme, which reflect the highly important function of this enzyme in cell survival. However, upon closer investigation, it became clear to us that there are still some conflicting findings and many questions which have not been addressed. One of these is the riddle why such an important enzyme would have a mechanism which apparently runs counter to its function.

We propose that the suicidal mechanism may have an as yet unknown biological function or it may possibly act as a protective mechanism against unknown detrimental effects of sustained high levels of altered MGMT in the cell. It is important to consider these questions, especially if these specific characteristics of the enzyme are to be exploited in research and in the development of chemotherapeutic drugs.

Our largely speculative thoughts regarding the peculiarities surrounding MGMT may be evaluated by further investigation of the effect of accumulating altered MGMT molecules on cellular integrity and functions. One possibility is to impair the degradation pathway or interfere with the ubiquitination of the alkylated molecule; however, it would be critical to avoid secondary effects of such an intervention. Other harmful molecules degraded through the same pathway may, for instance, also accumulate.

In conclusion, MGMT is an enzyme of paramount importance in the defence of the genome and is widely researched and used in the development of therapeutic drugs, once again highlighting its importance. However, its unusual mechanism of action does not appear to support its proposed function. We propose that not all functions of MGMT are known, and that this may have an impact on the way studies regarding this enzyme should be approached.

Conflicts of interest statement

The authors have no relevant conflict of interest.

Acknowledgements

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF. The study sponsor(s) played no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

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CHAPTER 3

THE DEVELOPMENT OF A HEREDITARY TYROSINEMIA TYPE 1 CELL CULTURE MODEL

3.1. Introduction and aim

As discussed in Chapter 1, developing a model for hereditary tyrosinemia type 1 (HT1) in cultured cells was deemed necessary to mimic the intracellular environment that is present in patients with this disease, and so allowing the study of the genetic and epigenetic changes which are present in a “true” HT1 environment. One of the objectives of this study was to establish this model via RNA interference (RNAi), using shRNA constructs which would inhibit the fumarylacetoacetate hydrolase (FAH) enzyme, mimicking the enzymatic defect observed in HT1 (Mitchell *et al.*, 2001). However, since low levels of this enzyme result in a near-normal phenotype, the knock-down has to be significant to result in a useful clinical phenotype, while a complete loss of activity of the FAH enzyme is fatal (Fernández-Cañón and Peñalva, 1995). Therefore, we decided to use a commercially produced inducible knock-down system, which can be controlled to establish a model which can be manipulated to yield cell lines representing a gradient of knock-down. This inducible HT1 model would then enable the observation of genetic and epigenetic changes over an extended period of time, in the presence of various degrees of this defect, seeing as different levels of FAH activity has been observed in HT1 patients.

3.2. Existing HT1 models

There are other HT1 models already described in the literature, including a fungal model and various mouse models. As alluded to in Chapter 1, these models and their shortcomings will now be discussed.

3.2.1. Fungal model

Aspergillus nidulans is an ascomycete which grows on, amongst others, phenylalanine to produce homogentisate, which in turn produces fumarylacetoacetate – the substrate for the FAH enzyme (Fernández-Cañón and Peñalva, 1995). This metabolic pathway is therefore similar to that of mammals, and Fernández-Cañón and Peñalva showed a 47% amino acid sequence conservation between the *A. nidulans* FAH, and its human homologue (Fernández-Cañón and Peñalva, 1995). They also showed that disruption of this gene resulted in succinylacetone production, illustrating that the fungus could be used as a model to study specific aspects of HT1. One such aspect would be toxicity studies of HT1-specific metabolites.

However, this model only provides a very basic tool, since it is based on a single cell system which lacks the complexity of the human metabolism. The cellular response to the accumulating

metabolite would also differ from actual HT1 hepatocytes, which would have an environment with several other amassed metabolites to interact with, or enhance the damage of, such as succinylacetone.

3.2.2. Worm model

Fisher and colleagues established that the same tyrosine catabolic pathway is present in *C. elegans* as in other eukaryotes (Fisher *et al.*, 2008). They also established the suitability of these worms as a model for HT1 through the knock-down of the K10C2.4 gene (a *FAH* homologue), which produced significant damage in the cells. They found that direct treatment of the worms with succinylacetone appeared to be toxic to the cells and activated the oxidative stress response pathways.

The advantages of this model lie in that it is a multi-cellular animal with most mammalian cell signalling and stress response pathways (Fisher *et al.*, 2008). The use of RNAi to control initiation of the disease also enabled some experimental flexibility. This model confirms that the use of RNAi to establish a disease model is practical and suitable, however, no further publications using this model were found in the literature.

3.2.3. Murine models

Various mouse models have been established for HT1, and these enabled studies of new treatment possibilities, including gene therapy (Grompe *et al.*, 1998, Russo *et al.*, 2001). A homozygous *Fah* deficient mouse model has been established through the disruption of its fifth exon, and extensive research has been done using this model (Grompe *et al.*, 1998, Orejuela *et al.*, 2008). The *Fah*^{-/-} mice are treated with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) to prevent neonatal fatality by inhibiting 4-hydroxyphenylpyruvate dioxygenase, and these treated mice survive into adulthood until the HT1 phenotype is induced through removal of the treatment (Grompe *et al.*, 1998, Nakamura *et al.*, 2007). Examples of studies using this model include the investigation of the AKT survival pathway activation in HT1 by Orejuela and associates (Orejuela *et al.*, 2008).

Aponte and associates recovered two *N*-ethyl-*N*-nitrosourea induced *Fah* gene point mutation mouse models based on the *Fah*^{-/-} mice (Aponte *et al.*, 2001). They found the *Fah*^{5961SB} mutation to mimic the acute form of human HT1, while the *Fah*^{6287SB} mice resembled the milder chronic phenotype. They proposed these models to be useful for therapy and diet assessment.

Manning and colleagues also developed a model based on the *Fah*^{-/-} mice, but aimed to rescue the lethal liver disease by incorporating *Hgd*^{aku} (Manning *et al.*, 1999). These *Fah*^{-/-}, *Hgd*^{aku}/*Hgd*^{aku} mice remained healthy with no NTBC treatment. This model is useful for further mutation studies.

A second mutant mouse strain which carries a FAH deficiency is the albino lethal c^{14CoS} mouse, developed by Glueckson-Waelsch in 1979 (Russell *et al.*, 1979, Grompe *et al.*, 1993). This strain has X-ray induced deletions of chromosome 7, and this results in lethality in this model a few hours after birth (Russell *et al.*, 1979, Grompe *et al.*, 1993). The mice have abnormal or absent expression of various hepatic enzymes, including FAH (Russell *et al.*, 1979, Klebig *et al.*, 1992,

Ruppert *et al.*, 1992, Grompe *et al.*, 1993). Although NTBC treatment of these FAH-deficient mice also avoids the early fatality, hepatocellular carcinomas still develop eventually and kidney damage is observed, indicating an incomplete suspension of the pathology (Nakamura *et al.*, 2007).

Since FAH-deficient mice have limited use, due to neonatal lethality and a different phenotype than HT1 patients, a double mutant mouse strain was created by Endo and colleagues, using the $c^{14\text{CoS}}$ mouse in which the *Hpd* gene was also mutated (Nakamura *et al.*, 2007). These *Fah*^{-/-}*Hpd*^{-/-} mice are viable, but the phenotype shows normal liver and kidney, with no carcinoma development (Nakamura *et al.*, 2007). This model would therefore not be suitable when studying this specific symptom and its development, as is the aim in this study. However, massive hepatocyte and tubular kidney cell death can be induced by a high dose of homogentisic acid (HGA) (Endo *et al.*, 1997, Kubo *et al.*, 1998).

When using models of non-human origin, unknown interspecies difference must be taken into account and care must be taken in extrapolating results between species (Mitchell *et al.*, 2001). For instance, the progression of HT1 in mouse models is significantly faster than in humans. Nevertheless, mouse models remain useful for pathophysiologic mechanism identification (Mitchell *et al.*, 2001).

3.2.4. Human cell culture model

Although cultured cells have been used to study HT1, and more specifically the effects of the accumulating metabolites, none of these models contained the *FAH* deficiency in its genome (Tanguay *et al.*, 1996, Jorquera and Tanguay, 1997, Jorquera and Tanguay, 1999, Van Dyk *et al.*, 2010). The alterations induced by the metabolites were assessed after direct exposure of the cells to the metabolites in solution (Jorquera and Tanguay, 1999, Van Dyk *et al.*, 2010). This approach, however, limits the time-span of the studies and it uses estimated concentrations of the metabolites and not the actual levels as produced in the cells. It may also not reflect all secondary or accumulating effects of the deficiency.

The use of cultured mammalian cells for a HT1 model has numerous advantages, but prior to RNAi technology development to enable such powerful gene activity perturbations, such a model was not possible (Echeverri and Perrimon, 2006). Through the use of HepG2 cells, a human HT1 cell model can be created, as opposed to less complex systems such as the fungal model. This will enable a more direct extrapolation of conclusions to human patients. Cell cultures are also easy to maintain, relatively cost-effective and it is simple to obtain sufficient amounts of material for analyses. The choice of HepG2 cells was based on their robust growth and because the liver is the organ most severely affected in HT1 (Mitchell *et al.*, 2001). Although the use of cancer cells for the model may be criticised, it was inevitable, since primary hepatocytes have limited availability, are problematic to culture and have a very limited number of cell divisions (Knasmüller *et al.*, 1998). Some primary cells have also been found to be unmanageable with standard transfection methods, such as lipid- or peptide-based reagents (Echeverri and Perrimon, 2006). HepG2 cells are also well characterised and have been used previously for knock-down experiments (Drobna *et al.*, 2006, Krones-Herzig *et al.*, 2006, Lin *et al.*, 2010), and HT1 studies (Jorquera and Tanguay, 1999, Van Dyk *et al.*, 2010).

3.2.5. Summary

To my knowledge, there are currently no human cell culture models available for hereditary tyrosinemia type 1, although validated commercially produced RNAi systems targeting *FAH* have recently become available (see Section 3.9). As discussed, such a cell model will have numerous advantages compared to previous fungal and mouse models, which makes it a worthwhile effort to establish such a model in HepG2 cultured cells.

It must be emphasized, however, that such a model would have to be inducible to ensure that the cell line can be established before the defect is “switched on” – very similar to the “rescue” of the mouse models (as discussed in Section 3.2.3.). The model should also contain stable knock-down of the *FAH* gene to ensure long-term evaluation of the model. It was decided to do this using a stable, inducible knock-down system from Clontech, which would enable the regulation of the level of knock-down achieved, and even its reversal. This, in turn, would facilitate long-term studies of the *in vivo* effects of the accumulating metabolite, since the knock-down can be controlled.

3.3. RNA interference

3.3.1. Overview

RNAi refers to a biological response to double-stranded RNA (dsRNA), which entails post-transcriptional gene silencing, brought about by this dsRNA, which induces destruction of a target gene’s mRNA – thereby silencing that gene (Hannon and Rossi, 2004, Echeverri and Perrimon, 2006, McIntyre and Fanning, 2006). Through a conserved endogenous cellular pathway, dsRNA and expressed dsRNAs (small hairpin RNAs or shRNA) are recognized by Dicer, a dsRNA-specific cytoplasmic RNase III, which then cleaves these molecules to produce smaller molecules called small interfering RNAs (siRNA) (Nykänen *et al.*, 2001, Hannon and Rossi, 2004, Echeverri and Perrimon, 2006, Lee and Sinko, 2006). An argonaute 2-containing RNA-induced silencing complex (RISC) is then assembled with the unwound siRNA strands (Echeverri and Perrimon, 2006). Through this process, one siRNA strand is selected as a guiding or antisense strand through thermodynamic lability principles (Nykänen *et al.*, 2001, Echeverri and Perrimon, 2006, Lee and Sinko, 2006). This guiding strand then targets its complementary mRNA sequence and marks it for a processive cycle of rapid degradation by RISC, leading to targeted gene silencing (Nykänen *et al.*, 2001, Hannon and Rossi, 2004, Sandy *et al.*, 2005, Echeverri and Perrimon, 2006, Lee and Sinko, 2006).

Gene expression can be silenced through several different mechanisms of short RNA production (Lee and Sinko, 2006). These include:

- Introduction of double stranded RNA into the cytoplasm – where it is cleaved by Dicer
- Direct introduction of chemically synthesized siRNAs – thereby sidestepping cleaving by Dicer
- Perfect duplex hairpin RNAs can be expressed and processed by Dicer to form siRNAs
- Imperfect duplex hairpin RNAs are processed into microRNAs.

When performing RNAi experiments in cultured mammalian cells, in order to choose the RNA molecule to target the mRNA and the method of its delivery, one must primarily consider the cell type and the extent of time the silencing is to be maintained (Sandy *et al.*, 2005). For this study, a small hairpin RNA (shRNA) knock-down system was chosen to target the *FAH* gene, since stable expression of the shRNA in the cell should enable continuous, long-term knock-down studies (Sandy *et al.*, 2005, McIntyre and Fanning, 2006). Using shRNA usually entails the design and production of appropriate target sequences in the form of two complementary oligonucleotides, which are then annealed and ligated into an appropriate expression plasmid or viral vector (McIntyre and Fanning, 2006). This plasmid or vector is then transfected into the selected cell line, in which the shRNA will then be expressed in the nucleus as a single-stranded RNA molecule which folds back on itself to produce a short hairpin which, after being processed, will silence the target mRNA (see Figure 3.2) (Lee and Sinko, 2006). There are several detailed reviews dedicated to this, and other shRNA construction methods and their advantages and disadvantages (Sandy *et al.*, 2005, McIntyre and Fanning, 2006).

3.3.2. The Clontech inducible knock-down system

The shRNA expression vector chosen for this study is part of the Knockout[®] inducible RNAi system (a double-stable inducible knock-down system) from Clontech. This system is based on one described by Gossen and Bujard, where a prokaryotic tetracycline repressor (*tetR*), providing DNA-binding activity depending on the presence of tetracycline, was fused with a transcriptional activating domain (TA) to form a transcription factor (tTA) (Gossen and Bujard, 1992). This tTA activates a minimal promoter by binding to a series of *tet* operator (*tetO*) sequences, which are tetracycline-responsive (Freundlieb *et al.*, 1999, Corbel and Rossi, 2002). Gossen and associates then developed a complementary system, where a reverse tetracycline controlled transactivator (rtTA) requires the antibiotic, doxycycline, to bind to *tetO* and thereby allows transcription (Gossen and Bujard, 1992, Gossen *et al.*, 1995, Corbel and Rossi, 2002). This system enables rapid induction, and gene activity can be regulated in a number of ways (Gossen *et al.*, 1995). Doxycycline (dox) is the most widely used tetracycline (small lipophilic compounds), and it penetrates eukaryotic cells by passive diffusion (Corbel and Rossi, 2002).

The Clontech system uses cell lines which are transfected with a vector enabling the cells to stably express a tetracycline-controlled transcriptional suppressor (tTS) (Freundlieb *et al.*, 1999). This enables the inducibility of the expression of the shRNAs contained in a separate expression vector, using the antibiotic dox to regulate expression from a tetracycline dependent mini-U6 promoter (see Figure 3.1) (Clontech, 2005).

The system enables long-term study of potentially potent or toxic gene suppression, since the concentration of dox can be varied to control the level of target gene expression, and even revert it to the uninduced state. To achieve this, a double-stable cell line must be established which stably expresses both the tTS regulatory protein and the shRNA. There are two vectors used in the system. The first is ptTS-Neo, a 6.4 kb expression vector which expresses a tTS regulatory protein (Clontech, 2006). The vector can be propagated and selected in bacteria, since it contains a bacterial origin of replication, and an *E.coli* Amp^r gene. Stable transformants can be selected due to the presence of a neomycin^r gene (Clontech, 2006). See Appendix A for a complete vector map. This vector is used to develop stable tet tTS cell lines. The second vector is a self-

inactivating retroviral expression vector, RNAi-Ready pSIREN-RetroQ-TetP which, under the control of the modified tet-responsive promoter, express a shRNA (Clontech, 2005). This vector can also be propagated and selected in bacteria, since it also contains a bacterial origin of replication, and an *E.coli* Amp^r gene, while stable transformants can be selected by the puromycin^r gene (see Appendix B).

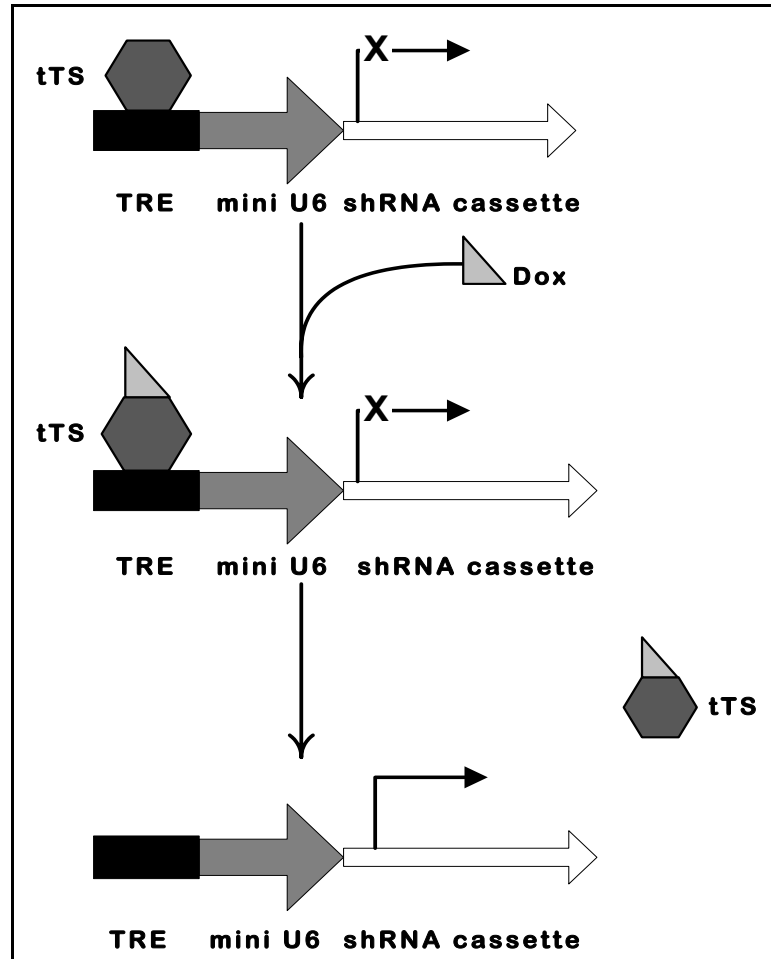


Figure 3.1. An illustration of the tetracycline-controlled inducible RNAi mechanism. In the absence of doxycycline (dox), stable tTS cell lines express the tTS regulatory protein. This protein binds in the Tet-Regulatory Element (TRE) of the response vector containing the shRNA, which inhibits transcription from the U6 promoter. Once dox is introduced to the cell line, it binds to the tTS molecule, causing it to dissociate from the TRE. Transcription is therefore no longer suppressed, and the shRNA can be expressed to silence the target gene (Adapted from (Clontech, 2005).

3.4. shRNA design and shRNA-pSIREN construct development

3.4.1. Introduction

Short hairpin RNAs (shRNAs) are double-stranded RNAs that contain 19- to 29-nucleotide sequences which are complimentary to the target gene mRNA, and is stably expressed from a cloned oligonucleotide template by an expression vector in the selected cell line (Clontech, 2005, Sandy *et al.*, 2005). These two RNA fragments are connected by a loop to enable formation of a stable, double-stranded stem-loop structure (see Figure 3.2) (Sandy *et al.*, 2005).

The shRNAs are synthesized *in vivo* from complementary DNA sequences, typically from Pol III promoters (Sandy *et al.*, 2005). The shRNA transcript has a distinct start site, and it contains a stretch of four or more thymidines (termination site), which after termination at position 2 results in 3'UU overhangs (Clontech, 2005, Sandy *et al.*, 2005). Post-transcriptional processing includes cleaving by Dicer to produce siRNAs which enters the RISC complex (Meister and Tuschl, 2004, Sandy *et al.*, 2005). Expressing the shRNA in such a manner results in a convenient and reproducible RNAi activation process in mammalian cell lines (Clontech, 2005).

3.4.2. shRNA design

There are various shRNA design tools available on the internet, and commercial constructs are available for numerous genes. For the *FAH* gene, however, no suitable or validated shRNA constructs were available at the start of this project, and it was therefore decided to use siRNA sequences designed by the Broad Institute, targeting the *FAH* gene (http://www.broadinstitute.org/rnai/public/gene/search_clones). They designed 21mer siRNA sequences for numerous genes and then performed all the bioinformatics evaluations needed for an RNAi experiment.

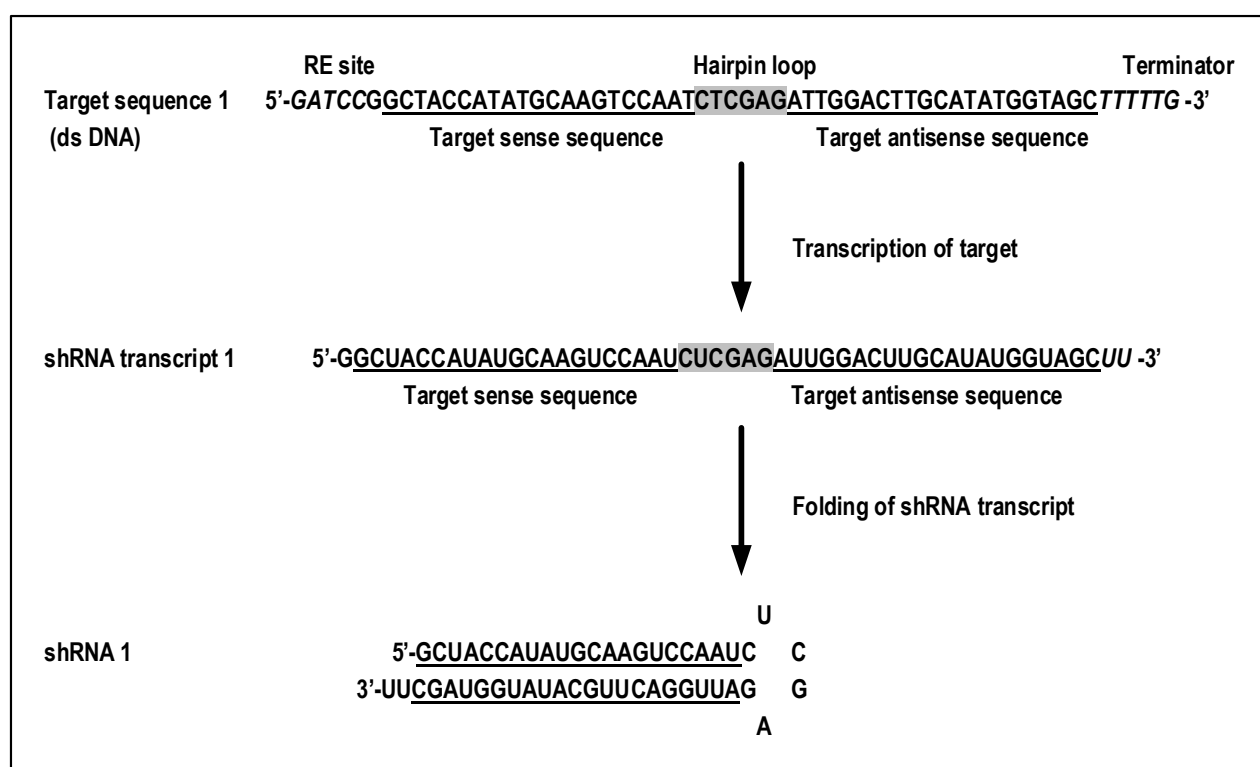


Figure 3.2. Generation of *FAH*-targeting small hairpin RNA construct 1 (*FAH*_shRNA1) using synthesized DNA oligonucleotides (Based on (Clontech, 2005)).

Although there were no validated shRNA sequences for silencing *FAH* available at the time, there were validated siRNAs available (Qiagen). The sequences of these siRNAs were not available unless purchased, however, the general location on the target sequence was indicated and those locations were matched with the target locations of the Broad institute siRNAs. They appeared to target the same region of the target mRNA. These siRNA sequences were adapted to make the ends compatible with the linearized vector, thereby changing the siRNA sequence to a shRNA

construct (see Table 3.1 and Figure 3.2). These 60mer oligonucleotides (with no phosphorylation) were synthesized and PAGE-purified by Sylvean Biotech, and subsequent analyses were performed upon receipt.

Table 3.1. Sequence information of the *FAH*-targeted shRNA oligonucleotides as adapted from the Broad Institute.

Oligonucleotide name	Sequence*
<i>FAH</i> _shRNA1_FRW	GATCCGGCTACCATATGCAAGTCCAATCTCGAGATTGGACTTGCATATGGTAGCTTTTTTG
<i>FAH</i> _shRNA1_REV	AATTCAAAAAGCTACCATATGCAAGTCCAATCTCGAGATTGGACTTGCATATGGTAGCCG
<i>FAH</i> _shRNA2_FRW	GATCCGGAGAGTGTTCCTTGCAGAACTTCTCGAGAAGTTCTGCAAGAACACTCTCTTTTTTG
<i>FAH</i> _shRNA2_REV	AATTCAAAAAGAGAGTGTTCCTTGCAGAACTTCTCGAGAAGTTCTGCAAGAACACTCTCCG
<i>FAH</i> _shRNA3_FRW	GATCCGCAGCATCATCAAGCACCTCTTCTCGAGAAGAGGTGCTTGATGATGCTGTTTTTG
<i>FAH</i> _shRNA3_REV	AATTCAAAAACAGCATCATCAAGCACCTCTTCTCGAGAAGAGGTGCTTGATGATGCTGCCG
<i>FAH</i> _shRNA4_FRW	GATCCGTGAAGTCATCATAACAGGGTACTCGAGTACCCTGTTATGATGACTTCATTTTTTG
<i>FAH</i> _shRNA4_REV	AATTCAAAAATGAAGTCATCATAACAGGGTACTCGAGTACCCTGTTATGATGACTTCACG

*The underlined sequences are the complementary target sequences, and the shadowed sequence forms the hairpin structure. The sequences forming the hairpin structure were designed to incorporate an *Xho* I recognition site - CTCGAG.

3.4.3. shRNA-pSIREN construct development

In this study, the shRNA templates were constructed by annealing two chemically synthesized DNA oligonucleotides to each other, resulting in a double-stranded fragment with overhangs complementary to the linearized RNAi-Ready pSIREN-RetroQ-TetP vector's ends. The fragments were then ligated into this expression vector. The commercially synthesized lyophilized shRNA oligonucleotides were resuspended in ddH₂O (Merck) to a final concentration of 100 µM. To anneal the two oligonucleotides for each fragment, 5 µl of each oligonucleotide of a pair were combined in a PCR tube, and incubated at 95 °C for 30 seconds, then 2 minutes each at 72 °C, 37 °C and 25 °C, after which it was incubated on ice. The annealed oligomers were diluted to approximately 0.5 µM and ligated directly into the RNAi-ready pSIREN-RetroQ-Tet-P vector with T4 ligase (Rapid DNA ligation kit; Fermentas), according to the manufacturer's instructions. All four *FAH*_shRNA constructs were synthesized in this manner.

The individual *FAH*_shRNA-pSIREN constructs were transformed into chemically competent JM109 *E.coli* cells (Promega), using a heat-shock at 42 °C, to propagate and purify the constructs. Following plasmid extraction with the PureYield™ plasmid midiprep system (Promega) as directed by the manufacturer, the constructs were screened via restriction endonuclease analysis with the enzyme *Xho* I (Fermentas). The empty vector would only be linearized once digested by the enzyme, while the correctly inserted *FAH*_shRNA fragments all contain a second recognition site for the enzyme. Therefore, constructs with the *FAH*_shRNA sequences correctly ligated will produce two fragments of approximately 1.2 kb and 5.3 kb upon digestion with *Xho* I. The electrophoretogram indicated several successful ligations of all four *FAH*_shRNA constructs (see Figure 3.3) and the sequence of one ligated product of each construct was confirmed with

sequencing by the Onderstepoort Veterinary Institute sequencing facility (see Appendix D). This step is important, since the frequency of false positives can be high in this method of shRNA construction (McIntyre and Fanning, 2006). Although this method of synthesizing two oligonucleotides and annealing them is relatively straightforward, the synthesis of such long oligonucleotides (50- to 70-nucleotides) is error prone (Sandy *et al.*, 2005).

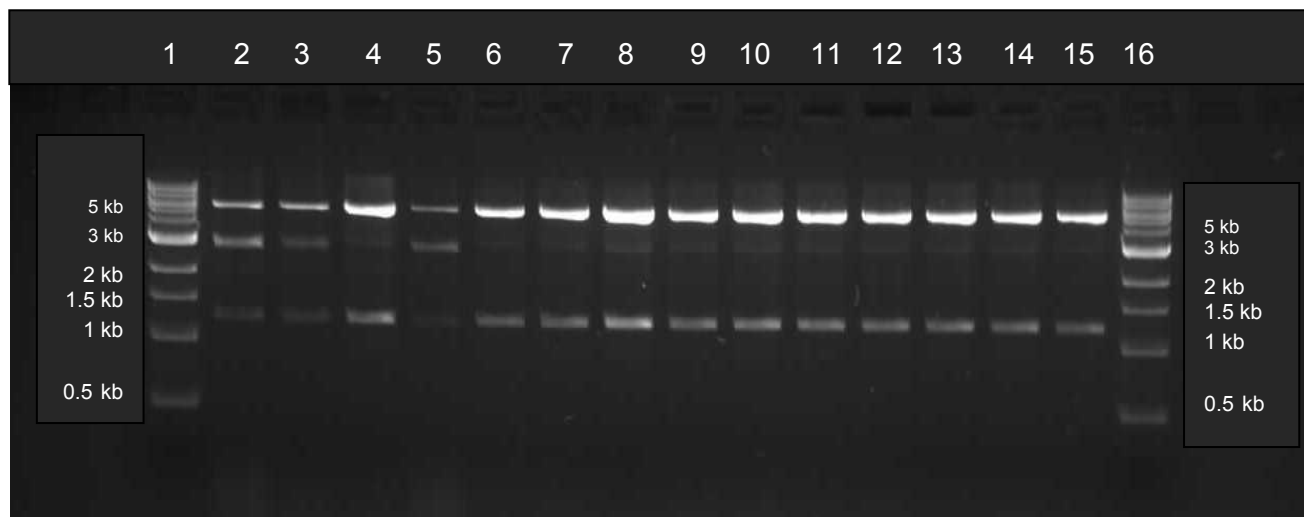


Figure 3.3. Electrophoretogram of restriction endonuclease digested pSIREN-RetroQ-Tet-P plasmid constructs containing *FAH*_shRNA sequences. Lanes 1 and 16 contain a 1 kb DNA ladder (New England Biolabs); lanes 2, 3 and 4 contain digested shRNA 1 constructs; lanes 5 to 8 contain digested shRNA 2 constructs; lanes 9 to 11 show digested shRNA 3 constructs and lanes 12 to 15 show digested shRNA 4 constructs. Electrophoresis was performed on a 1% agarose-TAE^e gel for 60 minutes at 80 V.

Unfortunately, some of the sequences obtained showed single nucleotide changes as is indicated in Appendix D. However, it was shown that sequence changes at some points in the shRNA sequence have no impact on knock-down potential, or can actually increase the knock-down efficiency of the construct (Czauderna *et al.*, 2003). Since miRNAs lacking perfect complementarity still recognize their targets, the same might apply to siRNAs (Hannon and Rossi, 2004). Also, the difficulty in the use of automated sequencing protocols to confirm the sequence of the hairpin region, remains a common drawback of constructing shRNA vectors, irrespective of the method used (McIntyre and Fanning, 2006). Therefore, we decided to continue with both the correct-sequence constructs and the constructs with one base change in the sequence.

3.4.4. Summary

In order to use the Knockout[®] inducible RNAi system, shRNA sequences specific to the target gene, *FAH*, were adapted from constructs designed by the Broad Institute. The altered oligonucleotides were annealed and ligated into the RNAi-ready pSIREN-RetroQ-Tet-P vector, and the subsequent constructs were propagated, purified and restriction enzyme digested to confirm successful ligation. The appropriate clones were then sequenced to determine if any mutations of the hairpin sequence occurred. In spite of some sequence changes, it was decided to continue with all four *FAH*_shRNA constructs, since some changes in the sequence may increase efficiency.

^e See appendix G for composition.

3.5. Preliminary optimization of cell culturing conditions

3.5.1. Cell culture and culturing conditions

The cell culture used for this study was human Caucasian hepatocyte carcinoma cells (HepG2), purchased from Sigma at passage 8. This cell line was chosen, since the liver is the most severely targeted organ in HT1 patients, and primary hepatocyte cell lines cannot be cultured indefinitely. The cells were propagated in Dulbecco's Modified Eagles Medium (DMEM) (HyClone medium; Thermo Scientific) with 10% (v/v) foetal bovine serum (FBS; Sigma), 1x MEM non-essential amino acids (Gibco), 2 mM [final] L-Glutamine (Gibco) and 1x penicillin/streptomycin (100 u pen./ml; 100 µg strep./ml; BioWhittaker). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were seeded in 6-well plates (Nunc) in 2 ml complete medium, unless stated otherwise.

3.5.2. Optimization of cell plating density and antibiotic concentrations

Prior to transfection, the optimal HepG2 cell plating density and antibiotic concentrations to be used for selection after transfection (G418 and puromycin) had to be determined. The optimal plating density was determined by plating between 100 000 and 400 000 cells in 6-well plates, and incubating these cells for 7 days. The optimal cell plating density was determined to be 200 000 cells per well of a 6-well culturing plate, 24 hours prior to the experiment, since this plating density resulted in the appropriate level of confluence after 3 days. The appropriate level of confluence entails that the growth surface of the well should be covered 60-80% with a mono-layer cells at the time of the experimental procedure. This ensures that transfection occurs in the logarithmic phase, and that selection will begin reducing the cells before the growth area is completely covered (which would result in contact inhibition and death of the cells). This plating density was subsequently used for the antibiotic kill curves.

Two antibiotics are used in the Knockout[®] inducible RNAi system; G418 (Sigma) to select the single-stable clones, and puromycin (Sigma) to select the double-stable cell lines. Antibiotic kill curves are necessary to determine the highest concentration of antibiotic that can be used for selection of a specific cell line, without having excessive levels of toxicity. Antibiotic kill curves were determined using concentration series ranging from 0 µg/ml to 1200 µg/ml for G418 (Figure 3.4) and 0 µg/ml to 6 µg/ml for puromycin (Figure 3.5). Cell death was estimated through visual approximation of confluence. The optimal selection concentrations were determined to be those which gave massive cell death within the first 5 days of selection, and complete cell death within two weeks (Clontech, 2005). However, the percentages given in Figures 3.4 and 3.5 are only approximate values as observed and not exact counts.

Although massive cell death was evident within 5 days at all G418 concentrations above 1000 µg/ml, complete cell death was only obtained with a concentration of 1200 µg/ml G418 (see Figure 3.4). The optimal concentration for selection with G418 in HepG2 cells was therefore taken as 1200 µg/ml. Massive cell death was present within 5 days at all puromycin concentrations above 0.5 µg/ml, however, 1.2 µg/ml and 1.5 µg/ml produced complete cell death within 4 to 6 days (see Figure 3.5). This indicated high levels of toxicity and these concentrations would not be suitable for selection. 1 µg/ml puromycin only induced complete cell death after

10 days, indicating acceptable levels of toxicity. The optimal concentration for selection with puromycin in HepG2 cells were therefore taken as 1 $\mu\text{g/ml}$.

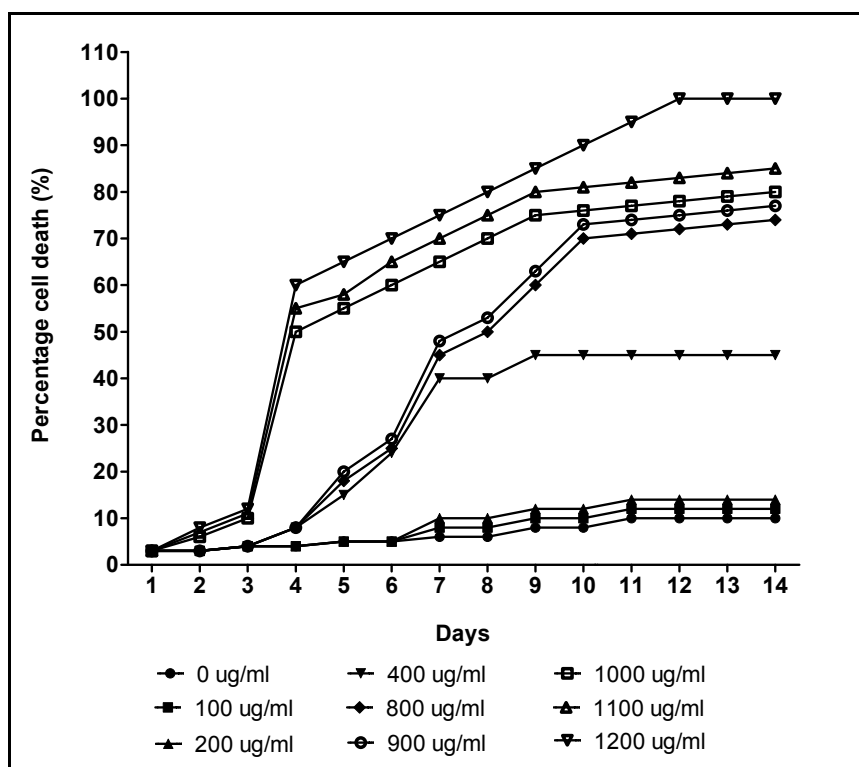


Figure 3.4. G418 antibiotic kill curves in HepG2 cultured cells. The number of days of incubation is represented on the X-axis, and the approximate percentage cell death on the Y-axis. Each line represents one concentration series of G418, as indicated.

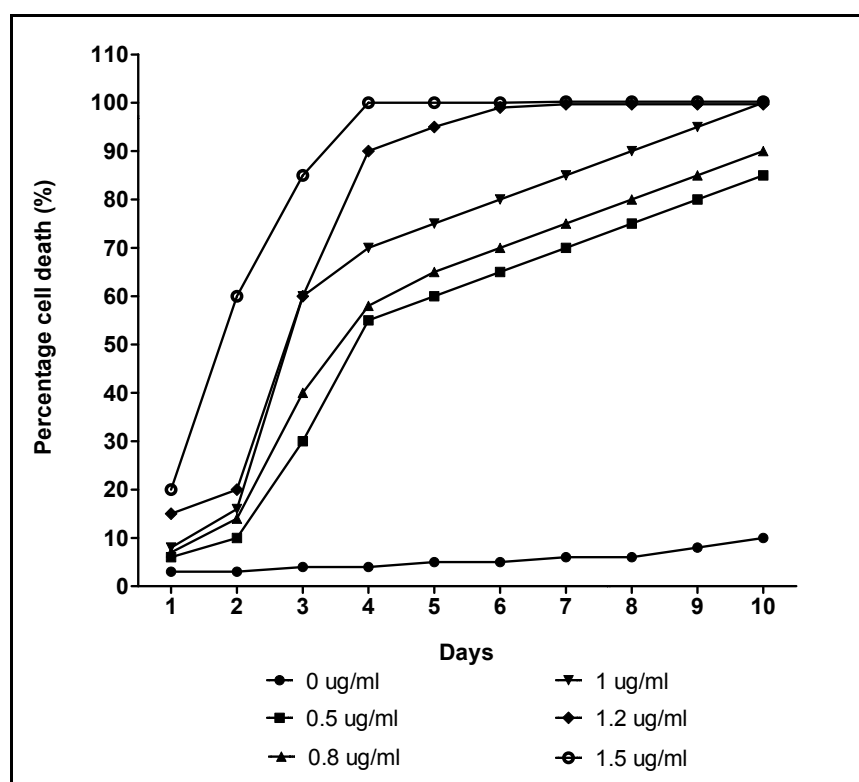


Figure 3.5. Puromycin antibiotic kill curves in HepG2 cultured cells. The number of days of incubation is represented on the X-axis, and the approximate percentage cell death on the Y-axis. Each line represents one concentration series of puromycin, as indicated.

3.6. Transfection optimization

3.6.1. Introduction

Transfection of a cell line entails the introduction of genetic material (usually by means of an expression vector) into cultured cells, using various chemical agents or electroporesis (Jordan and Wurm, 2004). The delivery method and protocol should be optimized for each cell type and cultivation condition to achieve the highest transfection efficiency possible (Jordan and Wurm, 2004).

3.6.2. Transfection with CalPhosTM reagent

The Knockout[®] inducible RNAi system user manual recommended the Clontech CalPhosTM transfection reagent to perform transfection of HepG2 cultured cells. Therefore, the initial transfections were performed with this reagent.

Transfections with the Clontech CalPhosTM transfection reagent were performed as prescribed by the manufacturer. This entailed plating 200 000 cells per well in a 6-well plate and incubating them overnight in 2 ml complete medium at 37 °C and 5% CO₂. The complete medium was then removed and substituted with DMEM containing no additives for 3 hours at 37 °C and 5% CO₂. Purified plasmid DNA (4 µg) was combined with 11.88 µl calcium-phosphate solution in a final volume of 120 µl (solution A). While 120 µl buffer HBSS (solution B) was mixed continuously, solution A was added very slowly. The mix was incubated at room temperature for 20 minutes, after which 240 µl of the mix was added very slowly to each well while gently mixing. After incubation for 5 hours at 37 °C, 2 ml DMEM containing 20% FBS was added to each well and incubated at 37 °C and 5% CO₂ for 12 hours. Each well was then washed with PBS and complete media was added.

The Clontech CalPhosTM transfection reagent uses calcium phosphate transfection, and is based on the formation of a calcium-DNA complex which precipitates on the surface of the cells by nucleation or by particle growth, and is ingested via endocytosis (Jordan and Wurm, 2004). The size of the complex crystals directly influences the transfection efficiency, as shown in Figure 2 in Jordan and Wurm, and the mixing procedure directly affects the precipitate size and structure (Jordan and Wurm, 2004).

It was observed that the antibiotic selection of the transfected cells did not result in massive cell death. Since the transfection reagent complexes form a layer of crystals covering the cells in the culture flask, it was proposed (assumed) that this layer could have interfered with the selection. It was also found that the transfection efficiency would be insufficient for this study. Transfection efficiency is an indication of the number of cells successfully transfected compared to the total number of cells present at the time of the transfection. This can be detected using a reporter, such as a vector expressing the pEGFP gene (Clontech, 1999) (see Appendix C), visible as a green signal when viewed with a fluorescence microscope (excitation maximum = 488 nm).

It is described in literature that the crystal complexes could be dissolved after transfection using 5 mM ethylene glycol tetraacetic acid (EGTA; Sigma) (Jordan and Wurm, 2004). This was applied

in these experiments by washing the cells 12 hours post-transfection with EGTA, prior to initiating selection with the relevant antibiotic. This, however, did not increase selection efficiency. It was therefore decided to use a different transfection reagent, since the CalPhosTM reagent did not produce sufficient transfection results.

3.6.3. Transfection with the FuGENE[®] 6 reagent

The FuGENE[®] 6 transfection reagent (Roche) consists of multiple components, including lipids, which forms complexes with the DNA to be transfected. These complexes are then transported into mammalian cells (Roche, 2006).

The transfection protocol was adapted from the instructions of the manufacturer, and entailed plating 200 000 cells per well in a 6-well plate and incubating them overnight at 37 °C and 5% CO₂ in 2 ml complete medium. The medium was removed, and the cells were washed with phosphate-buffered saline (PBS; Sigma). DMEM with no additives (2 ml) was added to each well, and incubated 2-4 hours at 37 °C and 5% CO₂. DMEM with no additives (92 µl) was placed in a microcentrifuge tube and 8 µl of the FuGENE[®] 6 reagent was added without touching the tube sides. The reagents were mixed and incubated at room temperature for 5 minutes. Purified plasmid DNA (2 µg) was then added, and mixed well. After the mixture was incubated at room temperature for 15 minutes, it was pipetted slowly into each well, while mixing gently. The cells were incubated 4 hours, and subsequently 2 ml DMEM containing 20% FBS was added to each well. They were then incubated overnight at 37 °C and 5% CO₂.

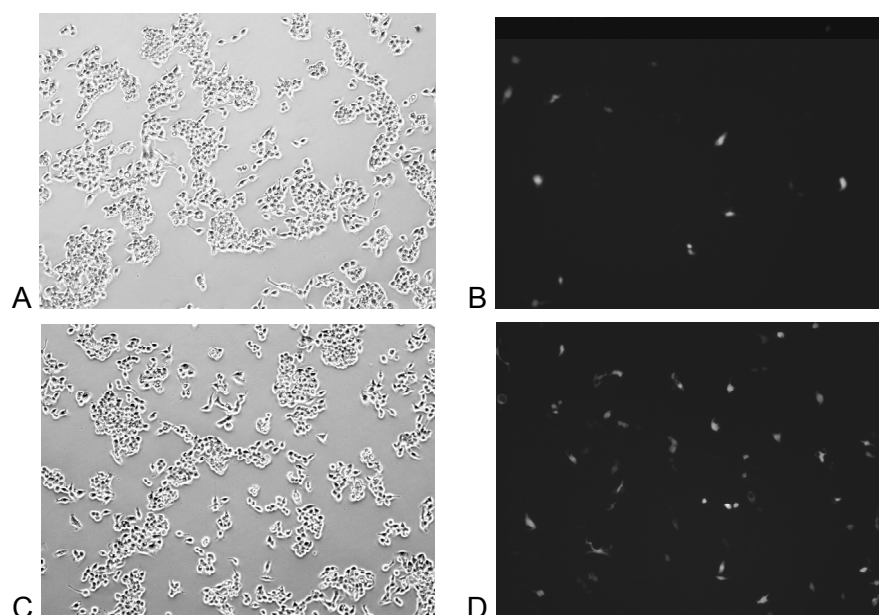


Figure 3.6. FuGENE[®] transfection efficiency optimization using a pEGFP expression vector. In A and C, all the cells present at the time can be seen; B shows fluorescence when a 6:2 ratio was used, while in D, an 8:2 ratio was used. 10x magnification, Nikon Eclips TE2000-s, Nikon DXM 1200C camera.

Various reagent:DNA ratios were investigated to determine the optimal conditions for transfection. The pEGFP-N1 expression vector was used again to determine the transfection efficiency, and expression of this gene was visualized with a fluorescence microscope (see Figure 3.6). An 8 µl

FuGENE® 6 and 2 µg plasmid DNA ratio (8:2) was determined as most efficient when compared to other ratios.

Even the most efficient transfection ratio did not result in sufficient levels of transfection (approximately 20%), leaving a great percentage of cells untransfected (as shown in Figure 3.6). This would result in inconsistent transient transfection results and an inadequate number of colonies after selection for stable transfected cells. To achieve the optimal amount of transfected cells, and subsequent stable cell lines, it was decided that a transfection efficiency of at least 50% was required. Therefore, a different transfection reagent was used.

3.6.4. Transfection with the ExGen 500 reagent

Transfection with ExGen 500 (Fermentas) is based on the protonation profile of aprotogenic polyethylenimine (PEI), which can increase from 20 to 45% due to the high amino nitrogen content which can be protonated (Fermentas, 2004). The reagent interacts with the DNA to form small, diffusible particles. This condenses and protects DNA before interacting with the cell membrane proteoglycans. The complexes are ingested through endocytosis, where the “proton sponge” effect causes endosomal swelling and rupture to prevent lysosomal DNA degradation. The DNA then translocates to the nucleus.

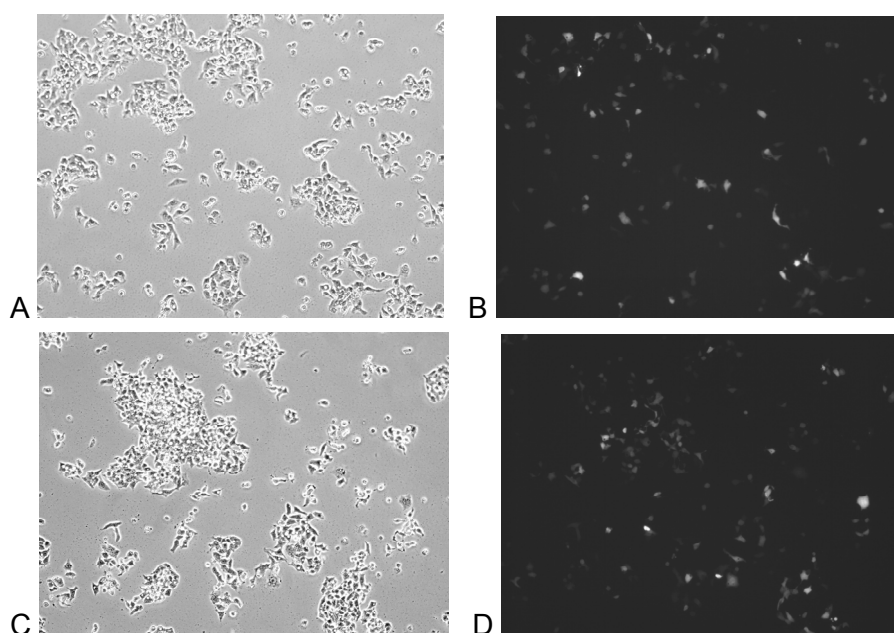


Figure 3.7. pEGFP fluorescence following transfection with ExGen 500. In A and C, all the cells present at the time can be seen, while the pEGFP signal from successfully transfected cells can be seen in B for an ExGen 500 ratio of 1:5 and D for an ExGen 500 ratio of 1:6. 10x magnification, Nikon Eclipse TE2000-s, Nikon DXM 1200C camera.

The transfection protocol prescribed by the manufacturer was adapted to the following: 200 000 cells were plated per well in a 6-well plate with 2 ml complete medium each, and incubated overnight at 37 °C and 5% CO₂. Once the complete medium was replaced with 2 ml DMEM containing 10% (v/v) FBS and no antibiotics, 3 µg purified plasmid DNA was diluted to a volume of 200 µl with 150 mM NaCl (Sigma) and vortexed. ExGen 500 reagent (8.23 µl) was added and vortexed immediately for 10 seconds. The mixture was incubated at room temperature

for 10 minutes, and subsequently 200 μ l of the mixture was added to each well and the plate was shaken lightly to mix. The plate was centrifuged at 280g for 5 minutes, and then incubated overnight at 37 °C and 5% CO₂.

A transfection efficiency of more than 50% was achieved with the ExGen 500 reagent (see Figure 3.7). When compared with the FuGENE[®] 6 reagent it was clear that the ExGen 500 reagent had significantly better transfection efficiency, with a much more efficient workflow, and it was seen that no significant difference in efficiency was observed between a 1:5 and a 1:6 ratio for the ExGen 500 reagent. It was therefore decided to proceed with this transfection reagent, using a 1:5 ratio.

3.6.5. Summary

Three different transfection reagents, based on three different transfection mechanisms, were tested. Although FuGENE[®] 6 had much higher transfection efficiency than the CalPhos[™] reagent, it was still unacceptably low (approximately 20%). ExGen 500, however, produced a much higher transfection efficiency (more than 50%), using a very efficient work-flow, and was therefore chosen as the optimal reagent to use in this study.

3.7. Development of tet tTS stable cell lines

3.7.1. Introduction

For this study, it was chosen to do consecutive transfections, rather than simultaneous transfection of the regulatory and response vectors needed for the inducible knock-down system. Performing consecutive transfections have the advantage of avoiding co-integration of the two plasmids. Consecutive transfections thus enable selection of the double-stable inducible RNAi cell line with the lowest background shRNA expression (Clontech, 2005). The first vector transfected into the HepG2 cell line was the tTS regulator plasmid, ptTS-Neo (see Appendix A). This vector provides resistance to neomycin, and therefore selection can be performed in the presence of G418.

3.7.2. HepG2 cell transfection with the ptTS-Neo vector

To obtain a single-stable HepG2 tTS cell line, HepG2 cells were transfected with the ptTS-Neo vector, followed by selection with 1200 μ g/ml G418. Growth media containing G418 was replaced every second day. After massive cell death was obtained, transfected resistant cells were maintained in the presence of G418 until they formed individual colonies. These colonies were isolated and propagated to establish new, individual HepG2 tTS cell lines. The aim was to generate a stable “tet” cell line which would provide a high level of tTS expression, and since this can be affected by the site of integration it is recommended by the supplier to select more than one clone for screening (Clontech, 2005). Four HepG2 tTS cell lines were established, but only two were used for the subsequent transfections, hereafter referred to as HepG2 tTS 10 and HepG2 tTS 12. It was observed that these two cell lines showed normal cell doubling time, while the other two cell lines had limited cell proliferation.

3.7.3. Transient *FAH* knock-down experiments

To screen the shRNA constructs before developing the double-stable cell lines, it is recommended by the supplier to perform transient transfections to measure the degree of knock-down achieved (Clontech, 2005). Transient transfections refer to the short-term evaluation (72 hours) of the knock-down achieved after transfection. This method does not contain a selection phase, and therefore an average result of the varying knock-down efficiencies in the cell population is measured. It should provide a general indication of the knock-down potential of each construct.

The transient transfection knock-down experiments were performed in untransformed HepG2 cells. The change in expression levels were measured 72 hours after transfection as follows: RNA was isolated from each well and cDNA synthesis performed (methods described in Section 4.6). Expression of the *FAH* gene was measured using relative quantification real-time PCR (qPCR) and the percentage knock-down achieved was calculated from the difference in expression between the samples relative to the normal, untransfected HepG2 cell line (see Section 4.7 for methods).

Since there is no ptTS-Neo vector encoding the regulatory protein expressed in the normal, untransformed HepG2 cells, transfection with the shRNA expression vector should result in the unregulated expression of the shRNAs. Therefore, the transient transfections should give an indication of the knock-down potential of each construct. The level of knock-down will, however, differ with each experiment due to the variation in transfection efficiency, as well as the variation in knock-down efficiency between cells. This variation is most probably due to different integration points of the shRNA construct in the cell genome (Clontech, 2005).

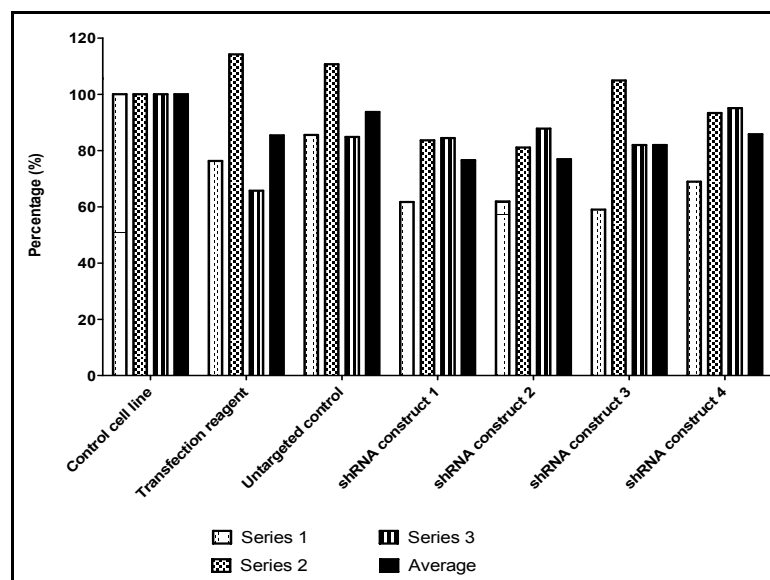


Figure 3.8. Changes in expression of *FAH* in HepG2 cells following transient transfections with the various shRNA constructs. Three repeats (and their average) of the transient knock-down experiments for each shRNA construct are compared to the control cell line, as well as a transfection reagent control and an untargeted control. For calculation purposes, a value of 100% was assigned to all control cell line samples.

As mentioned earlier, this variation in the expression of the *FAH* gene was observed in the results obtained (Figure 3.8). The level of expression for each construct differed between repeats, and these repeats also did not show a uniform trend in the increase or decrease in expression. The

level of knock-down of the *FAH* gene was also insufficient, with the highest percentage knock-down achieved being 40%. This does not necessarily indicate a lack of knock-down potential, since the level of knock-down in each cell differs, and only the mean expression across the total cell population is measured. When comparing the mean expression of the constructs with the untransformed control, all four shRNA constructs did result in some degree of *FAH* knock-down, with a knock-down of approximately 35% in series 1. However, comparing the expression levels to the transfection reagent control, different results are obtained. It could be argued that this is the true control for the experiment, and that all results should be viewed relative to the expression values of the transfection reagent control. But, as shown in Figure 3.8, the gene expression levels of *FAH* in these controls are rather inconsistent, differing with more than 40% in some cases. This could implicate some direct effect of the transfection reagent, or the transfection process itself, on gene expression levels in the cell cultures. Taking into account that the growth medium was changed only once between transfection and the expression assay, and no subculturing took place in this timeframe, it can be suggested that this effect would not impact the development of the double-stable cell lines. It does, however, appear to influence the results in transient knock-down experiments, since we observed marked variations in our results (see Figure 3.8). This underlines the limitation of transient knock-down experiments as an indicator of potential knock-down efficiencies due to variability, as discussed in this section.

It is to be expected that the level of knock-down in some of the double-stable inducible *FAH* knock-down HepG2 tTS cell lines would be higher than the levels obtained in this transient knockdown experiments, since individual transfected cells will be propagated to establish new double-stable cell lines. These cell lines will then be screened to find one with a suitable level of gene knock-down. One would, therefore, not measure the mean of different knock-down variations, but the actual knock-down potential of each clone.

3.7.4. Summary

Four single-stable HepG2 tTS cell lines were established successfully after transfection with the pTTS-Neo vector, and subsequent selection with G418. Two of these cell lines would be used for further transfections. Transient transfections were performed with the four shRNA constructs in an effort to identify the constructs with the most knock-down potential. The results of the transient knock-down experiments varied greatly though, and this was attributed to the variations in knock-down efficiency between the cells in the population. It was envisaged that higher and more consistent knock-down would be achieved once the double-stable cell lines have been established.

3.8. Development of a double-stable tet-inducible *FAH* knock-down cell line

3.8.1. Establishing individual colonies

To develop an inducible, double-stable HepG2 cell line with the *FAH* gene knocked down, only two of the four *FAH*_shRNA-pSIREN constructs were chosen to develop the double-stable cell lines. The *FAH*_shRNA-pSIREN_1 construct was chosen, since it showed the highest percentage knock-down in the transient transfection experiments (see Figure 3.8), while the *FAH*_shRNA-pSIREN_4

construct was chosen, since its sequencing indicated that it had no nucleotide changes from the designed sequence (see Appendix D).

Two of the single-stable HepG2 tTS cell lines (HepG2 tTS_10 and HepG2 tTS_12) were transfected with the two *FAH*_shRNA-pSIREN constructs. Following selection with 1 µg/ml puromycin, transfected cells were maintained in the presence of puromycin until single colonies developed. These were propagated into individual cell lines to be screened for potential inducible *FAH* knock-down.

3.8.2. Screening for knock-down efficiency in new double-stable cell lines

Each colony was manually transferred from the selection wells to individual 24-well plate wells for propagation. Once each new cell line colony was propagated to one confluent 6-well plate well, it was prepared for screening. The cell line was divided into three 6-well plate wells, of which one well was cultured further (in maintenance antibiotic concentrations of 200 µg/ml G418 and 0.2 µg/ml puromycin) and the other two were used for screening. These two wells were incubated for 3 hours in DMEM containing 10% FBS (no antibiotics) before adding 1 µg/ml doxycycline (dox; Sigma) to one of the two wells (induced). The medium and dox of each well was changed every 24 hours. RNA was isolated from each well 72 hours after induction, and cDNA synthesis performed (see Section 4.6). Expression of the *FAH* gene was measured using relative qPCR, and the percentage induced knock-down achieved was calculated from the difference in expression between the induced and uninduced samples (see Section 4.7).

3.8.3. Results and discussion

In total, 33 colonies were screened for *FAH* knock-down efficiency, including colonies transfected with an untargeted control-pSIREN construct and a pSIREN construct targeting a luciferase gene (unrelated control), the results of which are summarized in Table 3.2. The values in the table represent the normalized percentage decrease in *FAH* expression by each clone, relative to each of the untransfected single-stable control tTS cell lines, 10 and 12, respectively. The percentage expression of *FAH* by these two cell lines were taken as 100%.

The results in Table 3.2 indicated several clones with induced knock-down percentages of more than 70% in the HepG2 tTS 10 cell line. The HepG2 tTS 10 *FAH* 1/24 clone even achieved an induced *FAH* knock-down of more than 80%. The lowest induced knock-down potential was 1.1% in the HepG2 tTS 12 *FAH* 1/8 clone. However, there were some cell lines in which a marked increase in *FAH* expression was observed after induction.

Upon closer inspection though, it became clear that there was almost no induction of *FAH* knockdown measurable when comparing the percentage knockdown before and after induction. Since the percentages differed with less than 6% in the HepG2 tTS 10 cell line and with less than 23% in the HepG2 tTS 12 cell line, it is clear that induction did not result in increased knock-down. It was also observed in both the untargeted and unrelated control clones that the level of *FAH* knock-down observed was comparable with the knock-down observed in the *FAH*-targeting clones. This suggests that the decrease in expression of *FAH* was not a direct result of the targeted shRNA knock-down process, but rather a general consequence of the clone construction.

Table 3.2. Percentage *FAH* knock-down achieved in the propagated double-stable, inducible knock-down HepG2 tTS cell lines.

Colony name	Uninduced percentage knock-down*	Induced percentage knock-down [#]
HepG2 tTS 10 <i>FAH</i> 1/1	67.19826	70.35395
HepG2 tTS 10 <i>FAH</i> 1/3	74.41597	74.03733
HepG2 tTS 10 <i>FAH</i> 1/24	77.77434	81.82767
HepG2 tTS 10 <i>FAH</i> 1/25	72.26173	70.03106
HepG2 tTS 10 <i>FAH</i> 4/2	n.a.	44.65144
HepG2 tTS 10 <i>FAH</i> 4/3	84.22175	79.54614
HepG2 tTS 10 <i>FAH</i> 4/4	n.a.	53.01912
HepG2 tTS 10 <i>FAH</i> 4/5	74.11369	-302.388 [!]
HepG2 tTS 10 <i>FAH</i> 4/6	76.21594	78.43477
HepG2 tTS 10 untargeted control 3	63.46808	69.19634
HepG2 tTS 10 untargeted control 4	48.47537	43.98076
HepG2 tTS 10 untargeted control 6	72.05762	74.5802
HepG2 tTS 10 untargeted control 11	59.47672	65.19175
HepG2 tTS 10 unrelated control 1	n.a.	73.99721
HepG2 tTS 10 unrelated control 3	n.a.	77.51653
HepG2 tTS 10 unrelated control 7	70.50928	72.16626
HepG2 tTS 10 unrelated control 10	n.a.	73.70422
HepG2 tTS 12 <i>FAH</i> 1/1	17.46378	22.93276
HepG2 tTS 12 <i>FAH</i> 1/6	20.99562	25.73973
HepG2 tTS 12 <i>FAH</i> 1/8	49.3211	1.155634
HepG2 tTS 12 <i>FAH</i> 1/9	53.41063	38.39396
HepG2 tTS 12 <i>FAH</i> 4/1	22.28088	44.57588
HepG2 tTS 12 <i>FAH</i> 4/2	-7.89499 [!]	13.46238
HepG2 tTS 12 <i>FAH</i> 4/12	-99.6941 [!]	-106.614 [!]
HepG2 tTS 12 <i>FAH</i> 4/13	-0.96041 [!]	-0.30428 [!]
HepG2 tTS 12 <i>FAH</i> 4/15	56.3708	-209.508 [!]
HepG2 tTS 12 <i>FAH</i> 4/16	31.70156	42.23927
HepG2 tTS 12 <i>FAH</i> 4/22	47.77558	57.70516
HepG2 tTS 12 <i>FAH</i> 4/24	27.01113	32.71763
HepG2 tTS 12 <i>FAH</i> 4/29	-5.08139 [!]	-12.482 [!]
HepG2 tTS 12 untargeted control 5	n.a.	-4.87038 [!]
HepG2 tTS 12 unrelated control 7	n.a.	14.78048
HepG2 tTS 12 unrelated control 3	24.8939	47.16586

* Expression level of the uninduced sample relative to the untransfected, single-stable control cell line.

[#] Expression level of the dox-induced sample relative to the untransfected, single-stable control cell line.[!] A negative value indicates an upregulation in expression, i.e. higher than the control sample.

n.a. – not available. Amplification failed.

The results did raise the question of whether some type of induction “leakage” of the shRNA could be present in the system. This would explain why, in most cases, the difference in expression before and after induction were almost negligible. One possible cause of such a leak would be the presence of tetracycline in the serum used to culture the cells. Therefore, we performed an experiment using certified tet-free serum (Gibco), comparing it to the serum normally used. As is evident from Figure 3.9, the tet-free FBS made no significant difference to the uninduced level of expression of *FAH*, which remained lower than the reference cell lines. The level of expression after induction was also comparable with the expression level measured using the uncertified FBS. These results indicated that the down-regulation of *FAH* expression was not caused by a “leaky” expression of the shRNA due to the presence of tetracycline in the serum. It is, however, not clear whether the down regulation was a direct effect of shRNA expression, or simply an overall effect of the transfection/selection process itself.

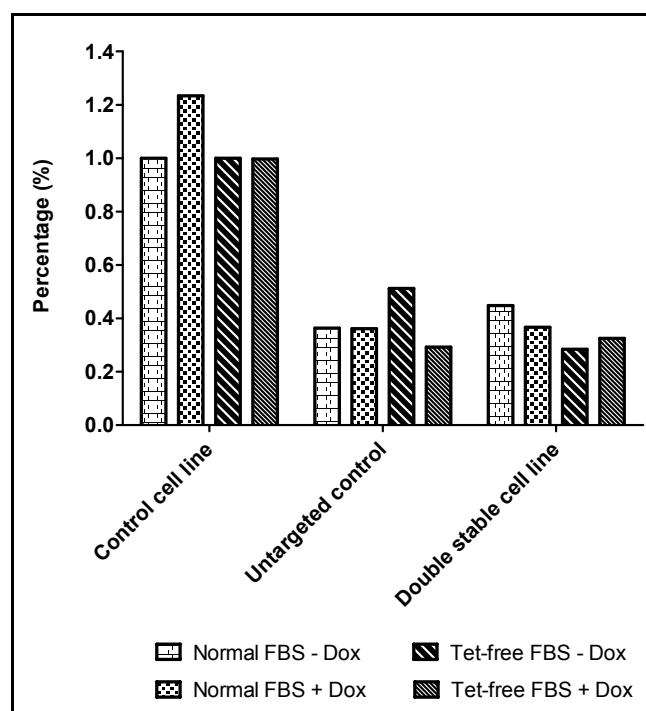


Figure 3.9. Changes in expression of *FAH* in HepG2 cells before and after induction, in the presence of normal FBS or certified tet-free FBS. All samples were normalized relative to the normal FBS – Dox sample.

The fact remains that in some of the clones markedly reduced levels of *FAH* expression was observed, some sufficiently enough to be useful in this study. Therefore, it was decided to propagate those cell lines with 30% *FAH* expression or less, to further characterize them. However, these cell lines proved to be problematic upon culturing. Some of the lines aggregated in clumps, and even after several trypsinization attempts the aggregations could not be separated into individual cells, or cultured in a monolayered fashion. These clump-forming tendencies resulted in a continuous contact-inhibition environment, which eventually resulted in cell death. Other clones simply ceased to proliferate after 8-10 passages (from colony selection), showing symptoms of severe distress in their morphology, while others could not withstand the trypsinization process itself. None of the cell lines selected for propagation could be cultured successfully.

3.8.4. Summary

From the results described in Section 3.8.3., it is clear that the model, as established at this time, is not yet suitable for the purposes it was intended for in this study. In the literature it has been suggested that even a low level of *FAH* gene-activity eliminates the HT1 phenotype (Fernández-Cañón and Peñalva, 1995) and, therefore, the level of knock-down achieved in several clones was not sufficient, and in the instances where it was sufficient enough, the cell lines were not viable. In the literature it was observed that primary hepatocytes from HT1 patients also divide poorly (Vogel *et al.*, 2004), which could suggest that the degree of control over the level of knock-down achieved was insufficient in the established cell lines. Since the induction part of the system appears not to be fully functional these cell lines can, therefore, not be “rescued” through the inhibition of the knock-down. The reason for the decrease in *FAH* expression remains unclear, and it could be a direct result of the presence of the shRNAs targeting the gene, or a different, unidentified factor could be involved. Should such an unknown factor be the cause, it would need further characterization of the model to establish other possible effects, including untargeted gene expression alterations.

3.9. Conclusion

One of the aims of this project was to establish a stable, inducible knock-down model for HT1 in cultured human cells. Four single-stable HepG2 tTS cell lines were established, of which the two with the best proliferation rates were used for subsequent transfections. Transient transfections of HepG2 cells with four *FAH*-targeting shRNA-pSIREN constructs produced inconsistent results, with very limited knock-down achieved. However, after transient transfections, only the average effect in all the cells are measured and, therefore, it does not show the optimal potential obtainable once individual cells are selected and propagated. Two of these *FAH*_shRNA-pSIREN constructs (1 and 4) were chosen for transfection into the two HepG2 tTS cell lines (10 and 12), followed by selection and propagation of individual double-stable inducible cell lines. These clones were screened to determine the level of knock-down achieved. Very promising levels of *FAH* knock-down were measured in several clones. However, the control constructs showed comparable levels of *FAH* expression reduction, suggesting an untargeted effect of the selection and propagation process.

In its current form, the model is not yet suitable for long-term studies of HT1, and I propose that the system should be refined. Also, some companies (OriGene, Sigma) made validated shRNA constructs targeting *FAH* recently available, and it may be worthwhile to invest in such constructs. Establishing this model would be of great importance for studies of this kind, and RNAi is the most effective method to establish such a model, as shown by Fisher and colleagues in the *C. elegans* model.

The double-stable cell lines established with high levels of *FAH* knock-down had some growth difficulties. This could be a direct result of the decrease in *FAH*, causing the accumulation of toxic metabolites. It could also be a result of untargeted gene expression alterations; it is therefore necessary to characterize these cell lines further to determine the origin and effect of the knock-down achieved. One option is to obtain culture medium containing no tyrosine or phenylalanine to

propagate the cell lines, much like diet restriction in patients (Mitchell *et al.*, 2001). This would hamper the accumulation of harmful metabolites, should the decreased FAH be the cause of the cell death. A second option would be to treat the cells with NTBC, much like the “rescue” of the mouse models. NTBC was, however, impossible for us to obtain.

The aim of this study was also to evaluate the model at each step in the production process to ensure that the process itself did not influence the characteristics of the cell culture pertaining to the study of HT1. This was important since previous studies have indicated that the process of transfection itself, as well as the introduction of a vector to a cell line, induced changes in DNA methylation on a global scale (Du Toit, 2010). This evaluation would have been achieved by performing all the analyses that was to be performed once the model was established, after each manipulation of the cell line. This was, however, not possible since the double-stable cell lines could not be propagated.

Concurrent with this part of the study, a direct exposure model was used to establish the abovementioned assays. This model entailed the direct exposure of cultured HepG2 cells with individual metabolites as previously reported in literature and done in our laboratory with great success (Jorquera and Tanguay, 1997, Jorquera and Tanguay, 1999, Van Dyk *et al.*, 2010). These experiments will be described in more detail in Chapters 4 and 5.

CHAPTER 4

ESTABLISHING ASSAYS TO MEASURE EPIGENETIC ALTERATIONS IN CULTURED CELLS

4.1. Introduction and aim

In several inborn errors of metabolism (IEMs) associated cancers can develop, probably as a result of numerous genetic and epigenetic alterations. The specific cause for each alteration and its subsequent result may give great insight into the associated pathologies and their treatment. As discussed in the previous chapters, hereditary tyrosinemia type 1 (HT1) and its characteristic development of hepatocellular carcinoma (HCC), is the model in which these alterations will be studied in this thesis. In concert with establishing the human cell culture model described in Chapter 3, a direct cell exposure model was used to standardise the assays and assess their application potential for measuring the effects of the accumulating HT1 metabolites on epigenetic mechanisms. The methods described in this chapter were to be applied after each selection step in the development of the inducible knock-down model, upon completion.

In HT1 there are various accumulating metabolites, of which succinylacetone (SA) and *p*-hydroxyphenylpyruvate (pHPPA) are but two which are detrimental to the cell, and the effects of both these metabolites have been studied to some extent in the literature (Prieto-Alamo and Laval, 1998, Van Dyk and Pretorius, 2005, Van Dyk *et al.*, 2010, Wentzel *et al.*, 2010). Other accumulating metabolites, such as fumarylacetoacetate (FAA) and maleylacetoacetate (MAA), have also been used in direct cell culture exposure experiments (Jorquera and Tanguay, 1997, Jorquera and Tanguay, 1999, Jorquera and Tanguay, 2001), but these metabolites are very unstable and are not commercially available. Therefore, only SA and pHPPA were used in this study. To establish what the effects of these metabolites may be on epigenetic mechanisms, and their role in the aetiology of HCC in HT1, cultured HepG2 cells were treated directly with these metabolites for various time intervals. A previous study established that the optimal concentrations of the metabolites for studies of this kind was 50 μ M [final] succinylacetone and 100 μ M [final] *p*-hydroxyphenylpyruvate (Van Dyk, 2005). This is a simplified model compared to the inducible knock-down model we aimed to establish in Chapter 3, but it is based on the same principles and was, therefore, considered suitable to establish the assays while the model was being developed. Following the treatment of the cells, various assays would be performed, each of which will be discussed in detail in this chapter. The cells were exposed to both metabolites simultaneously, since these metabolites usually accumulate concurrently in patients.

One of the enzymes to be studied is O⁶-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme reported to be regulated by methylation of its promoter region. Since the accumulating metabolites in HT1 are proposed to be alkylating agents (Jorquera and Tanguay, 1999, Jorquera and Tanguay, 2001), and MGMT is one of the main enzymes involved in the repair of this damage, it may be important in discovering the changes predominantly responsible for the

HT1 phenotype. This is supported by the suggestion of Major and Collier that, in the multistep process of HCC, defective MGMT is possibly an early or primary event and MGMT is known to be involved in the development of HCC (not of HT1) origin (Major and Collier, 1998, Zhang *et al.*, 2003).

In this chapter, all the assays used to evaluate changes on the epigenetic level, and other changes possibly linked to epigenetic alterations, will be described in detail. The results obtained with these assays will be shown and discussed in Chapter 5.

4.2. The use of HepG2 cells as *in vitro* model

4.2.1. Introduction

HepG2 cultured cells are routinely cultured human Caucasian hepatocyte carcinoma cells, purchased from Sigma at passage 8, and they are frequently used for various genetic and epigenetic studies (Jorquera and Tanguay, 1999, Drobna *et al.*, 2006, Krones-Herzig *et al.*, 2006, Lin *et al.*, 2010, Van Dyk *et al.*, 2010). Since primary hepatocytes are very problematic to obtain and culture, and have limited proliferation and a restricted lifespan, the use of cancer cells for liver cell studies is the best alternative (Ozturk *et al.*, 2009). HepG2 cells were chosen because they have been used in biological studies for many years, including similar studies interested in changes specific to the liver, and they are well characterized (Knasmüller *et al.*, 1998, Van Dyk *et al.*, 2010, Wentzel *et al.*, 2010). The inducible knock-down model we aimed to establish in Chapter 3 was also based on the use of HepG2 cells, and therefore the use of these cells in direct exposure experiments would be directly comparable with the model.

4.2.2. Cell culturing conditions

The cells were propagated in Dulbecco's Modified Eagles Medium (DMEM) (HyClone medium; Thermo Scientific) supplemented with 10% v/v foetal bovine serum (FBS; Sigma), 1x MEM non-essential amino acids (0.1 mM; Gibco), 2 mM [final] L-Glutamine (Gibco) and 1x penicillin/streptomycin (100 u pen./ml; 100 µg strep./ml; BioWhittaker). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were seeded at 200 000 cells per well in 6-well plates (Nunc) in 2 ml complete medium, with or without treatment. The amount of cells seeded was determined experimentally to ensure that cells continued to actively divide throughout the experiment, and that after 96 hours of growth, 70-80% coverage of growth surface was reached.

Prior to harvesting, the cells were washed with phosphate buffered saline (PBS; Sigma), and then either trypsinized, scraped or directly extracted. Trypsinization entailed incubation of the cells with 0.5 ml 1x trypsin (BioWhittaker) until cells were detached. The enzyme was then inactivated through the addition of culturing medium. Scraping was performed by manually scraping the cells from the surface in a 1 ml volume of PBS.

4.2.3. Treatment of HepG2 cells

HepG2 cells were seeded in 6-well plates and cultured under normal conditions, with or without treatment. Treatment entailed incubation of cells with 50 μM [final] succinylacetone^f (SA; Sigma) and 100 μM [final] *p*-hydroxyphenylpyruvate^g (pHPPA; ICN Biomedicals) simultaneously, which were added to the complete medium. These concentrations were determined in a previous study to be the optimal concentrations for these experiments (Van Dyk, 2005). Growth medium and metabolites were replaced every 24 hours, and cells were harvested after 24, 48, 72 and 96 hours. For positive oxidative stress controls, cells were treated with 2 mM [final] H_2O_2 (Pakmed) in the 2 ml complete medium for 3 hours prior to harvesting (Amer *et al.*, 2003).

4.2.4. Cell proliferation and viability of HepG2 cells

HepG2 cells were seeded in 6-well plates and cultured as described in Section 4.2.2 and treated as described in Section 4.2.3. After each 24 hour interval, a well of each of the treated and untreated cells were trypsinized and counted via the standard Trypan blue exclusion assay.

4.3. Genomic DNA extraction

4.3.1. Introduction

Genomic DNA was extracted from cultured cells for subsequent analyses, such as the cytosine extension assay and the real-time methylation-specific PCR analysis. DNA was extracted with the FlexiGene DNA kit (Qiagen), which is based on cell disruption, protein degradation by proteinase and ethanol precipitation. Subsequently, the DNA was analyzed to determine quantity and to ensure that the quality was suitable for further analyses.

4.3.2. Materials and method

Cells were harvested for DNA extraction via trypsinization, followed by the addition of 500 μl complete medium. Genomic DNA was isolated using a FlexiGene DNA kit (Qiagen), according to the manufacturer's guidelines. Precipitated DNA pellets were reconstituted in a suitable volume rehydration buffer (FG3), relative to the quantity of cells used for the extraction to ensure complete rehydration, while maintaining the highest concentration possible.

4.3.3. Quantification

Quantification of the samples was performed via spectrophotometric analysis (NanoDrop ND-1000; Thermo Scientific). The software (version V3.7.1) expresses the DNA quantity as $\mu\text{g}/\mu\text{l}$, calculated

^f See Appendix G for composition

^g See Appendix G for composition

from the 260 nm values measured, while the 260/280 ratio provides insight into the quality of the DNA. Ratios of 1.8 or higher are considered pure enough for use in the subsequent analyses.

4.4. Cytosine extension assay

4.4.1. Introduction

The cytosine extension assay (CEA) enables the assessment of global DNA methylation density changes, based on the simple method of restriction endonuclease digestion which leaves a 5' guanine overhang, followed by the incorporation of a radiolabeled [³H]dCTP. This incorporation is directly proportional to the number of cleaved CpG sites (Pogribny *et al.*, 1999). The use of different restriction endonucleases enables selection of the type of DNA methylation targeted, for instance, some enzymes predominantly recognize sequences outside CpG islands, while others specifically target the islands.

4.4.2. Materials and methods

Global DNA methylation was measured with the CEA as previously described (Pogribny *et al.*, 1999, Pogribny *et al.*, 2004, Wentzel *et al.*, 2010). The isolated DNA was digested with *Msp I* or *Hpa II* (Fermentas), respectively. Each reaction consisted of 500 ng DNA, 1x Tango buffer (Fermentas) and 10 u enzyme in a final volume of 20 µl. The reactions were incubated at 37 °C for 2 hours, followed by heat inactivation.

The CEA reaction consisted of 1x GoTaq® Flexi buffer (Promega), 1 mM [final] MgCl₂ (Promega), 5 u GoTaq® Flexi DNA polymerase (Promega) and 0.1 µl [³H]deoxycytidine triphosphate ([³H]dCTP; GE Healthcare) in a final volume of 20 µl. 5 µl digested DNA was added to each CEA reaction and incubated at 56 °C for 1 hour. Subsequently, the samples were placed on ice, and 200 µl PBS was added to stop the incorporation reaction. The samples were transferred to Whatman® DE-81 ion exchange filters (Sigma) and washed three times with PBS. The filters were air-dried prior to scintillation counting in 9 ml Ultima Gold™ XR (Perkin Elmer®). A background disintegrations per minute count (dpm) was subtracted from sample counts, and each sample was counted twice for 10 minutes or until sigma = 2%.

4.4.3. Calculations

The mean dpm was calculated for each sample. Subsequently, the global DNA methylation levels were calculated with the following equation:

$$\% \text{ methylated } 5'\text{-CCGG-}3' \text{ sites} = 100 - (Hpa \text{ II} / Msp \text{ I} \times 100) \quad (4.1)$$

4.5. Methylation-specific PCR assay

4.5.1. Introduction

The methylation-specific PCR (MSP) assay was first described by Herman and associates, and is based on the sodium-bisulfite treatment induced sequence differences between methylated and unmethylated DNA (Herman *et al.*, 1996). This treatment converts unmethylated cytosines to uracils, while methylated cytosines remain unchanged (Derks *et al.*, 2004). The bisulfite converted DNA is then used as a template in PCR amplification using two different sets of primers, specific for methylated versus unmethylated DNA (Herman *et al.*, 1996, Derks *et al.*, 2004).

The MSP assay is very sensitive, and it can detect abnormal methylation of a small DNA sequence surrounded by normal methylated DNA to a sensitivity of 0.1% methylated alleles of a specified CpG island locus (Herman *et al.*, 1996, Miyamoto and Ushijima, 2005). It also allows the examination of all CpG sites because it is not restricted by sequence recognition by methylation-sensitive restriction enzymes.

A variation of this assay was developed, enabling real-time PCR to be performed, resulting in a directly quantifiable measure of methylation (Derks *et al.*, 2004). Known as qMSP, this assay can quantify changes in the DNA methylation of a specific sequence, therefore eliminating the need for two sets of primers, since the unmethylated sequence is no longer measured. In addition, samples can be normalized in an experiment, and between experiments, through the addition of a reference gene. The reference gene is usually designed to contain no CpG sites in its primer sequences, but containing several cytosine bases. This renders the amplification of the reference gene sequence insensitive to DNA methylation changes in the sample, but enables it to act as a bisulfite conversion control. In this study β -actin (*ACTB*) was chosen, generally considered to be a “housekeeping gene”, and frequently used as reference gene for MSP studies (Eads *et al.*, 2001, Derks *et al.*, 2004).

MGMT is expressed in all human cells and tissues, but in varying degrees and it is predominantly regulated by DNA methylation, while loss of expression in tumours is rarely due to deletion or mutations, but rather because the promoter CpG island is hypermethylated (Esteller *et al.*, 1999, Bhakat and Mitra, 2003, Jacinto and Esteller, 2007). This hypermethylation has been correlated with the lack of MGMT protein, enzyme activity and mRNA expression (Jacinto and Esteller, 2007). Therefore, we chose to apply the qMSP assay to measure any DNA methylation changes in the promoter region of the *MGMT* gene.

4.5.2. Materials and method

Genomic DNA was isolated and treated with sodium bisulfite as prescribed by the manufacturer of the EpiTect[®] Bisulfite kit (Qiagen). This kit contains all the reagents needed for the bisulfite conversion, as well as the column based clean-up of the treated DNA. This produces bisulfite-converted DNA ready for further applications. Between 1 and 2 μ g DNA was treated, neutralized, washed and eluted with 40 μ l elution buffer (EB) per sample. One modification was made to the

manufacturer's protocol, by adding an equal volume 100% ethanol (Merck) to the sample when the BL buffer was added.

qMSP was performed using published TaqMan[®] primers and probes (see Appendix E) designed to target the methylated promoter sequence of the *MGMT* gene and the *ACTB* reference sequence. The reactions were multiplexed, with 1x TaqMan[®] universal PCR master mix (Applied Biosystems), 0.6 μ M [final] of each primer, 0.2 μ M [final] of each probe and 5.5 μ l converted DNA in a reaction volume of 25 μ l. All samples were amplified in duplicate on the ABI 7500 real-time PCR system (Applied Biosystems). The 7500 system SDS software version 1.3.1 (Applied Biosystems) process the amplification results, using the comparative cycle threshold method ($\Delta\Delta C_t$), to normalize all amplification data with the *ACTB* amplification results, and the *MGMT* promoter methylation levels are then expressed as a percentage change relative to the 24 hour control sample.

4.5.3. Validation

Both sets of primers and probes were validated using the purchased Epitect[®] PCR control DNA set (Qiagen), which included an unconverted, unmethylated sample, a converted unmethylated sample and a converted methylated DNA sample (results not shown).

4.6. RNA extraction and cDNA synthesis

4.6.1. Introduction

RNA was extracted from cultured cells for subsequent analysis of gene expression levels by means of the Nucleospin[®] RNA II kit (Macherey-Nagel). This kit is column-based, and yields high quantities, very pure RNA. This was confirmed after extraction with the NanoDrop, as discussed in Section 4.3.3. Subsequently, cDNA was synthesized using a reverse transcription kit.

4.6.2. Materials and method

Cells for RNA extraction were washed with PBS, followed by direct extraction with the Nucleospin[®] RNA II kit (Macherey-Nagel). Total RNA was isolated from the relevant material according to the manufacturer's instructions, although elution was performed twice with 40 μ l RNase-free water. Following spectrophotometric quantification, the RNA was stored at 4 °C for a maximum of 4 days prior to cDNA synthesis. cDNA was synthesized by reverse transcription with 1.5 μ g RNA as template, 1 μ M [final] Oligo(dT) 18mer and 1 μ M [final] 18S rRNA-specific reverse primer (see Appendix E), which were incubated at 65 °C for 2 minutes followed by 5 minutes on ice. Subsequently, 10 μ l of the enzyme mix (1x MMLV HP RT reaction buffer, 100 u MMLV high performance reverse transcriptase (Epicentre Biotechnologies), 4 μ M [final] dNTPs (New England Biolabs), 10 mM [final] DTT and 20 u recombinant RNasin[®] ribonuclease inhibitor (Promega)) was added. The reaction was performed overnight at 37 °C, after which the enzymes were deactivated at 85 °C for 5 minutes. The cDNA was diluted 250x prior to use.

4.7. Gene expression assay – *FAH*

4.7.1. Introduction

Gene expression levels can be determined by measuring the cDNA synthesized from the expressed mRNA of a target gene via real-time PCR (qPCR). The level of expression of fumarylacetoacetate hydrolase (*FAH*) is important when studying models of HT1, since it is the defective enzyme in these patients. The level of expression indicates the amount of the enzyme synthesized in the cells, at the point of analysis. Expression of a target gene is normalized relative to a reference gene. This reference gene is usually a type of “housekeeping” gene, and it is known to be expressed relatively stable in the cell or tissue type used (Thellin *et al.*, 1999). In this case, we chose the ribosomal protein 18S gene (*18S rRNA*) as our reference gene, since it is highly expressed in HepG2 cells, and is known to be very stably expressed and frequently used (Thellin *et al.*, 1999, Hermes *et al.*, 2004, Casorelli *et al.*, 2007). It was also necessary to choose a reference gene with no apparent connotation to any pathways which could be affected by the knock-down of the target gene, as this would compromise its stability and, therefore, its suitability as reference gene. This assay was applied in Chapter 3.

4.7.2. Materials and method

FAH expression was measured in a 20 μ l multiplex reaction via relative quantification using commercially available, validated TaqMan[®] assays (See Appendix E; Applied Biosystems), 1x TaqMan[®] universal PCR master mix (Applied Biosystems) and the ABI 7500 real-time PCR system (Applied Biosystems). The assays were chosen to be ones targeting exon-exon junctions, to exclude amplification of any residual genomic DNA in the sample. The amplification of the gene of interest was measured in conjunction with that of the reference gene, in this case *18S rRNA*. The reference gene was then used by the 7500 system SDS software version 2.0.5 (Applied Biosystems), using the comparative cycle threshold method ($\Delta\Delta C_t$), to normalize all amplification data between samples in an experiment and between experiments, and the expression of the gene of interest was expressed as a percentage change relative to a reference or control sample (Casorelli *et al.*, 2007).

4.7.3. Optimization

Initially the reference gene chosen was glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), but it was experienced that expression of this gene was not stable in HepG2 cells (results not shown). β -Globin was also considered, but expression levels were too low to use as a reference gene (results not shown). Most other regular reference genes had obvious connotations to the metabolic pathway altered by the gene knock-down proposed in Chapter 3.

18S rRNA was then selected and established to be expressed in very constant, high levels in HepG2 cells (see Figure 4.1). The use of this reference gene resulted in the adaptation of the cDNA synthesis protocol to include the use of an *18S rRNA* specific reverse primer, with the synthesis occurring at 37 °C, overnight. Because of the high abundance of *rRNA* genes in the cultured cells, the synthesised cDNA was diluted 250x for the qPCR assays.

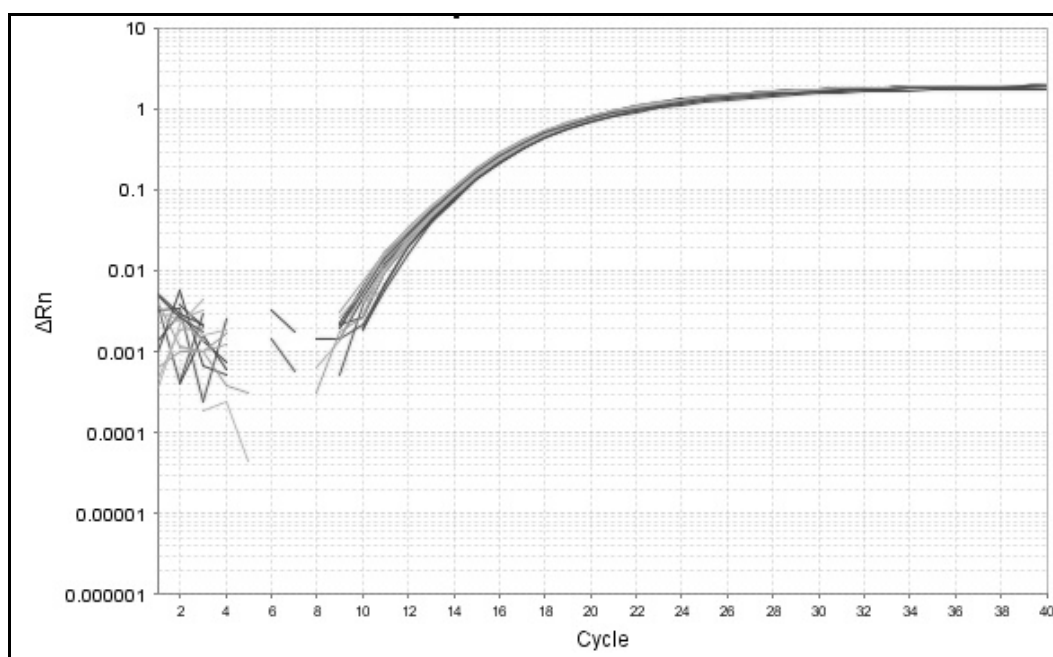


Figure 4.1. Real-time PCR amplification plot of 18S rRNA on the ABI 7500 real-time PCR system. 20 samples were amplified and their amplification (Y-axis) in the various cycles (X-axis) is presented on a logarithmic scale.

4.8. Gene expression assay - *MGMT*

4.8.1. Introduction

In HT1, one of the proposed detrimental effects of the accumulating metabolites is alkylation of DNA (Jorquera and Tanguay, 1997, Jorquera and Tanguay, 1999). O⁶-methylguanine-DNA methyltransferase (*MGMT*) is a DNA alkylation damage repair enzyme which removes adducts from O⁶-guanine bases (Pegg and Byers, 1992). It is therefore important to measure the level of expression in treated cells to determine whether the cells can cope with the damage induced. For this assay, *18S rRNA* was once again chosen as reference gene. Samples were normalized in and between experiments with the reference gene, and expression of the gene of interest was expressed as a percentage change relative to a reference or control sample.

4.8.2. Materials and method

Expression of the *MGMT* gene was determined a 20 µl multiplex reaction via relative quantification using a commercially available validated TaqMan[®] assay (see Appendix E; Applied Biosystems), 1x TaqMan[®] universal PCR master mix (Applied Biosystems) and the ABI 7500 real-time PCR system (Applied Biosystems). *MGMT* expression was measured in conjunction with the reference gene, *18S rRNA* and processed as discussed in Section 4.7.2, but with the 7500 system SDS software version 1.3.1 (Applied Biosystems). All measurements were expressed as percentage change in expression relative to the 24 hour control samples.

4.9. Flow cytometric analysis of oxidative stress

4.9.1. Introduction

Reactive oxygen species (ROS) are normal products in biological systems (Hawkins *et al.*, 2009). However, the production of ROS sometimes increase and can result in oxidative stress (Lee *et al.*, 2000). These increased oxidants have been shown to potentially damage all components of biological systems, and in view of the fact that it has been reported that some HT1 metabolites activate oxidative stress responses, it was deemed necessary to measure ROS when studying the effects of the HT1 metabolites (Dieter *et al.*, 2003, Vogel *et al.*, 2004, Langlois *et al.*, 2006).

Flow cytometry measures the properties of individual particles in solution (Robinson, 2004). The system separates particles into a single stream which moves past various light beams, resulting in a scattering of the light or fluorescence emission based on the presence of a fluorochrome (Nunez, 2001). Information about certain properties of the particles is then obtained (Rahman, 2006). The forward scatter channel (FSC) amass light scattered forward, and represents the particle's size (Nunez, 2001). The FSC intensity can also be used to distinguish between living cells and mere cellular debris. The granular content within a particle is represented in the side scatter channel (SSC). Each particle has a unique FSC and SSC, and it can be used to distinguish between cell types. Quantitative and qualitative data can be provided by fluorescence measurements when cell surface receptors or intracellular molecules are fluorochrome-labeled (Rahman, 2006). The flow cytometers usually have several fluorescence (FL-) channels with light detectors, and once a photodetector is excited, it generates a small current which is amplified and plotted graphically (Nunez, 2001, Rahman, 2006).

In this study, the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen) probe was used to quantify ROS, since ROS reacts with the probe to produce a fluorescent product, 2',7'-dichlorofluorescein (DCF) (Jorquera and Tanguay, 1997, Lee *et al.*, 2000, Jung *et al.*, 2009).

4.9.2. Materials and method

Free radical detection was performed as previously described (Jung *et al.*, 2009). All cells were seeded, cultured and treated, in duplicate, as described in Section 4.2, including the H₂O₂ treatment to produce positive controls for the ROS production. Other controls included cells not treated with the probe (cell control) and untreated cells treated with the probe (negative control). Prior to harvesting of the cells, 10 µM [final] DCFH-DA was added to each well and incubated 30 minutes at 37 °C in the dark. After incubation, the cells were washed with PBS and harvested by trypsinization. The medium added to inactivate the enzyme was removed after centrifugation at 1 000g for 3 minutes, and the cells were resuspended in 500 µl PBS for flow cytometric analysis.

DCF was measured using a 488 nm argon laser for excitation (green) on the FACSCalibur flow cytometer (Becton Dickinson) (Du Plessis *et al.*, 2010a, Du Plessis *et al.*, 2010b). The intensities of emitted fluorescence were collected with the FL1 photomultiplier tube, and in each

measurement a minimum of 50 000 events were collected in the gated positive population, and approximately 100 000 events in the rest of the population.

4.9.3. Data analysis

Data was acquired with the CELLQuest Pro Software (Becton Dickinson) and analysed using FCSExpress (version 3, De Novo Software). Flow cytometry produces distribution histograms, summarizing the number of cells over a range of fluorescence intensities (Overton, 1988). This data is then reduced to the number of fluorescent cells (positive population), and the mean fluorescence intensity (MFI). Size gates can also be established from the forward and side scatters, and debris excluded (Lee *et al.*, 2000). This was done as follows; in the FSC/SSC plot a “region of interest” was identified representing the positive or stained cell population and a size gate established. Only the information contained in this size gate is then represented in the histogram from which the geometric mean is calculated (see Figure 4.2).

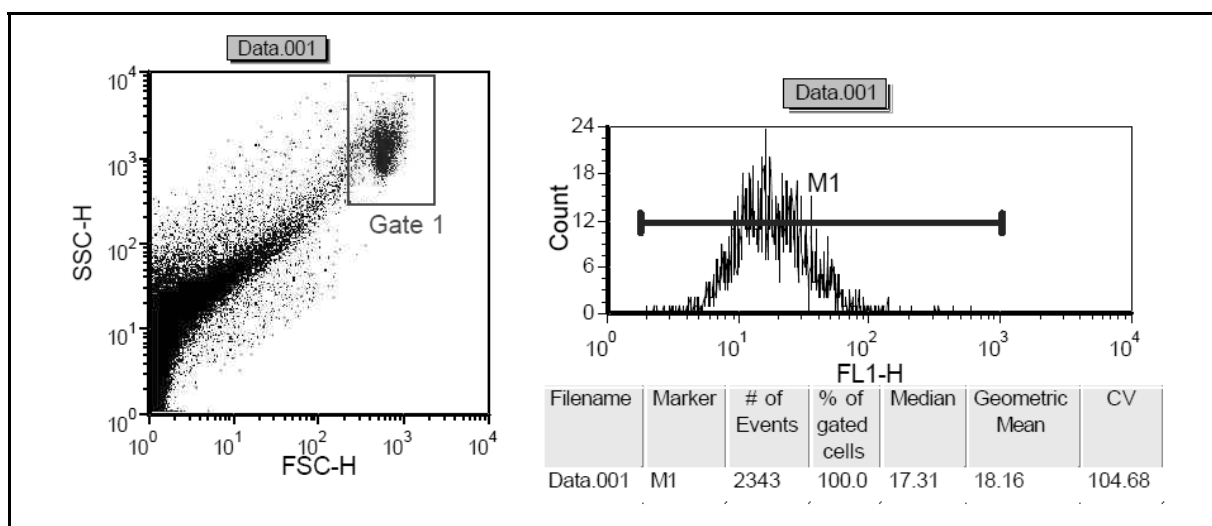


Figure 4.2. Flow cytometric analysis of size and granularity of DCF-stained HepG2 cells. The FSC channel is presented on the X-axis and the SSC channel on the Y-axis. The differentiated “positive” cells are selected in a size gate, and presented in a histogram (on the right).

The same can be done, taking into account the fluorescence measured (FL1), to confirm if the correct cell population was selected (see Figure 4.3). From the different graphs, it was observed that the different combinations of channels gave similar geometric mean values, confirming that the correct cell population was chosen. The horizontal distribution of cells in the FSC/FL1 graph (gate 3), however, distinguished this population best and was selected as the graph used for further calculations.

In this analysis, the assumption was made that only the cells in the size gate was fluorescently labelled, and all other cells were excluded from further calculations. A second assumption was made, in that there is a measure of background or auto-fluorescence present (Overton, 1988). Thus, the fluorescence of the cell control was subtracted from all other plots. The resulting MFI was then calculated as the average geometric mean of each duplicate sample.

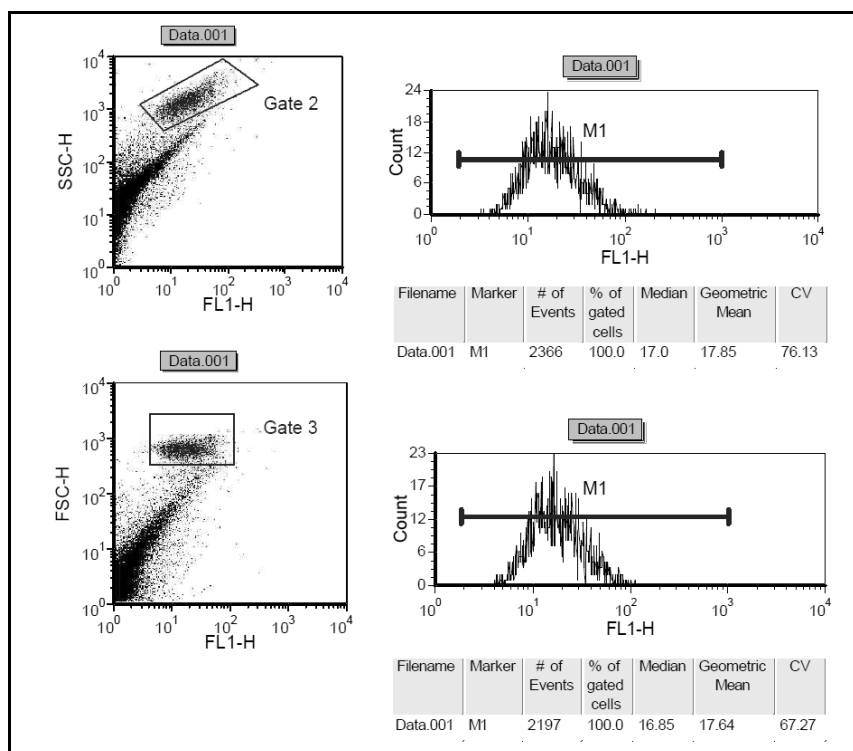


Figure 4.3. Flow cytometric analysis of size or granularity versus fluorescence of DCF-stained HepG2 cells. The FSC or SSC channel is presented on the Y-axis and the fluorescence on the X-axis. The differentiated “positive” cells are selected in a size gate, and presented in a histogram (on the right).

4.10. Protein carbonyl assay

4.10.1. Introduction

Reactive oxidants are generated in biological systems, and can damage all biological components, including DNA, RNA, proteins and lipids (Hawkins *et al.*, 2009). Since proteins are so abundant in the cells, it is a likely target and therefore an important level to measure oxidative damage at. Various radicals result in protein oxidation, and one of the general markers of this damage is protein carbonyl groups (Hawkins *et al.*, 2009). 2,4-dinitrophenylhydrazine (DNPH) reacts with these protein-bound carbonyl groups to give the corresponding hydrazone, and this yellow product can be quantified spectrophotometrically (Hawkins *et al.*, 2009).

4.10.2. Materials and method

Cells were washed with PBS and scraped before storage at -80 °C until use. The cells were thawed and 250 µl M-Per[®] mammalian protein extraction reagent (Thermo Scientific) was added to each sample to lyse the cells. The solution was mixed 5 minutes and then quantified spectrophotometrically (NanoDrop, Thermo Scientific) at 280 nm.

The assay was performed as previously described (Khan *et al.*, 2004, Hawkins *et al.*, 2009). This entailed the dilution of all samples to approximately 1.3 mg/ml prior to pipetting 100 µl of each sample into four 1.5 ml microcentrifuge tubes. 500 µl 10 mM DNPH (Fluka) was added to two tubes, while 500 µl 2 M HCl (Rochelle Chemicals) was added to the other two. All tubes were

vortexed and incubated at room temperature for 1 hour in the dark. Subsequently, 500 µl of a 20% (w/v) trichloroacetic acid (TCA, UnivAR) solution was added to each tube and vortexed. These solutions were then incubated at -20 °C for 20 minutes, prior to centrifugation at 9 500g for 10 minutes. After the supernatant had been discarded, the protein pellets were washed three times with 1 ml of an ice cold 1:1 ethanol/ethyl acetate (Merck) mixture, each time discarding the supernatant after centrifugation for three minutes. The pellets were then resuspended in 200 µl 6 M [final] guanidine-HCl^h and incubated for 30 minutes at 37 °C. The absorbance of the solutions was measured in flat-bottomed 96-well plates, at 280 nm and 370 nm, respectively, on the BioTek Synergy HT plate reader (Gen 5 version 1.05.11).

A standard concentration series was prepared by diluting a bovine serum albumin solution (BSA; Thermo Scientific) of a known concentration with 6 M guanidine-HCl. The series consisted of concentrations ranging between 0 and 2 mg/ml, in 0.4 mg/ml increments. The absorbance was measured at 280 nm.

4.10.3. Calculations

Protein concentration:

A straight line was fitted to the BSA series' absorbencies, with an expected R² value >0.995. The protein concentration of each sample (not containing DNPH) was then determined by referencing its absorbance to the standard curve.

Carbonyl concentration:

The protein carbonyl concentrations were determined with the following equation:

$\text{Carbonyl concentration (moles/L)} = (\text{Absorbance at 370 nm} - \text{background absorbance}) / 22\,000 \text{ (DNPH extinction coefficient)}$	4.2
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The carbonyl concentrations were then expressed as nmol of carbonyl per mg protein using the following equation:

$\text{nmol carbonyl per mg protein} = \text{carbonyls (nmol/ml)} / \text{protein (mg/ml)}$	4.3
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4.11. Senescence assay

4.11.1. Introduction

Cellular senescence is the ageing process of cells, characterized by the permanent loss of cell division (Suzuki *et al.*, 2002, Ozturk *et al.*, 2009). These cells have different morphology and gene expression patterns (Suzuki *et al.*, 2002). The degree of senescence in the cultured cells, with and

^h See Appendix G for composition.

without treatment, was assessed with the β -galactosidase assay. This assay measures the percentage cells expressing this enzyme which is associated with senescence (Pieper *et al.*, 1999).

4.11.2. Materials and method

The β -galactosidase assay was performed as previously described (Pieper *et al.*, 1999). Cells were washed twice with PBS (Sigma), prior to the addition of 1 ml fixating solution (PBS, 2% (v/v) formaldehyde, 0.2% (v/v) glutaraldehyde; Sigma). The cells were fixed at room temperature for 5 minutes, and then washed twice with PBS. Subsequently, 500 μ l freshly prepared staining solution (40 mM [final] citric acid/sodium phosphate buffer, pH 6, 5 mM [final] potassium ferrocyanide, 5 mM [final] potassium ferricyanide, 150 mM [final] NaCl, 2 mM [final] $MgCl_2$, 1 mM [final] X-gal in dimethylformamide) was added prior to incubation overnight at 37 °C (no CO_2). The percentage senescence-positive cells (coloured blue) were assessed using a 40x magnified microscope field.

4.12. Statistical analyses of data

For statistical interpretation the implemented statistical functions of GraphPad Prism[®] version 5.03 was used. Data was presented as the means of several series of experiments with its standard deviations, unless stated otherwise. To determine whether differences among multiple groups were statistically significant, two-way distributional assumptions of analysis of variance (two-way ANOVA) analyses, followed by the Bonferroni-adjusted sequential technique, were applied to the data. Comparisons between two samples were done using Student's *t* test. The differences with $p < 0.05$ were deemed statistically significant.

CHAPTER 5

MEASURING EPIGENETIC ALTERATIONS IN HT1 METABOLITE TREATED CULTURED CELLS

5.1. Introduction, aim and approach

5.1.1. Introduction and aim

In an effort to better understand the aetiology of hepatocellular carcinoma (HCC) in hereditary tyrosinemia type 1 (HT1), one must evaluate the effects of the disease on different levels in the cell. HCC is usually associated with an underlying disease, such as chronic Hepatitis B, and genetic alterations are thought in general to be HCC's basis (Lin *et al.*, 2001). Although several regulatory systems are in place to secure the homeostatic balance in cells, an accumulation of genetic and epigenetic changes can “free” the cells from these boundaries (Herceg and Hainaut, 2007, Franco *et al.*, 2008). It has become clear that epigenetic events are central to the development of several human cancers, and Mitchell and associates proposed that epigenic factors are involved in the clinical phenotype presented by HT1 patients (Mitchell *et al.*, 2001, Herceg and Hainaut, 2007). It has also been observed that in HT1 patients and mouse models, liver damage was associated with multiple gene expression alterations (Luijterink *et al.*, 2003), which also suggests the involvement of epigenetic mechanisms (Esteller, 2008).

One of the aims of this study was, therefore, to measure the changes induced by these accumulating metabolites on an epigenetic level, and to determine the role of these changes in the aetiology of HT1-associated HCC.

Epigenetic alterations generally refer to stable changes in gene expression potential which are mitotically and meiotically heritable, but do not involve changes in the DNA sequence itself (Vercelli, 2004, Ushijima, 2005, LeBaron *et al.*, 2010). This regulation is a dynamic process and, therefore, the epigenetic status of a gene can change or be modulated, which is essential for development (Vercelli, 2004, LeBaron *et al.*, 2010). For the purpose of this study, oxidative damage is also categorised as being “epigenetic” in nature, since it will be evaluated as a co-effector of DNA methylation changes. This is because scientists have shown that DNA methylation, and thereby gene expression, may be affected by oxygen radical (ROS) injury to DNA (Weitzman *et al.*, 1994, Franco *et al.*, 2008). It has also been shown that oxidative stress responses are activated in HT1, suggesting that ROS production may be increased in these patients (Dieter *et al.*, 2003, Luijterink *et al.*, 2003, Vogel *et al.*, 2004).

5.1.2. Approach

In this chapter, the various assays described in Chapter 4 were used to evaluate changes on an epigenetic level after direct exposure of cultured cells to the HT1 metabolites, SA and pHPA. HepG2 cells were used, since they were utilized to establish the HT1 model, and because they are

the most suitable cell line for this study (as discussed previously in Section 4.2.1). The results obtained with these assays will be described and discussed in detail, and specific conclusions will be made. The results will also be discussed in terms of the potential applications and/or limitations of such experiments in a cell culture model as the one described in Chapter 3.

In an effort to prevent confusion, the results were divided into different sections. There are three main focus areas, namely global DNA methylation, *MGMT* promoter methylation and expression, and finally oxidative stress and protein damage. Although the results are presented in three parts, they should be seen as components of one evaluation, since they are inter-connected and cannot really be dissociated. For that reason, the results of each section will be given and discussed briefly, after which various interactions between the sections will be evaluated and discussed further.

For each assay, the same treatment of cells was applied, unless stated otherwise. This entails that HepG2 cells were cultured under standard conditions as described in Section 4.2, followed by treatment with both of the HT1 metabolites, succinylacetone (SA; 50 μ M) and *p*-hydroxyphenylpyruvate (pHPPA; 100 μ M), for 96 hours in 24 hour intervals. The culture medium and metabolites were replaced every 24 hours to ensure adequate nutrition, waste removal and optimal metabolite exposure. The material required for each experiment was also prepared as described for that experiment in Chapter 4.

To ensure reliable results, the experiments were repeated several times. However, as some experiments contained incomplete data sets, only four “series” of experiments were selected and used for subsequent processing. Each series contain a minimum of two repeat analyses of each parameter, with a minimum of duplicate samples in each assay. For each result presented, the number of series used in the data processing will be stated. The original data for each series is compiled in Appendix F.

5.2. HepG2 proliferation

5.2.1. Introduction

HepG2 cells were seeded and cultured for 96 hours with or without HT1 metabolites, followed by harvesting by trypsinization at 24 hour intervals as described in Section 4.2. Following each harvest, the number of viable cells in a well was determined with Trypan blue staining (see Section 4.2.4).

5.2.2. Results and discussion

The HepG2 proliferation was plotted against the time points when they were harvested and counted.

Figure 5.1 represents the number of viable HepG2 cells with or without metabolite treatment as determined in 24 hour intervals. The growth appeared to follow a logarithmic pattern, and it was observed that a plateau was not yet reached which indicates that the cells were still actively

growing. However, the number of cells plated was determined experimentally in order to reach a growth area coverage of approximately 70-80% after 96 hours (results not shown). Therefore, cell density was already high at this time point, and the growth would presumably plateau within the next 24 hours.

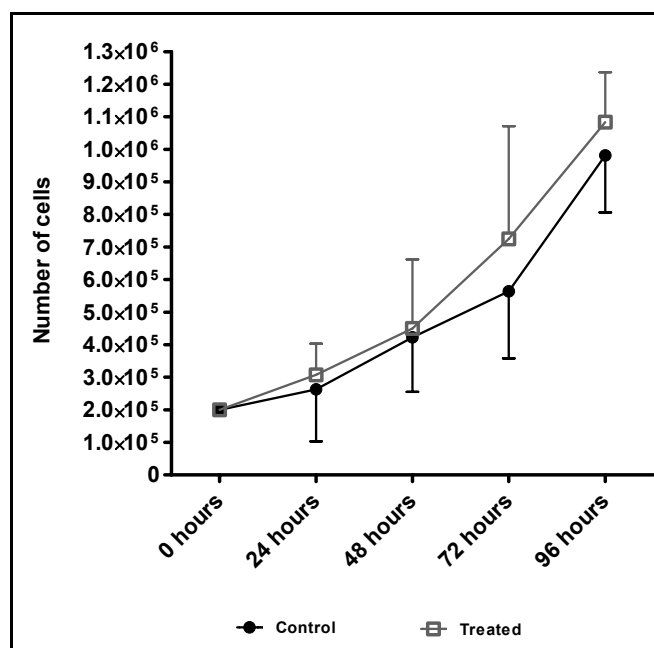


Figure 5.1. The influence of HT1 metabolite treatment on HepG2 proliferation. The number of HepG2 cells was determined over a 96 hour period from seeding, in intervals of 24 hours. The graph represents the mean cell count of three independent experimental series, and is expressed as mean with std. dev.

The number of cells for both the treated and untreated cultures increased significantly from seeding until the last time point ($p=0.0003$). It seemed that the treated HepG2 cells had a slightly higher doubling time than their untreated counterparts, although this difference was not quite statistically significant ($p=0.08$). Should this difference be considered biologically significant, however, it could suggest that SA and pHPPA may slightly induce cell death resistance. This cannot be discounted, since similar events have been observed in HT1 (Vogel *et al.*, 2004), and has been proposed to play a role in the pathophysiology of the disorder.

5.3. Global DNA methylation

5.3.1. Introduction

DNA methylation is the best-studied epigenetic mechanism, entailing the addition of a methyl group to a cytosine base on position C⁵ (Richardson, 2003, Herceg and Hainaut, 2007, LeBaron *et al.*, 2010). These methylated cytosines are mostly found in CG dinucleotides (CpG sites), and approximately 70-80% of these sites are methylated throughout the genome (Richardson, 2003, LeBaron *et al.*, 2010). Not only is DNA methylation important in the normal regulation of gene expression, it is also crucial for maintaining genome stability (Richardson, 2003, Esteller, 2008). Changes in the normal patterns of DNA methylation are associated with numerous cancers and

pathologies, and these changes generally include global hypomethylation and gene promoter associated hypermethylation (Herceg and Hainaut, 2007, Franco *et al.*, 2008).

DNA methylation can be measured on a whole genome scale using restriction endonucleases with different methylation sensitivities and the cytosine extension assay (CEA), as described in Section 4.4. Digestion with the enzymes leaves 5' guanine overhangs, followed by the incorporation of radiolabeled [³H]dCTP. Genomic DNA was extracted after each 24 hour interval and, subsequently, these samples were either digested with the restriction endonucleases *Msp I* or *Hpa II* for global DNA methylation. This incorporation of the [³H]dCTP into the DNA is directly proportional to the number of cleaved CpG sites (Pogribny *et al.*, 1999), therefore, this assay provides an indication of major changes in DNA methylation on a genome-wide level.

5.3.2. Global DNA methylation

CpG dinucleotides are spread out through the genome, and are frequently located in highly repetitive, non-coding sequences where they are usually methylated, as well as in retrotransposons where their methylated status is responsible for the inactivation of these sequences (Lin *et al.*, 2001, Richardson, 2003, LeBaron *et al.*, 2010). It has been shown, however, that in various cancers these CpG sites lose their methylation, leading to global DNA hypomethylation which could lead to genomic instability (Lin *et al.*, 2001, Esteller, 2008, Franco *et al.*, 2008). Genomic instability has been observed in HT1 (Jorquera and Tanguay, 2001).

The restriction endonuclease *Msp I* is methylation insensitive and, therefore, will digest 5'-CCGG-3' sequences independent of the methylation status of the CpG residue. *Hpa II*, on the other hand, will only digest this sequence if the second cytosine is not methylated. Through the use of these two enzymes in the CEA assay a ratio can be calculated, indicating the percentage of the CpG sites which are methylated. These enzymes have been shown to predominantly digest DNA outside CpG islands (Fazzari and Greally, 2004, Wentzel *et al.*, 2010), and it can be assumed that the results give an indication of changes in DNA methylation at CpG sites throughout the genome, rather than changes within CpG islands. The CEA was performed as described in Section 4.4.2, and the following results were obtained.

As shown in Figure 5.2, the relative global DNA methylation in untreated HepG2 cells after 24 hour incubation time, appeared to be approximately 45%. This methylation level changed minimally over incubation time and with increased cell density (see Section 5.2.2). Although a slight increase in DNA methylation was observed after 72 hours, it was rather insignificant ($p=0.64$). The levels of DNA methylation after treatment with the HT1 metabolites were shown to be almost identical to the control samples ($p=0.47$), except for the 72 hour interval, where a difference of approximately 10% was observed. But, once again, it was not statistically significant.

The standard deviations for some of the experimental repeats were somewhat large, specifically for the 24 hour time point. These variations were possibly a result of varying cell proliferation rates between experiments, as slight differences in the cell counts were also observed (see Figure 5.1). Another cause for this variation could be ascribed to possible discrepancies in the efficiencies of

the enzyme digestions or the polymerase activity during the CEA, resulting in inter-experimental differences.

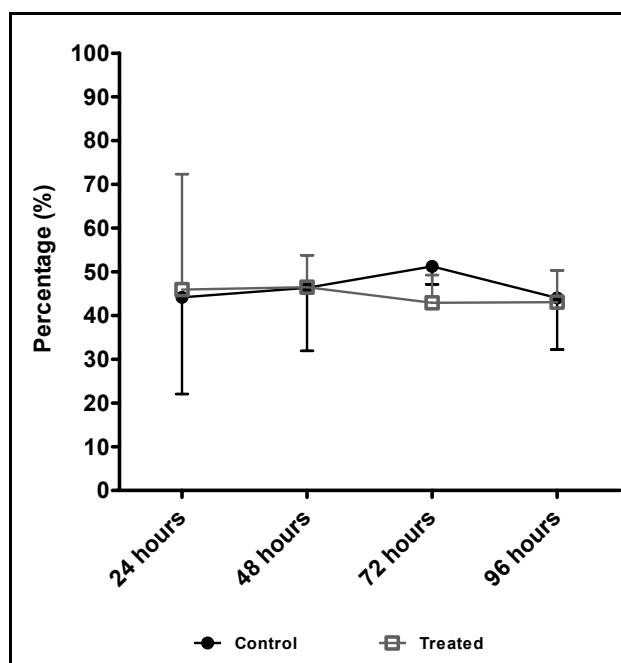


Figure 5.2. Global DNA methylation levels, following HT1 metabolite treatment. DNA methylation levels were determined over a 96 hour period in intervals of 24 hours. The graph represents the DNA methylation of three independent experimental series, and is expressed as mean with std. dev.

5.3.3. Discussion and conclusion

From the above described global DNA methylation investigation, it became apparent that treatment of the cultured HepG2 cells with the HT1 metabolites *per se* had no significant effect on the global DNA methylation levels observed. This, however, does not exclude the possibility that the metabolites could have localized effects in the genome, since the CEA only measures the average of all global DNA methylation changes in the genome.

The results presented here differ from a previous report suggesting CpG island hypermethylation and global non-CpG island hypomethylation in HT1 (Wentzel *et al.*, 2010). However, those results were obtained with the comet assay which measures changes on a single-cell level. The cells were also HepG2 cells, but contained a vector and treatment of the cells also consisted of only SA. The results also represent changes in the first 24 hours of exposure. In this more comprehensive study, the effect of longer-term exposure was investigated with the CEA assay, which measures the overall average changes in a cell population from the 24 hours exposure time point until 96 hours of exposure.

The results did indicate that growth conditions or cell density of cultured cells may be associated with slight changes in DNA methylation. This would indicate a level of adaptation in gene expression in response to growth conditions of the cultured cells, specifically HepG2 cells in this instance. In the literature it has been suggested that when cells are grown at high density, the processes that maintain genomic stability are down-regulated (Pieper *et al.*, 1999). Richards and associates showed that human cells kept at a high density had increased frameshift mutations (Richards *et al.*, 1997), while Pieper and associates proposed that not only genetic alterations were

induced by high density growth, but also epigenetic changes (Pieper *et al.*, 1999). They subsequently found an increase in the methylation of one CpG island, but it did not seem to be a global event. However, this study was performed in normal human fibroblast cultures, and can therefore not necessarily be directly correlated with the events in confluent HepG2 cells. Bertino and colleagues did suggest that the DNA methylation pattern they observed in neuronal cultures was growth condition related, although this observation was associated with apoptosis commitment (Bertino *et al.*, 1996). Wilson and Jones also found global hypomethylation over time in cultured fibroblasts, but more stable levels in immortalized cell lines (Wilson and Jones, 1983, Grafodatskaya *et al.*, 2010). It has been stated that DNA is demethylated during aging (Mazin, 2009), but this cannot be the case in this study, seeing as no senescence was present (as will be discussed in Section 5.6.5).

Taking these potential changes in global DNA methylation into account, it indicates that care must be taken at which time points or cell densities methylation studies are performed. This will be a factor to keep in mind when performing these types of analyses in the HT1 cell culture model, where a cell line will be cultured continuously and not only for 96 hours at a time. When designing studies for DNA methylation in such a cell model, one will have to ensure consistent cell conditions for repeatable results. On the other hand, the proposed HT1 model will be invaluable for further study of DNA methylation in HT1, since long-term studies will be possible.

A closer look at the results given in Figure 5.2 revealed a cyclical pattern (low frequency and small amplitude) in the global methylation over the time points investigated, for both the treated and untreated cultures, and it seemed as if treatment of the cells with the HT1 metabolites affected, although slightly, the synchronization of this pattern. This effect was also observed in other results, and will be discussed in Section 5.6.6.

5.4. *MGMT* promoter methylation and expression

5.4.1. Introduction

Promutagenic DNA base damage is involved in carcinogenesis initiation and, therefore, genetic changes can accumulate in cancer cells if DNA repair is defective (Major and Collier, 1998, Margison *et al.*, 2003). O⁶-methylguanine is one such a highly promutagenic DNA lesion, and it has been shown that a diminished capacity of its repair is a major carcinogenesis determinant (Major and Collier, 1998). O⁶-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme responsible for the removal of alkyl adducts from guanine bases, especially methyl adducts (Margison *et al.*, 2003, Zhang *et al.*, 2003). These adducts are potentially mutagenic lesions (Zhang *et al.*, 2003). As discussed in Chapters 2 and 4, MGMT is known to be involved in the development of HCC (not of HT1 origin), and has frequently been found to have altered promoter methylation patterns and/or expression patterns in these cancers (Zhang *et al.*, 2003). This involvement of MGMT in non-HT1 HCC aetiology compelled the investigation into the effect of accumulating HT1 metabolites on the promoter methylation and expression of this enzyme. It is also known that transcription of the *MGMT* gene is directly correlated with the levels of the protein in the cells, and the repair of each adduct requires one MGMT molecule (Major and Collier, 1998, Jacinto and Esteller, 2007).

A manuscript describing the results presented in this section was prepared for submission to *Journal of Inherited Metabolic Disease*, and is included at the end of this chapter.

5.4.2. *MGMT* promoter methylation

Loss of *MGMT* expression can be attributed mainly to *MGMT* promoter CpG island hypermethylation, as opposed to conventional deletions, mutations and rearrangements (Jacinto and Esteller, 2007, Kaina *et al.*, 2007, Sedgwick *et al.*, 2007). This epigenetic transcriptional regulation is suggested since *MGMT* mRNA levels usually parallel enzyme expression (Major and Collier, 1998). The methylation status of the *MGMT* promoter has been the focus of numerous studies, and hypermethylation has been associated with colorectal cancer, brain cancer and oesophageal adenocarcinoma (Esteller *et al.*, 2001a, Yin *et al.*, 2003, Baumann *et al.*, 2006). Bearzatto and colleagues, however, observed that in some cells hypermethylation of *MGMT*'s promoter did not result in decreased expression of *MGMT*, making this relationship between promoter methylation and transcription even more complex (Bearzatto *et al.*, 2000). Conversely, Hermes and associates have shown the *MGMT* promoter region to be partially hypermethylated in HepG2 cells (Hermes *et al.*, 2004).

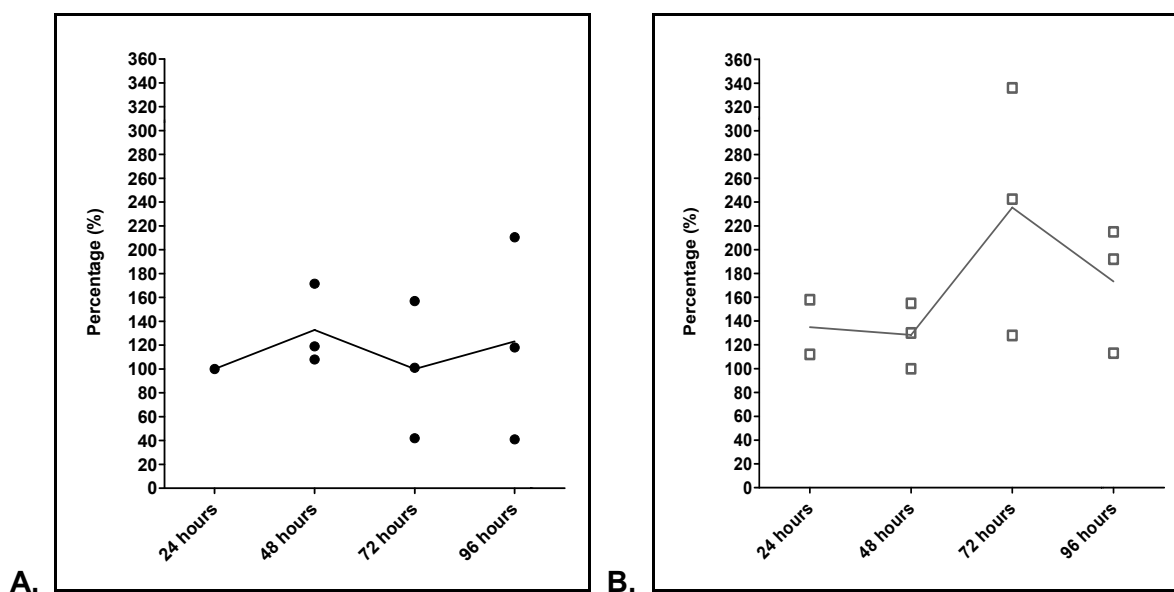


Figure 5.3. DNA methylation levels in the *MGMT* promoter in HepG2 cells with or without HT1 metabolite treatment. DNA methylation levels were determined over a 96 hour period in intervals of 24 hours. The graphs represent the changes in DNA methylation of three independent experimental series. Figure A represents the untreated cultures and each individual series is represented (solid circles), as well as the mean (solid line). Figure B represents the metabolite treated cultures and each individual series is represented (open squares), as well as the mean (solid line).

Gene-specific DNA methylation can be measured and quantified using the real-time methylation-specific PCR assay (qMSP), as described in Section 4.5. Genomic DNA was extracted after each 24 hour interval, and these samples were treated with sodium bisulfite to induce methylation-dependent sequence changes. These altered samples were then amplified and quantified via qMSP, measuring changes in the methylation pattern of the *MGMT* promoter sequence. The methylation data of the *MGMT* promoter was normalized using *ACTB* as reference gene (as discussed in Section 4.5.1). For the purpose of further calculations, a value of 100%

methylation was assigned to the 24 hour control samples, and all changes in the methylation levels of samples were expressed relative to this control.

The mean DNA methylation level of the *MGMT* promoter in the control samples, presented in Figure 5.3 A, appeared to change somewhat over time. These changes, however, were never more than approximately 30%, and did not follow a linear pattern of increase or decrease over the time period investigated ($p>0.05$). Instead, the same cyclical pattern was observed as in Figure 5.2. In this instance the pattern remained rather level, and did not show a marked decrease in DNA methylation.

The mean *MGMT* promoter methylation level of the HT1 metabolite treated samples (Figure 5.3 B) seemed to increase with time, with an overall increase of approximately 40% between intervals 24 hours and 96 hours. However, between 48 hours and 72 hours treatment, a dramatic increase of more than 100% was observed, followed by a less dramatic decrease to 96 hours. Even so, none of these changes appeared to be statistically significant ($p>0.05$). Once again no linear increase or decrease could be distinguished, although it did seem that the promoter methylation increased on average, albeit very slightly. Interestingly, the same cyclical pattern was once again observed, only more pronounced in the metabolite treated samples than in the untreated samples.

At the 72 hour time point of the treated sample, the methylation level seemed surprisingly high compared to the other time points, and the standard deviation for this time interval was also large. This raised the question of it simply being an outlier value. However, if one compares the data of the individual series, it is clear that two of the three sets showed the same marked increase at the 72 hour time point. One must therefore assume that it is a true phenomenon.

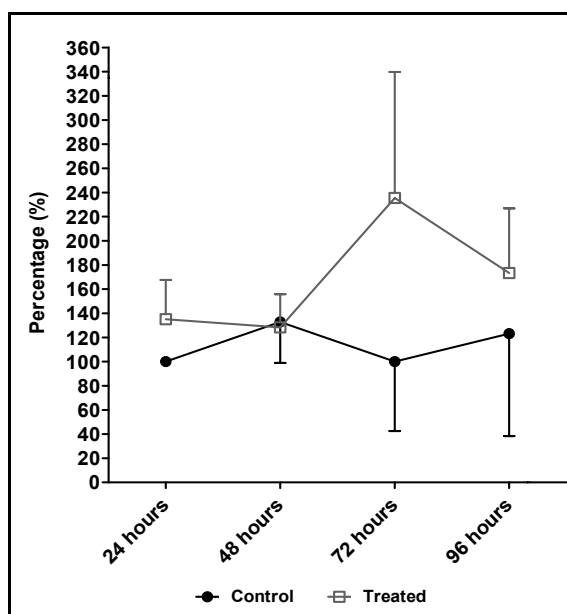


Figure 5.4. *MGMT* promoter methylation of HepG2 cells following HT1 metabolite treatment. DNA methylation levels were determined over a 96 hour period in intervals of 24 hours. The graph represents the DNA methylation of three independent experimental series, and is expressed as the mean with std. dev.

The mean *MGMT* promoter methylation of the untreated samples was plotted together with that of the treated samples in Figure 5.4, to further investigate the effect of the HT1 metabolites on the DNA methylation status of the *MGMT* promoter sequence.

The direct comparison of the HT1 metabolite treated and untreated samples showed a difference in not only the level of the *MGMT* promoter methylation, but also in the time-dependent pattern (see Figure 5.4). These differences were not statistically significant ($p=0.16$). Although both sample sets presented cyclical patterns, the HT1 metabolites resulted in increased levels of *MGMT* promoter methylation compared to the control samples, and it seems that the treatment shifted the cyclical pattern out of sync. After only 24 hours exposure to the metabolites, DNA methylation of the *MGMT* promoter was almost 40% higher than in the control samples. This difference increased to almost 50% after 96 hours. Should one consider this difference biologically significant, the increase in DNA methylation could suggest an upregulation of the DNMT enzymes, resulting in hypermethylation of the sequence. However, such an upregulation would have global effects, while this seemed to be a targeted effect.

5.4.3. *MGMT* expression

Expression of the *MGMT* enzyme directly affects the well-being of the cells by affecting the DNA repair capacity available to deal with any inflicted damage which might threaten its genomic integrity (Major and Collier, 1998). As was described in Section 5.4.1, the protein level of *MGMT* in a cell can be directly correlated with the mRNA content. Therefore, by measuring the *MGMT* mRNA expression in the HepG2 cells, one indirectly measures the protein production, and therefore the potential alkylation damage repair capacity of the cells.

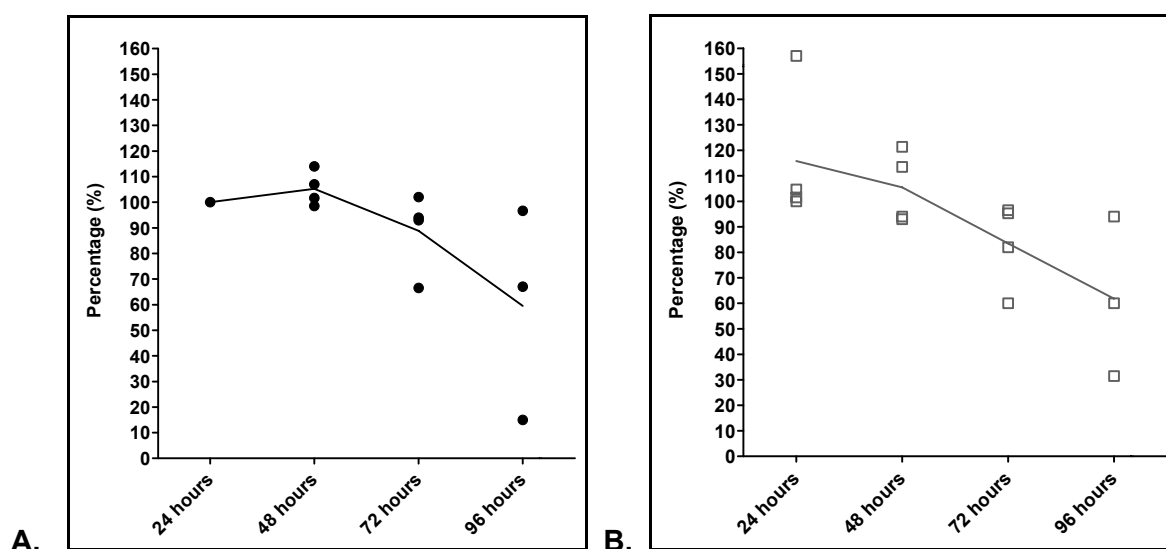


Figure 5.5. *MGMT* expression levels in HepG2 cells with or without HT1 metabolite treatment. *MGMT* expression levels were determined over a 96 hour period in intervals of 24 hours. The graphs represent both the untreated cultures (Figure A: the various data points (solid circles), as well as the mean expression levels of four independent experimental series (solid line)) and the treated cultures (Figure B: the various data points (open squares), as well as the mean expression levels of four independent experimental series (solid line)).

Expression of the *MGMT* gene was measured using relative quantification real-time PCR (qPCR), as described in Section 4.8. RNA was isolated at the same intervals as the genomic DNA, followed by reverse transcription cDNA synthesis and qPCR amplification. All expression levels measured in all the samples were normalized using a reference gene, *18S rRNA* (as discussed in Section 4.7.1), and were subsequently expressed relative to the 24 hour control samples (assigned the value of 100% expression for calculation purposes).

As is evident from Figure 5.5 A, the levels of *MGMT* expression appeared to decrease markedly (approximately 40%) between time points 48 hours and 96 hours in the control samples. This once again suggests a direct relationship between cell densities or growth conditions and cellular changes in HepG2 cell cultures. However, it appears as if *MGMT* is targeted by these cellular changes, resulting in dramatically decreased alkylation repair capability in the cell cultures.

The *MGMT* expression levels clearly decreased with more or less the same trend in the HT1 metabolite treated cultures (Figure 5.5 B), as it did in the untreated cultures (Figure 5.5 A). In the treated samples this decrease was visible from the 24 hour interval, and the expression levels were decreased with more than 50% after 96 hours of treatment. Clearly, the effect of the cell density or growth conditions influenced *MGMT* expression significantly ($p=0.01$).

To identify specific changes in the expression of *MGMT* as a result of the HT1 metabolites, the mean expression values of the control and treated samples were plotted together in Figure 5.6.

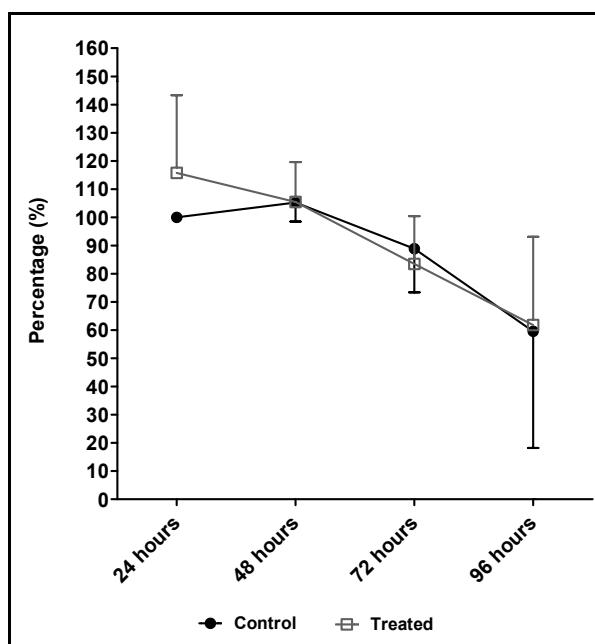


Figure 5.6. *MGMT* expression in HepG2 cells following HT1 metabolite treatment. *MGMT* expression levels were determined over a 96 hour period in intervals of 24 hours. The graph represents the expression levels in four independent experimental series, and is expressed as the mean with std. dev.

Expression of *MGMT* decreased significantly in both the untreated and the treated samples between the time intervals, once again suggesting a direct influence of the cell density or growth conditions on cellular processes in HepG2 cells. It is clear that this decrease was independent of metabolite treatment, which had no significant effect. However, in Figure 5.6 it was seen that the

expression of *MGMT* was almost 15% higher in the treated samples after 24 hours exposure than in the untreated samples, possibly indicating the induction of *MGMT* by the alkylating metabolites, although this difference was not deemed statistically significant ($p=0.33$). Nonetheless, this increase was only present at the 24 hours time point, after which the *MGMT* expression levels markedly decreased to the same level of expression after 96 hours as that of the control samples.

5.4.4. Discussion and conclusion

It is clear from the results that *MGMT* is greatly influenced by growth conditions or cell density on both a genetic and epigenetic level. Not only did methylation of the promoter region show a cyclical pattern between the time points, the expression of the gene also decreased significantly with time. In contrast, although DNA methylation is known to be directly involved in the regulation of gene expression (Esteller, 2008), in the case of *MGMT* in HepG2 cells, the findings appeared not to be directly correlated. This interaction will be further evaluated and discussed in Section 5.6.2.

Interestingly, Pieper and associates showed that the *MGMT* promoter associated CpG islands in normal human fibroblasts was highly hypermethylated during confluence, when compared with logarithmically growing cells (Pieper *et al.*, 1999). The results presented here (Figure 5.3 A) may differ from theirs as a result of the different cell line used, or because the cells used here were not yet fully confluent at time point 96 hours. It is not clear, however, whether *MGMT* expression in normal human fibroblasts is directly regulated by its promoter methylation, and if the increased methylation at high cell density results in decreased *MGMT* expression (Pieper *et al.*, 1999). Also, it has been shown in the literature that chronic inflammatory states, as observed in HCC, showed marked increases in CpG island methylation (Gallou-Kabani *et al.*, 2007), which correlates with the increase in *MGMT* promoter methylation observed in the treated cultures (Figure 5.4).

In addition, in literature it has been stated that *MGMT* is not inducible in HepG2 cells (Fritz *et al.*, 1991), while other publications stated that induction of *MGMT* varies with each different inducing agent and cell line used (Margison and Santib  n  z-Koref, 2002, Margison *et al.*, 2003). Although treatment with the HT1 metabolites seemed to induce higher expression of *MGMT* in this study (Figure 5.6), this induction was very slight, and unsustainable. Still, it is known that *MGMT* induction usually peaks between 24 and 48 hours post-exposure (Margison *et al.*, 2003), and this correlates with our findings. It is possible that the specific metabolites used in this experiment can, in fact, induce *MGMT* in HepG2 cells even if the “induction” was only by approximately 15%. Yet, it is in line with the literature, which stated *MGMT* to be only slightly inducible (Margison *et al.*, 2003).

Once again it must be highlighted that culturing conditions are of utmost importance in studies pertaining to DNA methylation and gene expression, and that this must be considered in the application of cell culture models as the one we described in Chapter 3. Clearly, *MGMT* expression significantly decreased in the cultured HepG2 cells with time, and this could render the cells vulnerable to alkylation damage. The specific cause for this decrease, be it cell density or ageing or an unknown factor, will need to be identified to ensure that a situation of impaired DNA repair is avoided.

5.5. Oxidative stress and protein damage

5.5.1. Introduction

ROS are highly reactive and unstable molecules generated in biological systems, which can damage all biological components, including DNA, RNA, proteins and lipids, and may contribute to malignant transformation (Cerdeira and Weitzman, 1997, Cecarini *et al.*, 2007, Hawkins *et al.*, 2009, Jung *et al.*, 2009). Once the ROS production exceeds the cellular antioxidant defences, oxidative stress ensues, compromising cellular homeostasis and function (Cecarini *et al.*, 2007, Franco *et al.*, 2008, Dunlop *et al.*, 2009). In addition, substantial evidence implicates oxidative stress in several stages of carcinogenesis (Franco *et al.*, 2008).

The tyrosine catabolites FAA and possibly MAA can react with, or even deplete, cellular glutathione (GSH), resulting in oxidative damage which appears to potentiate its mutagenicity (Jorquera and Tanguay, 1997, Mitchell *et al.*, 2001, Vogel *et al.*, 2004). Dieter and colleagues therefore suggested that various tyrosine intermediates are capable of oxidative stress induction, and that this was possibly causally linked to the ensuing liver pathology (Dieter *et al.*, 2003). They also proposed that HT1 patients on NTBC treatment are chronically exposed to oxidative stress, since the NTBC-rescued *14CoS/14CoS* mouse model had a significant oxidative stress response in the liver and kidneys (Dieter *et al.*, 2003). Several research groups have shown that oxidative stress response pathways are activated in HT1 models, but few of them directly assayed ROS production and its ensuing cellular damage (Dieter *et al.*, 2003, Vogel *et al.*, 2004, Bergeron *et al.*, 2006, Fisher *et al.*, 2008). Vogel and colleagues did indicate an increase in oxidative DNA damage in *Fah*^{-/-} mice (Vogel *et al.*, 2004), but Jorquera and Tanguay measured ROS production following direct exposure experiments with FAA, and did not detect any increase in its production (Jorquera and Tanguay, 1997). Bergeron and associates showed the launch of an endoplasmic reticulum (ER) stress response in HT1, and subsequently stated that it was unclear whether the previously observed induction of the stress-related proteins, HO-1 and Hsps, was a result of oxidative or ER stress (Bergeron *et al.*, 2006). As such, the source of the oxidative stress response in HT1 remains unclear, and needs further exploration.

A manuscript describing the results presented in this section was prepared for submission to *Journal of Inherited Metabolic Disease*, and is included at the end of this chapter.

5.5.2. ROS production

ROS is a by-product of aerobic respiration, P450 metabolism, nitric oxide synthesis and inflammation, as well as various exogenous sources (Cecarini *et al.*, 2007, Franco *et al.*, 2008). ROS can cause various DNA lesions, including the mutagenic 8-hydroxy-2-deoxyguanosine (8-OHdG) and O⁶-methylguanine (O⁶meG) (Franco *et al.*, 2008). These lesions can interfere with normal DNA methylation mechanisms, thereby changing the normal DNA methylation patterns of cells (Franco *et al.*, 2008). This potential interaction with the epigenetic profile of a cell compelled this investigation as to whether the HT1 metabolites affect intracellular ROS production in any way.

In this study intracellular ROS production was quantified with a probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA,) as described in Section 4.9. ROS production was quantified by measuring

its reaction with DCFH-DA to produce green fluorescent 2',7'-dichlorofluorescein (DCF), which was measured on the FACSCalibur flow cytometer (Lee *et al.*, 2000, Jung *et al.*, 2009, Du Plessis *et al.*, 2010a). The DCF test was used to quantify generic intracellular ROS production, and although some concerns regarding this method have been raised (Karlsson *et al.*, 2010), this method is a standard assay for ROS studies and has been used and validated extensively (Jung *et al.*, 2009, du Plessis *et al.*, 2010a, Kuznetsov *et al.*, 2011). All cells were seeded, cultured and treated in duplicate, as described in Section 4.2, including the H₂O₂ treatment to produce positive controls for the ROS production. Other controls included cells not treated with the probe (cell control) and untreated cells treated with the probe (negative control). In each measurement a minimum of 50 000 cells were analysed in the positive population, and approximately 100 000 cells in the rest of the population. The ROS production levels of the control vs. treated cells were presented as mean fluorescent intensity (MFI) in Figure 5.7.

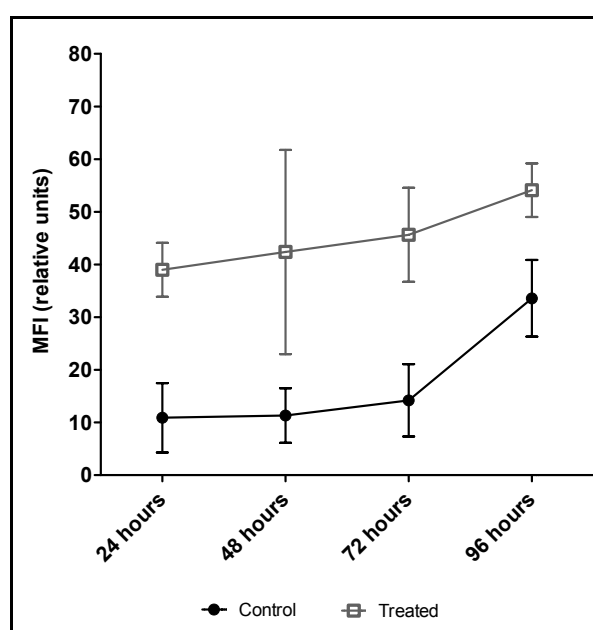


Figure 5.7. The effect of the accumulating HT1 metabolites on intracellular ROS production. DCF fluorescence was measured every 24 hours during continuous exposure to the metabolites for 96 hours. The graph represents the mean MFI of two independent experimental series for intervals 24 and 48 hours, and one series for intervals 72 and 96 hours, and is expressed as mean MFI $\pm$ std. dev.

Intracellular ROS production in the control HepG2 cells were quite low and stable during the first 72 hours of growth, followed by a sudden increase of approximately 20 units in the last 24 hours. This 3-fold increase was not statistically significant ($p > 0.05$). The intracellular ROS levels in the HT1 metabolite treated samples were significantly increased compared to the control samples ($p < 0.001$), after only 24 hours in the presence of the HT1 metabolites. This increased production was evident at all time points, and two-way ANOVA analysis showed the treated samples to differ significantly from the untreated samples ($p = 0.0016$). ROS production is a normal product of cell metabolism, however, when the production of these reactive molecules exceeds the cell's antioxidant capacity, a state of oxidative stress arises (Franco *et al.*, 2008, Dunlop *et al.*, 2009). This results in an environment where all cellular molecules can be damaged, including DNA, RNA and proteins (Cerdeira and Weitzman, 1997, Mary *et al.*, 2004, Cecarini *et al.*, 2007, Jung *et al.*, 2009). In normal, active dividing HepG2 cells with sufficient growth medium and limited accumulation of waste (medium was replaced every 24 hours), ROS was present, but in small

amounts. This was expected, since ROS is a normal by-product of cellular function (Kryston *et al.*, 2011). Although ROS production increased between the 72 and 96 hour time points, the change was not significant, and the ROS levels remained less than in the treated samples. This increase could be a result of the increased cell density during this time interval (see Figure 5.1).

Intracellular ROS levels increased significantly after exposure to the HT1 metabolites, SA and pHPPA, when compared to the control samples, which suggest that the metabolites induced increased intracellular ROS production or a decrease of the removal of these species (Cecarini *et al.*, 2007, Valko *et al.*, 2007, Kryston *et al.*, 2011). Interestingly, this increased ROS production seemed to follow the same pattern as in the control samples, implying that the effect of the metabolites on ROS production is not cumulative, but rather constant.

Increased ROS production, however, does not necessarily indicate oxidative stress. Since there are mechanisms in place to repair or counteract ensuing damage, it was important to measure the end-product of such damage to establish whether the increased ROS production was, in fact, detrimental to the cells. Therefore, protein carbonyl concentrations were determined.

5.5.3. Protein carbonyl damage

Since proteins are so abundant in the cells, it is a likely target and therefore an important level to measure oxidative damage at (Hawkins *et al.*, 2009). Oxidative protein damage can cause amino acid structure alterations and enzymatic activity changes, affecting cell signalling, cellular structure and metabolism (Cecarini *et al.*, 2007). These oxidized proteins have to be eliminated, but there are very few enzymatic repair systems available for these proteins and its degradation is the main mechanism of its removal (Mary *et al.*, 2004, Dunlop *et al.*, 2009). Intracellular proteases recognize this damage and removes it, but it appears that these proteolytic complexes can be directly affected by the oxidants, and in doing so compromise their function (Cecarini *et al.*, 2007, Dunlop *et al.*, 2009). Protein carbonyls are principal oxidation products and, therefore, generic markers of protein oxidation (Cecarini *et al.*, 2007, Hawkins *et al.*, 2009). These carbonyls can react with lysine residues, causing protein aggregate formation which cannot be degraded through the normal mechanisms (Cecarini *et al.*, 2007). Should this lack of degradation continue, proteolytic processes can be inhibited and cellular dysfunction enhanced (Cecarini *et al.*, 2007). Accumulation of oxidized proteins have been indicated in several human diseases (see (Dalle-Donne *et al.*, 2003) for references), and in several of these the increase in the disease activity corresponded with the carbonyl concentrations. It must be stressed, however, that the carbonyls do not indicate damage to specific proteins, information that requires further detailed investigations. In future, these observations can also be characterized on other levels such as DNA.

Protein-bound carbonyl groups react with 2,4-dinitrophenylhydrazine (DNPH) to produce a yellow hydrazone, which can be quantified spectrophotometrically (Hawkins *et al.*, 2009). The assay was performed as previously described in Section 4.10. Cells were lysed, quantified and diluted. Duplicate samples were then treated with either DNPH or HCl prior to precipitation with trichloroacetic acid. The pellets were resuspended and the absorbance was measured at both 280 nm and 370 nm. A standard BSA concentration series was prepared and a straight line was

fitted to the absorbance values. The protein concentration of each sample (not containing DNPH) was then determined by referencing it to the standard curve. Subsequently, the carbonyl concentrations were expressed as nmol of carbonyl groups per mg protein. A BSA sample was included in the assay as a negative control. The carbonyl concentrations in the control vs. metabolite treated cells are presented in Figure 5.8.

After 24 hours of growth, the untreated samples showed similar protein carbonyl concentrations as the negative control (see Figure 5.8). These levels remained low for the duration of the experiment, with a slight fluctuation at the 72 hour time point (statistically insignificant; $p=0.34$). These low levels of protein damage suggest that the level of intracellular ROS production measured in the untreated samples were normal cellular by-products, easily used or removed by the cell with minimal protein damage occurring. Although the ROS production in the untreated cultures increased during the 72 to the 96 hour increment, this increase was not reflected on the protein damage level, indicating active and sufficient removal of oxidized proteins by intracellular proteases, such as the proteasome complex, lysosomal cathepsins or cytosolic calpains (Cecarini *et al.*, 2007, Dunlop *et al.*, 2009).

In the HT1 metabolite treated cells, the concentrations of carbonyl groups were much the same as that of the untreated cells during the first 48 hours of exposure. However, in the next 48 hours the carbonyl concentrations increased 3-fold. Two-way ANOVA analysis revealed these changes to be statistically insignificant ($p=0.15$). Although this increase was not statistically significant, one can ask whether it could be biologically relevant, since it has been suggested that any increase in protein carbonyl concentrations are generally an indication of oxidative stress and protein dysfunction (Dalle-Donne *et al.*, 2003).

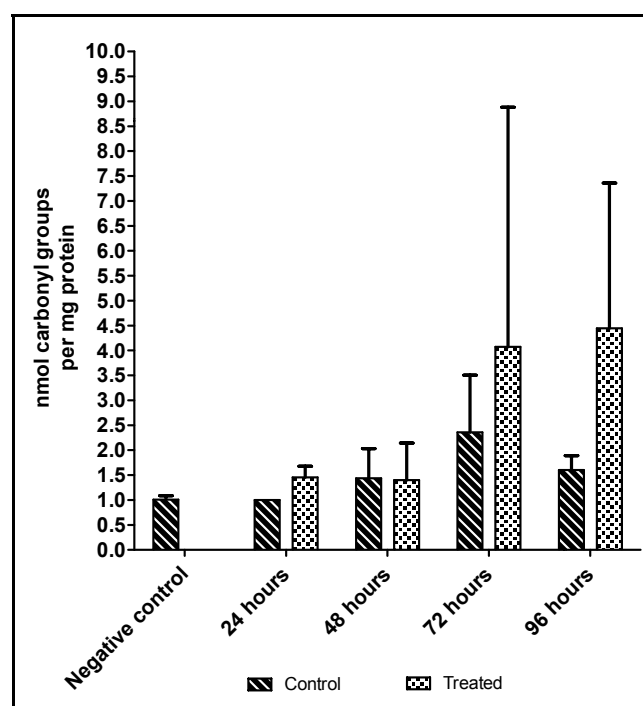


Figure 5.8. Protein carbonyl concentrations in HepG2 cells with and without HT1 metabolite treatment. Protein carbonyl concentrations were determined over a 96 hour period in intervals of 24 hours. The graph represents the concentrations of two independent experimental series, and is expressed as mean with std. dev.

Some variability was observed in the carbonyl concentrations after 72 and 96 hours exposure to the HT1 metabolites (as indicated by the error bars). This variation was not seen in any of the control samples or the first time points of the treated samples, indicating that the variation was not inter-experimental, but rather inter-cellular variations in carbonyl adduct formation and removal. This somewhat greater variation was most probably due to aging of the cells. As the cells grow older, the continual exposure to the metabolites may result in more variability in their ability to handle oxidative stress. This brings to mind whether this has anything to do with the clonal nature of HCC development in HT1 (Poudrier *et al.*, 1998)?

5.5.4. Discussion and conclusion

The HT1 metabolites clearly increased ROS production compared to the untreated samples (Figure 5.7). It also seemed that after 72 hours of growth, the production of ROS increased regardless of the presence of the metabolites, although this increase was not statistically significant. This once again suggests that growth conditions or cell density affects HepG2 cells.

It appears that the increased ROS production after metabolite exposure directly resulted in increased protein oxidation during sustained exposure. The lack of increased protein damage, in spite of increased ROS production, in the last 24 hours of the control samples suggests the active removal of the affected proteins, a process apparently inhibited or overwhelmed in the treated samples, hence the elevated levels of carbonylated proteins observed. It can be concluded that the increase in ROS production does result in a state of oxidative stress. The treated cells clearly sustain damage to proteins with prolonged exposure to the metabolites.

The increased production of intracellular ROS and the ensuing cellular damage is clearly an important occurrence in HT1, and requires further study to clarify the cause of the increased ROS and to establish the extent of the damage to the cells. The envisaged HT1 cell culture model would be of great value for such experiments, since more control of cell density and longer exposure times will be possible. The model will also enable a more complete study of the oxidative stress, since all the accumulating metabolites will be present, not only SA and pHPPA. Further characterization of the type of protein damage, as well as damage to other molecules such as DNA, will also provide more information as to the extent of the cellular damage.

5.6. Interactions between the investigated parameters

5.6.1. Introduction

No molecule, enzyme or mechanism in a cell can be studied independent from the complete intra- and extracellular environment. Changes observed in any of these can also not be interpreted completely if not analyzed in the context of its environment. Therefore, some of the results obtained were plotted in various combinations to identify any interactions or correlations between the different parameters investigated. All data sets were normalized relative to the 24 hour control samples to enable direct comparisons.

5.6.2. *MGMT* promoter methylation and gene expression

As stated in Section 5.4.3, DNA methylation of gene promoter regions is thought to be directly involved in the regulation of gene expression. As such, to determine whether this is true for the *MGMT* gene in HepG2 cells, the *MGMT* promoter methylation levels were plotted with the expression levels of *MGMT* in Figure 5.9. If the promoter methylation is involved in the regulation of the expression of the gene, one would expect to see an inverse correlation between the two, since increased methylation typically results in decreased expression.

Figure 5.9 indicated that, although an early increase in the DNA methylation levels was observed in the control samples, there was no corresponding decrease in expression of *MGMT*. In fact, the expression slightly increased. Also, in the second time interval both the DNA methylation levels and the expression of *MGMT* decreased. Only between the 72 and 96 hour time points were any inverse correlation between these two parameters observed, most likely this was coincidental. Although an inverse relationship was observed in the treated samples between the 48 and 72 hour time points, this effect was completely reversed in the following 24 hours.

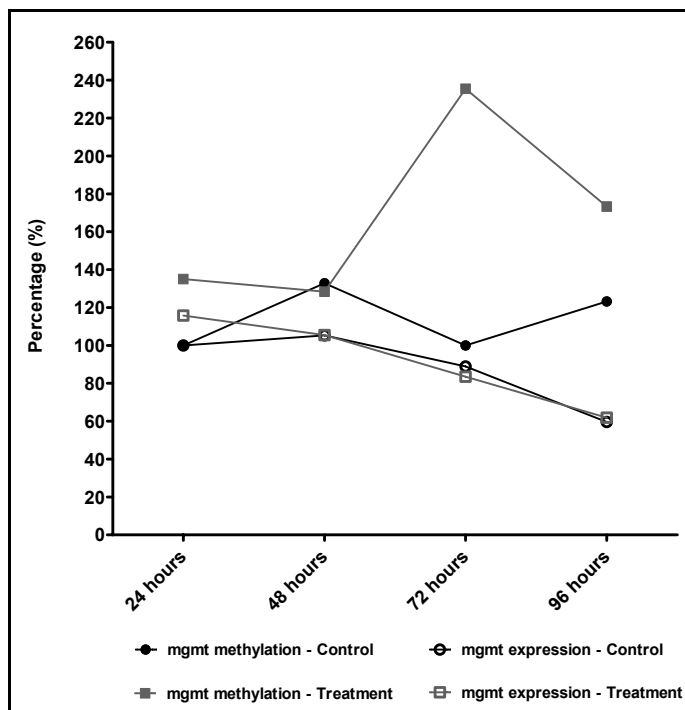


Figure 5.9. *MGMT* expression and *MGMT* promoter methylation in HepG2 cells following HT1 metabolite treatment. A value of 100% was assigned to the 24 hour control samples of both sample sets.

One can deduce that the DNA methylation status of the *MGMT* promoter region had no direct effect on its expression in HepG2 cells, in the time observed. Similar discordances between *MGMT* promoter methylation and mRNA expression have been reported (Bearzatto *et al.*, 2000, Everhard *et al.*, 2009). Actually there are some debate regarding the inverse correlation of *MGMT* CpG island methylation and *MGMT* gene expression. Recent studies pertaining to this relationship have shown that this association is frequent, but not absolute (Vlassenbroeck *et al.*, 2008, Everhard *et al.*, 2009, Kreth *et al.*, 2011). Furthermore, it has been proposed that other epigenetic mechanisms may be involved in *MGMT* expression regulation, and that DNA methylation is

indirectly involved (Vlassenbroeck *et al.*, 2008, Everhard *et al.*, 2009, Christmann *et al.*, 2011). This suggestion has been supported by other findings (Kreth *et al.*, 2011). The results obtained in this study further add to this debate, and highlights the uncertainty regarding the assumption that *MGMT* promoter methylation is directly responsible for translation repression and subsequent decreased expression of the *MGMT* gene. Some of these discordances may be attributed to the presence of different CpG islands in the *MGMT* promoter region (Everhard *et al.*, 2009, Christmann *et al.*, 2011).

It is clear, however, that *MGMT* expression was affected by the cell density or growth conditions, decreasing significantly with time. Once again, the proposed HT1 cell culture model could be useful for more elaborate study of these factors, since long-term studies and complete HT1 cell conditions may affect *MGMT* promoter methylation and expression differently. However, one must take into account the possible detrimental effect of decreased alkylation repair, should the cells be grown at cell densities affecting *MGMT* expression.

5.6.3. ROS production and cell proliferation

It was observed in Figure 5.7 that intracellular ROS production was increased between the 72 and 96 hour time points. In an effort to explain this sudden increase, the ROS levels were plotted together with the cell proliferation curve in Figure 5.10.

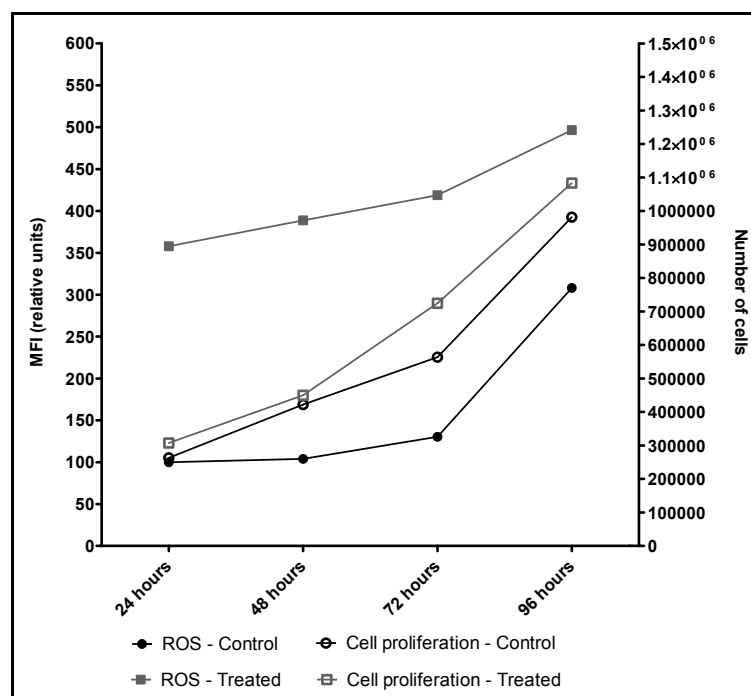


Figure 5.10. Changes in intracellular ROS production and cell proliferation in HepG2 cells following HT1 metabolite treatment. The MFI is presented on the left-hand Y-axis, while the number of cells is presented on the right-hand Y-axis.

It is clear from the cell proliferation curve that in the untreated samples there were a slight increase in the doubling time between the 72 and 96 hour time points compared to the first 3 time intervals, although it was not statistically significant. This coincided with the sudden increase in ROS production in the same time interval. In the treated samples, this increased

proliferation was already present from the 48 hour time point, which once again coincided with an increase in ROS production in the same time interval, although this increase was slightly less pronounced until the 72-96 hours time interval.

It does, therefore, appear that the time-dependent increase of intracellular ROS production in both the treated and untreated samples was a direct result of an increase in the cell density or change in growth conditions. One can deduce that these ROS levels can still be regarded as “normal”. Nevertheless, it does not change the fact that exposure of HepG2 cells to the HT1 metabolites markedly increased intracellular ROS production. Conversely, it has been shown that cell proliferation can be stimulated by the presence of oxidative stress, and that this increased growth was involved in tumour progression (Sasaki, 2006). One could thus propose that ROS production was not a result of increased cell proliferation, but that cell division was enhanced in response to increased ROS production.

5.6.4. Intracellular ROS production and *MGMT* expression

The change in *MGMT* expression over time could not be explained by the change in *MGMT* promoter methylation (see Section 5.6.2). Therefore, *MGMT* expression and ROS production in HepG2 cells were plotted together to investigate possible interactions between the two parameters.

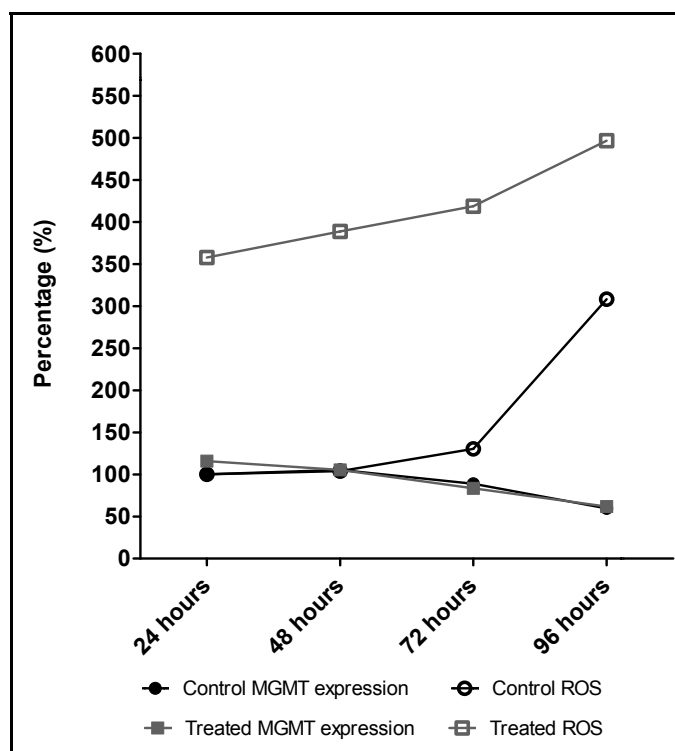


Figure 5.11. *MGMT* expression and intracellular ROS production in HepG2 cells following HT1 metabolite treatment. A value of 100% was assigned to both of the 24 hour control samples.

Once again, a directly inverse decrease in *MGMT* expression and increase in intracellular ROS production was observed (Figure 5.11). Interestingly, both of these parameters started to change notably from the 72 hour time point. This could indicate that the increase in ROS production somehow alters *MGMT* expression. The metabolite treatment, however, did not seem to affect this

correlation, even though the ROS production was greatly elevated as a result of the treatment. This could suggest that both ROS production and *MGMT* expression are influenced by the same factors, possibly increased cell proliferation or density, and that they do not necessarily directly affect each other. This conclusion was drawn from the fact that, although ROS production was already markedly increased after 24 hours exposure to the metabolites, *MGMT* expression was not decreased compared to the control samples.

5.6.5. Association between time related changes and cellular senescence

When cells stop dividing irreversibly, it is known as cellular senescence. These cells differ morphologically from normal cells and express senescence associated genes (Campisi, 1997, Suzuki *et al.*, 2002). The degree of senescence in the cultured cells, with and without treatment, was assessed with the β -galactosidase assay. This assay measures the percentage of cells expressing this enzyme which is associated with senescence (Pieper *et al.*, 1999).

The β -galactosidase assay was performed as previously described (see Section 4.11), and the percentage senescence-positive cells (coloured blue) were assessed using a 10x microscope field (results shown in Appendix F). The percentage senescent cells did not increase significantly with increased incubation time (age), and were never more than 5% of the total cell population. Also, almost no increase in cellular senescence was observed in the cultures treated with the HT1 metabolites compared to the control cultures.

It can be concluded that none of the changes observed in terms of cell growth conditions or cell density was a direct result of aging of the cultures. HT1 metabolite treatment clearly also had no effect on the rate of aging of the HepG2 cells in the time observed.

5.6.6. Cyclical DNA methylation patterns

Several of the results alluded to the presence of a cyclical DNA methylation pattern in HepG2 cells in the time intervals investigated. One does not want to over emphasize this phenomenon, but it was striking and needs further attention.

Although it is known that DNA methylation changes dramatically during development, it is generally thought that once established in a cell, the pattern remains stable and is only maintained (Brown *et al.*, 2007). In the literature, however, it has been observed that DNA methylation levels change in HeLa cells during its cell cycle (Brown *et al.*, 2007). Kangaspeska and colleagues also showed that cyclical CpG methylation variation was involved in the transcriptional regulation of several genes with a periodicity of approximately 100 minutes (Kangaspeska *et al.*, 2008, Métivier *et al.*, 2008).

It was also proposed that changes in gene expression as a result of oscillatory, circadian, and seasonal rhythms could be facilitated by epigenetic plasticity, giving rise to a so-called “circadian epiphenotype” (Gallou-Kabani *et al.*, 2007). This is because DNA methylation and expression can be affected by various environmental and genetic factors, although the circadian changes are thought to be mostly tissue-specific (Gallou-Kabani *et al.*, 2007). For instance, Ando and

associates showed that in the liver numerous genes were expressed rhythmically (Ando *et al.*, 2006). The cycling activity of promoters has thus far been divided into two classes, namely short-term interactions lasting up to tens of seconds, and longer term interactions which can last in the order of tens of minutes (Reid *et al.*, 2009).

Cyclical variation of CpG methylation is thought to provide an additional level of gene expression regulation in the genome (Métivier *et al.*, 2008). Gallou-Kabani and colleagues stated the following, “Even tiny changes in circadian gene expression patterns in particular tissues may result in functional asynchrony in the body as a whole”, and that various genetic or environmental triggers or pathophysiological conditions can also trigger epimutations (Gallou-Kabani *et al.*, 2007). Several factors, including shift work, travel or food restriction can cause a misalignment of these circadian phases. Such phase-shifts could eventually result in the prolonged presence of abnormal and unphased “locking” or “leakage” of gene expression (Gallou-Kabani *et al.*, 2007).

This leads to the question of whether the apparent cyclic patterns that were observed in this study (Figures 5.2 and 5.4) could be ascribed to the presence of an “oscillatory epiphenotype”, both on a gene-specific level and a global level, in HepG2 cells. However, these cyclic patterns were not reflected in the *MGMT* expression experiments (see Figure 5.6). This raises the question whether the cyclic patterns were a true phenomenon, or simply a result of the experimental design. The frequent observation of this pattern in the results, however, makes this improbable.

5.7. Summary

After direct exposure of HepG2 cells to the HT1 metabolites, SA and pHPPA, it was observed that global DNA methylation was not significantly increased or decreased as a result of the exposure during the time observed. However, the metabolites caused a clear shift in the cyclical pattern observed in the DNA methylation experiment, causing a misalignment of these “cyclical phases”.

MGMT has been associated with non-HT1 HCC in the literature, however, in this study no clear indication of inhibition of its expression could be observed. It must be stated, though, that this does not exclude possible changes in its function due to protein damage or depletion. It was also observed that in HepG2 cells there seemed to be no direct correlation between the promoter methylation status of the gene, and expression of *MGMT*. This suggests that *MGMT* was not directly regulated by DNA methylation alone, although this observation could be specific to the cell type.

It was clearly indicated that the HT1 metabolites resulted in a significant increase in intracellular ROS production, and it was subsequently shown that this increase directly resulted in a state of oxidative stress with associated protein damage. It was also observed that ROS levels increased as cell density increased, suggesting a direct correlation.

5.8. Manuscripts

The following manuscripts were prepared for publication, as mentioned in Section 1.5.

O⁶-methylguanine-DNA methyltransferase in the aetiology of hereditary tyrosinemia type 1 associated hepatocellular carcinoma

Chrisna Gouws and Pieter J. Pretorius

SUMMARY

In the metabolic disorder hereditary tyrosinemia type 1 (HT1) there is an accumulation of damaging tyrosine metabolites, such as succinylacetone (SA) and *p*-hydroxyphenylpyruvate (pHPPA). Hepatocellular carcinoma (HCC) is characteristic of the disease, although its molecular mechanism remains largely unknown. The HT1 metabolites have been proposed to be alkylating agents, which can lead to a plethora of adverse events and are thought to contribute to the HCC development. O⁶-methylguanine-DNA methyltransferase (MGMT), a DNA alkylation repair enzyme, has been found to have altered promoter methylation patterns and/or expression patterns in non-HT1 HCC. Defective MGMT alkylation repair is thought to be a fundamental event in multistep HCC, but this enzyme has not been studied in HT1-associated HCC. In this study, we investigated the effect of the accumulating HT1 metabolites SA and pHPPA on the promoter methylation and mRNA expression of the *MGMT* gene to elucidate its role in the associated liver pathophysiology. Although the loss of *MGMT* expression is usually attributed to *MGMT* promoter CpG island hypermethylation, the results obtained in this study suggest that other factors control *MGMT* expression in HepG2 cells, since no correlation could be observed. The results also indicated no significant change in either the promoter methylation or the expression of *MGMT* as a result of treatment with the accumulating metabolites. It is suggested MGMT should be investigated on a protein level to identify possible depletion or protein damage, in which case the ensuing alkylation damage accumulation could play a role in the associated liver pathophysiology.

Introduction

A deficient fumarylacetoacetate hydrolase enzyme (FAH; E.C.3.7.1.2) in the tyrosine catabolic pathway results in the metabolic disease hereditary tyrosinemia type 1 (HT1; OMIM ID: 276700) (Mitchell et al., 2001, Dreumont et al., 2004, Scott, 2006). Several damaging metabolites accumulate intracellularly, including fumarylacetoacetate (FAA), maleylacetoacetate (MAA), succinylacetone (SA) and to a lesser extent *p*-hydroxyphenylpyruvate (pHPPA) (Mitchell et al., 2001, Bergeron et al., 2006, Nakamura et al., 2007). In HT1 the liver is primarily affected, frequently resulting in hepatocellular carcinoma (HCC), the pathophysiology of which is still largely unresolved (Mitchell et al., 2001, Vogel et al., 2004, Scott, 2006). The accumulating HT1 metabolites have been proposed to be alkylating agents, and are thought to contribute to the frequent HCC development (Jorquera and Tanguay, 2001, Mitchell et al., 2001, Russo et al., 2001). These alkyl adducts can lead to a plethora of adverse events, even cancer and cell death, and it can potentially also change the normal DNA methylation patterns of cells (Hepburn et al., 1991, Mitchell et al., 2001, Jacinto and Esteller, 2007, Franco et al., 2008).

One of the most important DNA alkylation repair mechanisms involves the enzyme O⁶-methylguanine-DNA methyltransferase (MGMT; E.C.2.1.1.63), its main function being the removal of highly promutagenic and cytotoxic O⁶-alkyl adducts on guanine bases (O⁶-alkylG) in DNA (Liu and Gerson, 2006, Jacinto and Esteller, 2007, Kaina et al., 2007, Zhong et al., 2010). Cells deficient in MGMT have been shown to be more susceptible to the damage caused by alkylating agents (Grady and Ulrich, 2007, Kaina et al., 2007). Loss of *MGMT* expression can be attributed mainly to *MGMT* promoter CpG island hypermethylation, as opposed to conventional

deletions, mutations and rearrangements (Jacinto and Esteller, 2007, Kaina *et al.*, 2007, Hermes *et al.*, 2008, Vlassenbroeck *et al.*, 2008). This tight correlation suggests an important role for aberrant promoter hypermethylation of *MGMT* in primary human cancer, and *MGMT* has been demonstrated to have altered promoter methylation patterns and/or expression patterns in HCC (not of HT1 origin) (Esteller *et al.*, 1999, Zhang *et al.*, 2003, Vlassenbroeck *et al.*, 2008). Bearzatto and colleagues observed that in some cells, however, hypermethylation of its promoter did not result in decreased expression of *MGMT*, making this relationship between promoter methylation and transcription even more complex (Bearzatto *et al.*, 2000).

Expression of the *MGMT* enzyme directly affects the well-being of the cells by affecting the DNA repair capacity available to deal with any inflicted damage which might threaten its genomic integrity (Major and Collier, 1998, Hermes *et al.*, 2008). Since the protein level of *MGMT* in a cell can be directly correlated with the mRNA content (Major and Collier, 1998, Jacinto and Esteller, 2007), measuring the *MGMT* mRNA expression in the HepG2 cells gives a measure of the protein production, and therefore the potential alkylation damage repair capacity of the cells.

In spite of the well-known and widely publicised association between impaired *MGMT* and HCC development (Zhang *et al.*, 2003, Hermes *et al.*, 2008), and the apparent central role of O⁶-meG in the carcinogenesis initiation, this association has not been investigated in HT1. The liver is an important site for precarcinogenic agent metabolic activation, thereby possibly raising carcinogenic susceptibility, and Major and Collier proposed defective *MGMT* alkylation repair to be “an early, possibly pivotal, event in the multistep process of hepatocellular carcinogenesis” (Major and Collier, 1998). In this study, we investigated the effect of the accumulating HT1 metabolites SA and pHPA on the promoter methylation and mRNA expression of the *MGMT* gene to elucidate its role in the associated liver pathophysiology.

Materials and methods

Cell culturing

HepG2 cells were propagated in Dulbecco's Modified Eagles Medium (HyClone medium; Thermo Scientific) supplemented with 10% (v/v) foetal bovine serum (FBS; Sigma), 1 x MEM non-essential amino acids (0.1 mM; Gibco), 2 mM [final] L-glutamine (BioWhittaker) and 1 x Penicillin/Streptomycin (100 U Pen./ml; 100 µg Strep./ml; BioWhittaker). Culturing took place at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were seeded at 200 000 cells per well in 6-well plates (Nunc) in 2 ml complete medium, with or without metabolite treatment. The number of cells seeded was determined experimentally to ensure that cells continued to actively divide throughout the experiment, and that after 96 hours of growth 70-80% coverage of the growth surface was reached.

Treatment entailed the incubation of the cells in the simultaneous presence of 50 µM SA (Sigma; dissolved in ddH₂O) and 100 µM pHPA (ICN Biomedicals; dissolved in ddH₂O). Growth medium and metabolites were replaced every 24 hours, and cells were harvested after 24, 48, 72 and 96 hours. Harvesting consisted of the following: the cells were washed with phosphate buffered saline (PBS; Sigma), and then incubated with 1x trypsin (BioWhittaker) until the cells were detached. The enzyme was then inactivated by the addition of culturing medium.

Real-time methylation-specific PCR

Genomic DNA was isolated using a FlexiGene DNA kit (Qiagen), according to the manufacturer's guidelines, and between 1 and 2 µg was treated with sodium bisulfite as prescribed by the manufacturer of the EpiTect® Bisulfite kit (Qiagen). Real-time methylation-specific PCR (qMSP) was performed using published TaqMan® primers and probes (Harder *et al.*, 2008) designed to target the methylated promoter sequence of the *MGMT* gene and the *ACTB* reference sequence. The reactions were multiplexed, with 1 x TaqMan® universal PCR master mix (Applied Biosystems), 0.6 µM [final] of each primer, 0.2 µM [final] of each probe and 5.5 µl converted DNA in a reaction volume of 25 µl. All samples were amplified in duplicate on the ABI 7500 real-time PCR system (Applied Biosystems). The *MGMT* promoter methylation levels were expressed as a percentage change relative to the 24 hour control sample.

mRNA expression levels

Cells for RNA extraction were washed with PBS, followed by direct extraction with the Nucleospin® RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription with 1.5 µg RNA as template, 1 µM [final] Oligo(dT) 18mer and 1 µM [final] *18S rRNA*-specific reverse primer (5'-CGGACATCTAAGGGCATCAC-3'), which were incubated at 65 °C for 2 minutes followed by 5 minutes on ice. Subsequently, 10 µl of the enzyme mix (1 x MMLV HP RT reaction buffer, 100 U MMLV high performance reverse transcriptase (Epicentre Biotechnologies), 4 µM [final] dNTPs (New England Biolabs), 10 mM [final] DTT and 20 U recombinant RNasin® ribonuclease inhibitor (Promega)) was added. The reaction was performed overnight at 37 °C, after which the enzymes were deactivated at 85 °C for 5 minutes. The cDNA was diluted 250 x prior to use.

Expression of the *MGMT* gene was determined a 20 µl multiplex reaction via relative quantification using commercially available validated TaqMan® assays for *MGMT* (Hs00172470_m1; Applied Biosystems) and *18S rRNA* (Applied Biosystems), 1x TaqMan® universal PCR master mix (Applied Biosystems) and the ABI 7500 real-time PCR system (Applied Biosystems). *MGMT* expression was measured in duplicate, in conjunction with the reference gene, *18S rRNA* (Casorelli *et al.*, 2007) on the ABI 7500 real-time PCR system (Applied Biosystems). The *MGMT* expression levels were expressed as a percentage change relative to the 24 hour control sample by the 7500 system SDS software version 1.3.1 (Applied Biosystems).

Statistical methods

Data are presented as means ± std. dev. Comparisons among multiple groups were made by a two-way analysis of variance (ANOVA), followed by the Bonferroni sequential technique. Comparisons between individual samples were done using Student's *t* test. The differences with $p < 0.05$ were deemed statistically significant.

Results*MGMT promoter methylation*

HepG2 cells were seeded and cultured for up to 96 hours with or without HT1 metabolites, and harvested at 24 hour intervals. Following each harvest, the level of promoter methylation was determined. The methylation data of the *MGMT* promoter was normalized using *ACTB* as reference gene. For the purpose of further calculations, a value of 100% methylation was

assigned to the 24 hour control samples, and all changes in the methylation levels of samples were expressed relative to this control.

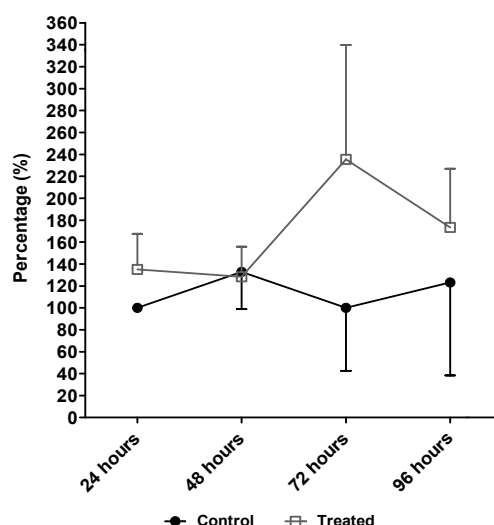


Fig. 1 The effect of HT1 metabolite treatment on *MGMT* promoter methylation in HepG2 cells. DNA methylation levels were determined over a 96 hour period in intervals of 24 hours. The graph represents the DNA methylation of three independent experimental series, and is expressed as the mean with std. dev.

The mean DNA methylation level of the *MGMT* promoter in the control samples appeared to change somewhat over time (Fig. 1). These changes, however, were never more than approximately 30%, and did not follow a linear pattern of increase or decrease over the time period investigated ($p > 0.05$). The mean *MGMT* promoter methylation level of the HT1 metabolite treated samples started from a higher level at 24 hours (35% higher than the untreated cells), and the methylation seemed to increase with time, with an overall increase of approximately 35% between intervals 24 hours and 96 hours. However, between 48 hours and 72 hours treatment, a dramatic increase of more than 100% was observed, followed by a less dramatic decrease to 96 hours. Even so, none of these changes appeared to be statistically significant ($p > 0.05$). Once again no linear increase or decrease could be distinguished, although it did seem that the promoter methylation increased on average, albeit very slightly. The comparison of the HT1 metabolite treated and untreated samples showed a difference in the level of the *MGMT* promoter methylation, but these differences were not statistically significant ($p = 0.16$).

At the 72 hour time point of the treated sample, the methylation level seemed surprisingly high compared to the other time points, and the standard deviation for this time interval was also large. This trend in *MGMT* promoter methylation was observed in two out of the three experiments that were performed, and in the other experiment the same pattern was present but much smaller deviations were observed.

MGMT expression in HepG2 cells

The expression levels of the *MGMT* gene were normalized using *18S rRNA* as reference gene. For the purpose of further calculations, a value of 100% expression was assigned to the 24 hour control samples, and all changes in the expression levels of samples were expressed relative to this control.

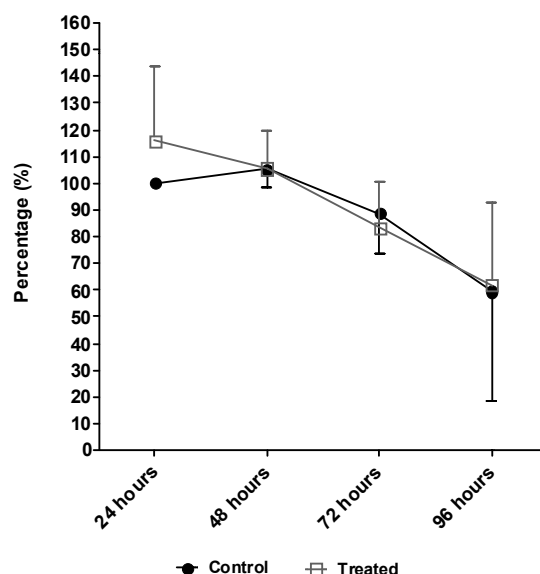


Fig. 2 *MGMT* expression in HepG2 cells following HT1 metabolite treatment. *MGMT* expression levels were determined over a 96 hour period in intervals of 24 hours. The graph represents the expression levels in four independent experimental series, and is expressed as the mean with std. dev.

The levels of *MGMT* expression appeared to decrease markedly (approximately 40%) between time points 48 hours and 96 hours in the control samples (Fig. 2). This could suggest a direct relationship between cell densities or growth conditions and cellular changes in HepG2 cell cultures. However, it appears as if *MGMT* is targeted by these cellular changes, resulting in dramatically decreased expression in the cell cultures. The *MGMT* expression levels clearly decreased with almost the same trend in the HT1 metabolite treated cultures as it did in the untreated cultures. In the treated samples this decrease was visible from the 24 hour interval, and the expression levels were decreased with more than 50% after 96 hours of treatment. Most probably, the effect of the cell density or growth conditions influenced *MGMT* expression, and this effect was significant ($p=0.01$).

The expression of *MGMT* was almost 15% higher in the treated samples after 24 hours exposure than in the untreated samples, possibly indicating the induction of *MGMT* by the alkylating metabolites, although this difference was not deemed statistically significant ($p=0.33$). Nonetheless, this increase was only present at the 24 hours time point, after which the *MGMT* expression levels markedly decreased to the same level of expression after 96 hours as that of the control samples.

Discussion

Although DNA methylation is known to be directly involved in the regulation of gene expression (Esteller, 2008), in the case of *MGMT* in HepG2 cells, the findings appeared not to be correlated. If the promoter methylation is involved in the regulation of the expression of the gene, one would expect to see an inverse relationship between the two, since increased methylation typically results in decreased expression. Although an early increase in the DNA methylation levels was observed in the control samples, there was no corresponding decrease in expression of *MGMT*. In fact, the expression slightly increased. Also, in the second time interval both the DNA methylation levels and the expression of *MGMT* decreased. Only between the 72 and 96 hour time points were any inverse association between these two parameters observed, most likely this was coincidental.

One can deduce that the DNA methylation status of the *MGMT* promoter region had no direct effect on its expression in HepG2 cells, in the time observed.

Similar discordances between *MGMT* promoter methylation and mRNA expression have been reported (Bearzatto et al., 2000, Everhard et al., 2009). Actually there are some debate regarding the inverse correlation of *MGMT* CpG island methylation and *MGMT* gene expression. Recent studies pertaining to this relationship have shown that this association is frequent, but not absolute (Vlassenbroeck et al., 2008, Everhard et al., 2009, Kreth et al., 2011). Furthermore, it has been proposed that other epigenetic mechanisms may be involved in *MGMT* expression regulation, and that DNA methylation is indirectly involved (Vlassenbroeck et al., 2008, Everhard et al., 2009). This suggestion has been supported by other findings (Kreth et al., 2011). The results obtained in this study further add to this debate, and highlights the uncertainty regarding the assumption that *MGMT* promoter methylation is directly responsible for translation repression and subsequent decreased expression of the *MGMT* gene.

Expression of *MGMT* decreased significantly in both the untreated and the treated samples between the time points. This decrease was independent of the metabolite treatment, which had no significant effect. It can be proposed that *MGMT* expression was greatly affected by an increase in cell density or altered growth conditions, decreasing significantly with time.

It has been stated that *MGMT* is not inducible in HepG2 cells (Fritz et al., 1991), while other publications stated that induction of *MGMT* varies with each different inducing agent and cell line used (Margison and Santibáñez-Koref, 2002, Margison et al., 2003). Although treatment with the HT1 metabolites seemed to induce higher expression of *MGMT* in this study, this induction was very slight, and unsustained. Still, it is known that *MGMT* induction usually peaks between 24 and 48 hours post-exposure (Margison et al., 2003), and this correlates with our findings. It is possible that the metabolites SA and pHPA used in this experiment can, in fact, induce *MGMT* in HepG2 cells even if the “induction” was only by approximately 15%. Yet, it is in line with the literature, which stated *MGMT* to be only slightly inducible (Margison et al., 2003). Also, it has been shown that chronic inflammatory states, as observed in HCC, showed marked increases in CpG island methylation (Gallou-Kabani et al., 2007), which correlates with the increase in *MGMT* promoter methylation observed in the treated cultures.

Alkylation damage may still increase in HT1 cells, even if the metabolites don't directly affect *MGMT* expression, since alkylation repair via *MGMT* is directly dependent on the amount of *MGMT* molecules available for repair and if damage exceeds synthesis, *MGMT* can become depleted. It is possible that *MGMT* can be impaired on the protein level, since SA is known to react with various amino acids and proteins, forming adducts (Manabe et al., 1985, Prieto-Alamo and Laval, 1998). *MGMT* can, therefore, still be involved in carcinogenesis in HT1, and further studies may shed more light on the involvement of *MGMT* in HT1-associated HCC.

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Accumulating tyrosinemia type 1 metabolites induce ROS production and protein oxidation*Chrisna Gouws, Lissinda du Plessis and Pieter J. Pretorius***ABSTRACT**

Damaging tyrosine metabolites accumulate in the metabolic disorder hereditary tyrosinemia type 1 (HT1), which is caused by a defective fumarylacetoacetate hydrolase enzyme. Characteristic of the untreated chronic form of this disease is the development of hepatocellular carcinoma, but its molecular mechanism remains largely unknown. Although oxidative stress responses are known to be activated in HT1, the direct effect of the accumulating metabolites succinylacetone (SA) and *para*-hydroxyphenylpyruvate (pHPPA) on the production of radical oxygen species (ROS) and their subsequent damage on a protein level have not been investigated. In this study, HepG2 cells were treated with both SA and pHPPA, and subsequently ROS levels were determined with a flow cytometric analysis. Protein carbonyl concentrations were also determined spectrophotometrically as a generic marker of oxidative protein damage. Our results clearly indicate that SA and pHPPA markedly increased intracellular ROS production, which possibly led to oxidative protein damage. Our results indicate that oxidative stress response activation in HT1 is probably a direct result of SA and pHPPA induced ROS production, and these metabolites may therefore play a more significant role in the development of HT1 associated liver disease than thought previously.

Introduction

Hereditary tyrosinemia type 1 (HT1) is a clinically severe autosomal recessive inherited metabolic disease (OMIM ID: 276700) caused by a deficiency of the fumarylacetoacetate hydrolase enzyme (FAH; E.C.3.7.1.2) in the tyrosine catabolism (Mitchell *et al.*, 2001, Dreumont *et al.*, 2004, Scott, 2006). Several upstream metabolites accumulate intracellularly, including fumarylacetoacetate (FAA), maleylacetoacetate (MAA), succinylacetone (SA) and to a lesser extent *para*-hydroxyphenylpyruvate (pHPPA) (Mitchell *et al.*, 2001, Bergeron *et al.*, 2006). The major affected organ in HT1 is the liver, often presenting with characteristic hepatocellular carcinoma (HCC) and somatic mosaicism (Mitchell *et al.*, 2001, Vogel *et al.*, 2004, Scott, 2006). The aetiology of HT1 associated HCC remains largely unknown, although some hypotheses involve the toxicity and possible alkylation potential of the accumulating metabolites (Mitchell *et al.*, 2001, Dreumont *et al.*, 2004, Bergeron *et al.*, 2006). The elucidation of the molecular mechanism of the cancer development remains critical, since patients receiving treatment can still develop HCC (Mitchell *et al.*, 2001, Vogel *et al.*, 2004).

Several common liver diseases associated with oxidative stress are prone to cancer development, which suggests that stress-induced malfunction of the death machinery may play a role in the pathophysiology of these disorders and could be related to the risk of developing HCC (Vogel *et al.*, 2004). FAA, and possibly MAA, can react with, or even deplete, cellular glutathione, resulting in oxidative damage which appears to potentiate its mutagenicity (Jorquera and Tanguay, 1997, Mitchell *et al.*, 2001, Vogel *et al.*, 2004, Langlois *et al.*, 2006). Dieter and colleagues therefore suggested that various tyrosine intermediates are capable of oxidative stress induction, and that this was possibly causally linked to the ensuing liver pathology (Dieter *et al.*, 2003). They also

proposed that HT1 patients on 2-(2-Nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) treatment are chronically exposed to oxidative stress, since the NTBC-rescued 14CoS/14CoS mouse model had a significant oxidative stress response in the liver and kidneys (Dieter *et al.*, 2003). Several research groups have since shown that oxidative stress response pathways are activated in HT1 models, but few of them directly assayed reactive oxygen species (ROS) production and its ensuing cellular damage (Dieter *et al.*, 2003, Vogel *et al.*, 2004, Bergeron *et al.*, 2006, Fisher *et al.*, 2008). Vogel and colleagues did indicate an increase in oxidative DNA damage in *Fah*^{-/-} mice (Vogel *et al.*, 2004), but Jorquera and Tanguay measured ROS production following direct exposure experiments with FAA, and did not detect any increase in its production (Jorquera and Tanguay, 1997). Bergeron and associates showed the launch of an endoplasmic reticulum (ER) stress response in HT1, and subsequently stated that it was unclear whether the previously observed induction of the stress-related proteins, HO-1 and Hsps, was a result of oxidative or ER stress (Bergeron *et al.*, 2006). As such, the source of the oxidative stress response in HT1 remains unclear, and needs further exploration.

Materials and methods

Cell culture conditions

To investigate the effect of the HT1 metabolites, SA and pHPA, on intracellular ROS production and its ensuing cellular damage, we directly exposed cultured hepatocytes to these metabolites. HepG2 cells were propagated in Dulbecco's Modified Eagles Medium (HyClone medium; Thermo Scientific), supplemented with 10% v/v foetal bovine serum (Sigma), 1% v/v MEM non-essential amino acids (0.1 mM; Gibco), 2 mM L-Glutamine (BioWhittaker) and 1% v/v Penicillin/Streptomycin (100 units Pen./ml; 100 µg Strep./ml; BioWhittaker). Culturing took place at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were seeded at 200 000 cells per well in 6-well plates (Nunc) in 2 ml complete medium, with or without metabolite treatment. The number of cells seeded was determined experimentally to ensure that cells continued to actively divide throughout the experiment, and that after 96 hours of growth 70-80% coverage of the growth surface was reached.

Treatment entailed simultaneous incubation of cells with 50 µM SA (Sigma; dissolved in ddH₂O) and 100 µM pHPA (ICN Biomedicals; dissolved in ddH₂O), which were added to the growth medium. Growth medium and metabolites were replaced every 24 hours, and cells were harvested after 24, 48, 72 and 96 hours. For positive oxidative stress controls, separate wells of cells were treated with 2 mM hydrogen peroxide (Pakmed) in 2 ml growth medium for 3 hours prior to harvesting. Harvesting consisted of the following: the cells were washed with phosphate buffered saline (PBS; Sigma), and then incubated with 1x trypsin (BioWhittaker) until cells were detached. The enzyme was then inactivated by the addition of culturing medium.

Flow cytometric ROS analysis

The 2',7'-dichlorodihydrofluorescein diacetate probe (DCFH-DA) was used to quantify ROS, since ROS reacts with the probe to produce a fluorescent product, 2',7'-dichlorofluorescein (DCF) (Jorquera and Tanguay, 1997, Lee *et al.*, 2000, Jung *et al.*, 2009, Kuznetsov *et al.*, 2011). ROS detection was performed as previously described (Lee *et al.*, 2000, Jung *et al.*, 2009, Du Plessis *et*

al., 2010a). Prior to harvesting of the cells, 10 μ M 2',7'-dichlorodihydrofluorescein diacetate probe (DCFH-DA) (Invitrogen, Molecular Probes) was added to each well and incubated for 30 minutes at 37 °C in the dark. After incubation, the cells were washed with PBS and harvested by trypsinization. Medium was added to inactivate the enzyme and the cells were collected by centrifugation at 1 000g for 3 minutes. The cells were then resuspended in 500 μ l PBS for flow cytometric analysis. Controls included positive controls for the ROS production, cells not treated with the probe (cell control) and untreated cells treated with the probe (negative control).

DCF fluorescence was measured using a 488 nm argon laser for excitation on the FACSCalibur flow cytometer (Becton Dickinson) (Lee *et al.*, 2000, Du Plessis *et al.*, 2010a). The intensities of emitted fluorescence were collected with the FL1 photomultiplier tube, and in each measurement a minimum of 50 000 events were collected in the gated positive population, and approximately 100 000 events in the total population. Forward- and side-scatters were used to establish size gates and exclude cellular debris from the analysis. Data was acquired with the CELLQuest Pro Software (Becton Dickinson) and analysed using FCSEXPRESS (version 3, De Novo Software). Relative change in the mean fluorescence intensity (MFI) of samples was calculated as the ratio between the MFI in the treated cells and that of the cell control.

Protein carbonyl assay

One of the general markers of protein oxidation damage is protein carbonyl groups (Hawkins *et al.*, 2009). 2,4-dinitrophenylhydrazine (DNPH) reacts with these protein-bound carbonyl groups to give the corresponding hydrazone, and this yellow product can be quantified spectrophotometrically (Hawkins *et al.*, 2009). Harvested cells were washed with PBS before storage at -80 °C until use. The cells were thawed and lysed with M-Per[®] mammalian protein extraction reagent (Thermo Scientific). The protein carbonyl detection assay was performed as previously described (Dalle-Donne *et al.*, 2003, Khan *et al.*, 2004, Hawkins *et al.*, 2009), with minor adaptations. This entailed the dilution of all samples to approximately 1.3 mg/ml protein prior to the addition of 500 μ l 10 mM 2,4-dinitrophenylhydrazine (DNPH) (Fluka) in 2 M HCl to 100 μ l of each sample (in duplicate), or 500 μ l 2 M HCl (Rochelle Chemicals) for corresponding sample reagent blanks (in duplicate). A bovine serum albumin (BSA; Thermo Scientific) sample was included as a negative control. All tubes were incubated at room temperature for 1 hour in the dark before 500 μ l of a 20% trichloroacetic acid (UnivAR) solution was added to each tube. These solutions were then incubated at -20 °C for 20 minutes, followed by centrifugation at 9 500g for 10 minutes at room temperature. The protein pellets were washed three times with 1 ml ice cold 1:1 ethanol/ethyl acetate (Merck), and resuspended in 200 μ l 6 M guanidine-HCl (Sigma). After incubation for 30 minutes at 37 °C, the absorbance of the solutions was measured in flat-bottomed 96-well plates, at 280 nm and 370 nm respectively, on the BioTek Synergy HT plate reader (Gen 5 version 1.05.11).

A standard concentration series was prepared by diluting a BSA solution with 6 M guanidine-HCl. The protein concentration of each reagent blank sample (not containing DNPH) was then determined by referencing its absorbance to the standard curve. The protein carbonyl concentrations were determined using the DNPH absorption coefficient of 22 000 M⁻¹cm⁻¹, and the carbonyl concentrations were expressed as nanomoles of carbonyl per milligram of protein.

Statistical methods

For statistical interpretation the implemented statistical functions of GraphPad Prism® (version 5.03) was used. Data was presented as the means of several series of experiments with its standard deviations, unless stated otherwise. To determine whether differences among multiple groups were statistically significant, two-way distributional assumptions of analysis of variance (two-way ANOVA) analyses, followed by the Bonferroni-adjusted sequential technique, were applied to the data. Comparisons between individual samples were done using Student's *t* test. The differences with $p < 0.05$ were deemed statistically significant.

Results

Intracellular ROS levels increase after exposure to HT1 metabolites

The effect of the accumulating HT1 metabolites, SA and pHPPA, on the production of intracellular ROS and oxidation of proteins in human hepatic cells was determined by continuously exposing HepG2 cells to both 50 μ M SA and 100 μ M pHPPA for 96 hours, in 24 hour intervals. ROS production was quantified by measuring its reaction with the DCFH-DA dye to produce green fluorescent DCF, which was measured on the FACSCalibur flow cytometer (Jung *et al.*, 2009, Du Plessis *et al.*, 2010a). The ROS production levels of the control vs. treated cells are presented as change in MFI in Fig. 1.

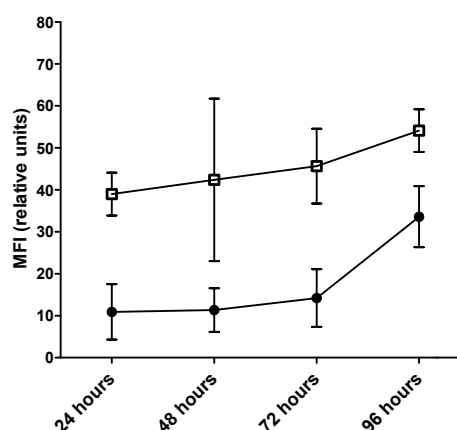


Fig. 1. The effect of the accumulating HT1 metabolites, SA and pHPPA, on intracellular ROS production in HepG2 cultured cells. Normal cells (solid circles); Cells + SA and pHPPA (empty squares). DCF fluorescence was measured every 24 hours during continuous exposure to the metabolites for 96 hours. The graph represents the mean MFI $\pm$ std.dev. of four repeats for time points 24 and 48 hours, and two repeats for time points 72 and 96 hours.

ROS production in the control HepG2 cells were quite low and stable during the first 72 hours of growth, followed by a sudden increase of approximately 20 units in the last 24 hours. This 3-fold increase was not statistically significant ($p > 0.05$). The intracellular ROS levels in the HT1 metabolite treated samples were significantly increased compared to the control samples ($p = 0.0016$), and this increased production was evident at all time points.

Protein carbonyl concentrations increase after exposure to HT1 metabolites

Protein carbonyl concentrations were determined spectrophotometrically following exposure of cellular proteins to DNPH, and were expressed as nmol of carbonyl groups per mg protein. A BSA

sample was included in the assay as a negative control. The carbonyl concentrations in the control vs. treated cells are presented in Fig. 2.

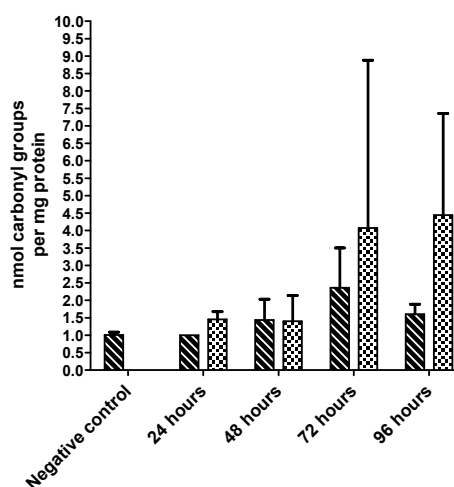


Fig. 2. HT1 metabolite induced carbonylation of intracellular proteins in HepG2 cultured cells. Untreated cells (diagonal stripes); Cells + SA and pHPPA (chequered). Carbonyl concentrations were measured every 24 hours during continuous exposure to the metabolites for 96 hours. The graph represents the mean nmol carbonyl groups with std.dev. of four repeat measurements.

After 24 hours of growth, similar protein carbonyl concentrations were observed in the untreated samples as in the negative control. These levels remained low for the duration of the experiment, with a slight fluctuation at the 72 hour time point (statistically insignificant; $p=0.34$). The HT1 metabolite treated samples initially presented the same, low concentrations of protein carbonyl groups, but at time points 72 and 96 hours, these concentrations increased approximately 3-fold. However, two-way ANOVA analysis revealed these changes to be not quite statistically significant ($p=0.15$).

Discussion

ROS is a normal product of cell metabolism, however, when the production of these reactive molecules exceeds the cell's antioxidant capacity, a state of oxidative stress can arise (Franco *et al.*, 2008, Dunlop *et al.*, 2009). This results in an environment where all cellular molecules can be damaged, including DNA, RNA and proteins (Cerdeira and Weitzman, 1997, Mary *et al.*, 2004, Cecarini *et al.*, 2007, Jung *et al.*, 2009). The DCF test was used to quantify generic intracellular ROS production, and although some concerns regarding this method have been raised (Karlsson *et al.*, 2010), this method is a standard assay for ROS studies and has been used and validated extensively (Jung *et al.*, 2009, du Plessis *et al.*, 2010a, Kuznetsov *et al.*, 2011).

In normal, active dividing HepG2 cells with sufficient growth medium and limited accumulation of waste (medium was replaced every 24 hours), intracellular ROS was present, but in small amounts. This was expected, since ROS is a normal by-product of cellular function (Cecarini *et al.*, 2007, Franco *et al.*, 2008). Although ROS production increased between the 72 and 96 hour time points, the change was not statistically significant, and the ROS levels remained less than in the treated samples. The increase can be a result of the increased cell density in this time interval. Intracellular ROS levels increased significantly after exposure to the HT1 metabolites, SA and

pHPPA, when compared to the control samples, which suggest that the metabolites induced increased intracellular ROS production or a decrease of the removal of these species (Cecarini *et al.*, 2007, Valko *et al.*, 2007). Interestingly, this increased ROS production seemed to follow the same pattern as in the control samples, implying that the effect of the metabolites on ROS production is not cumulative, but rather constant.

Proteins are likely oxidation targets and therefore an important level to measure such damage (Dalle-Donne *et al.*, 2003, Hawkins *et al.*, 2009). Oxidative protein damage can cause amino acid structure alterations, functional impairment and enzymatic activity changes, affecting cell signalling, cell structure and metabolism (Mary *et al.*, 2004, Cecarini *et al.*, 2007). Protein carbonyls are principal oxidation products, and therefore a common and generic marker of protein oxidation (Dalle-Donne *et al.*, 2003, Hawkins *et al.*, 2009). These oxidized proteins have to be eliminated, but there are very few enzymatic repair systems available for these proteins and degradation is the main mechanism of its removal (Mary *et al.*, 2004, Dunlop *et al.*, 2009). Accumulation of these oxidized proteins has been indicated in several human diseases (see (Dalle-Donne *et al.*, 2003) for references), and in several of these the increase in the disease activity corresponded with the carbonyl concentrations.

In the control samples, the protein carbonyl concentrations were comparable with the negative control, with insignificant fluctuations over time. This indicates that the level of intracellular ROS production measured in the untreated samples were normal cellular by-products, easily used or removed by the cell with minimal protein damage occurring. Although the ROS production in the untreated cultures increased during the 72 to the 96 hour increment, this increase was not reflected on the protein damage level, indicating active and sufficient removal of oxidized proteins by intracellular proteases, such as the proteasome complex, lysosomal cathepsins or cytosolic calpains (Cecarini *et al.*, 2007, Dunlop *et al.*, 2009).

In the HT1 metabolite treated cells, the concentrations of carbonyl groups were much the same as that of the untreated cells during the first 48 hours of exposure. However, in the next 48 hours the carbonyl concentrations increased 3-fold. Although this increase was not statistically significant, one can ask whether it could be biologically relevant, since it has been suggested that any increase in protein carbonyl concentrations are generally an indication of oxidative stress and protein dysfunction (Dalle-Donne *et al.*, 2003). Therefore, should it be considered biologically significant, it would suggest that the increased ROS production directly resulted in increased oxidative protein damage during sustained exposure to the metabolites. The lack of increased protein damage, in spite of increased ROS production, in the last 24 hours of the control samples suggests the active removal of the affected proteins, a process apparently inhibited or overwhelmed in the treated samples, hence the elevated levels of carbonylated proteins observed. It has been suggested that the proteolytic complexes can be functionally inhibited by free radicals (Cecarini *et al.*, 2007), and it could be that this is the case in the metabolite treated samples. It has also been proposed that only mildly oxidized proteins can be degraded, since heavily oxidized proteins tend to aggregate (Dunlop *et al.*, 2009).

Some variability was observed in the carbonyl concentrations after 72 and 96 hours exposure to the HT1 metabolites (as indicated by the error bars). This variation was not seen in any of the

control samples or the first time points of the treated samples, indicating that the variation was not inter-experimental, but rather inter-cellular variations in carbonyl adduct formation and removal. This somewhat greater variation was most probably due to aging of the cells. As the cells grow older, the continual exposure to the metabolites may result in more variability in their ability to handle oxidative stress. This brings to mind whether this has anything to do with the clonal nature of HCC development in HT1 (Poudrier *et al.*, 1998).

The oxidized proteins seems to be removed sub-optimally in the HT1 metabolite treated cells, and these proteins may accumulate and even aggregate, thereby altering cell function and promoting toxicity (Cecarini *et al.*, 2007). pHPPA has been thought to be an unlikely cause for the symptoms observed in HT1, since it is present in other disorders which do not show the same phenotype as HT1 (Jorquera and Tanguay, 1997, Mitchell *et al.*, 2001). We have previously shown, however, that pHPPA can impair DNA repair in liver cells and suggested its involvement in HT1 pathophysiology (van Dyk and Pretorius, 2005). We subsequently showed that both SA and pHPPA impaired base excision repair, and to a lesser extent nucleotide excision repair, by decreasing the recognition and incision efficiency of the initiating proteins (Van Dyk *et al.*, 2010). The specific mechanism of the impairment of the involved proteins is, however, unclear. The results obtained in this study suggest that SA and pHPPA induce oxidative damage of proteins, as well as the impairment of their removal.

In conclusion, the present study has shown for the first time that the HT1 metabolites SA and pHPPA significantly increase intracellular ROS production and possibly result in protein oxidation damage. Our results suggest that these metabolites are the cause of previously observed oxidative damage response activation in HT1, and it is probably a direct result of the increase in intracellular ROS levels. In the context of HT1-associated liver disease, we furthermore propose that the mechanism through which proteins involved in DNA repair are impaired after HT1 metabolite exposure, may involve oxidative damage to those proteins. These harmful effects of the metabolites may therefore be much more significant in the development of HCC in HT1 patients than previously thought. It should also be stressed that the results in this study were obtained during a limited time span, and the detrimental effects will most probably accumulate during prolonged exposure as is the case in the HT1 patients. This should, however, be confirmed with further studies.

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CHAPTER 6

CONCLUSION

6.1. Introduction

Hereditary tyrosinemia type 1 (HT1) is an inborn error of metabolism, with a frequent development of hepatocellular carcinoma (HCC) (Mitchell *et al.*, 2001). The molecular basis of this cancer development, however, remains unclear. In an effort to better understand the aetiology of HT1-associated HCC, one must evaluate the effects of the disease on different levels in the cell. Mitchell and associates, as well as Poudrier and colleagues, proposed that epigenetic factors could be involved in the clinical phenotype presented by HT1 patients (Poudrier *et al.*, 1998, Mitchell *et al.*, 2001, Herceg and Hainaut, 2007). It has also been observed that in HT1 patients and mouse models, liver damage was associated with several gene expression alterations (Luijterink *et al.*, 2003), which also suggests the involvement of epigenetic mechanisms (Esteller, 2008). Changes in normal DNA methylation patterns are associated with numerous cancers and pathologies, and these changes generally include global hypomethylation and gene promoter associated hypermethylation (Herceg and Hainaut, 2007, Franco *et al.*, 2008).

In this study, HT1 was chosen as a model to study the role of epigenetic changes in the aetiology of metabolic defect associated cancers. In an effort to elucidate the molecular basis of HCC in HT1 the aim of this study was, firstly, to develop a model for HT1 in cultured human cells via RNA interference (RNAi) (Mitchell *et al.*, 2001). Secondly, the aim was to study the epigenetic changes in an HT1 environment to establish its role in the aetiology of HT1-associated HCC. For the purpose of this study, oxidative damage was also categorised as being “epigenetic” in nature, since increased ROS production and its ensuing damage can alter DNA methylation (Weitzman *et al.*, 1994, Franco *et al.*, 2008).

The results obtained in this study are briefly summarized in this chapter, followed by a discussion of its significance in light of current literature. The chapter ends with some future prospects and a summary of the unique contributions of this thesis.

6.2. HT1 inducible knock-down model

Although several HT1 models have been described in the literature, including several mouse models, these models all lack some elements of a true human HT1 intracellular environment (discussed in Section 3.2). To better mimic the intracellular environment that is present in patients with HT1, it was proposed that a model for this disease should be developed in cultured human cells. One of the objectives of this study was to establish this model via RNAi, using small hairpin RNA (shRNA) constructs which would inhibit the expression of the fumarylacetoacetate hydrolase (FAH) enzyme, mimicking the enzymatic defect observed in HT1 (Mitchell *et al.*, 2001). The commercially produced Knockout[®] inducible RNAi system (a double-stable inducible knock-down system) from Clontech was chosen to establish a human cell culture HT1 model in HepG2 cells,

which would enable the observation of genetic and epigenetic changes over an extended period of time, in the presence of various degrees of this defect.

Although cultured cells have been used to study HT1, none of these models contained the *FAH* deficiency in its genome (Tanguay *et al.*, 1996, Jorquera and Tanguay, 1997, Jorquera and Tanguay, 1999, Van Dyk *et al.*, 2010). The approaches that were followed limit the time-span of the studies and it may not reflect all secondary or accumulating effects of the deficiency. A shRNA knock-down system was chosen to target the *FAH* gene, since stable expression of the shRNA in the cells should enable continuous, long-term knock-down studies (Sandy *et al.*, 2005, McIntyre and Fanning, 2006). This model would also be inducible to ensure that the cell line can be established before the defect is “switched on” – very similar to the “rescue” of the mouse models with NTBC, the treatment regularly used by HT1 patients. shRNA sequences specific to the target gene were adapted from constructs designed by the Broad Institute, and the altered oligonucleotides were annealed and ligated into the RNAi-ready pSIREN-RetroQ-Tet-P vector (Section 3.4). Two antibiotics were used in the system; G418 to select the single-stable clones, and puromycin to select the double-stable cell lines. Antibiotic kill curves were necessary to determine the highest concentration of antibiotic that can be used for selection of a specific cell line, without having excessive levels of toxicity. The optimal concentration for selection with G418 in HepG2 cells was established as 1200 µg/ml, and 1 µg/ml puromycin (see Figures 3.4 and 3.5). The optimal cell plating density was determined to be 200 000 cells per well of a 6-well culturing plate, 24 hours prior to the experiment.

Four single-stable HepG2 tTS cell lines were established successfully after transfection with the pTS-Neo vector, and subsequent selection with G418. Two of these cell lines were used for further transfections (Section 3.7). Transient transfections were performed with the four *FAH*-targeting shRNA constructs in an effort to identify the constructs with the most knock-down potential. The results of the transient knock-down experiments varied greatly though, and this was attributed to the variations in knock-down efficiency between the cells in the population. It was envisaged that higher and more consistent knock-down would be achieved once the double-stable cell lines were established.

To develop the inducible, double-stable HepG2 cell line with the *FAH* gene knocked down, the *FAH*_shRNA-pSIREN_1 and *FAH*_shRNA-pSIREN_4 constructs were transfected into two single-stable HepG2 tTS cell lines (HepG2 tTS_10 and HepG2 tTS_12). Following selection with puromycin, the transfected cells were maintained in the presence of puromycin until single colonies developed. These were propagated into individual cell lines and screened for potential inducible *FAH* knock-down. In total, 33 colonies were screened, including colonies transfected with an untargeted control-pSIREN construct and a pSIREN construct targeting a luciferase gene (unrelated control) (see Table 3.2). The results appeared to be quite positive, with several induced knock-down percentages of more than 70% in the HepG2 tTS 10 cell line. Upon closer inspection though, no induction could be detected. It was also observed that in both the untargeted and unrelated control clones the same level of *FAH* knock-down was observed as for the *FAH*-targeting shRNA constructs, suggesting that the decrease in expression of *FAH* was not a direct result of the targeted shRNA knock-down process, but rather a general result of the clone construction. However, some of the clones still had significantly reduced levels of *FAH* expression, some

sufficiently decreased to be useful in this study. It was therefore decided to propagate those cell lines with 30% *FAH* expression or less, to further characterize them. These cell lines, however, proved to be problematic upon culturing, and none of the cell lines selected for propagation could be cultured successfully.

In its current form, the model is not yet suitable for the purposes it was intended for in this study. In the clones where sufficient levels of knock-down were achieved, the cell lines were not viable. Since the induction part of the system appeared not to be functional, these cell lines could not be “rescued”. It was intended to evaluate the model at each step in the production process to ensure that the process itself did not influence the characteristics of the cell culture pertaining to the study of HT1. This would have been achieved by applying all the established analyses that was to be performed once the model was established, after each alteration of the cell line. This was not possible, since the double-stable cell line could not be propagated.

6.3. HT1 metabolite induced alterations in HepG2 cells

To establish what the effects of the accumulating HT1 metabolites, succinylacetone (SA) and *p*-hydroxyphenylpyruvate (pHPPA), may be on epigenetic mechanisms and their role in the aetiology of HCC in HT1, cultured HepG2 cells were exposed directly to these metabolites for various time intervals *in vitro*. This was a simplified model compared to the inducible knock-down model we aimed to establish, but it is based on the same principles and was considered suitable to establish all the assays which were to be applied to the knock-down model. This approach was also thought to be suitable to provide useful information regarding the aetiology of HT1 and to give some indication of the usefulness of the inducible model and the results one might expect with future applications.

For this direct exposure model, HepG2 cells were cultured under standard conditions and treated with SA and pHPPA, for 96 hours in 24 hour intervals. Several parameters were measured, including global DNA methylation, *MGMT* promoter methylation and expression, intracellular ROS production, protein oxidation, cell proliferation and senescence.

6.3.1. Global DNA methylation

Aberrant DNA methylation profiles can result in genomic instability and altered gene expression patterns, and these changes are often associated with cancer development (Richardson, 2003, Herceg and Hainaut, 2007, Esteller, 2008, Franco *et al.*, 2008). The involvement of such epigenetic changes in the pathophysiology of HT1 has been suggested (Mitchell *et al.*, 2001), and to our knowledge not yet demonstrated. It was therefore investigated whether direct exposure of HepG2 cells to HT1 metabolites over a 96 hour period would alter the global DNA methylation patterns.

Global DNA methylation in untreated HepG2 cells appeared to be approximately 45% after 24 hours incubation, changing minimally over time and with increased cell density

(see Figures 5.1 and 5.2). The level of DNA methylation after treatment with the HT1 metabolites were shown to be almost identical to the control samples, with insignificant fluctuations.

From the described global DNA methylation investigation, it became apparent that treatment of the cultured HepG2 cells with the HT1 metabolites *per se* had no significant effect on the global DNA methylation levels observed. Since the CEA only measures the average of semi-targeted global DNA methylation changes in the genome, it does not exclude the possibility that the metabolites could affect individual gene promoters or CpG islands differently. One can, nonetheless, assume that the mechanisms of DNA methylation and demethylation are not influenced by the metabolites on a genome-wide scale. One must also take into account that the experiment was only performed over a period of 96 hours, and it cannot entirely be excluded that these mechanisms may be affected over a longer period of accumulating metabolite exposure, as is found in HT1 patients, since tumour progression is a long-term process (Lin *et al.*, 2001). Although Lin and colleagues observed significant genome-wide hypomethylation in HCC patient tumours (not of HT1 origin), they did suggest that it was a “continuing physiological process that persists throughout the lifetime of the tumour cells rather than merely a historical event that occurs in the cell origins of the cancers” (Lin *et al.*, 2001). One would, however, have expected to see some indication of such changes in the time investigated. It is also possible that epigenetic mechanisms other than DNA methylation may be involved in the HT1 pathophysiology. For instance, the altered gene expression profiles, as observed in HT1 patients, could be a result of histone modifications since such alterations have been shown to be involved in gene expression regulation (Esteller, 2008).

The results did indicate that cell conditions or cell density of cultured cells can be associated with slight changes in DNA methylation. This suggests a level of adaptation in gene expression in response to growth conditions of the cultured cells, specifically HepG2 cells in this instance. It has been suggested that when cells are grown at high density, the processes that maintain genomic stability are down-regulated, including both genetic and epigenetic changes (Pieper *et al.*, 1999). Taking the observed change in global DNA methylation into account, it indicates that care must be taken at which time points or cell densities methylation studies are performed.

A close look at the results revealed a cyclical change in global DNA methylation over the time points investigated, for both the treated and untreated cultures, and it seemed as if treatment of the cells with the HT1 metabolites affected, although slightly, the synchronization of this pattern. DNA methylation changes dramatically during development, but it is generally thought that once established in a cell, the pattern remains stable and is only maintained (Brown *et al.*, 2007). It has been observed, however, that DNA methylation levels change in HeLa cells during its cell cycle (Brown *et al.*, 2007). It was also shown that epigenetic plasticity could facilitate changes in gene expression as a result of oscillatory, circadian, and seasonal rhythms (Gallou-Kabani *et al.*, 2007). This cyclical variation of CpG methylation is thought to provide an additional level of gene expression regulation in the genome (Métivier *et al.*, 2008), and it was proposed that misalignment of these circadian phases could eventually result in the prolonged presence of abnormal and unphased “locking” or “leakage” of gene expression (Gallou-Kabani *et al.*, 2007).

This led to the question of whether the apparent cyclic patterns that were observed in this study could be ascribed to the presence of these “circadian phases” in DNA methylation in HepG2 cells.

However, the cycles observed here were much longer, and lasted hours to days compared to the shorter times described above. This observation suggests that the cycling of DNA methylation may be present on a much larger scale than proposed until now. It was also clear from the results presented that the HT1 metabolites, SA and pHPPA, caused a misalignment of these cyclical phases. Should these cyclical patterns be ascribed to the presence of “circadian-like” phases in DNA methylation, such phase-shifts could be worthy of further investigation as a possible factor in the aetiology of HT1-associated HCC. This “long-term” cycling of DNA methylation could also be important in studies concerning DNA methylation, since it could imply obtaining different results depending on the time point of sampling. Care must be taken not to over emphasize the cyclical pattern observed because of their small amplitude and low frequency (Cheong and Levchenko, 2010). On the other hand, if it was random fluctuations, it is expected that different experiments would cancel out this cyclical pattern, and since it was not the case, this phenomenon can be accepted to a large degree as real. Further investigations are, however, necessary to confirm it beyond any doubt.

6.3.2. *MGMT* promoter methylation and expression

O⁶-methylguanine-DNA methyltransferase (*MGMT*) is a DNA repair enzyme responsible for the removal of potentially mutagenic alkyl adducts from guanine bases, especially methyl adducts (Zhang *et al.*, 2003). *MGMT* has been shown to be involved in the development of HCC (not of HT1 origin), and has frequently been found to have altered promoter methylation patterns and/or expression patterns in these cancers (Zhang *et al.*, 2003). This involvement of *MGMT* in non-HT1 HCC aetiology compelled the investigation into the effect of accumulating HT1 metabolites on the promoter methylation and expression of this enzyme.

The *MGMT* promoter methylation levels in the untreated cultures appeared to change somewhat over time. These changes did not follow a linear pattern of increase or decrease over the time period investigated, but instead followed the same cyclical pattern as was observed for the global DNA methylation. In this instance the pattern remained rather even, and did not show a marked increase or decrease in DNA methylation. The *MGMT* promoter methylation level of the HT1 metabolite treated samples also had no linear increase or decrease, although it did seem that the promoter methylation increased on average. Interestingly, the same cyclical pattern was once again observed, only more pronounced than in the untreated samples. The treated and untreated cultures showed a difference in not only the level of methylation of the *MGMT* promoter, but also in the time-dependent pattern (see Figure 5.4). Although both sample sets followed cyclical patterns, the HT1 metabolites clearly resulted in increased levels of *MGMT* promoter methylation compared to the control samples, and it seemed that the treatment shifted the cyclical pattern out of sync once again.

Expression of the *MGMT* enzyme directly affects the well-being of the cells by affecting the DNA repair capacity of the cells available to deal with damage which might threaten its genomic integrity (Jacinto and Esteller, 2007, Kaina *et al.*, 2007). Expression of *MGMT* decreased significantly in both the untreated and the treated samples between the time points, once again suggesting a direct influence of the cell density or growth conditions on cellular processes in HepG2 cells. This decrease was independent of the metabolite treatment, which had no significant effect. However,

in Figure 5.6 it was seen that the expression of *MGMT* was almost 15% higher in the treated samples after 24 hours exposure than in the untreated samples, possibly indicating the induction of *MGMT* by the alkylating metabolites, although this difference was not deemed statistically significant. However, it is known that *MGMT* is only slightly inducible, and induction usually peaks between 24 and 48 hours post-exposure (Margison *et al.*, 2003), and this correlates with our findings.

Figure 5.9 indicated no inverse correlation between the promoter DNA methylation and expression of *MGMT*. One can deduce that the DNA methylation status of *MGMT*'s promoter region has no direct effect on its expression in HepG2 cells. It is clear, however, that *MGMT*'s expression is affected by the cell density or growth conditions, decreasing significantly with time, and that this must be considered in the application of cell culture models as the one described in Chapter 3. This decrease in *MGMT* expression is probably the result of a different mechanism, since Margison and Santibáñez-Koref proposed that when transcription factor availability or chromatin structure changes result in a decrease in transcription, DNA methylation pattern differences could result as a secondary event (Margison and Santibáñez-Koref, 2002). The results obtained do highlight the complexity of the promoter methylation-mRNA expression interaction in *MGMT*. This complexity has also been the focus of various recent discussions (Everhard *et al.*, 2009, Christmann *et al.*, 2011). It should also be considered that although *MGMT* expression is considered to be highly regulated by methylation of its promoter, *MGMT* mRNA expression can also be regulated transcriptionally by p53 and other transcription factors (Christmann *et al.*, 2011). In the case of HepG2 cells this may, therefore, be the primary mode of expression regulation.

Alkylation damage may still increase in HT1 cells, even if the metabolites don't directly affect *MGMT* expression, since alkylation repair via *MGMT* is directly dependent on the amount of *MGMT* molecules available for repair and if damage exceeds synthesis, *MGMT* can become depleted (see article in Chapter 2). It is also possible that *MGMT* can be impaired on the protein level and *MGMT* can, therefore, still be involved in carcinogenesis in HT1.

6.3.3. Oxidative damage

Several common liver diseases associated with oxidative stress are prone to cancer development, which suggests that stress-induced malfunction of the death machinery may play a role in the pathophysiology of these disorders and could be related to the risk of developing HCC (Vogel *et al.*, 2004). Dieter and colleagues suggested that various tyrosine intermediates are capable of oxidative stress induction, and that this was possibly causally linked to the ensuing liver pathology (Dieter *et al.*, 2003). Several research groups have subsequently shown that oxidative stress response pathways are activated in HT1 models, but few of them directly assayed ROS production and its ensuing cellular damage (Dieter *et al.*, 2003, Vogel *et al.*, 2004, Bergeron *et al.*, 2006, Fisher *et al.*, 2008). Bergeron and associates showed the launch of an endoplasmic reticulum (ER) stress response in HT1, and subsequently stated that it was unclear whether the previously observed induction of the stress-related proteins, HO-1 and Hsps, was a result of oxidative or ER stress (Bergeron *et al.*, 2006). As such, the source of the oxidative stress response in HT1 remains unclear, and needed further exploration. ROS can cause various DNA lesions, which can interfere with normal DNA methylation mechanisms, thereby changing the normal DNA methylation

patterns of cells (Franco *et al.*, 2008). This potential interaction with the epigenetic profile of a cell compelled the investigation as to whether the HT1 metabolites affect intracellular ROS production in any way.

In normal, actively dividing HepG2 cells with sufficient growth medium and limited accumulation of waste (medium was replaced every 24 hours), ROS production was very limited, although present (Figure 5.7). This was expected, since ROS is a normal by-product of cellular function (Cecarini *et al.*, 2007, Franco *et al.*, 2008). Although ROS production increased between the 72 and 96 hour time points, the change was not significant, and the ROS levels remained less than in the treated samples. The increase could be a result of the increased cell density in that time interval. The significantly increased levels of intracellular ROS after exposure to the HT1 metabolites, compared to the control samples, suggested that the metabolites increased ROS production or decreased the removal of these species. Interestingly, this increased ROS production seemed to follow the same pattern as in the control samples, implying that the effect of the metabolites on ROS production was not cumulative, but rather constant.

Increased ROS production does not necessarily indicate oxidative stress, since there are mechanisms in place to repair or counteract oxidative damage. To establish whether the increased ROS production was detrimental to the cells, it was necessary to measure the extent of ensuing damage. Thus, protein carbonyl concentrations were determined. Protein carbonyls are principal oxidation products, and therefore common and generic markers of protein oxidation (Dalle-Donne *et al.*, 2003, Hawkins *et al.*, 2009). Accumulation of these oxidized proteins has been shown in several human diseases (see (Dalle-Donne *et al.*, 2003) for references), and in several of these the carbonyls increased in parallel with the development of the disease.

In the control samples, the protein carbonyl concentrations were comparable with the negative control, with insignificant fluctuations over time (Figure 5.8). Although the ROS production in the untreated samples increased during the 72 to the 96 hour increment, this increase was not reflected on the protein damage level, indicating active and sufficient removal of oxidized proteins by intracellular proteases, such as the proteasome complex, lysosomal cathepsins or cytosolic calpains (Cecarini *et al.*, 2007, Dunlop *et al.*, 2009). In the HT1 metabolite treated cells, the concentrations of carbonyl groups were much the same as that of the untreated cells during the first 48 hours of exposure. However, in the next 48 hours the carbonyl content increased more than 3-fold. Although this increase was not statistically significant, one can ask whether it could be biologically relevant, since it has been suggested that any increase in protein carbonyl concentrations are generally an indication of oxidative stress and protein dysfunction (Dalle-Donne *et al.*, 2003). Therefore, should it be considered biologically significant, it would suggest that the increased ROS production directly resulted in increased oxidative protein damage during continuous exposure to the HT1 metabolites.

The lack of increased protein damage, in spite of increased ROS production, in the last 24 hours of the control samples suggests the active removal of the affected proteins, a process apparently inhibited or overwhelmed in the treated samples, hence the elevated levels of carbonylated proteins observed. It has been suggested that the proteolytic complexes can be functionally inhibited by free radicals (Cecarini *et al.*, 2007), and it could be that this is the case in the

metabolite treated samples. It has also been proposed that only mildly oxidized proteins can be degraded, since heavily oxidized proteins tend to aggregate (Dunlop *et al.*, 2009).

The oxidized proteins seemed to be removed sub-optimally in the HT1 metabolite treated cells, and these proteins may accumulate and even aggregate, thereby altering cell function and promoting toxicity (Cecarini *et al.*, 2007). pHPPA has been thought to be an unlikely cause for the symptoms observed in HT1, since it is present in other disorders which do not show the same phenotype as HT1 (Jorquera and Tanguay, 1997, Mitchell *et al.*, 2001). Van Dyk and associates, however, have previously shown that pHPPA can impair DNA repair in liver cells and suggested its involvement in HT1 pathophysiology (Van Dyk *et al.*, 2010). They also showed that exposure of HepG2 cells to SA and pHPPA impaired base excision repair, and to a lesser extent nucleotide excision repair, by decreasing the recognition and incision efficiency of the DNA repair-initiating proteins (Van Dyk *et al.*, 2010). The mechanism of the impairment of the involved proteins is, however, unclear. The results obtained in this study suggest that SA and pHPPA induce oxidative damage of proteins, as well as the impairment of their removal. These harmful effects of the metabolites may therefore contribute directly to the development of HCC in HT1 patients.

A directly inverse decrease in *MGMT* expression and increase in intracellular ROS production was also observed (Figure 5.11). The metabolite treatment, however, did not seem to affect this correlation either, which could suggest that both ROS production and *MGMT* expression are simply influenced by the same factors. Another possibility could be the involvement of the p53 tumour suppressor protein. It has been shown that increased intracellular ROS can lead to the activation of p53 through several mechanisms (Sasaki, 2006). p53 activation conversely increases ROS generation, further increasing oxidative stress (Sasaki, 2006). In addition, studies have shown that over-expression of p53 leads to *MGMT* down-regulation (Sasaki, 2006). This suggests that the increase in ROS due to cell density or growth conditions can lead to the activation of p53, which in turn down-regulates *MGMT*. Should this be the case in HT1, this effect possibly has a biological limit, since the ROS increase due to the metabolite treatment did not increase the effect observed.

6.4. Final conclusion

The altered gene expression profiles and genetic instability observed in HT1 patients do not seem to be a direct result of aberrant DNA methylation. It is possible that epigenetic mechanisms other than DNA methylation may be involved in the HT1 pathophysiology. The present study have, however, indicated for the first time the possible presence of long-term cyclical phases in global DNA methylation, lasting in the order of days. It was also shown that the HT1 metabolites caused a clear shift in the cyclical patterns observed, causing a misalignment of the cyclical phases. Should these cyclical patterns be ascribed to the presence of “circadian-like” phases in DNA methylation, such phase-shifts could be involved in the pathophysiology of HT1 and are worthy of further investigation as a possible factor in the aetiology of HT1-associated HCC.

MGMT methylation and subsequent expression does not seem to be directly affected by the HT1 metabolites used in this study either. This does not exclude possible changes in its function due to protein damage or depletion. Alkylation damage may still increase in HT1 cells, even if the

metabolites don't directly affect *MGMT* expression, since alkylation repair via *MGMT* is directly dependent on the amount of *MGMT* molecules available for repair and if damage exceeds synthesis, *MGMT* can become depleted. It is possible that *MGMT* can be impaired on the protein level, since SA is known to react with various amino acids and proteins (Manabe *et al.*, 1985, Prieto-Alamo and Laval, 1998). *MGMT* can, therefore, still be involved in carcinogenesis in HT1, and further studies may shed more light on the involvement of *MGMT* in HT1-associated HCC.

The study has shown for the first time that the HT1 metabolites SA and pHPPA significantly increase intracellular ROS production and possibly result in protein oxidation damage. The results suggest that these metabolites are the cause of previously observed oxidative damage response activation in HT1, and it is probably a direct result of the increase in intracellular ROS levels caused by these metabolites. It is furthermore proposed that the mechanism through which proteins involved in DNA repair is impaired after HT1 metabolite exposure, may involve oxidative damage to those proteins. These harmful effects of the metabolites may, therefore, contribute directly to the development of HCC and could be much more significant in the pathophysiology of HT1 patients than previously thought. It appears, however, that in spite of indications in the literature of alterations in DNA methylation as a result of increased ROS damage to DNA, this association was not observed in this study.

The hypothesis, as presented in Section 2.8, was successfully tested. However, it appears that the DNA methylation patterns were not affected on a global scale, and gene expression alterations did not include a change in the expression of *MGMT*. Interestingly however, a dramatic increase in intracellular ROS production was observed, but this appeared not to affect the DNA methylation in the time span the observations were made, in spite of the known correlations between the two events. It is proposed that other epigenetic mechanisms may be involved, or that DNA methylation may be affected on a more region-specific level. It is also proposed that *MGMT* may still be impaired in HT1, although this damage may rather occur on a protein level.

6.5. Future prospects

The growth difficulties of the established double-stable cell lines could be a direct result of the decrease in *FAH*, causing the accumulation of toxic metabolites. It could also be a result of untargeted gene expression alterations; it is therefore of utmost importance to characterize these cell lines to determine the origin and effect of the knock-down achieved. One option is to obtain culture medium containing no tyrosine or phenylalanine to propagate the cell lines, much like diet restriction in patients (Mitchell *et al.*, 2001). This would hamper the accumulation of harmful metabolites, should the decreased *FAH* be the cause of the cell death. A second option would be to treat the cells with NTBC, much like the "rescue" of the mouse models. NTBC is, however, difficult to obtain and quite expensive

Alternative systems should also be investigated, and some companies made validated shRNA constructs targeting *FAH* available recently (OriGene, Sigma), and it may be worthwhile to invest in such constructs. I want to stress that establishing such a model would be of great importance for

studies of this kind, and that RNAi is the most effective method to establish such a model, as shown by Fisher and colleagues in the *C. elegans* model (Fisher *et al.*, 2008).

Epigenetic mechanisms other than DNA methylation may still be involved in the aetiology of HT1-associated HCC. Further study of other epigenetic mechanisms, such as histone modification, should be considered to clarify the role of epigenetic alterations in HT1. The proposed HT1 cell culture model would be invaluable for such studies, specifically for longer exposure and better control of cell growth conditions. The observed cyclical patterns could also be of interest, not only to researchers interested in metabolic disease associated carcinomas, but to all epigenetisists, since the cycling of DNA methylation may be present on a much larger scale than proposed until now. This is important, since the time point chosen for sampling can clearly influence the results, and the effect of the long-term cycling on the associated gene-expression remains unclear. The phase-shifts observed is also worthy of further investigation as a possible factor in the aetiology of HT1-associated HCC.

Long-term studies and complete HT1 cell conditions may affect *MGMT* promoter methylation and expression differently, and once again the proposed HT1 cell culture model could be useful for more elaborate study of these factors. It is also crucial that *MGMT* enzyme levels and activity in the cells be evaluated in such long-term studies to investigate if *MGMT* is depleted or inactivated, possibly increasing alkylation damage in HT1 cells.

The increased production of intracellular ROS and the ensuing cellular damage is clearly an important occurrence in HT1, and requires further study to clarify the mechanism through which the metabolites increased ROS, and to establish the extent of the damage to the cells. The envisaged HT1 cell culture model would be of great value for such experiments, since more control of cell density and longer exposure times will be possible. The model will also enable a more complete study of the oxidative stress, since all the accumulating metabolites will be present, not only SA and pHPA. Given that only generic ROS and protein oxidation were measured, it is necessary to characterize the nature of the oxidants produced, as well as the specific damage caused to all cellular molecules, to fully comprehend the role of this damage in the HT1 pathophysiology.

Better understanding of the pathophysiology of HT1 may enable identification of better therapeutic targets, possibly preventing HCC development entirely.

6.6. Unique contribution

To my knowledge, this is the first extensive study of epigenetic alterations in an inborn error of amino acid metabolismⁱ, and the potential role of such alterations in the cancers associated with many of these diseases. The research successfully tested the hypothesis that DNA methylation alterations could play a role in HT1-associated HCC development, and the conclusion was that changes in the levels of DNA methylation is probably not a significant contributing factor. This study did, however, identify long-term cyclic patterns in the DNA methylation, and identified a shift in these patterns to be present as a result of the metabolites SA and pHPPA. This thesis also presents the first direct evidence of ROS production in HT1, and successfully identified SA and pHPPA to be, at least in part, responsible for the oxidative stress present in HT1 patients. These results also confirm previous claims that SA and pHPPA may play a much more significant role in HT1 pathophysiology than previously thought.

Several manuscripts encapsulating the contribution of this thesis were prepared, and information concerning their publication status is given in Chapter 1.

ⁱ As per Pubmed search, using various combinations of the keywords “inborn error of metabolism”, “metabolic defect”, “amino acid metabolism”, “tyrosinemia”, “epigenetic”, “DNA methylation”.

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APPENDIX A.

Vector map of ptTS-Neo:

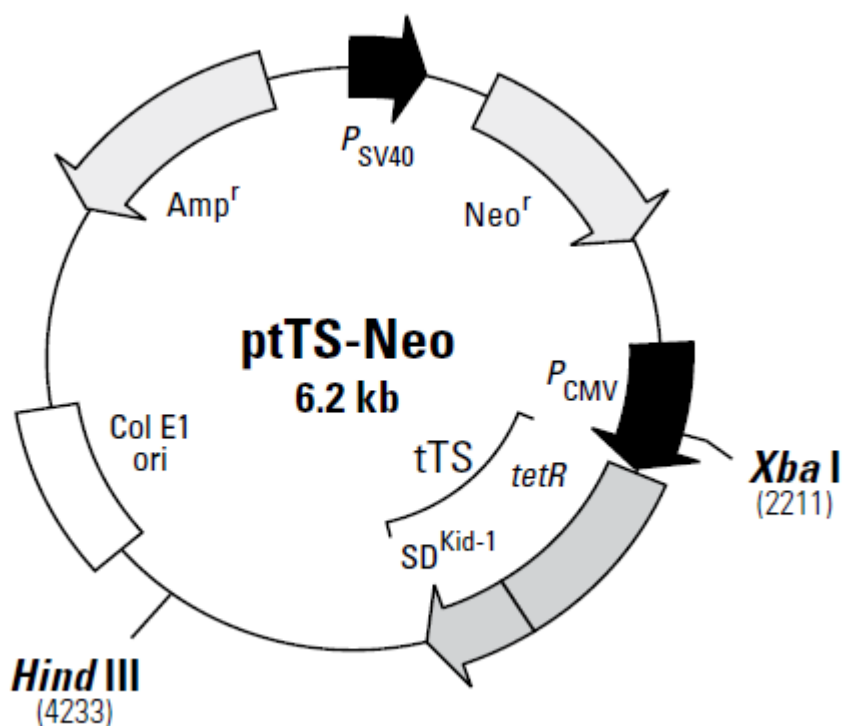


Figure A.1. Vector map of ptTS-Neo (Clontech).

APPENDIX B.

Vector map of RNAi-Ready pSIREN-RetroQ-TetP:

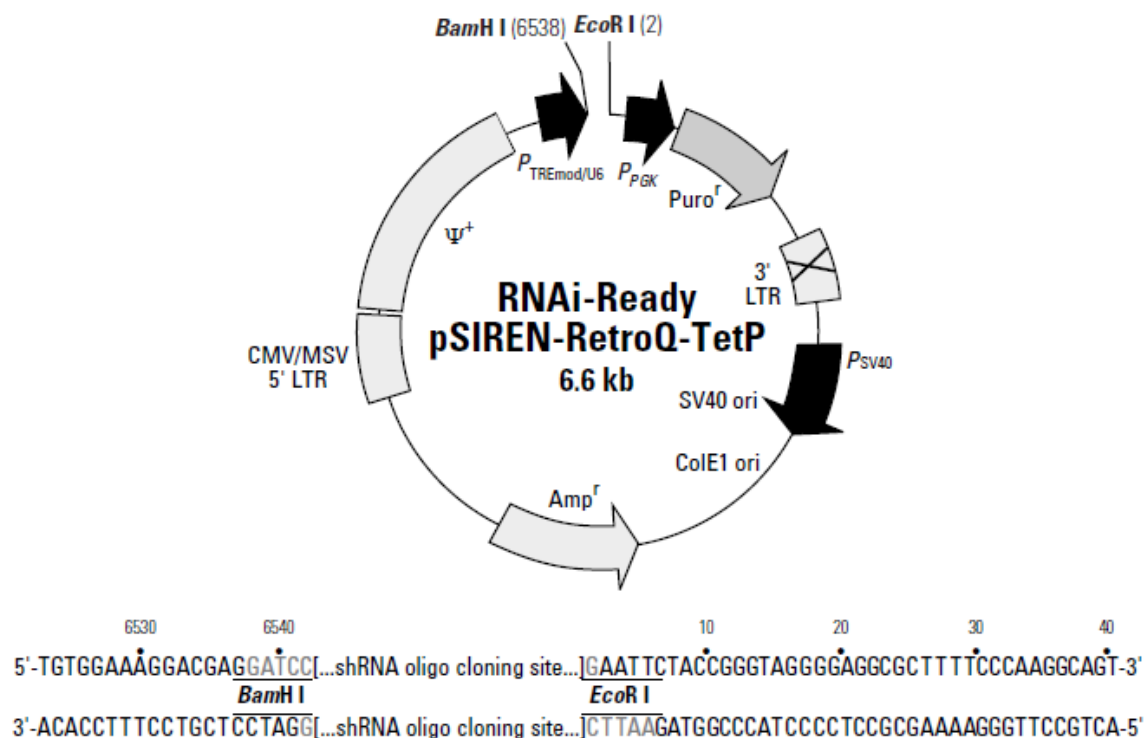


Figure B.1. Vector map of RNAi-Ready pSIREN-RetroQ-TetP. The sequence flanking the cloning site for the shRNA construct is shown (Clontech).

APPENDIX C.

Vector map of pEGFP:

pEGFP-N1 Vector Information

GenBank Accession #U55762

PT3027-5

Catalog #6085-1

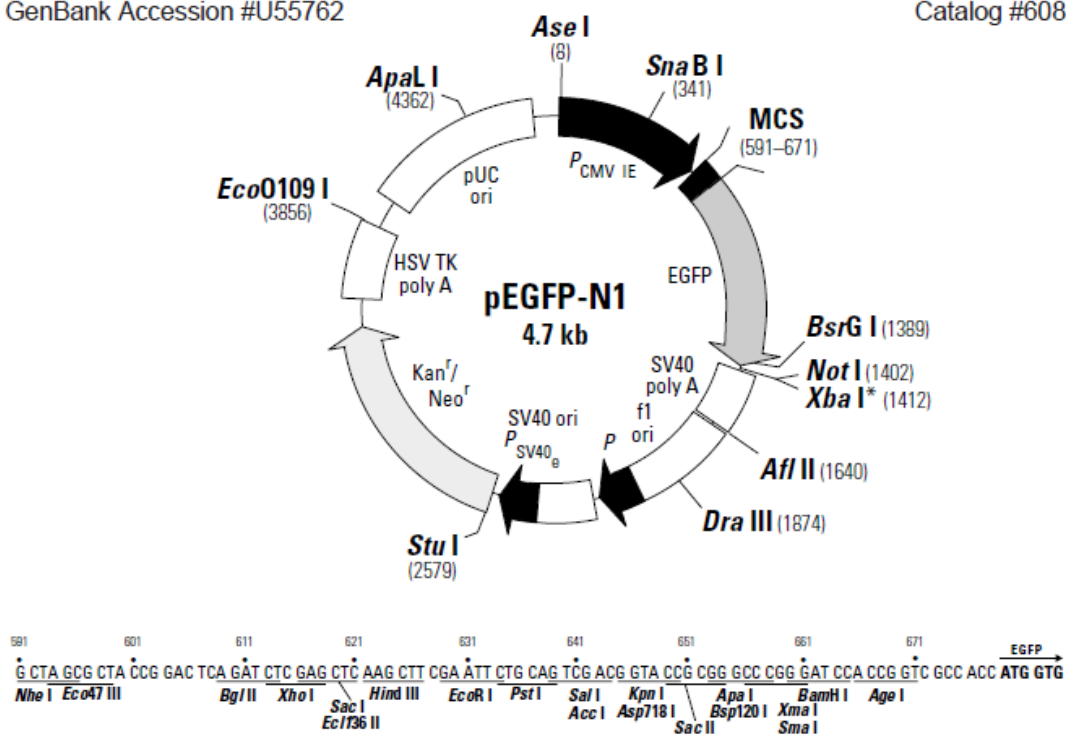


Figure C.1. Vector map of pEGFP-N1. The sequence of the multiple cloning site is shown (Clontech).

APPENDIX D.

Table D.1. Sequencing results of the ligated and propagated *FAH*-targeted shRNA constructs.

Construct	Sequencing result*
<i>FAH</i> _shRNA1_FRW	GATCCGGCTACCATATGCAAG G CCAATCTCGAGATTGGACTTGCATATGGTAGCTTTTTG
<i>FAH</i> _shRNA2_FRW	Sequencing failed
<i>FAH</i> _shRNA3_FRW	GATCCGCAGCATCATCAAGC C CTCTTCTCGAGAAGAGGTGCTTGATGATGCTGTTTTG
<i>FAH</i> _shRNA4_FRW	GATCCGTGAAGTCATCATAACAGGGTACTCGAGTACCCTGTTATGATGACTTCATTTTTG [#]

*The underlined sequences are the complementary target sequences, and the shadowed, bold nucleotides are changes in the construct as observed in the sequencing results.

[#] No sequence changes were observed.

APPENDIX E.

Primer sequences and information:

Table E.1. Real-time methylation specific PCR primer sequences.

Gene	Primer name	Sequence	Reference
MGMT	MGMT_FWD	5' -CGAATATACTAAAACAACCCGCG-3'	(Harder <i>et al.</i> , 2008)
	MGMT_REV	5' -GTATTTTTTTCGGGAGCGAGGC-3'	
	Probe	6FAM5' -AATCCTCGCGATACGCACCGTTTACG-3' TAMRA	
ACTB	ACTB_FWD	5' -TGGTGATGGAGGAGGTTTAGTAAGT-3'	
	ACTB_REV	5' -AACCAATAAAACCTACTCCTCCCTTAA-3'	
	Probe	6FAM5' -ACCACCACCCAACACACAATAACAAACACA-3' TAMRA	

Table E.2. Primers for RNA reverse transcription.

Primer	Sequence
Oligo(dT) 18mer	Fermentas
18S rRNA specific_reverse	5' -CGGACATCTAAGGGCATCAC-3'

Table E.3. Real-time PCR TaqMan[®] assay information.

Gene	Assay ID	Catalogue number
FAH (nm_000137.1)	Hs00908443_m1; Custom TaqMan [®] gene expression assays, non-inventoried	4351372
MGMT (nm_002412.2)	Hs00172470_m1; TaqMan [®] gene expression assays, GEx	4331182
18S rRNA	Predeveloped TaqMan [®] assay reagents	4319413E

Table E.4. Sequencing primer.

Name	Sequence
U6 Forward Sequencing Primer: 6105-6132	5' -CTTGAACCTCCTCGTTCGACCCCGCCTC-3'

APPENDIX F.

Original data sets for all experimental series:

Table F.1. Average number of HepG2 cells per 6-well plate well.

Time	Series 1		Series 2		Series 3	
	Control	Treated	Control	Treated	Control	Treated
0 hours	200000	200000	200000	200000	200000	-
24 hours	447222	375000	180000	240000	161250	-
48 hours	569444.3	600000	240000	300000	457500	-
72 hours	783333	969444	375000	480000	532500	-
96 hours	1172222	1191667	945000	975000	827500	-

Table F.2. Global DNA methylation.

Sample	Average dpm's		
	Series 1	Series 2	Series 3
K24 Hpa	1471.25	3089.25	580.5
M24 Hpa	2114	2812	318.75
K48 Hpa	1767.75	3162.75	251.625
M48 Hpa	1531	2642.5	528
K72 Hpa	1535.25	2971.25	119.625
M72 Hpa	1550.75	3156	288
K96 Hpa	266	363.5	149
M96 Hpa	150.5	3446.75	131.25
K24 Msp	1833.75	6145	1566.75
M24 Msp	2537.5	6012.25	994.5
K48 Msp	2568	6076.75	627.875
M48 Msp	1887	4757.25	1162.25
K72 Msp	2946	6725	239.125
M72 Msp	2408.25	5942.5	536.125
K96 Msp	401.5	2642.5	345.75
M96 Msp	238.5	7046.25	223.125

Table F.3. Average *MGMT* promoter methylation levels – normalized relative percentages.

Time	Average normalized percentages (%)					
	Series 1		Series 3		Series 4	
	Control	Treated	Control	Treated	Control	Treated
24 hours	100		100	112	100	158
48 hours	108	155	171.5	100	119	130
72 hours	101	336	157	242.5	42	128
96 hours	210.5	215	118	113	41	192

Table F.4. Average *MGMT* expression levels – normalized relative percentages.

<i>Average normalized percentages (%)</i>								
Time	Series 1		Series 2		Series 3		Series 4	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
24 hours	100	101.5	100	100	100	104.6667	100	157
48 hours	98.5	93	107	113.5	101.6667	121.3333	114	94
72 hours	102	82	94	96.5	93	95.33333	66.5	60
96 hours	15	31.5			96.66667	94	67	60

Table F.5. ROS production.

<i>Mean fluorescent intensity (arbitrary units)</i>								
Time	Series 1				Series 2			
	Control		Treated		Control		Treated	
24 hours	6.68	3.86	40.74	45.4	16.42	16.63	34.12	35.7
48 hours	10.11	18.99	55.93	62.08	8.09	8.14	25.3	26.17
72 hours	9.36	19.06	39.33	51.94				
96 hours	28.43	38.73	57.71	50.52				

Table F.6. Protein carbonyl concentrations.

<i>Concentrations (nmol carbonyls per mg protein)</i>				
Time	Series 1		Series 2	
	Control	Treated	Control	Treated
24 hours	5.050505	6.535948	3.109566	5.009577
48 hours	9.38072	9.71984	3.160556	2.730375
72 hours	16	3.400993	4.82429	23.23503
96 hours	7.072233	32.85871	5.61291	7.41763
Negative control	4.800817		3.308013	

Table F.7. Cellular senescence.

<i>Percentage cells (%)</i>				
Time	Series 1		Series 2	
	Control	Treated	Control	Treated
24 hours	1.00	-	0.00	0.00
48 hours	3.00	-	1.00	1.00
72 hours	4.00	-	3.00	3.00
96 hours	5.00	-	5.00	5.00

APPENDIX G.

Buffers and solutions:

- **1% Agarose gel (0.1 l; TAE buffer)**

1.0 g	Agarose
100 ml	1x TAE buffer
10 µl	Ethidium bromide

Running buffer: 1x TAE buffer.

- **0.4 M Citric acid (0.1 l)**

7.69 g	Citric acid (Mr = 192.43 g/mol)
80 ml	ddH ₂ O

Fill up to 0.1 litres with ddH₂O.

- **pH 6 Citrate-phosphate buffer (0.1 l)**

17.9 ml	0.4 M Citric acid
32.1 ml	0.8 M Sodium-phosphate buffer

Adjust to pH 6.0

Fill up to 0.1 litres with ddH₂O.

- **10 mM DNPH (0.1 l)**

0.2 g	DNPH (Mr = 198.14 g/mol)
100 ml	2 M HCl

Protect from light.

- **0.5 M EDTA (1 l)**

186.12 g	EDTA (Mr = 372.24 g/mol)
900 ml	ddH ₂ O

Adjust to pH 8.0 with 5 N NaOH

Fill up to 1 litre with ddH₂O.

- **10 mg/ml Ethidium Bromide (0.01 l)**

0.1 g Ethidium bromide

10 ml ddH₂O

Wrap container in aluminium foil.

<!-- Carcinogenic.

- **6 X gel-loading dye (0.01 l)**

0.25 % (w/v) Bromophenol blue

30% (v/v) Glycerol

Fill up to 10 ml with sterile ddH₂O.

- **6 M Guanidine-HCl buffer**

6 M Guanidine-HCl

0.2 M Acetic acid

- **40 mM H₂O₂ (0.001 l)**

24 µl H₂O₂ (6%)

976 µl ddH₂O

Dilute fresh every day.

- **2 M HCl (0.1 l)**

19.6 ml HCl (32%)

80.4 ml ddH₂O

- **5 N NaOH (0.5 l)**

100 g NaOH (Mr = 40 g/mol)

Fill up to 0.5 litres with ddH₂O.

<!-- Keep on ice while dissolving.

- **5 mM p-hydroxyphenylpyruvate (0.01 l)**

0.009 g p-hydroxyphenylpyruvate (Mr = 180.16 g/mol)

0.9 ml ddH₂O

Adjust pH with 5 N NaOH until fully dissolved.

Fill up to 0.01 litres with ddH₂O.

- **0.5 M Potassium cyanoferrate (II) (0.01 l)**

2.11 g $\text{C}_6\text{FeK}_4\text{N}_6 \cdot 3\text{H}_2\text{O}$ (Mr = 422.39 g/mol)

8 ml ddH₂O

Fill up to 0.1 litres with ddH₂O.

- **1 M Potassium cyanoferrate (III) (0.01 l)**

3.29 g $\text{C}_6\text{FeK}_3\text{N}_6$ (Mr = 329.24 g/mol)

8 ml ddH₂O

Fill up to 0.1 litres with ddH₂O.

- **0.8 M Sodium-phosphate buffer (0.1 l)**

14.24 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (Mr = 177.99 g/mol)

80 ml ddH₂O

Fill up to 0.1 litres with ddH₂O.

- **5 mM Succinylacetone (4,6-Dioxoheptanoic acid; 0.01 l)**

0.0079 g Succinylacetone (Mr = 158.16 g/mol)

8 ml ddH₂O

Fill up to 10 ml with sterile ddH₂O.

- **50x TAE buffer (1l)**

242 g Tris-base (Mr = 121.14 g/mol)

57.1 ml Glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

Fill up to 1 litre with ddH₂O.

- **20 mg/ml X-Gal (0.001 l)**

20 mg X-Gal

1 ml dimethylformamide

APPENDIX H.

Table H.1. Supplier companies and catalogue numbers of reagents used.

Reagent	Company	Catalogue number
1 kb DNA ladder	New England Biolabs	N3232S
6-Carboxy-2',7'-dichloro-dihydrofluorescein diacetate di(acetoxymethyl ester) (DCFH-DA)	Invitrogen	C2938
2,4-Dinitrophenyl hydrazine (DNPH)	Fluka	42210
2-Mercaptoethanol	Merck, BDH Biochemicals	44143
Agarose; Molecular grade low EEO	Hispanagar; Whitehead Scientific	D-1 LE
BCA™ Protein assay kit	Thermo Scientific	23227
CalPhos™ transfection reagent	Clontech	631312
Deoxynucleotide mix (dNTPs)	New England Biolabs	N0447S
Dimethylformamide	Fluka	40250
Doxycycline	Sigma	D9891
Dulbecco's Modified Eagles Medium (DMEM; HyClone)	Thermo Scientific	SH30243.FS
EDTA	Sigma	E5134
EGTA	Sigma	E3889
EpiTect® Bisulfite kit	Qiagen	59104
EpiTect® PCR control DNA set	Qiagen	59695
Ethanol absolute	Merck	1.00983.2500
Ethyl acetate	Merck	1.09623.2500
ExGen 500 in vitro transfection reagent	Fermentas	R0511
FlexiGene DNA kit	Qiagen	51204
Foetal Bovine Serum (FBS), Heat inactivated	Sigma	F4135
Foetal Bovine Serum (FBS), Certified	Gibco	16000
Formaldehyde	Merck	1.04003.2500
FuGENE® 6 transfection reagent	Roche	11 814 443 001
GeneRuler™ 50bp DNA ladder	Fermentas	SM0372

Reagent	Company	Catalogue number
GoTaq [®] Flexi DNA polymerase	Promega; Whitehead Scientific	M8305
[³ H]deoxycytidine triphosphate ([³ H]dCTP)	GE Healthcare	NET601A250UC
<i>Hpa</i> II	Fermentas	ER0512
Hydrogen chloride (HCl) 32%	Rochelle chemicals	-
Hydrogen peroxide (H ₂ O ₂)	PAKMED	-
Knockout RNAi Inducible System	Clontech	630926
L-Glutamine	BioWhittaker, Lonza	17-605E
Magnesium chloride (MgCl ₂)	Merck	8.14733.0100
MEM non-essential amino acids	Gibco, Invitrogen	11140
MMLV High performance reverse transcriptase	Epicentre Biotechnologies	RT80125K
M-PER [®] mammalian protein extraction reagent	Thermo Scientific	78503
<i>Msp</i> I	Fermentas	ER0541
Oligo(dT) 18mer	Fermentas	S0131
Neomycin (G418)	Sigma	A1720
Nucleospin [®] RNA II kit	Macherey-Nagel	740 955
Penicillin/Streptomycin	BioWhittaker, Lonza	17-602E
Phosphate buffered saline (PBS)	Sigma	P4417
p-hydroxyphenylpyruvate (pHPPA)	ICN Biomedicals	100434
Potassium hexacyanoferrate (II) trihydrate	Sigma	60279
Potassium hexacyanoferrate (III)	Sigma	60299
Propan-2-ol (Isopropanol)	Bio-zone chemicals	1184001
PureYield [™] plasmid midiprep system	Promega	A2492
Puromycin dihydrochloride	Sigma	P9620
Rapid DNA Ligation kit	Fermentas	K1421
Recombinant RNasin [®] ribonuclease inhibitor	Promega	N2515
Sodium chloride (NaCl)	Merck, pro Analysi	1.06404.0500
Sodium hydroxide	Sigma	S5881
Sodium phosphate	Merck, pro Analysi	6649

Reagent	Company	Catalogue number
Succinylacetone (SA)	Sigma	D1415
TaqMan [®] universal PCR master mix	Applied Biosystems	4324018
Trichloroacetic acid	univAR, Saarchem	611 05 00
Tris-chloride	Sigma	T5941
Trypsin	Invitrogen	15400-054
Trypan Blue	Sigma	T6146
Ultima Gold [™] XR	Perkin Elmer [®]	6013119
Water – HiPerSolv for HPLC [™]	Merck, BDH AnalaR [®]	1.15333.2500
Whatman [®] DE-81 ion exchange filters	Sigma	Z286591
<i>Xho</i> I	Fermentas	ER0691
X-Gal solution	Fermentas	R0949