Evaluation of selected dye compounds as inhibitors of monoamine oxidase

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DECLARATION

This dissertation is submitted in fulfilment of the requirements for the degree of *Master of Science in Pharmaceutical Chemistry*, at the North-West University Potchefstroom campus.

I the undersigned, Franciska de Beer, hereby declare that the dissertation with the title: "**Evaluation of selected dye compounds as inhibitors of monoamine oxidase**" is my own work and has not been submitted at any other University either whole or in part.

Ms. Franciska de Beer

Signed at Potchefstroom on the 14th day of November 2018.

LETTER OF PERMISSION

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To whom it may concern

CO-AUTHORSHIP ON RESEARCH ARTICLE

The undersigned are co-authors of the research article listed and hereby give permission to Miss F. de Beer to submit this article as part of the degree *Magister Scientiae* in Pharmaceutical Chemistry at the North-West University (NWU) Potchefstroom campus:

- **The monoamine oxidase inhibition properties of selected dye compounds**

Yours sincerely,

Prof. J.P. Petzer

Prof. Anél Petzer

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“As die HERE die huis nie bou nie, tevergeefs werk die wat daaraan bou; as die HERE die stad nie bewaar nie, tevergeefs waak die wagter.”

Psalm 127:1

ABSTRACT

Parkinson's disease (PD) and Alzheimer's disease (AD) are neurodegenerative diseases that significantly impact the quality of life of patients. The pathology of Parkinson's disease is characterised by neuronal death of the nigrostriatal dopaminergic nerve terminals as well as the formation of Lewy bodies. The activity of monoamine oxidase (MAO) B increases in patients diagnosed with Parkinson's diseases as they age resulting in oxidative stress, which in turn may lead to mitochondrial dysfunction as well as protein aggregation and misfolding. The pathology of Alzheimer's disease includes the formation of amyloid plaques and neurofibrillary tangles (NFT). The aetiologies of these neurodegenerative diseases are still unknown. Current treatments are based on the presenting pathogenesis and symptoms of the respective diseases. Therefore, there is no treatment that stops or reverses neurodegeneration in Parkinson's disease and Alzheimer's disease and current treatment focuses on alleviating the symptoms.

The MAO enzyme is involved in the pathogenesis of Parkinson's disease and Alzheimer's disease. MAO metabolises various neurotransmitters including noradrenaline (NA), dopamine (DA) and serotonin (5-HT). MAO is implicated in several neurodegenerative diseases due to the generation of reactive oxygen species (ROS) and participation in key pathological pathways. Monoamine oxidase type B (MAO-B) inhibition can be beneficial for the treatment of Parkinson's diseases as well as Alzheimer's disease. The current use of monoamine oxidase type A (MAO-A) inhibitors to treat depression is well documented. It is important to note that potent MAO-A inhibitors and serotonergic drugs should not be administered together as this can possibly induce serotonin toxicity. Caution should also be exercised with the use of potent irreversible inhibitors of MAO-A as they present with a higher occurrence of the "cheese reaction".

The dye compound methylene blue (MB), has shown promise as a therapeutic agent in studies conducted on Alzheimer's disease and depression. Of particular interest is the ability of methylene blue to inhibit MAO. It was found that methylene blue as well as azure B, the major metabolite of methylene blue, potently inhibit MAO-A. It was also found that certain structural analogues of methylene blue also are good potency MAO-A inhibitors. For example, the dye compounds cresyl violet (IC_{50} = 0.0037 µM), Nile blue (IC_{50} = 0.0077 µM) and 1,9-dimethyl methylene blue (DMMB) (IC_{50} = 0.018 µM) are high potency MAO-A inhibitors. Due to the high potency MAO-A inhibition associated with these dye compounds, the present study investigated 22 commercially available dyes, that are similar in structure to methylene blue, as potential human MAO-A and MAO-B inhibitors.

The inhibition potencies of the selected 22 dye compounds were determined by using recombinant human MAO-A and MAO-B enzymes. The IC_{50} values of the dyes were determined and were used as a measure of inhibition potency. Based on the IC_{50} values, acridine orange, oxazine 170 and Darrow red were identified as the highest potency inhibitors of this study. These compounds were subjected to further studies to determine the reversibility and mode of inhibition by means of dialysis and the construction of Lineweaver-Burk plots, respectively. It was found that acridine orange is a competitive and reversible inhibitor specific for MAO-A (IC_{50} = 0.017 µM). Oxazine 170 was identified as a competitive and reversible inhibitor specific for MAO-B (IC_{50} = 0.0065 µM). Darrow red exhibited competitive and reversible inhibition of MAO with specificity for neither of the isoforms (MAO-A, IC_{50} = 0.059 µM; MAO-B, IC_{50} = 0.065 µM).

When compared to methylene blue (MAO-A, IC_{50} = 0.07 µM; MAO-B, IC_{50} = 4.37 µM), acridine orange and Darrow red exhibited more potent inhibition of MAO-A. The MAO-B inhibition potencies of oxazine 170 and Darrow red were also found to be higher than that of methylene blue.

In conclusion, the MAO isoforms are implicated in the pathology of neurodegenerative and neuropsychiatric diseases such as Alzheimer's disease, Parkinson's disease and depression. Therefore, MAO is a viable and important target for possible medical intervention and drug treatments. The results of this study confirmed that some of the dye compounds evaluated in this study possess similar activity profiles to methylene blue. This can be explained by the structural similarity of the dye compounds with methylene blue. The dye compounds identified in this study can therefore be further investigated for possible preclinical development and be used as possible lead compounds to design future MAO inhibitors.

Keywords:

acridine orange, Alzheimer's disease, Darrow red, methylene blue, monoamine oxidase, inhibition, oxazine 170, Parkinson's disease, serotonin toxicity

OPSOMMING

Parkinson se siekte (PS) en Alzheimer se siekte (AS) is neurodegeneratiewe siektes wat die lewenskwaliteit van pasiënte aansienlik beïnvloed. Die patologie van Parkinson se siekte word gekenmerk deur die afsterwe van nigrostriatale dopamienergiese neurone sowel as die vorming van Lewy-liggame. Die aktiwiteit van monoamienoksidase (MAO) B is verhoog in die sentrale senuweestelsels van pasiënte wat met Parkinson se siekte gediagnoseer is. Hierdie verhoogde MAO-B aktiwiteit het oksidatiewe stres tot gevolg wat weer lei tot mitochondriale wanfunksie en aggregasie van proteïene. Die patologie van Alzheimer se siekte word gekenmerk deur die vorming van amiloïede plaak en neurofibrillêre bondels (NFB). Die etiologie van hierdie neurodegeneratiewe siektes is nog onbekend. Huidige behandeling vir hierdie neurodegeneratiewe siektes is gebaseer op die waarneembare patogenese en die simptome. Daar is dus geen behandeling wat neurodegenerasie in Parkinson se siekte en Alzheimer se siekte kan voorkom nie. Die huidige behandeling fokus slegs op die verligting van die simptome.

Die MAO-ensiem is betrokke by die patogenese van Parkinson se siekte en Alzheimer se siekte. MAO metaboliseer verskeie neuro-oordragstowwe insluitende noradrenalien (NA), dopamien (DA) en serotonien (5-HT). MAO speel ’n rol in verskeie neurodegeneratiewe siektes omdat MAO-katalise reaktiewe suurstofspesies (ROS) produseer wat neurone kan beskadig. Die inhibisie van MAO-B kan voordelig wees vir die behandeling van Parkinson se siekte sowel as Alzheimer se siekte. MAO-A-inhibeerders word tans gebruik in die behandeling van depressie. Dit is belangrik om daarop te let dat potente MAO-A-inhibeerders en serotonergiese middels nie saam toegedien moet word nie, aangesien dit moontlik serotonientoksisiteit kan veroorsaak. Verder moet potente, onomkeerbare MAO-A-inhibeerders vermy word aangesien hierdie middels die “kaasreaksie” kan veroorsaak.

Die kleurstof, metileenblou (MB), het potensiaal as 'n terapeutiese middel getoon in studies rakende Alzheimer se siekte en depressie. Van besondere belang is die vermoë van metileenblou om MAO te inhibeer. Daar is bevind dat metileenblou sowel as asuur B, die hoofmetaboliet van metileenblou, MAO-A baie potent inhibeer. Daar is ook gevind dat sekere strukturele analoë van metileenblou as potente MAO-A-inhibeerders optree. Strukturele analoë van metileenblou soos die kleurstowwe kresielviolet (IC_{50} = 0.0037 µM), Nylblou (IC_{50} = 0.0077 µM) en 1,9-dimetielmetileenblou (DMMB) (IC_{50} = 0.018 µM) is byvoorbeeld potente MAO-A-inhibeerders. As gevolg van die inhibisie van MAO-A wat met hierdie kleurstowwe geassosieer word, het die huidige studie 22 kommersieel beskikbare kleurstowwe as potensiële inhibeerders van menslike MAO-A en MAO-B ondersoek. Hierdie kleurstowwe toon meestal soortgelyke strukturele eienskappe aan metileenblou.

Die MAO-inhiberend eienskappe van die 22 kleurstowwe is ondersoek deur van rekombinante menslike MAO-A en MAO-B ensieme gebruik te maak. Die IC_{50}-waardes van die kleurstowwe is bepaal, en is gebruik om die potensie van inhibisie te definieer. Na vergelyking van die IC_{50}-waardes is daar gevind dat akridienoranje, oksasien 170 en Darrow rooi die mees potente inhibeerders van hierdie studie is. Hierdie verbindings is verder geëvalueer om die omkeerbaarheid en meganisme van inhibisie te bepaal deur van dialise en Lineweaver-Burk grafieke, onderskeidelik, gebruik te maak. Daar is gevind dat akridienoranje 'n kompeterende en omkeerbare inhibeerder van MAO-A (IC_{50} = 0.017 µM) is. Oksasien 170 is geïdentifiseer as 'n kompeterende en omkeerbare inhibeerder wat spesifiek MAO-B (IC_{50} = 0.0065 µM) inhibeer. Darrow rooi het kompeterende en omkeerbare inhibisie van MAO getoon met geen spesifisiteit vir enige van die isoforme (MAO-A, IC_{50} = 0.059 µM; MAO-B, IC_{50} = 0.065 µM) nie.

In vergelyking met metileenblou (MAO-A, IC_{50} = 0.07 µM; MAO-B, IC_{50} = 4.37 µM), is akridienoranje en Darrow rooi dus meer potente inhibeerders van MAO-A. Die potensies waarmee MAO-B deur oksasien 170 en Darrow rooi geïnhibeer is, was ook hoër as dié van metileenblou.

Ten slotte, die isoforme van MAO is betrokke by die patologie van neurodegeneratiewe en neuropsigiatriese siektes soos Alzheimer se siekte, Parkinson se siekte en depressie. Daarom is MAO 'n lewensvatbare en belangrike teiken vir die behandeling van hierdie siektetoestande. Die resultate van hierdie studie het bevestig dat sommige van die kleurstowwe wat geëvalueer is, oor soortgelyke aktiwiteitsprofiele beskik as metileenblou. Hierdie gevolgtrekking kan verklaar word deur die strukturele ooreenkomste van die kleurstowwe met metileenblou. Die kleurstowwe wat in hierdie studie geïdentifiseer is, kan dus verder ondersoek word vir moontlike prekliniese ontwikkeling en kan ook gebruik word as moontlike leidraadverbindings om toekomstige MAO-inhibeerders te ontwerp.

Sleutelterme:

akridienoranje, Alzheimer se siekte, Darrow rooi, metileenblou, monoamienoksidase, inhibisie, oksasien 170, Parkinson se siekte, serotonientoksisiteit

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ABBREVIATIONS

3xTg-AD	triple-transgenic mouse model of Alzheimer's disease
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine; serotonin
5-HT_{1A}	serotonin 1A receptor
5-HT_{2A}	serotonin 2A receptor
A	
Aβ	amyloid β peptides
$A\beta_{40}$	amyloid β peptide fragment 40
$A\beta_{42}$	amyloid β peptide fragment 42
ACh	acetylcholine
AChE	acetylcholinesterase
AChEI	acetylcholinesterase inhibitor
AD	Alzheimer's disease
ALDH	aldehyde dehydrogenase
ALS	amyotrophic lateral sclerosis
APP	amyloid precursor protein
ARJP	autosomal recessive juvenile Parkinsonism
αS	α-synuclein
ATP	adenosine triphosphate
B	
BAD	Bcl-2 associated death promoter
BAX	Bcl-2 associated X protein

Bcl-2	B-cell lymphoma 2
Bcl-Xl	B-cell lymphoma extra large
BuChe	butyrylcholinesterase

C

CAA	cerebral amyloid angiopathy
Ca^{2+}	calcium ion
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system

D

DA	dopamine
DAAD	Deutscher Akademischer Austausch Dienst
DLB	diffuse Lewy body disease
DMMB	1,9-dimethyl methylene blue
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOPAC	dihydroxyphenyl acetic acid

E

ETC	elthylthioninium chloride

F

FAD	flavin adenine dinucleotide
Fe^{2+}	ferrous iron

G

G6PD	glucose-6-phosphate-dehydrogenase deficiency

GSH	glutathione
H	
H_2O_2	hydrogen peroxide
I	
IFN-γ	interferon gamma
IL-1β	interleukin 1 beta
K	
K_i	dissociation constant
KCl	potassium chloride
L	
LeucoMB	leuco methylene blue
L-dopa	levodopa; L-3,4-dihydroxyphenylalanine
LMP	lysosomal membrane permeability
M	
MAO	monoamine oxidase
MAO-A	monoamine oxidase type A
MAO-B	monoamine oxidase type B
MAPK	mitogen-activated protein kinases
MB	methylene blue
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
N	

NA	noradrenaline
NaOH	sodium hydroxide
nAChRs	nicotinic acetylcholine receptors
NADPH	nicotinamide adenine dinucleotide phosphate
NFT	neurofibrillary tangles
NRF	National Research Foundation
NMB	new methylene blue
NMDA	N-methyl-d-aspartate
NO	nitric oxide
NR	neutral red

O

OLGs	oligodendroglia

P

PD	Parkinson’s disease
PHBH	p-hydroxybenzoate hydroxylase
PKC	protein kinase C

R

R-CHO	aldehyde species
R-COOH	carboxylic acid species
ROS	reactive oxygen species

S

sAPPα	soluble amyloid precursor protein alpha
SAR	structure-activity relationship

SD	standard deviation
SI	selectivity index
SNpc	substantia nigra pars compacta
SSRI	serotonin selective reuptake inhibitor

T

TDP-43	trans-activator regulatory DNA-binding protein 43
TNF-α	tumor necrosis factor alpha

U

UCH-L1	ubiquitin C-terminal hydrolase L1

CHAPTER 1 INTRODUCTION AND RATIONALE

1.1 GENERAL BACKGROUND

1.1.1 Monoamine oxidase

The monoamine oxidase enzyme (MAO) is found on the outer mitochondrial membrane and contains a flavin adenine dinucleotide (FAD) as cofactor (Youdim *et al.*, 2006). MAO can thus be categorised as a flavoenzyme (Youdim & Bakhle, 2006). MAO can be divided into two isoforms namely monoamine oxidase type A (MAO-A) and monoamine oxidase type B (MAO-B). These two isoforms can be differentiated by their substrate specificity for endogenous neurotransmitters as well as sensitivity to inhibitors such as clorgyline and selegiline (figure 1-1) (Youdim *et al.*, 2006). The endogenous neurotransmitters that are metabolised by MAO include tyramine, noradrenaline (NA), dopamine (DA) and serotonin (5-HT) (figure 1-2) (Youdim & Bakhle, 2006).

MAO is found in various tissues such as the brain, liver, intestinal mucosa and other organs (Boppana *et al.*, 2009). Noradrenaline, dopamine and serotonin are key components of the regulatory systems of the brain and can be targeted for the treatment of neuropsychiatric and neurodegenerative disorders. MAO-A inhibitors such as phenelzine, tranylcypromine, moclobemide and befloxatone have demonstrated antidepressant properties by elevating dopamine, noradrenaline and serotonin levels in the central nervous system (CNS) (Youdim *et al.*, 2006). MAO-B inhibitors have clinical relevance in the treatment of Parkinson's disease (PD), particularly as adjuvants to levodopa (L-dopa) (Birkmayer *et al.*, 1977). The mechanism of symptomatic relief in Parkinson's disease is not yet fully understood. However, it has been proposed that MAO-B inhibitors may elevate the CNS levels of dopamine as well as 2-phenylethylamine (Youdim *et al.*, 2006). MAO-B inhibitors are also being considered as a treatment option for Alzheimer's disease (AD). In Alzheimer's disease, amyloid β peptides (Aβ) pathology results in the formation of neurotoxic amyloid plaques (Mucke *et al.*, 2000). MAO inhibitors can potentially reduce the neurotoxic effects of Aβ pathology (Schedin-Weiss *et al.*, 2017). Furthermore, since components of tobacco smoke decrease MAO-B activity, it has been suggested that MAO inhibitors may aid in smoking cessation (Berlin *et al.*, 2002).

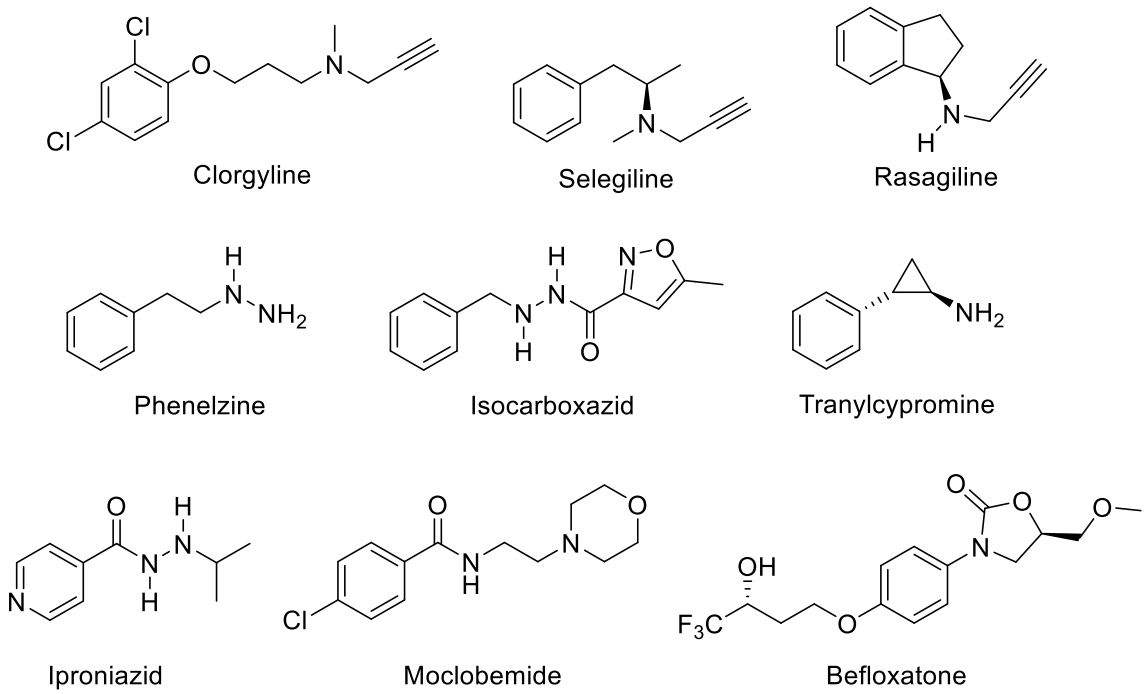


Figure 1-1: The structures of MAO inhibitors discussed in the text.

1.1.2 Oxidative stress

Oxidative stress is associated with the pathology of many neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. Oxidative stress can be defined as an imbalanced redox state caused when the antioxidant systems are not functioning properly or when an increased production of reactive oxygen species (ROS), such as hydroxyl radicals, occur and propagate free-radical chain reactions (Kim *et al.*, 2015; Sohal *et al.*, 1995). The brain tissue is susceptible to oxidative stress and vulnerable to free radical injury as it is high in lipid content and does not possess a strong antioxidant defence system (Mason *et al.*, 2000). Therefore, antioxidant therapy can be considered as a possible neuroprotective solution for neurodegenerative diseases. MAO-B inhibitors inhibit the MAO-catalysed reaction which generates hydrogen peroxide (H_2O_2) and aldehydes by amine oxidation (Youdim *et al.*, 2006). The reduction of H_2O_2 and aldehydes may result in neuroprotection. MAO inhibitors thus decrease the levels of ROS in neuronal tissue and protect against potential neurotoxicity. Since MAO-B activity increases in the brain as tissue ages, higher levels of H_2O_2 are formed by MAO-B in the brain (Youdim & Bakhle, 2006). Therefore, MAO-B inhibitors are particularly relevant as neuroprotective agents.

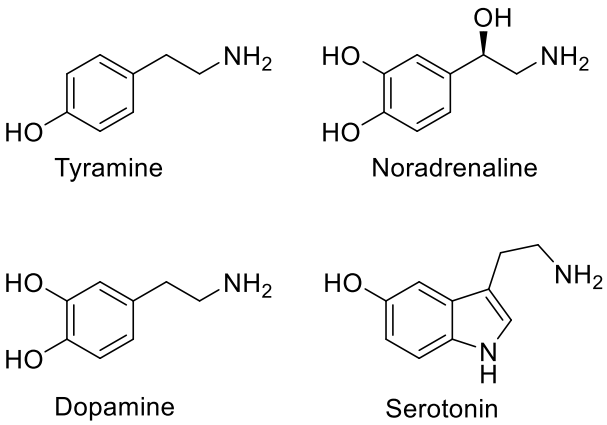


Figure 1-2: The structures of MAO substrates discussed in the text.

1.1.3 Monoamine oxidase inhibitors

MAO inhibitors include phenelzine, iscocarboxacid, tranylcypromine, iproniazid, clorgyline, selegiline and rasagiline (figure 1-1) (Youdim & Weinstock, 2002). MAO inhibitors can either be classified as reversible or irreversible inhibitors. Reversible inhibitors act competitive at the active site and are often structurally related to MAO substrates (Foley *et al.*, 2000). Irreversible or "suicide" inhibitors bind to the active site in a competitive manner, however they are subsequently oxidised by the enzyme to yield a reactive intermediate that covalently binds to the enzyme resulting in permanent unavailability of the active site. This results in the permanent inactivation of the active site for further binding and metabolism of substrates. Irreversible inhibitors have a longer effect and inhibition can only be overcome by *de novo* synthesis of new MAO enzymes (Foley *et al.*, 2000). Irreversible non-selective inhibitors of MAO-A possess a serious side effect known as the "cheese reaction". MAO-A inhibitors deactivate the MAO system in the peripheral tissues thereby preventing the metabolism of tyramine, an amine found is food such as cheese. The increased tyramine levels in the circulation induce the excessive release of noradrenaline from the peripheral adrenergic neurons resulting in a severe life-threatening hypertensive response (Mason *et al.*, 2000). This adverse effect is only observed with irreversibly acting MAO-A inhibitors. Reversible MAO-A inhibitors and inhibitors that are specific for MAO-B do not produce the cheese reaction and is considered safe for use (Youdim & Bakhle, 2006). This study will therefore aim to discover new reversible MAO-A and MAO-B inhibitors. For this purpose, commercial dye compounds will be selected and evaluated as potential MAO inhibitors. Certain dye compounds (figure 1-3), as exemplified by methylene blue (MB), are high potency MAO inhibitors which led to the hypothesis of this study that other dye compounds may also possess MAO inhibition properties.

1.1.4 The structure of monoamine oxidase

To discover MAO inhibitors, knowledge of the structures of the MAO-A and MAO-B active sites can be helpful. The active site of MAO-B consists of two cavities namely the entrance cavity (290 Å in volume) and substrate cavity (490 Å in volume) (Youdim *et al.*, 2006). The substrate cavity is characterised as flat and hydrophobic. The entrance cavity is separated from the substrate cavity by four residues: Tyr-326, Ile-199, Leu-171 and Phe-168 (Youdim *et al.*, 2006). The substrate cavity, containing the flavin moiety at the distal end, is covalently bound to Cys-397 by the tioether linkage at the 8α position of the flavin (Youdim *et al.*, 2006). The *cis* conformation of the amide linkage between the flavin moiety and the Cys-397 residue induces an "aromatic sandwich" structure with the phenolic side chains of Tyr-398 and Tyr-435. The amine groups of substrates are recognised for oxidation by this "aromatic sandwich". The MAO-A active site is similar to that of MAO-B with only six of sixteen active site residues differing between the two isoforms (Binda *et al.*, 2002; Son *et al.*, 2008). However, the MAO-A active site consists of a single cavity which in general accommodates smaller inhibitors better than larger inhibitors. In MAO-B the side chain of Ile-199 may rotate, forming a larger active site cavity by allowing the substrate and entrance cavities to fuse. This allows for larger inhibitors to bind and is known as a cavity-spanning mode of binding. Similar to MAO-B, the flavin moiety is located at the distal end of the MAO-A active site and is also bound to a cysteine residue (Cys-406) (Youdim *et al.*, 2006). In MAO-A the flavin also forms an "aromatic sandwich" structure with the phenolic side chains of Tyr-407 and Tyr-444, the site where the amine groups of substrates are recognised.

MAO inhibitors can be identified using a simple pharmacophore model of MAO (Boppana *et al.*, 2009; Shelke *et al.*, 2011). A potential pharmacophore model for MAO-A inhibitors includes a donor feature at one end of a molecule, involved in the formation of hydrogen bonding in the substrate cavity, and hydrophobic or aromatic features at the other end of the molecule, for establishing hydrophobic interactions and possibly Pi-stacking within the entrance of the MAO-A active site (Shelke *et al.*, 2011). These features are also of importance to the simple pharmacophore of MAO-B inhibitors (Boppana *et al.*, 2009). However the structure of the MAO-B inhibitor should be large enough for the donor feature to interact with the substrate cavity in proximity to the FAD, while the hydrophobic feature projects to and interacts with the entrance cavity of MAO-B. Such cavity spanning compounds are, in general, highly selective inhibitors of MAO-B over the MAO-A isoform since MAO-A accommodates smaller ligands better than larger compounds (Hubálek *et al.*, 2005; Legoabe *et al.*, 2012).

1.1.5 Methylene blue

Methylene blue was first used as a cotton dye and is considered to have various medical applications (figure 1-3). In recent years the focus has shifted to methylene blue as a potential antimalarial agent as well as a potential treatment of neurodegenerative disorders such as Alzheimer's disease (Oz *et al.*, 2009; Schirmer *et al.*, 2011). Methylene blue is a high potency inhibitor of MAO-A, inhibiting recombinant human MAO-A *in vitro* with an IC_{50} value of 0.07 µM (Harvey *et al.*, 2010; Ramsay *et al.*, 2007). Methylene blue is a much less potent MAO-B inhibitor with an IC_{50} value of 4.37 µM (Harvey *et al.*, 2010). MAO-A inhibition is a well-established mechanism of action for the MAO inhibitor class of antidepressants. Therefore, methylene blue's observed antidepressant action in preclinical models as well as in humans can, at least in part, be attributed to MAO-A inhibition. A number of methylene blue analogues and related dye compounds with similar structures have been evaluated as potential MAO inhibitors based on the high MAO-A inhibition potency exhibited by methylene blue. In a recent study, the human MAO inhibition properties of five methylene blue analogues namely neutral red (NR), Nile blue, new methylene blue (NMB), cresyl violet and 1,9-dimethyl methylene blue (DMMB) were investigated (figure 1-3). Analogues similar to methylene blue such as cresyl violet (IC_{50} = 0.0037 µM), Nile blue (IC_{50} = 0.0077 µM) and DMMB (IC_{50} = 0.018 µM) exhibited specific MAO-A inhibition properties more potent than methylene blue. Nile blue (IC_{50} = 0.012 µM) was also found to exhibit potent MAO-B inhibition properties (Delport *et al.*, 2017). An earlier study investigated the human MAO inhibition properties of methylene green, methylene violet, thionine, acriflavine and tacrine (figure 1-3). Among these, methylene green and acriflavine proved to be potent and specific MAO-A inhibitors with IC_{50} values of 0.25 µM and 0.43 µM, respectively (Harvey *et al.*, 2010).

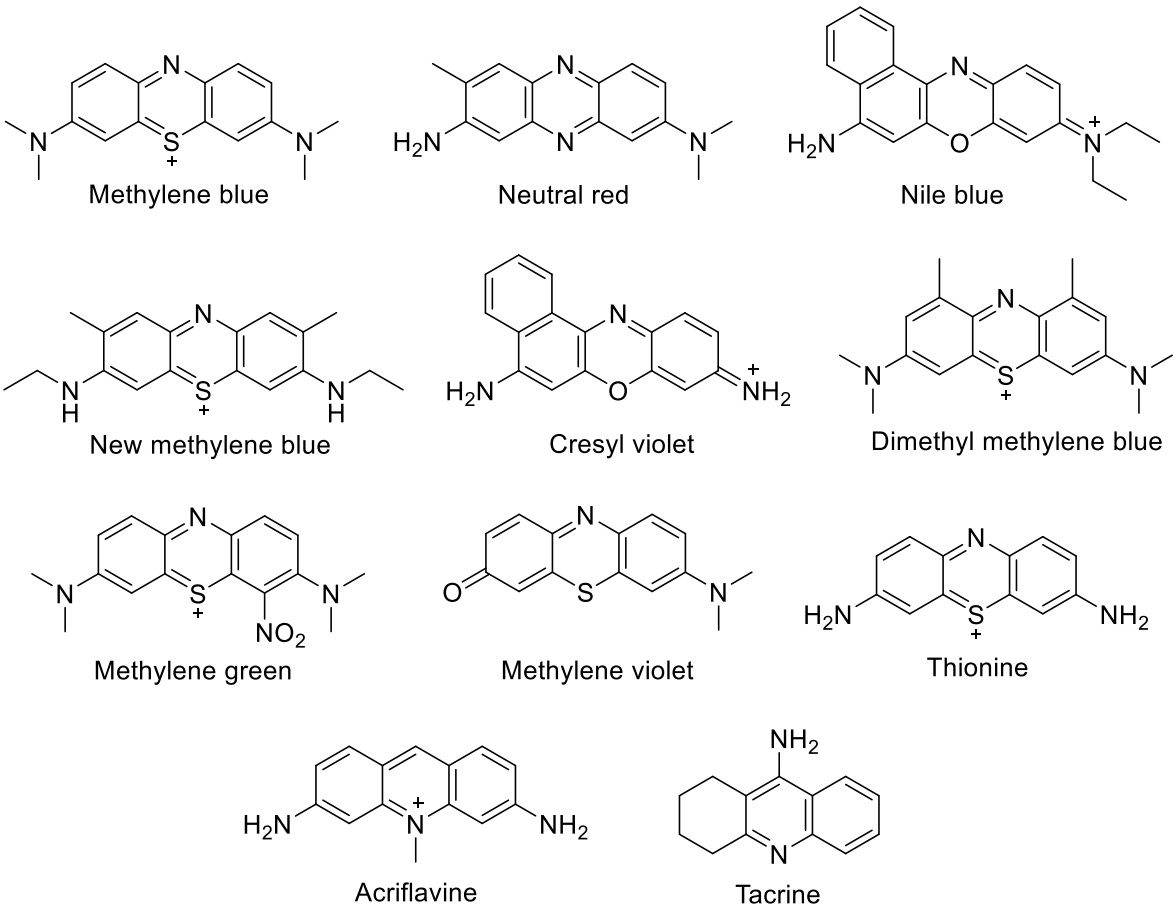
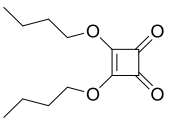
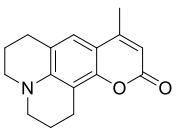
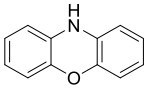
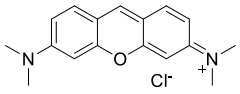
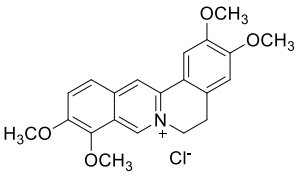
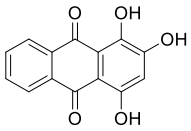
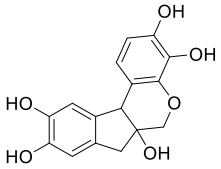
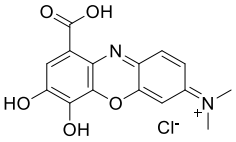
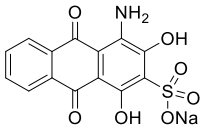
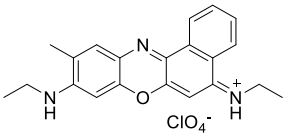
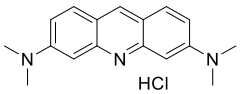
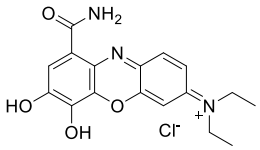
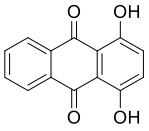
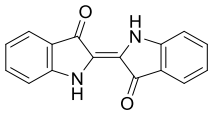
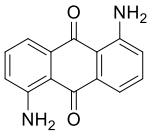
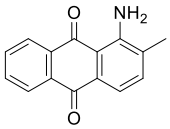
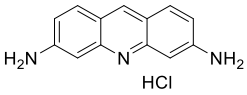


Figure 1-3: The structures of methylene blue and methylene blue analogues discussed in the text.

This study will attempt to discover potential inhibitors of the MAO enzymes among additional dye compounds. A list of commercially available dyes will be compiled and a subset of compounds will be selected based on visual inspection (based on the similarity to the structure of methylene blue). The selected dye compounds will be purchased and evaluated as potential inhibitors of recombinant human MAO-A and MAO-B enzymes. After the active inhibitors have been identified, the reversibility as well as the mode (i.e. competitive) of inhibition will be determined by appropriate enzyme experiments. Table 1-1 provides the structures of dye compounds that may be considered for this study.

Table 1-1: Structures of dye compounds that may be considered as potential inhibitors of recombinant human MAO-A and MAO-B during this study.

3,4-Dibutoxy-3-cyclobutene-1,2-dione (MW = 226.27)		Coumarin 102 (MW = 255.31)	
Phenoxazine (MW = 183.21)		Pyronin Y (MW = 302.80)	

Palmatine chloride hydrate (MW = 387.86)		Purpurin (MW = 256.21)	
Hematoxylin (MW = 302.28)		Gallocyanine (MW = 336.73)	
Nuclear fast red (MW = 357.27)		Oxazine 170 perchlorate (MW = 431.87)	
Acridine orange hydrochloride hydrate (MW = 301.81)		Celestine blue (MW = 363.80)	
1,4-Dihydroxyanthraquinone (MW = 240.21)		Indigo (MW = 262.26)	
1,5-Diaminoanthraquinone (MW = 238.24)		Disperse orange 11 (MW = 237.25)	
3,6-Diaminoacridine hydrochloride (MW = 245.71)			

1.2 RATIONALE

Methylene blue exhibits high potency inhibition of MAO-A and inhibits recombinant human MAO-A *in vitro* with an IC_{50} value of 0.07 µM. Furthermore, methylene blue also is an MAO-B inhibitor with an IC_{50} value of 4.37 µM. Recent studies with dye compounds that are structurally similar to methylene blue have discovered good potency MAO inhibitors. MAO inhibitors are of therapeutic interest as a possible treatment option of diseases such as depression, Parkinson's disease as well as Alzheimer's disease. This study will attempt to discover additional dye compounds that can serve as lead compounds for future studies that aim to develop MAO inhibitors.

1.3 HYPOTHESIS OF THIS STUDY

Based on the finding that methylene blue and several related dye compounds are good potency MAO inhibitors, it is postulated that additional dye compounds that have the potential to inhibit

the MAO enzymes may be discovered. Furthermore, it is postulated that the selection of dye compounds among commercially available dyes by simple visual inspection would yield compounds that are structurally similar to methylene blue with potentially good potency MAO inhibition.

1.4 OBJECTIVES OF THIS STUDY

The aims of this study are:

- To use visual inspection to select commercially available dye compounds that are similar in structure to methylene blue.
- To determine IC_{50} values of the dye compounds as inhibitors of human MAO-A and MAO-B.
- To determine the type of inhibition (reversible or irreversible) exhibited by active dye compounds by means of dialysis.
- To construct Lineweaver-Burk (double-reciprocal) plots. If the dye compounds exhibit reversible inhibition properties, the enzyme-inhibitor dissociation constants (K_i values) will also be determined.
- To determine the possible binding orientations and interactions of selected active dye compounds in the MAO active site using molecular modelling (i.e. molecular docking and dynamics simulation).

The objective of the study is:

- To discover MAO inhibitors from commercially available dyes.

1.5 SUMMARY

Methylene blue is a high potency inhibitor of MAO-A, inhibiting recombinant human MAO-A *in vitro* with an IC_{50} value of 0.07 µM. Methylene blue also is an MAO-B inhibitor with an IC_{50} value of 4.37 µM. Recent studies have shown that several dye compounds that are structurally related to methylene blue also act as good potency MAO inhibitors. Based on the therapeutic interest in MAO inhibitors, this study will attempt to discover additional dye compounds that have the potential to inhibit the MAO enzymes. Such compounds may be used in future studies as lead compounds for the development of drugs for the treatment of disorders such as depression, Parkinson's disease as well as Alzheimer's disease. The approach that will be followed includes the selection of dye compounds that are structurally related to methylene blue from commercially available dyes, and to evaluate them *in vitro* as potential MAO inhibitors using the commercially available recombinant human enzymes.

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CHAPTER 2 LITERATURE OVERVIEW

2.1 MONOAMINE OXIDASE

2.1.1 General background

The MAO enzyme is found attached to the outer mitochondrial membrane via a transmembrane α-helix that anchors the enzyme to the membrane while exposing the rest of the protein to the cytoplasm (Youdim *et al.*, 2006b). MAO metabolises neurotransmitters and exogenous arylalkylamines by means of oxidative deamination (Binda *et al.*, 2002). Oxygen has been identified as the electron acceptor and is converted to H_2O_2 by both enzymes (Binda *et al.*, 2002).

MAO is responsible for the metabolism of primary, secondary and tertiary amines but does not affect the metabolism of diamines such as histamine (Youdim *et al.*, 2006b; Youdim *et al.*, 1988). Substrates that are metabolised by MAO include noradrenaline, adrenaline, tyramine, dopamine and serotonin (Youdim & Bakhle, 2006). The MAO enzyme can be categorised into two isoforms namely MAO-A and MAO-B (Youdim & Bakhle, 2006). The difference in substrate as well as inhibitor specificities, distinguish the isoforms from each other. The isoforms also differ in the manner that they crystallise, with MAO-B crystallising as a dimer while MAO-A crystallises as a monomer (Binda *et al.*, 2007; Youdim *et al.*, 2006b). During the stages of development, MAO-A appears before MAO-B and as the ageing process continues MAO-B levels increase in the brain (Nicotra *et al.*, 2004; Strolin Benedetti *et al.*, 1992; Tsang *et al.*, 1986; Youdim *et al.*, 2006b).

The proportions of the MAO enzymes vary from tissue to tissue. In the central tissues, the level of MAO activity differs in the various brain regions and can be attributed to the fact that the two isoforms are not evenly distributed in the brain. MAO-B is the dominant form and is most commonly found in the basal ganglia (Collins *et al.*, 1970; Youdim *et al.*, 2006b). The highest level of MAO activity is found in the striatum of the basal ganglia as well as the hypothalamus (O'Carroll *et al.*, 1983). The lowest level of MAO activity is found in the cerebellum and neocortex (O'Carroll *et al.*, 1983). In the peripheral tissues, MAO-A is found in the intestine, liver, lungs and placenta (Youdim *et al.*, 2006b). In the tissues of the intestine, MAO-A metabolises dietary amines and thus regulates their entry into the circulation. MAO-B that is found in the microvessels of the blood-brain barrier, also acts as a metabolic barrier that contributes to this regulatory function (Youdim *et al.*, 2006b).

The discovery of the two isoforms of MAO resulted in the synthesis of inhibitors that are specific for the different isoforms. This yielded compounds that provide selective inhibition of either

MAO-A or MAO-B. Furthermore, based on kinetic studies inhibitors can be classified as irreversible and reversible inhibitors (Foley *et al.*, 2000). Reversible inhibitors are often similar in structure to the substrates of MAO and bind competitively and temporarily to the active site (Foley *et al.*, 2000). Irreversible inhibitors also bind competitively to the active site. However during the metabolism of the inhibitor, the inhibitor binds covalently to the FAD cofactor (Foley *et al.*, 2000). The unavailability of the cofactor results in the permanent inactivation of the enzyme and no amine metabolism can occur. Therefore, irreversible inhibitors have a prolonged effect which may last for weeks rather than hours as found in the case of reversible inhibitors (Foley *et al.*, 2000). The only way to overcome the effect of an irreversible inhibitor is by synthesising new enzyme by means of *de novo* synthesis (Foley *et al.*, 2000). Irreversible inhibitors that bind covalently to the FAD cofactor includes pargyline and rasagiline (Youdim *et al.*, 2006b).

2.1.2 The structure of MAO

The protein is anchored to the mitochondrial membrane by a C-terminal transmembrane polypeptide segment (Binda *et al.*, 2002). The protein containing the active site protrudes into the cytoplasm perpendicular to the mitochondrial membrane. Substrate and inhibitor entry into the active site of MAO occurs near the intersection of the enzyme with the membrane (Youdim *et al.*, 2006b).

The substrate cavity of human MAO-A, rat MAO and human MAO-B are surrounded by 16 residues (Son *et al.*, 2008). All 16 of the residues are the same for human and rat MAO-A and it may thus be concluded that the residues are conserved between species (Son *et al.*, 2008). Of these 16 residues only 6 are different when MAO-A and MAO-B are compared (Son *et al.*, 2008). In the active site of MAO, the loop conformation of residues 108-118 and 210-216 are of importance and determine the substrate or inhibitor specificities (De Colibus *et al.*, 2005; Edmondson *et al.*, 2007). Rat MAO-A and human MAO-B are nearly identical in this region whereas human MAO-A differ (De Colibus *et al.*, 2005). The residues lining the substrate cavity differ in human MAO-B and MAO-A. MAO-B is lined with Leu-171, Cys-172, Ile-199 and Tyr-326 whereas MAO-A is lined with Ile-180, Asn-181, Phe-208 and Ile-335 that corresponds with the residues mentioned for MAO-B (Son *et al.*, 2008). Certain residues are responsible for substrate and inhibitor specificity and distinguish MAO-A from MAO-B. In MAO-A, Ile-335 and Phe-208 are responsible for specificity whereas Tyr-326 and Ile-199 are the corresponding residues responsible for specificity in MAO-B (Son *et al.*, 2008). Ile-335 serves a similar function in MAO-A as Ile-199 in MAO-B. Ile-335 facilitates the induced fit conformation to allow accommodation of substrates or inhibitors (Son *et al.*, 2008).

FAD, contained in MAO-A and MAO-B, is the only redox co-factor required for catalysis (Edmondson *et al.*, 2004). The enzyme binds covalently to the co-factor at the C-terminal portion of the molecule via a thioether linkage between a cysteinyl residue and the 8α-methylene of the isoalloxazine ring (Kearney *et al.*, 1971). However, the cysteinyl residue bound to the 8α-methylene differs in MAO-A and MAO-B. In MAO-A the cysteinyl residue is Cys-406 and in MAO-B it is Cys-397 (Bach *et al.*, 1988).

2.1.2.1 The structure of MAO-A

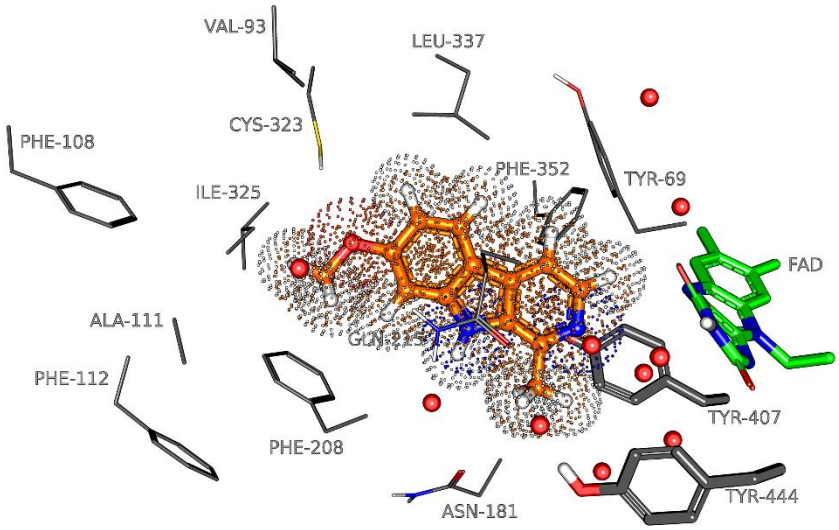


Figure 2-1: The 3D structure of the active site of MAO-A with key residues and the FAD cofactor (green). The co-crystallised ligand, harmine, is shown in the active site cavity of MAO-A. Figure generated with PyMOL.

The MAO-A substrate cavity, as shown in figure 2-1, is a single hydrophobic cavity that contains the active site and consists of a volume of 550 $Å^3$ (Youdim *et al.*, 2006b). Human MAO-A, as well as rat MAO-A, crystallises as monomers (De Colibus *et al.*, 2005). The structure of human MAO-A is similar to rat MAO-A. The human and rat MAO-A enzymes share an 87% amino acid sequence similarity (Son *et al.*, 2008). The amino acid sequence between residues 108-118 and 210-216 of human and rat MAO-A share an even higher similarity of 90% (Son *et al.*, 2008). In human MAO-A, the flavin-substituted Cys-406 is bound to Tyr-407 in a cis-conformation similar as is found in human MAO-B (Son *et al.*, 2008).

The C-terminal of monoclinic human MAO-A is still unresolved with only the positions of a few residues known. However, it has been established that rat MAO-A is attached to the mitochondrial membrane (Binda *et al.*, 2003; De Colibus *et al.*, 2005; Ma, 2004). The C-terminal of MAO-A can be described as a single one-turn helix attached perpendicularly to the mitochondrial membrane (Ma, 2004). The residues of the C-terminal of MAO-A are Arg-129,

His-148, Lys-151, Lys-163, Arg-493, Lys-503, Lys-520 as well as Lys-522, and interact with the mitochondrial membrane surface (Son *et al.*, 2008). The phospholipid hydrophilic head group on the mitochondrial membrane interacts with the positively charged residues (Son *et al.*, 2008).

Three loops containing Val-93 to Glu-95, Tyr-109 to Pro-112 and Phe-208 to Asn-212 surround the entrance of the MAO-A cavity (Son *et al.*, 2008). Studies conducted on the entry point of the MAO-A cavity found that during the steady-state of enzyme catalysis, entrance is not possible due to it being too narrow (Son *et al.*, 2008). As previously mentioned, the protein is attached by the C-terminal helix perpendicular to the mitochondrial membrane. Due to Brownian motion, the movement of the mitochondrial membrane and protein is not synchronised (Son *et al.*, 2008). These unsynchronised movements lead to structural fluctuations enlarging the entry point due to conformational changes to the three loops (Son *et al.*, 2008). Therefore, membrane anchoring as well as the flexibility of the loops, especially Gly-110 and loop 109-112 surrounding the entry point, are necessary to ensure effective substrate entry (Son *et al.*, 2008).

2.1.2.2 The structure of MAO-B

Studies done using C-terminal truncation confirmed that residues 461-520 found in the carboxyl terminal region anchors the MAO-B protein to the mitochondrial membrane via the 27-residue transmembrane α-helix (Edmondson *et al.*, 2004; Rebrin *et al.*, 2001). The 27-residue transmembrane α-helix is also responsible for the specificity of MAO-B for the mitochondrial membrane (Edmondson *et al.*, 2004). The surface of the C-terminal helix is lipophilic in nature which ensures successful insertion into the mitochondrial membrane (Edmondson *et al.*, 2004). C-terminal truncation decreases MAO-B activity but not inhibitor specificity leading to the conclusion that C-terminal anchoring to the membrane is essential for enzyme functionality (Rebrin *et al.*, 2001). The attachment of MAO-B can further be explained by additional membrane interactions resulting from residues such as Trp-157, the hydrophobic sequence of residues 481-488 and possibly Pro-109 and Ile-110 (Edmondson *et al.*, 2004). These additional membrane binding residues explain the remaining activity of MAO-B that occurs during C-terminal truncations. However, the additional membrane residues do not bind with the same strength or specificity to the mitochondrial membrane (Edmondson *et al.*, 2004).

As previously mentioned, MAO-B consists of two monomers that crystallise as a dimer (Edmondson *et al.*, 2004). The dimeric crystal structure presents as two crystal forms, namely orthorhombic and triclinic, and is not due to crystal packing (Binda *et al.*, 2002). Interactions between the two monomers are present and are represented by 2,095 $Å^2$ of the surface area that is lost when the dimer is formed (Binda *et al.*, 2002). As is seen with most flavoenzymes, MAO-B folds in the typical p-hydroxybenzoate hydroxylase (PHBH) formation (Edmondson *et al.*, 2004).

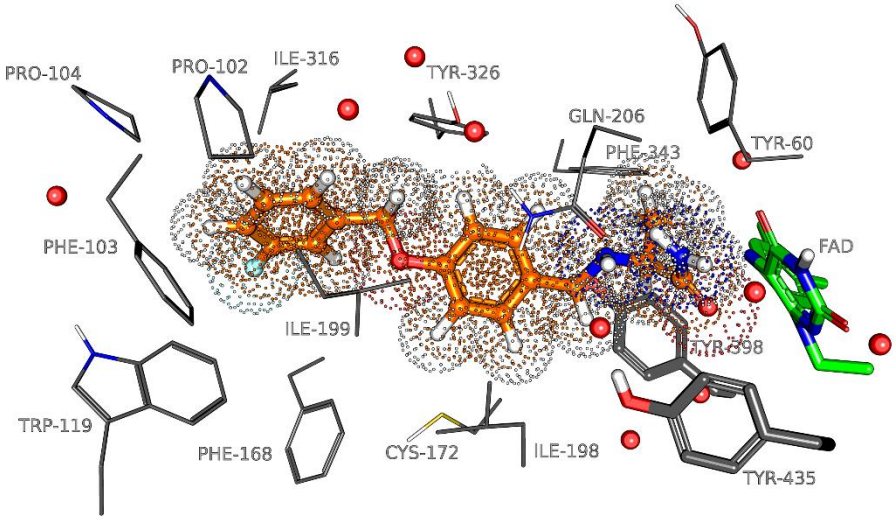


Figure 2-2: The 3D structure of the active site of MAO-B with key residues and the FAD cofactor (green). The co-crystallised ligand, safinamide, is shown in the active site cavity of MAO-B. Figure generated with PyMOL.

MAO-B differs from MAO-A as it consists of two cavities namely the substrate cavity and the entrance cavity. The entrance cavity of MAO-B has a volume of 290 $Å^3$ and is lined by residues Phe-103, Pro-104, Trp-119, Leu-164, Leu-167, Phe-168, Leu-171, Ile-199, Ile-316 and Tyr-326 (Binda *et al.*, 2002). The substrate cavity of MAO-B is a flat hydrophobic cavity lined with aromatic and aliphatic amino acids shaped as an elongated disc and has a volume of 420 $Å^3$ (figure 2-2) (Binda *et al.*, 2002). In this cavity, the FAD cofactor is located at the distal end and is responsible for the oxidation of amine substrates (Youdim *et al.*, 2006b). The substrate cavity also contains an “aromatic sandwich” which is generated by the *cis* conformation of the amide linkage between the flavin moiety and the Cys-397 residue (Youdim *et al.*, 2006b). This “aromatic sandwich” structure consists of phenolic side chains Tyr-398 and Tyr-435. The function of this “aromatic sandwich” is to recognise amine groups of substrates for oxidation and results in a hydrophobic environment. Therefore, it can be concluded that with an increase of the hydrophobicity of most substrates and inhibitors, the binding affinity of the substrate increases (Edmondson *et al.*, 2004). However, a decrease in binding affinity is often observed with an increased van der Waals volume due to the limited size of the MAO-B active site (Edmondson *et al.*, 2004).

Entry into the active site of the MAO-B enzyme occurs close to the mitochondrial membrane at the entrance cavity (Edmondson *et al.*, 2004). The mitochondrial membrane surface is negatively charged resulting in the attraction of amine substrates that are charged positively and thus facilitates entrance into MAO-B electrostatically (Edmondson *et al.*, 2004). Access is regulated by a loop of residues that covers the entrance cavity. This loop of residues consists of residues 99-112, which can be moved due to flexibility to allow for access to the entrance cavity.

This loop thus serves as the “gating switch” (Binda *et al.*, 2003). There is also a boundary that defines a separation between the entrance cavity and the substrate cavity. The four residues responsible for this boundary are Tyr-326, Ile-199, Leu-171 and Phe-168 (Youdim *et al.*, 2006b). The side chain of Ile-199 is significant as it can either rotate to an open or closed conformation. When Ile-199 is in the open conformation, the entrance and substrate cavities are fused which increases the volume available for occupation by a substrate or inhibitor to 700 $Å^3$ (Youdim *et al.*, 2006b). To reach the flavin ring at the distal end of the substrate cavity, the substrate travels a distance of 20 Å from the point of entry at the entrance cavity (Binda *et al.*, 2002). This phenomenon can be used to design MAO-B selective inhibitors. By manipulating the size of the inhibitor, larger compounds can be designed that are able to bind to MAO-B and not to MAO-A.

2.1.3 The genetic makeup of MAO

Human MAO-A and MAO-B share 70% amino acid sequence identity and are therefore similar (Son *et al.*, 2008). Human MAO-B consists of 520 amino acids whereas human MAO-A consists of 527 amino acids (Bach *et al.*, 1988).

Using complementary deoxyribonucleic acid (cDNA), the genetic makeup of MAO-A and MAO-B was examined (Bach *et al.*, 1988). It was found that the subunit molecular weight of MAO-A is 59,700 and MAO-B is 58,000 (Shih *et al.*, 1999). The isoforms of MAO are approximately 70% identical on the amino acid sequence level (Binda *et al.*, 2007; Youdim *et al.*, 2006b). However, the isoforms are not generated from the same protein but are different polypeptides (Shih *et al.*, 1999). Studies conducted on a number of mammalian species confirmed that separate genes for the encoding of MAO are located on the X chromosome (Xp11.23) (Kochersperger *et al.*, 1986; Pintar *et al.*, 1981).

cDNA specific fragments of MAO-A and MAO-B showed that the genetic makeup of the isoforms consists of 15 exons and identical exon-intron organisation (Grimsby *et al.*, 1991). This can be explained by MAO-A and MAO-B descending from a common ancestral gene (Grimsby *et al.*, 1991). This theory is further supported by the finding that 12 of the exon products are identical, resulting in a 93.9% similarity between MAO-A and MAO-B (Grimsby *et al.*, 1991). The amino acid sequence of MAO-A in humans, bovines and rats shows a > 87% similarity (Bach *et al.*, 1988; Hsu *et al.*, 1988; Kuwahara *et al.*, 1990; Powell *et al.*, 1989). Furthermore, the amino acid sequence of human and rat MAO-B exhibits an 88.3% similarity (Bach *et al.*, 1988; Ito *et al.*, 1988). The conservation of the MAO-A and MAO-B amino acid sequences across species can be interpreted as confirmation of the necessity of the physiological function of MAO (Shih *et al.*, 1999).

Even though studies suggest that MAO is not crucial for survival, gene deletion studies showed that MAO-A is essential during development (Lenders, 1996). In the absence of MAO-A function, a compulsive aggressive phenotype occurs (Brunner *et al.*, 1993). The absence of MAO-A mainly affects serotonin levels in the body which hampers neurobiological development. In MAO-A knockout mice, elevated levels of serotonin, noradrenaline and dopamine are found which correlates with aggressive behaviour (Shih *et al.*, 1999). This aggressive behaviour was also found in males with a lack of MAO-A activity as a result of gene deletion (Brunner *et al.*, 1993). This can be the result of increased levels of cortical serotonin causing structural changes in the somatosensory cortex (Cases *et al.*, 1996). In MAO-B knockout mice, only levels of phenylethylamine are increased (Shih *et al.*, 1999). Certain personality traits are correlated with low platelet MAO-B activity. These personality traits include sensation seeking, impulsive behaviour, extraversion and the possibility of substance abuse (Youdim *et al.*, 2006b). Aggression was not exhibited in MAO-B knockout mice (Shih *et al.*, 1999). Increased responses to stress were found in both MAO-A and MAO-B knockout mice (Shih *et al.*, 1999).

MAO-A and MAO-B differ in expression levels due to the genetic makeup of different tissues. These differences can be attributed to the core promoter regions (Shih, 2004; Wong *et al.*, 2002; Zhu *et al.*, 1994).

2.1.4 Biological function of MAO

2.1.4.1 Substrate specificities and metabolism of neurotransmitters

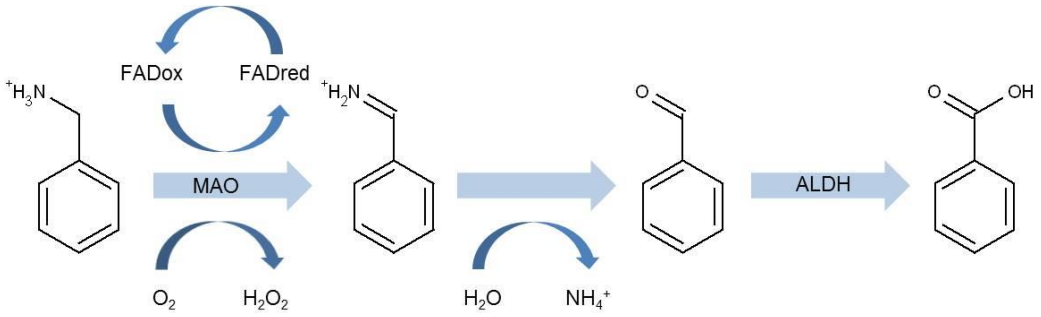


Figure 2-3: The oxidation pathway of neurotransmitters by MAO. Figure adapted from Gaweska and Fitzpatrick (2011).

MAO metabolises primary, secondary and tertiary amines to the corresponding imines by means of oxidation (figure 2-3). As is characteristic of flavoproteins, MAO catalyses substrates by means of two half-reactions consisting of a reductive and oxidative step. The first step is the reductive half-reaction in which the flavin cofactor accepts a hydride equivalent, where after the second step takes place when molecular oxygen reoxidises the flavin (Gaweska & Fitzpatrick, 2011). The imines are hydrolysed nonenzymatically to the corresponding aldehydes or ketones

(Edmondson *et al.*, 1993). The aldehydes or ketones are further oxidised by aldehyde dehydrogenase (ALDH) to the corresponding acids whereas aldehyde reductase metabolises the aldehydes to alcohols or glycols.

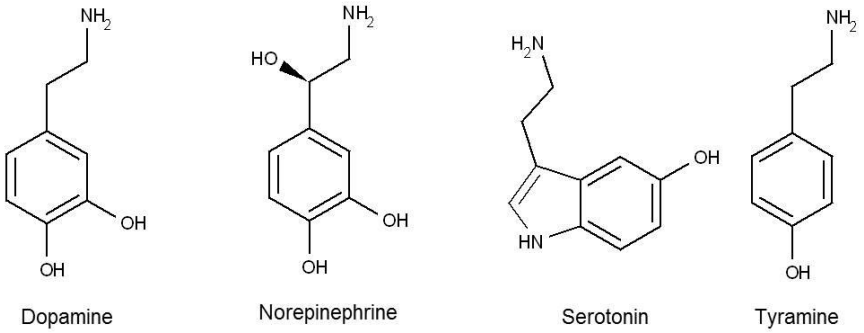


Figure 2-4: Substrates metabolised by MAO.

As previously mentioned, some amine substrates of MAO include neurotransmitters such as tyramine, dopamine, serotonin and noradrenaline (figure 2-4). MAO-A and MAO-B differ in their specificities for the metabolism of these neurotransmitters. MAO-A is responsible for the metabolism of tyramine, serotonin, noradrenaline and dopamine whereas MAO-B metabolises dopamine (Fowler & Benedetti, 1983; Hall *et al.*, 1969; McCauley & Racker, 1973). However, MAO-B can metabolise noradrenaline and serotonin at a slow rate (Fowler & Benedetti, 1983; Hall *et al.*, 1969; McCauley & Racker, 1973). Studies suggest that even with the full inhibition of one of the isoforms, the other isoform will metabolise dopamine successfully (Riederer & Youdim, 1986; Youdim *et al.*, 1972). This explains the unchanged levels of dopamine in the human striatum when MAO-A or MAO-B is selectively inhibited in comparison with the monoamines that are substrates for only one of the isoforms (Riederer & Youdim, 1986). However, selective inhibition by inhibitors such as moclobemide, clorgyline and rasagiline result in increased release of dopamine in the striatum of rodents (Haefely *et al.*, 1992). This shows that even though selective inhibition does not affect steady-state dopamine levels in the brain, the release of dopamine is modulated by selective MAO inhibition (Youdim & Bakhle, 2006).

As a result of the isoforms preference for certain neurotransmitters, MAO-A inhibitors are used as a treatment for depression and MAO-B inhibitors as a treatment for Parkinson's disease.

2.1.4.2 Metabolism of tyramine and the cheese reaction

The cheese reaction is a dangerous side effect commonly associated with MAO inhibitors. This side effect is the result of the unsuccessful metabolism of tyramine and other sympathomimetic amines. Tyramine and other sympathomimetic amines are found in fermented food such as cheese, and fermented drinks such as beer and wine (Da Prada *et al.*, 1988; Youdim & Bakhle, 2006). The cheese reaction is characterised by hypertensive crisis and haemorrhage in the

case of some inhibitors such as tranylcypromine (Youdim *et al.*, 1988). Many inhibitors exhibiting these side effects were withdrawn due to the possibility of death.

Normally tyramine and other dietary amines are metabolised by MAO found in the gut wall and the liver to such an extent that it does not enter the systemic circulation (Youdim & Bakhle, 2006). However, when MAO inhibitors prevent the metabolism of tyramine and other dietary amines, they enter the systemic circulation and potentiate sympathetic cardiovascular activity by inducing the release of noradrenaline from peripheral adrenergic neurons (figure 2-5) (Finberg & Tenne, 1982; Finberg *et al.*, 1981). The release of noradrenaline activates the cardiovascular system and results in a hypertensive crisis that can be fatal (Youdim & Bakhle, 2006).

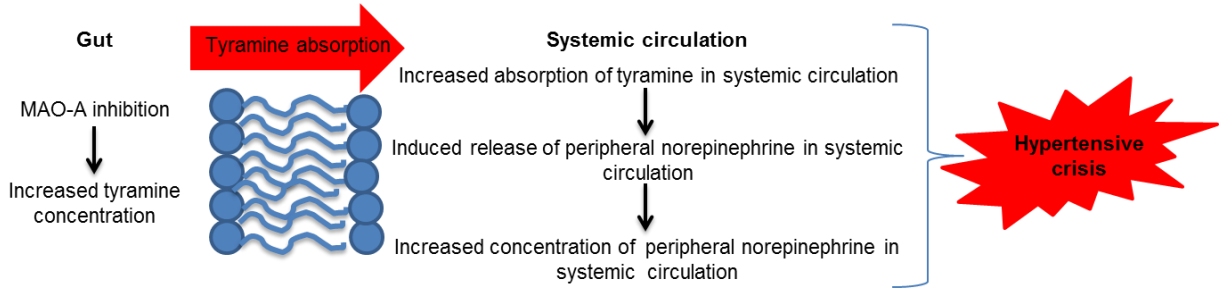


Figure 2-5: The pathway of the cheese reaction induced by MAO-inhibition.

To prevent the cheese reaction, selective inhibitors of MAO-B were explored. Since MAO-B is not expressed in the intestinal wall, tyramine and other sympathomimetic amines should be successfully metabolised by the MAO-A isoform that dominates the intestinal wall. This ensures that MAO-B selective inhibitors will not precipitate the cheese reaction (Knoll, 2000; Youdim & Weinstock, 2004). However, if irreversible MAO-B inhibitors are administrated at high doses, the inhibitor loses selectivity and may also inhibit the MAO-A isoform (Youdim *et al.*, 2006b). This would result in the cheese reaction.

The cheese reaction can also be prevented by using reversible MAO-A inhibitors. Reversible MAO-A inhibitors inhibit MAO-A sufficiently in the CNS to obtain an antidepressant effect. Tyramine is still metabolised successfully in the peripheral tissue since it competes with the reversible inhibitor at the active site for binding (Anderson *et al.*, 1993). This is done by displacing the inhibitor from the enzyme and ensuring successful metabolism by gut and liver MAO-A (Youdim & Bakhle, 2006). Inhibitors that rely on this mechanism are moclobemide and toloxatone (Da Prada, 1990). It may thus be concluded that the best approach to prevent the cheese reaction is by designing reversible competitive inhibitors of MAO and not relying on isoform selectivity or potency (Youdim & Bakhle, 2006).

2.1.4.3 Metabolism of serotonin and the serotonin toxicity

The inhibition of MAO-A increases serotonin in the brain. This would suggest that MAO-A is responsible for the metabolism of serotonin. Inhibitors of MAO-A are primarily classified as antidepressants (Youdim *et al.*, 2006b).

Serotonin toxicity, or as it was previously known serotonin syndrome, is caused by drug-drug interactions with a rapid onset and can cause death within 24 hours. Serotonin toxicity can be recognised by symptoms including altered mental status and neuromuscular and autonomic hyperactivity including headaches, dizziness, restlessness, tremors, seizures and diaphoresis (Volpi-Abadie *et al.*, 2013). The toxicity is caused by increased serotonin levels that overstimulate the peripheral and central postsynaptic serotonin 1A receptor (5-HT_{1A}) and serotonin 2A receptor (5-HT_{2A}) (Volpi-Abadie *et al.*, 2013). Serotonin toxicity can be life-threatening. Treatment for serotonin toxicity consists of supportive care to control the presenting symptoms.

Serotonin toxicity can be induced by administering a combination of MAO-A inhibitors (especially selective inhibitors) and drugs used in the treatment of depression such as uptake inhibitors [e.g. tricyclic antidepressant or serotonin-selective reuptake inhibitors (SSRIs)] (Boyer & Shannon 2005). All of these drug types lead to increased levels of serotonin in the CNS that result in overstimulation of serotonin receptors (figure 2-6). Fatalities as a result of the simultaneous use of an MAO-A inhibitor and an SSRI have been reported in cases with high doses of moclobemide and fluoxetine, citalopram or clomipramine. As a result, the simultaneous use of MAO inhibitors and SSRIs are contraindicated (Neuvonen *et al.*, 1993). As depression is treated with either MAO-A inhibitors or SSRIs, a washout period of two weeks is recommended if the patient changes between treatment options (Sternbach, 1991).

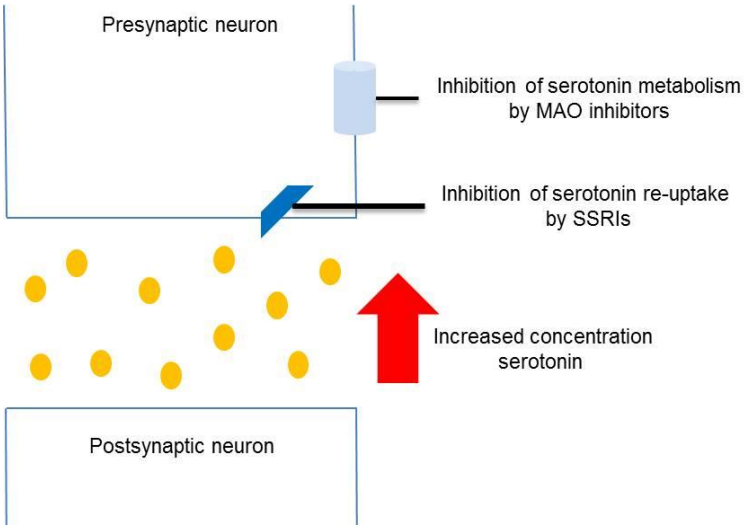


Figure 2-6: Precipitation of the serotonin toxicity.

The co-administration of SSRIs and methylene blue, an MAO-A inhibitor, can also lead to serotonin toxicity and therefore simultaneous use is also contraindicated (Boyer & Shannon 2005; Parlow & van Vlymen, 2008)

2.2 CLINICAL SIGNIFICANCE OF MAO IN PARKINSON'S DISEASE

2.2.1 Aetiology of Parkinson's disease

The exact cause of Parkinson's disease is unknown. Some possible causes include genetic factors that are precursors to endogenous toxins. Exposure to environmental toxins is also considered a possible trigger. Other theories are based on the formation of toxic by-products during the metabolism of neurotransmitters by MAO.

2.2.1.1 Genes associated with Parkinson's disease

Genes that have been linked to familial Parkinson's disease include α-synuclein (αS), parkin and ubiquitin C-terminal hydrolase L1 (UCH-L1) (Dauer & Przedborski, 2003; Zhang *et al.*, 2018). The mentioned genes are predominantly found in the substantia nigra pars compacta (SNpc) (Solano *et al.*, 2001). αS and parkin genes exhibit the same brain distribution suggesting a link in aetiology (Solano *et al.*, 2001). Missense mutations in αS and UCH-L1 is associated with autosomal dominant Parkinson's disease (Lücking *et al.*, 2000) whereas mutations of parkin are associated with autosomal recessive juvenile Parkinsonism (ARJP) (Farrer *et al.*, 1999; Leroy *et al.*, 1998).

αS may contain several point mutations. A base pair change at the position 209 of the fourth exon results in an exchange of Gly to Ala (Gly209Ala) (Polymeropoulos *et al.*, 1997). At position 53 of the amino acid chain, alanine is changed to threonine (Ala53Thr) (Polymeropoulos *et al.*, 1997) and at position 30 alanine is changed to proline (Krüger *et al.*, 1998). The original coding of the gene at Ala53Thr results in an α-helical formation bounded by a β sheet. However, the formation is disrupted when the base pair change occurs causing a disruption of the α helix resulting in the extension of the β sheet (Polymeropoulos *et al.*, 1997). Amyloid type structures can be formed by these β pleated sheets when self-aggregation of proteins occur (Polymeropoulos *et al.*, 1997). These mutations are commonly associated with early onset Parkinson's disease characterised by Lewy bodies and neuronal loss in the SNpc (Solano *et al.*, 2001). α, β, and γ proteins are encoded by synuclein genes (Clayton & George, 1998; Lavedan, 1998) and share 5 similar sized coding exons and several conserved residues (Lavedan, 1998). αS is the only protein of the three associated with familial Parkinson's disease (Polymeropoulos *et al.*, 1997). Lewy bodies were analysed and αS was found to be a major component of the inclusions (Irizarry *et al.*, 1998; Spillantini *et al.*, 1997).

The parkin gene containing deletions or point mutations in exons 3-7, is associated with ARJP (Hattori *et al.*, 1998; Kitada *et al.*, 1998; Lücking *et al.*, 1998). ARJP occurs at a young age and is characterised by neuronal loss in the SNpc, the absence of Lewy bodies and the occurrence of neurofibrillary tangles (NFT) (Rajput, 1999). These characteristics distinguish ARJP from idiopathic Parkinson's disease and confirm that the parkin gene mutation causes death to selective cells (Mori *et al.*, 1998). However, the molecular diagnosis of ARJP is difficult based on the numerous mutations that may occur in the parkin gene (Lücking *et al.*, 2000).

A mutation in the UCH-L1 gene was also identified in early-onset autosomal dominant Parkinson's disease (Leroy *et al.*, 1998). UCH-L1 proteins are also found in Lewy bodies and NFT associated with Alzheimer's disease (Lowe *et al.*, 1990). Mutations in both α–synuclein and UCH-L1 are thus associated with autosomal dominant Parkinson's disease (Farrer *et al.*, 1999; Gasser *et al.*, 1998).

Even though only a few documented cases of Parkinson's disease can be linked to genetic factors, the study of genetics can still be of use to understand the aetiology of the disease.

2.2.1.2 Endogenous and environmental neurotoxins

The catalytic pathway of MAO metabolises primary, secondary and tertiary amines to the corresponding aldehydes and free amines by means of oxidative deamination. The generation of an endogenous neurotoxin by MAO metabolism was considered after the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MAO converts MPTP to the active neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+), which is selectively toxic to dopaminergic neurons (figure 2-7) (Youdim *et al.*, 2006b).

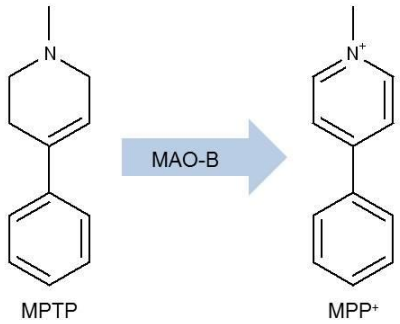


Figure 2-7: The metabolism of MPTP by MAO-B to yield MPP^+.

MPTP is selectively toxic to dopaminergic neurons in the substantia nigra and induces symptoms that are similar to those observed in Parkinson's disease when administered to animals and inadvertently to humans (Youdim & Bakhle, 2006). The neurotoxin MPP^+ enters the mitochondria and inhibits complex I of the electron transport chain (Nicklas *et al.*, 1985). This

leads to impaired oxidative phosphorylation and the disruption of the mitochondrial electron transport chain (Nicklas *et al.*, 1985; Tipton & Singer, 1993). Furthermore, complex I inhibition induces impaired calcium ion (Ca^{2+}) homeostasis (Kruman & Mattson, 1999; Mattson & Kroemer, 2003) and increases ROS production (Cleeter *et al.*, 1992; Dauer & Przedborski, 2003). This results in a decreased adenosine triphosphate (ATP) concentration in the striatum and ventral midbrain (Chan *et al.*, 1991; Fabre *et al.*, 1999). Dopaminergic nerve terminals are rich in synaptic mitochondria and are greatly affected by complex I inhibition (Dauer & Przedborski, 2003).

Studies in mice have shown not all MAO inhibitors exhibit neuroprotective properties against MPP^+ (Ansari *et al.*, 1993; Mytilineou, 1998). Of the selective inhibitors, only selegiline provided a protective effect while the MAO-A selective inhibitor, clorgyline, does not protect against MPTP-induced neurotoxicity (Heikkila *et al.*, 1984). This has led to the conclusion that if there is a neurotoxin that causes Parkinson's disease, it is metabolised and activated by MAO-B. These findings were corroborated by a study conducted on MAO-B knockout mice that showed resistance to MPTP (Shih *et al.*, 1999). Phenylethylamine also showed protective qualities by acting as a competitive substrate at the active site of MAO-B and thus preventing the oxidation of MPTP to the toxic metabolite, MPP^+ (Melamed & Youdim, 1985). Even though no toxin similar to the neurotoxin MPTP has been found to cause idiopathic Parkinson's disease, it is possible that the mechanism of neurodegeneration has been established through studies with MPTP (Youdim & Bakhle, 2006).

As previously mentioned, the theory that an endogenous or exogenous toxin may cause Parkinson's disease was centred on the discovery of the dopaminergic neurotoxin, MPTP (Langston *et al.*, 1983). Endogenous compounds such as isoquinoline derivatives, as well as exogenous compounds such as paraguat, were considered as possible toxins that may cause Parkinson's disease (Maruyama *et al.*, 2001). Herbicide and insecticide toxins such as paraquat and rotenone, are mitochondrial poisons (Dauer & Przedborski, 2003). However, it was found paraguat is not metabolised by MAO although industrial exposure to this pesticide increases the risk to develop Parkinson's disease (Uversky, 2004).

2.2.1.3 The generation of toxic by-products

Another theory derived from the effectiveness of MAO-B inhibitors as a treatment for neurodegenerative disorders, involves the possible generation of neurotoxic by-products as part of the metabolic pathway of MAO (figure 2-8) (Youdim *et al.*, 2006b).

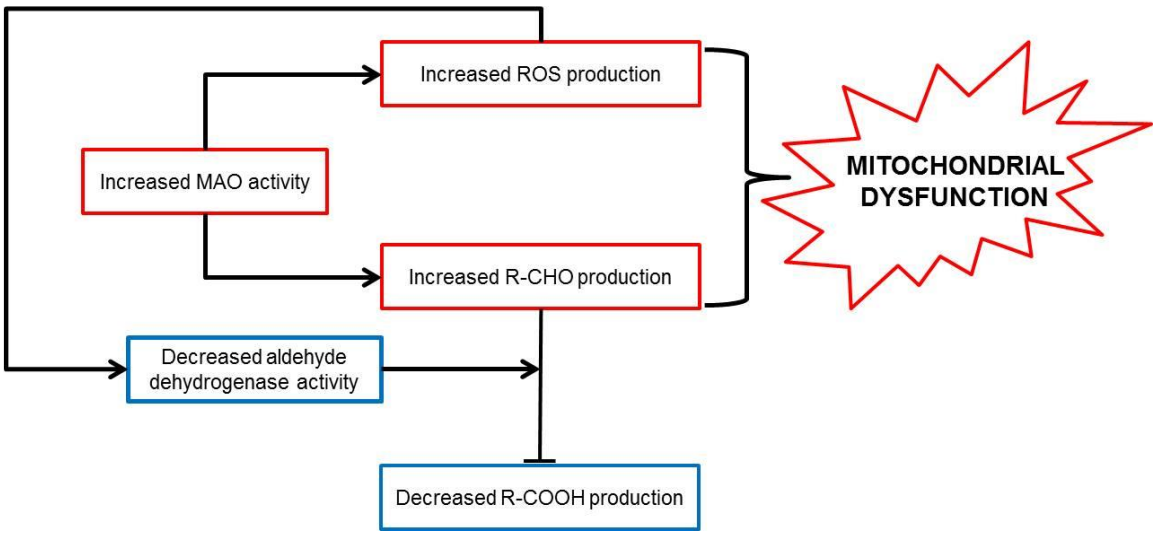


Figure 2-8: The metabolic pathway and resulting effects of increased MAO activity on mitochondrial function. Increased MAO activity results in the generation of neurotoxic by-products such as ROS. ROS suppresses the activity of ALDH. The inability of ALDH to convert aldehyde species (R-CHO) to carboxylic acid species (R-COOH) results in the accumulation of toxic R-CHO. Increased available concentrations of ROS and R-CHO cause mitochondrial dysfunction. Figure adapted from Kaludercic *et al.* (2014).

One of the by-products generated during MAO catalysis is H_2O_2, a species that may be converted to ROS. At high concentrations the products ammonia and H_2O_2 may be toxic to neuronal cells (Halliwell, 1992; Yang *et al.*, 2004). MAO-B inhibitors decrease the number of by-products such as H_2O_2 formed during amine oxidation and may thus protect against neurodegeneration in Parkinson's disease (Youdim *et al.*, 2006b).

The aldehyde by-products formed during dopamine oxidation by MAO are also considered neurotoxic as they may cause lesions of the dopamine neurons in the midbrain (Ansari *et al.*, 1993; Burke, 2003). Acidic metabolites are generated during the metabolism of the aldehydes by ALDH and include 5-hydroxyindole acetic acid (5-HIAA) generated from serotonin, or dihydroxyphenyl acetic acid (DOPAC) from dopamine. These metabolites are commonly used to measure MAO activity *in vitro* or *in vivo* (Youdim & Bakhle, 2006). In the brain tissue affected by Parkinson's disease, the aldehyde derived from dopamine is present in higher quantities compared to healthy brains (Lamensdorf, 2000). This effect may be attributed to reduced levels of ALDH in the substantia nigra as well as increased MAO-B activity in aged brain tissue (Galter *et al.*, 2003). The decrease in ALDH was confirmed with gene profiling of Parkinson's disease patients (Grünblatt *et al.*, 2004). This finding suggests that neurotoxic aldehydes derived from the metabolism of dopamine by MAO are able to accumulate since there is a reduced level of ALDH to facilitate neutralisation.

As previously discussed, H_2O_2 is generated by MAO oxidation. H_2O_2 is inactivated in the brain by glutathione peroxidase, with glutathione (GSH) acting as a co-factor (Youdim & Bakhle, 2006). It was found that glutathione levels are reduced in the SNpc of the brain in patients with Parkinson's disease and that ROS levels are increased (Riederer & Youdim, 1986; Sian *et al.*, 1994). This suggests that the primary protective mechanism against ROS is compromised and that H_2O_2 accumulates in the affected brain regions in Parkinson's disease.

The accumulated H_2O_2 is available to participate in the Fenton reaction and hydroxyl radicals are formed from H_2O_2 metabolism (Youdim & Bakhle, 2006). Iron participates in this reaction as the ferrous iron form (Fe^{2+}) (Youdim & Bakhle, 2006). After cellular antioxidants are depleted, the hydroxyl radical damages virtually all biomolecules including lipids, proteins and deoxyribonucleic acid (DNA). As a patient ages, iron and MAO activity increases in the SNpc resulting in the increased availability of the components of the Fenton reaction. This chain reaction increases hydroxyl radical formation (Youdim & Bakhle, 2006). By inhibiting MAO, the available monoamines are increased and the H_2O_2 levels available for hydroxyl formation are reduced. This lowers the potential for oxidative stress.

Based on these observations, it has been concluded that iron accumulation leads to an increase in oxidative stress, which potentiates neuronal damage (Youdim & Bakhle, 2006). The neuronal damage caused by iron is thus linked to the MAO-dependent formation of oxidative species (Symes *et al.*, 1969). An iron deficiency precipitates behavioural defects and also results in abnormal metabolism of monoamine neurotransmitters, especially dopamine (Youdim & Green, 1975). The theory that CNS dysfunction, such as Parkinson's disease, can be caused by abnormal iron metabolism is not widely accepted (Youdim & Bakhle, 2006). However many neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Huntington's disease, Friederich's ataxia and aceruloplasminemia show iron accumulation at sites of neuronal death (Mandel *et al.*, 2005).

2.2.2 Pathogenesis of Parkinson's disease

Two theories exist regarding the pathogenesis of Parkinson's disease. The first theory suggests that misfolding and aggregation of proteins lead to dopaminergic neuronal death (Dauer & Przedborski, 2003). The second theory claims that mitochondrial dysfunction results in oxidative stress, which in turn is the primary driver of neurodegeneration (Dauer & Przedborski, 2003). Some believe that these two mechanisms are linked and potentiate each other. An example of such an interaction is oxidative damage to αS that increases the probability that it may misfold and aggregate (Giasson *et al.*, 2000).

2.2.2.1 Protein aggregation and misfolding

Aggregated or misfolded proteins are indicative of neuronal diseases since misfolded proteins cause damage that leads to neuronal death (Dauer & Przedborski, 2003). Interestingly, some studies suggest that no relation between inclusion formation and neuronal death exists and that protein aggregation is simply a survival technique to remove toxic soluble misfolded proteins (Auluck *et al.*, 2002; Cummings *et al.*, 1999; Cummings *et al.*, 2001; Saudou *et al.*, 1998; Warrick *et al.*, 1999).

However, protein aggregation and inclusions could result in neuronal damage by causing deformation of cells, disrupting intracellular traffic or by isolating needed proteins (Dauer & Przedborski, 2003). Protein aggregation and misfolding are triggered by oxidative stress and ROS formation (Youdim *et al.*, 2006b). As age advances, more tissue is found with increased levels of oxidised proteins (Beckman & Ames, 1998). Therefore, it may be concluded that, with an advance in age, there exists an increased probability for the misfolding of proteins (Sherman & Goldberg, 2001).

2.2.2.2 Oxidative stress and mitochondrial dysfunction

The complexes of the electron transport chain are responsible for the transfer of electrons from donors to acceptors. This is done by redox reactions. Studies found that in Parkinson's disease, complex I is affected and behaves abnormally (Greenamyre *et al.*, 2001). This can be the result of the inhibition complex I causing a block of the mitochondrial electron transport chain (Nicklas *et al.*, 1987). It is also suggested that this defect is either the result of genetics that are inherited from the mitochondrial genome or toxicities leading to mutations in the mitochondrial DNA (Dauer & Przedborski, 2003). Neuronal loss can also be explained by the dysregulation of mitochondrial DNA homeostasis contributing to the pathogenesis of Parkinson's disease (Dölle *et al.*, 2016). The neurons in the substantia nigra of patients diagnosed with Parkinson's disease do not exhibit protective qualities to preserve mitochondrial DNA.

During the process of mitochondrial respiration, powerful oxidants are generated as by-products including H_2O_2 and superoxide radicals (Dauer & Przedborski, 2003). With complex I inhibited, the production of ROS is increased (Dauer & Przedborski, 2003). Increased ROS availability results in increased levels of toxic hydroxyl radical formation that leads to cellular damage since the hydroxyl radical is highly reactive and reacts with nucleic acids, proteins and lipids (Dauer & Przedborski, 2003). Some theorise that the electron transport chain is damaged by these reactive species, resulting in further mitochondrial damage and increased production of ROS (Cohen, 2000).

2.2.3 Neurochemical and neuropathological features of Parkinson's disease

In Parkinson's disease the nigrostriatal dopaminergic neurons undergo progressive degeneration (Dauer & Przedborski, 2003). Intraneuronal proteinaceous cytoplasmic inclusions, called Lewy bodies, are also formed (Dauer & Przedborski, 2003). It is also suggested that the neuronal death seen in Parkinson's disease, is a result of a dying back process in which the striatal dopaminergic nerve terminals are the primary targets of the degenerative process (Bernheimer *et al*., 1973). This theory is supported by studies with monkeys and mice that were treated with MPTP, a neurotoxin that induces a Parkinsonian syndrome in most mammals (Herkenham, 1991). In these studies, the dopaminergic neuronal loss was prevented by protecting the striatal terminals (Wu *et al*., 2003). The ageing process appears to be the major risk factor for the development of Parkinson's disease (Dauer & Przedborski, 2003). As the human brain ages, it is found that MAO-B activity increases. Since MAO-B is found in glial cells in the brain, this increase of MAO-B activity is a result of glial cell proliferation (Oreland & Gottfries, 1986). The increased levels of MAO-B lead to apoptosis in neuronal and kidney cells (Youdim *et al.*, 2006b). Interestingly, MAO-A activity does not increase in the brain with age (Youdim *et al.*, 2006b). An increase of MAO-B activity will result in increased production of ROS, lower concentrations of neurotransmitters and an increase in neuronal death.

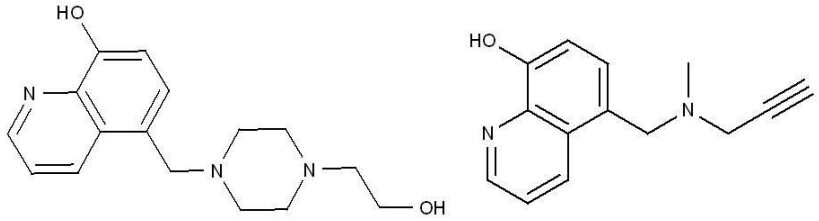
The neuronal loss in Parkinson's disease is not restricted to dopaminergic neurons only, the neuronal loss also occurs in noradrenergic, serotonergic and cholinergic systems (Hornykiewicz, 1998). This could explain the link between Parkinson's disease and depression.

2.2.4 Neuroprotection and neurorescue

The exact mechanism of neurodegeneration in Parkinson's disease is still being investigated. Increased ROS production has been identified in several neurodegenerative disorders and may contribute to neurotoxicity associated with neurodegenerative diseases (Andersen, 2004; Barnham *et al.*, 2004; Emerit *et al.*, 2004). Therefore, it may be hypothesised that compounds that exhibit neuroprotective properties often act as antioxidants (Youdim *et al.*, 2006b). This theory is supported by the increased capacity of cellular antioxidant ability, even if concentrations of the test compounds are too low for direct antioxidant activity (Carageorgiou *et al.*, 2003; Kitani, 2002; Mytilineou, 1998). Both MAO inhibition as well as iron chelation achieves the same neuroprotective effect by reducing oxidative stress and may be a key component in the process of neuroprotection (Youdim *et al.*, 2006b).

Other studies used a different approach to rectify the accumulation of Fe^{2+}. These approaches included designing iron chelators (e.g. desferal) to remove the excess free Fe^{2+} ions (Mandel *et al.*, 2005). Desferal was found to protect the nigrostriatal dopaminergic neurons from lesion

formation by 6-hydroxydopamine, a neurotoxic synthetic organic compound, as well as MPTP (Mandel *et al.*, 2005). Another compound, VK-28 (figure 2-9), is a dual iron chelator and MAO inhibitor that exhibits enhanced brain penetration as well as good neuroprotective abilities against MPTP and 6-hydroxydopamine (Mandel *et al.*, 2005). Unfortunately, when VK-28 was given systematically it did not inhibit MAO (Mandel *et al.*, 2005). M30 (figure 2-9) was designed by combining the iron chelating hydroxyquiniline pharmacophore of VK-28 with a propargylamine group, which is intended to inhibit MAO irreversibly. This resulted in a compound that chelates iron as well as potently inhibits MAO-A and MAO-B *in vitro* and *in vivo* (Gal *et al.*, 2005).



VK-28 M30

Figure 2-9: The structures of VK-28 and M30.

M30 and VK-28 show potential as a treatment for Alzheimer's disease by regulating the processing of the amyloid precursor protein (APP) of Aβ, a neurotoxic protein (Youdim & Bakhle, 2006). This is done by regulating the iron-responsive element on the APP messenger ribonucleic acid (mRNA) which allows cellular levels of iron to control translation as well as synthesis (Rogers *et al.*, 2002). M30 and VK 28 also increase the synthesis of a similar soluble amyloid precursor protein alpha (sAPPα) that exhibits neuroprotective abilities and stimulates neuronal growth as well (Youdim & Bakhle, 2006). MK30 and VK-28 can be seen as an alternative treatment option to the inhibition of β- or γ-secretase by influencing the synthesis of APP and lowering the levels of Aβ (Youdim & Bakhle, 2006).

Neurorescue can be defined as the prevention of neuronal death and neurodegeneration by the reversal of damage that otherwise would have led to neuronal death (Youdim *et al.*, 2006b). Some research suggests that the neurorescue and neuroprotective abilities of certain MAO inhibitors may reside in the propargylamine moiety (Youdim & Weinstock, 2001). Propargylamine itself also showed promise (Bar-Am *et al.*, 2005). The anti-apoptotic effects of propargylamine include the prevention of mitochondrial swelling, permeability-transition pore opening and cytochrome *c* release (Akao, 2002; Berry & Boulton, 2002; Tatton, 2002; Wadia, 1998).

2.3 CLINICAL SIGNIFICANCE OF MAO IN ALZHEIMER'S DISEASE

2.3.1 General background

Alzheimer's disease is a neurodegenerative disease resulting in the progressive decline of mental and learning abilities as well as significant behavioural changes (Anand *et al.*, 2014). Alzheimer's disease is responsible for 80% of dementia cases found in the elderly (Kumar *et al.*, 2015). Patients usually present with Alzheimer's disease symptoms at the age of 65 years. However, some cases in younger patients have been identified and have been deemed as early onset Alzheimer's disease. A rapid increase in Alzheimer's disease prevalence led to predictions that a patient may be diagnosed with Alzheimer's disease every 33 seconds by the year 2050 (Anon, 2015).

Autopsies conducted on the brains of Alzheimer's disease patients showed certain markers including amyloid plaques and NFT (Ramirez-Bermudez, 2012). This would suggest that amyloid plaques and NFT are involved in the pathology of the disease. Amyloid plaques are found in the brain parenchyma and can be described as the extracellular deposits of Aβ (Kumar *et al.*, 2015). The plaques can also be found in the cerebral blood vessels and are called congophilic or cerebral amyloid angiopathy (CAA) (Kumar *et al.*, 2015). The NFT consist of aggregated hyperphosphorylated tau proteins presenting in pairs of helical filaments and are responsible for neuronal and synaptic loss (Anand *et al.*, 2014). Patients with Alzheimer's disease also show decreased concentrations of acetylcholine (ACh) (Kumar *et al.*, 2015).

MAO has been identified to have a possible effect on the pathophysiology of Alzheimer's disease (Huang *et al.*, 2012; Zheng *et al.*, 2012b). MAO-B activity can cause cholinergic dysfunction as well as enhance the formation of amyloid plaques resulting in cognitive dysfunction (Delumeau *et al.*, 1994; Grailhe *et al.*, 2009; Huang *et al.*, 2012; Zheng *et al.*, 2012b). MAO levels found in Alzheimer's disease patients differ from those found in healthy individuals. The platelets of Alzheimer's disease patients show increased MAO-B activity which is considered as a possible biomarker associated with dementia and other behavioural changes (Bonuccelli *et al.*, 1990; Fischer *et al.*, 1994; Götz *et al.*, 1998; Mimica *et al.*, 2008; Parnetti *et al.*, 1994). MAO-A as well as MAO-B levels in brain tissue are also altered in Alzheimer's disease patients (Kennedy *et al.*, 2003). Increased MAO activity can also lead to NFT formation (Cai, 2014).

2.3.2 MAO and pathophysiology of Alzheimer's disease

As previously mentioned, markers for Alzheimer's disease include amyloidal plaques and hyperphosphorylated NFT (Kumar *et al.*, 2015). The cause of Alzheimer's disease is still unclear. Some theorise that there are multiple causative factors which result in the multifactorial

presentation of the disease (Kumar & Dogra, 2008). Some of these theories revolve around the cholinergic system, Aβ and tau protein formation as well as the process of neuroinflammation (Kurz & Perneczky, 2011). As previously mentioned, oxidative stress is caused by the increased activity of MAO as patients age. Increased oxidative stress has been associated with increased formation of Aβ resulting in the generation of amyloid plaques (Cai, 2014). Increased MAO activity can be linked to neuronal death and ultimately neurodegeneration by means of oxidative stress (Avramovich-Tirosh *et al.*, 2006; Hu *et al.*, 2008; Ono *et al.*, 2006; Weinreb *et al.*, 2009). Therefore, it may be concluded that MAO can be involved in the pathology of Alzheimer's disease.

2.3.2.1 Aβ formation

Theories regarding the involvement of Aβ fail to explain the complex pathophysiology of Alzheimer's disease (Hardy, 2009). New studies found that Aβ oligomers impair synaptic function which reduces brain functionality (Anand *et al.*, 2014; Dal Prà *et al.*, 2014; Galimberti *et al.*, 2013; Hardy, 2009). This decline in synaptic function may be the initial factor resulting in Alzheimer's disease rather than the formation of amyloid plaques which is only found in later stages of the disease (Dal Prà *et al.*, 2014). Studies showed that MAO plays a key role in the process of Aβ generation from APP (Bar-Am *et al.*, 2010; Weinreb *et al.*, 2006; Yogev-Falach *et al.*, 2006; Youdim *et al.*, 2006a). Aβ are formed from APP and under normal physiological circumstances APP is cleaved by α-secretase or β-secretase (Kumar *et al.*, 2015). The pathway which involves α-secretase does not yield Aβ and is considered as the non-amyloidogenic pathway (Liu *et al.*, 2010; Zhiyou *et al.*, 2009).

β-secretase is responsible for the first step in the formation of Aβ (figure 2-10) (Anand *et al.*, 2014). β-secretase cleaves APP extracellularly between the APP residues Met-671 and Asp-672 (Cai, 2014). This cleavage results in the formation of two fragments namely a soluble extracellular fragment as well as C99 (Cai, 2014). C99 is a membrane-bound fragment and the cleavage thereof by γ-secretase is the second step in the formation of Aβ (Cai, 2014). Aβ are released from the intracellular domain of APP by this second cleavage at Val-711 or Ile-713 within the hydrophobic transmembrane domain (Cai, 2014). This results in the increased formation of Aβ, and the inability to properly clear the increased concentrations successfully results in its accumulation (Salomone *et al.*, 2011). The accumulated Aβ aggregate to form soluble oligomers that fuse into fibrils in a β sheet formation resulting in plaque formation (Hardy, 2009).

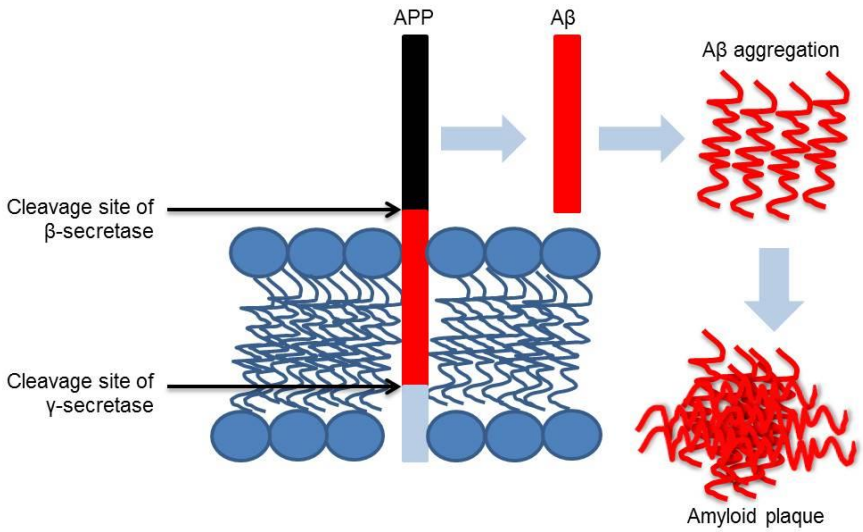


Figure 2-10: The proteolytic cleavage of APP. Figure adapted from Cai (2014).

Another oligomer has been studied for a possible contribution to the pathology of Alzheimer's disease. Amyloid β peptide fragment 42 ($A\beta_{42}$) oligomers have been associated with oxidative stress as well as increased formation of hyperphosphorylation tau proteins (Kumar & Dogra, 2008; Kurz & Perneczky, 2011). These factors lead to synaptic and mitochondrial damage (Kumar & Dogra, 2008; Kurz & Perneczky, 2011). Plaques formed by $A\beta_{42}$ also have the ability to attract microglia (Hanna, 2013). The activation of microglia leads to the formation of proinflammatory cytokines. After the release of cytokines, such as interleukin 1 beta (IL-1β), tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), they continue to stimulate astrocyte-neurons (Kumar *et al.*, 2015). The stimulated astrocyte-neurons continue to produce $A\beta_{42}$ oligomers propagating this chain reaction (Dal Prà *et al.*, 2014). This propagating chain reaction can be described as the amyloid cascade hypothesis (figure 2-11).

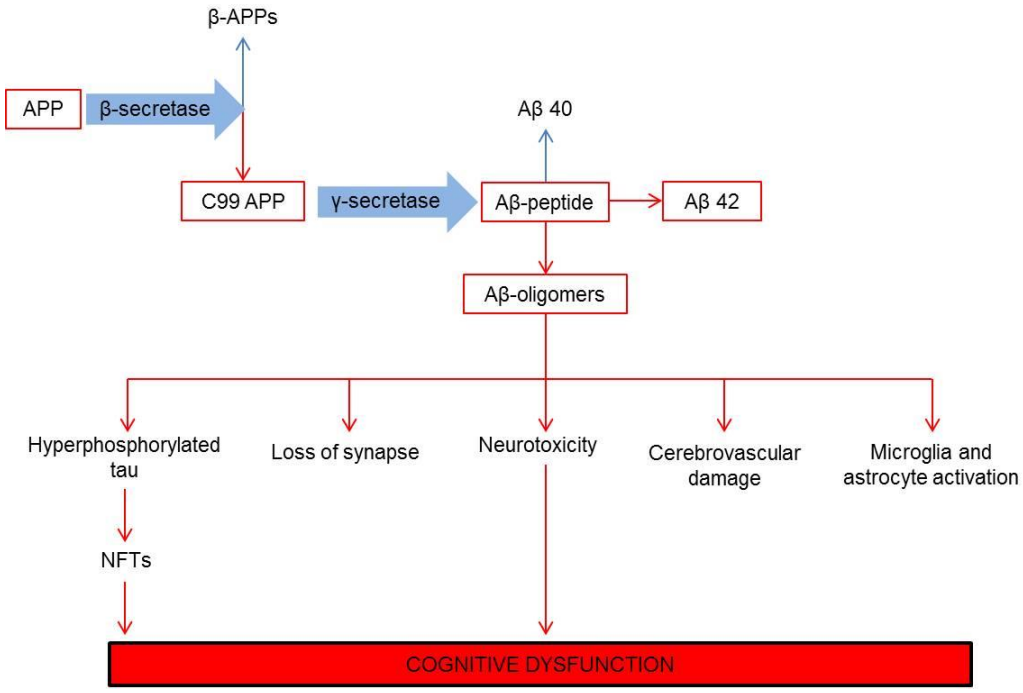


Figure 2-11: The amyloidogenic pathway induced by the metabolism of Aβ. Figure adapted from Kumar *et al.* (2015).

It has been hypothesised that the Aβ oligomers responsible for the formation of plaques result in the degeneration of neuronal and vascular structures associated with Alzheimer's disease (Roth *et al.*, 2005). Oligodendroglia (OLGs) generates the myelin sheath in the CNS that supports and provides insulation to axons and is therefore closely related to the neuron-astrocyte complex (Dal Prà *et al.*, 2014). Aβ oligomers destroy OLGs by means of oxidative stress (Antanitus, 1998). The ability of OLGs to scavenge oxygen radicals is impaired due to low concentrations of glutathione as well as high concentrations of iron found in the OLGs (Galimberti *et al.*, 2013). Another possible reason for the susceptibility of OLGs and myelin for $A\beta_{42}$ oligomers may be due to its cholesterol rich membranes (Roth *et al.*, 2005).

MAO inhibitors have been of value to prevent Aβ pathology and improve cognitive abilities. This is done by modulation of the proteolytic cleavage of APP by β-secretase and γ-secretase (Cai, 2014). The modulation is done by activating mitogen-activated protein kinases (p42/44 MAPK) and protein kinase C (PKC) signalling pathways (Bar-Am *et al.*, 2010; Yogev-Falach *et al.*, 2002; Youdim *et al.*, 2003; Youdim *et al.*, 2004). This results in the up-regulation of the metabolism of APP by α-secretase channelling proteins to the non-amyloidogenic pathway (Yang *et al.*, 2009). Studies conducted on the prevention of Aβ pathology using irreversible MAO-B inhibitors such as ladostigil, M30 and deprenyl confirmed the modulation of the proteolytic cleavage by p42/44MAPK (Bar-Am *et al.*, 2010; Yang *et al.*, 2009; Youdim *et al.*, 2003). Combination therapy with donepezil and selegiline resulted in the improvement of cognitive functionalities in Aβ-injected mice (Tsunekawa *et al.*, 2008).

2.3.2.2 MAO and neuroinflammation

As previously mentioned, Aβ pathology results in the release of cytokines and inflammatory mediators that can contribute to neuroinflammation as is seen in Alzheimer's disease patients (Calderon-Garciduenas *et al.*, 2012; Mazzulli *et al.*, 2011; McGeer & McGeer, 2010). Another contributing factor to neuroinflammation is the cycle induced by oxidative stress. This cycle consists of a self-induced inflammation further propagated by oxidative stress resulting in neurodegeneration and neuronal death (Hensley, 2010). Therefore, it can be concluded that targeting oxidative stress as well as inflammation can be of value in preventing the progression of Alzheimer's disease (Cai, 2014). In addition to reducing the production of H_2O_2 by MAO, MAO inhibitors prevent the release of proinflammatory cytokines by decreasing Aβ pathology and thus prevent the cascading effects that follow (Cai, 2014).

2.3.2.3 MAO and neurofibrillary tangles

The formation of NFT have been associated with oxidative stress. However, a direct link between increased MAO activity and NFT are yet to be established (Cai, 2014). As previously mentioned, NFT are formed by hyperphosphorylated aggregated tau proteins. However, increased MAO activity resulting in increased oxidative stress can be a contributing factor (Zheng *et al.*, 2012a). Oxidative stress due to MAO activity results in damage to mitochondria which causes neurodegeneration (Menazza *et al.*, 2010).

2.3.3 Alzheimer's disease treatment options

The current treatment for Alzheimer's disease includes acetylcholinesterase inhibitors (AChEIs) such as rivastigmine, galantamine, tacrine and donepezil to improve cognitive manifestations, as well as N-methyl-d-aspartate (NMDA) receptor antagonists such as memantine to treat the resulting symptoms (Auld *et al.*, 2002). These drugs increase the available concentrations of ACh by inhibiting its breakdown which results in improved memory (Kumar *et al.*, 2015). During the early stages of Alzheimer's disease rivastigmine, galantamine and donepezil are the most effective treatment options, whereas memantine has been designated for the later stages of the disease (Kumar *et al.*, 2015). The use of tacrine is not advisable due to potential liver damage caused by this drug (Kumar *et al.*, 2015). However, these drugs only treat the symptoms of Alzheimer's disease and do not slow the progression of the disease (Farlow *et al.*, 2008). Therefore, the search for disease-modifying drugs still continues. Possible targets for these disease-modifying drugs include the markers identified during the pathology of Alzheimer's disease. These markers are the Aβ peptide generated from APP, fibrils and plaques (Kurz & Perneczky, 2011).

Other treatment options that can be investigated include β- and γ-secretase inhibitors as well as Aβ vaccinations (Ramirez-Bermudez, 2012). Iron chelators and statins used for treating cholesterol can also be of use (Ramirez-Bermudez, 2012).

It may be argued that increased activity of MAO can play a role in the pathology of Alzheimer's disease (Cai, 2014). Therefore, MAO inhibitors can be of use in the treatment of Alzheimer's disease by affecting proteolytic cleavage, leading to a decrease in Aβ pathology with a resulting neuroprotective effect (Nedic *et al.*, 2010).

2.4 CLINICAL SIGNIFICANCE OF MAO IN OTHER DISEASES

2.4.1 Depression

The two monoamines, noradrenaline and serotonin, play key roles in depressive illness (Youdim & Bakhle, 2006). Therefore, the inhibition of MAO-A would be of importance in the treatment of depression. MAO-A inhibition in the CNS leads to increased levels of neurotransmitters such as dopamine, noradrenaline and serotonin (Youdim *et al.*, 2006b). The increased levels of these neurotransmitters result in the effective treatment of depression, especially in elderly patients (Gareri *et al.*, 2000). Positive results for the treatment of phobic anxiety, atypical depression, hypersomnia, bulimia, tiredness and impression of rejection with MAO-A selective and MAO non-selective inhibitors compared to amine-uptake inhibitors have been reported (Zisook, 1985).

MAO inhibitors that are used in the treatment of depression range from non-selective irreversible inhibitors such as phenelzine and tranylcypromine, to reversible MAO inhibitors such as moclobemide, befloxatone and toloxatone (Youdim *et al.*, 2006b). Irreversible inhibitors are avoided as a treatment for depression because of the 'cheese reaction', while reversible inhibitors that do not induce this reaction can be further investigated (Youdim & Bakhle, 2006). For the treatment of therapy-resistant depression, combinations of reversible MAO-A inhibitors such as moclobemide and reversible MAO-B inhibitors such as lazabemide can be of value (Youdim & Bakhle, 2006). However, combination therapy must be considered with caution because of the risk for the serotonin toxicity (Boyer & Shannon 2005).

MAO-B inhibitors do not possess antidepressant activity on their own (Youdim *et al.*, 2006b). The reason why MAO-B inhibition would contribute to the treatment of depression is not entirely established seeing that the substrates of MAO-A are primarily involved in depressive illness. Some hypothesise that the antidepressant effect of MAO-B inhibitors is due to an indirect modulation of serotonin action by dopamine (Youdim & Bakhle, 2006). This hypothesis may explain why there is an increased response during the treatment of bipolar depression with a combination of selegiline and a serotonin-enhancing agent instead of using each drug

separately (Mendlewicz & Youdim, 1983). This supports the possible use of a non-selective MAO inhibitor for the treatment of depression to increase dopamine as well as serotonin levels in the brain (Youdim & Bakhle, 2006).

This dual mechanism of action may also be beneficial in the treatment of Parkinson's disease since a significant amount of patients also show signs of depression (Youdim & Bakhle, 2006). Depression in Parkinson's disease patients can be related to the deficit in striatal dopamine, the significant reductions of noradrenaline in the locus coeruleus as well as reductions of serotonin in the raphe nucleus that have been recorded in patients with Parkinson's disease (Yamamoto, 2001). Further evidence supporting this theory was found when genetic studies showed that the same polymorphism in the serotonin transporter gene was observed in Parkinson's disease patients that presented with frequent and severe depressive episodes and patients with endogenous depression (Jay, 2002). However, MAO inhibitors are not considered to be the primary solution for depression because of the serious side effects that they may induce. This consideration led to the discovery of other antidepressants such as uptake inhibitors, tricyclic antidepressants and SSRIs (Youdim & Bakhle, 2006).

2.4.2 Cardiovascular disease and cerebral ischemia

As previously mentioned, free radicals cause oxidative stress, propagate and cause damage in various organ systems. However, the heart is not equipped with the same concentrations of free radical scavengers to neutralise the free radicals caused by oxidative stress (Chen *et al.*, 1994). This renders the heart vulnerable to damage.

The MAO-A isoform is expressed in higher concentrations in the myocardium of the heart and is responsible for ROS production (Kaludercic *et al.*, 2011). The ROS generated by MAO-A have been implicated in myocyte hypertrophy (Bianchi *et al.*, 2005b), ischemic reperfusion injury (Bianchi *et al.*, 2005a; Carpi *et al.*, 2009; Pchejetski *et al.*, 2007), heart failure as well as left ventricular remodelling (Kaludercic *et al.*, 2010; Villeneuve *et al.*, 2012). A study conducted on mice showed unsuccessful remodelling and left-ventricular dysfunction, caused by oxidative stress damage to the mitochondria as well as cardiomyocyte necrosis due to the activity of MAO-A in the heart (Kaludercic *et al.*, 2010; Villeneuve *et al.*, 2012). The increased effects witnessed in the myocardium of diabetic rats were not due to increased expression of MAO-A but rather an increase in MAO-A activity (Umbarkar *et al.*, 2015).

MAO-B is also found in high concentrations in the human heart but the effect it has on cardiac function is still unclear. However, MAO-B is required for the metabolism of catecholamines to ensure proper cardiac function (Kaludercic *et al.*, 2014). MAO-B activity results in increased oxidative stress resulting in reduced cardiac ability (Kaludercic *et al.*, 2014). The effects are

primarily witnessed when the heart undergoes stressful conditions such as pressure overload. During these stressful conditions increased oxidative stress is found due to increased MAO-B activity resulting in mitochondrial damage (Kaludercic *et al.*, 2014).

Inhibition of MAO-A by clorgyline have been found to prevent myocardial collagen increase, which improves left ventricle contraction (Umbarkar *et al.*, 2015). Other H_2O_2 scavengers such as catalase, glutathione peroxidase and peroxiredoxins were not responsible for the decrease in oxidative stress (Umbarkar *et al.*, 2015). The explanation for the reduced H_2O_2 activity is due to MAO-A inhibition (Umbarkar *et al.*, 2015). Therefore, MAO-A inhibition decreases apoptosis, fibrosis and cardiac oxidative stress in the heart (Umbarkar *et al.*, 2015). Studies conducted on pressure-overloaded mice showed that the inhibition of MAO-A circumvents heart failure (Kaludercic *et al.*, 2010).

Studies conducted on ROS and its impact on the cardiac muscles showed that ROS could be reduced by antioxidant therapy as well (Kumar *et al.*, 2013; Rösen *et al.*, 1995). Selegiline, a selective MAO-B inhibitor, has been shown to enhance recovery after stroke by decreasing the levels of H_2O_2 (Sivenius, 2001). Studies conducted on rat brains showed that selegiline protects neuronal tissue against oxidative stress damage caused during reperfusion (Seif-El-Nasr *et al.*, 2008). This can possibly be explained by reduced dopamine catabolism that results in a decrease of ROS. Furthermore, propargylamine structures show promising anti-apoptotic activity in cardiac myocytes and can possibly be used in cardiovascular medicine (Youdim & Buccafusco, 2005).

2.4.3 Smoking cessation

MAO inhibitors may be valuable as an aid to smoking cessation because it is hypothesised cigarette smoke inhibits MAO (Fowler, 1996; Fowler, 2003; Herraiz & Chaparro, 2005). The nicotine of cigarette smoke stimulates neurons to release monoamines via activation of presynaptic nicotinic acetylcholine receptors (nAChRs). This reinforces the rewarding effect of smoking (George & Weinberger, 2008). Selegiline and lazabemide have been found to aid in smoking cessation and persistent abstinence (Berlin, 1995; Houtsmuller *et al.*, 2002). The success of MAO-A inhibitors, such as moclobemide, in smoking cessation can be attributed to increased levels of serotonin in the brain (Berlin, 1995). However, it has not been established if smoking causes a reduction in MAO activity or whether enhanced MAO activity is associated with the susceptibility for smoking (Oreland, 2004). The effect of MAO inhibitors in smoking cessation can be attributed to the maintenance of certain levels of MAO inhibition (thus neurotransmitter levels) that smokers have come accustomed to.

2.4.4 Dementia

Dementia is most commonly caused by Alzheimer's disease and is also recognised as a common feature of Parkinson's disease. Patients exhibiting dementia are classified as having diffuse Lewy body disease (DLB) by postmortem investigation (Youdim & Bakhle, 2006). DLB patients receiving L-dopa therapy for their extrapyramidal disorders, responded cognitively to cholinesterase inhibitors that are used primarily in Alzheimer's disease, without loss of response to drugs treating Parkinson's disease (Youdim & Buccafusco, 2005). These observations initiated a search for a single bifunctional compound combining pharmacophores for MAO-B inhibition and for cholinesterase inhibition as a treatment for DLB patients. A series of compounds based on the selegiline and rasagiline pharmacophores and incorporating a carbamate moiety were synthesised and tested as inhibitors (Mandel *et al.*, 2005).

2.4.5 Amyotrophic lateral sclerosis (ALS)

ALS share common pathological features with most of the neurodegenerative diseases mentioned. These features include oxidative stress, iron accumulation, excitotoxicity, inflammatory processes and the misfolding of toxic proteins that cannot be degraded after ubiquitination (Youdim *et al.*, 2006b). However selegiline is not a successful treatment option for the treatment of ALS, but in mouse models using rasagiline and CGP 3466, both MAO-B inhibitors, effectiveness was shown (Lange, 1998; Sagot, 2000; Waibel *et al.*, 2004).

2.5 METHYLENE BLUE

2.5.1 Biochemistry of methylene blue

Methylene blue (chemical name tetramethylthionone chloride) is a deep blue cation and is metabolised as part of a catalytic redox cycle by nicotinamide adenine dinucleotide phosphate (NADPH) or thioredoxin (Wainwright & Amaral, 2005). Methylene blue presents as a redox couple in equilibrium and forms part of a reversible-oxidation reduction system (Oz *et al.*, 2009). The metabolism of methylene blue results in an uncharged colourless leuco methylene blue (leucoMB) which is spontaneously reoxidised by molecular oxygen to yield methylene blue again (figure 2-12) (Schirmer *et al.*, 2011). Interestingly, methylene blue dissolves in water as well as organic solvents (Wagner *et al.*, 1998). The oxidised form of methylene blue has a maximum absorption at 609 nm and 688 nm while leucoMB does not absorb light in the visible region (Ramsay *et al.*, 2007).

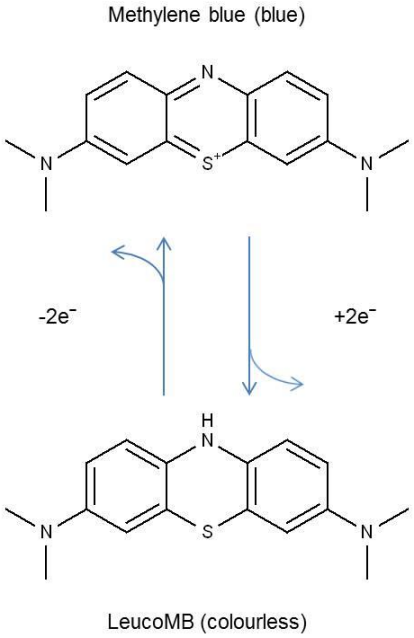


Figure 2-12: The interchangeable forms of methylene blue.

The metabolism of methylene blue results in two metabolites namely azure A and azure B, each with their own pharmacological effects (figure 2-13) (Culo *et al.*, 1991; Warth *et al.*, 2009). Methylene blue can therefore be classified as a prodrug of these N-demethylated metabolites. The oxidised form of azure B can transform into a neutral form which can cross membranes by means of diffusion (Schirmer *et al.*, 2011). This characteristic of azure B is advantageous in comparison to the oxidised form of methylene blue which is unable to diffuse through membranes. Therefore, it can be concluded that the pharmacological effects attributed to methylene blue may, at least in part, be a result of the azure B metabolite (Schirmer *et al.*, 2011).

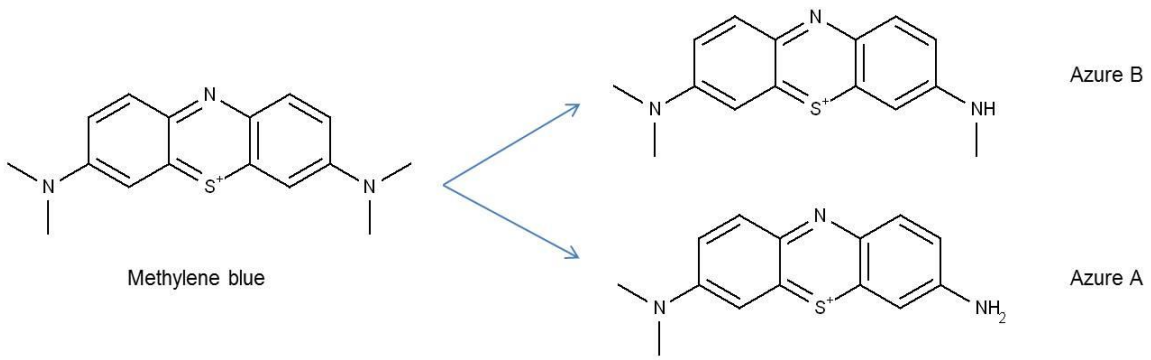


Figure 2-13: The metabolites generated from methylene blue.

A mixture of methylene blue, leucoMB, azure B and azure A (demethylated metabolites) are excreted in the urine resulting in a clear green-blue colour (Gaudette & Lodge, 2005).

Methylene blue exhibits multi-compartmental pharmacokinetics (when administered intravenously) and has a half-life of 10 hours and a bioavailability of 73% (Schirmer *et al.*, 2011).

The organ distribution of methylene blue is determined by the route of administration and differs when oral and intravenous routes are compared. Higher concentrations are found in the intestinal tissue and liver after oral administration while intravenous administration results in higher concentrations in the brain (Peter *et al.*, 2000). After methylene blue is administered to rats, regardless of the route (intravenously, intraperitoneally or intraduodenally), it accumulates in the brain (Jinwal *et al.*, 2009; Peter *et al.*, 2000; Walter-Sack *et al.*, 2009). This would suggest that methylene blue can permeate the blood-brain barrier specifically in rats.

MAO-A, nitric oxide (NO) synthases, methemoglobin, guanylate cyclase, acetylcholinesterase (AChE) and disulphide reductases (including glutathione reductase and dihydrolipoamide dehydrogenase) are targets for methylene blue and azure B (Buchholz *et al.*, 2008; Harvey *et al.*, 2010; Juffermans *et al.*, 2010). Methylene blue is a substrate as well as an inhibitor for flavin-dependent disulphide reductases (Schirmer *et al.*, 2011). The flavoenzyme reduces methylene blue to yield leucoMB that is reoxidised by molecular oxygen to give methylene blue again.

2.5.2 Medicinal uses of methylene blue

In the past methylene blue has been used in the treatment of malaria as well as to identify malaria parasites, *Mycobacterium tuberculosis* and the structural organisation of nerve tissues by means of staining (Barcia, 2007; Fleischer, 2004; Garcia-Lopez *et al.*, 2007). The antidepressant properties of methylene blue were discovered when methylene blue was used to monitor compliance of psychiatric patients by means of the discolouration of urine samples (Harvey *et al.*, 2010). As a result chlorpromazine and tricyclic antidepressants were designed based on the structure of methylene blue (Schirmer *et al.*, 2011).

Current medical indications for methylene blue include inherited and acute methemoglobinemia, ifosfamide-induced neurotoxicity in cancer patients, prevention of urinary tract infections in elderly patients, vasoplegic adrenaline-resistant shock, Alzheimer's disease and paediatric malaria (Cawein *et al.*, 1964; Kupfer *et al.*, 1994; Pelgrims *et al.*, 2000; Warth *et al.*, 2009). Methylene blue is also used to visualise nerves, nerve tissues, endocrine glands and pathologic fistulae (Jinwal *et al.*, 2009; Schirmer *et al.*, 2011). Nitric oxide synthase, as well as soluble guanylate cyclase, are selectively inhibited by methylene blue (Schirmer *et al.*, 2011). This inhibition results in nitric oxide-mediated vasodilation and is used in the treatment of catecholamine-refractory septic shock (Schirmer *et al.*, 2011). A previously mentioned,

methylene blue is also used in the treatment of hereditary methemoglobinemia to prevent the build-up of methemoglobin in red blood cells (Cawein *et al.*, 1964).

Methylene blue is contraindicated in patients taking serotonin reuptake inhibitors and in patients with hereditary glucose-6-phosphate-dehydrogenase deficiency (G6PD) (Ramsay *et al.*, 2007). Studies conducted on rats revealed that methylene blue increases extracellular levels of serotonin regardless of the administration route (local or systematic) (Wegener *et al.*, 2000). Methylene blue inhibits MAO-A thereby preventing the metabolism of serotonin which may lead to increased central serotonin levels. This increases the risk of serotonin toxicity when methylene blue is combined with serotonergic drugs (Oz *et al.*, 2009).

2.5.3 Clinical significance of methylene blue in neurodegenerative diseases

The primary mechanism for the effect of methylene blue in neurodegenerative diseases is yet to be established. For example, the multiple effects of methylene blue in Alzheimer's disease may be attributed to the inhibition and modulation of tau aggregation and Aβ peptides as well as interactions with the mitochondria resulting in a possible antioxidant effect (Schirmer *et al.*, 2011). Cellular and mitochondrial membranes are easily penetrated by methylene blue and result in its accumulation within mitochondria (Hassan & Fridovich, 1979). Methylene blue acts as an electron acceptor and prevents the formation of ROS (Kelner *et al.*, 1988; Salaris *et al.*, 1991).

Methylene blue can be of use in the treatment of Alzheimer's disease by modulation and prevention of the aggregation of tau protein and Aβ peptides, thus reducing the formation of amyloid plaques (Chang *et al.*, 2009; Necula *et al.*, 2007a). Amyloid plaques are made up of soluble monomers, oligomers and fibrillar deposits that form in the extracellular space and results in Aβ aggregation (Oz *et al.*, 2009). Methylene blue inhibits $A\beta_{42}$ oligomerization by increasing $A\beta_{42}$ fibrilization (Necula *et al.*, 2007a; Taniguchi *et al.*, 2005). However, another study reported that methylene blue has the ability to inhibit amyloid β peptide fragment 40 ($A\beta_{40}$) fibrilization at different concentrations (Taniguchi *et al.*, 2005). This effect was also witnessed with certain phenothiazine-class compounds (Necula *et al.*, 2007a; Necula *et al.*, 2007b; Taniguchi *et al.*, 2005). Therefore, further studies regarding the mechanism of oligomerization and fibrillization are necessary as they may be dependent on different cellular processes (Oz *et al.*, 2009). Even though tau accumulation and phosphorylation was not improved in triple-transgenic mouse models of Alzheimer's disease (3xTg-AD) treated with methylene blue, soluble Aβ clearance as well proteasome activity improved (Medina *et al.*, 2011).

Higher temporal an spatial concentrations of hyperphosphorylated tau protein has been associated with increased neuronal cell damage and dementia (Oz *et al.*, 2009). The

hyperphosphorylated tau protein bound in microtubules form paired helical and straight filaments resulting in intracellular neurofibrillary lesions (Wischik *et al.*, 1996). Studies conducted using methylene blue, azure A, azure B and certain phenothiazines showed a decrease in the formation of aggregates consisting of truncated tau proteins (Wischik *et al.*, 1996). Further studies showed that the N-unsubstituted phenothiazine ring of methylene blue prevents tau protein from forming filaments by inhibiting heparin-induced assembly without preventing the interaction of tau protein with microtubules (Taniguchi *et al.*, 2005).

Methylene blue also showed the potential to prevent the aggregation of trans-activator regulatory DNA-binding protein 43 (TDP-43) identified as a key protein in frontotemporal dementias (Yamashita *et al.*, 2009). This modulation by methylene blue is done by inhibiting proteolytic processing and phosphorylation. Azure B is more effective in preventing protein aggregation than methylene blue (Schirmer *et al.*, 2011). Therefore, it can be concluded that the amount of azure B present as an impurity in methylene blue can influence pharmacological data obtained when using methylene blue (Schirmer *et al.*, 2011).

The ability of methylene blue to prevent neurodegeneration with age is based on the interaction of the oxidised and reduced forms of methylene blue with the mitochondria and cytochrome c oxidase (Atamna & Kumar, 2010; Atamna *et al.*, 2008). The activity of cytochrome c oxidase plays a role in ATP production as well as neuronal metabolism, and declines in patients diagnosed with Alzheimer's disease (Pratico, 2008). Low doses of methylene blue improved behaviour and memory in studies conducted on rats with a minimum of side effects (Riha *et al.*, 2005). These positive effects translated into the results seen in doses administered to humans at 300 mg/day or 4 mg/kg (Naylor *et al.*, 1986). Methylene blue improves the function of cytochrome c oxidase found at the terminal of the electron transport chain and thus improves memory (Callaway *et al.*, 2004). Cytochrome c activity is most improved in the areas of the brain where memory consolidation takes place (Gonzalez-Lima & Bruchey, 2004). The effects of improved cytochrome c oxidase functionality lead to increased ATP formation and improved neuronal oxidative ability (Zhang *et al.*, 2006). Methylene blue also improves heme synthesis and delays deterioration due to age (Atamna *et al.*, 2008).

Methylene blue also is a cholinesterase inhibitor and synergistically potentiates the effects of rivastigmine (an AChEI) (Pfaffendorf *et al.*, 1997). The side effects of rivastigmine can be prevented by administering low additional doses of methylene blue with lower doses of rivastigmine (Deiana *et al.*, 2009). Therefore, it can be concluded that methylene blue will interact with other cholinesterase inhibitors used in the treatment of Alzheimer's disease (Schirmer *et al.*, 2011). Methylene blue inhibits both butyrylcholinesterase (BuChE) and AChE.

2.6 INHIBITORS OF MAO-B

2.6.1 Irreversible inhibitors of MAO-B

Selegiline, pargyline, rasagiline and ladostigil are examples of irreversible inhibitors of MAO-B (figure 2-14). As previously mentioned, irreversible MAO-B inhibitors are void of the cheese reaction at low doses and pose little risk of serotonin toxicity.

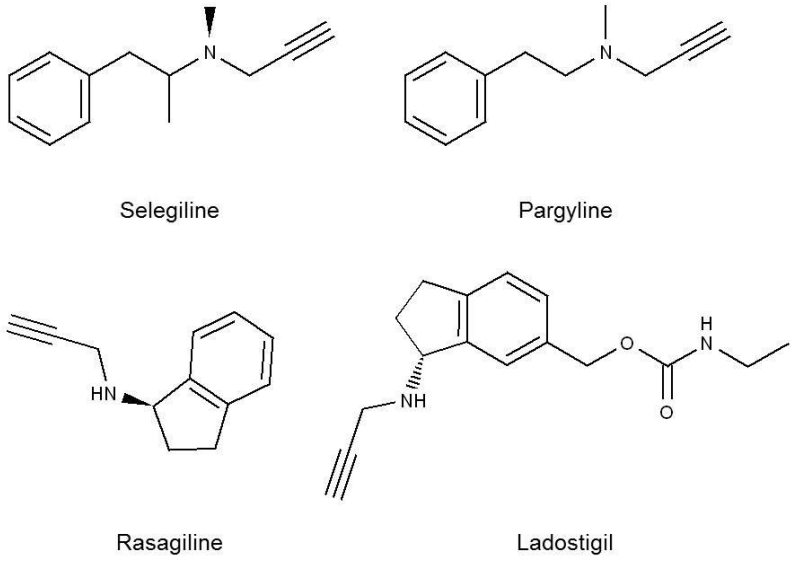


Figure 2-14: Irreversible inhibitors of MAO-B.

2.6.1.1 Selegiline

Selegiline contains the propargylamine group which results in irreversible inhibition of MAO-B (Youdim & Bakhle, 2006). Selectivity of selegiline is achieved at low doses and primarily inhibits the oxidative deamination of dopamine, phenylethylamine and benzylamine. At low doses selegiline does not inhibit the MAO-A-catalysed oxidative deamination of noradrenaline or serotonin. When high doses of selegiline are used selectivity for MAO-B is lost and the inhibition of MAO-A as well as MAO-B may occur (Youdim & Bakhle, 2006).

Selegiline was first considered for the treatment of depression but it was later discovered through experimental findings that selegiline is more effective in the treatment of Parkinson's disease (Birkmayer *et al.*, 1975; Green *et al.*, 1977; Green & Youdim, 1975). Selegiline is particularly effective when used in combination with L-dopa and selectively inhibits MAO-B in the basal ganglia of the brain (Collins *et al.*, 1970; Youdim *et al.*, 1972). Since selegiline does not inhibit MAO-A at low doses, it is devoid of the cheese reaction (Birkmayer *et al.*, 1975). It was shown that, when selegiline is used in combination with L-dopa, the survival rate of the patients improves compared to when only L-dopa is used as treatment (Birkmayer *et al.*, 1985).

An explanation for this can be that selegiline exhibits neuroprotection and may slow dopaminergic neurodegeneration. However, selegiline is considered an amphetamine derivative and selegiline and metabolites of selegiline may exhibit *in vivo* sympathomimetic activity similar to methamphetamine compounds (Blandini, 2005).

Selegiline shows promise for reversing the decline of cellular ageing and increasing neuronal survival (Tipton, 1994). This effect of selegiline may lead to an increase in the duration of the lifespan of patients diagnosed with Parkinson's disease by slowing the progression of dopaminergic neurodegeneration (Birkmayer, 1985). The neuroprotective effects of selegiline have been validated by the discovery of the synthetic dopaminergic proneurotoxin MPTP and its neurotoxic metabolite MPP^+. In this instance selegiline protects against MPTP-induced neurotoxicity in experimental animals by inhibition of the MAO-B-catalysed activation of MPTP to MPP^+ (Youdim *et al.*, 2006b). It may be hypothesised that degeneration of dopaminergic neurons induced by similar neurotoxins can be prevented by selegiline (Tatton & Greenwood, 1991). Selegiline also contributes to neurorescue by preventing the death and degeneration of neurons. This effect reverses damage that would have been lethal to neurons (Youdim *et al.*, 2006b). Selegiline also protects against cell death caused by oxidative stress as well as 6-hydroxydopamine (De La Cruz, 1996; Salonen *et al.*, 1996).

2.6.1.2 Pargyline

Pargyline is an irreversible inhibitor of MAO-B (Youdim *et al.*, 2006b). This inhibitor does not contain a hydrazine group, which overcomes the liver toxicity exhibited by hydrazine containing inhibitors such as iproniazid (Youdim *et al.*, 2006b). Pargyline has been used for the treatment of depression even though it is not devoid of the cheese reaction (Youdim *et al.*, 2006b).

2.6.1.3 Rasagiline

Rasagiline does not contain the amphetamine structure of selegiline and therefore does not exhibit any sympathomimetic activity (Finberg & Youdim, 1985). The aminiondan ring structure results in the existence of two isomers namely the R(+)- and S(-)-enantiomers. The R(+)-enantiomer (known as rasagiline) is a threefold more potent irreversible inhibitor of MAO-B than the S(-)-enantiomer (Youdim *et al.*, 2006b; Youdim *et al.*, 2001).

When rasagiline is administered in combination with L-dopa the levels of dopamine increase in the basal ganglia as shown by microdialysis (Finberg *et al.*, 1998). Rasagiline is also superior to selegiline regarding neuroprotection as showed with neuronal cultures (Mandel *et al.*, 2005). The neuroprotective action of rasagiline can be attributed to the activation of anti-apoptotic pathways through the activation of B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra large (Bcl-XL), as well as downregulation of Bcl-2 associated death promoter (BAD) and Bcl-2

associated X protein (BAX) on the mitochondrial level (Youdim *et al.*, 2005). Further structure-activity relationship (SAR) studies conducted with rasagiline analogues revealed that the propargyl moiety is crucial for neuroprotective activity. This was reinforced by similar neuroprotective activities recorded for the S(-)-enantiomer and the propargyl moiety itself (Mandel *et al.*, 2005).

Rasagiline also shows promise for cardiovascular medicine since this compound exhibits anti-apoptotic properties in cultures of cardiac myocytes (Youdim & Buccafusco, 2005).

2.6.1.4 Ladostigil

Ladostigil is a non-selective MAO inhibitor that increases levels of all three monoamines, noradrenaline, serotonin and dopamine, in the hippocampus and striatum of rats and mice. This compound also exhibits antidepressant activity in animal models (Sagi *et al.*, 2005). Ladostigil may be similar to rasagiline in structure but it does not inhibit MAO-A or MAO-B *in vitro* or acutely *in vivo*. But after chronic treatment of approximately 1-8 weeks it was found that, in the brain, both isoforms are inhibited although inhibition is not observed in the gut or liver. This selective tissue inhibition allows ladostigil to bypass the cheese reaction while irreversible inhibition of all MAO activity in the brain occurs (Mandel *et al.*, 2005; Sagi *et al.*, 2005). The mechanisms resulting in this tissue selectivity as well as the difference between acute and chronic effects have yet to be established (Gal *et al.*, 2005; Youdim & Buccafusco, 2005). It has been postulated that during the metabolism of ladostigil, active metabolites are generated which are responsible for these effects (Youdim & Bakhle, 2006). Studies with cultures of neuronal cells showed that ladostigil exhibits neuroprotective and anti-apoptotic activity (Mandel *et al.*, 2005).

2.6.2 Reversible inhibitors of MAO-B

Lazabemide, isatin and safinamide are examples of reversible inhibitors of MAO-B (figure 2-15). The use of reversible MAO-B inhibitors does not induce the cheese reaction or serotonin toxicity.

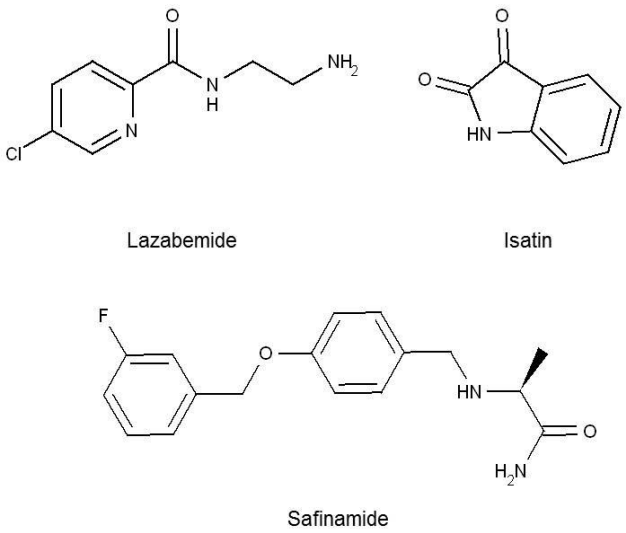


Figure 2-15: Reversible inhibitors of MAO-B.

2.6.2.1 Lazabemide

Lazabemide is classified as a highly selective and potent MAO-B inhibitor. In comparison with selegiline, it is 1000-fold more potent (Berlin, 2002). Lazabemide was also found to prevent dopaminergic neurotoxicity induced by MPTP. Furthermore, lazabemide potentiates the activity of L-dopa. Lazabemide is a fully reversible inhibitor and no inhibition is observed 24 hours after drug treatment has been terminated (Berlin, 2002).

Lazabemide has been shown to prevent oxidative damage by means of the inhibition MAO-B (Mason *et al.*, 2000). Lazabemide surpasses the antioxidant activity of selegiline and Vitamin E (Mason *et al.*, 2000). The antioxidant activity can be explained by the chemical structure of lazabemide. Lazabemide possesses an amino group that can donate a proton that neutralises free radicals. The unpaired electron of the amino group is then stabilised within the resonance structure. This ultimately stops the propagation of the free radicals (Mason *et al.*, 2000). Importantly, lazabemide is devoid of the cheese reaction because it is a reversible MAO inhibitor (Da Prada, 1990).

2.6.2.2 Isatin

Isatin is a heterocyclic reversible MAO-B inhibitor and can be used to synthesise quinolones, and indoles (Priyanka & Pritam, 2015). It is die oxidised product of indigo and bright orange of colour. Isatin and isatin derivatives may act as potential antipsychotics, anticonvulsants as well as anti-anxiety drugs due to their sedative properties (Bhattacharya *et al.*, 1991; Priyanka & Pritam, 2015).

2.6.2.3 Safinamide

Safinamide is a reversible α-aminoamide MAO-B inhibitor that exhibits both dopaminergic and non-dopaminergic properties (Ramirez-Bermudez, 2012). The non-dopaminergic properties include the blockage of sodium and calcium channels (Blair & Dhillon, 2017). Safinamide reduces daily dosages to once daily due to its long half-life (Blair & Dhillon, 2017). Animal studies using safinamide showed that it possesses neuroprotective and neurorescuing properties that may be dependent on the inhibition of MAO-B as well as its non-dopaminergic activity (Dézsi & Vécsei, 2014). Safinamide is used as an adjuvant to L-dopa therapy in Parkinson's disease patients (Blair & Dhillon, 2017).

2.7 INHIBITORS OF MAO-A

2.7.1 Irreversible inhibitors of MAO-A

Clorgyline, tranylcypromine, phenelzine, moclobemide and iproniazid are examples of irreversible inhibitors of MAO-A (figure 2-16). Irreversible MAO-A inhibitors can precipitate the cheese reaction due to the unsuccessful metabolism of tyramine as well as induce serotonin syndrome when used with other serotonergic drugs and should therefore be used with caution.

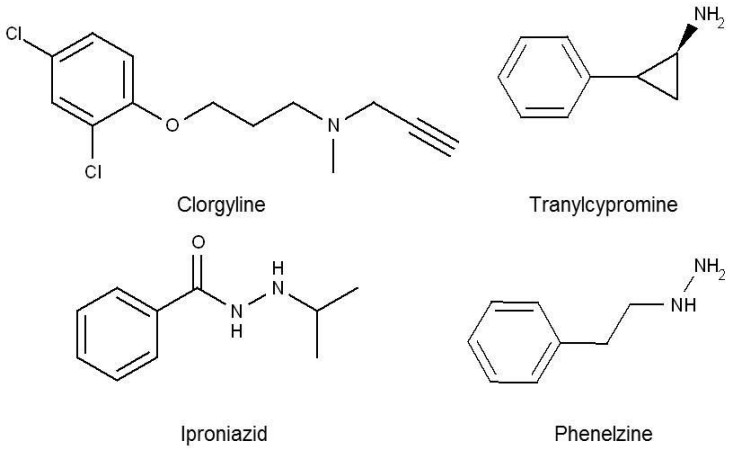


Figure 2-16: Irreversible inhibitors of MAO-A.

2.7.1.1 Clorgyline

Clorgyline also possesses neurorescue and neuroprotective abilities as a result of the propargylamine moiety (De Girolamo *et al.*, 2001; Seymour *et al.*, 2003). Clorgyline is a selective MAO-A inhibitor. Since clorgyline is a potent and irreversible inhibitor of MAO-A, it increases the levels of noradrenaline and serotonin in the brain and is considered as a potential antidepressant (Youdim & Bakhle, 2006). The activity of clorgyline was tested in clinical trials

but it was abandoned as an inhibitor when the cheese reaction was observed (Youdim & Bakhle, 2006).

2.7.1.2 Iproniazid and phenelzine

Iproniazid was initially used to treat tuberculosis but was later used as an antidepressant when its irreversible MAO-A inhibiting properties were discovered (Youdim *et al.*, 2006b). This led to the design of other hydrazine derivatives as potential MAO inhibitors such as phenelzine (Youdim *et al.*, 2006b). However, the hydrazine containing inhibitors are associated with liver toxicity (Youdim & Bakhle, 2006) and also possess side effects such as the cheese reaction which may lead to haemorrhage. These side effects could result in death and lead to the withdrawal of many of the hydrazine-containing MAO inhibitors.

2.7.1.3 Tranylcypromine

Tranylcypromine is an irreversible non-selective MAO inhibitor used as an antidepressant (Youdim *et al.*, 2006b). This inhibitor does not possess the hydrazine group and thus does not lead to liver toxicity. As mentioned previously, hydrazine containing compounds such as iproniazid are associated with liver toxicity (Youdim *et al.*, 2006b). However, since tranylcypromine is an irreversible MAO inhibitor, it is not devoid of the cheese reaction (Youdim *et al.*, 1988).

2.7.2 Reversible inhibitors of MAO-A

Moclobemide, toloxatone and befloxatone are examples of reversible inhibitors of MAO-A (figure 2-17). Reversible MAO-A inhibitors can be used without fear of inducing the cheese reaction. The competitiveness at the binding site allows for the successful metabolism of tyramine peripherally. However, the selectiveness of reversible MAO-A inhibitors can still cause serotonin toxicity and should be used with caution when used with other serotonergic drugs.

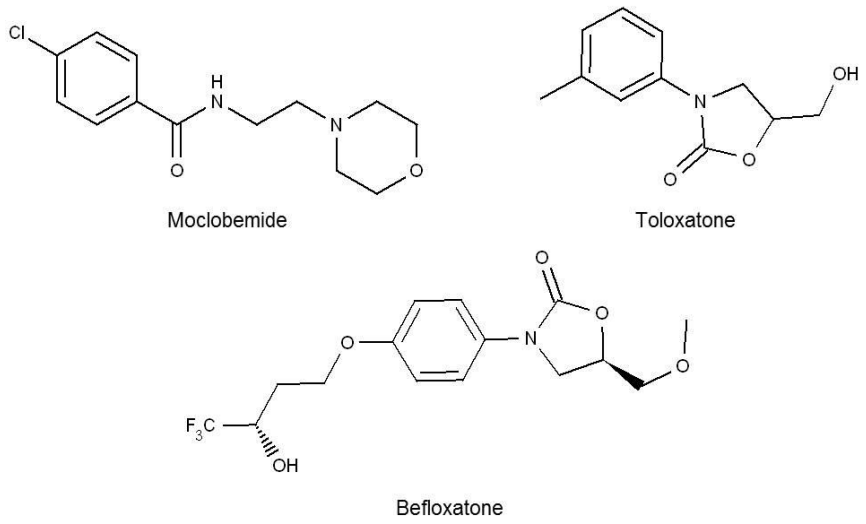


Figure 2-17: Reversible inhibitors of MAO-A.

2.7.2.1 Moclobemide

Moclobemide is an MAO-A inhibitor which does not exhibit the cheese reaction since it is a reversible inhibitor (Da Prada, 1990). Moclobemide is used as a mild antidepressant in older patients and is devoid of major side effects (Bonnet, 2003). Clinical studies conducted with moclobemide as add-on therapy to L-dopa and dopaminergic agonists showed a slight symptomatic improvement in the motor functions of Parkinson's disease patients (Youdim & Weinstock, 2004). It has been proposed that by administering a higher dose, a better clinical response could be achieved (Youdim & Bakhle, 2006).

2.7.2.2 Toloxatone

Toloxatone is a phenyloxazolidinone derivative and a reversible MAO-A inhibitor that is used as a treatment for depression. Due to the reversibility of MAO inhibition by toloxatone, it does not precipitate the cheese reaction and is safe to use even at high doses (Berlin *et al.*, 1989; Bieck *et al.*, 1989; Mann *et al.*, 1984). Toloxatone reaches a peak plasma concentration after 1 hour and is metabolised by the liver and excreted in the urine.

2.7.2.3 Befloxatone

Befloxatone, similar to toloxatone, is also a phenyloxazolidinone derived inhibitor and does not contain an amino functional group. Befloxatone is a reversible MAO-A inhibitor which is devoid of the cheese reaction and is used to treat depression (Caille *et al.*, 1996). The metabolism of befloxatone yields an O-demethylated metabolite that possesses weaker MAO-A inhibition potency compared to befloxatone (Rosenzweig *et al.*, 1998).

2.8 SUMMARY

MAO exists as isoforms with differences in tissue distribution. The physiological effects of MAO activity are extensive due to the various neurotransmitters that act as substrates for MAO. MAO activity thus affects many metabolic pathways. Due to these considerations, MAO is an important drug target. MAO has been implicated in the pathology of diseases such as Parkinson's disease, Alzheimer's disease as well as depression; therefore MAO inhibition may be beneficial in the treatment of these disorders. MAO inhibitors may also act as potential neuroprotective agents by reducing the formation of toxic metabolic by-products associated with MAO metabolism. MAO-A inhibition has been associated with an antidepressant effect whereas MAO-B inhibition is valuable in the treatment of symptoms associated with Parkinson's disease. MAO inhibitors are not free of adverse effects and caution should be exercised to prevent serotonin toxicity as well as the cheese reaction. Dye compounds are of special interest due to the high potency MAO inhibition properties of methylene blue.

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CHAPTER 3 ARTICLE

3.1 THE MONOAMINE OXIDASE INHIBITION PROPERTIES OF SELECTED DYE COMPOUNDS

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3.1.1 Abstract

It was recently discovered that the cotton dye, methylene blue, is a reversible, competitive monoamine oxidase (MAO) A inhibitor. Azure B, the major metabolite of methylene blue, as well as various other structural analogues of methylene blue also retained the ability to inhibit MAO-A. Numerous dye compounds have since been shown to inhibit MAO-A potently and include compounds such as cresyl violet (IC_{50} = 0.0037 µM), Nile blue (IC_{50} = 0.0077 µM) and DMMB (IC_{50} = 0.018 µM). Based on the high potency inhibition of MAO-A reported for these dye compounds, the present study evaluated 22 commercially available dyes, many of which bear a structural resemblance to methylene blue, as potential inhibitors of human MAO-A and MAO-B. The results highlighted three dye compounds as good potency MAO inhibitors: acridine orange, oxazine 170 and Darrow red. For these compounds, the reversibility of MAO inhibition was further investigated by dialysis while the modes of inhibition were determined by the construction of Lineweaver-Burk plots. Acridine orange was found to be a competitive and reversible MAO-A specific inhibitor (IC_{50} = 0.017 µM), whereas oxazine 170 is a competitive and reversible MAO-B specific inhibitor (IC_{50} = 0.006 µM). Darrow red was found to be a competitive and reversible inhibitor of MAO (MAO-A, IC_{50} = 0.059 µM; MAO-B, IC_{50} = 0.065 µM) and displayed no isoform specificity. Acridine orange and Darrow red are more potent inhibitors of MAO-A compared to methylene blue (MAO-A, IC_{50} = 0.07 µM; MAO-B, IC_{50} =4.37 µM), with oxazine 170 and Darrow red also exhibiting higher MAO-B inhibition than methylene blue. It may thus be concluded that active dye compounds such as those described above exhibit similar activity profiles to methylene blue due to the structural similarity with methylene blue. These compounds may be advanced for further testing and preclinical development, or be used as possible lead compounds for the future design of MAO inhibitors. MAO is an important drug target since the MAO isoforms play a key role in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, as well as in neuropsychiatric diseases such as depression. MAO-B inhibitors are currently used for the symptomatic treatment of Parkinson's

disease while MAO-A inhibitors are used as antidepressants. Potent MAO-A inhibitors should be used with caution due to the possibility of inducing serotonin toxicity when combined with serotonergic drugs.

Keywords: methylene blue, acridine orange, oxazine 170, Darrow red, monoamine oxidase, inhibition, serotonin toxicity

Abbreviations: AD, Alzheimer's disease; DMMB, 1,9-dimethyl methylene blue; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; ETC, elthylthioninium chloride; G6PD, glucose-6-phosphate-dehydrogenase deficiency; KCl, potassium chloride; LMP, lysosomal membrane permeability; MAO, monoamine oxidase; NMB, new methylene blue; NR, neutral red; PD, Parkinson's disease; SAR, structure-activity relationship; SD, standard deviation; SSRI, serotonin selective reuptake inhibitor.

3.1.2 Introduction

The medicinal properties of dye compounds have been of interest for many years and some dye compounds have even found application in the treatment of certain diseases in humans. One of these dye compounds is methylene blue. Methylene blue was first employed in 1876 as a synthetic dye for colouring cotton (Oz *et al.*, 2009; Schirmer *et al.*, 2011). In 1891, methylene blue was used medicinally for the first time as an antimalarial agent (Guttmann & Ehrlich, 1960). Methylene blue also exhibits antibacterial properties and may be used for the treatment of tuberculosis (Wainwright & Crossley, 2002). Its main indication, however, is for the treatment of methemoglobinemia as well as ifosfamide-induced encephalopathy associated with the neurotoxicity of ifosfamide in cancer patients (Cawein *et al.*, 1964; Kupfer *et al.*, 1994; Pelgrims *et al.*, 2000). Methylene blue is also of use as a preventative measure for urinary tract infections in elderly patients as well as vasoplegic adrenaline-resistant shock (Schirmer *et al.*, 2011; Warth *et al.*, 2009). Other uses of methylene blue include the staining of various structures such as malaria parasites, *Mycobacterium tuberculosis*, nerve tissues, endocrine glands as well as pathologic fistulae (Barcia, 2007; Fleischer, 2004; Garcia-Lopez *et al.*, 2007).

Of interest to this study is the observation that methylene blue exhibits inhibition of the monoamine oxidases, MAO-A and MAO-B. *In vitro* studies conducted with methylene blue showed that this dye inhibits MAO-A with an IC_{50} value of 0.07 µM (Harvey *et al.*, 2010; Ramsay *et al.*, 2007) while an IC_{50} value of 4.37 µM was recorded for the inhibition of MAO-B (Harvey *et al.*, 2010). These results show that methylene blue is a more potent inhibitor of MAO-A than MAO-B. Since MAO-A inhibition is a key mechanism of action of many antidepressant agents, the inhibition of MAO-A by methylene blue may explain, at least in part, its antidepressant effect in animals as well as in clinical trials with humans (Harvey *et al.*, 2010). Methylene blue may

also be of importance in the treatment of neurodegenerative disorders such as Parkinson's disease (PD) as well as Alzheimer's disease (AD) since MAO inhibitors are reported to prevent or reduce neuronal death in neurodegenerative disorders (Wischik *et al.*, 2008).

One of the side-effects of methylene blue is the discolouration of urine. This side-effect was used to monitor the compliance of psychiatric patients with medication regimens and led to the discovery of the antidepressant properties of methylene blue (Ehrlich & Leppmann, 1890; Harvey *et al.*, 2010). Further studies of methylene blue showed that it inhibits nitric oxide synthase, soluble guanylate cyclase as well as MAO (Aeschlimann *et al.*, 1996; Harvey *et al.*, 2010; Ramsay *et al.*, 2007; Schirmer *et al.*, 2011). These pharmacological actions of methylene blue may all be associated with an antidepressant effect (Brand *et al.*, 2015; Harvey *et al.*, 2010). As mentioned above, pre-clinical studies and clinical trials have confirmed the antidepressant properties of methylene blue and have also suggested an advantageous anxiolytic effect (Alda *et al.*, 2011; Eroglu & Çaglayan, 1997; Harvey *et al.*, 2010; Narsapur & Naylor, 1983; Naylor *et al.*, 1986; Naylor *et al.*, 1987). This resulted in the use of methylene blue as a lead compound for the design of several tricyclic antidepressants as well as chlorpromazine (Schirmer *et al.*, 2011).

An important adverse effect observed during the use of methylene blue is linked to an increase of extracellular serotonin levels in the brain (Wegener *et al.*, 2000). Increased serotonin levels due to MAO-A inhibition may lead to serotonin toxicity in patients also using serotonergic drugs such as serotonin reuptake inhibitors (SSRIs) (Gillman, 2006a; Gillman, 2006b; Isbister *et al.*, 2003; Oz *et al.*, 2009; Ramsay *et al.*, 2007; Stanford *et al.*, 2009). Therefore, the simultaneous use of methylene blue and serotonergic drugs is contraindicated. Methylene blue is also contraindicated in patients diagnosed with hereditary glucose-6-phosphate dehydrogenase deficiency (G6PD) (Ramsay *et al.*, 2007).

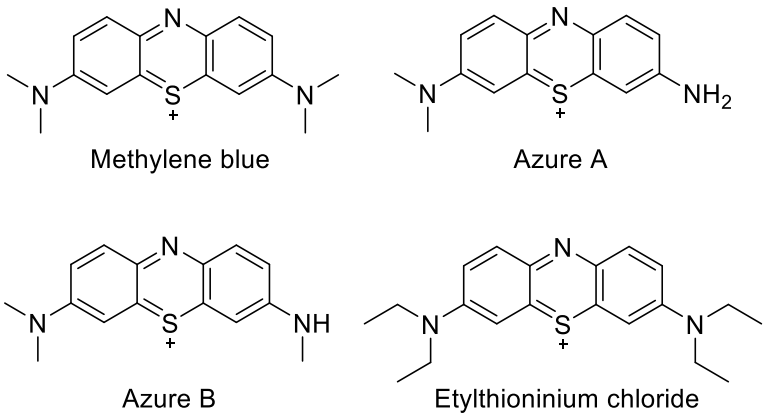


Figure 3-1: The structures of methylene blue, azure A and azure B and elthylthioninium chloride (ETC).

Methylene blue may also be considered to be a prodrug, yielding several N-demethylated metabolites *in vivo*. The principal metabolites of methylene blue are azure A and azure B, which exhibit similar pharmacological activities compared to methylene blue (figure 3-1). Among these is the ability to inhibit the MAO enzymes (Culo *et al.*, 1991; Warth *et al.*, 2009). Azure B is characterised as a potent reversible MAO-A inhibitor (IC_{50} = 0.01 µM) as well as an MAO-B inhibitor (IC_{50} = 0.97 µM) (Petzer *et al.*, 2012). A structural analogue of methylene blue namely elthylthioninium chloride (ETC) also exhibits MAO-A (IC_{50} = 0.51 µM) and MAO-B (IC_{50} = 0.59 µM) inhibition activities (Delport *et al.*, 2014). The antidepressant properties of azure B and ETC were evaluated and confirmed by studies conducted with animal models (Delport *et al.*, 2014).

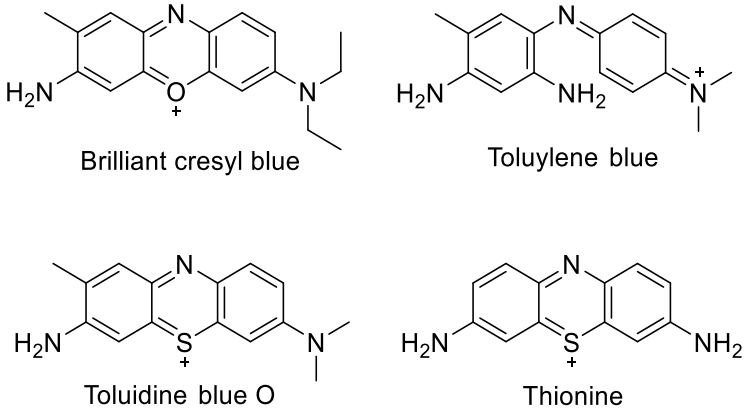


Figure 3-2: The structures of brilliant cresyl blue, toluylene blue, toluidine blue O and thionine.

Brilliant cresyl blue, toluylene blue, toluidine blue O and thionine are similar in structure to methylene blue and are also classified as redox dyes (figure 3-2). These compounds have been evaluated as MAO inhibitors, and similar to methylene blue exhibit reversible MAO inhibition with a higher potency for MAO-A (Oxenkrug *et al.*, 2007). The success of methylene blue as a dye compound led to the discovery and use of other synthetic dyes such as Nile blue and Nile red in 1896 (Möhlau & Uhlmann, 1896). These dye compounds as well as others that share similar structures to methylene blue have been evaluated as potential MAO inhibitors in recent studies. Compounds such as 1,9-dimethyl methylene blue (DMMB), new methylene blue (NMB), Nile blue, neutral red (NR) as well as cresyl violet have thus been studied (figure 3-3). From these studies it was found that DMMB (IC_{50} = 0.018 µM), Nile blue (IC_{50} = 0.0077 µM) and cresyl violet (IC_{50} = 0.0037 µM) are more potent inhibitors of MAO-A than methylene blue (IC_{50} = 0.07 µM) (Delport *et al.*, 2017). It was also found that Nile blue (IC_{50} = 0.012 µM) is a high potency MAO-B inhibitor compared to methylene blue (IC_{50} = 4.37 µM) (Delport *et al.*, 2017).

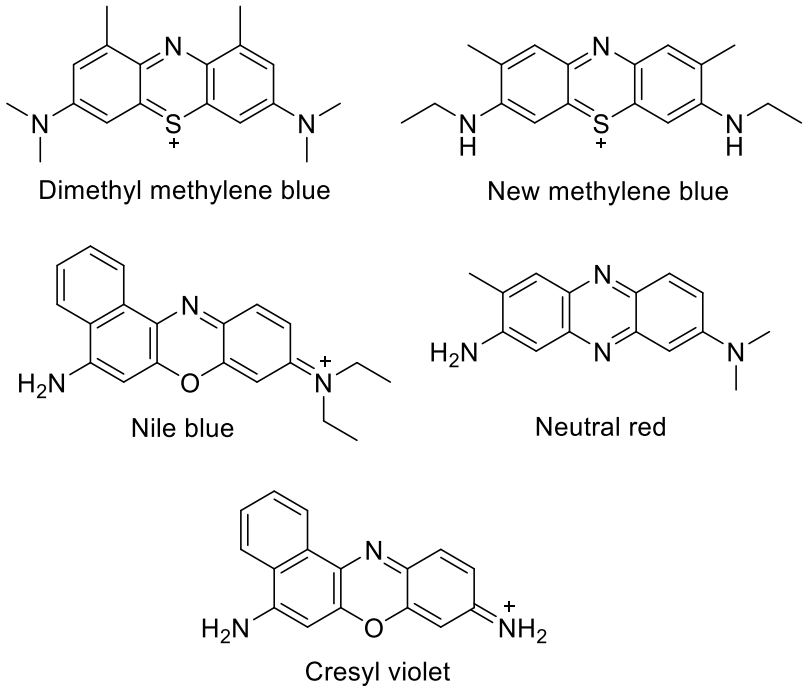


Figure 3-3: The structures of 1,9-dimethyl methylene blue (DMMB), new methylene blue (NMB), Nile blue, neutral red (NR) and cresyl violet.

Other studies with structural analogues of methylene blue investigated the MAO inhibition properties of tacrine, acriflavine, thionine, methylene green as well as methylene violet (figure 3-4). These studies showed that methylene green (IC_{50} = 0.25 µM) and acriflavine (IC_{50} = 0.43 µM) are potent MAO-A inhibitors (Harvey *et al.*, 2010). Further studies conducted with animal models confirmed the antidepressant properties of methylene green (Harvey *et al.*, 2010).

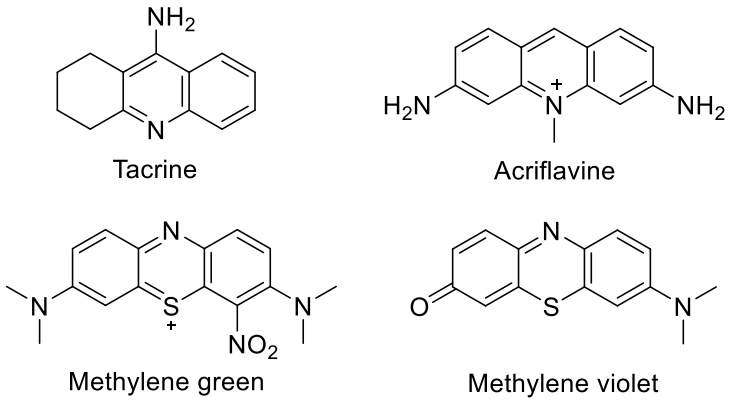
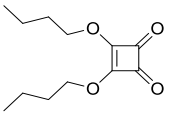
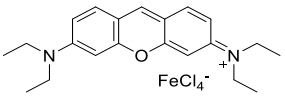
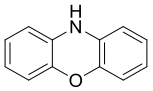
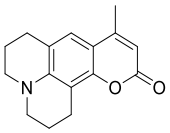
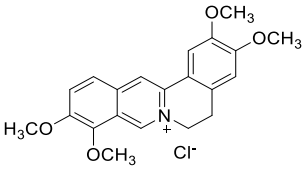
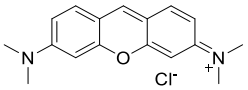
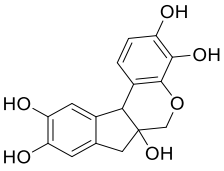
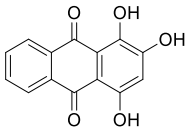
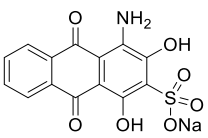
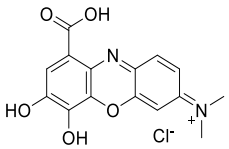
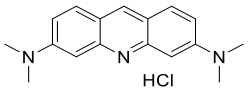
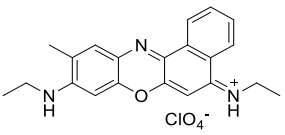
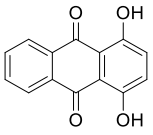
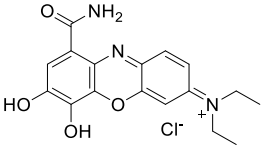


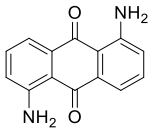
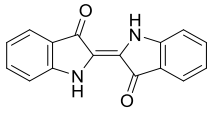
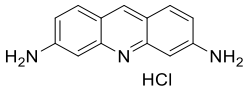
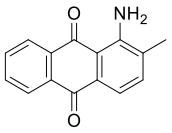
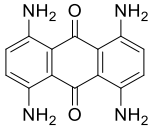
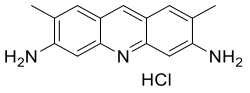
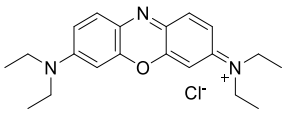
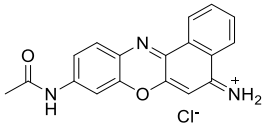
Figure 3-4: The structures of tacrine, acriflavine, methylene green and methylene violet.

Based on the MAO inhibition exhibited by several dye compounds and the therapeutic applications associated with MAO inhibition, this study attempts to identify other dye compounds

that may act as potent MAO inhibitors. Such dye compounds may find future use as lead compounds for the design of therapeutic agents for neurodegenerative and neuropsychiatric disorders or may be developed as drugs for the treatment of depression, Alzheimer's disease and Parkinson's disease. Additional analogues of methylene blue with, for the most part, similar structures were selected from commercially available dye compounds and evaluated as *in vitro* inhibitors of the human MAO enzymes. The structures that were selected are shown in table 3-1 and are: 3,4-dibutoxy-3-cyclobutene-1,2-dione, phenoxazine, palmatine, hematoxylin, nuclear fast red, acridine orange, 1,4-dihydroxyanthraquinone, 1,5-diaminoanthraquinone, 3,6-diaminoacridine, disperse blue 1, basic blue 3, pyronin B, coumarin 102, pyronin Y, purpurin, gallocyanine, oxazine 170, Celestine blue, indigo, disperse orange 11, acridine yellow G and Darrow red.

Table 3-1: Structures of dye compounds considered as potential inhibitors of human MAO-A and MAO-B in this study. The salt forms and molecular weights of each dye are also given.

3,4-Dibutoxy-3-cyclobutene-1,2-dione (MW = 226.27)		Pyronin B (MW = 521.11)	
Phenoxazine (MW = 183.21)		Coumarin 102 (MW = 255.31)	
Palmatine chloride hydrate (MW = 387.86)		Pyronin Y (MW = 302.80)	
Hematoxylin (MW = 302.28)		Purpurin (MW = 256.21)	
Nuclear fast red (MW = 357.27)		Gallocyanine (MW = 336.73)	
Acridine orange hydrochloride hydrate (MW = 301.81)		Oxazine 170 perchlorate (MW = 431.87)	
1,4-Dihydroxyanthraquinone (MW = 240.21)		Celestine blue (MW = 363.80)	

1,5-Diaminoanthraquinone (MW = 238.24)		Indigo (MW = 262.26)	
3,6-Diaminoacridine hydrochloride (MW = 245.71)		Disperse orange 11 (MW = 237.25)	
Disperse Blue 1 (MW = 268.27)		Acridine yellow G (MW = 273.76)	
Basic blue 3 (MW = 359.89)		Darrow red (MW = 339.78)	

Most of the selected dye compounds are similar in structure to methylene blue as they are also planar, aromatic and possess possible redox chemistry. Commercial availability also influenced the selection process. This study will not only discover new lead compounds for MAO inhibition but will also increase the understanding of the structure-activity relationships (SARs) for MAO inhibition by methylene blue analogues. Furthermore, based on their abilities to inhibit MAO-A, this study will also discuss the possibility that some of these dye compounds may possess a risk for serotonin toxicity when co-administered with serotonergic drugs.

3.1.3 Results

3.1.3.1 IC_{50} values of MAO inhibition

The 22 dye compounds were evaluated to determine their IC_{50} values for the inhibition of recombinant human MAO-A and MAO-B. The non-specific MAO substrate, kynuramine, was used as substrate for the studies with both MAO-A and MAO-B. Kynuramine is metabolised by the MAOs to yield the intermediate iminium which is converted to the corresponding aldehyde intermediate [3-(2-aminophenyl)-3-oxo-propionaldehyde] after hydrolysis (figure 3-5). After closing of the ring, 4-hydroxyquinoline is obtained, a compound which fluoresces in basic media.

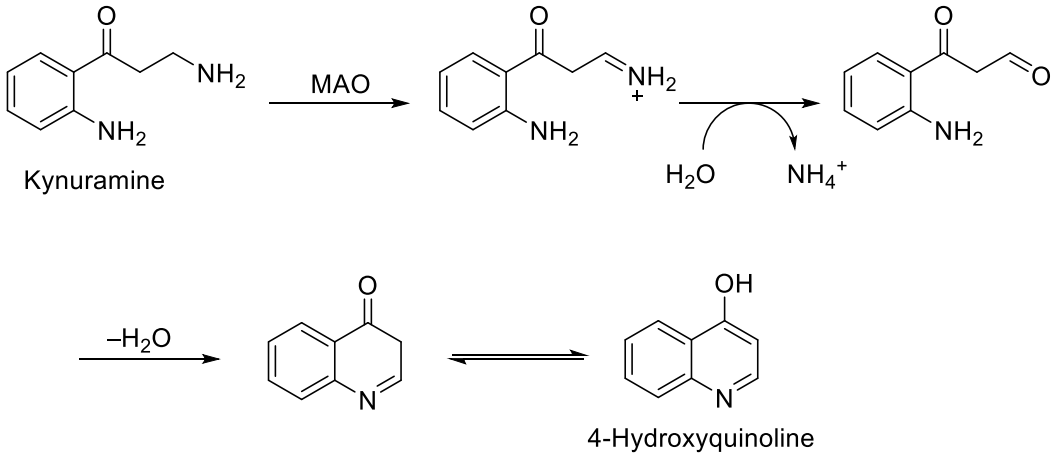


Figure 3-5: The oxidation of kynuramine by MAO to ultimately yield 4-hydroxyquinoline.

By measuring the fluorescence of 4-hydroxyquinoline after the enzyme reactions have been terminated at the endpoint (after 20 minutes) with sodium hydroxide (NaOH), the formation of 4-hydroxyquinoline was quantitated and the rate of MAO catalysis calculated. The catalytic rates of MAO-A and MAO-B were measured in the absence and presence (0.003–100 µM) of the dye compounds and the rate data was fitted to the one site competition model incorporated into the Prism 5 software package (GraphPad). IC_{50} values were estimated from the resulting sigmoidal curves of catalytic rate versus the logarithm of inhibitor concentration (log[I]). Examples of sigmoidal curves for the inhibition of MAO by Darrow red, acridine orange, oxazine 170 and phenoxazine are shown in figure 3-6 (MAO-A inhibition) and figure 3-7 (MAO-B inhibition).

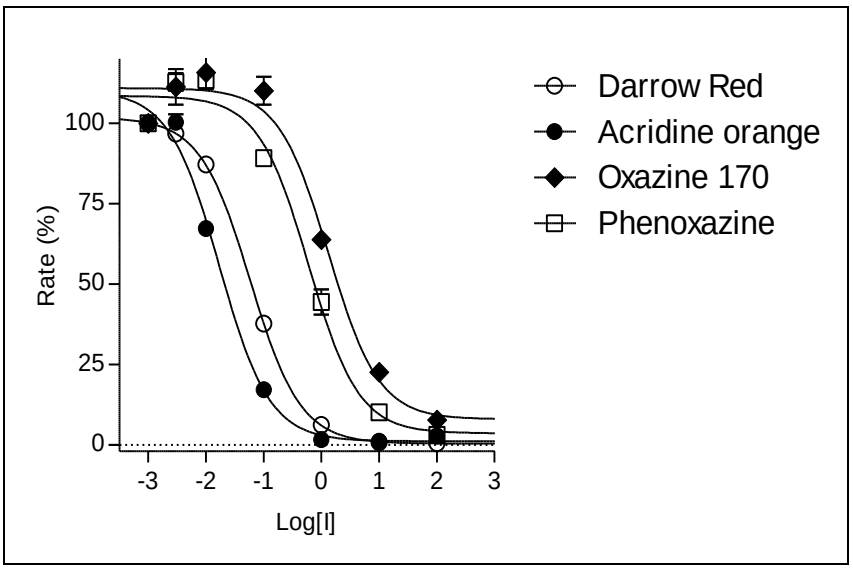


Figure 3-6: Sigmoidal curves of MAO-A catalytic rate versus the logarithm of inhibitor concentration for the inhibition of MAO-A by selected dye compounds.

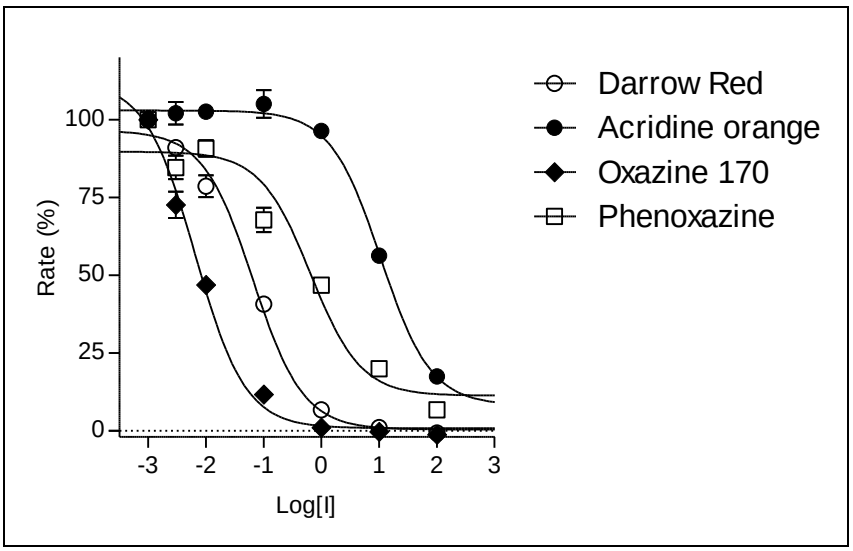


Figure 3-7: Sigmoidal curves of MAO-B catalytic rate versus the logarithm of inhibitor concentration for the inhibition of MAO-B by selected dye compounds.

The IC_{50} values of the dye compounds are summarised in table 3-2 and given as the mean ± standard deviation (SD) of triplicate determinations.

Table 3-2: The IC_{50} values of inhibition of recombinant human MAO by selected dye compounds

Compound	MAO-A IC_{50} (µM) ± SD	MAO-B IC_{50} (µM) ± SD	SI
3,4-Dibutoxy-3-cyclobutene-1,2-dione	>100	43.2 ± 3.12	<0.43
Phenoxazine	0.625 ± 0.104	0.819 ± 0.021	1.3
Palmatine	44.4 ± 5.11	34.6 ± 5.70	0.78
Hematoxylin	56.8 ± 5.48	65.6 ± 9.45	1.2
Nuclear fast red	30.2 ± 2.01	48.3 ± 2.55	1.6
Acridine orange	0.017 ± 0.001	12.4 ± 1.60	729
1,4-Dihydroxyanthraquinone	0.887 ± 0.102	>100	>113
1,5-Diaminoanthraquinone	0.585 ± 0.084	>100	>171
3,6-Diaminoacridine	0.507 ± 0.035	32.5 ± 4.69	64
Disperse blue 1	7.58 ± 0.287	>100	>13
Basic blue 3	7.45 ± 0.476	3.51 ± 0.682	0.47
Pyronin B	4.04 ± 0.225	11.2 ± 1.17	2.8
Coumarin 102	10.1 ± 1.15	>100	>9.9
Pyronin Y	0.293 ± 0.038	12.2 ± 0.245	42
Purpurin	7.99 ± 0.759	40.2 ± 5.42	5.0
Gallocyanine	46.56 ± 2.97	57.6 ± 9.06	1.2
Oxazine 170	1.45 ± 0.013	0.0065 ± 0.0005	0.0045
Celestine blue	23.1 ± 1.44	29.9 ± 4.19	1.3

Indigo	>100	>100	–
Disperse orange 11	0.450 ± 0.001	>100	>222
Acridine yellow G	0.350 ± 0.031	11.9 ± 1.52	34
Darrow red	0.059 ± 0.003	0.065 ± 0.004	1.1

Based on the IC_{50} values, the selectivity index (SI) for each dye compound may be calculated. This value provides an indication of the specificity of inhibition of the MAO-A isoform. Among the dye compounds evaluated, nine compounds inhibited MAO-A with IC_{50} values < 1 µM, while only three compounds exhibited submicromolar IC_{50} values for MAO-B. Based on the SI values, acridine orange, 3,6-diaminoacridine, coumarin 102, pyronin Y, purpurin and acridine yellow G may be viewed as specific inhibitors of the MAO-A isoform with SI > 5. No inhibition of MAO-B at a maximal tested concentration of 100 µM was observed for 1,4-dihydroxyanthraquinone, 1,5-diaminoanthraquinone, disperse blue 1 and disperse orange 11, which shows that these compounds are highly specific inhibitors of MAO-A. Inhibitors that are more specific for the MAO-B isoform included palmatine, basic blue 3 and oxazine 170, with the latter exhibiting the highest degree of specificity. 3,4-Dibutoxy-3-cyclobutene-1,2-dione, palmatine, hematoxylin, nuclear fast red, gallocyanine, Celestine blue and indigo are weak inhibitors of both MAO-A and MAO-B with IC_{50} values > 23.1 µM. These compounds thus show little potential as MAO inhibitors. Phenoxazine and Darrow red showed potential as inhibitors of both MAO-A and MAO-B and can be classified as nonspecific inhibitors.

As shown by the IC_{50} values, this study discovered high potency inhibitors of both MAO-A and MAO-B. The most potent MAO-A inhibitors were acridine orange and Darrow red with IC_{50} values of 0.017 µM and 0.059 µM, respectively. These values are within the same range as that reported for methylene blue (IC_{50} = 0.07 µM). The most potent MAO-B inhibitors of this study were oxazine 170 and Darrow red with IC_{50} values of 0.0065 µM and 0.065 µM, respectively. These compounds are approximately two orders of magnitude more potent MAO-B inhibitors compared to methylene blue (IC_{50} = 4.37 µM). Based on their high potency MAO inhibition, acridine orange, oxazine 170 and Darrow red were selected for further evaluation regarding reversibility and mode of inhibition.

3.1.3.2 Reversibility of inhibition of MAO

The reversibility of MAO inhibition by acridine orange, Darrow red and oxazine 170 was investigated. Each of the dye compounds (at a concentration of 4 × IC_{50}) was combined with the MAO enzymes and pre-incubated for a period of 15 minutes. After the incubation was completed, the mixtures were dialysed for 24 hours as described in the experimental section. After the dialysis was completed, the residual MAO catalytic activities were measured and compared to the negative control value (100%), which is the MAO activity recorded after dialysis

of incubations containing no inhibitor. As a positive control, MAO was pre-incubated and dialysed in the presence of the irreversible MAO-A and MAO-B inhibitors, pargyline and selegiline, respectively. For irreversible inhibitors, dialysis is not expected to restore enzyme activity, while for reversible inhibitors, enzyme activity will be recovered, at least partially by dialysis.

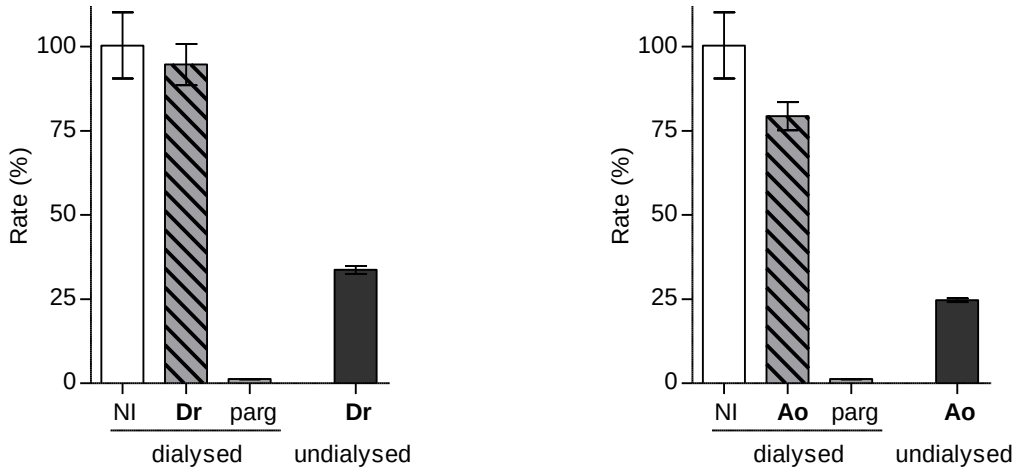


Figure 3-8: Reversibility of MAO-A inhibition by Darrow red and acridine orange. MAO-A was pre-incubated in the presence of the inhibitors (at 4 × IC_{50}) for 15 minutes, dialysed for 24 hours and the residual enzyme activity was measured (Dr/Ao – dialysed). Similarly, MAO-A was pre-incubated and dialysed in the absence of the inhibitor (NI – dialysed) and in the presence of the irreversible MAO-A inhibitor, pargyline (parg – dialysed). The residual activities of undialysed mixtures of MAO-A and Darrow red or acridine orange (Dr/Ao – undialysed) were also measured for comparison.

As can be seen in figure 3-8, after incubation with Darrow red and acridine orange, MAO-A catalytic activity is almost fully restored by dialysis compared to the negative control (100%). The residual activities are restored by dialysis to 95% and 79% for the incubations containing Darrow red and acridine orange, respectively. The residual activity of the undialysed mixture containing Darrow red and MAO-A is 34%, which is significantly lower than the negative control value. The residual catalytic activity of the undialysed mixture containing acridine orange and MAO-A (25%) was also significantly lower when compared to the negative control. After incubation of MAO-A with the irreversible MAO-A inhibitor, pargyline, dialysis did not restore catalytic activity with the residual activity at 1.2%. Based on the observation that MAO-A inhibition by both Darrow red and acridine orange is reversed by dialysis, it may be concluded that these inhibitors act reversibly.

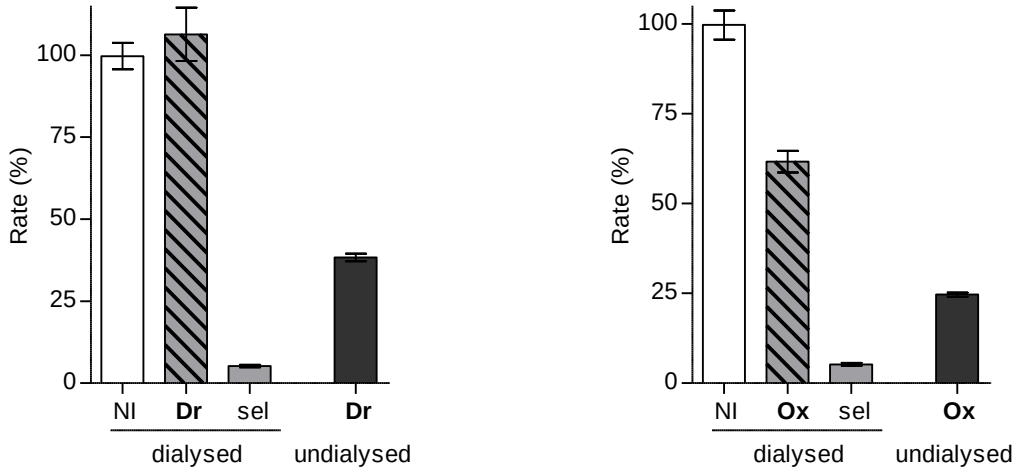


Figure 3-9: Reversibility of MAO-B inhibition by Darrow red and oxazine 170. MAO-B was pre-incubated in the presence of the inhibitors (at 4 × IC_{50}) for 15 minutes, dialysed for 24 hours and the residual enzyme activity was measured (Dr/Ox – dialysed). Similarly, MAO-B was pre-incubated and dialysed in the absence of the inhibitor (NI – dialysed) and in the presence of the irreversible MAO-B inhibitor, selegiline (sel – dialysed). The residual activities of undialysed mixtures of MAO-B and Darrow red or oxazine 170 (Dr/Ox – undialysed) were also measured for comparison.

To evaluate the reversibility of inhibition of MAO-B by oxazine 170 and Darrow red, similar dialysis studies were carried out (figure 3-9). After the dialysis of mixtures containing oxazine 170 and MAO-B, the enzyme activity was partially recovered to 62% of the negative control value (100%). For mixtures containing Darrow red and MAO-B, complete recovery of catalytic activity was observed (106%). In undialysed mixtures of MAO-B and oxazine 170 as well as undialysed mixtures of MAO-B and Darrow red, inhibition persists with the enzyme activities at 25% and 38%, respectively. The irreversible inhibitor, selegiline, was included as a positive control in this study. After dialysis of mixtures containing MAO-B and selegiline, enzyme activity is not recovered with the residual activity at 5%. Based on the observation that MAO-B inhibition by both oxazine 170 and Darrow red is reversed by dialysis, it may be concluded that these inhibitors act reversibly.

3.1.3.3 Mode of inhibition of MAO

To investigate the mode of inhibition (e.g. competitive inhibition) of MAO, sets of Lineweaver-Burk plots for the inhibition of the MAOs by acridine orange, Darrow red and oxazine 170 were constructed. Each set consisted of six lines which were constructed in the presence of six

different inhibitor concentrations (0 µM, ¼ × IC_{50}, ½ × IC_{50}, ¾ × IC_{50}, 1 × IC_{50} and 1¼ × IC_{50}). For each line, kynuramine at eight different concentrations (15–250 µM) was used as substrate.

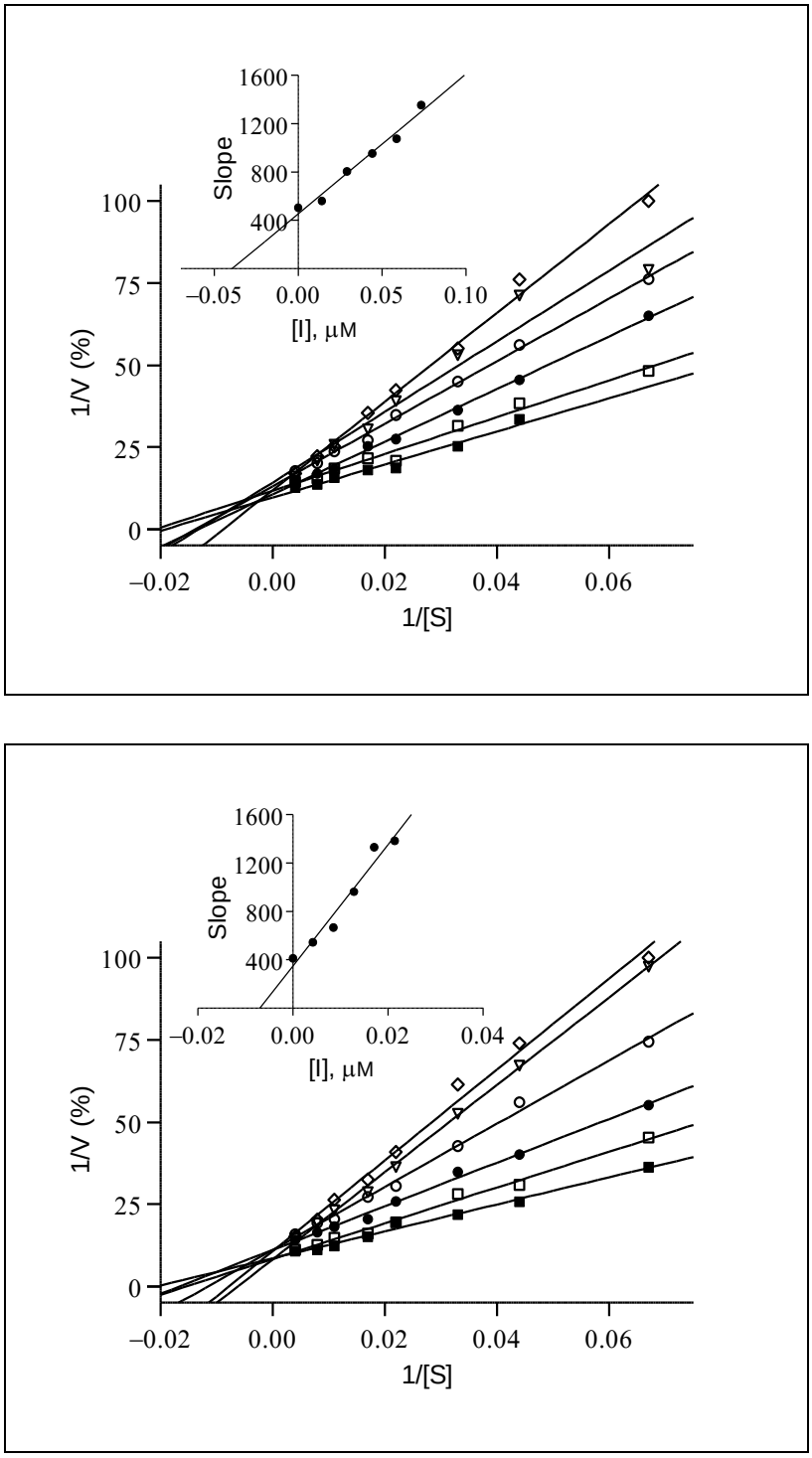
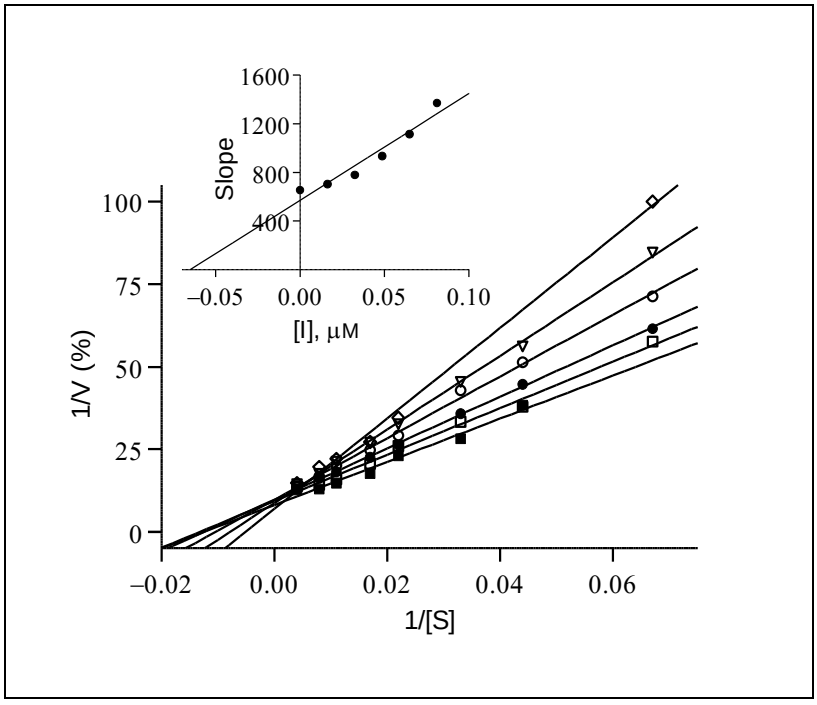


Figure 3-10: Lineweaver-Burke plots of MAO-A catalytic activities in the absence (filled squares) and presence of various concentrations of Darrow red (top, K_i = 0.039 µM) and acridine orange (bottom, K_i = 0.0069 µM). Inhibitor concentrations used were equal to ¼ × IC_{50}, ½ × IC_{50}, ¾ × IC_{50}, 1 × IC_{50} and 1¼ × IC_{50}. The inserts are plots of the slopes of the Lineweaver-Burk plots

versus inhibitor concentration. From these replots, the K_i values were estimated.

The Lineweaver-Burke plots constructed for the inhibition of MAO-A by acridine orange and Darrow red were linear and intersected on the y-axis (figure 3-10). This suggests that these inhibitors act as competitive MAO-A inhibitors. Lineweaver-Burk plots of competitive inhibitors converge and intersect on the y-axis whereas noncompetitive inhibitors intersect on the x-axis. Acridine orange exhibited a K_i value of 0.0069 µM and Darrow red exhibited a K_i value of 0.039 µM. The K_i value is indicative of the strength of the binding of an inhibitor to the enzyme, with low K_i indicating high binding affinity.



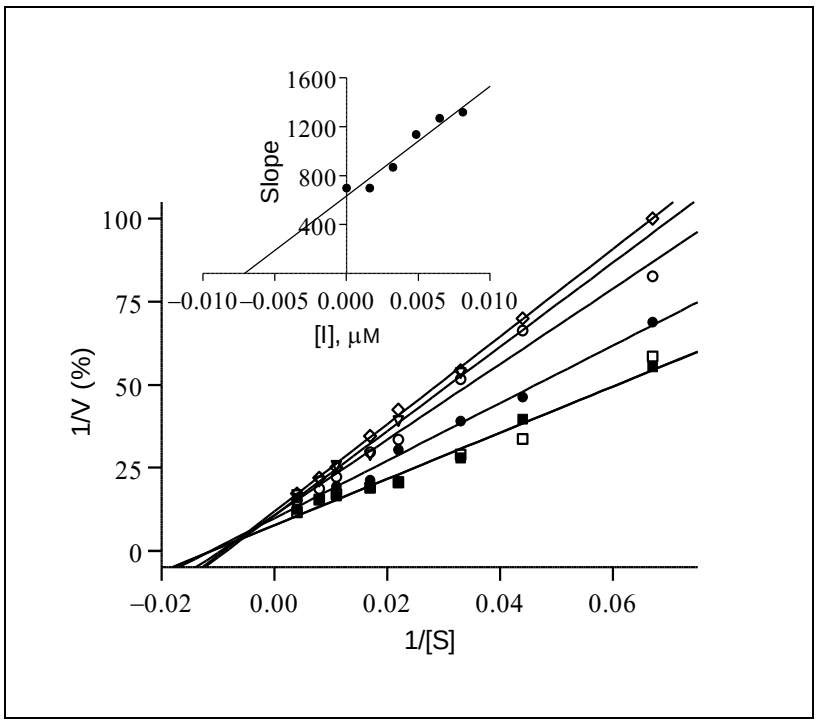


Figure 3-11: Lineweaver-Burke plots of MAO-B catalytic activities in the absence (filled squares) and presence of various concentrations of Darrow red (top, K_i = 0.065 µM) and oxazine 170 (bottom, K_i = 0.007 µM). Inhibitor concentrations used were equal to ¼ × IC_{50}, ½ × IC_{50}, ¾ × IC_{50}, 1 × IC_{50} and 1¼ × IC_{50}. The inserts are plots of the slopes of the Lineweaver-Burk plots versus inhibitor concentration. From these replots, the K_i values were estimated.

Lineweaver-Burke plots were also constructed for the inhibition of MAO-B by oxazine 170 and Darrow red (figure 3-11). For both inhibitors, the plots were linear and intersected on the y-axis. It may thus be concluded that oxazine 170 and Darrow red are competitive inhibitors of MAO-B with K_i values of 0.007 µM and 0.065 µM, respectively.

3.1.3.4 Molecular modelling

To investigate possible binding orientations and interactions of Darrow red and methylene blue in MAO-A and MAO-B, molecular docking experiments were carried out. Darrow red was selected for this study since it is a potent inhibitor of MAO-A and MAO-B, while methylene blue was selected since it is the lead compound for this study. Molecular docking was carried out with the Discovery Studio 3.1 modelling software (Accelrys). The X-ray crystal structures of human MAO-A co-crystallised with harmine (PDB entry: 2Z5X) (Son *et al.*, 2008) and human MAO-B co-crystallised with safinamide (PDB entry: 2V5Z) (Binda *et al.*, 2007) were selected and molecular docking was carried out according to the reported protocol with the CDOCKER application of the Discovery Studio (Mostert *et al.*, 2015). The protein models were prepared by first calculating of the pKa values and protonation states of the amino acid residues, which were

followed by an energy minimisation with the protein backbone, constrained. The structures of the ligands were drawn in Discovery Studio, and after docking with CDOCKER, the docking orientations were refined using *in situ* ligand minimisation. For each ligand, the highest ranked orientation was selected among the ten solutions generated.

The results show that Darrow red binds to MAO-A with the acetamide moiety directed towards the entrance of the MAO-active site and the exocyclic amine binding in proximity to the FAD (figure 3-12). Darrow red forms an extensive network of hydrogen bonding and Pi-interactions with MAO-A (figure 3-13). The exocyclic amine is hydrogen bonded to Asn-181, Tyr-444 and a water molecule, while the acetamide carbonyl oxygen is hydrogen bonded to Val-210. The aromatic rings of Darrow red undergo a variety of Pi-interactions with residues including Cys-323, Phe-208, Ile-335, Ile-180 and Tyr-407. Methylene blue forms less hydrogen bond interactions with MAO-A, while also undergoing a variety of Pi-interactions with Cys-323, Phe-208, Ile-335, Gln-215 and Leu-337 (figure 3-13). Interestingly, methylene blue undergoes Pi-interactions with the FAD. These extensive interactions may explain the high potency MAO-A inhibition of Darrow red and methylene blue, with hydrogen bonding (in addition to Pi-interactions) possibly contributing significantly to the stabilisation of Darrow red while Pi-interactions are mostly involved in the binding of methylene blue. It is noteworthy that the sulphur atom of methylene blue interacts directly with the amidic N of Gln-215 (figure 3-12), while Phe-208 form T-shaped Pi-interactions with both inhibitors. This may be significant since Phe-208 is replaced by an isoleucine residue (Ile-199) in MAO-B.

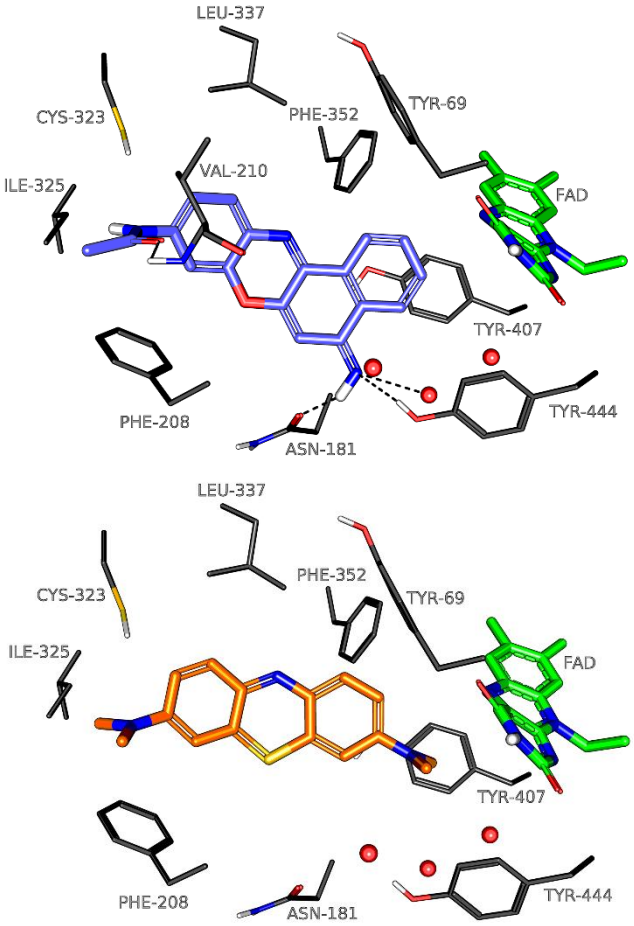


Figure 3-12: Three-dimensional representation of the interactions of Darrow red (top) and methylene blue (bottom) with MAO-A.

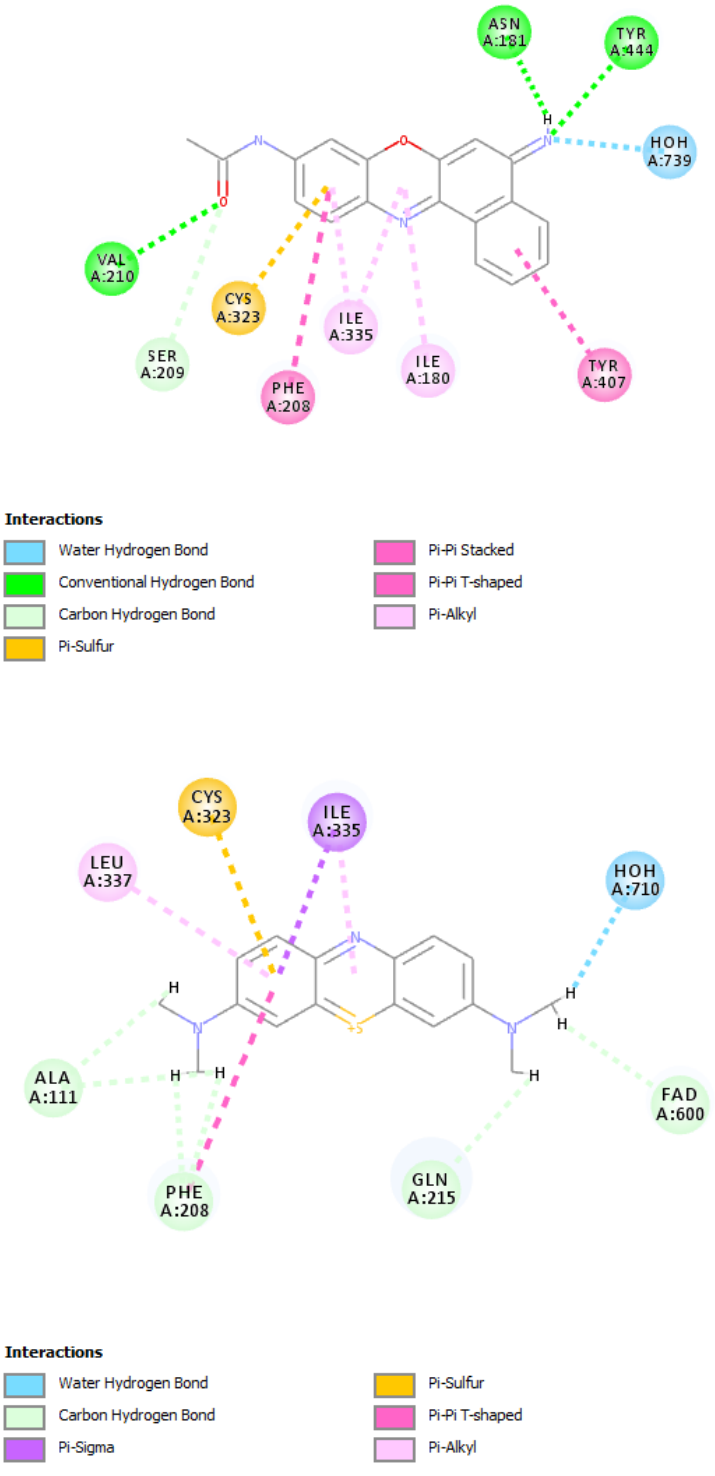


Figure 3-13: Two-dimensional representation of the interactions of Darrow red (top) and methylene blue (bottom) with MAO-A.

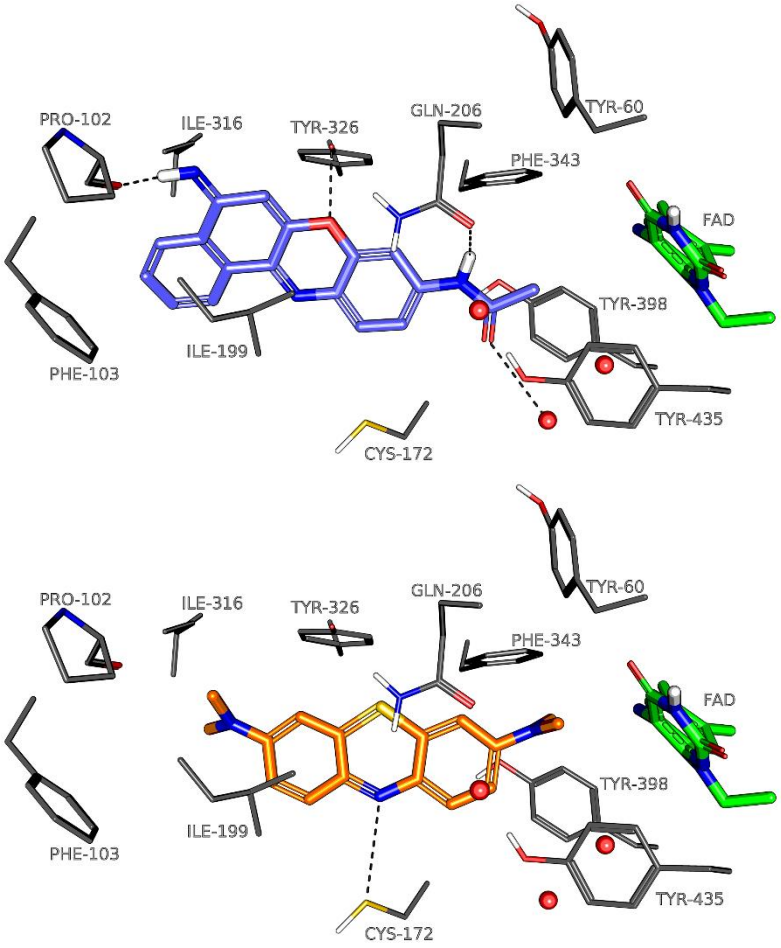


Figure 3-14: Three-dimensional representation of the interactions of Darrow red (top) and methylene blue (bottom) with MAO-B.

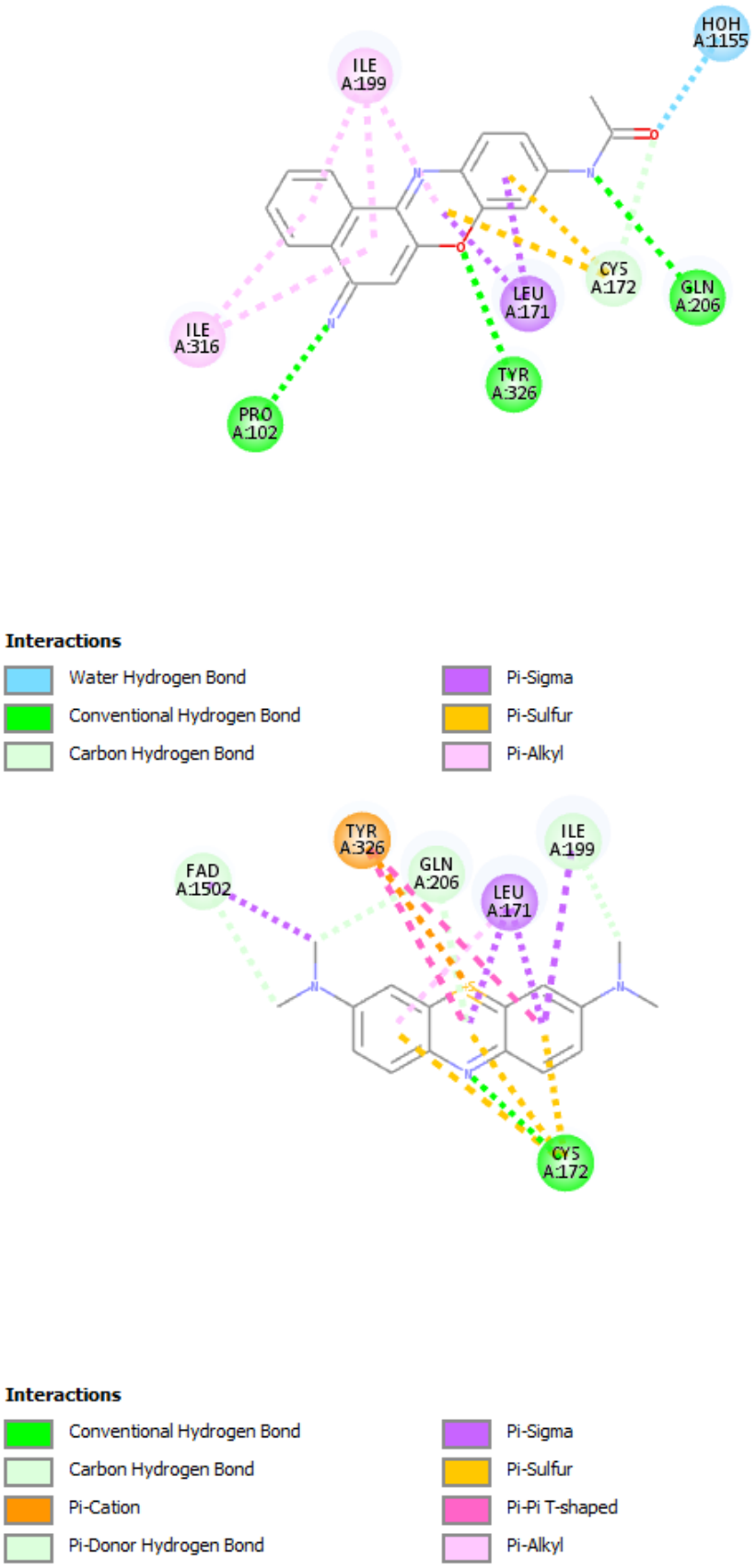


Figure 3-15: Two-dimensional representation of the interactions of Darrow red (top) and methylene blue (bottom) with MAO-B.

In MAO-B, Darrow red exhibits a reversed binding orientation with the acetamide directed towards the FAD (figure 3-14). The inhibitor also binds much more distant from the FAD. In spite of this, Darrow red forms many interactions with the MAO-B active site (figure 3-15), and undergoes hydrogen bonding with Pro-102, Tyr-326, Gln-206 and a water molecule. These interactions involve the exocyclic amine, the acetamide carbonyl and the oxazine oxygen. Pi-interactions are formed with Ile-199, Ile-316 and Leu-171. This interaction network may explain why Darrow red also is a potent MAO-B inhibitor. Methylene blue also exhibits a different binding orientation in MAO-B compared to MAO-A (figure 3-14). In MAO-B, the sulphur is directed towards the top of the active site, in the direction of Tyr-326. Hydrogen bonding occurs with Cys-172, while a variety of Pi-interactions are formed which include interactions with Tyr-326, Leu-171, Ile-199 and Cys-172. A Pi-interaction is also formed with the FAD. Although methylene blue undergoes many interactions with MAO-B (figure 3-15), it is a much weaker MAO-B inhibitor compared to MAO-A. Key interactions that occur in MAO-A and not MAO-B may explain the lower MAO-B inhibition of methylene blue, possibly the interaction of the sulphur atom of methylene blue with the amidic N of Gln-215. More investigation is necessary to determine the exact molecular basis for the specificity of MAO-A inhibition by methylene blue.

3.1.4 Discussion and conclusion

Recent studies have shown that various dye compounds are good potency MAO inhibitors. This is exemplified by methylene blue, a high potency MAO-A inhibitor. Based on these observations, the current study evaluated the MAO inhibition properties of 22 commercially available dye compounds, most of which are structurally similar to methylene blue. The aim of this study was to discover new dyes with good potency MAO inhibition and to contribute to the SAR of MAO inhibition by dye compounds.

The results documented that among the dye compounds there are a number of high potency MAO inhibitors. Nine compounds inhibited MAO-A with IC_{50} values < 1 µM, while three compounds inhibited MAO-B with IC_{50} values < 1 µM. The most potent MAO-A inhibitors are acridine orange and Darrow red with IC_{50} values of 0.017 µM and 0.059 µM, respectively. These values are within the same range as that reported for methylene blue (IC_{50} = 0.07 µM). The most potent MAO-B inhibitors of this study are oxazine 170 and Darrow red with IC_{50} values of 0.0065 µM and 0.065 µM, respectively. These inhibitors are significantly more potent MAO-B inhibitors that methylene blue (IC_{50} = 4.37 µM). It is interesting to note that Darrow red is a benzophenoxazine compound similar to cresyl violet and Nile blue, compounds that are high potency MAO-A inhibitors with IC_{50} values of 0.0037 µM and 0.0077 µM, respectively (Delport *et al.*, 2017). Cresyl violet and Nile blue are also good potency MAO-B inhibitors with IC_{50} values of 0.592 µM and 0.012 µM, respectively. Since Darrow red was found to be a potent MAO-B inhibitor, it may be concluded that benzophenoxazines are a general class of potent MAO

inhibitors. Another benzophenoxazine, oxazine 170, was the most potent MAO-B inhibitor of this study. Interestingly, this compound was a much weaker MAO-A inhibitor (IC_{50} = 1.45 µM).

As mentioned above, acridine orange is of note since it is one of the most potent MAO-A inhibitors of the current study. This potent activity of acridine orange may be explained by its close structural similarity to methylene blue. The demethylated analogue 3,6-diaminoacridine (IC_{50} = 0.507 µM) also is a potent MAO-A inhibitor, although 30-fold less potent than acridine orange. Another acridine compound, acridine yellow G (IC_{50} = 0.350 µM), also presented with potent MAO-A inhibition, showing that the acridine class of compounds are in general good potency MAO-A inhibitors.

Phenoxazine dyes, such as brilliant cresyl blue, have also been investigated as MAO inhibitors (Oxenkrug *et al.*, 2007). The present study investigated the MAO inhibition properties of additional phenoxazines such as phenoxazine, basic blue 3, Celestine blue and gallocyanine. Among these, only phenoxazine and basic blue 3 are notable MAO inhibitors, both exhibiting inhibition of MAO-A and MAO-B. In fact, phenoxazine inhibits both MAO isoforms with IC_{50} < 1 µM. Basic blue 3 may be considered to be the phenoxazine analogue of ETC, a phenothiazine compound. Since ETC (IC_{50} = 0.51 µM) is a significantly more potent MAO-A inhibitor than basic blue 3 (IC_{50} = 7.45 µM), it may be concluded that the phenothiazine moiety of ETC is more suitable for MAO-A inhibition that the phenoxazine.

Several anthraquinone compounds have been included in this study namely 1,4-dihydroxyanthraquinone, 1,5-diaminoanthraquinone, nuclear fast red, disperse blue 1, purpurin and disperse orange 11. Among these, 1,4-dihydroxyanthraquinone (IC_{50} = 0.887 µM), 1,5-diaminoanthraquinone (IC_{50} = 0.585 µM) and disperse orange 11 (IC_{50} = 0.450 µM) are notable as submicromolar inhibitors of MAO-A with no inhibition of MAO-B observed at 100 µM. These compounds may thus be considered as potent and specific inhibitors of MAO-A. Disperse blue 1 also is a specific MAO-A inhibitor (IC_{50} = 7.58 µM) that did not present with MAO-B inhibition, although less potent.

Pyronin B and pyronin Y were also found to inhibit the MAOs with specificity for the MAO-A isoform. Pyronin Y is a particularly noteworthy MAO-A inhibitor with an IC_{50} value of 0.293 µM. This good potency MAO-A inhibition may be expected since pyronin Y is a close structural analogue of methylene blue. Although several coumarin analogues are well known to inhibit MAO-B potently, coumarin 102 only moderately inhibited MAO-A (IC_{50} = 10.1 µM), with no inhibition of MAO-B observed at 100 µM. Hematoxylin and palmatine were weak MAO inhibitors which may be due to the non-planarity of their structures. All other compounds of this study that presented with good potency MAO inhibition possess planar structures consisting of 3- or 4-fused aromatic or heteroaromatic rings. Modelling suggests that fused aromatic systems are

able to undergo a variety of Pi-interactions with the MAO active sites, which will contribute greatly to binding affinity and inhibition potency.

The most potent inhibitors of MAO-A and MAO-B discovered in this study, acridine orange, oxazine 170 and Darrow red, were further evaluated for reversibility and mode of inhibition. These results showed that Darrow red and acridine orange are reversible and competitive inhibitors of MAO-A with K_i values of 0.039 µM and 0.0069 µM, respectively. Darrow red and oxazine 170 were found to be reversible and competitive inhibitors of MAO-B with K_i values of 0.065 µM and 0.007 µM, respectively.

Acridine orange was synthesised in 1979 and can be classified as a cationic fluorescence cytochemical (Lyles & Cameron, 2002). Uses of acridine orange include the staining of intracellular acidic vesicles such as cell nuclei containing deoxyribonucleic acid (DNA), measuring lysosomal membrane permeability (LMP) and the detection of autophagy in order to study apoptosis (Singh *et al.*, 2012; Traganos & Darzynkiewicz, 1994). Oxazine 170 is used as a laser dye due to its luminescent properties as well as a dye during fluorescence quenching when imaging certain structures (Miluski, 2017; Tan *et al.*, 2013). Darrow red is certified to stain sections of brain and spinal cord tissue frozen in formalin or preserved by paraffin to identify neuronal cell bodies (Penney *et al.*, 2002; Powers *et al.*, 1960).

In conclusion, most of the dye compounds were found to be MAO-A specific inhibitors with nine compounds inhibiting MAO-A with IC_{50} values < 1 µM. This is similar to methylene blue which confirms that planar structures consisting of fused aromatic rings are often good potency MAO-A inhibitors. This study revealed numerous good potency MAO inhibitors with potential as lead compounds for future drug design. Among these compounds acridine orange, oxazine 170 and Darrow red are particularly noteworthy. As mentioned, MAO-B inhibitors may be of use in the treatment of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. MAO-A inhibitors, in turn, are important drugs in the management of depression. Therefore, the active dye compounds from this study may have a role in the development of future MAO inhibitors. However, caution should be exercised with the simultaneous use of specific MAO-A inhibitors and serotonergic drugs, such as tricyclic antidepressants and SSRIs, as this may lead to serotonin toxicity.

3.1.5 Materials and methods

3.1.5.1 Biological and chemical reagents

Microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg protein/mL) and kynuramine dihydrobromide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Selegiline, pargyline, 3,4-dibutoxy-3-cyclobutene-1,2-dione, phenoxazine, palmatine,

hematoxylin, nuclear fast red, acridine orange, 1,4-dihydroxyanthraquinone, 1,5-diaminoanthraquinone, 3,6-diaminoacridine, disperse blue 1, basic blue 3, pyronin B, coumarin 102, pyronin Y, purpurin, gallocyanine, oxazine 170, Celestine blue, indigo, disperse orange 11, acridine yellow G and Darrow red were obtained from Sigma-Aldrich. The dye compounds were in the salt forms shown in table 3-1 and used without further purification (70–90% dye content).

3.1.5.2 Instrumentation

A Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, USA) was used to determine fluorescence intensities.

3.1.5.3 IC_{50} determinations

IC_{50} values for the inhibition of the MAOs were determined using the method reported in literature (Mostert *et al.*, 2015; Mostert *et al.*, 2016). Recombinant human MAO-A and MAO-B were used as enzyme sources and all reactions were carried out in potassium phosphate buffer [pH 7.4, 100 mM, made isotonic with potassium chloride (KCl)] in white 96-well microtiter plates (Eppendorf, Hamburg, Germany). The reactions contained kynuramine (50 µM), MAO-A (0.0075 mg protein/mL) or MAO-B (0.015 mg protein/mL) and the test inhibitor (0.003–100 µM), and were prepared to a final volume of 200 µL. Dimethyl sulfoxide (DMSO) was used to prepare stock solutions of the test inhibitors and was added to the reactions to yield a 4% concentration of DMSO. Stock solutions of the substrate and enzyme were prepared in the reaction buffer. As negative controls, reactions carried out in the absence of inhibitor were included. The reactions comprised of reaction buffer (92 µL), substrate (50 µL), test inhibitor (8 µL) and enzyme (50 µL). The reactions were initiated with the addition of the MAO enzymes, and after incubation at 37 ˚C for 20 minutes in a convection oven, the reactions were terminated with the addition of 80 µL of NaOH (2 N). The oxidation of kynuramine by MAO yields 4-hydroxyquinoline as ultimate product. Employing fluorescence spectrophotometry (λ_{ex} = 310 nm; λ_{em} = 400 nm), the concentration of 4-hydroxyquinoline in the reactions were measured at the endpoint. To quantitate 4-hydroxyquinoline, a linear calibration curve was constructed with authentic 4-hydroxyquinoline (0.047–1.56 µM) (figure 3-16). The rates of MAO catalysis were calculated and the rate data was fitted to the one site competition model incorporated into the Prism 5 software package (GraphPad, San Diego, CA, USA). This yielded sigmoidal plots of enzyme catalytic rate versus the logarithm of inhibitor concentration from which IC_{50} values were determined. The IC_{50} values were measured in triplicate, and are given as the mean ± SD.

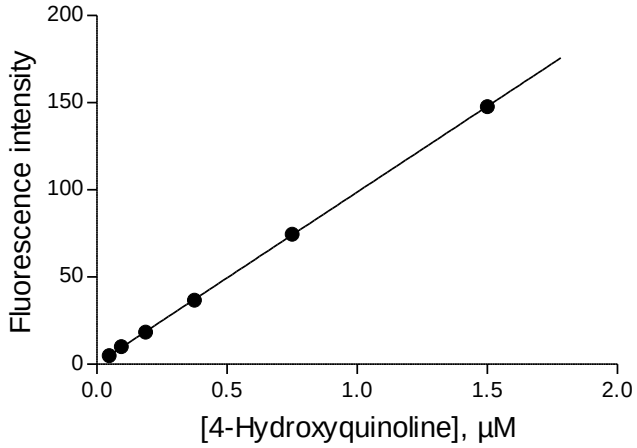


Figure 3-16: A calibration curve constructed for the quantitation of 4-hydroxyquinoline by fluorescence spectrophotometry.

3.1.5.4 Reversibility of inhibition by dialysis

The protocol to investigate the reversibility of inhibition by dialysis was obtained from literature (Mostert *et al.*, 2015; Mostert *et al.*, 2016). The reversibility of MAO inhibition by acridine orange, oxazine 170 and Darrow red was investigated by dialysis. Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Waltham, MA, USA) with a molecular cut-off of 10 000 and a 0.5–3 mL sample volume were used for dialysis. The test inhibitor (at a concentration equal to 4 × IC_{50}) and the MAO enzyme (0.03 mg protein/mL) were dissolved in 0.8 mL dialysis buffer, which consisted of potassium phosphate buffer (100 mM, pH 7.4) containing 5% sucrose. Stock solutions of the inhibitors were prepared in DMSO and added to the dialysis samples to yield a final concentration of 4% DMSO. The samples were pre-incubated at 37 ˚C for 15 minutes and then dialysed in 80 mL dialysis buffer at 4 ˚C for 24 hours. The dialysis buffer was replaced at 3 hours and 7 hours after the start of dialysis with fresh buffer. As positive controls for MAO-A and MAO-B, similar dialysis of the enzymes was carried out in the presence of the irreversible inhibitors, pargyline (IC_{50} = 13 µM) and selegiline (IC_{50} = 0.079 µM), respectively (Strydom *et al.*, 2012). As a negative control, dialysis of the enzymes was carried out in the absence of inhibitor. After dialysis, the dialysis samples (250 µL) were diluted twofold with the addition of 250 µL kynuramine to yield the following final concentrations: kynuramine (50 µM), MAO (0.015 mg protein/mL) and test inhibitor (2 × IC_{50}). The reactions were incubated at 37 ˚C for 20 minutes in a water bath where after 400 µL NaOH (2 N) was added to terminate the reactions. After the addition of 1000 µL water, the concentration of 4-hydroxyquinoline was measured using fluorescence spectrophotometry (λ_{ex} = 310 nm; λ_{em} = 400 nm) using a 3.5 mL quartz cuvette (pathlength 10 × 10 mm). Using authentic 4-hydroxyquinoline (0.047–1.56 µM), a linear calibration curve was constructed for the quantitation of 4-hydroxyquinoline. For

comparison, undialysed samples containing the MAO enzyme and test inhibitors were maintained at 4 ˚C for 24 hours and diluted and assayed as described above. All the dialysis reactions were carried out in triplicate. The resulting residual enzyme catalytic rates were determined and are reported as the mean ± SD.

3.1.5.5 Lineweaver-Burk plots

The mode of inhibition of MAO was determined using the literature protocol for the construction of Lineweaver-Burk plots (Mostert *et al.*, 2015; Mostert *et al.*, 2016). For each test inhibitor, a set consisting of six Lineweaver-Burk plots were constructed. The six plots were constructed in the absence of inhibitor and presence of five different inhibitor concentrations (¼ × IC_{50}, ½ × IC_{50}, ¾ × IC_{50}, 1 × IC_{50} and 1¼ × IC_{50}). Eight different concentrations of kynuramine (15–250 µM) were used for each plot. The enzyme reactions were thus carried out to a volume of 500 µL as described above for the dialysis studies and contained kynuramine (15–250 µM), the test inhibitor (0–1¼ × IC_{50}) and the MAO enzyme (0.015 mg protein/mL). The measurement of the concentration of 4-hydroxyquinoline was carried out as described above for the dialysis studies, and the Prism 5 software package was used for linear regression analyses.

3.1.5.6 Molecular docking

The Discovery Studio 3.1 software package (Accelrys) was used to conduct molecular modelling. The Brookhaven Protein Data Bank served as a source for the structures of both human MAO-A (PDB code 2Z5X) (Son *et al.*, 2008) and human MAO-B (PDB code 2V5Z) (Binda *et al.*, 2007). After the pKa values and protonation states of the ionisable amino acids were calculated, as well as the valences of the FAD cofactors (oxidised state) and co-crystallised ligands were corrected, hydrogen atoms were added to the models at pH 7.4. The Momany and Rone CHARMm forcefield was used to type the protein models automatically and a fixed atom constraint was applied to the backbone. The Smart Minimiser algorithm was employed to minimise the energy of the models with the maximum amount of steps set to 50000 and using the implicit generalised Born solvation model with molecular volume. The co-crystallised ligands, waters and backbone constraints were removed from the models and the active sites were identified from the cavities present in the proteins. Three active site waters present in the MAO-A (HOH 710, 718 and 739) as well as MAO-B (HOH 1155, 1170 and 1351 in the A-chain) were retained. Discovery studio was used to construct the structures of the ligands and a Dreiding-like forcefield (5000 iterations) was used to optimise the geometries of the generated structures before they were submitted to the Prepare Ligands protocol. The Momany and Rone CHARMm forcefield was used to assign the necessary atom potential types and partial charges to the ligands. The CDOCKER algorithm was used to dock the test inhibitors into the MAO models. Ten random ligand conformations were generated, the heating target

temperature was set to 700 K and full potential mode was employed. Finally the Smart Minimizer algorithm was used to refine the docking solutions by means of *in situ* minimisation.

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CHAPTER 4 CONCLUSION

Possible treatment options for neurodegenerative diseases have been investigated for many years. Unfortunately no cures to prevent the progression of diseases such as Parkinson's disease and Alzheimer's disease have been found. However, the treatment of the resulting symptoms of neurodegenerative diseases can significantly increase the quality of life for patients.

The mitochondrial bound enzyme, MAO, presents as two isoforms namely MAO-A and MAO-B (Youdim *et al.*, 2006). MAO metabolises various neurotransmitters such as tyramine, noradrenaline, dopamine and serotonin by means of oxidative deamination of these amine substrates (Binda *et al.*, 2002). The tissue distribution of the two isoforms of MAO differs, with MAO-B being predominantly present in the platelets and glial cells of the brain whereas MAO-A is primarily located in the heart, intestines and placenta (Youdim *et al.*, 2006). The MAO enzyme is of importance in the pathology of several neurodegenerative disorders including Parkinson's disease and Alzheimer's disease as well as neuropsychiatric diseases such as depression.

The primary cause or trigger of Parkinson's disease is still unidentified. Possible causes that have been studied range from environmental toxins, genetic factors, endogenous toxins and the formation of toxic by-products due to MAO metabolism. The pathogenesis and dopaminergic neuronal death in Parkinson's disease may be related to the misfolding and aggregation of proteins and oxidative stress due to mitochondrial dysfunction (Dauer & Przedborski, 2003). Another risk factor to be considered in the pathology of Parkinson's disease is the increase of MAO-B activity in brain tissue due to glial cell proliferation as a patient ages (Oreland & Gottfries, 1986). Increased MAO-B activity depletes available neurotransmitters and increases metabolically produced concentrations of ROS, such as H_2O_2, as well as neurotoxic aldehydes which may act as neurotoxins. The accumulation of these neurotoxic by-products associated with the MAO-B metabolism of neurotransmitters accelerates neuronal death in Parkinson's disease.

MAO-B inhibition is beneficial in the treatment of Parkinson's disease by increasing the availability of dopamine (symptomatic) and by preventing further neurodegeneration. MAO-B inhibition not only increases the availability of neurotransmitters, but also decreases the formation of toxic by-products by MAO-B in the brain. MAO-B inhibition can also protect against possible unknown pro-neurotoxins by preventing the MAO-B-catalysed metabolic activation to produce the active neurotoxic metabolites. By reducing the production of H_2O_2, MAO-B inhibition will reduce damage to the mitochondria as well as decrease protein aggregation and misfolding, which are both beneficial in the treatment of Parkinson's disease.

The brain tissue of patients diagnosed with Alzheimer's disease is characterised by amyloid plaques as well as NFT and can therefore be considered as part of the pathology of the disease (Ramirez-Bermudez, 2012). Increased MAO-B activity associated with Alzheimer's disease initiates the amyloid cascade hypothesis resulting in oxidative stress, increased Aβ formation, generation of amyloid plaques as well as increased NFT formation (Cai, 2014). All of these pathological effects lead to cognitive dysfunction as a result of neurodegeneration.

MAO-B inhibition is beneficial in the treatment of Alzheimer's disease by reducing oxidative stress and preventing the amyloid cascade hypothesis. The decrease of oxidative stress and modulation of the proteolytic cleavage of APP prevents the formation of Aβ (Cai, 2014). Therefore, MAO inhibition reduces the generation of amyloid plaques as well as NFT formation preventing neuroinflammation, which may result in a neuroprotective effect (Cai, 2014) (Nedic *et al.*, 2010).

MAO-A inhibition has been used in the treatment of depression for many years. Increased availability of dopamine, noradrenaline and serotonin, due to reduced MAO-A-catalysed metabolism, effectively treats depressive illness (Gareri *et al.*, 2000). MAO inhibition can also be of use in the treatment of cardiovascular disease, cerebral ischemia, smoking cessation, dementia as well as ALS.

The cotton dye, methylene blue, has been used for the treatment of various ailments throughout many years. Recently, the potential effects of methylene blue on neurodegenerative diseases such as Alzheimer's disease and neuropsychiatric diseases such as depression have been studied (Oz *et al.*, 2009; Schirmer *et al.*, 2011). The ability of methylene blue to inhibit MAO is of special interest. Methylene blue exhibits reversible MAO inhibition with an IC_{50} value of 0.07 µM for MAO-A and an IC_{50} of 4.37 µM for MAO-B.

This study was based on the positive results obtained in recent studies on the MAO inhibition properties of dye compounds. Based on the high potency MAO-A inhibition of methylene blue, 22 other dye compounds similar in structure were selected and evaluated for MAO inhibition. The structures selected for the study included: 3,4-dibutoxy-3-cyclobutene-1,2-dione, phenoxazine, palmatine, hematoxylin, nuclear fast red, acridine orange, 1,4-dihydroxyanthraquinone, 1,5-diaminoanthraquinone, 3,6-diaminoacridine, disperse blue 1, basic blue 3, pyronin B, coumarin 102, pyronin Y, purpurin, gallocyanine, oxazine 170, Celestine blue, indigo, disperse orange 11, acridine yellow G and Darrow red.

This study discovered several high potency inhibitors with the majority of the tested dye compounds specific for the inhibition of MAO-A. Nine compounds exhibited submicromolar (IC_{50} < 1 µM) inhibition of MAO-A. Acridine orange and Darrow red exhibited IC_{50} values for the

inhibition of MAO-A of 0.017 µM and 0.059 µM, respectively, and are the most potent MAO-A inhibitors of this study. Three compounds were found to inhibit MAO-B in the submicromolar range (IC_{50} < 1 µM). Oxazine 170 (IC_{50} = 0.006 µM) and Darrow red (IC_{50} = 0.065 µM) were the most potent MAO-B inhibitors identified in this study. Based on the IC_{50} values, it may be concluded that acridine orange and Darrow red has similar MAO-A inhibition potencies compared to methylene blue (IC_{50} = 0.07 µM). Oxazine 170 and Darrow red are significantly more potent inhibitors of MAO-B than methylene blue (IC_{50} = 4.37 µM).

Previously tested compounds such as cresyl violet and Nile blue can be classified as benzophenoxazine dye compounds and are high potency MAO-A inhibitors with IC_{50} values of 0.0037 µM and 0.0077 µM, respectively, and also exhibit good potency MAO-B inhibition with IC_{50} values of 0.592 µM and 0.012 µM, respectively (Delport *et al.*, 2017). The MAO-A and MAO-B inhibition potencies of Darrow red, another benzophenoxazine, is thus similar to cresyl violet and Nile blue. Benzophenoxazines can therefore be classified as a general class of potent MAO inhibitors. Interestingly, the most potent MAO-B inhibitor identified in this study, oxazine 170, also is a benzophenoxazine but showed much weaker MAO-A inhibition (IC_{50} = 1.45 µM).

As previously mentioned, acridine orange is the most potent MAO-A inhibitor among the dye compounds evaluated in this study. The demethylated analogue of acridine orange, 3,6-diaminoacridine (IC_{50} = 0.507 µM), was also tested in this study and exhibited potent MAO-A inhibition. However, 3,6-diaminoacridine is 30-fold less potent than acridine orange. Another acridine compound, acridine yellow G, was also found to exhibited potent MAO-A inhibition with an IC_{50} value of 0.350 µM. These compounds are similar to methylene blue in structure, which may explain their potency for the inhibition of MAO-A. Therefore, the acridine class of compounds can be classified as general good potency MAO-A inhibitors.

In past studies brilliant cresyl blue, a phenoxazine dye, has been investigated for MAO inhibition (Oxenkrug *et al.*, 2007). The MAO inhibition properties of other phenoxazine dyes such as phenoxazine, basic blue 3, Celestine blue and gallocyanine were tested in this study. It was found that phenoxazine and basic blue 3 showed inhibition of both MAO-A and MAO-B with phenoxazine, the most potent inhibitor of this class, inhibiting both of the isoforms with an IC_{50} value of < 1 µM. The phenothiazine compound, ETC share structural similarities with basic blue 3 and can therefore be considered to be its phenoxazine analogue. However ETC (IC_{50} = 0.51 µM) exhibited more potent inhibition of MAO-A when compared to basic blue 3 (IC_{50} = 7.45 µM). This would suggest that the phenothiazine moiety of ETC is more beneficial for MAO-inhibition than the phenoxazine of basic blue 3.

1,4-Dihydroxyanthraquinone, 1,5-diaminoanthraquinone, nuclear fast red, disperse blue 1, purpurin and disperse orange 11 can be classified as anthraquinone compounds and were also

investigated in this study. 1,4-Dihydroxyanthraquinone (IC_{50} = 0.887 µM), 1,5-diaminoanthraquinone (IC_{50} = 0.585 µM) and disperse orange 11 (IC_{50} = 0.450 µM) inhibit MAO-A in the submicromolar ranges while not inhibiting MAO-B at a maximal tested concentration of 100 µM. Due to these characteristics, 1,4-dihydroxyanthraquinone, 1,5-diaminoanthraquinone and disperse orange 11 can therefore be classified as potent and specific MAO-A inhibitors. Disperse blue 1 also did not inhibit MAO-B and can thus be classified as a specific MAO-A inhibitor (IC_{50} = 7.58 µM). However, disperse blue 1 (IC_{50} = 7.58 µM) exhibited a lower MAO-A inhibition potency compared to the other tested anthraquinone compounds.

Pyronin B and pyronin Y inhibited both isoforms of MAO with specificity for MAO-A. The good potency for the inhibition of MAO-A exhibited by pyronin Y (IC_{50} = 0.293 µM) can possibly be explained by the similarity in structure to methylene blue. Coumarin 102 showed moderate MAO-A inhibition properties (IC_{50} = 10.1 µM) and exhibited no inhibition of MAO-B at 100 µM. Coumarin 102 does not conform to the normally observed potent MAO-B inhibition profile exhibited by other coumarin analogues (Orhan & Gulcan, 2015). The weak MAO inhibition properties of hematoxylin and palmatine can be explained by the non-planarity of their structures as all the other compounds exhibiting good MAO inhibition potency are planar structures containing 3- or 4-fused aromatic or heteroaromatic rings.

Acridine orange, oxazine 170 and Darrow red were identified as the most potent inhibitors of MAO and were further investigated to determine the reversibility as well as the mode of inhibition. It was found that Darrow red and acridine orange presented as reversible and competitive MAO-A inhibitors with K_i values of 0.039 µM and 0.0069 µM, respectively. Darrow red and oxazine 170 were identified as reversible and competitive MAO-B inhibitors with K_i values of 0.065 µM and 0.007 µM, respectively.

Acridine orange is a cationic fluorescence cytochemical that was synthesised in 1979 (Lyles & Cameron, 2002). Acridine orange can be used to stain intracellular acidic vesicles including cell nuclei containing DNA, to determine LMP as well as studying apoptosis by means of autophagy detection (Singh *et al.*, 2012; Traganos & Darzynkiewicz, 1994). The luminescent properties of oxazine 170 are of use as a laser dye as well as imaging certain structures during fluorescence quenching (Miluski, 2017; Tan *et al.*, 2013). Darrow red is a certified stain used to identify neuronal cell bodies in frozen brain and spinal cord tissue preserved in paraffin (Penney *et al.*, 2002; Powers *et al.*, 1960).

The planar structure of acridines results in its intercalation with DNA (Wainwright, 2001). Due to the shared planar structure across all of the test compounds, toxicity due to intercalation with DNA can be of concern. However the planar nature of the structures does not necessarily guarantee DNA intercalation. This can be seen by the difference in the intercalation properties

witnessed with variances of acridine based compounds (Wainwright, 2001). Furthermore, the DNA intercalation of acridine compounds was tested at subchronic levels and it was found that the test animals did not experience any toxic side effects (Moir *et al.*, 1997). Therefore the toxicity of Darrow red, oxazine 170 and acridine orange must be studied to determine the exact toxicity profile of each.

Certain precautions should be exercised when using MAO inhibitors. To reduce the possibility of the occurrence of the cheese reaction it is beneficial to use reversible MAO-A inhibitors as well as selective MAO-B inhibitors (Knoll, 2000; Youdim & Weinstock, 2004). These inhibitors do not reduce the ability of the peripheral tissues to metabolise tyramine (Anderson *et al.*, 1993). Furthermore, the concurrent use of MAO-A inhibitors and serotonergic agents can result in serotonin toxicity, as observed for methylene blue, and should be avoided.

The aims and objectives of this study were met as stated in Chapter 1 and the stated hypothesis has been proved: Using visual inspection to determine the similarity in structure to methylene blue, other dye compounds were identified and confirmed as potent inhibitors of MAO. This can be attributed to the fused aromatic rings found within the planar structures as observed for methylene blue, the lead compound of this study. These structural characteristics can alert drug discovery scientists for potential good potency MAO-A inhibition.

Further studies regarding the safety profile as well as the chemical properties of acridine orange, oxazine 170 as well as Darrow red should be conducted to determine if the dye compounds can qualify as viable treatment options for use in patients suffering from Parkinson's disease, Alzheimer's disease and depression. The additional information can ensure possible development and clinical testing. Furthermore, the results collected as part of this study revealed possible lead compounds that may be of use in the design of future MAO-B inhibitors as part of the pursuit of possible treatment options for neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. MAO-A inhibitors discovered in this study, in turn, can be of use in the treatment of depression. This study discovered new dye compounds with good potency MAO inhibition that may affect the development of future MAO inhibitors.

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