The synthesis and evaluation of imidazopyridines as adenosine receptor antagonists

R. Lefin
21684758

Dissertation submitted in partial fulfillment of the requirements for the degree Masters of Science in Pharmaceutical Chemistry at the Potchefstroom Campus of the North-West University

Supervisor: Prof. G. Terre’Blanche
Co-supervisor: Dr. M.M. van der Walt

September 2017
The financial assistance of the National Research Foundation (NRF) towards this study is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.
ACKNOWLEDGEMENTS

I want to take this opportunity to sincerely thank and express my appreciation to the following individuals/institutions for their contributions and support during this study:

- My supervisor, Prof. G. Terre’Blanche and co-supervisor, Dr. M. M. van der Walt for all their guidance and support throughout this study and writing of this dissertation.

- The following individuals for performing the different analyses of this study: Mr. A. Joubert (NMR), Dr. J. Jordaan (MS), Prof. J. Du Preez and Mr. K. Malebe (HPLC), as well as Dr. M. M. van der Walt (radioligand binding assays).

- The North-West University, Potchefstroom Campus, for providing all the training required and facilities necessary to complete this study.

- The National Research Foundation (NRF) and the Medical Research Council (MRC) for providing the funding used for this study.

- To my friends for always being a wonderful support system. Your support and encouragement mean the world to me.

- To Justin and Nevaeh; thank you for inspiring me in your own unique ways.

- My parents, Ronnie and Lynette Lefin, for always believing in me, for their unconditional love and support, which without I would not have become the person I am today. I want to take this opportunity to convey my sincerest appreciation towards them and all that they have done for me by dedicating this dissertation to them.

- I want to take this opportunity to thank God, for without Him none of this would be possible.

***

Even when the journey to fulfill your dreams is filled with more obstacles than those of others, does not mean it is not worth pursuing or that it is unattainable. Although the journey will be more challenging, the destination will be extraordinary.

Lovies
Roslyn xxx
ABSTRACT

Alzheimer’s disease (AD) is the most common occurring neurodegenerative disorder worldwide and includes deficiencies in memory and cognitive impairment. Both the hippocampus and cortex are important neuronal areas in the regulation of cognitive function, while the hippocampus is central in memory processing. Treatment that is currently available aims at restoring the acetylcholine imbalance and includes antioxidants, cholinesterase inhibitors and psychotropic drugs for the symptomatic treatment of AD. These drugs, however, fail to prevent further disease progression and neurodegeneration from occurring, therefore necessitating the need to explore and develop alternative treatments.

Parkinson’s disease (PD) is a chronic, age-related neurodegenerative disorder that may be characterised pathologically by the loss of dopaminergic neurons in the nigrostriatal pathway, causing dopamine in the striatum to decrease. Thus far no curative treatment for the disorder exists with the only available treatment aiming to restore the dopamine deficiencies in the brain. L-dopa remains the gold standard treatment of PD, whilst dopamine agonists, selective monoamine oxidase-B inhibitors, and anticholinergic drugs are used for the symptomatic treatment of PD. None of the treatment currently available slow, terminate or prevent the neurodegeneration from occurring, thus the development of disease modifying drugs are essential.

Adenosine plays an important role in neurodegenerative disorders such as AD and PD. There are four receptor subtypes of adenosine and they are classified as A\(_1\), A\(_{2A}\), A\(_{2B}\), and A\(_3\). The adenosine A\(_1\) receptors are important for cognitive function and are found copiously throughout the hippocampus and cortex. In turn the adenosine A\(_{2A}\) receptors are highly expressed in the striatum and play an important role in motor function and neuroprotection.

In the case of AD and PD, adenosine A\(_{2A}\) receptor antagonists have neuroprotective properties by preventing β-amyloid neurotoxicity in AD and protecting nigrostriatal dopaminergic neurons from neurodegeneration in PD. Furthermore, selective adenosine A\(_1\) receptor antagonists may improve cognitive functions due to their expression in the hippocampus and cortex and selective adenosine A\(_{2A}\) receptor antagonists may also improve motor function due to the expression of the adenosine A\(_{2A}\) receptors in the striatum. Depression is a common neuropsychiatric symptom in both AD and PD and remains inadequately treated with current drugs available.
Adenosine A\textsubscript{2A} receptor antagonists have displayed antidepressant effects in rodent models of depression and may find therapeutic value to improve depressive symptoms. Therefore development of non-selective adenosine receptor antagonists are attractive for the treatment of both AD and PD as they improve the cognitive and motor function, prevent further neurodegeneration and improve the depressive symptoms in both disorders.

Previous research has shown that bicyclic 6:5-fused heteroaromatic compounds with two N-atoms have variable degrees of adenosine A\textsubscript{1} antagonistic activity. Prompted by this a pilot study was undertaken, where imidazo[1,2-\alpha]pyridine analogues were synthesised, characterised and evaluated for their adenosine A\textsubscript{1} and A\textsubscript{2} antagonistic activity as possible treatment agents for AD and PD. Radioligand binding studies were performed to determine the adenosine binding affinities and the most promising adenosine A\textsubscript{1} receptor analogue was subjected to a GTP shift assay to determine whether or not the compound has agonistic or antagonistic functionality.

Imidazo[1,2-\alpha]pyridine analogues (4a–i) were synthesised by means of a modified catalyst-and-solvent-free method by reacting cyclohexyl isocyanide, 2-aminopyridine and the appropriate aldehyde at a suitable temperature. Compounds 1 and 3a–e were obtained commercially and used to compare the effect of substitution on position C2 alone as well as position C2 in combination with position C3 on adenosine A\textsubscript{1} and A\textsubscript{2A} receptor affinity.

Imidazo[1,2-\alpha]pyridine, the parent scaffold, was found devoid of affinity for the adenosine A\textsubscript{1} and A\textsubscript{2A} receptors. The influence of substitution on position C2 showed no improvement for either adenosine A\textsubscript{1} or A\textsubscript{2A} receptor affinity. The addition of an amino or a cyclohexylamino group to position C3 also showed no improvement of adenosine A\textsubscript{1} or A\textsubscript{2A} receptor affinity. Surprisingly para-substitution on the phenyl ring at position C2 in combination with a cyclohexylamino group at position C3 led to adenosine A\textsubscript{1} receptor affinity in the low micromolar range with compound 4d (para-methyl) showing the highest affinity for the adenosine A\textsubscript{1} receptor with a $K_i$ value of 2.06 µM. Compound 4d behaved as an adenosine A\textsubscript{1} receptor antagonist in the GTP shift assay performed with rat whole brain membranes expressing adenosine A\textsubscript{1} receptors.

This pilot study concludes that para-substituted 3-cyclohexylamino-2-phenyl-imidazo[1,2-\alpha]pyridine analogues represent an interesting scaffold to investigate further structure-activity relationships in the design of novel imidazo[1,2-\alpha]pyridine-
based adenosine $A_1$ receptor antagonists for the treatment of neurodegenerative disorders such as AD and PD.

**Keywords:** Alzheimer's disease, Parkinson's disease, imidazo[1,2-α]pyridine analogues, adenosine $A_1$ receptor antagonists, adenosine $A_{2A}$ receptor antagonists.
OPSOMMING

Alzheimer se siekte (AS) is die mees algemene neurodegeneratiewe siekte wêreldwyd en sluit geheue tekortkominge en kognitiewe inkorting in. Beide die hippocampus en korteks is belangrike neuronale areas om kognitiewe funksie te reguleer, terwyl die hippocampus sentraal is vir geheue verwerking. Huidige behandeling poog om die wanbalans van asetielcholien te herstel en sluit in: antioksidante, choliesterase inhibeerders en antipsigotiese middels vir die simptomatiese behandeling van AS. Hierdie middels, versuim egter om verdere progressie van die siekte en senuweesel verval te voorkom, en daarom die groot behoefte om alternatiewe geneesmiddels vir behandeling te verken en te ontwikkel.

Parkinson se siekte (PS) is ‘n chroniese, ouderdom-verwante neurodegeneratiewe siektetoestand wat patologies gekenmerk word deur die verlies van dopaminergiese neurone in die nigrostriatale baan, wat ‘n verlies van dopamien in die striatum tot gevolg het. Tot dusver is daar geen genesende behandeling vir die siektetoestand nie, terwyl huidige behandeling daarop gemik is om die dopamien tekortkominge in die brein te herstel. L-dopa bly die basis vir die behandeling van PS, terwyl dopamien agoniste, selektiewe monoamienoksidase-B-inhibeerders en anticholinergiese middels gebruik word vir die simptomatiese behandeling van PS. Huidige behandeling wat tans beskikbaar is, vertraag, stop of voorkom nie neurodegenerasie nie, en daarom is die ontwikkeling van siektemodifiserende geneesmiddels noodsaaklik.


neuropsigiaatriese simptoom in beide AS en PS en word tans onvoldoende behandel. Die adenosien A2A-reseptor antagonistie het antidepressiewe effekte getoon in knaagdiërmodele vir depressie en kan van terapeutiese waarde wees om depressiewe simptome te verlig. Die ontwikkeling van nie-selektiewe adenosien reseptor antagonistie is dus ’n belowe behandelingsterapie in beide AS en PS aangesien dit kognitiewe en motor funksies verbeter, verdere neurodegenerasie verhoed en die depressiewe simptome in beide siektetoestande verhoed.

Vorige navorsing het getoon dat bisikliiese 6:5-gekondenseerde heteroaromatiese verbings met twee N-atome varierende adenosien A1-antagonistiese aktiwiteit besit. Na aanleiding hiervan is ’n loodsstudie onderneem waar imidazo[1,2-α]piridien analoë gesintetiseer, gekarakteriseer en geëvalueer is vir hul adenosien A1- en A2A-antagonistiese aktiwiteite as moontlike behandeling vir AS en PS. Radioligandbindingstudies is uitgevoer om die analoë se bindingsaffiniteite te bepaal en die mees belowe analog van die adenosien A1-reseptor is onderwerp aan ’n GTP-verskuiwingstoets om te bepaal of die verbinding agonistiese of antagonistiese funksionaliteit besit.

Imidazo[1,2-α]piridien analoë (4a–i) is gesintetiseer deur middel van ’n gemodifiseerde katalis-en-oplosmiddel-vrye metode deur sikloheksiel isosianied, 2-aminoiridien en ’n gepaste aldehyd te reageer by ’n gesikte temperatuur. Verbindings 1 en 3a–e is kommersieel verkry om sodoende die effek van substitusie op posisie C2 alleen, sowel as posisie C2 in kombinasie met posisie C3 op die affiniteit vir adenosien A1- en A2A-reseptore te bepaal.


Hierdie loodsstudie kom tot die gevolgtrekking dat para-gesubstitueerde 3-sikloheksielamino-2-feniel-imidazo[1,2-α]piridien analoë ’n belowe
kernstruktuur voorstel om verdere struktuur-aktiwiteitsverwantskappe te ondersoek vir die ontwerp van nuwe imidazo[1,2-α]piridien-gebaseerde adenosien A₁-resceptor antagoniste vir die behandeling van neurodegeneratiewe afwykings soos AS en PS.

**Sleutelwoorde:** Alzheimer se siekte, Parkinson se siekte, imidazo[1,2-α]piridien analoë, adenosien A₁-receptorantagoniste, adenosien A₂A-receptorantagoniste.
# ABBREVIATIONS

## Summary/ Opsomming

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AS</td>
<td>Alzheimer se siekte</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PS</td>
<td>Parkinson se siekte</td>
</tr>
</tbody>
</table>

## Chapter 1

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>Carbon</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>$^1$H</td>
<td>Proton</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>mp</td>
<td>Melting point</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectrometry</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationships</td>
</tr>
<tr>
<td>ZM-241385</td>
<td>4-[2-[7-amino-2-(2-furyl[1,2,4]-triazolo[2,3-α][1,3,5]triazin-5-yl]amino]ethyl]phenol</td>
</tr>
</tbody>
</table>

## Chapter 2

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-Hydroxydopamine</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
</tbody>
</table>
APOE  Apolipoprotein
ChEIs  Cholinesterase inhibitors
CNS   Central nervous system
COMT  Catechol-O-methyltransferase
COX-1  Cyclooxygenase 1
COX-2  Cyclooxygenase 2
CPT   8-cyclopentyl-1,3-dimethylxanthine
DA    Dopamine
DAT   Dopamine transporter
LBs   Lewy bodies
L-dopa  L-3,4-dihydroxyphenylalanine
MAO   Monoamine oxidase
MAO-A  Monoamine oxidase isoform A
MAO-B  Monoamine oxidase isoform B
MPTP  1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NE    Norepinephrine
NSAID's  Non-steroidal anti-inflammatory drugs
PD    Parkinson's disease
PSEN 1  Presenilin 1
PSEN 2  Presenilin 2
SN    Substantia nigra
SNpc  Substantia nigra pars compacta
SNpr  Substantia nigra pars reticulata
SSRI's Selective serotonin reuptake inhibitors
STN   Subthalamic nucleus
βAPP Beta amyloid precursor protein
### Chapter 3

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-Hydroxodopamine</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ASP 5854</td>
<td>5-[5-Amino-3(4-fluorophenyl)pyrazin-2yl]-1-isopropylpyridine-2(1H)-one</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BG-9719</td>
<td>1,3-dipropyl-8-[2-(5,6-epoxynorbornyl)xanthine</td>
</tr>
<tr>
<td>CGS-15943</td>
<td>9-Chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPX</td>
<td>8-cyclopentyl-1,3-dipropylxanthine</td>
</tr>
<tr>
<td>CSC</td>
<td>8-(3-chlorostyryl)caffeine</td>
</tr>
<tr>
<td>DMPX</td>
<td>3,7-dimethyl-1-propagylxanthine</td>
</tr>
<tr>
<td>DPCPX</td>
<td>8-cyclopentyl-1,3-dipropyl-xanthine</td>
</tr>
<tr>
<td>KF-17837</td>
<td>(E)-1,3-dipropyl-8-3,4-dimethoxystyril)-7-methyl-3,7-dihydo-1H-purine-2,6-dione</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>KW-3902</td>
<td>1,3-dipropyl-8-(3-noradamantyl)xanthine</td>
</tr>
<tr>
<td>KW-6002</td>
<td>(E)-1,3-diethyl-8-(3,4-dimethoxystyril)-7-methyl-3,7-dihydo-1H-purine-2,6-dione</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>SCH-63390</td>
<td>N$^8$-substituted pyrazolo-triazolo-pyrimidines.</td>
</tr>
<tr>
<td>ZM-241385</td>
<td>4-[2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-α][1,3,5]triazin-5-y1]amino]ethyl]phenol</td>
</tr>
</tbody>
</table>

### Chapter 4

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3]$H]DPCPX</td>
<td>Radioligand 1,3-dipropyl-8-cyclopentylxanthine</td>
</tr>
<tr>
<td>$^13$C</td>
<td>Carbon</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric-pressure chemical ionisation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>CPA</td>
<td>N°-cyclopentyladenosine</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets</td>
</tr>
<tr>
<td>ddd</td>
<td>double doublet of doublets</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMSO-d6</td>
<td>deuterated dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPCPX</td>
<td>1,3-dipropyl-8-cyclopentylxanthine</td>
</tr>
<tr>
<td>dt</td>
<td>doublet of triplets</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>(^1)H</td>
<td>Proton</td>
</tr>
<tr>
<td>HRMS</td>
<td>High-resolution mass spectrometry</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant</td>
</tr>
<tr>
<td>(K_d)</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>(K_i)</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>mp</td>
<td>Melting point</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>s</td>
<td>Singlet</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationships</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>t</td>
<td>Triplet</td>
</tr>
<tr>
<td>td</td>
<td>triplet of doublets</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>(\delta)</td>
<td>chemical shift</td>
</tr>
</tbody>
</table>
Chapter 5

\[^{3}\text{H}]\text{DPCPX}\] Radioligand 1,3-dipropyl-8-cyclopentylxanthine

\[^{3}\text{H}]\text{NECA}\] Radioligand 5'-N-Ethylcarboxamidoadenosine

\(^{13}\text{C}\) Carbon

AD Alzheimer's disease

DA Dopamine

GTP Guanosine-5'-triphosphate

\(^{1}\text{H}\) Proton

ITAs N-acyl-(7-substituted-2-phenylimidazo[1,2-α][1,3,5]triazin-4-yl)amines

\(K_i\) Dissociation constant

MS Mass spectrometry

NMR Nuclear magnetic resonance

PD Parkinson's disease
2.2.2.3.2 Apathy .............................................................................................................. 19
2.2.2.3.3 Anxiety ............................................................................................................. 19
2.2.2.3.4 Psychosis ......................................................................................................... 19
2.2.2.3.5 Agitation ........................................................................................................... 20
2.2.3 EPIDEMIOLOGY................................................................................................... 20
2.2.3.1 Genetic risk factors ........................................................................................... 20
2.2.3.2 The cholinergic hypothesis of AD ...................................................................... 20
2.2.3.3 The β-amyloid cascade hypothesis of AD ................................................................. 21
2.2.3.4 The β-amyloid oligomer hypothesis of AD ................................................................. 21
2.2.3.5 The tau hypothesis of AD ...................................................................................... 22
2.2.3.6 Cellular dysfunction ............................................................................................ 22
2.2.4 TREATMENT ....................................................................................................... 22
2.2.4.1 Cholinesterase inhibitors ...................................................................................... 23
2.2.4.2 Antipsychotic drugs ............................................................................................. 23
2.2.4.3 Antioxidants ........................................................................................................... 24
2.2.4.4 Ginko biloba ........................................................................................................... 24
2.2.4.5 Oestrogen replacement therapy ............................................................................. 24
2.2.4.6 Non-steroidal anti-inflammatory drugs .................................................................. 25
2.2.4.7 Neuroprotective therapy ....................................................................................... 25
2.2.5 SUMMARY ............................................................................................................. 25
2.3 PARKINSON’S DISEASE (PD) ................................................................................. 26
2.3.1 PATHOLOGY ....................................................................................................... 26
2.3.1.1 The nigrostriatal dopaminergic pathway ............................................................... 26
CHAPTER 3

ADENOSINE AND ADENOSINE RECEPTORS ................................................................. 66

3.1 INTRODUCTION ........................................................................................................ 66

3.2 ADENOSINE AND THE CHOLINERGIC SYSTEM .............................................. 67

3.3 ADENOSINE AND THE DOPAMINERGIC SYSTEM ............................................ 67

3.4 ADENOSINE A_{2A} RECEPTORS .......................................................................... 68

3.4.1 ADENOSINE A_{2A} RECEPTOR ANTAGONISTS IN THE TREATMENT OF AD AND PD .... 68

3.4.1.1 Neuroprotective therapy ........................................................................... 68

3.4.1.2 Depression .................................................................................................... 70

3.4.1.3 Motor function ........................................................................................... 70

3.5 ADENOSINE A_{1} RECEPTORS ......................................................................... 71

3.5.1 ADENOSINE A_{1} RECEPTOR ANTAGONISTS IN THE TREATMENT OF AD AND PD .... 71

3.5.1.1 Cognitive dysfunction ................................................................................ 71

3.5.1.2 Motor function ........................................................................................... 72

3.6 DUAL ACTING ADENOSINE A_{1} AND A_{2A} RECEPTOR ANTAGONISTS .......... 73

3.7 STRUCTURES OF ADENOSINE A_{2A} RECEPTOR ANTAGONISTS ................. 74
4.2.2.3 3-Cyclohexylamino-2-(4’-methoxyphenyl)imidazo[1,2-α]pyridine (4c) .......... 101
4.2.2.4 3-Cyclohexylamino-2-(4’-methylphenyl)imidazo[1,2-α]pyridine (4d) .......... 101
4.2.2.5 3-Cyclohexylamino-2-(4’-bromophenyl)imidazo[1,2-α]pyridine (4e) .......... 102
4.2.2.6 3-Cyclohexylamino-2-(4’-chlorophenyl)imidazo[1,2-α]pyridine (4f) .......... 102
4.2.2.7 3-Cyclohexylamino-2-(4’-fluorophenyl)imidazo[1,2-α]pyridine (4g) .......... 102
4.2.2.8 3-Cyclohexylamino-2-[(4’-(trifluoromethyl)phenyl]imidazo[1,2-α]pyridine (4h) .... 103
4.2.2.9 3-Cyclohexylamino-2-(4’-nitrophenyl)imidazo[1,2-α]pyridine (4i) .......... 103

4.2.3  PHYSICAL CHARACTERISATION ........................................................................ 103

4.2.3.1 3-Cyclohexylamino-2-phenylimidazo[1,2-α]pyridine (4a) .......... 103
4.2.3.2 3-Cyclohexylamino-2-(4’-hydroxyphenyl)imidazo[1,2-α]pyridine (4b) .......... 104
4.2.3.3 3-Cyclohexylamino-2-(4’-methoxyphenyl)imidazo[1,2-α]pyridine (4c) .......... 104
4.2.3.4 3-Cyclohexylamino-2-(4’-methylphenyl)imidazo[1,2-α]pyridine (4d) .......... 104
4.2.3.5 3-Cyclohexylamino-2-(4’-bromophenyl)imidazo[1,2-α]pyridine (4e) .......... 105
4.2.3.6 3-Cyclohexylamino-2-(4’-chlorophenyl)imidazo[1,2-α]pyridine (4f) .......... 105
4.2.3.7 3-Cyclohexylamino-2-(4’-fluorophenyl)imidazo[1,2-α]pyridine (4g) .......... 105
4.2.3.8 3-Cyclohexylamino-2-[(4’-(trifluoromethyl)phenyl]imidazo[1,2-α]pyridine (4h) .... 106
4.2.3.9 3-Cyclohexylamino-2-(4’-nitrophenyl)imidazo[1,2-α]pyridine (4i) .......... 106

4.3  BIOLOGICAL ASSAY .......................................................................................... 107

4.3.1 MATERIALS AND INSTRUMENTATION .............................................................. 107
4.3.2 TISSUE PREPARATION ...................................................................................... 107
4.3.3 RADIOLIGAND BINDING ASSAY PROTOCOL FOR THE ADENOSINE A₂A RECEPTORS 108
4.3.4 RADIOLIGAND BINDING ASSAY PROTOCOL FOR THE ADENOSINE A₁ RECEPTORS .... 108
4.3.5 GTP SHIFT ASSAY .......................................................................................... 109
Table 4-1: The dissociation constant ($K_i$) values for the binding of the imidazo[1,2-α]pyridine analogues to rat adenosine $A_1$ and $A_{2A}$ receptors. .......................................................... 114
LIST OF FIGURES

Figure 1-1: Imidazopyridine analogues exhibiting adenosine A$_1$ receptor affinity (Reutlinger et al., 2014). ................................................................. 5

Figure 1-2: The proposed para-phenyl substituted imidazo[1,2-α]pyridine analogues that will be synthesised, characterised and analysed during this study........................................................................................................................................ 6

Figure 2-1: Cholinergic innervation in the brain, known as the cholinergic pathways (Breedlove & Watson, 2013). ........................................................................................................ 16

Figure 2-2: Schematic representation demonstrating the functions of the cerebral cortex (Waugh & Grant, 2009). ................................................................. 16

Figure 2-3: Atrophy of the brain in patients with Alzheimer's disease compared to the brains of healthy individuals (NIH National Institute on aging, 2016)............................................................................................................ 17

Figure 2-4: A schematic representation of the basal ganglia and the different brain structures that it consists of, illustrated in the right hemisphere of the brain. The SN neurons that innervate the caudate nucleus and the putamen are illustrated in the left hemisphere of the brain. Abbreviations: SN (Substantia Nigra); STN (Subthalamic Nucleus) (Guilarte, 2010). .................. 27

Figure 2-5: Image (A) of the normal nigrostriatal dopaminergic pathway is represented in red. Neuromelanin containing dopaminergic neurons move from the SNpc towards the caudate nucleus and the putamen which make up the striatum. Image (B) of the nigrostriatal dopaminergic pathway in PD is degenerated, modestly in the caudate nucleus and much more evidently in the putamen. Depigmentation of the SNpc is due to the loss of neuromelanin containing dopaminergic neurons. Abbreviations: SNpc (Substantia Nigra pars compacta). (Dauer & Przedborski, 2003)........... 28

Figure 4-1: Imidazo[1,2-α]pyridine. .............................................................................. 96

Figure 4-2: The imidazo[1,2-α]pyridine scaffold. ................................................................. 97
Figure 4-3: The lead compound used to design the investigated imidazopyridines of the current study (Reutlinger et al., 2014). .......................... 97

Figure 4-4: Test compounds commercially available from Sigma-Aldrich® ..................... 98

Figure 4-5: The sigmoidal-dose response curves of compound 4d (Panel A) and CPA (Panel B) displaying the binding affinity to adenosine A\textsubscript{1} receptors in the absence and presence of GTP. Panel C displays the binding affinity of DPCPX to adenosine A\textsubscript{2\textalpha} receptors. .............................. 113

Figure 4-6: Illustrating the adenosine A\textsubscript{1} and A\textsubscript{2\textalpha} receptor binding affinity of imidazo[1,2,α]pyridine (1), 2-phenyl-imidazo[1,2-α]pyridine-3-yl-amine (3), and the targeted imidazo[1,2,α]pyridine analogues (4). Abbreviations: AR (adenosine receptor). ....................................................... 115

Figure 5-1: The adenosine binding affinities of selected ITA analogues (Novellino et al., 2002) ........................................................................................................ 124

Figure 5-2: Proposed future structural modifications to compound 4d ....................... 125
LIST OF SCHEMES

Scheme 4-1: The catalyst- and solvent-free synthetic procedure that was utilised to obtain the corresponding imidazo[1,2-α]pyridines. **Reagents and conditions:** (a) heated at 120°C (4a, 4b, 4c, 4d, 4e, 4f, 4g, 4i) or 60°C (4h), (b) reflux for the appropriate time. ................................................... 98

Scheme 4-2: A possible mechanism for the cyclisation pathway of the targeted imidazo[1,2-α]pyridine analogues by means of compound 4a as an example (Sarkar *et al.*, 2016). ............................................................... 100
CHAPTER 1

RESEARCH RATIONALE AND AIMS

1.1 BACKGROUND

Neurodegenerative disorders such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) are associated with progressive pathological changes in the brain (Armstrong et al., 2005). With disease progression, an increasing number of neuronal regions become affected causing cognitive dysfunction (Zlokovic, 2008). As the severity of cognitive dysfunction increases, the affected individuals experience a declined quality of life and increased caregiver burden (Leroi et al., 2012; Serrano-Aguilar et al., 2006).

AD is a progressive, age-related neurodegenerative disorder accompanied with memory, decision-making, language, proprioception, and judgement difficulties (Alzheimer, 1911). Pathologically AD is associated with a progressive loss of cholinergic neurons in the hippocampus and cortex resulting in a volumetric reduction as well as a decrease of acetylcholine (ACh) in these areas (Bartus et al., 1982; Cummings, 2001). The hippocampus processes short-term and long-term memory, thus the volumetric reduction of the hippocampus is consistent with the memory deficiencies seen in AD (Devanand et al., 2007; Du et al., 2001; Morris et al., 1989; Morris et al., 1993; Morris, 1997). The cerebral cortex and hippocampus are important regions for cognitive function (Fredholm et al., 1999). Current treatment regimens of AD provide symptomatic relief, however, the effectiveness decreases with disease progression (Cummings, 2001). Additionally, it does not prevent further cognitive decline, necessitating the development of alternative treatment agents (Cummings, 2001).

The primary pathology of PD is evident on motor function. PD is recognised by a decrease of dopamine (DA) in the brain due to dopaminergic neuronal loss in the striatum. The current treatment of PD predominantly aims at replenishing the waning DA levels and although this treatment is effective as symptomatic treatment initially, prolonged treatment is not only ineffective but is also associated with treatment-related complications (Olanow et al., 2009). The direct and indirect pathways are two parallel pathways of the basal ganglia (Bolam et
Activation of the direct pathway facilitates movement whereas activation of the indirect pathway inhibits movement (Kravitz et al., 2010). The DA D1 receptors function within the direct pathway whilst the DA D2 receptors function within the indirect pathway (Cepeda et al., 1993; Gerfen et al., 1990). The motor symptoms characteristic of PD may be due to an imbalance between the direct and indirect pathways (Albin et al., 1989). Because the current treatment of PD is unsatisfactory, modulation of the direct and indirect pathways may present a valuable strategy for the future treatment of PD.

Adenosine has a role in neurodegenerative disorders such as AD and PD. The adenosine receptors are classified into four receptor subtypes namely A1, A2A, A2B, and A3 (Fredholm et al., 1994). The adenosine A1 receptors are important for cognitive function and are densely expressed throughout the hippocampus, cortex, cerebellum, dorsal horn of the spinal cord and gamma-aminobutyric neurons (Fredholm et al., 2001; Onodera & Kogure, 1988; Rivkees et al., 1995) and antagonism of these receptors may improve cognition (Mihara et al., 2007). Furthermore the adenosine A2A receptors are expressed in the striatopallidal neurons, striatum, nucleus accumbens, olfactory tubercle, olfactory bulb, and hippocampus. (Fredholm et al., 2001). Selective adenosine A2A receptor antagonists may improve motor function due to the expression of the adenosine A2A receptors in the striatum (Kuwana et al., 1999) and are also attractive as neuroprotective therapeutic agents in both AD and PD (Chen et al., 1999; Geiger et al., 2006; Ikeda et al., 2002; Monopoli et al., 1998). A common neuropsychiatric symptom of AD and PD is depression. Selective adenosine A2A receptor antagonists have displayed antidepressant effects in rodent models of depression (Yacoubi et al., 2001; Yacoubi et al., 2003) and therefore these agents may be beneficial for treating depressive symptoms in patients with AD and PD.

For the abovementioned reasons dual-acting adenosine A1 and A2A receptor antagonists have valuable potential as treatment agents for AD and PD as it will synergistically improve motor function by means of adenosine A1 and A2A receptor antagonists, enhance cognitive function through adenosine A1 receptor antagonism, provide neuroprotective properties to sufferers by means of adenosine A2A receptor antagonism, and alleviate depressive symptoms via adenosine A2A receptor antagonism.
1.2 RATIONALE

AD and PD are the two most common occurring neurodegenerative disorders in the world (Goedert & Spillantini, 2006; Olanow et al., 2009), yet both disorders still lack effective, disease modifying and neuroprotective treatment (Francis et al., 1999; Kakkar & Dahiya, 2015; Shook & Jackson, 2011). For these reasons investigating and developing non-dopaminergic and non-cholinergic disease modifying and neuroprotective treatment, agents are essential (Shook & Jackson, 2011). The adenosine receptors have displayed potential in the treatment of neurodegenerative disorders, with the adenosine $A_1$ and $A_{2A}$ receptors being the most predominant (Gomes et al., 2011). The adenosine compounds can be grouped into two chemical classes i.e. the xanthine derivatives and the amino-substituted heterocyclic compounds (Cristalli et al., 2003; Dhalla et al., 2003; Klotz, 2000; Muller, 2003; Soudijn et al., 2003).

![Xanthine](image)

Various amino-substituted heterocyclic analogues have been investigated for their affinity for the adenosine $A_1$ and $A_{2A}$ receptors. Although the majority of these analogues are considered amino-substituted heterocyclic compounds, they may also be thought of as extensions of the xanthine scaffold. The 1H-imidazo[4,5-c]quinolin-4-amine analogues were among the first amino-substituted heterocyclic compounds to display affinity for the adenosine $A_1$ receptor based on predictions from early ligand modeling (van Galen et al., 1991).

![1H-Imidazo[4,5-c]quinolin-4-amine analogues](image)

Furthermore the triazolo-purinones, a tricyclic extension of the xanthine scaffold, were synthesised and showed promising affinity at the adenosine $A_1$ receptor.
with a significant degree of selectivity for the adenosine $A_1$ receptor versus the adenosine $A_{2A}$ receptor (Gaida et al., 1997; Moro et al., 2006).

![Triazolo-purinones](image)

Another class of simplified analogues structurally related to the xanthine scaffold is the pyrozolo[1,5-$\alpha$]pyridine analogues which showed favorable affinity and selectivity for the adenosine $A_1$ receptor compared to the adenosine $A_{2A}$ receptor (Akahane et al., 1996).

![Pyrozolo[1,5-$\alpha$]pyridine analogue](image)

The first promising scaffold for the adenosine $A_{2A}$ receptors was the pyrozolo-triazolo-pyrimidines. Unfortunately, these analogues presented poor water solubility and as such poor bioavailability. ZM-241385, (4-[2-[[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-$\alpha$][1,3,5]triazin-5-yl]-amino]ethyl]phenol) was the solution to the bioavailability problem, but had low affinity for the adenosine $A_1$ receptor. Not only did ZM-241385 have favourable water solubility, it was also a potent adenosine $A_{2A}$ receptor antagonist (Poucher et al., 1995) with a dissociation constant ($K_i$) value of 0.8 nM (Ongini et al., 1999).
Compounds containing the imidazo[1,2-α]pyridine ring system have been shown to possess a broad range of useful pharmacological properties, including antibacterial, antifungal, anthelmintic, antiviral, antiprotozoal, anti-inflammatory, anticonvulsant, anxiolytic, hypnotic, gastrointestinal, antiulcer, and immunomodulatory activities (Abignente et al., 1992; Al-Tel et al., 2011, Gueiffier et al., 1998; Lange et al., 2001). Until recently imidazopyridines have only been described in patent literature for their selective adenosine A₁ antagonistic properties (Beresis et al., 2003). Reutlinger and colleagues (2014) synthesised two imidazopyridine compounds (Figure 1–1) which possess affinity for the adenosine A₁ receptor. These compounds displayed an 84% and 89% binding affinity at 100 µM respectively.

Figure 1-1: Imidazo[1,2-α]pyridine analogues exhibiting adenosine A₁ receptor affinity (Reutlinger et al., 2014).

The compound with the highest binding affinity (89% at 100 µM for the adenosine A₁ receptor) served as the lead compound in this pilot study, and was used to design and synthesise a series of para-phenyl substituted imidazo-[1,2-α]pyridine analogues.
1.3 AIMS AND OBJECTIVES

The main objective of this pilot study is to gain insight into the optimisation of existing imidazo[1,2-α]pyridine structural templates to obtain other new potent adenosine receptor antagonists that may be used as symptomatic and disease modifying treatments in AD and PD. The aims and objectives of this study are summarised below:

- Imidazo[1,2-α]pyridine analogues (Figure 1–2) will be synthesised using a modified catalyst-free three-compound synthesis reaction (Adib et al., 2007).
- The synthesised imidazo[1,2-α]pyridine analogues will be verified with proton (\(1^H\)) and carbon (\(13^C\)) nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) and melting points (mp).
- The purities of the synthesised imidazo[1,2-α]pyridine analogues will be determined by high-performance liquid chromatography (HPLC).
- Commercially bought imidazo[1,2-α]pyridine analogues will be used to further explore the structure-activity relationships (SAR) of the imidazo[1,2-α]pyridine scaffold at position C2 alone and position C2 in combination with position C3.
- The affinities for both the adenosine A\(_1\) and A\(_{2A}\) receptor subtypes for all the test compounds (both synthesised and commercially bought) will be determined \textit{in vitro} using radioligand binding studies described in the literature (Van der Walt & Terre'Blanche, 2015).
- The compound with the most promising affinity for the adenosine A\(_1\) receptor will be subjected to a GTP shift assay in order to determine its functionality as either an adenosine A\(_1\) receptor agonist or antagonist.

![Figure 1-2: The proposed para-phenyl substituted imidazo[1,2-α]pyridine analogues that will be synthesised, characterised and analysed during this study.](image-url)
1.4 CONCLUSION

The remainder of this dissertation is set out as follows. Chapter 2 is a literature review describing cognitive dysfunction in neurodegenerative disorders, specifically AD and PD. The literature reviews of AD and PD will describe each disorder’s pathology, epidemiology and treatment. Chapter 3 is a literature review of adenosine, the adenosine receptors and the role of adenosine in the treatment of AD and PD. Chapter 4 describes the experimental section of this study including the synthesis and biological evaluation of the selected imidazo[1,2-α]pyridine analogues. The final chapter, Chapter 5, contains the final conclusions based on the results obtained and proposals for future studies.
1.5 REFERENCES


CHAPTER 2

NEURODEGENERATIVE DISORDERS

2.1 INTRODUCTION

Neurodegenerative disorders are associated with distinct pathological changes in the brain and comprise of cellular inclusions, extracellular protein inclusions, and deviations in cellular morphology (Armstrong et al., 2005). These disorders are generally diagnosed when an individual displays a specific clinical profile in conjunction with the presence or absence of a specific legion associated with a particular neurodegenerative disorder (Alzheimer, 1911; Armstrong et al., 2005; Lewy, 1912). Neurodegenerative disorders include: Alzheimer's disease (AD), Parkinson's disease (PD), Pick's disease, dementia with Lewy bodies, fatal familial insomnia and multiple system atrophy (Armstrong et al., 2005). A brief overview of AD and PD is provided in this chapter.

2.2 ALZHEIMER'S DISEASE (AD)

AD is an age-related, progressive neurodegenerative disorder and was first described by Alois Alzheimer in 1906 (Alzheimer, 1911). This disorder causes the irreversible loss of neurons in the hippocampus and cortex (Alzheimer, 1911). Clinically the disorder is recognised as the progressive deterioration of memory, decision-making, language, proprioception and judgement (Alzheimer, 1911; McKhann et al., 1984). AD affects approximately 1% of individuals 65-69 years of age with the prevalence's increasing to as much as 50% in individuals 95 years and older (Hy & Keller, 2000). There appears to be a lag period of one or two decades from the pathological process to the time symptoms of AD becomes evident (Dubois et al., 2010; Jack et al., 2010; Mormino et al., 2009; Perrin et al., 2009). The average age of disease onset is approximately 80 years but early-onset AD disease may occur in individuals as young as 60-65 years of age (Campion et al., 1999; Helmer et al., 2001). The majority of AD cases are idiopathic in nature with only a small percentage being caused by a familial history (Campion et al., 1999).
2.2.1 Pathology

Pathologically AD is known for the formation of senile plaques containing β- amyloid in the extracellular space and neurofibrillary tangles that contain hyperphosphorylated tau proteins within neurons (Goedert et al., 1988; Wischik, 1988). The extensive range of structural and functional alterations in the brain may act as prognostic and diagnostic biomarkers that may be important in identifying AD. A definitive diagnosis of AD can only be made post-mortem, thus a patient is thought to have AD if they display the clinical hallmarks of the disorder and other causes can be ruled out (Clark et al., 1998).

The pathological hallmarks of AD start in the entorhinal cortex and hippocampus, but as the disease progresses the temporal, parietal and frontal cortices are included (Braak & Braak, 1991; Braak & Braak, 1996; Hyman et al., 1984). The majority of the cholinergic receptors (Figure 2–1) in the hippocampus and cerebral cortex originate from the cholinergic cells of the ventral forebrain (Growdon, 1992). The neuronal loss within the basal forebrain nuclei causes a reduction in the production of acetyltransferase ultimately contributing to the cholinergic deficiencies in AD (Cummings, 2001). This may be attributed to the enzymatic activity of acetyltransferase that is transported from the basal forebrain to the cortex producing acetylcholine (Ach). These cells form part of the presynaptic cholinergic system. The postsynaptic cholinergic receptors remain predominantly undamaged (Cummings, 2001). Degeneration of these neuronal cells takes place relatively early on in the disorder (Growdon, 1992). Therefore the behavioural, memory and cognitive deficiencies are caused by the loss of cholinergic neurons in the basal forebrain (Cummings, 2001; Francis et al., 1999).

ACh is however not the only neurotransmitter that is involved in AD. The neuronal loss in AD also leads to a decline in other neurotransmitters such as norepinephrine (NE) and 5-hydroxytryptamine (5-HT) (Cummings, 2001; Price et al., 1998). The reduction of NE and 5-HT are brought on by the death of the noradrenergic and serotonergic neurons that contribute to the behavioural changes seen in individuals with AD (Cummings, 2001).
2.2.2 SYMPTOMATOLOGY

The symptomatology of AD includes deficiencies in recent memory, visuospatial disturbances and language abnormalities that may range from mild to moderate (Figure 2–2) (Cummings, 2001).

Figure 2-1: Cholinergic innervation in the brain, known as the cholinergic pathways (Breedlove & Watson, 2013).

Figure 2-2: Schematic representation demonstrating the functions of the cerebral cortex (Waugh & Grant, 2009).
2.2.2.1 Memory deficiencies

Memory may be defined as the process of encoding, keeping and recovering information from various stimuli (Jahn, 2013). AD patients are usually the first to notice deficiencies in their recent memory and will seek help from clinicians. As the disorder progresses, the memory deficiencies may develop into greater cognitive impairment (Jahn, 2013). The loss of cholinergic neurons causes atrophy of the hippocampus and cerebral cortex (Figure 2–3) (Perry et al., 1978). The hippocampus is responsible for processing short- and long-term memory, and the atrophy of this region is consistent with the deficiencies in memory seen in individuals with AD and mild cognitive impairment (Devanand et al., 2007; Du et al., 2001; Jahn, 2013).

The adenosine A_1 receptors are highly expressed in the hippocampus, which play a significant role in memory formation (Costenla et al., 1999; Rivkees et al., 1995). Antagonists of the adenosine A_1 receptor have displayed cognitive improvements in rodent models of AD (Maemoto et al., 2004).

Figure 2-3: Atrophy of the brain in patients with Alzheimer's disease compared to the brains of healthy individuals (NIH National Institute on aging, 2016).
2.2.2 Cognitive dysfunction

AD is the most prevalent neurodegenerative disorder in the world and presents with deficiencies in memory together with the presence of cognitive defects. Cognitive defects are an inclusive term describing impairment in memory, language, visuospatial skills, decision-making, problem-solving and planning (Albert et al., 2011).

The adenosine A₁ receptors are highly expressed in the neocortex, prefrontal cortex, cerebellum, the dorsal horn of the spinal cord and the CA1 and CA3 regions of the hippocampus. These brain areas are important regions for cognitive function (Fredholm et al., 2001; Onodera & Kogure, 1988; Ribeiro et al., 2002). In AD the hippocampus and striatum show reduced levels of the adenosine A₁ receptor (Fastbom et al., 1987; Jaarsma et al., 1991; Ulas et al., 1993). Current treatment regimens of AD lack the ability to effectively improve cognitive dysfunction, thus necessitating the development of other treatment approaches (Mohs et al., 1985; Friedman et al., 1999).

2.2.2.3 Neuropsychiatric symptoms

There are various neuropsychiatric symptoms that may accompany the recognisable symptoms of AD and include: depression, apathy, anxiety, psychosis, hallucinations, agitation, sleep disturbances and wandering.

2.2.2.3.1 Depression

Depression may occur in approximately half of the patients with AD. Major depression is far less frequent with only approximately 6-10% of patients being affected (Cummings, 2001; Devanand et al., 1997; Greenwald et al., 1989). The depressive symptoms are most commonly treated with the selective serotonin reuptake inhibitors (SSRI’s) (Cummings, 2001; Nyth & Gottfries, 1990; Taragano et al., 1997). Venlafaxine (a combined noradrenergic and serotonergic reuptake inhibitor) and nortriptyline (a tricyclic antidepressant) have also been used (Petracca et al., 1995; Reifler et al., 1989). Adverse effects of the SSRI’s include drowsiness, fatigue, orthostatic hypotension, decreased sexual arousal and sexual dysfunction (Taragano et al., 1997), while the tricyclic antidepressants may cause confusion, disorientation, nausea and diarrhoea (Katona et al., 1998).
Adenosine $A_{2A}$ receptor antagonists have displayed antidepressant properties in rodent models of depression (Yacoubi et al., 2001) and may find therapeutic value in AD-related depression. The antidepressant effect is most likely due to the interaction these receptors have with the dopaminergic neurotransmission in the frontal cortex (Yacoubi et al., 2003). The adenosine-dopamine receptor-receptor interactions will be discussed in more detail in Chapter 3.

2.2.2.3.2 Apathy

Apathy is defined as a lack of motivation that persists over a period of time and is the most common neuropsychiatric symptom in AD occurring in up to 65-92% of patients (Mega et al., 1996). AD patients that experience apathy has a greater level of functional disability thus increasing caregiver burden (Landes et al., 2001). Apathy symptoms may manifest as reduced initiation and persistence as well as loss of interest, indifference, little social engagement, lack of insight and a diminished emotional response. These symptoms may be present at early stages of AD, deteriorating as the disease progresses, possibly due to neuronal damage of the frontal lobes or their subcortical connections (Bózzola et al, 1992; Burns et al., 1990; Cummings & Back, 1998). Apathy may be improved with cholinesterase inhibitors (Cummings et al., 2008; Herrmann et al., 2008; Padala et al., 2007).

2.2.2.3.3 Anxiety

Anxiety may occur in 40-50% of AD patients and the majority of these patients do not require pharmacological intervention (Cummings, 2001). When pharmacological interventions are required, the non-benzodiazepine anxiolytics like buspirone are favoured whereas the benzodiazepines are avoided due to their effects on cognition (Cummings, 2001).

2.2.2.3.4 Psychosis

Psychosis may be experienced by up to 50% of patients with AD (Cummings, 2001). As the disease progresses the incidence of delusions and psychosis increases too (Devanand et al., 1997). Neuroleptic drugs such as haloperidol and risperidone may help alleviate the psychotic symptoms (Katz et al., 1999; Schneider et al., 1990).
2.2.3.5 Agitation

Agitation occurs in up to 70% of AD sufferers. The frequency and intensity increase as the disease progresses (Cummings, 2001). Medications that may be helpful in the management of anxiety include anxiolytics, anticonvulsants with mood stabilising effects, antipsychotics, trazodone, and beta-blockers (Cummings, 2001). Neuroleptics, anticonvulsants and trazodone have shown to be the most efficacious in the treatment of AD-related agitation (Cummings, 2001).

2.2.3 EPIDEMIOLOGY

The cause of AD remains unknown although various contributing mechanisms have been proposed. These mechanisms include metabolic, inflammatory, mitochondrial, neuronal, cytoskeletal and age-related alterations that increase an individual's risk of developing the disorder (Herrup, 2010; Pimplikar et al., 2010).

2.2.3.1 Genetic risk factors

A small number of AD cases are linked to genetic factors. One of the genetic risk factors is deviations of the apolipoprotein (APOE) E4 genotype (Strittmatter & Roses, 1996). Patients with deviations in the presenilin (PSEN) 1 and PSEN 2 genes result in early-onset of AD (before 65 years of age) (Schellenberg, 2006; Selkoe, 2001). Contradictory to that, deviations in the β-amyloid precursor protein (βAPP) and APOE E4, cause late-onset of the disorder (Bertram et al., 2010; Saunders et al., 1996).

2.2.3.2 The cholinergic hypothesis of AD

The cholinergic hypothesis proposes that the cognitive symptoms associated with AD and old age are due to the cholinergic dysfunction in the central nervous system (CNS) (Bartus et al., 1982). The use of tacrine in the treatment of AD, supported the cholinergic hypothesis of AD. First of all it suggested that the symptoms of AD can be improved by pharmaceutical drugs, secondly it emphasised the association between the cholinergic function and cognitive symptoms of AD and lastly it revealed the need for the development of superior cholinesterase inhibitors (Davis et al., 1992). Although the cholinergic hypothesis has merit, it is not supported since scopolamine-induced cholinergic blockade does not correlate with the cognitive symptoms of AD (Beatty et al., 1986; Flicker
et al., 1992; Litvan et al., 1995). Treatment with the cholinesterase inhibitors do not halt the progression of AD, however, it does delay patient institutionalisation and as such decreases the cost of living with AD and prolong a patient’s quality of life (Bartus et al., 1985; Bartus, 2000).

2.2.3.3 The β-amyloid cascade hypothesis of AD

Amyloid is a general term used to describe fibrillar protein assemblies that bind to dyes and are visible under polarised light (Ferreira et al., 2007). As mentioned earlier, AD is pathologically known for the accumulation of extracellular senile plaques that contain β-amyloid and neurofibrillary tangles. (Cummings, 2001). When the βAPP is enzymatically cleaved by proteases enzymes, especially the γ-secretase enzyme, the β-amyloid peptide has the ability to form insoluble toxic fibrils that accumulate in the senile plaques in AD patients (Iwatsubo et al., 1994).

The amyloid cascade hypothesis of AD suggests that accumulation of β-amyloid peptides in senile plaques in the brains of individuals with AD are the driving force for the pathology seen in AD (Hardy & Selkoe, 2002). It is thought that the deposition of tau proteins in neurofibrillary tangles only occur after the deposition of β-amyloid plaques (Hardy et al., 1998). Studies have shown that the accumulation of β-amyloid is the chief stimulus in AD and that the remaining pathology results because of the imbalance between β-amyloid production and clearance (Bales et al., 1997; Lewis et al., 2001; Olson et al., 2001; Rapoport et al., 2002). Although many studies support this hypothesis there are various limitations, for example the degree of β-amyloid deposits do not show a direct relationship with the cognitive dysfunction experienced by patients (Lue et al., 1999; McLean et al., 1999; Wang et al., 1999).

2.2.3.4 The β-amyloid oligomer hypothesis of AD

The CNS neurotoxin, β-amyloid oligomer, inhibits the functional synaptic plasticity of neurons resulting in AD-related memory loss. Persistent exposure to this neurotoxin leads to neuronal death (Lambert et al., 1998). The oligomer hypothesis of AD suggests that memory loss early on in the disorder is caused by the accumulation of β-amyloid oligomer, with later stages of the disorder being attributed to the neurodegenerative effect of the oligomer (Lambert et al., 2009). Individuals with type 2 diabetes, traumatic brain injuries or patients
receiving general anaesthesia have shown increased β-amyloid oligomers. The latter observations indicate possible risk factors for the development of AD (Eckenhoff et al., 2004; Johnson et al., 2009; Luchsinger, 2008).

### 2.2.3.5 The tau hypothesis of AD

In addition to β-amyloid oligomer contributing to memory deficiencies in AD, it also contributes to the neuropathology, for example, instigating the hyperphosphorylation of tau proteins (Klein, 2006).

The neurofibrillary tangles that contain the hyperphosphorylated microtubule-associated tau proteins are conformed in paired helical filaments making them insoluble neurofibrillary tangles that contribute to the pathology of AD (Grundke-Iqbal et al., 1986; Selkoe, 2001). The tau hypothesis of AD postulates that under pathological conditions, tau proteins self-aggregate into paired helical filaments and as AD progresses these paired helical filaments turn into neurofibrillary tangles, ultimately resulting in neuronal death (Berger et al., 2007; Delacourte, 2006). After neuronal death, tau oligomeric species and filaments are released causing the activation of microglia. The activation of microglia causes an unusual reactivation cycle to occur through a positive feedback mechanism thus activating an inflammatory cascade and causing neuronal death (Morales et al., 2010; Fernandez et al., 2008).

### 2.2.3.6 Cellular dysfunction

The brain is highly susceptible to damage caused by oxidative stress (Butterfield et al., 2001; Markesbery, 1997) and research has shown that oxidative stress plays a role in neurodegenerative disorders, ischemia and cancers (Butterfield et al., 2007). Mitochondrial dysfunction is present in AD and leads to oxidative stress (Beal, 2005; Blass, 2000; Cardoso et al., 2004; Lin & Beal, 2006) in the hippocampus and parietal cortex and contributes to the pathology of AD (Butterfield, 2002; Hensley et al., 1995; Markesbery, 1997; Perry et al., 2002).

### 2.2.4 Treatment

The symptomatic treatment of AD mainly includes three pharmaceutical classes namely antioxidants, cholinesterase inhibitors (ChEIs) and psychotrophic drugs (Cummings, 2001; Francis et al., 1999). Although cognitive decline and disease progression continue after treatment has started, the functionality and quality of...
life of patients receiving treatment are better than patient’s who do not receive treatment (Cummings, 2001).

2.2.4.1 Cholinesterase inhibitors

The role of ChEIs in AD is the inhibition of the intrasynaptic activity of acetylcholine esterase. Hereby the elimination of ACh in the synapses is prevented and the probability of postsynaptic stimulation is increased (Cummings, 2001; Nordberg & Svensson, 1998). The following ChEIs have been approved for the treatment of AD: tacrine, donepezil, rivastigmine and galantamine (Cummings, 2001). Tacrine is linked to hepatotoxicity and as such regular liver function tests are required (Cummings, 2001), whilst rivastigmine is associated with weight loss and monitoring of a patient’s weight is necessary (Corey-Bloom et al., 1998; Cummings, 2001).

Patients receiving treatment with ChEIs show improvement in cognition, memory, behaviour, activities of daily living and even neuropsychiatric symptoms (Cummings, 2001). The beneficial use of the ChEIs are limited as their efficiency decrease with the deterioration of the presynaptic neurons (Cummings, 2001).

\[
\text{Tacrine} \quad \text{Donepezil}
\]

\[
\text{Rivastigmine} \quad \text{Galantamine}
\]

2.2.4.2 Antipsychotic drugs

The antipsychotic drugs risperidone, olanzapine and haloperidol have shown reductions in behavioural symptoms (Cummings, 2001). The most common side effect of antipsychotics is their sedative effect (Cummings, 2001).
Antioxidants may be beneficial in the treatment of AD, since they reverse the neurotoxic damage caused by oxidative stress (Behl & Sagara, 1997; Cummings, 2001; Halliwell & Gutteridge, 1985; Yoshida et al., 1985). Studies have shown that treatment with antioxidants such as selegiline or vitamin E prolong the time of deterioration that is caused by disease progression. However, simultaneous treatment with selegiline and vitamin E showed no improvement compared to single treatment (Cummings, 2001). The treatment with vitamin E may be associated with a proneness to bruises and bleeding (Cummings, 2001). Selegiline may cause anxiety, insomnia, syncope, falls and psychosis (Sano et al., 1997).

Ginko biloba has displayed minor improvement in cognitive function. An extract of the subtropical tree’s leaf has presumably antioxidant, neurotrophic and anti-inflammatory properties (Oken et al., 1998).

In a study conducted by Tang and co-workers (1996), they found that women receiving oestrogen replacement therapy for at least a year had a significantly reduced risk of developing AD. This may suggest that oestrogen therapy in women with AD may be beneficial, although other studies have shown that oestrogen has no beneficial effects on behaviour or cognition (Cummings, 2001).
2.2.4.6 Non-steroidal anti-inflammatory drugs

Inflammation plays a role in AD (Herrup, 2010; Pimplikar et al., 2010). Non-steroidal anti-inflammatory drugs (NSAIDs) are inhibitors of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), leading to a decrease in the synthesis of pro-inflammatory prostaglandins (Rao & Knaus, 2008). A study conducted by Daniels and colleagues (2016) found that the fenamate class of NSAIDs displayed an improvement of cognitive impairments in rodent models of AD.

2.2.4.7 Neuroprotective therapy

Although various AD treatment regimes are available to target a specific mechanism, rescuing the susceptible neurons from neurodegeneration are still lacking (Kim et al., 2007).

The adenosine A\textsubscript{2A} receptor antagonists are attractive as neuroprotective therapeutic agents in AD because they display neuroprotection of the cortical neurons (Geiger et al., 2006). Caffeine, a non-selective antagonist of the adenosine A\textsubscript{1} and A\textsubscript{2A} receptors, displayed neuroprotective properties in rodent models of AD by preventing β-amyloid-induced neurotoxicity (Maia & Mendonça, 2002). The neuroprotective effect may be attributed to the antagonism of the adenosine A\textsubscript{2A} receptors, rather than the adenosine A\textsubscript{1} receptors (Dall'Igna et al., 2007).

2.2.5 SUMMARY

AD is a devastating and debilitating disorder affecting an increasing number of individuals. Current treatment is limited to symptomatic treatment but does not prevent neurodegeneration or disease progression. The adenosine receptor antagonists have potential in the treatment of AD as it displays neuroprotective and cognitive enhancing effects. The role of the adenosine A\textsubscript{1} receptor antagonists as cognitive enhancing therapy and the adenosine A\textsubscript{2A} receptor antagonists as neuroprotective and antidepressant therapy in AD will be discussed in more detail in Chapter 3.
2.3 PARKINSON’S DISEASE (PD)

PD, initially named shaking palsy or paralysis agitans, was first described by James Parkinson in his 1817 Essay; *An Essay on the Shaking Palsy*. PD can be described as a chronic, slow progressing, age-related neurodegenerative disorder (Parkinson, 2002). It is the second most common neurodegenerative disorder following AD with approximately 1-2% of the middle-aged and elderly population suffering from the disorder (De Lau & Breteler, 2006; Olanow et al., 2009). The disorder typically affects individuals older than 60 years and as the life expectancy of the population increases it is anticipated that prevalences of the disorder will also increase (Olanow et al., 2009). Individuals diagnosed with PD younger than 50 years of age are known as early-onset PD. In a small number of patients, PD is genetic and where there is no obvious genetic link it is known as sporadic or idiopathic PD (Tanner et al., 1999).

2.3.1 PATHOLOGY

Pathologically PD is recognised by the intense and progressive loss of pigmented DA neurons in the substantia nigra *pars compacta* (SNpc). The loss of dopaminergic neurons lead to a decrease in DA levels in the striatum. The loss of DA is also accompanied by the presence of interneuronal, cytoplasmic, aggregated and phosphorylated protein structures, known as Lewy bodies (LBs) (Lewy, 1912; Savitt et al., 2006).

2.3.1.1 The nigrostriatal dopaminergic pathway

The basal ganglia control the voluntary movement of the body by integrating sensorimotor information into voluntary movement (Alexander et al., 1986). The basal ganglia consist of the striatum, globus pallidus, subthalamic nucleus (STN), SNpc, substantia nigra *pars reticulata* (SNpr) and the thalamus. The striatum is composed out of the caudate nucleus and the putamen, whilst the globus pallidus consist of the internal and external segments (*Figure 2–4*) (Graybiel, 2000; Guilarte, 2010).

The structures of the basal ganglia are predominantly regulated by DA. When a loss of dopaminergic neurons occur altered responses of these structures takes place, giving rise to the key motor symptoms of PD (Sapir et al., 2014).
Figure 2-4: A schematic representation of the basal ganglia and the different brain structures that it consists of, illustrated in the right hemisphere of the brain. The SN neurons that innervate the caudate nucleus and the putamen are illustrated in the left hemisphere of the brain. Abbreviations: SN (Substantia Nigra); STN (Subthalamic Nucleus) (Guilarte, 2010).

The nigrostriatal system controls cognition and motivation, whilst playing an important role in the release of hormones and motor functions. These functions of the nigrostriatal system are commenced when DA is released presynaptically from the axon terminals of neurons in the striatum or from dendrites in the substantia nigra (SN) into the synaptic cleft. From there, the released DA interacts with DA receptors that are located postsynaptically. These functions are terminated by the dopamine transporter (DAT) when DA is taken up (Amara & Sonders, 1998; Iversen, 1975).

The DAT has the ability to transport DA as well as neurotoxins into dopaminergic neurons, for example 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) (Kitayama et al., 1992; Pifl et al., 1993). This reuptake of neurotoxins into the dopaminergic neurons may lead to PD-associated symptoms (Lehmensiek et al., 2002).

Within the normal nigrostriatal dopaminergic pathway (Figure 2–5 A) the cell bodies of neurons that contain DA are located within the SNpc (Bertler & Rosengren, 1959; Dauer & Przedborski, 2003) and they mostly ascend alongside the nigrostriatal axons towards the striatum (Andén et al., 1964). There are noticeable amounts of neuromelanin present in the nigrostriatal neurons, giving the SNpc a pigmented appearance (Marsden, 1982).
Individuals suffering from PD manifest with a noticeable degeneration of the nigrostriatal dopaminergic pathway with a modest loss of the dopaminergic neurons that projects from the SNpc towards the caudate nucleus and a more obvious loss of the dopaminergic neurons from the SNpc towards the putamen (Figure 2–5 B) (Bernheimer et al., 1973; Marsden, 1982).

![Image](image.png)

**Figure 2-5:** Image (A) of the normal nigrostriatal dopaminergic pathway is represented in red. Neuromelanin containing dopaminergic neurons move from the SNpc towards the caudate nucleus and the putamen which make up the striatum. Image (B) of the nigrostriatal dopaminergic pathway in PD is degenerated, modestly in the caudate nucleus and much more evidently in the putamen. Depigmentation of the SNpc is due to the loss of neuromelanin containing dopaminergic neurons. *Abbreviations: SNpc (Substantia Nigra pars compacta). (Dauer & Przedborski, 2003).*

### 2.3.1.2 Lewy bodies

First described in 1912 by Friederich Lewy, LBs can be described as round, eosinophilic, intracytoplasmic, protein aggregate inclusions. LBs are found in normal aging brains as well as in the brains of patients with neurodegenerative diseases such as AD, PD and dementia with LBs (Lewy, 1912).
Proteins that are found copiously in the brain are proteins called synucleins. The human synuclein proteins consist of α-synuclein, β-synuclein and γ-synuclein (Galvin et al., 1999; Lavedan, 1998; Spillantini et al., 1995). In idiopathic PD α-synuclein forms a major component of LBs (Irizarry et al., 1998). Before α-synuclein can aggregate and accumulate in LBs it has to be transformed into insoluble protofibrils (El-Agnaf et al., 1998). The formation and accumulation of misfolded protein aggregates are thought to be the reason for the degeneration of dopamine neurons in PD, hereby indicating α-synuclein as a plausible cause of PD (Recasens & Dehay 2014).

2.3.2 SYMPTOMATOLOGY

PD is primarily a chronic and progressive motor system disorder with the principal symptoms being: tremor, rigidity, bradykinesia and postural instability (Parkinson, 2002). The diagnosis of PD is based on the presence of two or more of the principal motor symptoms and by excluding other causes of parkinsonism (Hughes et al., 1993; Louis et al., 1997; Rajput et al., 1991).

2.3.2.1 Motor symptoms

2.3.2.1.1 Bradykinesia

Bradykinesia is one of the symptoms necessary for the diagnosis of PD and may be the most disabling and distressing symptom, as this symptom is unpredictable and prevents patients from doing routine movements swiftly. It may be described as a loss of autonomic and spontaneous movement causing movements to be delayed even more (Goldenberg, 2008; Lees et al., 2009). It seems that there is a direct relationship between the dopaminergic deficiency in the striatum and the extent of bradykinesia (Vingerhoets et al., 1997).

2.3.2.1.2 Tremor

Tremor or trembling usually originates in the hand although the arms, legs and jaw are similarly affected. Tremor is seldom disabling and in 75% of patients, only one part or side of the body is initially affected, with a more widespread tremor occurring during later stages of the disease (Goldenberg, 2008).
2.3.2.1.3 Rigidity

Rigidity affects most patients with PD and appears when the delicate balance of opposing muscles is disturbed. This disruption causes the muscles to stay contracted and tense, causing the person to feel stiff, weak and results in aching muscles (Goldenberg, 2008; Nelson et al., 2005).

2.3.2.1.4 Postural instability

Postural instability causes patients to have impaired balance and coordination. This impairment causes them to either lean forward or backward and this can easily lead to falls. Postural instability typically develops during the late stages of the disease (Ahlskog, 2001; Nelson et al., 2005).

2.3.2.2 Non-motor symptoms

The non-motor symptoms or neuropsychiatric symptoms are very common in PD. The presence of these symptoms is accompanied by an acceleration of both cognitive and functional decline (Chaudhuri et al., 2006). Furthermore, it has a negative effect on a patient’s quality of life, causing earlier institutionalisation and a faster mortality rate (Aalten et al., 2005; Geda et al., 2013; Nelson et al., 2005; Steinberg et al., 2008). Evidence suggests that the ‘total’ burden and thus the effect on a patient’s quality of life is due to the occurrence of non-motor symptoms as a whole and not just the occurrence of one single neuropsychiatric symptom (Chaudhuri et al., 2011).

2.3.2.2.1 Depression

Depression may transpire months or even years prior to the symptoms characteristic of PD and is the most frequent non-motor symptom affecting PD patients (Reijnders et al., 2008; Remy et al., 2005; Zesiewicz et al., 1999). Although it is possible that some patients may develop depression because they have trouble adjusting to the diagnosis, this is doubtful in most cases (Witjas et al., 2002).

2.3.2.2.2 Anxiety

During the progression of PD, anxiety is present in 30-50% of patients (Leentjens et al., 2011). Clinically significant anxiety frequently co-occurs with PD-associated depression. The simultaneous manifestation of both anxiety and
depression may lead to more motor impairment, dyskinesias, increased incidences of motor fluctuations and also further reduce the patient’s quality of life (Dissanayaka et al., 2010; Leentjens et al., 2011; Pontone et al., 2009).

2.3.2.2.3 Dementia

In approximately 24-31% of patients substantial and dramatic deviations take place in the patient’s capability to reason, recollect and concentrate (Aarsland et al., 2005; Emre, 2007; Galvin, 2006; Reid et al., 2011). Dementia in PD is associated with memory and attention impairments, as well as deficiencies in the visuospatial functions that are related to behavioural symptoms, hallucinations and apathy (Emre et al., 2007).

2.3.2.2.4 Cognitive deficits

The cognitive changes may disrupt a patient’s daily life as much if not more than the motor symptoms of PD and includes thinking, memory and language defects (Marsh, 2008).

2.3.3 EPIDEMIOLOGY

As soon as DA producing neurons within the SNpc become impaired, degenerate or die, patients are unable to control their muscle activity normally, thus leading to disability and a decreased quality of life (Khudados et al., 1999; Schneider et al., 1982). Although the exact mechanisms that cause or at least contribute to the development of PD and its accompanying pathology are still unclear, various mechanisms have been proposed.

2.3.3.1 Genetic risk factors

Several studies have displayed a positive correlation between a familial history of PD and a risk of developing the disorder especially if the patient has an early age of disease onset, however, a distinct manner of inheritance has yet to be established (Lücking et al., 2000; Valente et al., 2004). Although genetic variations of PD are quite similar to idiopathic PD it does display features not typical of idiopathic PD. These include a young age of disease onset, dystonia, responsiveness to the treatment with L-3,4-dihydroxyphenylalanine (L-dopa) and usually LB pathology does not occur (Ishikawa & Takahasi, 1998; Mori et al., 1998).
2.3.3.2 **Cellular dysfunction**

Damage caused by reactive oxygen species and oxidative stress may initiate a protein metabolism dysfunction (Swerdlow et al., 1996). As seen in PD, LBs comprise of α-synuclein that has been modified by oxidation and this modification increases α-synuclein’s susceptibility to aggregate. Once proteins misfold, the cells respond by producing chaperones and when these chaperones fail to refold the proteins correctly, they undergo proteasomal degradation via polyubiquitination. As individuals age the ability of cells to produce an assortment of chaperones becomes compromised, the proteasomal degradation decreases and as a result causes an accumulation of aggregated proteins in cells (Dauer & Przedborski, 2003; Giasson et al., 2000; Jenner, 2003; Muchowski, 2002; Swerdlow et al., 1996).

2.3.3.3 **Environmental risk factors**

In 1983 individuals developed the typical symptoms of PD following the intravenous injection of drugs that contained MPTP. This phenomenon led to the hypothesis that exposure to environmental toxins may be related to the risk of developing PD (Langston & Ballard, 1983). Other environmental toxins include 6-OHDA, paraquat, and rotenone (Betarbet et al., 2000; Langston & Ballard, 1983). Occupational exposures to heavy metals (iron, copper, manganese, lead, aluminium, amalgam and zinc) have been suggested as possible risk factors for the development of PD, as these metals may accumulate within the substantia nigra (SN) leading to increased levels of oxidative stress, although evidence is still inconclusive (Jankovic, 2005; Racette et al., 2001; Seidler et al., 1996).

Living in rural areas and drinking from wells may contribute to the development of idiopathic PD albeit to a small extent (Koller et al., 1990). Individuals that use neuroleptic drugs such as haloperidol also present with parkinsonian symptoms as these medications increase both DA release and metabolism thus decreasing the amount of DA in the brain (Zetterström et al., 1984).

2.3.4 **Factors that decrease the risk of developing PD**

Curiously lifestyle choices including smoking, caffeine consumption and using NSAIDs do not contribute to the risk of developing PD, but may rather exert a protective effect.
Various epidemiological studies have shown an inverse relationship between smoking and the risk of developing PD (Grandinetti et al., 1994; Hernán et al., 2002). The possible neuroprotective effect of smoking probably involves the nicotine component present in these products (Quik, 2004).

The active component of coffee is caffeine. Caffeine is a non-selective adenosine receptor antagonist. This antagonism is associated with a decreased risk of developing PD and research has also shown improved motor function in a mouse model of PD (Grandinetti et al., 1994; Ross et al., 2000).

2.3.5 TREATMENT

Treatment is generally aimed at restoring the DA deficiency since there is still no curative treatment for PD. Initially patients respond well to L-dopa and other dopaminergic therapies, however, after 5-10 years patients develop treatment-related complications (Brooks, 2000; Hely et al., 2005). The aim of pharmacological treatments is to maintain a patient’s quality of life whilst simultaneously safeguarding their socioeconomic status for as long as possible (Aminoff, 2006; Clarke et al., 2011; Ferreira et al., 2013; Schapira & Obeso, 2006).

The symptomatic treatment of early PD currently consists of three main classes of dopaminergic drugs: L-dopa, DA agonists and selective monoamine oxidase B (MAO-B) inhibitors (Fox et al., 2011; Sprenger & Poewe, 2013). Generally the selective MAO-B inhibitors and DA receptor agonists are the first treatment options in patients not displaying cognitive impairment and with a young age of onset. The initiation of L-dopa therapy is preferred for the elderly since the risk of developing treatment-related motor complications are minimised (Hauser et al., 2007; Holloway et al., 2004; Rascol et al., 2000; Schapira & Olanow, 2008).

2.3.5.1 L-dopa

The most efficacious treatment for PD remains L-dopa, the metabolic precursor of DA (Goldenberg, 2008). Treatment with L-dopa greatly improves the motor symptoms, however, continuous treatment leads to fluctuations in treatment efficacy. This is called the ‘wearing off’ phenomenon. Initially, it can be improved by increasing the frequency and dosage of L-dopa but as L-dopa levels are elevated excessively, abnormal involuntary movements may develop known as dyskinesias (Goldenberg, 2008). The L-dopa-induced motor complications are
still poorly understood, however, factors that may contribute to the development of these treatment-related complications are possibly the short half-life of L-dopa, the erratic absorption of L-dopa with subsequently irregular delivery of the drug in the striatum and presynaptic nigral DA depletion (Hametner et al., 2010; Leenders et al., 1986).

When L-dopa is administered, most of the drug is metabolised peripherally by aromatic L-amino acid decarboxylase and not much of the drug enters the cerebral circulation and CNS (See section 2.3.5.3). For this reason it is preferred to prescribe L-dopa in combination with carbidopa or benserazide (inhibitors of aromatic L-amino acid decarboxylase) (Goldenberg, 2008).

\[
\begin{align*}
\text{L-dopa} & & \text{Carbidopa} & & \text{Benserazide} \\
\end{align*}
\]

2.3.5.2 Dopamine receptor agonists

In an attempt to minimise the incidence and severity of treatment-related motor complications as a result of long-term treatment with L-dopa, the DA receptor agonists were developed in the 1970's.

DA receptor agonists act directly on striatal DA (D₂) receptors and enzymatic conversion of these agents are not required compared to L-dopa (Sprenger & Poewe, 2013). The first agent that displayed a beneficial effect on the motor complications was the ergot derived bromocriptine (Calne et al., 1974). Examples of DA receptor agonists include the ergot derived compounds: bromocriptine, cabergoline, pergolide, lisuride and α-dihydroergocryptine and the non-ergot derived compounds: pramipexole, ropinirole, rotigotine, apomorphine and piribedil (Sprenger & Poewe, 2013). The non-ergot DA receptor agonists decrease motor fluctuations and dyskinesias without the risk of developing pulmonary and cardiac valvular fibrotic reactions as occasionally seen with the ergot derived compounds (Sprenger & Poewe, 2013).

It is hypothesised that the beneficial effects with DA receptor agonist treatment, may be attributed to the activation of the DA D₁ receptors by residual
endogenous DA that is formed by the nigrostriatal terminals not yet degenerated by the disease progression (Brooks, 2000).

![Molecular structures of Pergolide, Ropinirole, and Pramipexole]

Adverse effects that may be experienced include nausea, headaches, hallucinations, vivid dreams, delusions, mood alterations, paranoid psychosis, peripheral oedema, sleep attacks, daytime somnolence, orthostatic hypotension, and the development of impulse control disorders (Fox et al., 2011; Holloway et al., 2004; Oertel et al., 2006; Potenza et al., 2007; Rascol et al., 2000).

### 2.3.5.3 Catechol-o-methyltransferase inhibitors

Catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) is responsible for the catabolism of L-dopa and DA. COMT causes L-dopa to be transformed into 3-O-methyl-dopa and DA into 3-O-methoxytyramine (Goldenberg, 2008). The majority of the L-dopa is catabolised by aromatic L-amino acid decarboxylase into DA and decrease the amount of L-dopa that cross the blood-brain barrier. This can be prevented by the concomitant administration of carbidopa, leading to an increase of L-dopa that can be methylated by COMT. The use of COMT inhibitors such as tolcapone and entacapone inhibit the peripheral methylation of L-dopa, thus improving both the plasma half-life of L-dopa and the amount that reaches the CNS (Kurth et al., 1997). Side effects of COMT inhibitors include nausea, orthostatic hypotension, hallucinations, confusion, vivid dreams, diarrhoea, increased serum alanine aminotransferase and aspartate transaminase, while tolcapone has the ability to induce hepatotoxicity (Goldenberg, 2008).
2.3.5.4 Selective monoamine oxidase-B inhibitors

There are two isoenzymes of monoamine oxidase namely isoform A (MAO-A) and isoform B (MAO-B). MAO-B is the main isoform present in the striatum and is responsible for inactivating DA through oxidative metabolism in the brain (Goldenberg, 2008). The selective MAO-B inhibitors, for example selegiline and rasagiline, are effective as initial monotherapy to improve motor symptoms for patients experiencing mild disability and as adjunctive therapy with L-dopa when motor symptoms are no longer efficiently controlled with MAO-B inhibitors alone (Fox et al., 2011; Teo & Ho, 2013).

Generally, the MAO-B inhibitors are well tolerated by patients. An adverse effect that may be experienced when treated with selegiline is insomnia as selegiline is metabolised into amfetamine and methamphetamine (Wimbiscus et al., 2010).

2.3.5.5 Anticholinergic drugs

Another approach to treat PD is utilising anticholinergic drugs. The use of these drugs aims to restore the equilibrium between the dopaminergic and cholinergic inputs on the basal ganglia (Brocks, 1999; Schapira, 2005). The anticholinergic drugs or muscarinic receptor antagonists were used for the treatment of PD prior to the discovery of L-dopa (Brocks, 1999, Sprenger & Poewe, 2013; Goldenberg, 2008). The anticholinergic drugs that are presently still in use include trihexyphenidyl, benztpine mesylate and diphenhydramine. These drugs are used during early stages of the disease as add-on therapy to dopaminergic
treatment or to control the degree of tremor in younger patients (Goldenberg, 2008). The anticholinergic drugs may have the following adverse effects: constipation, urinary retention, and blurred vision (Schapira, 2005).

![Diphenhydramine](image)

### 2.3.5.6 Amantadine

Amantadine, an antiviral drug, is mostly used for the prevention and treatment of influenza A (Hubsher et al., 2012). Since amantadine changes DA release in the striatum and possess anticholinergic activity, it is also used to treat PD (Goldenberg, 2008). Amantadine is mostly used for the treatment of mild PD and as adjunct therapy for patients experiencing motor fluctuations and dyskinesias due to L-dopa treatment (Fox et al., 2011; Goldenberg, 2008). Amantadine improves dyskinesias likely due to its ability to block the action of the N-methyl-D-aspartate glutamate receptor, without worsening the symptoms of PD (Goldenberg, 2008; Reis et al., 2006; Wolf et al., 2010). Amantadine can be effective as long-term treatment, however, nausea, dizziness, light-headedness, livedo reticularis, leg oedema and insomnia can be experienced. (Goldenberg, 2008; Sprenger & Poewe, 2013)

![Amantadine](image)

### 2.3.5.7 Non-dopaminergic treatment

The deficiencies that are experienced with the current dopaminergic treatments emphasise the necessity to develop novel treatment regimes that not only provide symptomatic relief but also offer neuroprotection and disease altering properties.
2.3.5.7.1 Neuroprotective therapy

A neuroprotective drug has the potential to affect the pathogenesis of a disorder, thus causing a delay in the progression of the disorder or prevent the disorder completely by inhibiting the death of neuronal cells (Koller, 1997).

The selective MAO-B inhibitor, selegiline, was the first pharmaceutical drug investigated as a neuroprotective drug for PD (Koller, 1997). Caffeine, a non-selective adenosine receptor antagonist displayed neuroprotective properties in MPTP and 6-OHDA neurotoxin-induced rodent models of PD (Chen et al., 2001). Various selective adenosine A2A receptor antagonists have displayed similar clinically significant neuroprotective properties in MPTP and 6-OHDA neurotoxin-induced rodent models of PD, these include KW-6002, KF-15372, KF-18446 and SCH-58261 (Chen et al., 2001; Ikeda et al., 2002). The adenosine A2A receptor antagonists are attractive as potential neuroprotective treatments in PD as it provides neuroprotection without causing the treatment-related complications associated with the dopaminergic agents (Ikeda et al., 2002).

The role of the adenosine A2A receptor antagonists as a neuroprotective treatment in PD will be discussed in more detail in Chapter 3.

2.3.5.7.2 Adenosine A2A and A1 receptor antagonists

The adenosine receptor antagonists are currently being investigated for their effectiveness in the treatment of PD. (Aoyama et al., 2000; Shook & Jackson, 2011). The striatum, nucleus accumbens, and the olfactory tubercle are enriched with the adenosine A2A receptors, which plays an important role in the control of motor function (Kuwana et al., 1998).

KW-6002 is a xanthine-based antagonist of the adenosine A2A receptors and is currently being used as adjunctive therapy for the treatment of PD in Japan (Dungo & Deeks, 2013), after displaying efficacy in both primate and rodent animal models without producing the treatment-related complications associated with the dopaminergic drugs (Kanda et al., 1998; Shiozaki et al., 1999). In a study conducted by Ikeda and colleagues (2002), the adenosine A2A receptor antagonists displayed neuroprotective properties of the nigrostriatal dopaminergic neurons in both MPTP and 6-OHDA neurotoxin-induced rodent models of PD. Therefore, when the adenosine A2A receptors are antagonised,
the treatment of PD is twofold as it offers both motor dysfunction improvement and neuroprotection (Ikeda et al., 2002).

**KW-6002**

Adenosine A₁ receptors are also expressed in the motor regions of the brain (cerebellum and motor cortex) (Rivkees et al., 1995). When the selective adenosine A₁ receptor antagonist, 8-cyclopentyl-1,3-dimethylxanthine (CPT), was administered systemically in 6-OHDA neurotoxin-induced rodent model of PD, CPT significantly potentiated the motor functions of these animals (Popoli et al., 1996). Although adenosine A₂A receptor antagonists are the main target for the motor activating effects of caffeine in rats (Karcz-Kubicha et al., 2003), Jacobson and co-workers (1993) also found evidence of the occurrence of synergism between the motor-activating effects of adenosine A₁ and A₂A receptor antagonists. Furthermore the adenosine A₁ receptor antagonists also offer enhanced cognitive function in animal models of PD (Mihara et al., 2007).

**CPT**

Mihara and colleagues (2007) found that a dual acting adenosine A₁ and A₂A receptor antagonist, ASP 5854 (see section 3.6), improved motor function and prevented neurodegeneration through its ability to antagonise the adenosine A₂A receptors, whilst at the same time the antagonistic activity of the adenosine A₁ receptors improved cognitive dysfunction.

The role of the adenosine A₁ receptor antagonists as cognitive enhancing therapy as well as the adenosine A₂A receptor antagonists as neuroprotective and antidepressant therapy in PD will be discussed in more detail in Chapter 3.
2.3.6 **SUMMARY**

PD is a distressing and costly disorder that will affect a greater number of individuals in the future. The current available treatment only provides symptomatic relief, offering no neuroprotective or disease modifying effects. Additionally, continuous use of current treatment produces debilitating treatment-related complications, thus further decreasing a patient’s quality of life. The adenosine receptor antagonists have exhibited neuroprotective effects as well as improvement of both the motor and non-motor symptoms of PD.

2.4 **CONCLUSION**

In conclusion, both AD and PD have devastating effects on the lives of those individuals affected by these neurodegenerative disorders. An increasing number of individuals will become affected as the life expectancy of the global population increases. Although the current available treatments for both AD and PD merely provide symptomatic relief, neither the cholinergic nor the dopaminergic agents offer disease modifying or neuroprotective effects. The aforementioned deficiencies in the treatment regime of AD and PD thus necessitate future research and drug development to focus on the development of non-cholinergic and non-dopaminergic treatments. These treatments will not only provide symptomatic relief but also offer neuroprotective effects and improve cognitive function. The adenosine receptor antagonists have displayed potential in both AD and PD treatment as they have symptomatic and disease modifying effects.

The role of adenosine receptor antagonists in the treatment of AD and PD will be discussed in more detail in Chapter 3.
2.5 REFERENCES


Pramipexole vs levodopa as initial treatment for Parkinson disease: a 4-year randomized controlled trial. *Archives of neurology*, 61(7):1044-1053.


activity of adenosine A$_{2A}$ antagonists in experimental models. Advances in neurology, 80:121-123.


hippocampal formation, cerebral cortex, cerebellum, and basal ganglia. *Brain research*, 677(2):193-203.


3.1 INTRODUCTION

Adenosine is an endogenous purine ribonucleoside that is formed when adenosine triphosphate (ATP) is metabolised within the extracellular space \(\text{(Fredholm et al., 2001)}\). Adenosine has many physiological functions throughout the body as well as a role in numerous nervous system disorders including cognitive disorders, epilepsy, ischemia, stroke, AD and PD \(\text{(Gomes et al., 2011)}\). Adenosine is metabolised either by adenosine deaminase through deamination or by adenosine kinase through phosphorylation \(\text{(Hagberg et al., 1987; Zetterström et al., 1982)}\).

\[
\text{Adenosine}
\]

The effects of adenosine are mediated by four metabotropic G protein-coupled receptors and they are classified as \(A_1\), \(A_{2A}\), \(A_{2B}\) and \(A_3\) adenosine receptor subtypes \(\text{(Fredholm et al., 1994; Fredholm, 2001)}\). These adenosine receptors consist of seven transmembrane α-helical domains that are connected through intracellular and extracellular loops \(\text{(Fredholm et al., 1994; Fredholm, 2001; Lefkowitz, 2004)}\). The role of the adenosine receptors in neurodegenerative disorders are predominantly mediated by the adenosine \(A_1\) and \(A_{2A}\) receptors \(\text{(Stone et al., 2009)}\). To follow is a discussion highlighting the role of the adenosine \(A_1\) and \(A_{2A}\) receptors in AD and PD.
3.2 ADENOSINE AND THE CHOLINERGIC SYSTEM

The adenosine system has an inverse relationship with the cholinergic system as described in several studies. In these studies, acetylcholine (ACh) release was found to be decreased with age whereas the concentration of adenosine was increased (Cunha et al., 2001). Patients with AD compared to elderly controls show a significant reduction in choline acetyltransferase activity (Wilcock et al., 1982). Biopsies performed on AD patients have shown a reduced synthesis of ACh (Bowen et al., 1982). Furthermore, individuals with AD have displayed a severe loss of the cholinergic neurons in the neuronal region that forms the main cholinergic input into the cortex (Whitehouse et al., 1981). In a study conducted by Phillis and co-workers (1980) the administration of theophylline (a non-selective adenosine receptor antagonist) enhanced the release of ACh in the rat cerebral cortex. Rodent models have shown that the administration of caffeine increased the prefrontal cortex concentration of ACh (Acquas et al., 2002). This effect is most likely brought on by blockade of the adenosine A<sub>1</sub> receptors, such as seen within in vitro and in vivo studies displaying that the adenosine A<sub>1</sub> receptors have multiple regulation sites of cholinergic neuronal activity (Broad & Fredholm, 1996).

3.3 ADENOSINE AND THE DOPAMINERGIC SYSTEM

Since dopamine plays a role in both the central nervous system (CNS) and peripheral functions, it is not surprising that changes in dopaminergic signalling are associated with various disorders, such as PD, depression, dyskinesias, schizophrenia and hypertension (Boyd & Mailman, 2012). Furthermore, dopamine-mediated responses may be modulated by adenosine receptors and it is suggested that they play a major role in the control of PD motor behaviour (Pinna et al., 2005).

The mechanism by which the adenosine agonists and antagonists can modulate the motor activity may be a result of the antagonistic intramembrane A<sub>2A</sub>-D<sub>2</sub> and A<sub>1</sub>-D<sub>1</sub> interactions (Ferré et al., 1991; 1997; 1998). Two pathways exist in the striatum that is important for motor behaviour, namely the direct and indirect pathway. The adenosine A<sub>2A</sub> receptors are co-localised with the dopaminergic D<sub>2</sub> receptors in the indirect pathway and the adenosine A<sub>1</sub> receptors are co-localised with the DA D<sub>1</sub> in the direct pathway (Fuxe et al., 2007a,b). Stimulation
of the direct pathway leads to motor activation (initiate movement) and stimulation of the indirect pathway leads to motor inhibition, whereas inhibition of the indirect pathway will alleviate motor inhibition (Fuxe et al., 2007a,b).

A patient with PD has depleted DA neurons in the striatum leading to a surplus excitation of the neurons in the direct pathway and an over-inhibition of the thalamocortical and brainstem motor centres (Kravitz et al., 2010). Thus, it is hypothesised that the motor symptoms of PD may occur due to an overactive indirect pathway and underactive direct pathway (Albin et al., 1989; Mallet et al., 2006). Bilateral excitation of the medium spiny projection neurons of the indirect pathway provokes a parkinsonian state, meaning a person will experience more freezing and bradykinesia with reduced levels of locomotor activity. Whereas the activation of the medium spiny projection neurons of the direct pathway will reduce freezing of movement and bradykinesia and improve locomotion, thus providing an antiparkinsonian effect (Kravitz et al., 2010). By modulating the direct pathway within the basal ganglia through the antagonistic adenosine-dopamine receptor interaction, the adenosine receptor antagonists may have potential as an effective therapeutic strategy for improving the motor symptoms of PD (Ferré et al., 1991; Kravitz et al., 2010).

3.4 ADENOSINE A2A RECEPTORS

The adenosine A2A receptors are densely expressed in the basal ganglia predominantly within the striatum where it is co-localised with the DA D2 receptors (Fredholm et al., 2003; Shook & Jackson, 2011; Svenningsson et al., 1999). The adenosine A2A receptors are also located in the striatopallidal neurons, olfactory bulb, olfactory tubercle, astrocytes, microglia and blood vessels of the brain (Fredholm et al., 2001; Ribeiro et al., 2002).

3.4.1 ADENOSINE A2A RECEPTOR ANTAGONISTS IN THE TREATMENT OF AD AND PD

3.4.1.1 Neuroprotective therapy

As mentioned previously adenosine has a role in nervous system disorders such as AD and PD (Gomes et al., 2011). A possible explanation for the neuroprotective effects of the adenosine A2A receptor antagonists might be due to their anti-inflammatory properties (Geiger et al., 2006). Furthermore, when these receptors are inhibited, they have neuroprotective functions preventing the
apoptosis of neuronal cells (Cunha, 2005; Silva et al., 2007) and have the ability to modulate the release of other neurotransmitters. One such a neurotransmitter is glutamate. Glutamate has the ability to induce excitotoxic neuronal damage during ischemic as well as non-ischemic circumstances (Choi, 1994). Antagonists of the adenosine A$_2A$ receptor may thus provide neuroprotection by inhibiting the release of glutamate (Popoli et al., 1995). Neuroprotection by means of the adenosine A$_2A$ receptor antagonists may also be provided through other mechanisms of action besides regulating glutamate release and include: vasodilatation, preventing the formation of neutrophil superoxide, preventing platelet aggregation, inhibiting cytokine release and reducing microglia activation (Ongini et al., 1997).

The adenosine A$_2A$ receptors have an increased expression within the hippocampus, cerebral cortex and microglial cells in individuals with AD (Angulo et al., 2003). The adenosine A$_2A$ receptors may have a neuroprotective effect in AD since it increase the resistance of neurons to insults and the adenosine A$_2A$ receptor blockade prevents the neurotoxicity caused by β-amyloid (Dall'Igna et al., 2003). The consumption of caffeine containing beverages (such as coffee) has displayed a protective effect when it comes to the development of AD and PD. Caffeine, a non-selective adenosine antagonist, exhibited a neuroprotective effect probably due to the antagonism of the adenosine A$_2A$ receptors. (Dall'Igna et al., 2007).

Dopaminergic neuronal damage in animal models of PD (induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA)) has shown that the antagonism of the adenosine A$_2A$ receptors also exert neuroprotective effects (Chen et al., 2001; Ikeda et al., 2002). Further studies have shown that blocking the adenosine A$_2A$ receptors reduces the nigrostriatal dopaminergic damage caused by the administration of the irreversible mitochondrial complex II inhibitor, 3-nitropropionic acid (Blum et al., 2003). Alfinito and colleagues (2003) also demonstrated the neuroprotective function of adenosine A$_2A$ receptor antagonists after malonate (a mitochondrial complex II inhibitor) induced neuronal damage.

The adenosine A$_2A$ receptor antagonists may be used for the treatment of AD and PD because it offers a disease-modifying therapeutic approach that current treatment is still lacking (Schwarzschild et al., 2006).
3.4.1.2 Depression

Although PD is mainly a motor deficit disorder, various non-motor symptoms such as anxiety and depression are also present. These symptoms are still inefficiently treated in PD and as such contribute to a more rapid progression of the disease, a decreased quality of life and higher mortality rates (Gallagher & Schrag, 2012).

As mentioned before adenosine has many physiological functions in the CNS which include mood alterations such as anxiety and other cognitive processes (El Yacoubie et al., 2000; Kopf et al., 1999). When adenosine receptors are stimulated it induces a state of ‘learned helplessness’ within animal models of depression (Minor et al., 1994). Furthermore, it has been found that classic antidepressants reverse this adenosine-induced depression in mice (Kulkari & Mehta, 1985). The depressant effect of the adenosine receptors is likely due to the antagonistic $A_{2A}-D_2$ intramembrane receptor-receptor interaction that takes place within the striatum as both are elevated in patients with depression (D’haenen & Bossuyt, 1994; Laasonen-Balk et al., 1999).

In a study conducted by Yacoubi and co-workers (2001) inhibition of the adenosine $A_{2A}$ receptors showed potential for the treatment of depression. In their study, the adenosine $A_{2A}$ receptor antagonist induced an antidepressant effect in mice at lower doses than used with traditional antidepressants like fluoxetine and imipramine. Moreover, their study found that adenosine $A_{2A}$ receptor antagonists decrease the immobility time of mice during both the forced swim test and the tail suspension test.

3.4.1.3 Motor function

The adenosine $A_{2A}$ receptors are involved in motor function and the development of habitual behaviours (Yin & Knowlton, 2006). The adenosine $A_{2A}$ receptor antagonists have potential as non-dopaminergic treatment for individuals suffering from PD, because of the antagonistic intramembrane $A_{2A}-D_2$ receptor-receptor interactions in the brain, causing improved postsynaptic dopaminergic activity (see section 3.3) (Fuxe et al., 2007a,b). When the adenosine $A_{2A}$ receptors are blocked by means of adenosine $A_{2A}$ receptor antagonists it improves the motor impairment experienced by patients suffering from PD. The improvement of the motor symptoms may be due to the inhibition of the
adenosine A$_{2A}$ receptor that augments the response to DA on a postsynaptic level (Shook & Jackson, 2011). This has been shown in animal models, where blocking the adenosine A$_{2A}$ receptor repaired the motor deficits by enabling the receptor signalling of DA (Schwarzschild et al., 2006).

### 3.5 ADENOSINE A$_1$ RECEPTORS

The adenosine A$_1$ receptors are found predominately in the neocortical and limbic areas and are also densely expressed in the basal ganglia, cerebellum, the majority of nuclei in the brain stem, the dorsal horn of the spinal cord and the hippocampus (Fastbom et al., 1987). The expressions of the adenosine A$_1$ receptors in the hippocampus and prefrontal cortex, which are important areas for cognitive function, make adenosoine A$_1$ receptor antagonists an attractive therapy to induce cognitive enhancement in both AD and PD (Jacobson & Gao, 2006).

#### 3.5.1 ADENOSINE A$_1$ RECEPTOR ANTAGONISTS IN THE TREATMENT OF AD AND PD

The adenosine A$_1$ receptor antagonists present a beneficial strategy in the treatment of AD and PD since they have cognitive enhancing and memory improving abilities that are still inadequately treated with available treatment in both disorders.

##### 3.5.1.1 Cognitive dysfunction

Cognition may refer to various cognitive processes; including the acquisition and processing of information, the storage and recollection of memories as well as the planning and execution of actions (Takahashi et al., 2008). Adenosine A$_1$ receptors are expressed in the hippocampus and prefrontal cortex which are important areas for cognitive function (Onodera & Kogure, 1988). When these receptors are inhibited by adenosine A$_1$ receptor antagonists, the hippocampus increases the release of neurotransmitters and these neurotransmitters have shown augmented learning and memory performance within animal models (Hauber & Bareiss, 2001; Pereira et al., 2002).

AD patients have altered expressions of adenosine A$_1$ receptors (Angulo et al., 2003) and reduced levels of adenosine A$_1$ receptors in the striatum and hippocampus (Ikeda et al., 1993). Post mortem studies of patients with AD have
shown a 40-60% reduction of adenosine A\textsubscript{1} receptors in the hippocampus (Hyman \textit{et al.}, 1986). The adenosine A\textsubscript{1} receptors have been implicated as a potential drug target for AD to enhance cognitive function (Hameleers \textit{et al.}, 2000), prevent cognitive impairment (Arendash \textit{et al.}, 2006) as well as stimulating the release of various neurotransmitters including ACh (Rahman, 2009).

PD patients also experience cognitive dysfunction (Chaudhuri \textit{et al.}, 2011) and it was found that those consuming coffee regularly had less pronounced symptoms compared to those who did not drink coffee (Martyn & Gale, 2003). Although caffeine is a non-selective adenosine A\textsubscript{1}/A\textsubscript{2A} receptor antagonist, it was found that selective adenosine A\textsubscript{1} receptor antagonism improved the cognitive symptoms of PD (Schwarzschild \textit{et al.}, 2002).

This ability of the adenosine A\textsubscript{1} receptor antagonists to improve cognition in animal models offer a non-dopaminergic treatment approach to treat the cognitive impairments related to PD and AD.

3.5.1.2 Motor function

Antagonistic activity of the adenosine A\textsubscript{1} receptor enhance presynaptic DA release while the postsynaptic response to DA is enhanced through the antagonistic activity of the adenosine A\textsubscript{2A} receptor (Ballarin \textit{et al.}, 1995; Moore \textit{et al.}, 2003).

Initially adenosine A\textsubscript{1} receptors were recognised as the main mediator of the motor-inducing effects of caffeine (Snyder \textit{et al.}, 1981), however after the discovery of the high concentration of adenosine A\textsubscript{2A} receptors in the striatum, several experiments proved that selective adenosine A\textsubscript{2A} receptor antagonism is the main target for the motor activating effects of caffeine (Karcz-Kubicha \textit{et al.}, 2003). The motor function of adenosine A\textsubscript{1} receptors is speculated to be due to a synergistic mechanism with the adenosine A\textsubscript{2A} receptors (Jacobson \textit{et al.}, 1993; Shook \textit{et al.}, 2010).
3.6 DUAL ACTING ADENOSINE A\textsubscript{1} AND A\textsubscript{2A} RECEPTOR ANTAGONISTS

In the literature examples exist of dual acting adenosine A\textsubscript{1}/A\textsubscript{2A} receptor antagonists. Caffeine evokes its stimulating effects via blockade of adenosine A\textsubscript{1} and A\textsubscript{2A} receptors (Brunyé et al., 2010). These effects include stimulatory actions on alertness, attention, cognitive performance and reduced sleep (Landolt et al., 2004; Prediger & Takahashi, 2005).

![Caffeine](image)

The ability of caffeine to improve motor function is attributed to antagonism of the adenosine A\textsubscript{1} and A\textsubscript{2A} receptors in the brain especially in regions where DA is found abundantly (Brunyé et al., 2010). Johnson-Kozlow and co-workers (2002) found that elderly women who drank large amounts of coffee (caffeine) over their lifetimes had a better performance on memory and other cognitive tests than non-coffee drinkers. This was also confirmed in other studies showing that the psychostimulant properties of caffeine reduced the risk of cognitive impairment in aged woman without dementia (Johnson-Kozlow et al., 2002; Maia & de Mendonça, 2002; Ritchie et al., 2007). The cognitive effects of caffeine are mostly due to its ability to antagonise adenosine A\textsubscript{1} receptors. Adenosine A\textsubscript{1} receptor antagonists depolarize postsynaptic neurons and presynaptically enhance the release of a number of neurotransmitters, eg. ACh, glutamate, 5-Hydroxytryptamine (5-HT) and norepinephrine (NE) (Suzuki, 1992a). These neurotransmitters have been implicated in learning and memory and are also reduced in AD (Rahman, 2009). The release of these neurotransmitters has the potential for treating cognitive deficits such as those associated with AD and PD.

One example of a potent dual acting antagonist of the adenosine A\textsubscript{1} and A\textsubscript{2A} receptors is 5-[5-Amino-3(4-fluorophenyl)pyrazin-2yl]-1-isopropylpyridine-2(1H)-one (ASP-5854). This compound displayed dual antagonism of the adenosine A\textsubscript{1} and A\textsubscript{2A} receptors and as such improved motor deficits and cognitive function in animal models of PD. Furthermore, the antagonism of the adenosine A\textsubscript{2A} receptors improved neurodegeneration (Mihara et al., 2007).
3.7 STRUCTURES OF ADENOSINE A<sub>2A</sub> RECEPTOR ANTAGONISTS

Compounds that present with affinity for the adenosine A<sub>2A</sub> receptors are generally classified as either xanthine derivatives (see 1.2) or non-xanthine derivatives. The non-xanthine derivates are usually aromatic heterocyclic compounds.

3.7.1 XANTHINE DERIVATIVES

Caffeine and other methylxanthines like theophylline act as non-selective adenosine receptor antagonists by binding to the adenosine A<sub>1</sub> and A<sub>2A</sub> receptors in the brain (Fredholm, 1995; Snyder et al., 1981). The first xanthine derivative with significant affinity for the adenosine A<sub>2A</sub> receptors in comparison to the adenosine A<sub>1</sub> receptors was the 8-unsubstituted 1-propagylxanthine (Müller & Stein, 1996). Substitution on position 1, 3 and 8 of xanthine led to the discovery of potent and selective adenosine A<sub>2A</sub> receptor antagonists which include: (E)-1,3-dipropyl-8-3,4-dimethoxystyryl)-7-methyl-3,7-dihydo-1H-purine-2,6-dione (KF-17837), (E)1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydo-1H-purine-2,6-dione (KW-6002), 8-(3-chlorostyryl)caffeine (CSC) and 3,7-dimethyl-1-propagylxanthine (DMPX) (Ongini et al., 2001).

\[
\text{CSC}
\]

KF-17837 is a selective and potent antagonist of the adenosine A<sub>2A</sub> receptor \((K_i = 1 \text{ nM})\) (Nonaka et al., 1994) that has displayed potential in the treatment of PD in various studies using animal models (Correa et al., 2004; Kanda et al., 1994).
Due to the low water solubility and consequently low bioavailability of the xanthine derivatives, polar groups on the phenyl ring as well as phosphate prodrugs have been suggested as possible ways to improve solubility and bioavailability (Müller et al., 1998). KW-6002 is a selective and potent ($K_i = 7.94$ nM) antagonist of the adenosine $A_{2A}$ receptors (Van der Walt et al., 2013). Another adenosine $A_{2A}$ receptor antagonist is CSC ($K_i = 26.2$ nM) (Van der Walt et al., 2013) that is often used as a reference adenosine $A_{2A}$ receptor antagonist in pharmacological studies (Vlok et al., 2006).

### 3.7.2 Non-Xanthine Derivatives

9-Chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS-15943) was the first non-xanthine compound that displayed affinity for the adenosine $A_{2A}$ receptors but showed no selectivity for these receptors in comparison with the other adenosine receptors (Francis et al., 1988; Kim et al., 1996; Williams et al., 1987).

The selectivity for the adenosine $A_{2A}$ receptors improved with the synthesis of $N^8$-substituted pyrazolo-triazolo-pyrimidines. SCH-58261 ($K_i = 2$ nM) (Ongini & Fredholm, 1996) and SCH-63390 ($K_i = 2.4$ nM) (Cheong et al., 2011) are two $N^8$-substituted pyrazolo-triazolo-pyrimidine compounds that displayed potent and selective affinity for the adenosine $A_{2A}$ receptors in rodent and human models (Baraldi et al., 1996; Baraldi et al., 1998). Although these compounds displayed high affinity and selectivity, their bioavailability was low because of their low water solubility. In an effort to increase the water solubility and consequently, the
bioavailability a hydroxyl group was substituted on the phenyl ring in the \textit{para} position. This modification in the chemical structure not only improved water solubility, it also increased the affinity and selectivity of these compounds for the adenosine \(A_{2A}\) receptors (Todde \textit{et al.}, 2000). A non-xanthine derived compound named ZM-241385 is another example of a potent adenosine \(A_{2A}\) receptor antagonist showing favourable water solubility (Poucher \textit{et al.}, 1995). Moreover, a series of 5-carboxamidoimidazopyridines also displayed affinity for the adenosine \(A_{2A}\) receptors with the optimal compound having a \(K_i\) value of 12 nM (McGuinness \textit{et al.}, 2010).

\begin{center}
\begin{tikzpicture}
\node[below] {ZM-241385};
\end{tikzpicture}
\end{center}

### 3.8 STRUCTURES OF ADENOSINE A\textsubscript{1} RECEPTOR ANTAGONISTS

The adenosine \(A_1\) receptor antagonists can be divided into xanthine derivatives and non-xanthine heterocyclic compounds with the xanthine derivatives still being the most selective for the adenosine \(A_1\) receptor (Beraldi \textit{et al.}, 2008). The heterocyclic compounds have different structural classes including mono (non-fused), bi-or-tri-cyclic heteroaromatic compounds. (Chang \textit{et al.}, 2004; Müller, 1997).

#### 3.8.1 XANTHINE DERIVATIVES

Initially xanthine derivatives acted as non-selective adenosine receptor antagonists for example caffeine and theophylline that display affinity for the adenosine \(A_1\) and \(A_{2A}\) receptors in the micromolar range (Fredholm, 1995). Various potent and selective adenosine \(A_1\) receptor antagonists have now been identified and contain substitutions that are large and lipophilic at the 1-, 3-, 8-positions (Peet \textit{et al.}, 1993; Shimada \textit{et al.}, 1991; Shimada \textit{et al.}, 1992). For example DPCPX (8-cyclopentyl-1,3-dipropyl-xanthine) (Bruns \textit{et al.}, 1987) displayed high selectivity for the adenosine \(A_1\) receptors compared to adenosine \(A_{2A}\) receptors in a rat model, however, was less selective in a human model (Shamin \textit{et al.}, 1988).
Other examples of the xanthine derived selective adenosine A₁ receptor antagonists are 8-cyclopentyl-1,3-dipropylxanthine (CPX), 1,3-dipropyl-8-(3-noradamanatyl)xanthine (KW-3902) and 1,3-dipropyl-8-[2-(5,6-epoxynorbornyl)xanthine (BG-9719) (Pfister et al., 1997; Suzuki et al., 1992b).

BG-9719 is a highly potent and selective antagonist of the adenosine A₁ receptor ($K_i = 0.43$ nM). The synthesis of this compound involved introducing a 2-(5,6-epoxy)norborny moiety on the 1,3-dipropylxanthine nucleus at the C8 position (Pfister et al., 1997).

3.8.2 NON-XANTHINE DERIVATIVES

The majority of the non-xanthine heterocyclic compounds may be considered as extensions of the xanthine scaffold (Gaida et al., 1997).

3.8.2.1 Monocyclic heteroaromatic rings (non-fused rings)

This class of compounds mainly consist of monocyclic substituted rings and are rarely found to act as adenosine A₁ receptor antagonists (Chang et al., 2004). However an investigation identified two five-membered heterocycles, thiazoles and thiadiazoles with affinities in the low nanomolar range (Chang et al., 2004; Van Muijlwijk-Koezen et al., 2001).
Fused heteroaromatic ring systems consist of two (bicyclic) or three (tricyclic) fused rings.

The bicyclic heteroaromatic systems have shown potential as non-xanthine derivatives with varying degrees of adenosine $A_1$ receptor antagonistic activity. These systems include 6:5-fused, 6:6-fused and 5:7-fused rings, containing either two, three, four or five N-atoms and bicyclic heterocyclic compounds isolated from natural compounds that contained no N-atoms (Chang et al., 2004).

The tricyclic heteroaromatic systems consist of 6:6:5-fused, 6:5:6-fused and 5:6:5-fused ring systems that contain various N-atoms (Chang et al., 2004). These tricyclic ligands have a remarkable number of similarities and are further developments of the bicyclic heteroaromatics described above.

For the purpose of this pilot study, we focussed on the bicyclic 6:5-fused N-containing heteroaromatic compounds with two N-atoms.
3.8.2.3 6:5-Fused N-containing heteroaromatic ring system with two N-Atoms in the five-membered ring

The class of 6:5-fused N-containing heteroaromatic compounds can be divided into three groups: pyrazolo[1,5-α]pyridines, imidazo[1,2-α]pyridines and the benzimidazoles with the pyrazolo[1,5-α]pyridines being the most investigated class (Chang et al., 2004).

Pyrazolo[1,5-α]pyridine Imidazo[1,2-α]pyridines Benzimidazoles

FK-453 is derived from pyrazolo[1,5-α]pyridines, structurally related to the xanthine core, and display high affinity and selectivity for the adenosine A₁ receptors (Akahane et al., 1996). The phenyl group at the C2 position and the α, β-unsaturated amide at the C3 position forms the distinctive features of this compound (van Muijlwijk-Koezen et al., 2001). Alterations of the dihydropyridazinyl group with 2-substituted cyclohexane analogous displayed low nanomolar affinities for the adenosine A₁ receptor (Kuroda et al., 2000). When CH₂ spacers with different functional groups were attached to the dihydropyridazinyl group the affinity for the adenosine A₁ receptor increased to the sub-nanomolar range (Kuroda et al., 2001). It was also found that amino substitution is essential for the compounds to cross the blood-brain barrier (Kuroda et al., 2001).

Compounds containing the imidazo[1,2-α]pyridine ring system have been shown to possess a broad range of useful pharmacological properties, including antibacterial, antifungal, anthelmintic, antiviral, antiprotozoal, anti-inflammatory, anticonvulsant, anxiolytic, hypnotic (e.g., zolpidem), gastrointestinal, antiulcer,
and immunomodulatory activities (Abignente et al., 1992; Al-Tel et al., 2011; Gueiffier et al., 1998; Lange et al., 2001).

Zolpidem

The imidazo[1,2-α]pyridines and the benzimidazoles have thus far only been described in patent literature (Beresis et al., 2003). In a study conducted by Reutlinger and co-workers (2014), a G protein-coupled receptor ligand was identified with affinity for the adenosine A₁/A₂B and adrenergic α₁A/B receptors. Two imidazo[1,2-α]pyridine derivatives (Chapter 1, Figure 1-1) were predicted as good adenosine A₁ receptor scaffolds with 84% and 89% binding at 100 µM, respectively.

Imidazo[1,2-α]pyridines

Based upon the findings of Reutlinger and co-workers (2014), this pilot study aims to synthesise a series of para-phenyl substituted imidazo[1,2-α]pyridines that was previously synthesised by Adib and co-workers (2007). These compounds possess the imidazo[1,2-α]pyridine scaffold and will be synthesised via a one-pot synthesis and screened for adenosine A₁ and A₂A receptor affinity.
3.9 CONCLUSION

In conclusion non-selective adenosine A$_1$ and A$_{2A}$ receptor antagonists display potential in the treatment of AD and PD as antagonism of the adenosine A$_1$ receptor improves cognition and memory while working synergistically with the adenosine A$_{2A}$ receptor antagonists to improve motor function. Furthermore, the adenosine A$_{2A}$ receptor antagonists have neuroprotective properties and have displayed antidepressant effects. Thus multiple aspects of each disorder will be addressed if dual-targets are developed. In the following chapter the experimental procedure of this study will be described. Analogues of the imidazo[1,2-α]pyridines will be synthesised using a procedure described by Adib and colleagues (2007) and screened for dual acting adenosine A$_1$/A$_{2A}$ receptor antagonistic activity.
3.10 REFERENCES


Correa, M., Wisniecki, A., Betz, A., Dobson, D.R., O'Neill, M.F., O'Neill, M.J. & Salamone, J.D. 2004. The adenosine A₂A antagonist KF17837 reverses the locomotor suppression and tremulous jaw movements induced by haloperidol in


Yacoubi, M.E., Ledent, C., Parmentier, M., Bertorelli, R., Ongini, E., Costentin, J. & Vaugeois, J.M. 2001. Adenosine A$_{2A}$ receptor antagonists are potential


SYNTHESIS AND BIOLOGICAL EVALUATION

4.1 INTRODUCTION

The imidazo[1,2-α]pyridine analogues consist of a 6:5-fused N-containing heteroaromatic system. Derivatives of the imidazo[1,2-α]pyridine scaffold have shown affinity for the adenosine $A_1$ receptors, as mentioned in Chapter 3. The main aim of this pilot study was to evaluate selected imidazo[1,2-α]pyridine analogues for their binding affinities to adenosine $A_1$ and $A_{2A}$ receptors via radioligand binding assays. The compound displaying the most promising affinity for the adenosine $A_1$ receptor will be subjected to a GTP (Guanosine-5'-triphosphate) shift assay in order to determine its functionality as an adenosine $A_1$ receptor agonist or antagonist. The selected imidazo[1,2-α]pyridine analogues were either obtained commercially (1, 3a–e) or synthesised (4a–i) according to literature procedures. Compound 1, imidazo[1,2-α]pyridine, is seen as the parent compound of the study (Figure 4–1). The structure-activity relationships (SAR) of the imidazo[1,2-α]pyridine scaffold was further explored via substitution at the C2 position alone and in combination with position C3. The C2 position was substituted with a phenyl ring alone (3a) as well as para-substitution with OH- (3b), OCH$_3$- (3c) and Br- (3d). The simultaneous substitution of C2 and C3 included C2 phenyl substitution in combination with an amino- (3e) and cyclohexyl amino-group (4a). The effect of para-substitution at C2 of compound 4a was further investigated by substitutions at position C4' with halogens (Br- (4e), Cl- (4f), F- (4g)) as well as OH- (4b) OCH$_3$- (4c), CH$_3$- (4d), CF$_3$- (4h), NO$_2$- (4i), groups (Figure 4–2; Table 4–1).

Figure 4-1: Imidazo[1,2-α]pyridine.
Reutlinger and co-workers (2014) reported that compound 2 possessed affinity for the adenosine A₁ receptor (Figure 4–3). Substitution at position C2 and position C3 of the parent scaffold, compound 1, resulted in compound 2. Thus, compound 2 has been identified as the lead compound for this study.

![C2 phenyl substitution, with C4' para-substitution](image)

Figure 4-2: The imidazo[1,2-α]pyridine scaffold.

X= H, OH, OCH₃, CH₃, Br, Cl, F, CF₃, NO₂

Compounds 1 and 3a–e were bought commercially from Sigma-Aldrich®. In turn, compounds 4a–i was synthesised by means of a previously documented protocol (Adib et al., 2007). A detailed discussion of the synthetic preparation can be found in section 4.2. The aforementioned compounds (1, 3a–e) (Figure 4–4) were used to compare the effect of substitution on position C2 and simultaneous substitution at positions C2 and C3 via the synthesised compounds (4a–i) to evaluate their adenosine A₁ and A₂A receptor affinity.

Figure 4-3: The lead compound used to design the investigated imidazopyridines of the current study (Reutlinger et al., 2014).
Figure 4-4: Test compounds commercially available from Sigma-Aldrich®.

4.2 SYNTHESIS

Scheme 4-1: The catalyst- and solvent-free synthetic procedure that was utilised to obtain the corresponding imidazo[1,2-α]pyridines. 

Reagents and conditions: (a) heated at 120°C (4a, 4b, 4c, 4d, 4e, 4f, 4g, 4i) or 60°C (4h), (b) reflux for the appropriate time.

A modified catalyst-free three-compound reaction was used to synthesise compounds 4a–i of this study. No solvent was used during the synthesis of these test compounds (Adib et al., 2007). The reaction was performed by refluxing a mixture of cyclohexyl isocyanide (12 mmol), the appropriate aldehyde (10 mmol) and 2-aminopyridine (10 mmol) at 120°C or 60°C for the appropriate time. After the reaction was completed the crude product was filtered and washed with ethyl acetate and recrystallised from either hexane:ethyl acetate (4a, 4d, 4e, 4f, 4g, 4h) or ethyl acetate:ethanol (4b, 4c, 4i) to yield the desired imidazo-[1,2-α]pyridine analogues.

4.2.1 MATERIALS AND INSTRUMENTATION

All reagents, except the cyclohexyl isocyanide, were acquired from Sigma-Aldrich®. Cyclohexyl isocyanide was obtained from Merck®. All reagents were used without further purification. The synthesised compounds in this study were monitored with thin layer chromatography (TLC) during the reaction.
process and confirmed by nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) and the melting points (mp) of each test compound.

4.2.1.1 Thin layer chromatography (TLC)

During the synthesis of the test compounds, the reactions were monitored by means of TLC. Silica gel 60 TLC (Merck®) with UV\textsubscript{254} fluorescent indicator plates were used as the TLC indicator. The mobile phase consisted of one of the following: 60% ethyl acetate and 40% petroleum ether (4a, 4d, 4e, 4f, 4g), or 100% ethyl acetate (4b, 4c, 4h, 4i). The TLC plates were visualised under a UV light at a wavelength of 254 nm.

4.2.1.2 Nuclear magnetic resonance (NMR)

All synthesised compounds were verified with proton (\textsuperscript{1}H) and carbon (\textsuperscript{13}C) NMR which is recorded using a Bruker Ultrashield Plus Avance III 600 spectrometer in deuterated dimethyl sulfoxide (DMSO-d\textsubscript{6}) (Merck®). The chemical shifts (\(\delta\)) are measured in parts per million (ppm) comparative to the residual solvent signals which were 2.50 ppm for the proton, 3.30 ppm for the water of DMSO-d\textsubscript{6} and 39.5 ppm for the carbon (Gottlieb et al., 1997). The spin multiplicities are as follows: singlet (s), doublet (d), doublet of doublets (dd), double doublet of doublets (ddd), triplet (t), doublet of triplets (dt), triplet of doublets (td), or multiplet (m). See Annexure A for the NMR of compounds 4a–i.

4.2.1.3 Mass spectrometry (MS)

High-resolution mass spectrometry (HRMS) was used to determine the mass spectrometry of each test compound. The data was recorded on a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionisation (APCI) mode. See Annexure B for the MS of compounds 4a–i.

4.2.1.4 Melting points (mp)

The mp of each synthesised compound was determined on a Buchi M-454 melting point apparatus and is uncorrected.
4.2.2 DETAILED SYNTHETIC APPROACH

As previously mentioned the imidazo[1,2-α]pyridine analogues consist of a 6:5-fused N-containing heteroaromatic system. A possible mechanism for the cyclisation pathway of compounds \(4a-i\) is illustrated in Scheme 4–2 with compound \(4a\) as an example (Sarkar et al., 2016). Thus, it is expected that a similar reaction mechanism will take place to obtain compounds \(4b-i\).

Scheme 4-2: A possible mechanism for the cyclisation pathway of the targeted imidazo[1,2-α]pyridine analogues by means of compound \(4a\) as an example (Sarkar et al., 2016).
4.2.2.1 3-Cyclohexylamino-2-phenylimidazo[1,2-α]pyridine (4a)

A mixture of 2-aminopyridine (0.941 g, 10 mmol), benzaldehyde (1.060 g, 10 mmol) and cyclohexyl isocyanide (1.310 g, 12 mmol) was stirred and refluxed at 120°C for 24 hours. The crude product was filtered and washed with ethyl acetate. TLC was used to monitor the reaction until completion. The eluent consisted of 60% ethyl acetate and 40% petroleum ether. After completion the product was recrystallised from hexane:ethyl acetate (3:1), filtered and washed with ethyl acetate (twice) to afford a white solid.

4.2.2.2 3-Cyclohexylamino-2-(4'-hydroxyphenyl)imidazo[1,2-α]pyridine (4b)

A mixture of 2-aminopyridine (0.941 g, 10 mmol), 4-hydroxybenzaldehyde (1.221 g, 10 mmol) and cyclohexyl isocyanide (1.310 g, 12 mmol) was stirred and refluxed at 120°C for 24 hours. The crude product was filtered and washed with ethyl acetate. TLC was used to monitor the reaction until completion. The eluent consisted of 100% ethyl acetate. After completion the product was recrystallised from ethyl acetate:ethanol (2:1), filtered and washed with ethyl acetate to afford a light brown solid.

4.2.2.3 3-Cyclohexylamino-2-(4'-methoxyphenyl)imidazo[1,2-α]pyridine (4c)

A mixture of 2-aminopyridine (0.941 g, 10 mmol), 4-methoxybenzaldehyde (1.360 g, 10 mmol) and cyclohexyl isocyanide (1.310 g, 12 mmol) was stirred and refluxed at 120°C for approximately 48 hours (2 days). The crude product was filtered and washed with ethyl acetate. TLC was used to monitor the reaction until completion. The eluent consisted of 100% ethyl acetate. After completion the product was recrystallised from ethyl acetate:ethanol (2:1), filtered and washed with ethyl acetate to afford light brown crystals.

4.2.2.4 3-Cyclohexylamino-2-(4'-methylphenyl)imidazo[1,2-α]pyridine (4d)

A mixture of 2-aminopyridine (0.941 g, 10 mmol), 4-methylbenzaldehyde (1.202 g, 10 mmol) and cyclohexyl isocyanide (1.310 g, 12 mmol) was stirred and refluxed at 120°C for approximately 72 hours (3 days). The crude product was filtered and washed with ethyl acetate. TLC was used to monitor the reaction until completion. The eluent consisted of 60% ethyl acetate and 40% petroleum ether. After completion the product was recrystallised from
hexane:ethyl acetate (3:1), filtered and washed with ethyl acetate to afford a white solid.

4.2.2.5 3-Cyclohexylamino-2-(4'-bromophenyl)imidazo[1,2-α]pyridine (4e)

A mixture of 2-aminopyridine (0.941 g, 10 mmol), 4-bromobenzaldehyde (1.850 g, 10 mmol) and cyclohexyl isocyanide (1.310 g, 12 mmol) was stirred and refluxed at 120°C for 24 hours. The crude product was filtered and washed with ethyl acetate. The product was recrystallised from hexane:ethyl acetate (3:1). TLC was used to monitor the reaction until completion. The eluent consisted of 60% ethyl acetate and 40% petroleum ether. After completion the product was recrystallised from hexane:ethyl acetate (3:1), filtered and washed with ethyl acetate (twice) to afford a light yellow solid.

4.2.2.6 3-Cyclohexylamino-2-(4'-chlorophenyl)imidazo[1,2-α]pyridine (4f)

A mixture of 2-aminopyridine (0.941 g, 10 mmol), 4-chlorobenzaldehyde (1.406 g, 10 mmol) and cyclohexyl isocyanide (1.310 g, 12 mmol) was stirred and refluxed at 120°C for 24 hours. The crude product was filtered and washed with ethyl acetate. The product was recrystallised from hexane:ethyl acetate (3:1). TLC was used to monitor the reaction until completion. The eluent consisted of 60% ethyl acetate and 40% petroleum ether. After completion the product was recrystallised from hexane:ethyl acetate (3:1), filtered and washed with ethyl acetate (twice) to afford an off-white solid.

4.2.2.7 3-Cyclohexylamino-2-(4'-fluorophenyl)imidazo[1,2-α]pyridine (4g)

A mixture of 2-aminopyridine (0.941 g, 10 mmol), 4-fluorobenzaldehyde (1.241 g, 10 mmol) and cyclohexyl isocyanide (1.310 g, 12 mmol) was stirred and refluxed at 120°C for 24 hours. The crude product was filtered and washed with ethyl acetate. TLC was used to monitor the reaction until completion. The eluent consisted of 60% ethyl acetate and 40% petroleum ether. After completion the product was recrystallised from hexane:ethyl acetate (3:1), placed on ice after which it precipitated out, filtered and washed with ethyl acetate to afford light brown crystals.
4.2.2.8 3-Cyclohexylamino-2-[(4’-(trifluoromethyl)phenyl]imidazo[1,2-α]pyridine (4h)

A mixture of 2-aminopyridine (0.941 g, 10 mmol), 4-(trifluoromethyl)benzaldehyde (1.741 g, 10 mmol) and cyclohexyl isocyanide (1.310 g, 12 mmol) was stirred and refluxed at 60°C for approximately 96 hours (4 days). TLC was used to monitor the reaction until completion. The eluent consisted of 100% ethyl acetate. After completion the product was recrystallised from hexane:ethyl acetate (3:1), filtered and washed with ethyl acetate to afford an off-white solid.

4.2.2.9 3-Cyclohexylamino-2-(4’-nitrophenyl)imidazo[1,2-α]pyridine (4i)

A mixture of 2-aminopyridine (0.941 g, 10 mmol), 4-nitrobenzaldehyde (1.511 g, 10 mmol) and cyclohexyl isocyanide (1.310 g, 12 mmol) was stirred and refluxed at 120°C for 24 hours. The crude product was filtered and washed with ethyl acetate. TLC was used to monitor the reaction until completion. The eluent consisted of 100% ethyl acetate. After completion the product was recrystallised from ethyl acetate:ethanol (3:1), filtered and washed with ethyl acetate to afford an orange solid.

4.2.3 PHYSICAL CHARACTERISATION

See Annexure A for the $^1$H and $^{13}$C NMR and Annexure B for the MS of compounds 4a–i.

4.2.3.1 3-Cyclohexylamino-2-phenylimidazo[1,2-α]pyridine (4a)

The title compound was prepared from 2-aminopyridine, benzaldehyde and cyclohexyl isocyanide in a yield of 1%. mp: 183.5-184.2 °C (Lit: 176-178 °C; Bode et al., 2011). $^1$H NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-$d_6$) δ 1.06 (t, 3H, J= 8.8 Hz), 1.21–1.29 (m, 2H,), 1.47 (s, 1H), 1.60 (d, 2H, J= 4.9 Hz), 1.69 (d, 2H, J= 12.1 Hz), 2.77–2.84 (m, 1H), 4.78 (d, 1H, J= 5.8 Hz), 6.86 (t, 1H, J= 6.7 Hz), 7.13–7.17 (m, 1H), 7.25 (t, 1H, J= 7.3 Hz), 7.41 (t, 2H, J= 7.7 Hz), 7.45 (d, 1H, J= 9.0 Hz), 8.21 (d, 2H, J= 7.4 Hz), 8.30 (d, 1H, J= 6.8 Hz); $^{13}$C NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-$d_6$) δ 24.5, 25.4, 33.5, 56.4, 111.3, 116.7, 123.4, 123.8, 125.8, 126.4, 126.8, 128.2, 134.6, 134.8, 140.5; APCI-HRMS m/z: calculated for C$_{19}$H$_{22}$N$_3$, 292.1808, found (MH$^+$) 292.1823. HPLC purity determination: 100 %.
4.2.3.2 3-Cyclohexylamino-2-(4'-hydroxyphenyl)imidazo[1,2-α]pyridine (4b)

The title compound was prepared from 2-aminopyridine, 4-hydroxybenzaldehyde and cyclohexyl isocyanide in a yield of 29%. mp: 178.8-180.3 °C. $^1$H NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-d6) δ 1.01–1.27 (m, 5H), 1.48 (s, 1H), 1.61 (d, 2H, J=4.3 Hz), 1.68 (d, 2H, J= 12.1 Hz), 2.74–2.87 (m, 1H), 4.68 (d, 1H, J= 5.5 Hz), 6.79 (d, 2H, J= 8.6 Hz), 6.83 (t, 1H, J= 6.4 Hz), 7.11 (t, 1H, J= 6.8 Hz), 7.40 (d, 1H, J= 9.0 Hz), 8.02 (d, 2H, J= 8.6 Hz), 8.25 (d, 1H, J= 6.8 Hz), 9.46 (s, 1H); $^{13}$C NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-d6) 24.5, 25.5, 33.5, 56.3, 111.1, 115.1, 116.5, 123.1, 123.3, 124.3, 125.7, 127.9, 135.4, 140.3, 156.5; APCI-HRMS $m/z$: calculated for C$_{19}$H$_{22}$N$_3$O, 308.1757, found (MH$^+$) 308.1736. HPLC purity determination: 100%.

4.2.3.3 3-Cyclohexylamino-2-(4'-methoxyphenyl)imidazo[1,2-α]pyridine (4c)

The title compound was prepared from 2-aminopyridine, 4-methoxybenzaldehyde and cyclohexyl isocyanide in a yield of 3%. mp: 153.7-155.3 °C (Lit: 149-154 °C; Bode et al., 2011). $^1$H NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-d6) δ 0.99–1.16 (m, 3H), 1.16–1.33 (m, 2H), 1.49 (s, 1H), 1.62 (dd, 2H, J= 4.4, 8.5 Hz), 1.71 (d, 2H, J= 12.2 Hz), 2.76–2.88 (m, 1H), 3.80 (s, 3H), 4.73 (d, 1H, J= 5.6 Hz), 6.86 (td, 1H, J= 6.7, 1.1 Hz), 6.96–7.03 (m, 2H), 7.14 (dd, 1H, J= 9.0, 6.6, 1.2 Hz), 7.44 (dt, 1H, J= 9.0, 1.0 Hz), 8.13–8.21 (m, 2H), 8.29 (dt, 1H, J= 6.8, 1.0 Hz); $^{13}$C NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-d6) δ 24.9, 25.9, 34.0, 55.1, 56.8, 111.6, 114.1, 116.9, 123.7, 123.9, 125.1, 127.8, 128.2, 135.5, 140.9, 159.7; APCI-HRMS $m/z$: calculated for C$_{20}$H$_{24}$N$_3$O, 322.1914, found (MH$^+$) 322.1917. HPLC purity determination: 100%.

4.2.3.4 3-Cyclohexylamino-2-(4'-methylphenyl)imidazo[1,2-α]pyridine (4d)

The title compound was prepared from 2-aminopyridine, 4-methylbenzaldehyde and cyclohexyl isocyanide in a yield of 26%. mp: 163.3-164.0 °C (Lit: 166-169 °C; Adib et al., 2011). $^1$H NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-d6) δ 1.02–1.13 (m, 3H), 1.20–1.29 (m, 2H), 1.49 (s, 1H), 1.58–1.66 (m, 2H), 1.70 (d, 2H, J= 11.7 Hz), 2.33 (s, 3H), 2.77–2.85 (m, 1H), 4.75 (d, 1H, J= 5.8 Hz), 6.87 (td, 1H, J= 6.7, 1.1 Hz), 7.15 (ddd, 1H, J= 9.0, 6.6, 1.2 Hz), 7.23 (d, 2H, J= 7.9 Hz), 7.45 (d, 1H, J= 9.0 Hz), 8.12 (d, 2H, J=
8.2 Hz), 8.30 (d, 1H, J = 6.8 Hz); $^{13}$C NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-d$_6$) δ 21.3, 24.9, 25.9, 34.0, 56.9, 111.7, 117.09, 123.7, 124.0, 125.8, 126.9, 129.3, 132.5, 135.4, 136.4, 140.9; APCI-HRMS m/z: calculated for C$_{25}$H$_{24}$N$_3$, 360.1965, found (MH$^+$) 360.1973. HPLC purity determination: 100%.

4.2.3.5 3-Cyclohexylamino-2-(4′-bromophenyl)imidazo[1,2-α]pyridine (4e)

The title compound was prepared from 2-aminopyridine, 4-bromobenzaldehyde and cyclohexyl isocyanide in a yield of 12%. mp: 171.2-172.4 °C (Lit: 166-168 °C; Bode et al., 2011). $^1$H NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-d$_6$) δ 1.09 (s, 3H), 1.26 (dd, 2H, J = 21.4, 12.7 Hz), 1.5 (s, 1H), 1.63 (dd, 2H, J = 9.3, 4.3), 1.71 (d, 2H, J = 12.3 Hz), 2.77–2.84 (m, 1H), 4.84 (d, 1H, J = 5.9 Hz), 6.90 (t, 1H, J = 6.4 Hz), 7.16–7.21 (m, 1H), 7.47 (d, 1H, J = 9.0 Hz), 7.61 (d, 2H, J = 8.5 Hz), 8.19 (d, 2H, J = 8.5 Hz), 8.31 (d, 1H, J = 6.9 Hz); $^{13}$C NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-d$_6$) δ 24.9, 25.9, 34.0, 57.0, 111.9, 117.2, 120.3, 123.9, 124.6, 126.6, 128.8, 131.6, 134.0, 134.5, 141.1; APCI-HRMS m/z: calculated for C$_{19}$H$_{21}$N$_3$Br, 370.0921, found (MH$^+$) 370.0898. HPLC purity determination: 95 %.

4.2.3.6 3-Cyclohexylamino-2-(4′-chlorophenyl)imidazo[1,2-α]pyridine (4f)

The title compound was prepared from 2-aminopyridine 4-chlorobenzaldehyde and cyclohexyl isocyanide in a yield of 15%. mp: 188.7-191.8 °C (Lit: 198-199 °C; Bode et al., 2011). $^1$H NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-d$_6$) δ 1.07 (s, 3H), 1.23 (dd, 2H, J=20.2, 10.8 Hz), 1.48 (s, 1H), 1.56–1.64 (m, 2H), 1.69 (d, 2H, J = 12.3 Hz), 2.75–2.83 (m, 1H), 4.83 (d, 1H, J = 5.9 Hz), 6.88 (t, 1H, J = 6.7 Hz), 7.12–7.20 (m, 1H), 7.46 (t, 3H, J = 8.1 Hz), 8.23 (d, 2H, J = 8.6 Hz), 8.30 (d, 1H, J = 6.8 Hz); $^{13}$C NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-d$_6$) δ 24.5, 25.4, 33.5, 56.5, 111.5, 116.8, 123.5, 124.1, 126.0, 128.1, 128.3, 131.2, 133.5, 133.7, 140.6; APCI-HRMS m/z: calculated for C$_{19}$H$_{21}$ClN$_3$, 326.1419, found (MH$^+$) 326.1404. HPLC purity determination: 100 %.

4.2.3.7 3-Cyclohexylamino-2-(4′-fluorophenyl)imidazo[1,2-α]pyridine (4g)

The title compound was prepared from 2-aminopyridine, 4-fluorobenzaldehyde and cyclohexyl isocyanide in a yield of 4%. mp: 171.1-171.9 °C (Lit: 167-169 °C;
4.2.3.8 3-Cyclohexylamino-2-\{4'-\(\text{trifluoromethyl}\)phenyl\}imidazo[1,2-\(\alpha\)]pyridine (4h)

The title compound was prepared from 2-aminopyridine, 4-(trifluoromethyl)benzaldehyde and cyclohexyl isocyanide in a yield of 17%. mp: 149.6-152.1 °C \(\text{Lit}: 138-140 °C; \text{Bode et al.}, 2011\). \(^1\)H NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-\(d_6\)) \(\delta\) 1.09 (s, 3H), 1.28 (dt, 2H, J= 14.3, 7.1 Hz), 1.49 (s, 1H), 1.63 (dd, 2H, J= 9.4, 4.3 Hz), 1.73 (d, 2H, J= 12.5 Hz), 2.77–2.89 (m, 1H), 4.95 (d, 1H, J= 6.2 Hz), 6.92 (td, 1H, J= 6.8, 1.0 Hz), 7.21 (ddd, 1H, J= 9.0, 6.6, 1.2 Hz), 7.50 (d, 1H, J = 9.0 Hz), 7.78 (d, 2H, J= 8.3 Hz), 8.35 (d, 1H, J= 6.9 Hz), 8.45 (d, 2H, J= 8.2 Hz); \(^{13}\)C NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-\(d_6\)) \(\delta\) 25.0, 25.8, 34.0, 57.2, 112.2, 117.5, 124.1, 124.9, 125.6, 125.9, 127.2, 127.3, 127.6, 133.4, 139.3, 141.3; APCI-HRMS \(m/z\): calculated for C\(_{20}\)H\(_{21}\)F\(_3\)N\(_3\), 360.1682, found (MH\(^+\)) 360.1685. HPLC purity determination: 100%.

4.2.3.9 3-Cyclohexylamino-2-\{4'-\(\text{nitrophenyl}\)\}imidazo[1,2-\(\alpha\)]pyridine (4i)

The title compound was prepared from 2-aminopyridine 4-nitrobenzaldehyde and cyclohexyl isocyanide in a yield of 12%. mp: 213.2-213.6 °C \(\text{Lit}: 203-205 °C; \text{Bode et al.}, 2011\). \(^1\)H NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-\(d_6\)) \(\delta\) 1.09 (s, 3H), 1.29 (dd, 2H, J= 20.0, 10.0 Hz), 1.49 (s, 1H), 1.59–1.65 (m, 2H), 1.73 (d, 2H, J= 12.2 Hz), 2.79–2.86 (m, 1H), 5.04 (d, 1H, J= 6.6 Hz), 6.94 (t, 1H, J= 6.4 Hz), 7.24 (ddd, 1H, J= 8.9 Hz), 7.51 (d, 1H, J= 9.0), 8.30 (d, 2H, J= 8.9), 8.35 (d, 1H, J= 6.9 Hz), 8.48 (d, 2H, J= 8.9 Hz); \(^{13}\)C NMR (Bruker Ultrashield Plus Avance III 600 spectrometer in DMSO-\(d_6\)) \(\delta\) 25.0, 25.8, 34.0, 57.4, 112.5, 117.6, 124.2, 124.2, 125.5, 127.3, 128.9, 132.5, 141.5, 141.9,
146.0; APCI-HRMS $m/z$: calculated for $\text{C}_{19}\text{H}_{21}\text{N}_4\text{O}_2$, 337.1659, found (MH$^+$) 337.1664. HPLC purity determination: 93%.

4.3 BIOLOGICAL ASSAY

The affinities for both the adenosine $A_1$ and $A_{2A}$ receptor subtypes were determined using the method described by Van der Walt and Terre'Blanche (2015).

4.3.1 MATERIALS AND INSTRUMENTATION

The reagents that were commercially available were acquired from various manufacturers (Ascent Scientific®, Merck® and Sigma-Aldrich®) and were used without further purification. The radioligand 5'-N-Ethylcarboxamidoadenosine [$^3$H]NECA (specific activity 25 Ci/mmol) was obtained from Amersham Biosciences®, whilst the radioligand 1,3-dipropyl-8-cyclopentylxanthine [$^3$H]DPCPX (specific activity 120 Ci/mmol) was obtained from PerkinElmer®. The following reagents were obtained from Sigma-Aldrich®: Sigma-cote® (silicone solution); adenosine deaminase (8.3 mg protein/mL, 141 units/mg protein); anhydrous magnesium chloride ($\text{MgCl}_2$); Trizma-base; Trizma-hydrochloride; unlabelled N$^6$-cyclopentyladenosine (CPA) and unlabelled DPCPX. Furthermore, the filter-count scintillation fluid and scintillation vials were obtained from PerkinElmer®, while the Whatman GF/B 25 mm diameter filters and dimethyl sulfoxide (DMSO) solvent were purchased from Merck®. A Packard Tri-CARB 2810 TR liquid scintillation counter was used to count radio activities.

4.3.2 TISSUE PREPARATION

The tissue samples that were necessary for the radioligand binding and GTP shift assays were obtained from male Sprague-Dawley rats after the study was approved by the Research and Ethics Committee of the North-West University (NWU-0035-10-A5). The tissue samples included whole brains (excluding the cerebellum and brain stem) that expressed the adenosine $A_1$ receptor and striatal membranes that were used for the adenosine $A_{2A}$ receptor binding assays. Following the dissection, the tissue samples were snap frozen without delay by means of liquid nitrogen and stored at -70°C. On the day of use, the tissue samples were prepared in accordance with the protocol as described in the literature (Van der Walt et al., 2015). The protein content of all the tissue
samples was determined by utilising a Bradford reagent as described in the literature (Bradford, 1979).

### 4.3.3 Radioligand Binding Assay Protocol for the Adenosine A$_{2A}$ Receptors

The stock solutions of each test compound were prepared with DMSO solvent. Incubations consisted of 120 µg of striatal membranes, 4 nM $[^{3}H]$NECA, 50 nM CPA, 10 mM MgCl$_2$, 0.2 units/mL adenosine deaminase, the test compound and 1% DMSO (Bruns et al., 1986; Van der Walt et al., 2015). The final volume of each incubation consisted of 1mL 50 mM Tris-HCl buffer (pH 7.7 at 25°C). The assays were performed as documented in the literature (Bruns et al., 1986; Van der Walt et al., 2015). The order of addition was as follows: test compound (0.01 mL), membrane suspension (0.79 mL), 500 nM CPA (0.1 mL) and radioligand (0.1 mL). The CPA is added to the adenosine A$_{2A}$ receptor binding assays to selectively eliminate the adenosine A$_{1}$ receptors as the radioligand $[^{3}H]$NECA bind to both the adenosine A$_{1}$ and A$_{2A}$ receptors in the striatal membranes. The addition of CPA will thus cause occupancy of approximately 98% of the adenosine A$_{1}$ receptors, whilst sparing nearly 93% of the adenosine A$_{2A}$ receptors (Bruns et al., 1986). The membrane suspension was prepared with 50 mM Tris-HCl buffer, the rat striatal membranes previously prepared, MgCl$_2$ and adenosine deaminase. The role of the MgCl$_2$ is to increase the binding of the adenosine A$_{2A}$ receptors, whilst simultaneously decreasing non-specific binding (Bruns et al., 1986). The control incubation contained 1% DMSO, demonstrating that DMSO did not affect the specific binding of the assay (Bruns et al., 1986). In order to define non-specific binding, CPA was added at a final concentration of 100 µM (Bruns et al., 1986; Van der Walt et al., 2015). The order of addition for the non-specific binding was 10 mM CPA (0.01 mL), membrane suspension (0.79 mL), 500 nM CPA (0.1 mL) and radioligand (0.1 mL). The test compounds were initially screened at 1, 10 and 100 µM concentrations. The dissociation constant ($K_i$) values were determined for those test compounds that exhibited a percentage displacement of the radioligand $[^{3}H]$NECA superior to 25% at the initial screening of the maximum tested concentration of 100 µM.

### 4.3.4 Radioligand Binding Assay Protocol for the Adenosine A$_{1}$ Receptors

All stock solutions of the test compounds were prepared with DMSO solvent. Incubations contained 120 µg of whole brain membranes, 0.1 nM $[^{3}H]$DPCPX, 0.1 units/mL adenosine deaminase, the test compound and 1% DMSO (Bruns et
al., 1987; Van der Walt et al., 2015). After preparation, the incubations contained a final volume of 1mL 50 mM Tris-HCl buffer (pH 7.7 at 25°C). The assays were conducted in accordance with the method described in the literature (Van der Walt et al., 2015). The order of addition was as follows: test compound (0.01 mL), radioligand (0.1 mL) and membrane suspension (0.89 mL). The membrane suspension was prepared with 50 mM Tris-HCl buffer, the rat whole brain tissue preparation and adenosine deaminase. During the adenosine A₃ receptor binding assays adenosine deaminase is added to exclude endogenous adenosine from binding to the receptor. Control incubations received 1% DMSO, indicating that DMSO had no effect on specific binding (Bruns et al., 1986), whilst the non-specific binding was defined by adding a final concentration of 100 µM CPA to the incubation (Van der Walt et al., 2015). Therefore, the order of addition for the non-specific binding was 10 mM CPA (0.01 mL), radioligand (0.1 mL) and membrane suspension (0.89 mL). The test compounds were initially screened at 1, 10 and 100 µM final concentrations. The $K_i$ values were determined for those compounds exhibiting a percentage displacement of the radioligand $[^3]H$DPCPX superior to 25% at the initial screening of the maximum tested concentration of 100 µM.

4.3.5 GTP SHIFT ASSAY

The same method was followed as described in section 4.3.2 of the adenosine A₃ radioligand binding assay tissue collection.

The GTP shift assay was performed with rat whole brain membranes. The final assay volume contained 1 mL of 50 mM Tris-HCl buffer (pH 7.7 at 25°C.) (Bruns et al., 1987; Van der Walt et al., 2015). The $[^3]H$DPCPX ($K_d = 0.36$ nM) radioligand was used at a final concentration of 0.1 nM (Van der Walt et al., 2015). Each incubation consisted of 120 µg whole brain membrane 0.1 nM $[^3]H$ DPCPX, 0.1 mM GTP, the test compound and 1% DMSO. The addition of GTP to the final concentration of 0.1 mM was performed according to the specifications reported in the literature (Van der Walt et al., 2015). The order of addition was test compound (0.01 mL), radioligand (0.1 mL), 10 mM GTP (0.1 mL) and membrane suspension (0.79 mL). The membrane suspension was prepared with 50 mM Tris-HCl buffer, rat whole brain membranes, and adenosine deaminase. Each control incubation received 1% DMSO and did not affect the specific binding. The non-specific binding was defined by a final concentration of 10 µM unlabelled DPCPX. The order of addition of the non-
specific binding was as follows: 1 mM DPCPX (0.01 mL), radioligand (0.1 mL), 10 mM GTP (0.1 mL) and membrane suspension (0.79 mL).

4.3.6 Data Analysis

As mentioned above, each test compound was initially screened at a final concentration of 1, 10, and 100 µM for the adenosine A1 and A2A receptor binding assays. The screening results were then expressed as a calculated percentage displacement of the radioligand with regards to the control. The specific binding is defined as the total binding minus the non-specific binding (Bruns et al., 1986, Van der Walt & Terre'Blanche, 2015). The non-specific binding of [3H]DPCPX and [3H]NECA was defined as binding in the presence of a final concentration of 100 µM CPA (Bruns et al., 1986, Van der Walt et al., 2015). Thus, the percentage displacement was calculated by dividing the specific binding of the test compound by the specific binding of the control (1% DMSO) times 100. The results are documented in Table 4–1. Based on the screening results the $K_i$ values were determined for those test compounds that exhibited a percentage displacement of the radioligand ([3H]DPCPX or [3H]NECA) superior to 25% at the initial screening of the maximum tested concentration of 100 µM.

The $K_i$ values were obtained by determining the IC$_{50}$ values from sigmoidal-dose response curves by means of the Graphpad Software Inc. package. The corresponding $K_i$ value for the competitive inhibition by the test compounds of [3H]DPCPX ($K_d = 0.36$ nM) (Bruns et al., 1987) or [3H]NECA ($K_d = 15.3$ nM) (Bruns et al., 1986) were subsequently calculated from the IC$_{50}$ values. The sigmoidal-dose response curves were obtained by plotting the specific binding (i.e. the eight concentrations of each test compound ranging between 0 µM and 100 µM) versus the logarithm of the test compound’s concentrations (Van der Walt et al., 2015). The sigmoidal-dose response curves of the reference compounds CPA and DPCPX are provided in Figure 4–5. The $K_i$ values obtained from the latter compounds are in accordance with the literature and validate our radioligand binding and GTP shift assays.

For the adenosine A1 radioligand binding studies the $K_i$ values of the test compounds were calculated by using the Cheng and Prussof equation below (Cheng & Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{radioligand}]}{K_d}}$$
[Radioligand] = concentration of [\(^3\)H]DPCPX (0.1 nM)

\(K_d\) = dissociation constant of [\(^3\)H]DPCPX (0.36 nM)

However, because the adenosine A\(_{2A}\) radioligand binding studies were performed in the presence of CPA, an adapted version of the Cheng-Prusoff equation (Bruns et al., 1986), as indicated below, was used to calculate the \(K_i\) values:

\[
K_i = \frac{L \cdot C_{50}}{1 + \frac{L}{K_d} + \frac{C}{K_C}}
\]

\(C\) = concentration of CPA (50 nM)

\(K_C\) = dissociation constant of CPA (685 nM)

\(L\) = concentration of [\(^3\)H]NECA (4 nM)

\(K_d\) = dissociation constant of [\(^3\)H]NECA (15.3 nM)

Figure 4–5 illustrates the sigmoidal-dose response curves of compound 4d, (Panel A) indicating the binding to the adenosine A\(_1\) receptor in the presence and absence of GTP and that compound 4d acts as an adenosine A\(_1\) receptor antagonist, while the sigmoidal-dose response curves of CPA (Panel B) is provided in the presence and absence of GTP showing a calculated GTP shift of 6.5 due to CPA acting as an adenosine A\(_1\) receptor agonist. The sigmoidal-dose response curve of DPCPX (Panel C) indicates the binding to the adenosine A\(_{2A}\) receptors.

Furthermore, the \(K_i\) values are expressed in concentrations of \(\mu\)M. The lower the \(K_i\) value, the higher the affinity for the receptor. All incubations were carried out in triplicate and the \(K_i\) values are expressed as mean ± standard error of mean (SEM) (Van der Walt & Terre’Blanche, 2015).

### 4.4 RESULTS AND DISCUSSION

The target compounds (4a–i) were successfully synthesised and the structures confirmed by NMR spectroscopy and supported by MS results. Both the \(^1\)H and \(^{13}\)C NMR of each test compound corresponded with the proposed structures and the spectra reported in the literature (Bode et al., 2011). The adenosine A\(_1\) and A\(_{2A}\) receptor affinity for all the test compounds (1, 3a–e, 4a–i) were determined by means of radioligand binding assays described previously (Van der Walt & Terre’Blanche, 2015).
The in vitro results of the adenosine A_1 and A_{2A} receptor affinities of compounds 1, 3a–e and 4a–i are provided in Table 4–1.

Previous research (Reutlinger et al., 2014) identified 2-phenylimidazo-[1,2-α]pyridine (2) as a lead for designing compounds with adenosine A_1 receptor affinity. Imidazo[1,2-α]pyridine (1), the parent scaffold, is unsubstituted at positions C2 and C3 and was devoid of affinity for the adenosine A_1 and A_{2A} receptors. The influence of position C2 substitution was further investigated by comparing compound 1 to compounds 3a–d. Although compounds 3a–d showed improved specific binding percentages compared to compound 1, the unsubstituted phenyl ring (3a) and the para-substituted phenyl ring (3b–d) compounds showed no improvement for either adenosine A_1 or A_{2A} receptor affinity. The addition of an amino group to position C3 (3e vs 3a) also showed no improvement of adenosine A_1 or A_{2A} receptor affinity. Similarly, replacing the primary amine (3e) with a cyclohexylamino group (4a) exhibited no improvement in either adenosine A_1 or A_{2A} receptor affinity. Surprisingly para-substitution on the phenyl ring at position C2 in combination with a cyclohexylamino group at position C3 led to adenosine A_1 receptor affinity in the low micromolar range (4a vs 4b, 4c, 4d, 4e). Unfortunately, this substitution pattern did not favour adenosine A_{2A} receptor affinity. Good adenosine A_1 receptor affinity was found with the more bulky electron donating groups (-OH, -OCH_3, -CH_3), with the exception of the bulky electron withdrawing bromo group which also showed good adenosine A_1 receptor affinity. Other electron withdrawing groups (-Cl, -F, -CF_3, -NO_2) showed no affinity for the adenosine A_1 receptor (Figure 4–6).
Figure 4-5: The sigmoidal-dose response curves of compound 4d (Panel A) and CPA (Panel B) displaying the binding affinity to adenosine $A_1$ receptors in the absence and presence of GTP. Panel C displays the binding affinity of DPCPX to adenosine $A_{2A}$ receptors.
Table 4-1: The dissociation constant ($K_i$) values for the binding of the imidazo[1,2-α]pyridine analogues to rat adenosine $A_1$ and $A_{2A}$ receptors.

![Chemical structures](image)

<table>
<thead>
<tr>
<th>Compd</th>
<th>X</th>
<th>$A_1$ vs $[^3\text{H}]$DPCPX</th>
<th>$A_{2A}$ vs $[^3\text{H}]$NECA</th>
<th>$A_1 + \text{GTP}^c$ vs $[^3\text{H}]$DPCPX</th>
<th>GTP Shift$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>&gt;100 (100%)$^b$</td>
<td>&gt;100 (75%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3a</td>
<td>H</td>
<td>&gt;100 (49%)$^b$</td>
<td>&gt;100 (52%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3b</td>
<td>OH</td>
<td>&gt;100 (47%)$^b$</td>
<td>&gt;100 (85%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3c</td>
<td>OCH$_3$</td>
<td>&gt;100 (76%)$^b$</td>
<td>&gt;100 (61%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3d</td>
<td>Br</td>
<td>&gt;100 (86%)$^b$</td>
<td>&gt;100 (87%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3e</td>
<td>-</td>
<td>&gt;100 (52%)$^b$</td>
<td>&gt;100 (86%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4a</td>
<td>H</td>
<td>&gt;100 (57%)$^b$</td>
<td>&gt;100 (77%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4b</td>
<td>OH</td>
<td>5.53 ± 0.86$^a$</td>
<td>&gt;100 (27%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4c</td>
<td>OCH$_3$</td>
<td>7.61 ± 1.25$^a$</td>
<td>&gt;100 (55%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4d</td>
<td>CH$_3$</td>
<td>2.06 ± 0.08$^a$</td>
<td>&gt;100 (83%)$^b$</td>
<td>2.00 ± 0.24$^a$</td>
<td>1$^e$</td>
</tr>
<tr>
<td>4e</td>
<td>Br</td>
<td>3.90 ± 0.65$^a$</td>
<td>&gt;100 (54%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4f</td>
<td>Cl</td>
<td>&gt;100 (61%)$^b$</td>
<td>&gt;100 (64%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4g</td>
<td>F</td>
<td>&gt;100 (63%)$^b$</td>
<td>&gt;100 (90%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4h</td>
<td>CF$_3$</td>
<td>&gt;100 (76%)$^b$</td>
<td>&gt;100 (74%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4i</td>
<td>NO$_2$</td>
<td>&gt;100 (99%)$^b$</td>
<td>&gt;100 (69%)$^b$</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>DPCPX</th>
<th>CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.006$^e$</td>
<td>0.530$^e$</td>
</tr>
<tr>
<td></td>
<td>0.015$^e$</td>
<td>0.331$^e$</td>
</tr>
</tbody>
</table>

$^a$ All $K_i$ values are determined in triplicate and expressed as mean ± SEM.
$^b$ Percentage displacement of the radioligand at a maximum tested concentration (100 µM).
$^c$ GTP shift assay, where 100 µM GTP was added to the adenosine $A_1$ receptor radioligand binding assay.
$^d$ GTP shift is calculated by dividing the $K_i$ in the presence of GTP by the $K_i$ in the absence of GTP.
$^e$ Literature value obtained from Van der Walt & Terre'Blanche, 2015.
Figure 4-6: Illustrating the adenosine A₁ and A₂A receptor binding affinity of imidazo[1,2-α]pyridine (1), 2-phenyl-imidazo[1,2-α]pyridine-3-yl-amine (3), and the targeted imidazo[1,2-α]pyridine analogues (4).

Abbreviations: AR (adenosine receptor).
Compound 4d, with a para-methyl substituent, showed the best adenosine A1 receptor affinity with a $K_i$ value of 2.06 µM. Compound 4e with a para-bromo substituent ($K_i = 3.90$ µM) was found to possess the second highest adenosine A1 receptor affinity. Compounds 4b (p-OH) and 4c (p-OCH$_3$) displayed $K_i$ values of 5.53 µM and 7.61 µM, respectively. The above structural modifications did not, however, significantly modify the affinity for the adenosine A$_{2A}$ receptor, but para-substitution on the C2 phenyl ring together with C3 cyclohexylamino substitution afforded selective adenosine A1 receptor affinity indicating that both the substitution on positions C2 and C3 is needed for adenosine A1 receptor affinity (Figure 4–6).

In order to demonstrate if the compound possessing the highest adenosine A1 receptor binding affinity, compound 4d, acted as an agonist or antagonist, a GTP shift experiment was performed. Generally, a shift to the right of the binding curve in the presence of GTP (due to an uncoupling of the adenosine A1 receptor from its G$_i$ protein) is expected for an adenosine A1 receptor agonist. In the case of an adenosine A1 receptor antagonist, no significant shift is anticipated in the presence of GTP (Gütschow et al., 2012; Van der Walt & Terre’Blanche, 2015). As expected compound 4d showed no significant shift of the binding curve in the presence of GTP, thus compound 4d may be considered an antagonist of the adenosine A1 receptor Table 4–1 and Figure 4–5. Based on the structural similarity of compound 4d to compounds 4b, 4c and 4e, the latter compounds are also expected to act as adenosine A1 receptor antagonists.

4.5 CONCLUSION

This chapter describes the successful synthesis of selected imidazo[1,2-α]pyridine analogues (4a–i). The structures of the synthesised compounds were confirmed by $^1$H and $^{13}$C NMR as well as MS, whilst the purities of each synthesised compound was determined via HPLC analysis. All test compounds (both synthesised and commercially bought) were then subjected to radioligand binding assays to determine the affinities for the adenosine A1 and A$_{2A}$ receptors. The most potent compound (4d) possessed a para-substituted methyl functional group and displayed a $K_i$ value of 2.06 µM for the adenosine A1 receptor. Compound 4d was then subjected to a GTP shift assay indicating that it is an adenosine A1 receptor antagonist. Unfortunately, none of the test compounds (1, 3a–e, 4a–i) displayed significant $K_i$ values below 100 µM for the adenosine
A$_{2A}$ receptor. In conclusion, the 3-cyclohexylamino-2-phenyl-imidazo-[1,2-α]pyridine analogues (4a–i) may be considered an ideal scaffold to design novel imidazo[1,2-α]pyridine-based adenosine A$_{i}$ receptor antagonists in future studies.
4.6 REFERENCES


Adenosine and the adenosine receptors are involved in neurodegenerative disorders such as Alzheimer’s (AD) and Parkinson’s disease (PD) (Ribeiro et al., 2002). The effects of adenosine in neurodegenerative disorders are predominately mediated by the adenosine A₁ receptors expressed in the hippocampus and the adenosine A₂A receptors expressed in the striatum (Fredholm et al., 2001; Rivkees et al., 1995; Sebastiao & Ribeiro, 1996). The adenosine A₂A receptors are important for motor function (Kuwana et al., 1998) and display neuroprotective (Chen et al., 2001; Geiger et al., 2006; Monopoli et al., 1998) and antidepressant effects (Yacoubi et al., 2001), whereas the adenosine A₁ receptors play an important role in cognitive function (Mihara et al., 2007). Furthermore, the adenosine A₁ and A₂A receptors exert a synergistic effect on motor function as the adenosine A₁ receptors enhance the presynaptic release of dopamine (DA) (Fuxe et al., 2007a) while the adenosine A₂A receptors improve the postsynaptic DA activity (Fuxe et al., 2007b; Schiffmann et al., 2007).

Compounds with affinity for the adenosine A₁ and A₂A receptors are generally divided into xanthine derivatives and the amino-substituted heterocyclic compounds (non-xanthine) (Gaida et al., 1997; Francis et al., 1988; Müller & Stein, 1996; Pfister et al., 1997). Compounds containing the imidazo-[1,2-α]pyridine ring system have shown to possess a broad range of useful pharmacological properties (Abignente et al., 1992; Gueiffier et al., 1998; Lange et al., 2001) and their affinity for the adenosine A₁ receptors has been described by Beresis and colleagues (2003). Recently Reutlinger and co-workers (2014) screened the imidazopyridine scaffold for adenosine affinity and two imidazo-[1,2-α]pyridines were identified with good binding affinities for the adenosine A₁ receptor. Based on these findings a pilot study was undertaken to synthesise and evaluate a series of 3-cyclohexylamino-imidazo[1,2-α]pyridine analogues for their adenosine A₁ and A₂A receptor affinity.

The target compounds (4a–i) were successfully synthesised and confirmed with nuclear magnetic resonance (NMR) and mass spectrometry (MS). Furthermore,
the proton (\(^1\)H) and carbon (\(^{13}\)C) NMR of the targeted compounds corresponded with the spectra reported in the literature (Bode et al., 2011). The adenosine A\(_1\) and A\(_{2A}\) receptor affinities for both the synthesised and commercially bought compounds (1, 3a–e, 4a–i) were determined through radioligand binding assays as described in the literature (Van der Walt et al., 2015). In short, the adenosine A\(_1\) receptor affinities were determined with rat whole brain membranes in the presence of the radioligand 1,3-dipropyl-8-cyclopentylxanthine ([\(^3\)H]DPCPX), whereas the adenosine A\(_{2A}\) receptor affinities were determined with rat striatal membranes in the presence of the radioligand 5'-N-Ethylcarboxamidoadenosine [\(^3\)H]NECA. The test compound with the highest affinity at the adenosine A\(_1\) receptor was subsequently subjected to a GTP shift assay to determine whether or not the compound has agonistic or antagonistic functionality (Van der Walt et al., 2015).

Imidazo[1,2-α]pyridine (1), the parent scaffold of this pilot study, is unsubstituted at positions C2 and C3 and was devoid of adenosine A\(_1\) and A\(_{2A}\) receptor affinity. The influence of C2 substitution on adenosine A\(_1\) and A\(_{2A}\) receptor affinity was investigated by comparing compound 1 to compound 3a–d containing a C2 phenyrling. Although the specific binding percentages of these compounds improved compared to compound 1, they did not display improved adenosine A\(_1\) and A\(_{2A}\) receptor affinity. The addition of an amino group at position C3 (3e vs. 3a) also showed no improvement for the adenosine A\(_1\) and A\(_{2A}\) receptor affinity. Alternatively the combination of a para-substituted phenyrling at position C2 together with a cyclohexylamino group at position C3 displayed adenosine A\(_1\) receptor affinity in the low micromolar range (4a vs. 4b, 4c, 4d, and 4e).

However, no significant adenosine A\(_{2A}\) receptor affinity was obtained. It seems that the more bulky electron donating groups (-OH, -OCH\(_3\), -CH\(_3\)) displayed good adenosine A\(_1\) receptor affinity, while the electron withdrawing groups (-Cl, -F, -CF\(_3\), -NO\(_2\)) showed no adenosine A\(_1\) receptor affinity with the exception of the bromo group that possessed good adenosine A\(_1\) receptor affinity (Figure 4–6).
The compound documented with the best adenosine $A_1$ receptor affinity, was compound 4d with a $\text{para}$-methyl substituent ($K_i = 2.06 \mu M$). Compound 4e (the $\text{para}$-bromo substituent) displayed the second highest adenosine $A_1$ receptor affinity ($K_i = 3.90 \mu M$), followed by the $\text{para}$-hydroxy (compound 4b) and $\text{para}$-methoxy (compound 4c) analogues, exhibiting $K_i$ values of 5.53 $\mu M$ and 7.61 $\mu M$ respectively for the adenosine $A_1$ receptor. Unfortunately, none of these structural modifications favoured adenosine $A_{2A}$ receptor affinity. Furthermore, selective adenosine $A_1$ receptor affinity was only obtained with $\text{para}$-substitution of the phenyl ring at position C2 in combination with a cyclohexylamino substitution on position C3, thus requiring substitution of both position C2 and C3 to obtain adenosine $A_1$ receptor affinity (Figure 4–6).

Compound 4d was subsequently subjected to a GTP shift assay to determine whether or not it has agonistic or antagonistic functionality. When a compound has agonistic functionality of the adenosine $A_1$ receptor, the binding curve displays a rightward shift in the presence of GTP and no significant shift of the binding curve in the presence of GTP is expected for antagonistic functionality (Gütschow et al., 2012; Van der Walt & Terre’Blanche, 2015). Compound 4d displayed no significant shift of the binding curve in the presence of GTP, and may be considered as an antagonist of the adenosine $A_1$ receptor (Table 4–1, Figure 4–5). Based on the structural similarities of compounds 4b, 4c and 4e to compound 4d, it is expected that these compounds will also be antagonists of the adenosine $A_1$ receptor.

For future optimization of the 3-cyclohexylamino-imidazo[1,2-α]pyridine scaffold the results of a study by Novellino and co-workers (2002) gave valuable insight for future scaffold modifications. They explored the adenosine $A_1$ affinity of novel N-alkyl- and N-acyl-(7-substituted-2-phenylimidazo[1,2-α][1,3,5]triazin-4-yl)amines (ITAs). Their findings (Figure 5–1) highlighted the importance of a CO linker. The CO linker enhanced adenosine $A_1$ receptor affinity; however the affinity of the adenosine $A_{2A}$ receptor remained decreased. When an acetyl group was introduced at the N4 position, it reduced the binding affinity of the adenosine $A_1$ receptor, while the binding affinity of the adenosine $A_{2A}$ receptor remained unchanged. The adenosine $A_1$ receptor affinity was improved when a CO-cyclohexyl and CO-phenyl group was introduced although the binding affinity of the adenosine $A_{2A}$ receptors showed no improvement. The addition of a methylene spacer between the N4 position and the acetyl group enhanced
adenosine $A_1$ receptor affinity. When a CO-cyclopentyl was introduced, it enhanced the affinity for the adenosine $A_1$ receptor and affinity for the adenosine $A_{2A}$ receptor was gained. Substitution of the C7 position with a phenyl moiety rather than a methyl group maintained affinity of the adenosine $A_1$ receptor, while adenosine $A_{2A}$ receptor affinity was increased (Novellino et al., 2002).

A possible explanation for our low affinity can be ascribed, in part to the research done by Gillespie and co-workers (2009a), where the increased number of nitrogens in the heterocyclic ring (from a pyridine to a pyrimidine) enhanced both adenosine $A_1$ and $A_{2A}$ receptor affinities. They compared the affinities of triazine, pyrimidine and pyridine scaffolds and observed that the pyridine scaffold was sevenfold less potent than the triazine and 45-fold less potent than the corresponding aminopyrimidine, thereby concluding that two nitrogens in the ring are optimum for both adenosine $A_1$ and $A_{2A}$ receptor affinity (Gillespie et al., 2009a,b). This trend was also observed in the ITA analogues synthesised by Novellino and co-workers (2002), where the 6:5 fused bicyclic rings containing four nitrogen atoms, displayed better adenosine receptor affinity in comparison to the investigated imidazo[1,2-α]pyridine analogues containing two nitrogens in the fused heterocyclic rings.
Figure 5-1: The adenosine binding affinities of selected ITA analogues (Novellino et al., 2002).
Therefore we hypothesise that the introduction of additional nitrogens in the heterocyclic ring may increase adenosine $A_1$ and $A_{2A}$ receptor affinity (Figure 5–2). Moreover the findings of Novellino and co-workers (2002) can be used for further structural modifications (Figure 5–2) to 3-cyclohexylamino-2-(4'-methylphenyl)imidazo[1,2-α]pyridine (compound 4d) to optimise the scaffold for improved adenosine $A_1$ and $A_{2A}$ receptor affinity (Figure 5–2). These modifications include the replacement of the cyclohexyl ring with a cyclopentyl ring and the insertion of a CO group and a methylene spacer between the NH and the CO group.

Figure 5-2: Proposed future structural modifications to compound 4d.

In conclusion, the newly proposed structural optimization of the investigated imidazo-[1,2-α]pyridine analogues may be implemented in future studies to potentially improve the adenosine $A_1$ receptor affinity and gain adenosine $A_{2A}$ receptor binding. Among the test compounds 4d was identified as the best selective adenosine $A_1$ receptor antagonist ($K_i = 2.06 \, \mu M$), thereby emphasizing the importance of the imidazo[1,2-α]pyridine-based scaffold for further structure-activity relationship studies to design novel imidazo-[1,2-α]pyridine-based adenosine $A_1$ receptor antagonists for the treatment of neurodegenerative disorders such as AD and PD.
5.1 REFERENCES


characterization of a novel, potent adenosine A\textsubscript{1} and A\textsubscript{2A} receptor dual antagonist, \(5-[5\text{-}amino\text{-}3\text{-}(4\text{-}fluorophenyl)\text{ pyrazin\text{-}2\text{-}yl}\text{-}1\text{-}isopropylpyridine\text{-}2\text{(1H)}\text{-}one}\) (ASP5854), in models of Parkinson's disease and cognition. *Journal of pharmacology and experimental therapeutics*, 323(2):708-719.


ANNEXURE A NMR DATA

$^1$H NMR: Compound 4a (DMSO-$d_6$)

$^{13}$C NMR: Compound 4a (DMSO-$d_6$)
**$^1$H NMR:** Compound 4b (DMSO-$d_6$)

![NMR Spectrum Image]

**$^{13}$C NMR:** Compound 4b (DMSO-$d_6$)

![NMR Spectrum Image]
\textbf{\textsuperscript{1}H NMR:} Compound 4c (DMSO-\textit{d6})

\textbf{\textsuperscript{13}C NMR:} Compound 4c (DMSO-\textit{d6})
$^{1}H\text{ NMR: Compound 4d (DMSO-d}_6$)

\[\text{Diagram of }^{1}H\text{ NMR spectrum for Compound 4d.}\]

$^{13}C\text{ NMR: Compound 4d (DMSO-d}_6$)

\[\text{Diagram of }^{13}C\text{ NMR spectrum for Compound 4d.}\]
C NMR:

13C NMR: Compound 4e

H NMR: Compound 4e
$^1$H NMR: Compound 4f

$^{13}$C NMR: Compound 4f
$^{13}$C NMR: Compound 41

$^1$H NMR: Compound 41
ANNEXURE B MS DATA

Compound: 4a

Compound: 4b
Compound: 4i