Radioligand binding assays of selected A_1/A_{2A} adenosine antagonists using Rat Membrane Cells and Chinese Hamster Cells expressing Human adenosine receptors

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ABSTRACT

Alzheimer's and Parkinson's disease are considered to be the first and second most common neurological diseases, which contribute to mortality in the elderly population, reduces quality of life and increase socioeconomic difficulties. Despite numerous neuro-pharmacological products on the market and others that are currently being researched, A_1 and A_{2A} adenosine receptor (AR) antagonists are of high interest in controlling Alzheimer's and Parkinson's disease related cognitive deficits (A_1 AR subtype) and Parkinson's disease motor dysfunction (A_{2A} AR subtype).

When using animal experiments to ascertain A_1 AR affinity, there are limitations translating these results to humans; as some functions observed with the rat model might not be obtained in humans. Our aim was to establish a radioligand binding assay (RBA) using Chinese hamster ovary (CHO) cells expressing human A_1 ARs. In addition, known 7-azaindole derivatives were screened as novel A_1 AR antagonists using both rat brain membrane cells and CHO cells expressing human A_1 ARs.

An adapted method of Klotz and co-workers (1997) were used to established the A_1 AR radioligand binding assay with CHO cells transfected with human A_1 ARs. Reference compounds DPCPX (A_1 antagonist) and istradefylline (A_{2A} antagonist) were used leading to successful binding competition curves, which corresponded with literature values. The 7-azaindole derivatives, previously screened for protein kinase inhibition, were found to be potential A_1 AR antagonists with 7-azaindole-chalcone conjugated compound **1a** showing the highest binding affinity for A_1 ARs in rat and human models (hA_1K_i = 0.92 µM; rA_1K_i = 0.90 µM). The 7-azaindole-indanone conjugated compound **1e** showed the second highest affinity (hA_1K_i = 1.7 µM; rA_1K_i = 1.1 µM). The 7-azaindole-α-tetralone fused compounds (**1b**, **1c**, **1d**) showed a two- to three-fold lower binding affinity for A_1 ARs in rat and human.

In conclusion, the A_1 AR radioligand binding assay, using transfected CHO cells, was successfully established and affinity values of reference compounds correlated with literature. Therefor reducing the challenges in ethics by minimizing the harvesting of rat brains and high costs of rat models. Further, the 7-azaindole-chalcone core was identified as a promising scaffold to explore modifications for improved A_1 AR affinity, which may play a therapeutic role in Alzheimer's and Parkinson's disease with regards to improved cognitive deficits associated with neurodegenerative diseases.

Key Words:

Alzheimer's disease, Parkinson's disease, A_1 adenosine receptor antagonists, 7-azaindole-chalcone, Chinese hamster ovary cells

ABBREVIATIONS

[^{3}H]DPCPX [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine/1,3-[^{3}H]-Dipropyl-8-cyclopenthylxanthineα-syn alpha-synuclein

A

Aβ amyloid-beta

AChEIs acetylcholinesterase inhibitor

AC adenyl cyclase

ADA adenosine deaminase

AR(s) adenosine receptor(s)

ATP adenosine 5'-triphosphate

B

BG basal ganglia

BBB blood-brain barrier

BChE butyrylcholinesterase

BDN-F brain-derived neurotrophic factor

BPT 4-(1-benzylpiperidin-4yl) thiosemicarbazone

C

cAMP cyclic adenosine monophosphate

CHO Chinese hamster ovary

CNS central nervous system

COMT catechol-O-methyltransferase

CPA N6-cyclopentyladenosine

CPT 8-cyclopentyltheophylline

CSC	8-(3-chlorostyryl)caffeine
CSF	cerebrospinal fluid

D

DA	dopamine
DDQ	diethyl(3,-4-dihydroxyphene thylamino)quinolin-4-yl)methylphosphonate
DMEM/F12	Dulbecco's modified eagles medium with nutrient mixture F12
DMPX	3, 7-dimethyl-1-propargylxanthine
DMSO	dimethyl sulfoxide
DPCPX	8-cyclopentyl-1,3-dipropylxanthine

F

FBS	fetal bovine serum
FDA	Food and Drug Administration
FST	forced swim test

G

GABAB	gamma-aminobutyric acid B
GBA	glucocerebrosidase
GCS	glucosylceramide synthase
GPe	external segment of globus pallidus
GPi	internal segment of globus pallidus
Gs	stimulatory G-protein
GSTO2	glutathione S-transferase omega-2
GTP	guanosine triphosphate

H

h	human

I

IL-1b	interleukin 1 beta
IL-6	interleukin 6
IFN-g	interferon gamma

K

*K*i	inhibition constant
Kd	dissociation constant
KO	knockout
KW-6002	istradefylline

L

LAMB	Laboratory for Analytical and Molecular Biology
LB	Lewy body
LPS	lipopolysaccharide
LN	Lewy neurite
L-dopa	levo-dopa/L-3,4-dihydroxyphenylalanine

M

MAO	monoamine oxidase
MAO-A	monoamine oxidase type A
MAO-B	monoamine oxidase type B
MAPK	mitogen-activated protein kinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

N

N	nitrogen
NEAA	non-essential amino acids
NFTs	neurofibrillary tangles
NM	neuromelanin
NMDA	N-methyl-D-aspartate
NSAIDs	nonsteroidal anti-inflammatory drugs

O

OCH3	methoxy
OH	hydroxy

P

PBS	Phosphate buffered saline
PD	Parkinson’s disease
Pen-Strep	Penicillin-Streptomycin
PET	positron emission tomography
PINK1	phosphatase and tensin homolog (PTEN)-induced putative kinase 1
PON1	paraoxonase 1
PSEN-1	presenilin 1
PSEN-2	presenilin 2
PTEN	phosphatase and tensin homolog -induced putative kinase 1 (PINK1)

R

r	rat
RBA	radioligand binding assay
REM	rapid eye movement

ROS	reactive oxygen species

S

SAR	structure activity relationship
SEM	standard error of mean
Sirt1	sirtuin 1
SNpc	substantia nigra *pars compacta*
SNr	substantia nigra *pars reticulata*
STN	subthalmic nucleus

T

TNF-a	**tumour necrosis factor alpha**
TrkB	tyrosine kinase B

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CHAPTER 1: INTRODUCTION

1.1 Background

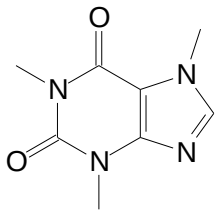
Alzheimer's disease (AD) and Parkinson's disease (PD) are two common age-related chronic neurodegenerative disorders that affect the elderly population over 65 years of age (Calne & Peppard, 1987; Nussbaum & Ellis, 2003). AD and PD are described as the first and second leading neurodegenerative diseases, respectively, and clinically present with cognitive dysfunction in AD and PD, and classical motor dysfunction in PD (Calne & Peppard, 1987, Nussbaum & Ellis, 2003). The prevalence of AD is estimated to be about 1-2% in the age of 65 years and doubles every five years to 35% or more around the age of 85 years, while the prevalence of PD is 1% over the ages of 65 years and climb to 4% at the age of 80 years (Shalash *et al.,* 2017).

AD is characterized by progressive damage of memory and other cognitive abilities leading to dementia (Nussbaum & Ellis, 2003). The cause of AD is unknown. The accumulation of extracellular amyloid plaques containing amyloid-beta (Aβ) peptide and formation of intraneuronal neurofibrillary tangles in the hippocampus, entorhinal cortex, amygdala and cerebral cortex are classical hallmarks of AD (Hauw *et al.*, 1996; Kumar & Clark, 2005). On the other hand, PD is characterized by the progressive deterioration of the basal ganglia dopaminergic nigrostriatal pathway from the substantia nigra pars compacta (SNpc) (Calne & Peppard, 1987). The dopaminergic deficit in PD arises from a loss of the neurons in the SNpc that provide innervation to the striatum (caudate and putamen) - leading to dysregulation of striatal function which account for the symptoms observed in PD; for example resting tremor, muscular rigidity and bradykinesia (Ahlskog & Muenter, 2001). Non-motor symptoms of PD also include cognitive impairment (Simola *et al.*, 2014).

Although scientists have tried to find effective symptomatic treatment, there is, at present, no cure for PD and AD (Di Stefano *et al.*, 2011; Sowell *et al.*, 2009). PD motor symptoms have been reduced by using levodopa (L-3,4-dihydroxyphenylalanine), which is a precursor to dopamine (DA) and remains the drug of choice for the treatment of PD (Ahlskog & Muenter, 2001). Administration of levodopa presents two disadvantages; namely, the onset of motor fluctuations (wearing-off and on-off phenomena) and motor complications (dyskinesias) after prolonged treatment, ultimately leading to disease progression (Ahlskog & Muenter, 2001; Obeso *et al.*, 2000). Other drug options include selective DA receptor agonists, catechol-O-methyltransferase (COMT) inhibitors (LeWitt, 2000), and monoamine oxidase-B (MAO-B) inhibitors (Tabakman *et al.,* 2004). While AD symptomatic treatment options include acetylcholinesterase inhibitor (AChEIs) and N-methyl-D-asperate (NMDA) receptor antagonists (Di Stefano *et al.*, 2011), these

compounds only slow disease progression in its early stage but do not help with end stage AD (Di Stefano *et al.*, 2011).

A unique drug that targets all challenges presented by these neurodegenerative diseases is needed urgently to improve quality of life and cut the burden associated with patient care and socio-economic factors (Van Bulck *et al.*, 2019). A systematic approach where a compound targets multiple sites is more likely to succeed as a treatment option for AD and PD to alleviate symptoms (Ribeiro & Sebastião, 2010). Adenosine receptors (ARs) may be the solution to the challenge, since a number of epidemiological studies have shown an inverse relationship between coffee consumption and the prevalence of AD and PD (Lindsay *et al.,* 2002; Martyn & Gale, 2003). Caffeine is a xanthine derivative and acts as a non-selective A_1 and A_{2A} AR antagonist (Schwarzschild *et al.*, 2002).



Caffeine

Adenosine is a neuromodulator that facilitates signalling via different neurotransmitters and receptors, which include four G-protein coupled ARs, namely A_1, A_{2A}, A_{2B}, and A_3 generally found all over the human body (Burnstock, 2006). The number of A_1 and A_{2A} ARs in the brain is high which increase the affinity of adenosine. Adenosine acts through inhibitory (A_1 and A_3) or stimulatory (A_{2A} and A_{2B}) AR pathways (Chen *et al.,* 2014; Palmer & Stiles, 1995). Co-expression (i.e. heteromers) occurs between A_1 and A_{2A} ARs in the presynaptic membranes and A_{2A} and dopamine (D_2) receptors localized around GABAergic striato-pallidal neurons (Azdad *et al.,* 2009; Trifilieff *et al.*, 2011). The activation/blockade of A_1 and A_{2A} AR produce a spectrum of pathophysiological activities in the central nervous system (CNS) ranging from cognition (A_1 AR), locomotion (A_{2A} AR), behaviour (A_{2A} AR) and neurodegeneration (A_{2A} AR) (Chen *et al.*, 2014).

Modification of the adenosine structure has been explored to identify new AR agonists, whereas the xanthine scaffold of caffeine was used for the design of new AR antagonists. However, xanthines display low water solubility, thus limiting their *in vivo* application (Müller *et al.,* 2002). Nitrogen-free heterocyclic ring systems, such as chalcones, are considered a privileged scaffold in medicinal chemistry, seeing that they have attracted attention not only from synthetic and biosynthetic perspectives but also due to their wide-ranging biological activities, such as antimicrobial, antiviral, antifungal, antimalarial, antileishmanial, anti-inflammatory and anticancer

properties (Gaonkar & Vignesh, 2017; Zhuang *et al.,* 2017). Chalcones are a member of the flavonoid family which have been explored as AR antagonists (Van der Walt & Terre'Blanche, 2018), and justly, a chalcone is nothing but an open-chain flavonoid consisting of two aromatic rings linked by an aliphatic three carbon chain. In addition, several chalcone-coumarin derivatives have shown to possess AR affinity (Vazquez-Rodriguez *et al.,* 2013; Vazquez-Rodriguez *et al.,* 2020) and recently C3 amino-substituted chalcone derivatives with a bromo substitution on benzylidene ring B were reported to possess selective adenosine A_1 AR affinity in the micromolar range (Janse van Rensburg, *et al.,* 2020) (**Figure 1-1**).

Radioligand binding assays (RBAs) are widely used for the screening of new potential receptor ligands and are ideally suitable for structure-activity relationship analysis and molecular modelling studies. The basic concept of RBAs comprises of a radioligand that possesses high affinity for a specific receptor. Thus, the radioligands are used to measure the affinity that a test compound possesses for a specific receptor. 1,3-[^{3}H]-Dipropyl-8-cyclopenthylxanthine ([^{3}H]DPCPX) is an example of a radioligand used to determine a compound's affinity for the adenosine A_1 AR subtype. The adenosine A_1 RBAs are performed with rat whole brain membranes that express the A_1 AR subtype (Van der Walt & Terre'Blanche, 2015). The above-mentioned RBA is frequently performed using rat membranes. However, using rat membranes are costly and the use of animals in scientific and therapeutic research has been a subject of hot discussion for many years. There are strong opposing opinions among enthusiasts and sceptics about the relevance of animal data for humans and the likelihood of successful cross-species translation.

Prompted by the above discussion, the current pilot study undertook to establish the A_1 AR RBA using Chinese hamster ovary (CHO) cells expressing the human A_1 AR. In addition, reference compounds DPCPX and istradefylline (KW-6002) were included for comparison with literature values. Further several known 7-azaindole hybrids, previously evaluated as potential protein kinase inhibitors (Qhobosheane *et al.,* 2020b), were chosen to be screened as novel adenosine A_1 AR antagonists. **Figure 1-1** represents the structural similarity of the 7-azaindole scaffold compared to the chalcone moiety.

Firstly, this chapter provides the background, rationale, research problem and aims and objectives of the current pilot study. Chapter 2 and Chapter 3 contain a literature review of PD, AD and ARs. Chapter 4 describes the experimental procedures and the results and discussion are presented in Chapter 5. Lastly, Chapter 6 summarises the present study and suggests future research.

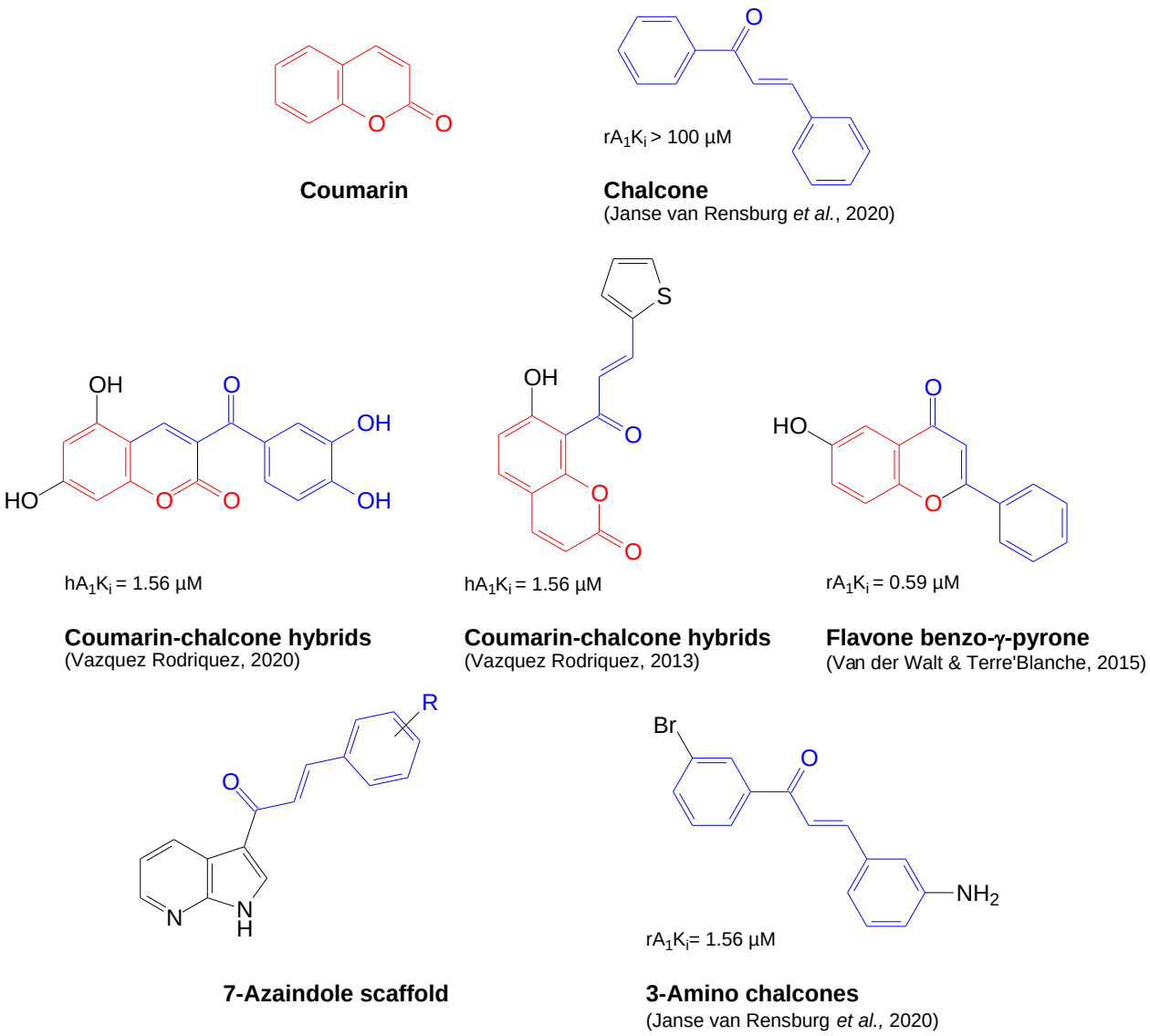


Figure 1--1: Structural similarities of different chalcone based derivatives.

1.2 Research Problem

Understanding how humans respond to drugs is an essential question in drug research. Often this question cannot be addressed because it is not possible to perform some experiments in human volunteers. An alternative can be the generation of responses in animal models and "translating' those results to humans. The limitations are far from clear and further assessment of the potential to extrapolate animal data to humans are needed (Jacobson & Gao, 2006). Several A_1 AR antagonists were screened by Maemoto and co-workers (1997), showing that there were no major differences in inhibition constant (K_i) values between human and rat tissue using [^{3}H]DPCPX as ligand. Contradictory, the adenosine A_1 antagonist, DPCPX, has a K_i value of 3 nM in human compared to a value of 0.5 nM in rat using [^{3}H]DPCPX as ligand (Alnouri *et al.,* 2015; Maemoto *et al.,* 1997), whereas no such species difference was found for theophylline (Klotz *et al.,* 1997). Supporting these findings Fredholm and co-workers (2001) compared

literature values of antagonists showing the following: DPCPX (human 3.9 nM vs rat 0.3 nM), XAC (human 29 nM vs rat 2.8 nM) and ZM 241385 (human 260 nM vs rat 2 nM).

It is clear that some of the functions observed with one animal model might not be obtained in other animal models and in humans. Cross-species testing is thus important to validate the receptor function or effects of agonists and antagonists. Therefore, the question arises whether the compounds in our library which show high affinity and selectivity at rat A_1 AR will exhibit similar affinity and selectivity for their human orthologues.

1.3 Aims and Objectives

Since there is a contradiction in literature between rat and human adenosine receptor affinity data together with the costs and ethics involved in using rats, the aim of this pilot study is to establish the RBA using CHO cells expressing human A_1 ARs. In addition, the affinities of known 7-azaindole-chalcone hybrids will be tested as novel adenosine A_1 AR antagonists using CHO cells expressing human A_1 AR.

In short, the objectives of this study are:

- Growing and harvesting of transfected CHO cells expressing human A_1 AR
- Establishing RBA using expressed CHO cells
- Comparing affinity values of reference compounds DPCPX and istradefylline in both rat brain membrane cells and CHO cells expressing the human A_1 AR
- Full RBA using CHO cells and promising 7-azaindole-chalcones previously screened in rat RBA (unpublished results)
- To ascertain structure activity relationships of 7-azaindole-chalcones which govern A_1 AR affinity

CHAPTER 2: ALZHEIMER'S AND PARKINSON'S DISEASE AND ADENOSINE RECEPTORS

2.1 General Background

Alzheimer's disease (AD) and Parkinson's disease (PD) are known as the most progressive neurodegenerative disorders, holding the first and second titles, respectively (Xie *et al.*, 2014). These neurological conditions both present a spectrum of clinical features and neuropathological findings.

In 1907 Dr Alois Alzheimer discovered and described "presenile dementia" (Jarvik & Greenson, 1987), Alzheimer described the neurohistopathology and distinguished the pathological hallmark, namely "plaque-only". This hallmark entailed insoluble amyloid-beta (Aβ) protein in the brain parenchyma, the cerebral blood vessels and neurofibrillary tangles (NFTs) consisting of precipitates of hyperphosphorylated forms of microtubules-associated protein tau. The cognitive impairment in patients with AD is associated with synaptic loss in the neocortex and limbic system (Ittner & Götz, 2011). AD is the most common cause of dementia in the elderly, which presents as learning and memory impairment leading to executive dysfunction and, ultimately, interferes with daily life activities (Scheltens *et al.*, 2016).

On the other hand, PD was first described by James Parkinson in 1817 in an essay entitled: "An essay on the shaking palsy" (Parkinson, 2002). The onset of PD is characterized by the degeneration of dopamine neurons in the substantia nigra pars compacta (SNpc) within the basal ganglia (BG), as well as Lewy neurite intracellular inclusions composed of α-synuclein (α-syn) proteins (Xie *et al.*, 2014). Classically known as a movement disorder, PD presents with motor symptoms such as tremor, rigidity, bradykinesia and imbalance (Jankovic, 2008). Non-motor symptoms include sleep disorders, psychiatric symptoms, olfactory dysfunction, gastrointestinal and cognitive dysfunction (Simola *et al.*, 2014).

Both AD and PD present common features such as protein aggregation, oxidative stress, progressive neuronal degeneration, systemic- and neuroinflammation. Additionally, AD and PD boast an array of treatment options which do not modify disease progression but only treat symptoms (Van Bulck *et al.*, 2019).

The prevalence of AD and PD continue to increase because there is, at present, no cure (Prince *et al.*, 2015; Rocca, 2018). This assertion is substantiated in the literature, and AD is commonly known as the most prevalent and PD the second most prevalent neurodegenerative disorders in the world (Alam *et al.*, 2016). In 2015, there were 46.8 million AD patients worldwide with direct

and indirect costs to society of 81,800 million USD (Prince *et al.,* 2015), and in 2016 there were 6.1 million individuals with PD worldwide (Rocca, 2018). The incidence of AD and PD throughout the world is expected to triple by 2050 (Weiner *et al.,* 2015).

2.2 Neuropathology

AD is the most common form of dementia in the elderly and is characterized by the presence of Aβ in senile (diffuse and neuritic) plaques, NFTs in the brain and a major loss of synaptic connections (Duyckaerts & Dickson, 2011). Senile plaques that are found in the cerebral grey matter are made of Aβ deposition, surrounded by abnormal arranged neurites (Ferrer *et al.*, 2004; Perl, 2010). NFTs consist of abnormal accumulation of hyperphosphorylated tau proteins found within the perikaryal cytoplasm, including the entorhinal cortex, subiculum, hippocampus, amygdala, Meynert nucleus and neocortex (Ferrer *et al.,* 2004). The presence of dementia and major neurocognitive disorders are highly linked to intermediate and high level neuropathological changes in AD. Clinical diagnosis of symptomatic AD in living patients depend on signs of dementia and positive biomarkers of Aβ from positron emission tomography (PET) and Aβ or tau protein levels from cerebrospinal fluid (CSF) (McKhann *et al.*, 2011), while a definite diagnosis of AD may only be made after death (Šimić *et al.*, 2017).

PD pathology is underlined by degeneration and loss of nigrostriatal dopaminergic neurons, with the presence of Lewy bodies (LB) (Braak *et al.,* 2006). LB or Lewy neurites (LN) are intracytoplasmic neuronal inclusions of the SNpc and largely composed of α-syn (Xie *et al.,* 2014). Additionally, pale staining inclusions can also be found in the amygdala and neocortex (Dickson, 2018). This neurodegeneration affects the noradrenergic locus coeruleus (oral parts) and motor vagal nucleus, the dopaminergic mesocorticolimbic system, the serotonergic raphe nuclei, the cholinergic nucleus basalis of Meynert, pedunculopontine nucleus, Westphal-Edinger nucleus, and many peptidergic brainstem nuclei containing cholecystokinin, met enkephalin, substance P, somatostatin and neuropeptide Y (De Erausquin *et al.,* 1994; Jenner *et al.*, 1992). Braak and co-workers (2006) found that PD pathology extend beyond the substantia nigra where PD starts in the medulla and then moves to the olfactory bulb, pons, substantia nigra, basal forebrain, amygdala, medial temporal lobe and lastly to the cortical area (Di Stefano *et al.*, 2011; Dickson, 2018).

2.3 Aetiology

According to literature the aetiology factors of AD and PD are not known or are unclear (Di Stefano *et al.*, 2011; Dickson, 2018), while other studies indicate that environmental factors and genetics play a major role in the cause of AD and PD (**Figure 2-1**) (Chin-Chan *et al.*, 2015). AD and PD,

are both related to old-age and low-grade inflammatory status of the immune system, commonly referred as inflammaging (Franceschi *et al.*, 2000), which might be the most important aetiology factor of these neurodegenerative diseases. These diseases come in two forms: namely familial (genetic cause) and idiopathic (unknown cause). The familial form of PD (various genetic mutations before the age of 65 years) accounts for 5% and the idiopathic form accounts for 95% of all AD and PD cases (Chakrabarti *et al.*, 2015; Grünblatt *et al.*, 2018). Shared common genetic determinants have been hypothesized to be involved in both AD and PD. Xie and colleagues (2014) reported that common genetic mutations of paraoxonase 1 (PON1), glutathione S-transferase omega-2 (GSTO2) and NEDD9 genes are implicated in and shared between AD and PD (Allen *et al.*, 2012; Chapuis *et al.*, 2008). The use of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to induce neurotoxicity can also cause a syndrome closely related to PD (Langston *et al.*, 1983). Pesticides and herbicides are implicated to cause PD, while exposure to rotenone (a broad-spectrum insecticide, piscicide and pesticide) are also connected to the development of PD in humans (Dauer & Przedborski, 2003).

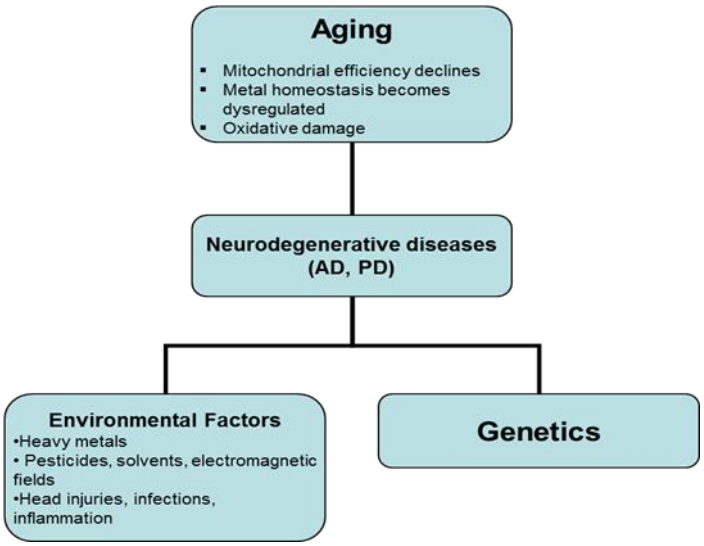


Figure 2-1: Etiology of AD and PD neurodegeneration

2.4 Pathogenesis and/or Mechanism of Neurodegeneration in AD and PD

The pathogenic mechanisms of these two neurodegenerative diseases are interrelated with no claim of sole responsibility for disease progression in AD and PD. The pathogenic mechanisms may be attributed to misfolding and aggregation of proteins, mitochondrial dysfunction, oxidative stress, inflammation and apoptosis among others see **Figure 2-2** (Van Bulck *et al.*, 2019).

2.4.1 Misfolding Protein

In AD Aβ aggregates around postsynaptic compartments and produce toxic species including dimers, oligomers and fibrils, which block complex 4-dependent respiration leading to mitochondrial impairment (Ittner & Götz, 2011). Secondly, hyperphosphorylation of tau interferes with neuronal function by impairing mitochondrial complex 1 of the respiratory chain. In PD, LB are largely composed of α-syn which accumulate in the neurogenic region where adult neurogenesis occur, and impairs olfactory bulb and hippocampal neurogenesis (Horgusluoglu *et al.*, 2017).

2.4.2 Mitochondrial Dysfunction

Mitochondria participate in neurodegenerative disorders by different mechanisms for example misfolding and aggregation of proteins which directly damage mitochondrial DNA, impairment to organelle trafficking and dynamics, bioenergetics/mitophagy processes, apoptosis, oxidative stress, cell metabolism and mitochondria-dependent cell death (Fiorito *et al.*, 2018). PD-related gene mutations are also implicated in mitochondrial quality control through phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) and Parkin proteins (Truban *et al.*, 2017). AD-related gene mutations alter mitochondrial activity through PSEN-1 and PSEN-2 proteins (Sarasija & Norman, 2018)

2.4.3 Oxidative Stress

It is well reported in literature how AD and PD are associated with oxidative stress via mitochondrial dysfunction and neuroinflammation. At a molecular level, oxidative stress causes peroxidation of lipids and formation of 4-hydroxynonenal, which lead to mutation and destabilization of nucleic acid (Cheignon *et al.*, 2018). These biochemical changes result in a loss of membrane integrity, metabolism, mutation of DNA, a decrease of ATP levels, altered mitochondrial function and activation of pro-apoptotic pathways leading to neuronal death (Hroudov *et al.*, 2014).

2.4.4 Neuroinflammation

Inflammation in AD and PD progression is defined by the accumulation of activated glial cells (astrocytes and microglia) in damaged regions of the brain (Alam *et al.*, 2016), leading to chronic inflammation. Chronic inflammation stimulation may lead to undesired injury which may result in continuous eroding of surrounding tissues causing neuronal death (Akiyama *et al.*, 2000). With aging, prolonged and impaired activation of macrophage and microglia can induce reactive oxygen species (ROS) production (Chinta *et al.*, 2015).

In AD, an autoimmune reaction in the brain activates microglial cells which have both positive and negative effects on AD pathogenesis, where the negative outweighs the positive. Cytokines including **tumour necrosis factor alpha** (TNF-a), interleukin 1 beta (IL-1b) and interleukin 6 (IL-6) act directly on cholinergic neurons and induce their apoptosis (Liang *et al.*, 2017). In PD, autoreactive neuromelanin (NM) specific T cells participate in dopamine neuronal damage and activation of microglia (Koutsilieri *et al.*, 2013). Intraneuronal aggregation of α-syn protein promotes activation of microglia and astrocytes which produce cytokines TNF-a, IL-1b, IL-6, interferon gamma (IFN-g) and ROS (Hirsch *et al.*, 2012; Liang *et al.*, 2017).

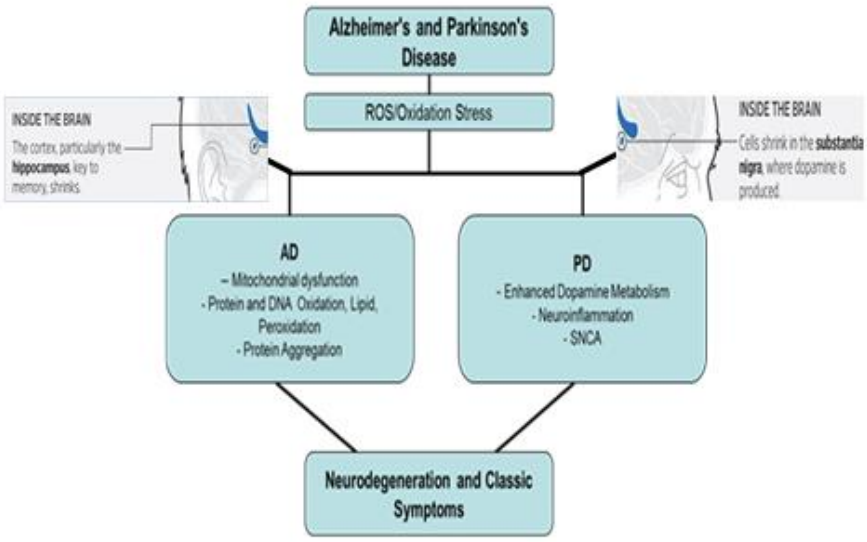


Figure 2-2: Pathogenesis and mechanism of neurodegeneration of Alzheimer's (AD) and Parkinson's (PD) diseases. Adapted from Tan (2018).

2.5 Current Treatment Strategies in AD

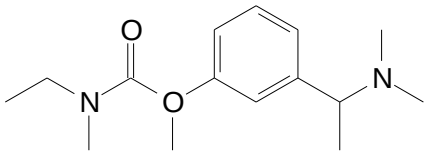
The therapeutic options that are usually selected for AD exploits a symptomatic attack using acetylcholinesterase inhibitors (AChEIs) and an N-methyl-D-aspartate (NMDA) receptor antagonist (Di Stefano *et al.*, 2011). Only four drugs are approved by the United States Food and Drug Administration (FDA); rivastigmine, galantamine and donepezil (AChEIs) and memantine (a NMDA receptor antagonist) (Di Stefano *et al.*, 2011). Cumming and colleagues reported recently that 132 agents were in clinical trials for the treatment of AD (Cummings *et al.*, 2019). Despite the robust development of drugs for the treatment of AD, only symptoms of AD can be addressed by presently approved drugs which intend to improve cognitive function via two different mechanisms: firstly, agonism of the cholinergic system and secondly, antagonism of the NMDA receptor (Di Stefano *et al.*, 2011). The degree of cholinergic loss due to acetylcholine-synthesizing enzyme, namely choline acetyltransferase, in the hippocampus and cortex is related to the level of cognitive impairment and density of amyloid plaques (Bassil & Grossberg, 2009; Perry *et al.*,

1978). During the first year of treatment, AChEIs have the tendency to stabilize cognitive performance and daily functioning, but after the first year the disease, unfortunately, progresses (Scheltens *et al.*, 2016). Just like other AChEIs, donepezil, and most 4-(1-benzylpiperidin-4yl) thiosemicarbazone (BPT) analogues, have the potential to act as moderate AChEIs (Van Bulck *et al.*, 2019). These pyridoxal BPT analogues were tested on five major hallmarks associated with AD and proved to be most effective (Van Bulck *et al.*, 2019). These analogues have shown the ability to inhibit Cu^{2+} mediated $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ aggregation and cellular iron uptake (Amor *et al.*, 2014).

2.5.1 Acetylcholinesterase Inhibitors

2.5.1.1 Rivastigmine

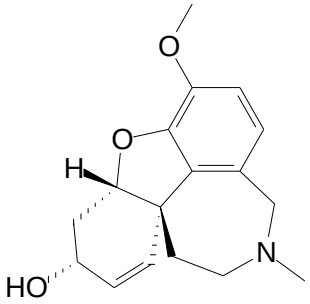
Rivastigmine's inhibitory effect on acetylcholinesterase is termed 'pseudo-irreversible' due to its persistent action after plasma levels of the drug has decreased, while it also inhibits butyrylcholinesterase (Polinsky, 1998). Early clinical trials have shown that rivastigmine exhibits a dose dependent effect – with a higher dose range improving cognition and functionality (Herrmann *et al.*, 2011). Some of the advantages of rivastigmine are less gastrointestinal cholinergic side effects (Massoud *et al.*, 2011).



Rivastigmine

2.5.1.2 Galantamine

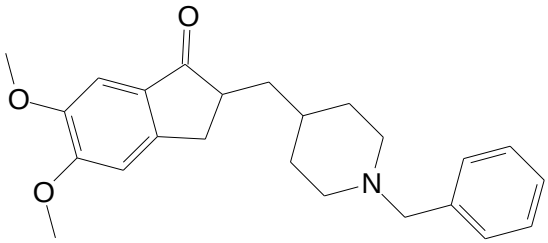
Galantamine is a tertiary alkaloid that is a reversible and competitive cholinesterase inhibitor and nicotinic acetylcholine receptor modulator that improves nicotine transmission (Robinson & Plosker, 2006). In comparison to other AChEIs, galantamine has an inconsistent dose dependence effect, but still remains statistically significant for the improvement of cognition, functionality and behaviour (Herrmann *et al.*, 2011; Massoud *et al.*, 2011).



Galantamine

2.5.1.3 Donepezil

Donepezil binds to acetylcholinesterase in a reversible and non-competitive manner and is hydrolysed instead of acetylcholine (Herrmann *et al.*, 2011). Research has shown that donepezil can protect cortical neurons against glutamate toxicity, prevent apoptotic cell death, increase expression of nicotinic receptors and decrease Aβ-induced toxicity (Herrmann *et al.*, 2011; Mangialasche *et al.*, 2010). Clinical trials have shown that donepezil-treated patients have increased livelihood, but its effects on behaviour were inconsistent (Herrmann *et al.*, 2011; Holmes *et al.*, 2004).



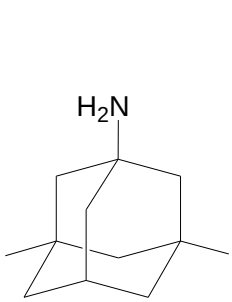
Donepezil

2.5.2 NMDA Receptor Antagonists

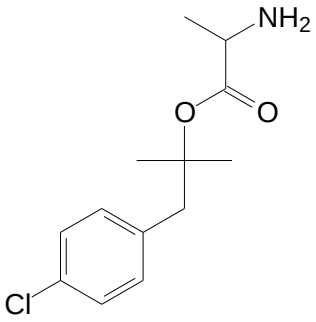
2.5.2.1 Memantine

Memantine works as a non-competitive antagonist with moderate affinity for NMDA receptors that target glutamatergic dysfunction (Lipton, 2006). Inhibition of the NMDA receptor is believed to inhibit excitotoxicity of Ca^{2+} influx and cell death, while other researchers say the efficacy of memantine is inconclusive (Lipton, 2006). The voltage-dependency and rapid kinetics of memantine allow the reduction in receptor blocking at regular levels which increase blocking at pathological levels and this improves both synaptic function, memory, and cognition (Wenk, 2006). Overall, in clinical trials memantine has shown positive effects on patients' cognition and function, but the effect on the patient's quality of life is unclear (Wilkinson, 2012).

Another non-competitive NMDA receptor antagonist, A03, has a similar effect as memantine on reducing excitotoxicity with additional benefit on sirtuin 1 (Sirt1) expression reduction (Van Bulck *et al.*, 2019). Treatment with A03 increases Sirt1 expression which plays a major role in tau pathology a risk factor for AD (Campagna *et al.*, 2018; Van Bulck *et al.*, 2019).



Memantine



AO3

2.5.3 Emerging Therapies

2.5.3.1 Cerebrolysin

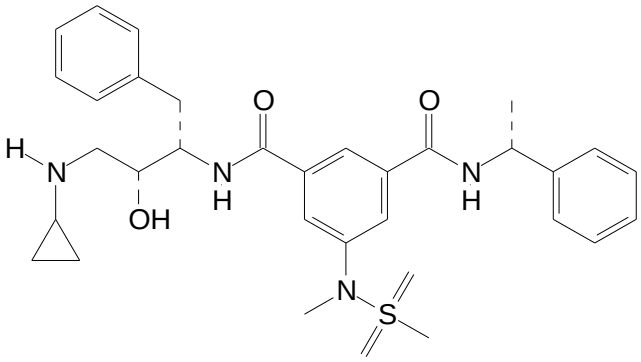
Cerebrolysin is a non-cholinergic treatment, which is manufactured using purified brain proteins (Herrmann *et al.*, 2011). It has neurotrophic effects, similar to endogenous nerve growth factors (Veinbergs *et al.*, 2000) and acts as a preserver of neural function and structure under conditions of stress and promotes neuronal plasticity and differentiation (Rockenstein *et al.*, 2007; Veinbergs *et al.*, 2000). The suggestions provided by studies highlight interactions with inhibitory neurotransmitter receptors such as gamma-aminobutyric acid B (GABAB) and adenosine A_1 receptors (Herrmann *et al.*, 2011; Xiong *et al.*, 1996; Xiong *et al.*, 1995). In clinical trials cerebrolysin has shown to have similar efficacy as donepezil, and combination therapy of these drugs possess synergistic properties (Herrmann *et al.*, 2011).

2.5.3.2 Targeting Misfolding Protein Aggregation

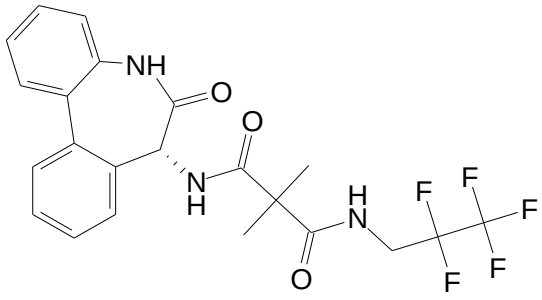
IRL-1620 is a synthetic analogue that activates endothelin B receptors which are highly expressed in the central nervous system (CNS) and which play a role in synaptogenesis and neurogenesis (Van Bulck *et al.*, 2019). The clearance of both Aβ toxicity as well as increased cerebral blood flow is stimulated by IRL-1620 which can also increase neural growth factors and Synapsin I expression (Briyal *et al.*, 2015; Gulati *et al.*, 2018).

Further research has shown firstly, that reduction of Aβ production can be stimulated by β-secretase inhibitors, γ-secretase inhibitors and α-secretase enhancers (Van Bulck *et al.*, 2019). Secondly, decreasing Aβ aggregation by suppressing the accumulation and reducing the stability

of Aβ oligomers was achieved by using a nutraceutical (Vivimind™) (Haas, 2012). This nutraceutical has since been marketed, despite no changes in cognitive function (Van Bulck *et al.*, 2019). Lastly, facilitating Aβ clearance is shown to be induced by two strategies involving antibody mediation: active immunization (which include Aβ peptide) and passive immunization (by inserting immunoglobulins) (Van Bulck *et al.*, 2019).



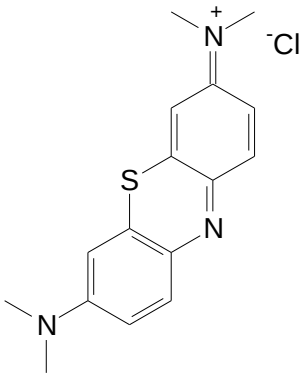
Beta-Secretase



Gamma-secretase inhibitor

2.5.4 Other Treatment Approaches

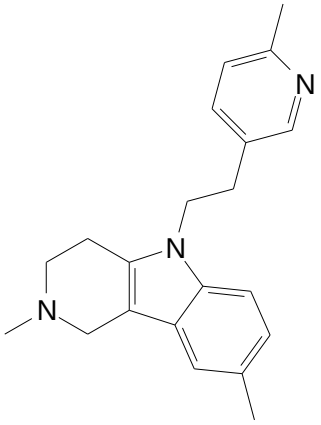
Treatment that targets the pathology of tau, include lithium and valproate which have been used as inhibitors of glycogen synthase kinase-3 and both of these drugs are currently undergoing phase 2 and phase 3 clinical trials, respectively (Tariot & Aisen, 2009). On the other hand methylthioninium chloride has been used to dissolve tau filaments in phase 3 clinical trials (Van Bulck *et al.*, 2019).



Methylthioninium chloride

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been related to a reduction in the incidence of AD (Szekely *et al.*, 2004). However a number of neuroinflammatory drugs used in clinical trials have shown no significant improvement of cognition in AD patients (Herrmann *et al.*, 2011).

Mitochondrial dysfunction can be targeted by the use of latrepirdine (also known as dimebon), prescribed in Russia as an anti-histamine. Latrepiridine showed significant cognitive improvement in phase 2 clinical trials, but in a phase 3 multi-national connection trial no improvement in cognition was shown (Doody *et al.*, 2008; Herrmann *et al.*, 2011). Another mitochondrial intervention compound was developed with neuroprotective effects, namely diethyl(3,-4-dihydroxyphene thylamino)quinolin-4-yl) methylphosphonate (DDQ). DDQ reduced $A\beta_{(1-42)}$ levels, and increased $A\beta_{(1-40)}$ levels, mitochondrial ATP, and cytochrome oxidase activity. Further it also reduced free radicals and oxidative stress in AD patients (Kuruva & Reddy, 2017).



Latrepirdine

2.5.5 Current Treatment Strategies in PD

PD is a devastating neurodegenerative disorder, which requires immediate treatment that will prolong the health of neurons while retaining neuronal functions. The aim of the current PD treatment is to alleviate symptomatic presentation by amplification of dopamine signalling in the striatum by mechanisms that (Díaz-Cabiale *et al.*, 2002; Xu *et al.*, 2005):

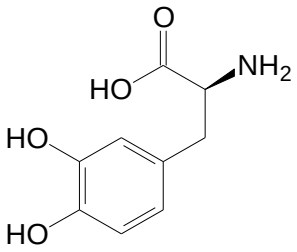
- increase formation of dopamine (DA) (by the DA precursor levodopa)
- stimulate DA receptors (via DA receptor agonists)
- block the degradation of DA (with MAO-B Inhibitors, and COMT inhibitors)

The common goals of these drugs are to restore the equilibrium of DA in the brain that is affected by dopaminergic cell loss (Borovac, 2016). The antiparkinsonian drugs pose challenges due to debilitating side effects that reduce the patient's quality of life.

2.5.5.1 Levodopa (L-Dopa)

The presence and regional distribution of DA in the human brain as well as it's deficiency in the striatum of PD patients were described in 1959 and 1960 (Nagatsu & Sawada, 2009). PD results as the level of DA decreases in the striatum of the brain; DA is synthesized from L-3,4-dihydroxyphenylalanine (L-dopa).

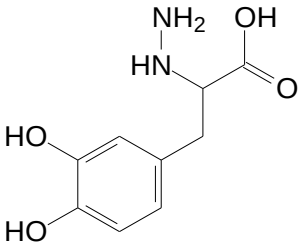
After prolonged treatment with L-dopa, many patients developed motor complications such as motor fluctuations and abnormal involuntary movement (dyskinesia) (Fabbrini *et al.*, 2007; Pahwa *et al.*, 2006).



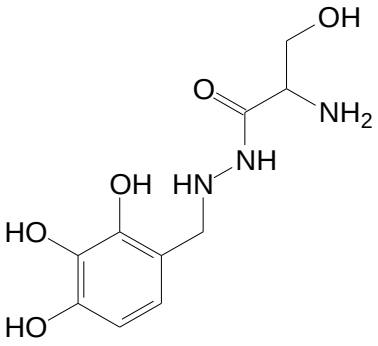
L-Dopa

2.5.5.2 Carbidopa and Benserazide

L-dopa is generally combined with benserazide or carbidopa; which are aromatic amino acid decarboxylases inhibitors which do not cross the blood-brain barrier (BBB) but prevent the conversion of L-dopa to dopamine peripherally (Münchau & Bhatia, 2000). Consequently, adverse effects are minimized, central delivery is improved and the dosage of L-dopa can be reduced (Almeida *et al.*, 2004). Other than L-dopa's adverse effects, a major concern is that L-dopa could be neurotoxic, since L-dopa is metabolised to toxic metabolites and free radicals, both possible mechanisms of neurodegeneration in PD (Basma *et al.*, 1995; Graham *et al.*, 1978).



Carbidopa

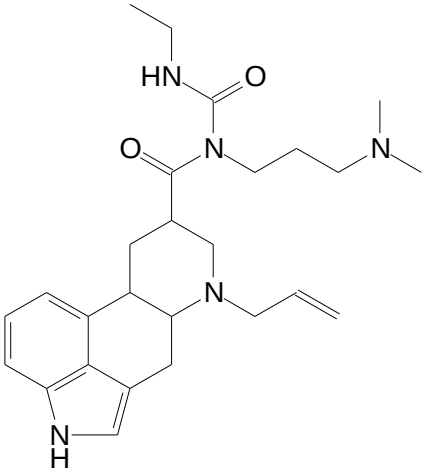


Benserazide

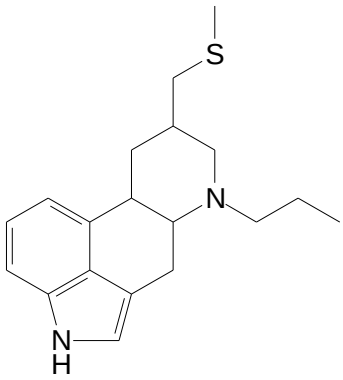
2.5.5.3 Dopamine Agonists

A number of reports argue that the treatment of PD should start with dopamine agonists, as L-dopa leads to the onset of dyskinesia (Montastruc *et al.*, 1999). Two types of drugs are commonly used as dopamine agonists: ergoline and non-ergoline derivative agonists (Borovac, 2016). Ergoline derivatives are first generation DA receptor agonists and also have interactions with receptors other than the D_2 family (Kvernmo *et al.*, 2006); Examples of ergoline agonists are: bromocriptine, cabergoline, pergolide and lisuride (Borovac, 2016). The two most used DA

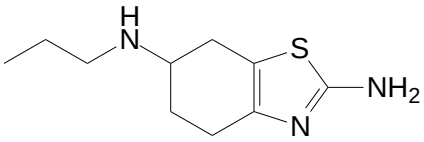
receptor agonists are cabergoline and pergolide which can be used as monotherapy in the early stages of PD (Borovac, 2016). Non-ergoline agonists bind to D_2 and D_3 receptors with high affinity (Borovac, 2016; Frampton, 2014). The most commonly prescribed non-ergoline drugs in the USA are pramipexole and ropinirole (Borovac, 2016). Ergoline agonists have diverse side effects compared to non-ergoline agonists. The side effects of ergoline agonists include constipation, nausea, headache, heart failure, daytime sleepiness and risk of cancer (Pagano *et al.*, 2015; Tholfsen *et al.*, 2015).



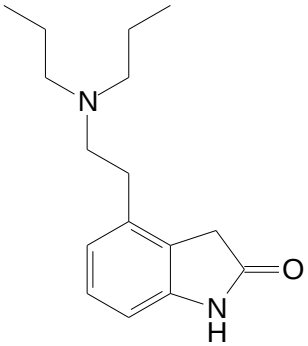
Cabergoline



Pergolide



Pramipexole

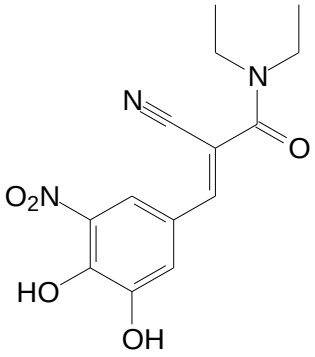


Ropinirole

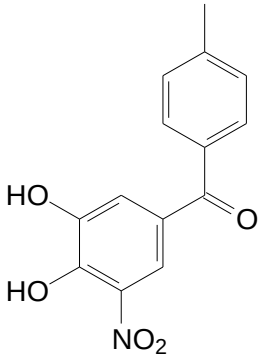
2.5.5.4 Catechol- O- methyltransferase (COMT) Inhibitors

The physiological substrates of COMT include L-dopa, catecholamines (DA, norepinephrine and epinephrine), their hydroxylated metabolites, and catechol estrogen (Ball & Knuppen, 1980). The general function of COMT is to eliminate the biologically active or toxic catechols and other hydroxylated metabolites. The regulation of active DA and norepinephrine in various parts of the brain are associated with COMT and apparently it is associated with moods and other mental

processes (Mannisto, 1999). Studies done on rats have shown that the combination of L-dopa and COMT inhibitors provide increased DA formation and release in the brain (Mannisto, 1999). Entacapone and tolcapone are commonly used COMT inhibitors which prolong the duration of L-dopa effects, thereby increasing DA delivery to the brain. Unfortunately these drugs cause the onset of nausea, anorexia, vomiting, orthostatic hypertension, sleep disorder and hallucination (Kaakkola, 2000).



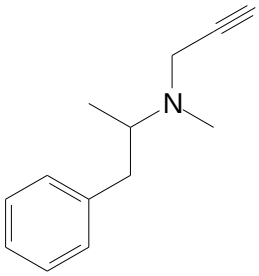
Entacapone



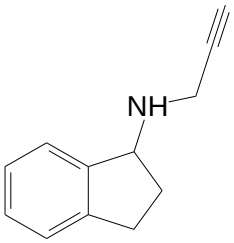
Tolcapone

2.5.5.5 Monoamine Oxidase (MAO) Inhibitors

MAO inhibitors prevent the breakdown of biogenic amine neurotransmitters such as DA and thus increases the concentration of DA in the synaptic cleft and postsynaptic receptor sites. MAO-B inhibitors like selegiline and rasagiline are used to treat akinesia and motor fluctuations in PD as monotherapy or in combination with L-dopa and decarboxylase inhibitors. Selegiline causes sleepiness, nausea, vomiting and dizziness in 2-5% of patients, while rasagiline's side effects are nausea, vomiting, orthostatic hypotension, somnolence, hallucination and dyskinesia (Riederer & Laux, 2011).



Selegiline

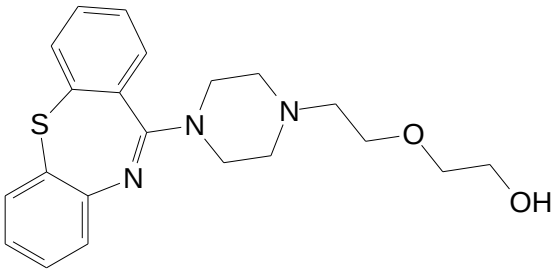


Rasagiline

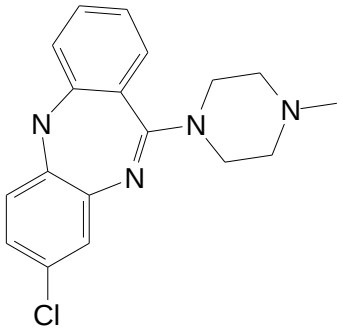
2.5.5.6 Anticholinergic Drugs

Anticholinergic drugs provide an alternative mechanism of action which alleviates some of the troublesome symptoms experienced in PD, in particular the involuntary resting tremor. Clozapine

and quetiapine are common anticholinergic drugs which can be used as monotherapy in the early stage of PD, and synergistically with L-dopa in more advanced stages of PD (Brocks, 1999). These drugs have the potential to delay the need of L-dopa use, thus reducing the dose of L-dopa required, and further extend the use of L-dopa. Furthermore, anticholinergic drugs decrease extrapyramidal side effects associated with the use of antipsychotic agents (Brocks, 1999). Some reports have shown the relationship between anticholinergic drugs use and cognitive decline (Ehrt *et al.*, 2010) and mental confusion (de Smet *et al.*, 1982).



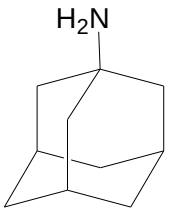
Quetiapine



Clozapine

2.5.5.7 Amantadine

Results from clinical trials with amantadine and a placebo group showed more benefits in relieving akinesia and rigidity than tremors (Dallos *et al.*, 1970). L-dopa induced dyskinesias can be counteracted by amantadine (anti-dyskinetic), however it was reported that the effect wears off after 9 months of treatment (Wolf *et al.*, 2010). Amantadine is initially prescribed as mild symptomatic therapy either as monotherapy or in combination with L-dopa. It enhances the release of DA, the reuptake of DA, anticholinergic effects and blockade of NMDA glutamate receptors (Lees, 2002).



Amantadine

2.5.5.8 Surgery

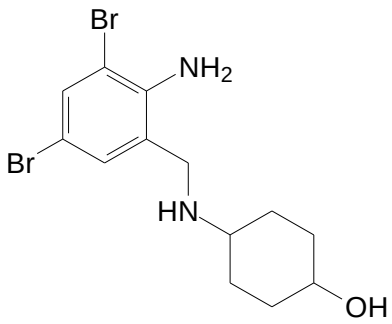
Implantation of electrodes into the bilateral subthalamic nucleus of the globus pallidus for deep brain stimulation in patients with advanced PD have shown significant motor benefits (Deep-Brain Stimulation for Parkinson's Disease Study Group, *et al.*, 2001; Hertel *et al.*, 2006). This procedure

reduced dyskinesia and motor fluctuations in patients, but stimulation has shown to mirror the effects of a destructive lesion whereby high risk of intracranial bleeding occurs (Deep-Brain Stimulation for Parkinson's Disease Study Group; J.A. Obeso *et al.*, 2001).

2.5.6 Emerging Therapies

2.5.6.1 Targeting α-syn Aggregation

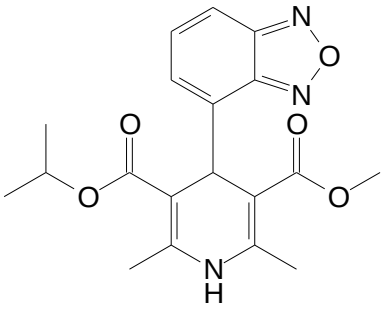
As mentioned earlier α-syn aggregation is one of the hallmarks of PD and there are a number of mechanisms to avoid the detrimental effects of α-syn (Van Bulck *et al.*, 2019). Protein accumulation can be avoided by increasing its degradation. A decrease in the glucocerebrosidase (GBA) protein activity leads to accumulation of α-syn. GBA enhancer drugs (e.g. ambroxol - currently in phase 2 clinical trials) can be used to increase GBA's activity. Alternatively, the inhibition of glucosylceramide synthase (GCS), a substrate of GBA, can be inhibited by GZ/SAR40261 which is in phase 2 clinical trials (Wong & Krainc, 2017).



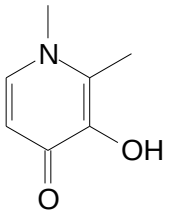
Ambroxol

2.5.6.2 Targeting Calcium Channel Blocking

Cytosolic Ca^{2+} overload may lead to oxidative stress and alleviating increased Ca^{2+} load in DA cells can be achieved by L-type voltage gated calcium channel blockers. For instance, isradipidine which specifically blocks voltage gated calcium 1.3 channels and is currently in phase 3 clinical trials (NCT02168842). Some developing drugs that target metal ion homeostasis alteration in PD are also used in AD (Van Bulck *et al*., 2019). Deferiprone, which is an iron chelator in phase 3 clinical trials is used as possible disease-modifying drug against PD (Belaidi & Bush, 2016).



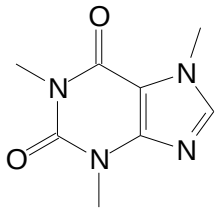
Isradipine



Deferiprone

2.5.6.3 A_1/A_{2A} Antagonist Treatment in AD and PD

The first observation of AR signalling, dates back to 1927 after intravenous adenosine caused temporary heart block (Drury & Szent-Györgyi, 1929). Then only after cloning of purinergic (adenosine) receptors in the 1990's the therapeutic potential of A_1, A_{2A}, A_{2B} and A_3 ARs were explored (Ralevic & Burnstock, 1998), with the emphasis on A_1 and A_{2A} ARs as drug targets for neurological diseases (Burnstock, 2017). Antagonism of both A_1 and A_{2A} ARs are reported to improve cognitive and memory decline in AD (Agostinho *et al.*, 2010; Fredholm *et al.*, 1999), as well as cognitive and motor impairments in PD (Mihara *et al.*, 2007). Case control and prospective studies have associated the consumption of the non-selective A_1/A_{2A} AR antagonist caffeine with a decreased risk of AD (Lindsay, 2002; van Boxtel *et al.*, 2003) and PD (Ascherio *et al.*, 2001; Ross, 2000).



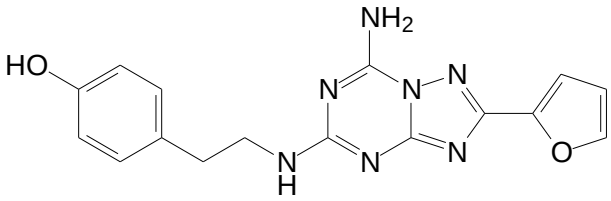
Caffeine

2.5.6.4 A_1/A_{2A} Antagonist Therapy for AD

Angulo and colleagues (2003) clearly showed that AD patients has a change in the pattern of expression and redistribution of ARs compared with samples from controls. Therefore, the location of A_1 ARs in neurodegenerative structures such as neurofibrillary tangles and of A_{2A} ARs in the microglia of patient's hippocampus, suggest that ARs can play a role in regulating the events along the development of AD.

A novel, potent and selective A_1 AR antagonist FR194921 exerts both cognitive-enhancement and anxiolytic activity (Maemoto *et al.*, 2004). Further investigations on rodents were done by intraperitoneal injection of caffeine, an A_1 and A_{2A} AR antagonist, and ZM 241385, a selective A_{2A}

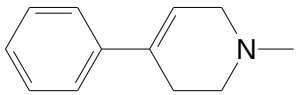
AR antagonist. Both caffeine and ZM 241385 prevented learning and memory impairment by attenuating neuronal damage caused by Aβ toxicity in AD (Dall'Igna *et al.*, 2003).



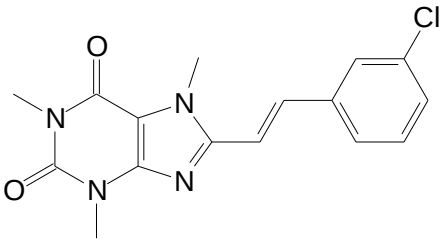
ZM241385

2.5.6.5 A_1/A_{2A} Antagonist Therapy in PD

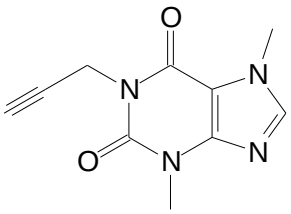
Reports have shown that PD patients with dyskinesia have an increased number of A_{2A} ARs in their putamen, postulating that L-dopa induced dyskinesia is linked to the A_{2A} AR (Chen *et al.*, 2013; Mishina *et al.*, 2011; Ramlackhansingh *et al.*, 2011). Studies of induced toxicity of MPTP, showed either xanthine based compounds such as 8-(3-chlorostyryl)caffeine (CSC), 3, 7-dimethyl-1-propargylxanthine (DMPX) or istradefylline (KW-6002) or non-xanthine like SCH-58261, to attenuate dopamine-induced neurotoxicity (Field *et al.*, 2011; Lopes *et al.*, 2011). Further results showed the neuroprotective abilities of A_{2A} AR antagonists, while A_1 AR antagonists did not show any neuroprotection after multiple doses with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Lopes *et al.*, 2011). A different study by Mihara and colleagues (2007), showed that dual A_1/A_{2A} AR antagonist ASP-5854 reversed memory loss induced by scopolamine in the rat model, whereas specific A_{2A} AR antagonist, KW-6002, did not. In addition, ASP-5854 improved haloperidol-induced catalepsy in rats and therefore it was concluded that ASP-5854 improves motor deficits and is neuroprotective through A_{2A} AR antagonism, and enhances cognitive function through A_1 AR antagonism. KW-6002 is the only A_{2A} AR antagonist treatment that has been approved in Japan since March 2013, while the FDA still needs statistically significant efficacy in the decrease of symptoms of the disease before approving it in the USA (Mishina *et al.*, 2011; Lopes *et al.*, 2011); however, the FDA recently approved KW-6002 as adjunctive treatment for parkinsonian motor symptoms.



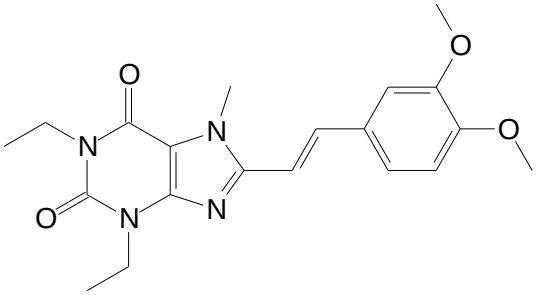
MPTP



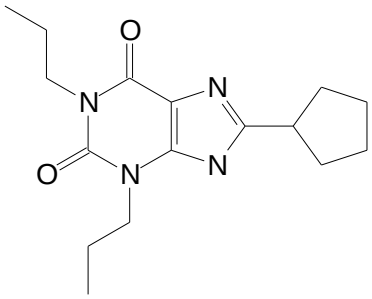
8-(3-Chlorostyryl)caffeine



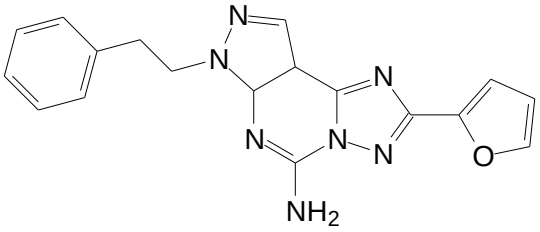
DMPX



Istradefylline



DPCPX



SCH-58261

2.6 Summary

Tremendous efforts have been made to combat neurodegenerative diseases such as AD and PD through different treatment strategies. As discussed in this chapter, PD and AD can also share the same aetiology mechanism, while presenting different symptoms. The challenges concerning different treatments were briefly discussed, while it is important to note that the current treatments only alleviate symptoms with added side effects and no cure. Research has shown that dual A_1/A_{2A} AR antagonism may be used for the pharmacological treatment of AD and PD symptoms with the addition of A_{2A} AR antagonists possessing neuroprotective properties. A multi-drug target approach involving manipulation of AR signalling in combination with L-dopa has been postulated, which reduces the risk of developing dyskinesia presented by L-dopa in PD treatment (Lopes *et al.,* 2011). A detailed discussion of ARs and their antagonists follows in Chapter 3.

CHAPTER 3: ADENOSINE RECEPTORS

3.1 General Background

Adenosine is a nucleoside in the brain which is released by neurons and astrocytes into extracellular spaces to act as a neuromodulator, whereby it facilitates signalling by various neurotransmitters and receptors (Chen *et al.*, 2014). The four G-protein coupled adenosine receptors (ARs), namely A_1, A_{2A}, A_{2B} and A_3 are generally found throughout the body, A_1 and A_{2A} ARs are predominantly expressed in the brain and both possess a high affinity for adenosine (Chen *et al.*, 2014). These AR subtypes usually modulate cAMP formation: the A_1 and A_3 ARs inhibit adenyl cyclase and the A_{2A} and A_{2B} ARs stimulate adenyl cyclase (Londos *et al.*, 1980, van Calker *et al.*, 1979)

A_1 ARs are distributed throughout the brain, and are most abundant in the limbic and neocortical regions and present in the basal ganglia and cerebellum, and in most nuclei of diencephalons of the brain stem (Yuzlenko & Kiec-Kononowicz, 2006). A_{2A} ARs are predominantly distributed in the basal ganglia as well as limbic and neocortical regions in the brain (Yuzlenko & Kiec-Kononowicz, 2006). Co-expression (heteromers) of A_1 and A_{2A} (A_1/A_{2A}) ARs is usually found in presynaptic membranes, while A_{2A} and dopamine D_2 receptor (A_{2A}/D_2) heteromers are selectively localized on GABAergic striato-pallidal neurons (Azdad *et al.*, 2009, Ciruela *et al.*, 2006, Trifilieff *et al.*, 2011). Locomotive control is facilitated by regulation of dopamine (D_1 and D_2) neurotransmission, which either face motor depression or activation as result of A_1 and A_{2A} (agonist or antagonist) effect (Popoli *et al.*, 1996; Armentero *et al.*, 2011). As a substrate of activation both A_1 and A_{2A} ARs display a spectrum of pathophysiological activities in the central nervous system which may lead to memory and cognitive decline, depression, motor impairment, neuroinflammation and neurodegeneration in Alzheimer's disease (AD) and Parkinson's disease (PD) (Gomes *et al.*, 2011).

3.2 Adenosine Receptors in AD and PD

Stimulation of A_1 and A_{2A} ARs by endogenous adenosine enables dilapidation of intercorporate cognition and memory, as experienced in AD (Cunha & Agostinho, 2010). As mentioned above A_1 and A_{2A} ARs are mainly located around synaptic terminals where these receptors control the release of acetylcholine and glutamate neurotransmitters, involved in memory and other cognitive functions (Cunha, 2005, Ribeiro *et al.*, 2002).

A commonly used therapeutic strategy in AD is through inhibition of acetylcholinesterase (AChE) and N-methyl-D-aspartate (NMDA) (Van Bulck *et al.*, 2019). An A_1 AR antagonist can block the release of AChE thereby acting as a cognitive enhancer (Ribeiro & Sebastiao, 2010). Additionally,

the A_{2A}–AR antagonists SCH58261 and ZM 241385 have shown to enhance social memory and halt Aβ induced synaptic loss in animal studies of AD (Kopf *et al.*, 1999, Prediger *et al.*, 2005). Experimental studies have shown that caffeine improves memory and cognitive abilities, reduce Aβ production, stabilize blood-brain barrier (BBB) integrity, due to its anti-inflammatory effects that stimulate the pro-survival cascade and inhibit the pro-apoptotic pathway (Chen *et al.*, 2010, Rivera-Oliver & Díaz-Ríos, 2014). Caffeine is a non-selective A_1/A_{2A} AR antagonist and may be advantageous for AD as it leads to reduced cognitive impairment while minimizing Aβ accumulation (Arendash *et al.*, 2009, Dall'Igna *et al.*, 2007). However, some conflicting reports state that prolonged caffeine exposure can cause desensitization as well as inverse effects that resemble AR agonists (Jacobson *et al.*, 1993).

The involvement of ARs in the development of PD may be as follows: (1) A_1 ARs are commonly found in presynaptic striatal neurons where their stimulation inhibits glutamate, dopamine, and acetylcholine release which contribute to motor and non-motor dysfunctions experienced in PD (Ambrósio *et al.*, 1997, Fredholm & Dunwiddie, 1988). (2) Several studies have highlighted the role of A_{2A} AR in the pathophysiology of PD namely: a). the physiological role in motor control; b) ability to regulate glutamatergic transmission; c) increased mediated activity in PD; d) eventual involvement in neuroinflammation in substantia nigra pars compacta (SNpc); e) ability to control metabolism and mitochondrial functioning (Gomes *et al.*, 2011).

Although phase 3 clinical trials revealed that caffeine provides no clinical improvement of motor manifestation in PD (Postuma *et al.*, 2017), there is alarming evidence of prospective and case-control studies that show caffeine intake is related to a reduction in the risk of PD (Palacios *et al.*, 2012). Currently, the first line pharmaco-therapeutic strategy in PD targets restoring dopamine levels and/or effects by the use of a dopamine precursor, dopamine agonists and inhibitors of enzymatic degradation of dopamine. These drugs mainly alleviate motor symptoms and limitations include long-term side effects (in particular, motor disability, including dyskinesia) and inability to stop the ongoing degenerative process (Gomes *et al.*, 2011).

3.3 Adenosine Receptor Antagonists for the Treatment of AD and PD Symptoms

3.3.1 Cognitive Dysfunction

Caffeine is related to the regulation of neurotransmitter release and increased synaptic plasticity through A_1 and A_{2A} AR antagonism that affects neuronal processes associated with mood and cognition (Gomes *et al.*, 2011). Human studies demonstrated the effects of caffeine in preventing and delaying the onset of old-aged cognitive decline (Eskelinen *et al.*, 2009, Jarvis, 1993, Maia & de Mendonça, 2002).

Various pre-clinical studies support the use of A_1 AR antagonists for the treatment of cognitive dysfunction. As shown with the administration of the A_1 AR antagonist rolofylline (KW-3902) to pro-aggregant-tau transgenic mice which restored the spatial memory deficits and normalized basic (synaptic transmission) neuronal activity (Dennissen *et al.*, 2016). The A_1 AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) seemed to prevent morphine-induced impairment in regaining partial reference memory and further enhanced learning and memory in animal models (Lu *et al.*, 2010, Maemoto *et al.*, 2004). Further scopolamine-induced memory loss was alleviated by orally active FR19492, an A_1 AR antagonist, which also displayed anxiolytic effects in the elevated plus-maze test (Maemoto *et al.*, 2004). In animal studies, both acute and chronic caffeine intake has shown to improve social recognition, olfactory memory, and emotional behaviour; which could be due to suppression of α- and γ-secretase in old rats (Panza *et al.*, 2015, Prediger *et al.*, 2005, Sallaberry *et al.*, 2013).

Growing evidence also supports the A_{2A} AR as a major player in memory performance as could be seen in both spatial water maze and radial arm maze test of A_{2A} AR knockout (KO) mice (Gimenez-Llort *et al.*, 2007, Zhou *et al.*, 2009). Various brain insults that resulted in memory impairment has been shown to be reversed by blocking A_{2A} ARs, while alleviating deposition of Aβ aggregates (Dai *et al.*, 2010, Gelber *et al.*, 2011). The effect of A_{2A} AR antagonism through processes such as reduction of glutamate overdrive release, increase of brain-derived neurotrophic factor (BDN-F) and tyrosine kinase B (TrkB), also improved synaptic plasticity (Armentero *et al.*, 2011, Sallaberry *et al.*, 2013).

3.3.2 Neuroinflammation

Caffeine and A_{2A} AR antagonists can inhibit hippocampal lipopolysaccharide (LPS) induced neuroinflammation in aged mice (Brothers *et al.*, 2010, Rebola *et al.*, 2011) by acting as an anti-inflammatory, anti-rheumatic and immunosuppressive drug. Different studies have shown that consumption of caffeine down-regulate neuroinflammatory responses and nitric oxide production (Salvemini *&* Mollace., 2013, Tsutsui *et al.*, 2004, Yaday *et al.*, 2012).

A_1 ARs, found around non-neuronal spaces, are able to regulate both for astrocytic and microglia-related functions in brain tissue (Cupino & Zabel, 2013, Haselkorn *et al.*, 2010, Tsutsui *et al.*, 2004), which explain the evacuation of Aβ accumulation caused by A_1 AR antagonism in and around cerebral blood vessels (Cupino & Zabel, 2013). Additionally, studies on A_1 AR KO mice indicate the anti-inflammatory function of these receptors (Wei *et al.*, 2011).

Pre-treatment of MPTP mice with KW-6002 (A_{2A} AR antagonist), present low microglia activation, which further decreased neurotoxic factors, noxious astrogliosis and mitogen-activated protein

kinase (MAPK) p38 in the SNpc and striatum (Ikeda *et al.*, 2002, Pinna *et al.*, 2010, Yasuda *et al.*, 2011). The blockade of the A_{2A} AR shows a pattern of neuroprotection that consistently link decrease of inflammatory events in AD and PD patients (Armentero *et al.*, 2011, Brambilla *et al.*, 2003, Yu *et al.*, 2008)

3.3.3 Depression

Different studies have shown that consumption of coffee in both men and women decrease depression at doses similar to dementia protection (Lucas *et al.*, 2011, Pham *et al.*, 2013, Ruusunen *et al.*, 2010). The implication of the adenosine system in the pathophysiology of depression was shown by the antidepressant action of A_1 (DPCPX) and A_{2A} (ZM 241385) AR antagonists and, also, caffeine which reduced the depressant effects of Zinc in rodents (Lobato *et al.*, 2008). Moreover, A_1 and A_{2A} AR KO mice showed anxiogenic-like behaviour (Gimenez-Llort *et al.*, 2002, Johansson *et al.*, 2001).

Adenosine has been shown to produce antidepressant like effects in the forced swim test (FST) in mice through the A_1 AR (Kaster *et al.*, 2004, Lobato *et al.*, 2008), which is contradictory to other studies that have shown high immobility times in the FST when mice were treated with adenosine (Porsolt *et al.*, 1977). Endogenous adenosine, has limited long-term depression effects through the activation of A_1 ARs, while the inhibition of the A_1 AR present anxiolytic properties (de Mendonça *et al.*, 1997, Maemoto *et al.*, 2004).

The A_{2A} AR antagonist istradefylline (KW-6002), is not only effective in the treatment of the motor symptoms of PD, but may also induce antidepressant-like effects (Preti *et al.*, 2015). KW-6002 alone or co-administered with currently available antidepressants may be useful for the treatment of depression as well as motor symptoms of PD (Yamada *et al.*, 2013). Various studies have shown that the antidepressant effect of A_{2A} AR antagonists such as CSC, SCH-58261, KW-6002, ZM 241385 and caffeine reduced immobility time in the FST and tail suspension test in animal models of depression (El Yacoubi *et al.*, 2003, Huang *et al.*, 2004, Pechlivanova *et al.*, 2012).

3.3.4 Neuroprotection

The chronic consumption of caffeine provides neuroprotection by reversing excitotoxicity caused by induced hypoxia and ischemia in animal models. (de Mendonça *et al.*, 2000), and furthermore, protects BBB disruption in both AD and PD (Xuesong *et al.*, 2010). Another mechanism of caffeine-induced neuroprotection results from the increase of pro-BDNF (increasing neurotrophin ratio) which increase neuronal survival and reduction of glutamate overdrive (Armentero *et al.*, 2011, Chen & Chern, 2011).

Various research evidently show that chronic administration of caffeine displayed neuroprotective effects in rat cerebellar neurons where it reduces Aβ-induced neurotoxicity via A_{2A} AR antagonists (Arendash *et al.*, 2009, Cao *et al.*, 2009). The selective A_{2A} antagonist ZM 241385 showed similar neuroprotection effects to that of caffeine, while the selective A_1 AR antagonist 8-cyclopentyltheohylline (CPT) did not show any neuroprotection (Dall'Igna *et al.*, 2003). The direct injection of the A_{2A} AR antagonist ZM 241385 into the hippocampus of male Wistar rats, reduced kainate-induced neuronal damage, while direct injection of A_{2A} AR agonist CGS21680 into the hippocampus failed to provide protection (Jones *et al.*, 1998).

Based on various studies A_{2A} AR antagonists can conversely protect the brain from damage after ischemia (Chen *et al.*, 1999), excitotoxicity (Popoli *et al.*, 2000), traumatic brain injury (Dai *et al.*, 2010) and PD (Chen *et al.*, 2001, Lopes *et al.* 1999). Caffeine and A_{2A} AR antagonists mediate the excessive release of neuroinflammatory responses and promote neuroprotection (Ikeda *et al.*, 2002a). Moreover, A_{2A} AR antagonists provide broad-spectrum neuroprotection via its regulation of glutamate neurotransmission and mitochondrial toxicity (Chen *et al.*, 2001)

3.3.5 Motor Function in PD

The gradual loss of dopamine in the ventral midbrain and subsequent loss of dopamine input to the forebrain (striatal) motor structures is the major cause of motor impairment in PD (Fink *et al.*, 1992, Schiffmann *et al.*, 1991). PD is a basal ganglia associated disorder. The basal ganglia is made up of input nuclei (caudate, putamen and accumbens), output nuclei (globus pallidus interna (GPi), substantia nigra pars reticulate (SNr), and intrinsic nuclei (globus pallidus externa (GPe), subthalamic nucleus (STN) and SNpc, that facilitate planning and initiation of movement (Armentero *et al.*, 2011). There are two pathways that coordinate information from the striatum to the basal ganglia - firstly the direct pathway, facilitating excitation through A_1 ARs and DA type 1 (D_1) receptors ending in GPi/SNr nuclei, and secondly the indirect pathway, facilitate inhibition through A_{2A} AR and DA type 2 (D_2) receptors projecting to GPe (Armentero *et al.*, 2011, Mori, 2014). A_1 ARs are localized presynaptically of dopamine axon terminals and inhibition of these AR facilitates DA release in the striatum, whereas A_{2A} AR antagonism potentiates the postsynaptic response to DA (Shook & Jackson, 2011).

Restoration of motor activity was demonstrated by the effect of A_{2A} AR blockade in different animal models of PD, which includes modulated behaviour in unilateral 6-OHDA lesioned rodents, reduction of motor impairment in MPTP-treated non-human primates; as well as reversion of haloperidol-induced catalepsy (Simola *et al.*, 2008, Xu *et al.*, 2005). Mono-administration of the A_{2A} AR antagonist KW-6002 as well as co-administration with L-dopa did not present dyskinesia or any side effects, thereby showing synergy between these compounds (Grondin *et al.*, 1999,

Kanda *et al.*, 2000). Recently reports showed that administration of an A_{2A} AR antagonist only show a reduction in the OFF time in dyskinesia but not a significant improvement in dyskinesia (Zheng *et al.*, 2018).

Furthermore, several studies indicated that consumption of caffeine and A_{2A} AR antagonist administration is able to attenuate dopaminergic neurotoxicity and neurodegeneration (Chen *et al.*, 2001, Ikeda *et al.*, 2002, Xu *et al.*, 2002). In genetically susceptible patients, caffeine intake also reduced the risk of developing PD (Kumar *et al.*, 2015), supporting a previous meta-analysis of 26 observational studies which showed a decreased risk of developing PD with caffeine intake (Costa *et al.*, 2010). Unfortunately, after prolonged treatment with caffeine, rapid tolerance develops, but this tolerance is not shown by selective A_{2A} AR antagonists SCH-58261(Halldner *et al.*, 2000, Popoli *et al.*, 2000).

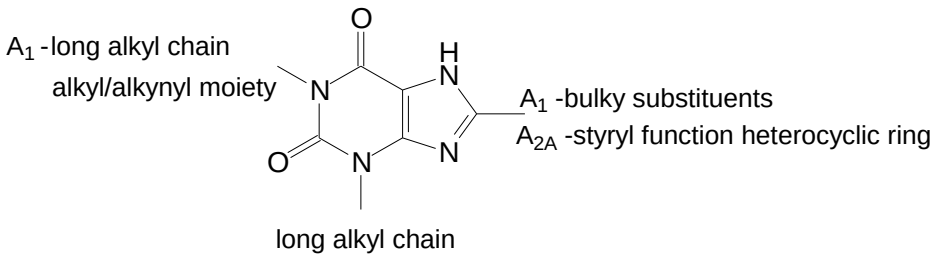
3.4 Potential Adenosine Receptor Antagonists

3.4.1 Xanthine A_1 Adenosine Receptors Antagonists

Xanthine derivatives present some physico-chemical disadvantages which include water solubility, CNS penetration and bioavailability (Klotz, 2000, Moro *et al.*, 2006). It was also found that the AR affinity of xanthine based antagonists differ between species, unlike non-xanthine derivatives which display similar AR affinity between different species (Maemoto *et al.*, 1997, Maemoto *et al.*, 2004).

Xanthine based compounds with good A_1 AR affinity, possess bulky C8-substituents as well as long alkyl chains in positions 1 and 3, whereas high affinity of A_{2A} AR ligands are C8-styryl substituted with N(1)-alkyl/alkynyl moiety or fused tricyclic xanthines with heteroatoms (Yuzlenko & Kiec-Kononowicz, 2006) (**Figure 3-1**). Promising novel A_1 and A_{2A} AR antagonists are generally based on position C8 substitutions with aryl or cycloalkyl groups (Baraldi *et al.*, 2008). Non-xanthine AR antagonists are commonly non-fused monocyclic, fused bi-and tricyclic analogues made of nitrogen, oxygen and sulphur where most often A_1 AR ligands are adenine based with amino group substitution and adjustable nitrogen atoms in the molecule, while A_{2A} AR ligands possess good affinity when a furanyl function is present for binding capacity (Yuzlenko & Kiec-Kononowicz, 2006).

Xanthine derivatives



Non-xanthine derivatives

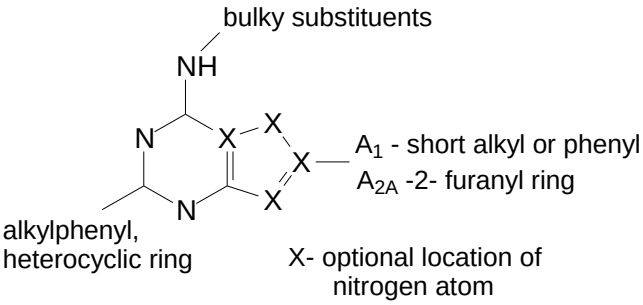
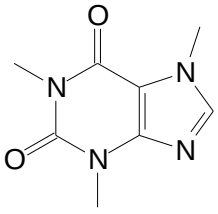


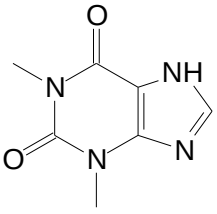
Figure 3-1: Summary of the general features of xanthine and non-xanthine based A_1 and A_{2A} AR antagonists (Yuzlenko & Kiec-Kononowicz, 2006)

Natural xanthines such as caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine) are known to be non-selective A_1/A_{2A}antagonists and through different manipulations initiated the discovery of some of the most potent and selective A_1 and A_{2A}AR antagonists (Yuzlenko & Kiec-Kononowicz, 2006). The xanthine core provided a number of possibilities for xanthine derivatives as they present N1, N3, N7 and C8 positions for substitution to form key pharmaceutically active compounds (Singh *et al.*, 2018). Several studies found that propyl and ethyl substitutions at N1, N3 and N7 positions appear to enhance affinity at A_1 AR when compared to methyl substitution (Van Der Walt & Terre'Blanche, 2015).



Caffeine

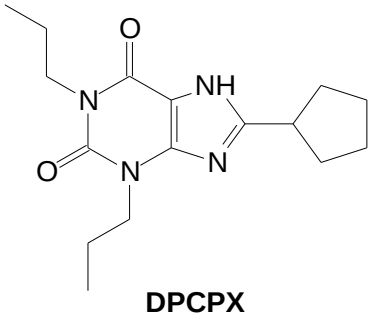
hA_1K_i = 44 900 nM $hA_{2A}K_i$ = 23 000 nM
rA_1K_i = 18 800 nM $rA_{2A}K_i$ = 43 000 nM



Theophylline

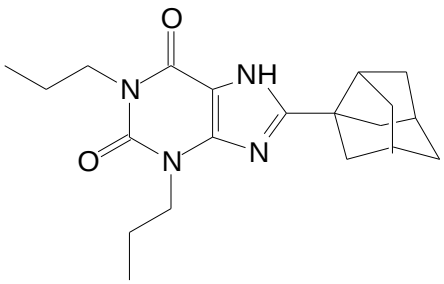
hA_1K_i = 6 200 nM $hA_{2A}K_i$ = 4 200 nM
rA_1K_i = 8 500 nM $rA_{2A}K_i$ = 25 000 nM

Manipulation of the xanthine nucleus initiated different potent and selective antagonists, which contain bulky lipophilic substituents leading to the selective A_1 AR antagonist DPCPX. DPCPX has high affinity and selectivity for rat brain A_1 ARs and a 10 fold less affinity for human A_1 ARs (Fredholm *et al.*, 2001). Because of its high affinity and selectivity for A_1 ARs, [^{3}H]DPCPX became a ligand of choice in A_1 AR radioligand binding assays (Maemoto *et al.*, 1997). The selective A_1 AR antagonist KW 3902, (rolofylline) showed high affinity for rat and human A_1 ARs and improved presynaptic dysfunction and restored neuronal activity (Dennissen *et al.*, 2016; Müller & Jacobson, 2011).



DPCPX

hA_1K_i = 3.9 nM
rA_1K_i = 0.4 nM



KW 3902

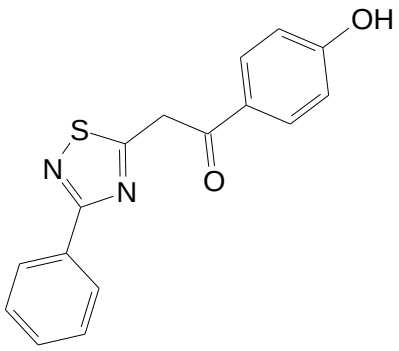
hA_1K_i = 8 nM
rA_1K_i = 0.19 nM

3.4.2 Non-xanthine A_1 Adenosine Receptor Antagonists

Non-xanthine ligands are non-fused monocyclic or fused bi- and tricyclic derivatives with the nitrogen, oxygen and sulphur heteroatoms (Yuzlenko & Kiec-Kononowicz, 2006).

3.4.2.1 Monocyclic

Usually, monocyclic heteroaromatic rings are very rare and most of them have low affinity with only few exceptions of anti-inflammatory action and generally consist of two five-membered heterocycles, thiazoles or thiadiazoles (Chang *et al.*, 2004, Yuzlenko & Kiec-Kononowicz, 2006). Modification of 1,2,4-thiadiazol by substitution of a phenol group resulted in the potent antagonist N-(3-phenyl-1,2,4-thiadiazol-5-yl)-4-hydroxybenzamide (LUF-5437) (Yuzlenko & Kiec-Kononowicz, 2006)



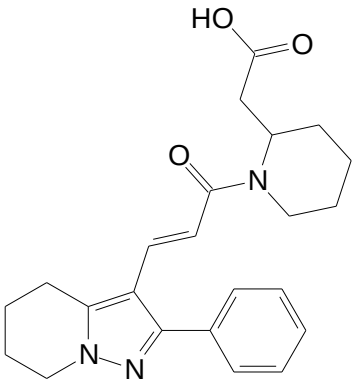
LUF-5437

rA_1K_i = 7 nM

3.4.2.2 Bicyclic Fused Heteroaromatic System

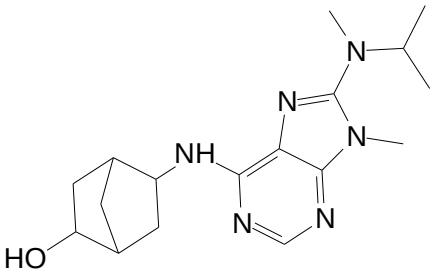
Bicyclic 6:5 fused heteroaromatic compounds form the largest group of published and synthesized non-xanthine A_1 AR antagonists (Yuzlenko & Kiec-Kononowicz, 2006). Pyrazolo[1,5-α]pyridines have the advantage of water solubility as salts with high selectivity for A_1 ARs and (R)-1-[(E)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)acryloyl]pipendin-2yl acetic acid (FK-352) was identified as a high affinity selective A_1 AR antagonist (Maemoto *et al.*, 1997). Further structure-activity relationships with substitution in the 8-position of adenine afforded the isopropyl-methyl-amine moiety 8-(N-methyl isopropyl)amino-N6-(5′-endohydroxy-endonorbornyl)-9-methyladenine) WRC-0571 with improved A_1 AR affinity (Robeva *et al.*, 1996).

Research by van der Walt and co-workers (2013) showed that by introducing aryl-substituents at position C8, the xanthine structure resulted in improved A_1 AR affinity for (8-(3-phenylpropyl)-1,3,7-triethylxanthine.



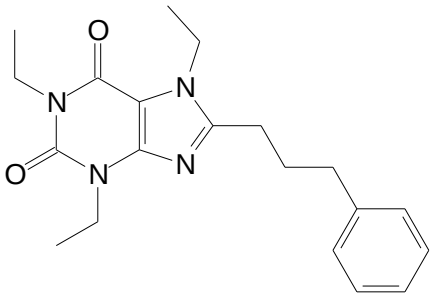
FK-352

hA_1K_i = 7.57 nM
rA_1K_i = 7.52 nM



WRC-0571

hA_1K_i = 1.7 nM

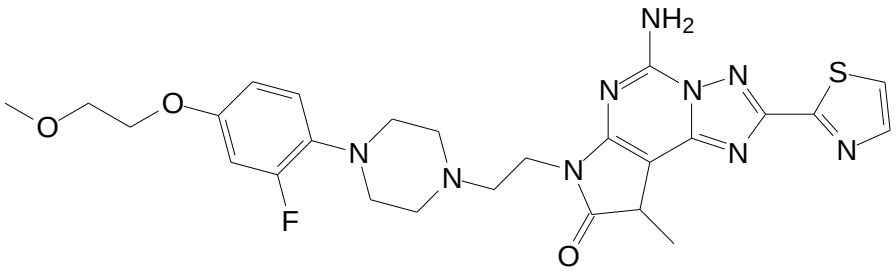


8-(3-phenylpropyl)-1,3,7-triethylxanthine

rA_1K_i = 164 nM

3.4.2.3 Tricyclic Fused Heteroaromatic System

Tricyclic fused heteroaromatic rings are usually composed of 6:6:5 fused, 6:5:6 fused and 5:6:5 fused rings with different number and arrangement of nitrogen atoms (Yuzlenko & Kiec-Kononowicz, 2006). Most 6:6:5 fused N-heteroaromatic systems have similarities in the number of ring arrangements, and favourable cyclopentyl group presence (Chang *et al.*, 2004).



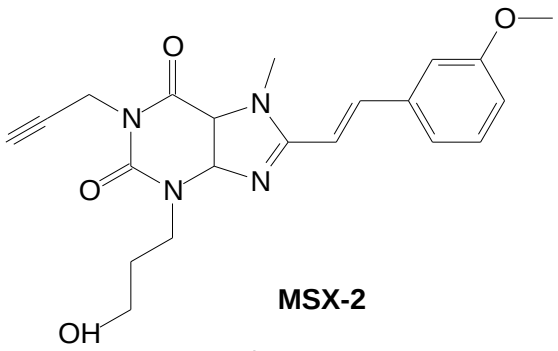
5-amino-[1,2,4]triazolo-[5,1-f]purin-2-one

hA_1K_i = 1.5 nM

3.4.3 Xanthine and Non-xanthine A_{2A} AR Antagonists

Based on the core xanthine structure some A_{2A} AR antagonists present good affinity, with some of these A_{2A} antagonists undergoing clinical trials and only one drug approved for the adjunctive treatment of PD (Yuzlenko & Kiec-Kononowicz, 2006).

The first highly selective A_{2A} ligands were 8-styryl derivatives e.g. MSX-2 which contains a propargyl group. 3-(3-Hydroxypropyl)-8-(m-methoxystryl)-7-methyl-1-propargyl xanthine MSX-2 further showed high affinity in radioligand binding assays at the human recombinant A_{2A} AR (Cacciari *et al.*,2003; Yuzlenko & Kiec-kononowicz, 2006).

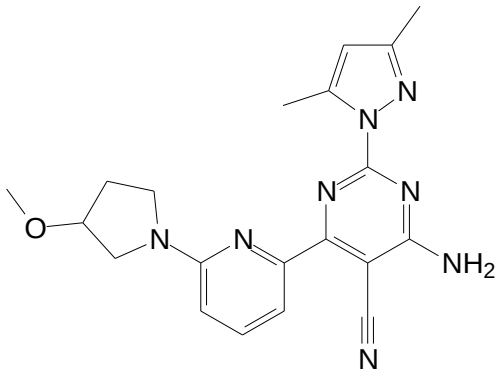


MSX-2

$hA_{2A}K_i$ = 5 nM
$rA_{2A}K_i$ = 8 nM

3.4.3.1 Monocyclic Fused Heteroaromatic System

The monocyclic A_{2A} antagonist (R)-4-amino-2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-(6-(3-methoxypyrrolidin-1-yl)pyridin-2-yl)pyrimidine-5-carbonitrile showed high affinity against A_{2A} ARs and emerged as potential treatment for PD (Yang *et al.*, 2016).



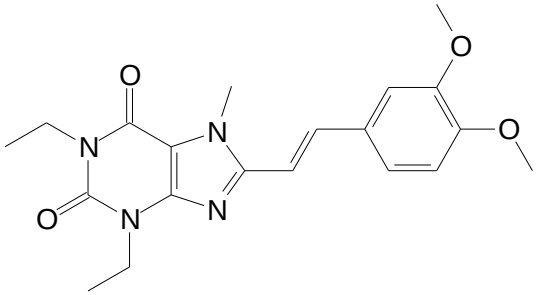
(R)-4-Amino-2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-(6-(3-hydroxypyrrolidin-1-yl) pyridin-2-yl)pyrimidine-5-carbonitrile

$hA_{2A}K_i$ = 1.0 nM

.

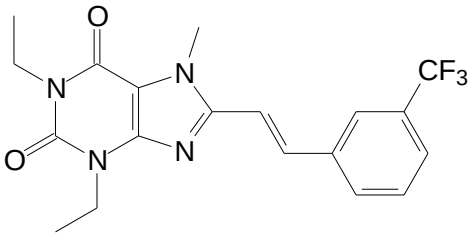
3.4.3.2 Bicyclic Fused Heteroaromatic System

KW-6002, reported as the most advanced A_{2A} antagonist, received marketing approval in Japan under the name NOURIAST™ in 2013 and was also recently approved by the United States FDA. Exploration of the 1,3-diethyl-8-(3,4-dimehtoxystryryl)-7-methylxanthine scaffold which is substituted by two ethyl groups on the xanthine nucleus (Zheng *et al.*, 2014; Hockemeyer *et al.*, 2004; Van der Walt *et al.* 2013), led to the discovery of the structurally related (*E*)-1,3-diethyl-7-methyl-8-[(3-trifluoromethyl)styryl]xanthine which also showed high binding affinity against rat striatal A_{2A} ARs.



KW-6002

$hA_{2A}K_i$ = 12 nM
$rA_{2A}K_i$ = 2 nM

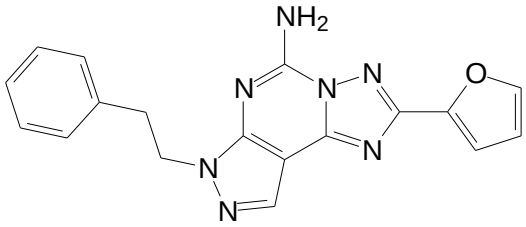


(*E*)-8-styrylxanthine-1,3-diethyl-7-7methylxanthine

$rA_{2A}K_i$ = 11.9 nM

3.4.3.3 Tricyclic Fused Heteroaromatic System

[1,2,4]triazolo[5,1-f]purin-2-one derivatives, synthesized by Basu and co-workers (2017), showed significant affinity for A_{2A} ARs and proved to be good therapeutical drugs for the potential future treatment of PD. Additionally, a series of 5-amino-2-furylopyrazolotriazolo-pyrimidines showed good affinity and selectivity for A_{2A} ARs, but displayed poor water solubility (Baraldi *et al.* 2002). Interestingly, the report by Lopes and co-workers. (1999) on another tricyclic fused heteroatomic ring system namely SCH-58261 clearly showed how the intraperitoneal injection of either caffeine or the aforementioned selective A_{2A} AR antagonist (SCH-58261) prevent learning deficit and the expression of Aβ toxicity, which may be helpful in AD.

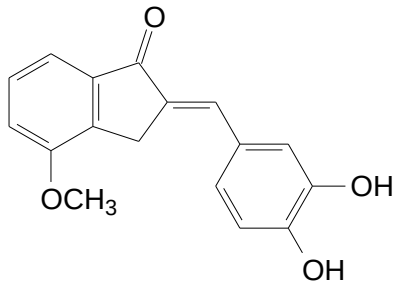


SCH 58261

$hA_{2A}K_i$ = 1.1 nM
$rA_{2A}K_i$ = 1.1 nM

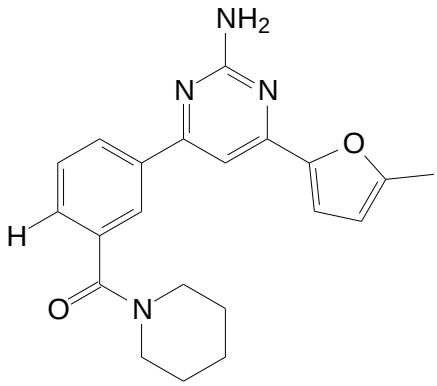
3.4.4 Dual Target A_1/A_{2A} AR Antagonists

Despite its low affinity in both A_1 and A_{2A} ARs, the effects of caffeine and coffee consumption are significant in patients with AD and PD, as seen in different studies (Jacobson *et al.*, 1993, Kim *et al.*, 2014, Rodrigues *et al.*, 2015). Recent research on structure-activity relationships of flavonoids and structurally related compounds, lead to the discovery of dual A_1 and A_{2A} AR antagonists of the benzylidene indanone family with high affinity in the nanomolar range (Janse van Rensburg *et al.*, 2020, Van Der Walt & Terre'Blanche, 2015). Another dual compound was 4-(5-methylfuran-2-yl)-6-[3-(piperidine-1-carbonyl)phenyl]pyrimidin-2-amine which displayed high affinity with low K_i values against A_1/A_{2A} ARs in rat brain membranes (Robinson *et al.*, 2015).



2-(3,4-dihydroxybenzylidene)-4-methoxy-2,3-dihydro-1H-inden-1-one

rA_1K_i = 42 nM
$rA_{2A}K_i$ = 78 nM



4-(5-methylfuran-2-yl)-6-[3-(piperidine-1-carbonyl)phenyl]pyrimidin-2-amine

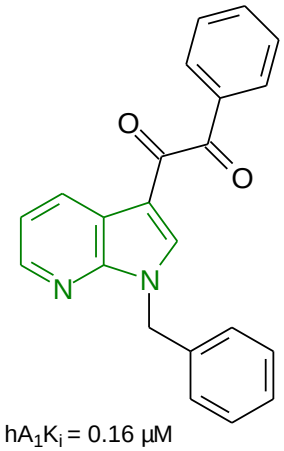
rA_1K_i = 9.54 nM
$rA_{2A}K_i$ = 6.34 nM

3.4.5 Chalcone Based Adenosine Antagonists

Chalcones are potent organic scaffolds that forms part of the flavonoid group. Chalcones are composed of two aromatic rings that are linked by a three carbon α,β-unsaturated carbonyl system and are considered a privileged scaffold in medicinal chemistry. They have attracted attention not only from synthetic and biosynthetic perspectives but also due to their wide-ranging biological activities, such as antimicrobial, antiviral, antifungal, antimalarial, antileishmanial, anti-inflammatory and anticancer properties (Gaonkar & Vignesh, 2017; Zhuang *et al.*, 2017). Chalcones are a member of the flavonoid family which have been explored as AR antagonists (Van der Walt & Terre'Blanche, 2018). Chalcones have also showed potential in the treatment of neurological conditions by acting as antidepressants and anxiolytics, as well as the inhibition of acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and MAO (Mathew *et al.*, 2019). In addition, several chalcone-coumarin derivatives have shown to possess AR affinity (Vazquez-Rodriguez *et al.*, 2013; Vazquez-Rodriguez *et al.*, 2020) and recently C3 amino-substituted

chalcone derivatives with a bromo substitution on benzylidene ring B were reported to possess selective adenosine A_1 AR affinity in the micromolar range (Janse van Rensburg, *et al.,* 2020).

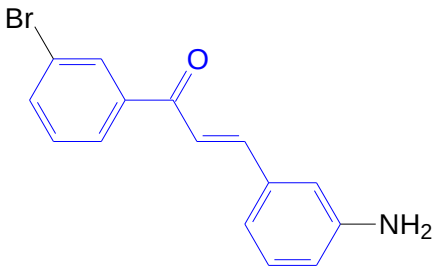
Compounds structurally related to chalcones include benzylidene tetralones, benzylidene indanones, aurones, benzoyl benzofurans, as well as isoxazole and pyrazole derivatives. Due to the open-chain model and scaffold modification of chalcones, a new class of organic compounds such as isoxazole-, pyrazole- and indole-based chalcones was synthesized. In a recent review by Taliani and co-workers (2020) they exploited the indole scaffold to design compounds binding to different pharmacological targets and highlighted. the affinity of 1-benzyl-3-ketoindole derivatives with 1-(1-Benzyl-1*H*-indol-3-yl)-2-phenylethane-1,2-dione exhibiting submicromolar affinity for the A_1 AR of 161 nM. Qhobosheane and co-workers (2020a) synthesized novel C3 substituted chalcone-based derivatives of 7-azaindole and evaluated their protein kinase inhibition activity. Due to the structural similarity of the 7-azaindoles compared to chalcones and indoles it was decided to screen these compounds for novel A_1 AR affinity.



hA_1K_i = 0.16 µM

1-(1-Benzyl-1H-indol-3-yl)-2-phenylethane-1,2-dione

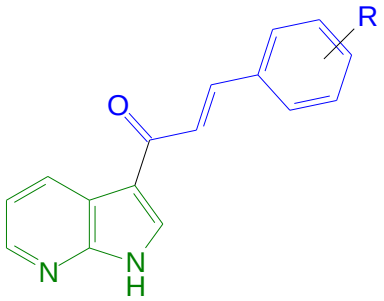
(Taliani *et al.*, 2020)



rA_1K_i= 1.56 µM

3-Amino chalcones

(Janse van Rensburg *et al.,* 2020)



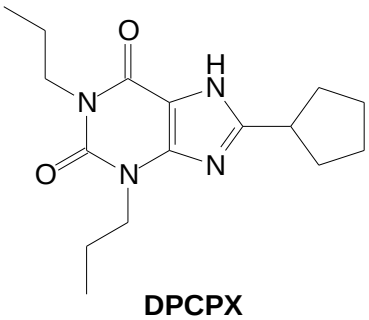
7-Azaindole scaffold

(Qhobosheane et al., 2020)

3.5 Species Difference in Adenosine Receptor Affinity

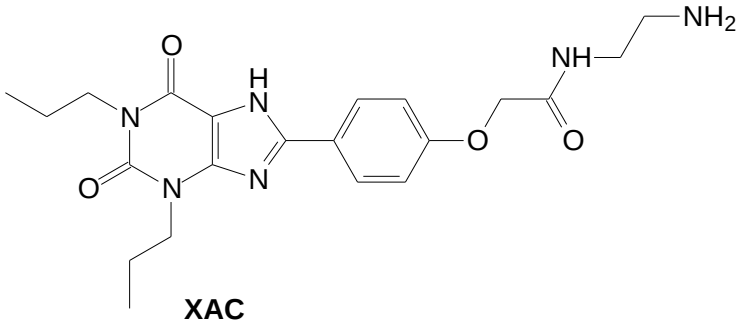
Preclinical studies are mostly performed in mice or rats. The A_1 AR of human, rat, and mouse comprises of 326 amino acids and the similarity of the amino acid sequence of the three species is as follows: human vs. rat 95%, human vs. mouse 95%, and rat vs. mouse 98% (Alnouri *et al.,* 2015). In a study by Maemoto and co-workers (1997) xanthine based AR antagonists exhibited higher A_1 affinity for [^{3}H]DPCPX binding sites in rat cortical membranes, compared with human brain membranes. In contrast to xanthine antagonists, pyrazolopyridine derivatives displayed similar affinity for [^{3}H]DPCPX in both rat and human membranes.

Supporting these findings Fredholm and co-workers (2001) compared literature values of xanthine antagonists showing the following: DPCPX (human 3.9 nM vs rat 0.3 nM), XAC (human 29 nM vs rat 2.8 nM) ZM 241385 (human 260 nM vs rat 2 nM). Non-xanthine AR antagonist, ASP-5854 (5-[5-Amino-3(4-fluorophenyl) pyrazin-2yl]-1-isopropylpyridine-2(1H)-one), further showed similar binding affinity for A_1 ARs in humans, rats, and mice (Mihara *et al.*, 2007). In addition, Klotz and co-workers (1991) also reported xanthine derivatives DPCPX and XAC to be 10-30 times less potent at the human A_1 AR compared to the rat A_1 AR. Interestingly no significant species difference was found for the xanthine, theophylline (Klotz *et al.* 1991).



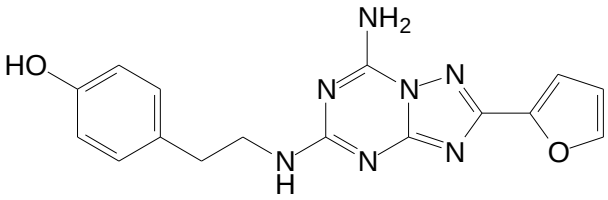
DPCPX

hA_1K_i = 3.9 nM
rA_1K_i = 0.4 nM



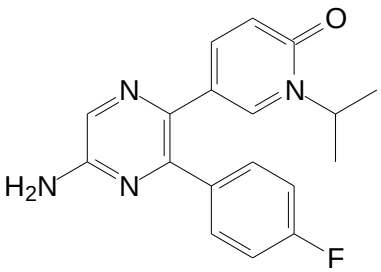
XAC

hA_1K_i = 29 nM
rA_1K_i = 2.8 nM



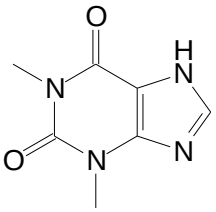
ZM 241385

hA_1K_i = 260 nM
rA_1K_i = 2 nM



ASP-5854

hA_1K_i= 9 nM
rA_1K_i= 12.5 nM



Theophylline

hA_1K_i = 6 200 nM
rA_1K_i = 8 500 nM

Regardless of the nature of A_1 AR from different species, above mentioned research provides a caution against extrapolating profiles of potencies for AR antagonist from one species to another.

3.6 Summary

In this chapter, we highlighted the importance and the physiological role of A_1 and A_{2A} ARs in neurological diseases such as AD and PD, together with the positive effects of the xanthine derivative caffeine on motor and non-motor (cognitive and depression) dysfunctions in PD. A_1 and A_{2A} AR antagonists were explored as rational drug treatment for AD and PD, and dual A_1/A_{2A} antagonists may offer a solution in alleviating motor symptoms, depression and cognitive dysfunction and provide neuroprotection simultaneously. The affinity of the AR antagonists is very important and shows the potential of the compound as a drug; however, drug affinity tests done only on rat brain models are not ideal for a number of reasons. As highlighted in chapter 1, there is a strong opposition and debate among the scientific community about the relevance of animal data for humans and the likelihood of successful cross-species translation. The current pilot study will aid in establishing a radioligand binding assay to determine A_1 AR affinity using CHO cells transfected with human A_1 AR. This will enable future studies using CHO cells transfected with human A_{2A}, A_{2B} and A_3 ARs in order to test compounds against all the adenosine subtypes. In addition, this study also aims to identify the 7-azaindole scaffold as a promising target for the development of novel compounds for AR affinity.

CHAPTER 4: RADIOLIGAND BINDING ASSAY USING CHO CELLS EXPRESSING HUMAN ADENOSINE A_1 RECEPTORS

4.1 Introduction

The A_1 AR radioligand binding assays provide a means for assessing the degree of binding affinity that selected test compounds may possess toward the A_1 ARs. Typically, a radioligand binding study is conducted using proteins that express the desired receptor in the presence of a radioligand known to exhibit a high affinity towards the receptor in question.

RBAs are based upon competitive binding between an analyte and a radiolabelled ligand at a certain target receptor. By using this method, the amount of the ligand bound to a target receptor (ligand-receptor-complex) can be measured in order to determine the radiolabelled ligand's binding affinity for certain receptors, after which the degree of selectivity for the receptor can be calculated by comparing various affinities of receptor subtypes. Any receptor can be used to perform RBAs as long as it has a selective ligand which can be labelled with radioactive isotopes. Currently, determination of adenosine receptor affinity is mainly accomplished by performing RBAs with ligands radiolabelled with either tritium or iodine

Competition binding experiments determines the relative affinity of an unlabelled compound or drug for its target receptor. During these experiments a single concentration of a radioligand is measured in the presence of increasing concentrations of unlabelled compounds with the same target receptor during a state of equilibrium. The concentration of bound radioligand wil decrease as the concentration of the unlabelled ligand increases. The affinity constant (K_i) is determined, and indicates the affinity of the unlabelled compound or drug for its target receptor. Further, the radioligands bound to receptors and those free of receptors are separated by the use of filters after incubation, for example a Hoffeler filtration system (Van der Walt, *et al.*, 2013). An acceptable amount of ligand needs to be bound to their receptors in order to prevent losing too much after the unbound ligand are washed away during the process of separation. In order to achieve this, the ligand has to be given an adequate amount of time to bind with their respective receptors. Liquid scintillation counting is used to count the bound radioactivity trapped in the filters after placing the filters in scintillation fluid. For the present study, a Packard Tri-Carb 2100 TR liquid was used, and the scintillation count expressed as counts per minute (CPM).

All cell culture work and radioligand binding assays were done at the North-West University's Laboratory for Analytical and Molecular Biology (LAMB). Furthermore, all materials and reagents were commercially available and purchased from various manufacturers. The Chinese hamster

ovary (CHO) cells expressing the human A_1 AR cells were kindly donated by Prof KN Klotz from the University of Würzburg, Germany.

4.2 Ethics

The use of CHO cells for the A_1 AR radioligand binding assay was approved by the Health Sciences Ethics Office for Research, Training and Support, North-West University, application number: NWU-00585-19-A5 (Annexure).

4.3 Cloning of Adenosine Receptor and Stable Transfection of Cells

Chen and Okayama (1987) were the first to describe the transfection protocol of mammalian cells by plasmid DNA, while Freund *et al*. (1994) clearly illustrated transfection of the rat A_1 AR into CHO cells. CHO A_1 cells were prepared at the University of Würzburg, Germany; specifically cloning of the human A_1 AR and stable transfection of CHO cells cDNAs were verified according to GenBank entries after sequencing and comparison and found to correspond with other published sequences for the A_1 AR (Klotz *et al.*, 1998).

4.4 Culturing of CHO A_1 AR Cells

First and foremost, all cell culture work was done in a sterile environment; all apparatus and equipment were always disinfected with 70% ethanol prior to use.

4.4.1 Apparatus and Equipment

- Tube racks
- Spray bottle with 70% ethanol
- Paper towels
- Centrifuge tubes (15 cm^2/50 cm^2)
- Cell culture flasks (25 cm^2/75 cm^2)
- Serological pipettes (5 mL; 10 mL)
- Waste containers (one for liquid waste, one for plastic waste)
- Pipettor
- Laminar flow hood
- CO_2 incubator
- Water bath
- Light microscope
- Centrifuge

4.4.2 Materials and Reagents

- Growth medium: Dulbecco's Modified Eagles Medium with nutrient mixture F12 (DMEM/F12) (PAN Biotech) as well as added 10% Fetal Bovine Serum (FBS) (Gibco), 1% L-Glutamine (Lonza), 1% Non-Essential Amino Acids (NEAA) (Lonza), 1% Penicillin-Streptomycin (Pen-Strep) (Lonza), 0.2mg/mL (0.4%) Geneticin (Gibco)
- Phosphate buffered saline (PBS) (HyClone)
- Trypsin-Versene™-Trypsin-EDTA Cell Culture Reagent (Lonza)

4.5 Procedure for Culturing

This procedure is valid for the routine culturing of CHO A_1 cells in 25 and 75 cm^2 culture flasks donated from the University of Würzburg, Germany as described by Klotz *et al.* (1998). CHO A_1 cells between passages 4 and 14 were used for the experiments.

4.5.1 Reviving Frozen Cell Stocks

The following procedure is effective for the standard revival of CHO A_1 cells after storage at -150°C for long periods:

The cryovial was removed from the cryogenic refrigerator and rapidly thawed at 37 °C in the water bath, and once thawed 60–80% (presenting a slush), disinfected and placed in the laminar flow hood. (Please note that the cap of the cryovial must never be submerged in the water bath.) To

the thawed cryovial containing the selected cell stock, 1 mL preheated growth medium was slowly added, and subsequently, transferred to a sterile 15 cm^2 tube already containing 4 mL growth medium. The cell-medium mixture was then centrifuged at 140 *g* for 5 minutes; the resulting supernatant was removed by pipetting. The pellet was re-suspended in 5 mL growth medium. The cell-medium mixture was transferred to a sterile 25 cm^2 cell culture flask (taking care to distribute the said mixture evenly over the growth surface) and placed in the CO_2 incubator overnight. The following day, attachment of cells to the growth surface was determined using a light microscope. The spent growth medium was removed from the cell culture flask by pipetting and new growth medium was added to and distributed in the flask, and then, the cell culture flask was returned to the CO_2 incubator.

- For a 25 cm^2 cell culture flask, 5 mL growth medium was added
- For a 75 cm^2 cell culture flask, 15 mL growth medium was added

4.5.2 Subculturing Cells

Of note, CHO A_1 cells were subcultured between 80–90% confluences. Again, the spent growth medium was removed from the cell culture flask by pipetting. The flask was then rinsed twice with preheated PBS.

- For a 25 cm^2 cell culture flask, 5 mL PBS was added twice
- For a 75 cm^2 cell culture flask, 10 mL PBS was added twice

Subsequently, preheated Trypsin-Versene™ was added to and distributed evenly in the flask and the flask was then promptly returned to the CO_2 incubator at 37 °C for 2 minutes. Thereafter, the flask was removed from the incubator and tapped in order to detach the cells from the growth surface.

- For a 25 cm^2 cell culture flask, 1 mL Trypsin-Versene™ was added
- For a 75 cm^2 cell culture flask, 3 mL Trypsin-Versene™ was added

Once the cells were detached, preheated growth medium was added to the flask.

- For a 25 cm^2 cell culture flask, 5 mL growth medium was added
- For a 75 cm^2 cell culture flask, 6 mL growth medium was added

The cell-medium mixture was transferred to a sterile 15 cm^2 tube and centrifuged at 140 *g* for 5 minutes; the resulting supernatant was removed by pipetting. The pellet was re-suspended in 5 mL preheated growth medium, and upon mixing, the cell suspension was divided between the appropriate number of 25 $cm^2$and/or 75 cm^2 cell culture flasks. (Subculture in a ratio between 1:8

and 1:20. A subculture ratio of 1:20 takes about 1 week to reach 80% confluence). Preheated growth medium was then added to each flask to obtain the desired final volume. Finally, the cell culture flasks were put in the CO_2 incubator.

- Final volume of growth medium for a 25 cm^2 cell culture flask is 5 mL
- Final volume of growth medium for a 75 cm^2 cell culture flask is 15 mL

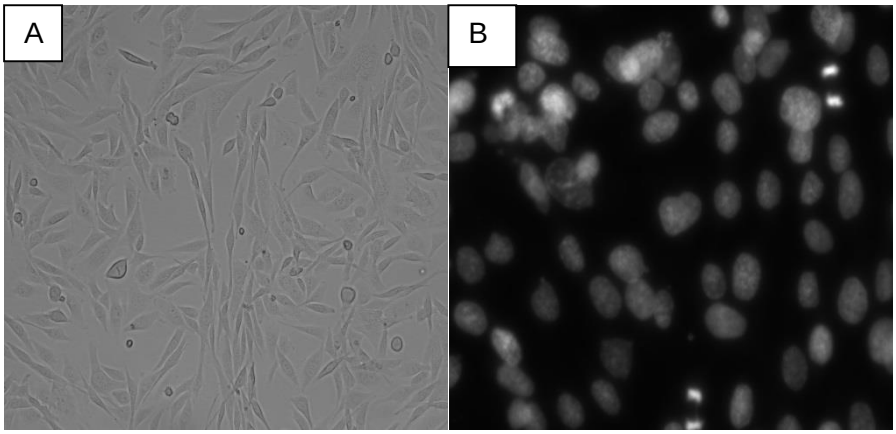


Figure 4-1: Illustrates the (A) CHO A_1 cells at 50-60 % confluence. (B) Clean CHO A_1 growth without mycoplasma contamination.

4.6 Membrane Storage

4.6.1 Apparatus and Equipment:

- Tube racks
- Spray bottle with 70% ethanol
- Paper towels
- Centrifuge tubes (15 cm^2/50 cm^2)
- Cell culture flasks (25 cm^2/75 cm^2)
- Serological pipettes (5 mL; 10 mL)
- Waste containers (one for liquid waste, one for plastic waste)
- Pipettor
- Cell scraper
- Laminar flow hood
- CO_2 incubator
- Water bath
- Light microscope
- Centrifuge

- Freezer

4.6.2 Materials and Reagents

- PBS
- 50 mM Tris/HCl (pH 7.7 at 25 °C)

4.6.3 Method

For CHO A_1 cells between 80-90% confluences, the spent growth media was removed from the cell culture flask by pipetting. The flask was then rinsed twice with preheated PBS.

- For a 25 cm^2 cell culture flask, 5 mL PBS was added twice
- For a 75 cm^2 cell culture flask, 10 mL PBS was added twice

The cells attached to the growth surface of the cell culture flask were then scraped off in ice-cold hypotonic buffer and transferred to a sterile 50 cm^2 tube (repeated two times). These tubes were then frozen at -20 °C until day of membrane preparation.

- For a 25 cm^2 cell culture flask, 5 mL (50 mM Tris/HCl (pH 7.7 at 25 °C) was added twice
- For a 75 cm^2 cell culture flask, 10 mL (50 mM Tris/HCl (pH 7.7 at 25 °C) was added twice

4.7 Membrane Preparation

4.7.1 Apparatus and Equipment

- Centrifuge tubes (15 cm^2/50 cm^2)
- Serological pipettes (5 mL; 10 mL)
- Pipettor
- Dispersing instrument
- Centrifuge
- Freezer

4.7.2 Materials and Reagents

- 50 mM Tris/HCl buffer (pH 7.7 at 25 °C)(Merck)
- GF/B filters (Whatman®)
- Liquid scintillation counter (PerkinElmer)

4.7.3 Method

Membrane proteins were prepared by thawing frozen CHO A_1 cells on ice. The resulting cell suspension was homogenized on ice (2 x 15 s at full speed) and the homogenate was then spun for 10 min (4 °C) at 1,000 *g*. The pellet was discarded, and the supernatant was then centrifuged for 30 min at 100,000 *g*. The final membrane pellet was resuspended in 50 mM Tris/HCl buffer (pH 7.7 at 25 °C) at a protein concentration of 1.2 mg/ml and stored at -80 °C (Klotz *et al.*, 1998).

4.8 Protein Concentration

The protein concentration was determined according to the Bradford assay, using bovine serum albumin as reference standard (Bradford, 1976).

4.9 Adenosine A_1 Receptor Radioligand Binding Assays

The degree of binding affinity the test compounds showed toward the human A_1 AR was determined via radioligand binding assays which were adapted from Klotz *et al.* (1998) and Van der Walt and Terre'Blanche (2015). The A_1 AR radioligand binding assay used membrane proteins expressing the human A_1 AR (see sections 4.1–4.6) (Klotz *et al.*, 1998) and 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) as radioligand (Bruns *et al.*, 1987).

4.9.1 Apparatus and Equipment

- Tube racks
- Microcentrifuge tubes (1.5 cm^2)
- Centrifuge tubes (15 cm^2/50 cm^2)
- Polyvials
- GF/B filters
- Serological pipettes (5 mL; 10 mL)
- Pipettor
- Pippette tips (1000 µL, 100 µL, 10 µL, 1 µL)
- Pipettes (1000 µL, 100 µL, 10 µL, 1 µL)
- Hoffeler vacuum system
- Shaking water bath
- Liquid scintillation counter

4.9.2 Materials and Reagents

- Dimethyl sulfoxide (DMSO) (Sigma)

- 50 mM Tris/HCl buffer (pH 7.7 at 25 °C) (Merck)
- 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) (PerkinElmer)
- Adenosine deaminase (ADA)(Sigma)
- CHO A_1 membrane proteins

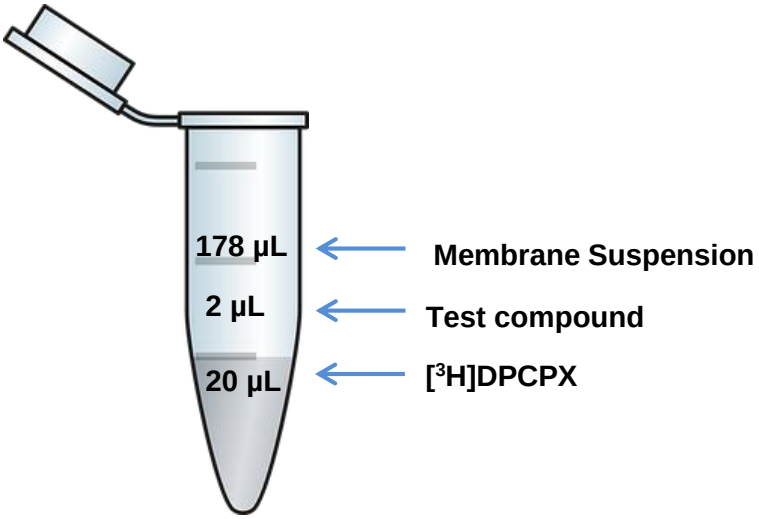


Figure 4-2: Illustration of 2 mL tube with final order of addition: (1) 20 µL radioligand solution (containing [^{3}H]DPCPX) (2) 2 µL test compound (at the desired concentration ranging from 0 µM to 100 µM) and (3) 178 µL membrane suspension (containing CHO A_1 membranes).

4.9.3 Method

Each incubation consisted of (in order of additions): (1) 20 µL radioligand solution, (2) 2 µL test compound (at the desired concentration ranging from 0 µM to 100 µM) and (3) 178 µL membrane suspension. The radioligand solution had a concentration of 1 nM [^{3}H]DPCPX (2) and the membrane suspension was equivalent to 20 µg/200 mL membrane proteins (expressing the A_1 AR) and 0.2 units/mL adenosine deaminase (ADA). The final volume of all incubations contained 200 µL 50 mM Tris/HCl buffer (pH 7.7 at 25 °C) and 1% DMSO (Van der Walt & Terre'Blanche, 2015). After the additions were made, each sample was vortexed and incubated in a shaking water bath at 25 °C for 1 hour. Thirty minutes, post-commencement of incubation, each sample was vortexed again and returned to the shaking water bath. Incubation was terminated by filtering each sample through 25 mm GF/B filter and the pertaining microcentrifuge tube was washed twice with 1 mL Tris/HCl buffer (pH 7.7 at 25 °C). Filtration occurred under reduced pressure using a Hoffeler vacuum system and upon completion each filter was placed into a scintillation vial, along with 4 mL scintillation fluid. The vials were subjected to agitation before being left in a stationary position for two hours. At that time, the residual radioactivity of each filter was measured with a

liquid scintillation counter. Non-specific binding of [^{3}H]DPCPX for the radioligand binding assay was defined as binding in the presence of 10 µM DPCPX (Bruns *et al.*, 1987; Van der Walt *et al.*, 2015). Specific binding was defined as the total binding minus the non-specific binding (Van der Walt *et al.*, 2015).

4.10 Statistical Data Analysis

Briefly, all statistical data analyses were done using Microsoft Excel and GraphPad Prism Software based on non-linear curve fitting procedures. Sigmoidal dose response curves, from which IC_{50} values were calculated, were obtained by plotting the specific binding of [^{3}H]DPCPX against the logarithm of the test compounds' concentrations. Subsequently, the IC_{50} values were used to calculate the inhibition constant (K_i) values for the competitive inhibition of [^{3}H]DPCPX against membrane proteins by means of the Cheng-Prusoff equation, where [C^*] is the concentration of the radioligand and K_d its dissociation constant (**Equation 4–1**). The equilibrium dissociation constant (K_d) value for the radioligand [^{3}H]DPCPX was taken as 3.86 nM (Klotz *et al.*, 1998). All calculated K_i values were determined in triplicate and given as mean ± standard error of the mean (SEM).

$$\boldsymbol{K_i = IC_{50} \,/\, (1 + [C^*] \,/\, K_d)} \qquad \textbf{Equation 4-1}$$

IC_{50} test compound / (1 + [Concentration radioligand [^{3}H]DPCPX] / K_d value radioligand [^{3}H]DPCPX)

Table 4-1: Summary of A_1 AR radioligand binding assay using CHO cells.

Component	Function
DMSO	Used as solvent for test compounds
50 mM Tris.HCl	Served as buffer solution
Scintillation fluid	Used to dissolve GF/B filters containing bound radioligand in order to measure radioactivity with a scintillation counter.
A_1 AR membrane suspension	Contains CHO membranes expressing the A_1 AR as well as ADA.
[^{3}H]DPCPX	Selective A_1 AR antagonist used as radioligand (Bruns *et al.*, 1987; Van der Walt & Terre'Blanche, 2015)
ADA	Breakdown endogenous adenosine and prevents binding of endogenous adenosine to ARs (Bruns *et al.*, 1987)

CHAPTER 5: RESULTS AND DISCUSSION

5.1 Introduction

As mentioned earlier, the current pilot study will aid in establishing the radioligand binding assay using CHO cells transfected with the human A_1 AR. This will enable future studies using CHO cells transfected with human A_{2A}, A_{2B} and A_3 ARs in order to test compounds against all the adenosine subtypes. In addition, this study also aimed to identify the 7-azaindole scaffold as a promising target for the development of novel compounds with AR affinity.

5.2 Establishing Standard Radioligand Binding Assay for CHO Cells Expressing the A_1 AR

The radioligand binding assay utilizing rat A_1 ARs was adapted, as seen in **Table 5-1**, to establish the assay using CHO A_1 cell membranes.

Table 5-1: Radioligand binding assay parameters utilizing either rat or CHO A_1 AR

Parameters	Rat A_1 AR RBA	Human A_1 AR RBA
Final volume	1000 µL	200 µL
Membrane suspension	Rat whole brain: 120 µg/890 µL ADA: 0.1 units/890 µL	CHO A_1 cell: 17.8 µg/178 µL ADA: 0.04 units/178 µL
Radioligand solution	[3H]DPCPX: 0.1 nM	[3H]DPCPX: 1 nM
Solvent (DMSO)	1%	1%
Buffer	1000 µL 50 mM Tris–HCl buffer (pH 7.7 at 25 °C)	200 µL 50 mM Tris–HCl buffer (pH 7.7 at 25 °C)
Non-specific binding	10 µM DPCPX	10 µM DPCPX
Tube for incubation:	5 mL round-bottom polypropylene tubes	2 mL microcentrifuge tube
Tubes rinsed 2 x with:	4 mL 50 mM Tris–HCl buffer (pH 7.7 at 25 °C)	1 mL 50 mM Tris–HCl buffer (pH 7.7 at 25 °C)
Rinsed filter with:	4 mL 50 mM Tris–HCl buffer (pH 7.7 at 25 C)	4 mL 50 mM Tris–HCl buffer (pH 7.7 at 25 C)
Scintillation fluid	4 mL	4 mL
Performed in:	triplicate	triplicate

In this study two reference compounds (DPCPX and KW-6002) were used to establish the method and the results are shown in **Table 5-2**. The radioligand binding assays were validated with

DPCPX (A_1 antagonist) and istradefylline (A_{2A} antagonist) as reference compounds and results were in accordance with literature values.

As reported previously by Maemoto and co-workers (2004) as well as Bulicz and colleagues (2006), the present study also found that the affinity of reference compounds DPCPX and istradefylline have significantly reduced activity against human versus rat A_1 ARs. Affinity at the human A_1 AR binding of DPCPX is almost tenfold lower than rat whole brain membranes expressing the A_1 AR. This phenomenon, is apparently, often observed with xanthine derivatives such as DPCPX and istradefylline (Maemoto *et al.*, 2004).

Table 5-2: Inhibition constant (K_i) values for the binding affinity of reference compounds against rat (r) and human (h) A_1 ARs

	K_i ± SEM (µM)[a]	
Compound	**rA_1[b] vs [^{3}H]DPCPX[c]**	**hA_1[d] vs [^{3}H]DPCPX[e]**
DPCPX (A_1 antagonist)	(0.0005)[f] (0.0004)[g] (0.0003)[h]	0.004 ± 0.0002 (0.001)[i] (0.003)[j]
Istradefylline (A_{2A} antagonist)	(0.230)[k]	1.7 ± 0.56 (0.841)[l]

[a] All inhibition constant (K_i) values were determined in triplicate and expressed as mean ± standard error of the mean (SEM) in µM.

[b] Rat receptors were used (rA_1)

[c] 0.1 nM [^{3}H]DPCPX

[d] Human receptors were used (hA_1).

[e] 1 nM [^{3}H]DPCPX

[f] Literature value obtained from (Van der Walt & Terre'Blanche, 2015)

[g] Literature value obtained from (Janse van Rensburg *et al.*, 2017)

[h] Literature value obtained from (Lohse *et al.*, 1987)

[i] Literature value obtained from (Maemoto *et al.*, 2004)

[j] Literature value obtained from (Bulicz *et al.*, 2006)

[k] Literature value obtained from (Müller & Jacobson, 2011)

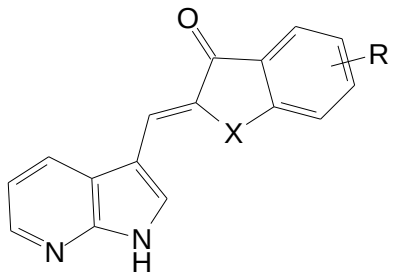
[l] Literature value obtained from (Müller & Jacobson, 2011)

Therefore, the current study demonstrated the reliable *in vitro* assessment of radioligand binding affinity in human A_1 AR transfected CHO cells. The already designed method of radioligand binding assays using transfected CHO cells, expressing A_1 AR has already been validated (Klotz *et al.*, 1998), and is now established at the NWU's LAMB as a tool to measure the binding affinity of novel compounds.

5.3 Novel 7-Azaindole-Chalcone Core Binding against A1 AR in Rat and Human

The degree of binding affinity that the test compounds showed toward human (h) A_1 and rat (r) A_1 ARs were determined via radioligand binding assays in triplicate (inhibition constant K_i, µM) and expressed as mean ± standard error of the mean (SEM) (**Table 5-3**). The 7-azaindole derivatives under investigation were previously synthesized and evaluated as protein kinase inhibitors (Qhobosheane *et al.*, 2020a; Qhobosheane *et al.*, 2020b). Thirty-five 7-azaindole compounds (Qhobosheane *et al.*, 2020a; Qhobosheane *et al.*, 2020b) were screened (at concentrations of 100 µM, 10 µM and 1 µM) and five compounds warranted full RBA against rat A_1 ARs (**Table 5-3**). Thereafter, the affinity of these compounds were determined with the CHO (h) A_1 AR assay and the inhibition constant (K_i) value in µM was calculated by methods reported previously (Klotz *et al.*, 1998; Van der Walt & Terre'Blanche, 2015) (**Table 5-3**). These radioligand binding assays were carried out under the parameters shown in **Table 5-1**.

Table 5-3: Inhibition constant (K_i) values for the binding affinity of 7-azaindole derivatives against rat (r) and human (h) A_1 ARs



			K_i ± SEM (µM)[a]	
Compound	**(X)**	**R**	**rA_1[b] vs [^{3}H]DPCPX[c]**	**hA_1[d] vs [^{3}H]DPCPX[e]**
1a	-Cinnamoyl		0.90 ± 0.07	0.92 ± 0.13
1b	$(CH_2)_2$	-	1.7 ± 0.12	1.9 ± 0.67
1c	$(CH_2)_2$	6-OH	3.5 ± 0.28	2.7 ± 0.28
1d	$(CH_2)_2$	6-OCH_3	1.9 ± 0.11	2.5 ± 0.26
1e	CH_2	5,6-diOCH_3	1.1 ± 0.07	1.7 ± 0.43

[a] Inhibition constant (K_i) values were determined in triplicate and expressed as mean ± standard error of the mean (SEM) in µM.

[b] Rat receptors were used (rA_1)

[c] 0.1 nM [^{3}H]DPCPX

[d] Human receptors were used (hA_1)

[e] 1 nM [^{3}H]DPCPX

The competition binding curve (**Figure 5-1**), demonstrates the successful binding of compound **1a** to CHO (h) A_1 AR cells, showing the affinity of 7-azaindole-chalcone core by calculating the

concentration of 50% inhibition of radioligand binding (IC_{50}) and using equation 4-1 to calculate affinity (K_i).

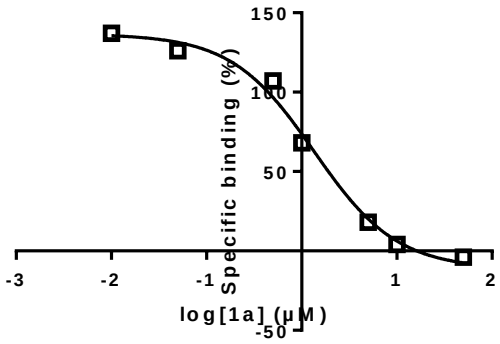


Figure 5-1: **Typical dose-response curve for our *in vitro* model validation. The average binding-response curve for compound 1a on our human A_1 determined via a radioligand binding assay using CHO cell membranes expressing the human A_1 AR with [^{3}H]DPCPX as radioligand.**

The evaluation of 7-azaindole conjugated (chalcone/tetralone/indanone) scaffolds showed promising affinity for A_1 ARs in both rat and human species in the low micromolar range. Compound **1a** recorded the best A_1 AR affinity among the test compounds with rat A_1 K_i value of 0.90 µM and a human A_1K_i value of 0.92 µM, showing similar affinities in rat compared to human A_1 ARs (**Table 5-3**). This is also consistent with the nature of non-xanthine derivatives and previous reports by Maemoto and co-workers (2004), that non-xanthine ligands exhibit similar affinity for [^{3}H]DPCPX binding sites in the brain membranes of different species – including rat and human. The second best affinity was the 7-azaindole-indanone derivative **1e** with dimethoxy substitution on the fused 6- and 5-membered rings, showing A_1 AR affinity of 1.1 µM in rat and a K_i value of 1.7µM in human A_1 ARs (CHO cells) (**Table 5-3**). The same trend was observed with similar affinities in rat compared to human A_1 ARs for compounds **1b**, **1c** and **1d**. The 1-indanone scaffold has also been reported by Janse van Rensburg and colleagues (2019a, 2019b), showing moderate affinity towards A_1 ARs.

Three compounds (**1b**, **1c**, **1d**) comprising of a conjugated 7-azaindole-α-tertralone structure were compared to **1a** with a conjugated 7-azaindole-chalcone structure and **1e** with a conjugated 7-azaindole-indanone structure. Compounds **1b** (unsubstituted tetralone), **1c** (hydroxyl substituted tetralone) and **1e** (methoxy substituted tetralone) showed a two- to three-fold decrease in affinity for both the rat and human A_1 ARs (**Table 5-3**) compared to **1a** and **1e.** The α-tetralone scaffold has also been shown to possess affinity for the A_1 ARs (Janse van Rensburg *et al.*, 2017; Legoabe *et al.,* 2018). However, seeing that the difference between K_i values are

small and only a limited number of derivatives were evaluated, these structure-activity relationships should be seen as preliminary. In general, the conjugated 7-azaindole-chalcone derivative **1a** showed better affinity than the indanone (**1e**) and tetralone (**1b, 1c**, **1d**) derivatives. These limited results show that the 7-azaindole moiety may hold promise in the design of A_1 AR ligands; where the 7-azaindole moiety is conjugated with a chalcone, tetralone or indanone moiety (**Figure 5-2**).

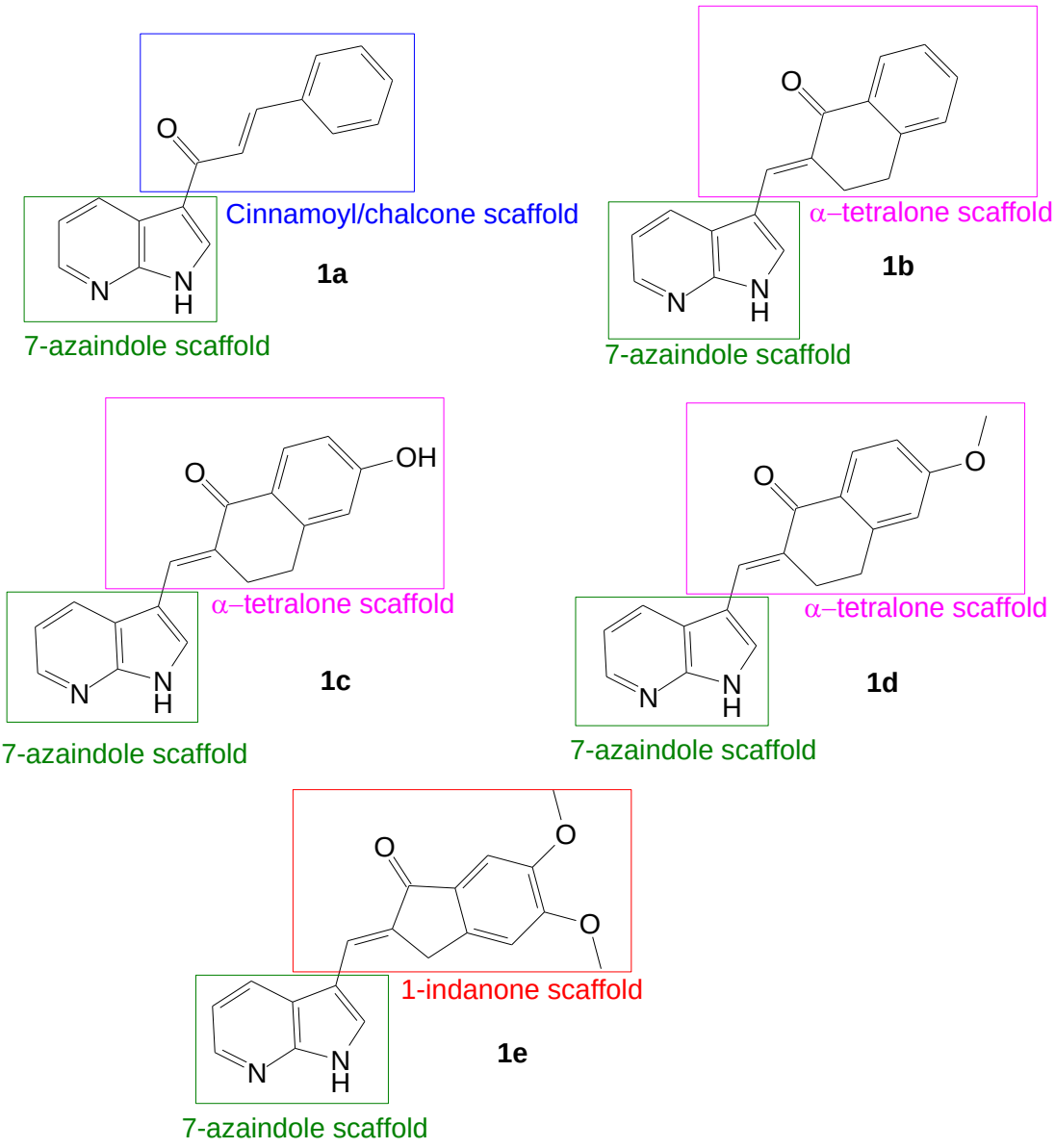


Figure 5-2: **Structural relationships of 7-azaindole derivatives containing either a chalcone, tetralone or indanone moiety.**

CHAPTER 6: CONCLUSION

Animal research played a vital role in scientific and medical research the past century and continues to aid our understanding of various diseases and the development of new drugs. Understanding how humans respond to drugs is an essential question in drug research and often this cannot be addressed because it is not possible to perform some experiments in human volunteers. An alternative is usually to generate responses in animal models and translating it to humans.

RBAs are widely used for the screening of new potential receptor ligands and are ideally suitable for structure-activity relationship (SAR) analysis and molecular modelling studies. The adenosine A_1 RBAs are routinely performed with rat whole brain membranes that express the adenosine A_1 receptor subtype (Van der Walt & Terre'Blanche, 2015). However, using rat membranes are costly and the use of animals in scientific and therapeutic research has been a subject of hot discussion for many years. There are strong opposing opinions among enthusiasts and sceptics about the relevance of animal data for humans and the likelihood of successful cross-species translation.

Furthermore, cell-culture based tests have considerably reduced the use of rodents in the initial screening of potential new drugs, while speeding up the process so that more compounds can be screened in the same period of time. Prompted by the above discussions, the current pilot study undertook to establish the A_1 AR RBA using CHO cells expressing human A_1 ARs. Reference compounds DPCPX and istradefylline (KW-6002) were included for comparison with literature values. Further, five known 7-azaindole hybrids, previously evaluated as potential protein kinase inhibitors (Qhobosheane, *et al.,* 2020b), were screened as novel adenosine A_1 AR antagonists in the rat (unpublished results) and then selected to be evaluated as A_1 AR antagonists in CHO cells. The A_1 AR radioligand binding assay, using transfected CHO cells, was successfully established and affinity values of reference compounds correlated with literature values studies, thus showing that the above radioligand binding assay is reliable and reduced the challenges in ethics by minimizing the harvesting of rat brains and high costs.

Since the discovery of AR and their connection to various neurodegenerative diseases, the development of xanthine analogues has shown shortcomings of poor oral bioavailability, Blood-brain barrier (BBB) penetration and species differences. On the other hand, non-xanthine antagonists display similar affinity for A_1 ARs in both rat and human A_1 AR membranes. The five non-xanthine 7-azaindole derivatives displayed A_1 AR affinity in the low micromolar range and showed similar affinity for both rat and human A_1 AR membranes.

The 7-azaindole-chalcone conjugated compound **1a** showed the highest binding affinity for A_1 ARs in rat and human (hA_1K_i = 0.92 µM; rA_1K_i = 0.90 µM). The 7-azaindole-indanone conjugated compound **1e** showed the second highest affinity (hA_1K_i = 1.7 µM; rA_1K_i = 1.1 µM). The 7-azaindole-α-tetralone fused compounds (**1b**, **1c**, **1d**) showed a two- to three-fold lower binding affinity for A_1 ARs in rat and human.

In conclusion, the A_1 AR radioligand binding assay, using transfected CHO cells, was successfully established and affinity values of reference compounds correlated well with the results in literature. It also reduced challenges in ethics by minimizing the harvesting of rat brains and high costs. Although the compounds were limited for extensive SARs, the 7-azaindole-chalcone core appears to be the most promising lead for the development of compounds with improved A_1 AR affinity with regards to improved cognitive deficits associated with neurodegenerative diseases.

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ANNEXURE

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North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

18 September 2019

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 18/09/2019, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Radioligand binding assays of selected A1/A2A adenosine antagonists using rat membrane cells and Chinese Hamster Ovary- cells expressing human adenosine receptors
Principal Investigator/Study Supervisor/Researcher: Prof G Terre'Blanche
Student: T Tutubala - 23368322

Ethics number:

N	W	U	-	0	0	5	8	5	-	1	9	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single
Commencement date: 18/09/2019
Expiry date: 30/09/2020

Risk: Category 0

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of September annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal*

without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.

- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by Christiaan B Brink
Date: 2019.09.18 16:34:28 +02'00'

Prof Tiaan Brink
Chairperson NWU-AnimCareREC

Digitally signed by Prof Minrie Greeff
Date: 2019.09.18 20:13:34 +02'00'

Prof Minrie Greeff
Head of the Faculty of Health Sciences Ethics Office

Current details:(23239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
20 August 2019

File Reference: 9.1.5.4.2