

**Synthesis and evaluation of 7-deazapurine
derivatives as ligands of adenosine A1 and A2A
receptors**

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PREFACE

This dissertation is submitted in an article format in accordance with the General Academic Rules (A.13.7.3) of the North-West University. An article in the form of a manuscript is included in this dissertation.

Chapter 1: Introduction

Chapter 2: Literature Study

Chapter 3: Article

Pyrrolopyrimidine analogues with adenosine rA_1 and/or rA_{2A} receptor affinity in the low micromolar range

This article will be submitted to the journal, Molecular Diversity, and was prepared according to the author guidelines which can be accessed via the following link:

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Chapter 4: Conclusion

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"For I know the plans I have for you," declares the Lord, "plans to prosper you and not to harm you, plans to give you hope and a future."

Jeremiah 29:11

ABSTRACT

The search for treatment options for neurodegenerative disorders is ongoing. These disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD), have been conjectured to have similar pathogenic pathways, which include mitochondrial dysfunction, oxidative stress, and neuroinflammation. The cognitive decline can be targeted with A₁ adenosine receptor (AR) antagonism, and the depression and locomotor degeneration, accompanying disease progression and levodopa-induced dyskinesia (LID), can be ameliorated with A_{2A} adenosine receptor (AR) antagonistic efforts. Antagonism can counterbalance the surplus levels of adenosine triphosphate (ATP), adenosine and the increased expression of A_{2A} ARs prevalent in neuropathology. The pyrrolopyrimidines and -pyridines of interest, 7-deazapurine- and 7-azaindole scaffolds, can be optimised for dual A₁- and A_{2A} AR binding via Suzuki cross-coupling synthetic methods on the fourth and sixth positions. These scaffolds are derived from the adenine structure and several xanthine compounds share these structural features but have poor physicochemical properties; except for caffeine, a non-selective AR antagonist, due to the methylation of the heteroatoms. Evaluation of radioligand binding assay results indicate that two heteroatoms are apt for binding as the 7-azaindole derivatives have higher binding affinities for the A₁ ARs (**2b**: rA₁K_i: 0.16 ± 0.02 μM; [³H]NECA = 38% specific binding and, **2d**: rA₁K_i: 0.44 ± 0.04; rA_{2A}K_i: [³H]NECA = 57% specific binding); whereas the 7-deazapurine derivatives have increased dual A₁- and A_{2A} activity (**2g**: rA₁K_i: 0.19 ± 0.02 μM; rA_{2A}K_i: 0.43 ± 0.01 μM). The monosubstituted test compounds (**2b** and **2g**), with the phenylboronic acid substituents on the 4th position of both scaffolds have the best combined *in vitro* and *in silico* dual binding affinity. The evaluation of molecular modelling results showcased that test compound **2c** (rA₁K_i: 75% specific binding *in vitro*), with disubstituted chlorophenyl groups, has the best human A₁- and A_{2A} AR binding *in-silico* with binding affinities of -9.2 and -8.4 kcal, in that order. (Protein Databank Identifications (PDB IDs: 6D9H & 3PWH)). These findings prompt further evaluation and optimisation of the scaffolds and functionalisation thereof, as well as safety and efficacy testing in preclinical- and clinical settings. Thus, the pyrrolopyrimidines' potential and versatility for A₁ and/or A_{2A} AR binding can be of use for future investigations.

Key terms: pyrrolopyrimidines; 7-deazapurine derivatives; 7-azaindole derivatives; neurodegenerative disorders; Alzheimer's disease; Parkinson's disease; A₁ adenosine receptors; A_{2A} adenosine receptors; antagonists.

OPSOMMING

Die soeke na behandelingsopsies vir neurodegeneratiewe afwykings duur voort. Hierdie versteurings, soos Alzheimer se siekte (AD) en Parkinson se siekte (PD), het vermoedelik soortgelyke patogene bane wat mitochondriale disfunksie, oksidatiewe spanning en neuro-inflammasie insluit. Die kognitiewe agteruitgang kan geteiken word met A_1 -adenosienreseptor (AR) -antagonisme en die depressie en lokomotoriese degenerasie, wat gepaard gaan met die siekteprogressie en levodopa-geïnduseerde dyskinesie (LID), kan verbeter word met A_{2A} adenosienreseptor (AR) antagonistiese pogings. Antagonisme kan die surplusvlakke van adenosientrifosfaat (ATP), adenosien en die verhoogde uitdrukking van A_{2A} adenosienreseptore (ARs), wat algemeen in neuropatologie voorkom, normaliseer. Die pirrolopirimidiene en -piridienne van belang, 7-deazapurien- en 7-azaindool-steiers, kan geoptimaliseer word vir dubbele A_1 - en A_{2A} AR-binding via Suzuki-kruiskoppelende sintetiese metodes op die vierde en sesde posisies. Hierdie steiers is afgelei van die adenienstruktuur en verskeie xantienverbindinge deel hierdie strukturele kenmerke, maar het swak fisiese-chemiese eienskappe; behalwe kafeïen, 'n nie-selektiewe AR-antagonis, as gevolg van die metilering van die heteroatome. Evaluasie van die resultate van radioligandbinding studies dui aan dat twee heteroatome geskik is vir binding, aangesien die 7-azaindool-derivate hoër bindingsaffiniteite vir die A_1 AR's het (**2b**: rA_1K_i : $0,16 \pm 0,02 \mu\text{M}$; [^3H]NECA = 38% spesifieke binding en, **2d** : rA_1K_i : $0,44 \pm 0,04$; $rA_{2A}K_i$: [^3H]NECA = 57% spesifieke binding); terwyl die 7-deazapurien-derivate die dubbele A_1 - en A_{2A} -aktiwiteit verhoog het (**2g**: rA_1K_i : $0,19 \pm 0,02 \mu\text{M}$; $rA_{2A}K_i$: $0,43 \pm 0,01 \mu\text{M}$). Die monosubstitueerde toetsverbindinge (**2b** en **2g**), met die fenielboronsuursubstituente op die vierde posisie van beide steiers, het die beste saamgevoegde *in vitro* en *in-silico* dubbele bindingsaffiniteit. Die evaluering van molekulêre modelleringsresultate het aangedui dat toetsverbinding **2c** (rA_1K_i : 75% spesifieke binding *in vitro*), met dubbele substitusie met chlorofenielgroepe, die beste menslike A_1 - en A_{2A} AR-binding *in silico* uitbeeld met bindingsaffiniteite van -9.2 en -8.4 kcal, in daardie volgorde (proteïen databank identifikasie (PDB ID: 3PWH & 6D9H). Hierdie bevindings kan lei tot verdere evaluering en optimalisering van die steiers en die funksionalisering daarvan, sowel as die veiligheid- en doeltreffendheidstoetsing in prekliniese- en kliniese omgewings. Dus kan die pirrolopirimidiene se potensiaal en veelsydigheid vir A_1 en/of A_{2A} AR binding van nut wees vir toekomstige ondersoeke.

Sleuteltermes: pirrolopirimidiene; 7-deazapurien-derivate; 7-azaindole-derivate; neurodegeneratiewe afwykings; Alzheimer siekte; Parkinson se siekte; A_1 adenosienreseptore; A_{2A} adenosienreseptore; antagonist.

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LIST OF ABBREVIATIONS

7-azaindole	1,7-dideazapurine or 1H-pyrrolo[2,3-b]-pyridine or 1,7-diazaindene or 7-aza-1-pyridine or pyrrolo[2,3-b]pyridine
7-deazapurine	pyrrolo[2,3-d]pyrimidine
7-NI	7-nitroindazole
Å	angstrom
μM	micromolar
α-LA	alpha-lipoic acid
Aβ	amyloid beta
AβP	amyloid beta protein
AC	adenylyl cyclase
ACh	acetylcholine
AChEI	acetyl cholinesterase inhibitor
AD	Alzheimer's disease
ADs	antidepressants
ADA	adenosine deaminase
ADP	adenosine diphosphate
AIM	abnormal involuntary movements
AK	adenylate kinase
AKT	protein kinase B
ALS	amyotrophic lateral sclerosis

AMP	adenosine monophosphate
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APCI	atmospheric pressure chemical ionisation
APP	amyloid precursor protein
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
AR(s)	adenosine receptor(s)
BBB	blood-brain barrier
BChE	butyryl cholinesterase
BCKDH	branched chain α -ketoglutarate dehydrogenase
BD	bipolar disorder
BDNF	brain-derived neurotrophic factor
$^{\circ}\text{C}$	degrees Celsius
Ca^{2+}	calcium ion
^{13}C NMR	carbon nuclear magnetic resonance
cAMP	cyclic adenosine monophosphate
CD39	ectonucleoside triphosphate diphosphohydrolase-1
CD73	ecto-5'-nucleotidase

CGS15943	9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine
CGS21680	3-(4-(2-((6-amino-9-((2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9H-purin-2-yl)amino)ethyl)phenyl)propanoic acid
c-Met	tyrosine protein kinase
CNS	central nervous system
CNTs	concentrative nucleoside transporters
COMT	catechol-O-methyltransferase
COX	cyclooxygenase
CREB	c-AMP-responsive element binding protein
CRF	corticotropin-releasing factor
CRH	corticotropin-releasing hormone
CUS	chronic unpredictable stress
d	doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
DA D ₂	dopamine D ₂ receptor
DAWS	dopamine agonist withdrawal syndrome
DCM	dichloromethane
DDS	dopamine dysregulation syndrome
DLB	dementia with Lewy Bodies
DNA	deoxyribonucleic acid

DMSO	dimethyl sulfoxide
DMPX	3,7-dimethyl-1-propargylxanthine
DPCPX	dipropylcyclopentylxanthine or 8-cyclopentyl-1,3-dipropylxanthine
DRT	dopamine replacement therapy
dt	doublet of triplets
EAA	excitatory amino acids
ENTs	equilibrative nucleoside transporters
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
EtOAc	ethyl acetate
FSTs	forced swim tests
FTLD	frontotemporal lobar degeneration
GIT	gastrointestinal tract
Glu	glutamic acid
GPCRs	G-protein-coupled receptors
GPI	internal segment of the globus pallidus
GTP	guanosine triphosphate
hA ₁ AR	human A ₁ adenosine receptor
hA _{2A} AR	human A _{2A} adenosine receptor
H-bond	hydrogen bond
HD	Huntington's disease

[³ H]DPCPX	[³ H]-8-cyclopentyl-1,3-dipropylxanthine
His	histidine
[³ H]NECA	5'-N-[³ H]-ethylcarboxamideadenosine
¹ H NMR	hydrogen nuclear magnetic resonance
H ₂ O ₂	hydrogen peroxide
Hz	Hertz
IC ₅₀	half-maximal inhibitory concentration
IL	interleukin
IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthase
Ile	Isoleucine
<i>J</i>	coupling constant
JNK	c-Jun N-terminal kinase
KGDH	α-ketoglutarate dehydrogenase
<i>K_d</i>	equilibrium dissociation constant
<i>K_i</i>	inhibition constant
KW6002	istradefylline
Leu	Leucine
LID	Levodopa-induced dyskinesia
LTD	long-term depression
LTP	long-term potentiation
m	multiplet

M	Molar concentration
MAO	monoamine oxidase
MAOIs	monoamine oxidase inhibitors
Met	methionine
MDD	major depressive disorder
mmol	millimole
mp	melting point
MPP ⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	mass spectra
MS	multiple sclerosis
m/z	mass to charge ration
NADPH	nicotinamide adenine dinucleotide phosphate
NF-kB	nuclear factor kappa B
NFTs	neurofibrillary tangles
NIHL	noise-induced hearing loss
NKA	Na ⁺ /K ⁺ ATPase
NLRP3	NLR family pyrin domain containing 3
nM	nanomolar
NMDA	<i>N</i> -methyl- <i>D</i> -aspartate
NMR	nuclear magnetic resonance
NO	nitric oxide

NOS	nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
NTs	nucleoside transporters
O_2^-	superoxide anion
OBX	bilateral olfactory bulbectomy
$OH^\bullet$	hydroxyl radicals
OS	oxidative stress
P2X7R	P2X purinoceptor 7
P2YR	P2Y purinoceptor
PD	Parkinson's disease
PDB	protein data bank
PDH	pyruvate dehydrogenase
$Pd(PPh_3)_4$	tetrakis(triphenylphosphine)palladium(0)
PE	petroleum ether
PGE_2	prostaglandin E_2
Phe	phenylalanine
PKA	c-AMP-dependent kinase
PKG	protein kinase G
PLC	phospholipase C
ppm	parts per million
PUFAs	polyunsaturated fatty acids
rA_1 AR	rat A_1 adenosine receptor

rA _{2A} AR	rat A _{2A} adenosine receptor
RCS	reactive carbonyl species
REM	rapid eye movement
RLS	restless legs syndrome
RNA	reactive nitrogen species
ROS	reactive oxygen species
s	singlet
SAH	S-adenosyl-homocysteine
SANS	South African National Standard
SAR	structure activity relationship
SCH412348	7-[2-[4-2,4-difluorophenyl]-1-piperazinyl]ethyl]-2-(2-furanyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine
SCH58261	7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
Ser	serine
SI	selectivity index
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SNRIs	serotonin and norepinephrine reuptake inhibitors
SOD	superoxide dismutase
SPs	senile plaques
SSRIs	selective serotonin reuptake inhibitors

ST1535	2-butyl-9-methyl-8-(2H-1,2,3-triazol-2-yl)-9H-purin-6-ylamine
TBI	traumatic brain injury
TCAs	tricyclic antidepressants
td	triplet of doublets
TLC	thin-layer chromatography
TMs	transmembranes
TNF- α	tumor necrosis factor <i>alpha</i>
TRD	treatment-resistant depression
TRKs	receptor tyrosine kinases
Tris/HCl	hydroxymethyl hydrochloride
Trp	tryptophan
TSTs	tail suspension tests
Tyr	tyrosine
US FDA	United States Food and Drug Administration
V81444	ciforadenant
Val	valine
XO	xanthine oxidase
ZM241385	4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazine-5-ylamino)ethyl)phenol

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CHAPTER 1 INTRODUCTION

1.1 Background

The G-protein-coupled receptors (GPCRs) modulate an array of physiological processes and consist of several groups. The adenosine receptors (ARs) constitute part of the GPCRs; the ARs have rhodopsin-like characteristics and interact with G-proteins (Fredholm *et al.*, 1994:5). The ARs can be further extrapolated into four subtypes, each with unique properties and mechanisms: A₁, A_{2A}, A_{2B} and A₃ ARs (Fredholm *et al.*, 2001:447). Selective and/or dual binding to the A₁ and/or A_{2A} ARs, will be explored in this study.

Adenosine is an endogenous purine nucleoside widely found in nature that modulates many physiological processes (Fredholm *et al.*, 1994:1-2; Sawynok, 2006:301) by modulating the activity of ARs (A₁, A_{2A}, A_{2B} and A₃) (Haskó *et al.*, 2008:1-2). Adenosine is composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) via a β -N⁹-glycosidic bond (Fredholm *et al.*, 1994:19; Sawynok, 2006:301-320). Adenosine accumulation and AR activation in the brain leads to an array of pathological conditions and the figure below (**Figure.1–1**) showcases the different ARs that are involved in central nervous system (CNS) disorders and the regions of the brain that can be affected (Wei *et al.*, 2011:1371).

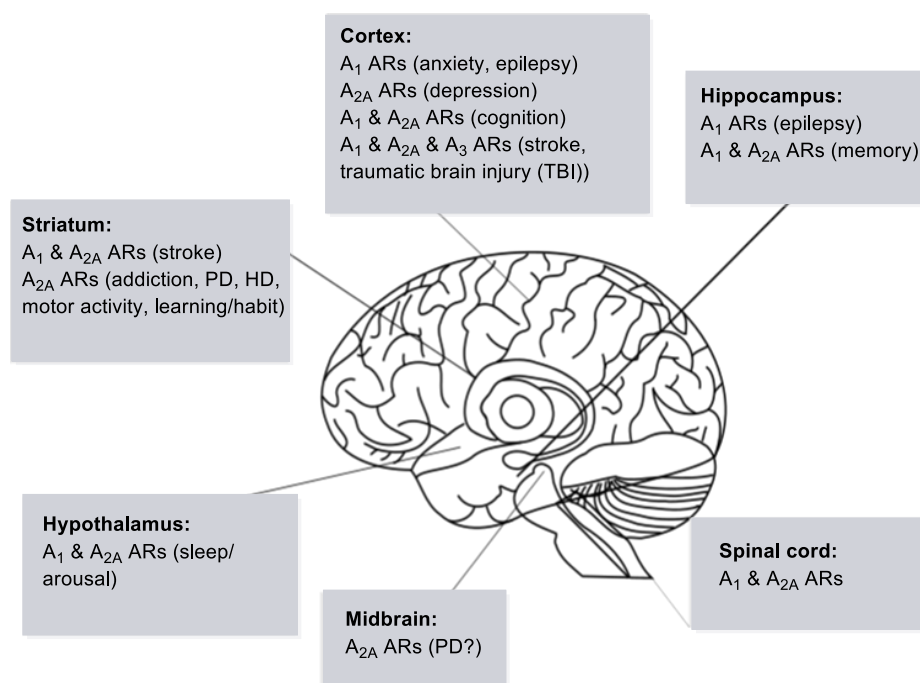


Figure 1–1: Adenosine's controversial involvement in pathophysiological conditions, adapted from Wei *et al.* (2011:1371).

The ARs play a role in anti-inflammatory processes, energy homeostasis and immunomodulation (Jespers *et al.*, 2017:1-2). The mitochondria has several complexes and activity of complex I has been reported to be reduced in the substantia nigra pars compacta (SNc) and frontal cortex of Parkinson's disease (PD) patients' autopsies (Schapira *et al.*, 1989:1269; Parker *et al.*, 2008:87-83). Mitochondrial dysfunction can lead to excitotoxicity and cause a reduction in cellular adenosine triphosphate (ATP) levels, an increase in cellular Ca^{2+} , or a combination of both (Sherer *et al.*, 2002: 194). ATP can be viewed as molecular currency and consumption without its replacement can lead to an energetic crisis and maintaining a homeostatic equilibrium is critical since a surplus amount of ATP is apparent during pathological conditions (Kepp *et al.*, 2017:83). There exists an overlap where adenosinergic/purinergic signalling coincides with mitochondrial dysfunction, oxidative stress and neuroinflammation (which will be explored more in depth in **Chapter 2**). These incidents manifest cell death and neurodegeneration. Neuroinflammation is a 'double-edged sword' and has a hand in cognitive decline and various neurological disorders (Lyman *et al.*, 2014:6).

Due to high levels of expression of the inhibitory A_1 AR in the hippocampus, cortex and cerebellum, A_1 AR antagonists were studied as a possible treatment option for CNS conditions, such as cognitive deficits associated with Alzheimer's disease (AD) and PD (Giovannoni *et al.*, 2010:7890-7894; Ribeiro *et al.*, 2002:387; Lopes *et al.*, 2011:3-10). The elevation of extracellular acetylcholine by A_1 AR inhibition at the cholinergic terminals is significant in cognitive processing (Van der Walt *et al.*, 2015:6641-6642; Glukhova *et al.*, 2017:867). Memory and learning processes are regulated by the plasticity and/or interactions of A_1 and A_{2A} ARs (Takahashi *et al.*, 2008:2614-2615). Furthermore, adenosine catabolism prevented cognitive impairment in scopolamine-treated zebrafish (Bortolotto *et al.*, 2015:118), which suggests that attenuation of adenosine activity might reduce cognitive function impairment.

A_{2A} ARs, on the other hand, are mainly distributed in the striatum, basal ganglia and the shell region of the nucleus accumbens of the brain (Cacciari *et al.*, 2018:1-4; Soliman *et al.*, 2018:662-663). Inspired by the fact that A_{2A} ARs were found to be connected in an inhibitory manner to the dopamine (DA) D_2 receptors, A_{2A} AR antagonists have been explored as possible treatment options for neurodegenerative disorders such as PD (Marcellino *et al.*, 2010:465) where increased dopaminergic activity is required as part of treatment. In fact, A_{2A} AR antagonists (Basu *et al.*, 2017:835) are currently being developed as a non-dopaminergic alternative for the treatment of PD (Chou, 2008:65-83), especially due to their potential in ameliorating motor disorders associated with PD (Armentero *et al.*, 2011:281). In addition, the A_{2A} ARs antagonists are documented to have antidepressant effects with chronic administration (Padilla *et al.*, 2018:7-9).

Over the years, various A₁ and/or A_{2A} AR antagonists have been synthesised and evaluated successfully. The A_{2A} AR antagonists can be divided into two groups, namely:

- **Xanthine derivatives:** Xanthine has previously been utilised as a scaffold for the design of A₁ and A_{2A} AR ligands (Van der Walt *et al.*, 2013:53; Van der Walt *et al.*, 2015:6645). Xanthine derivatives have extremely low water solubility, which may be problematic (Gütschow *et al.*, 2012:3331-3333). Caffeine is a non-selective AR antagonist. A_{2A} AR antagonists and caffeine show neuroprotective properties and inversely correlate with memory deterioration and depression (Kaster *et al.*, 2015:7833).
- **Nonxanthine derivatives:** Most non-xanthine A_{2A} AR antagonist compounds are flat, aromatic structures (Ongini & Fredholm, 1996:368). They are usually tricyclic heteroaromatic structures that contain nitrogen with an amino group (exocyclic). The amino group plays a significant role in binding affinity (Poucher *et al.*, 1995:1096-1101).

Istradefylline (A_{2A} K_i = 12 nM) and ZM-241385 (A_{2A} K_i = 0.8 nM) has been developed as potent and selective A_{2A} AR antagonists (**Figure 1-2**) (De Lera Ruiz *et al.*, 2013:3626-3627; Poucher *et al.*, 1995:1101). Due to its anti-depressive effects (Yamada *et al.*, 2013:5-6) and motor effects, istradefylline is indicated for PD (and has recently been approved by the United States Food and Drug Administration (US FDA) as add-on treatment for PD), although its application is limited by its physicochemical properties associated with xanthine derivatives (Chang *et al.*, 2004:1593; Davies *et al.*, 1984:2117-2128). Most xanthine derivatives bring forth problems associated with bioavailability, inadequate selectivity over the other ARs and poor water solubility, except for caffeine due to methylation of the nitrogen atoms (Chang *et al.*, 2004:2593; Davies *et al.*, 1984:2117-2128).

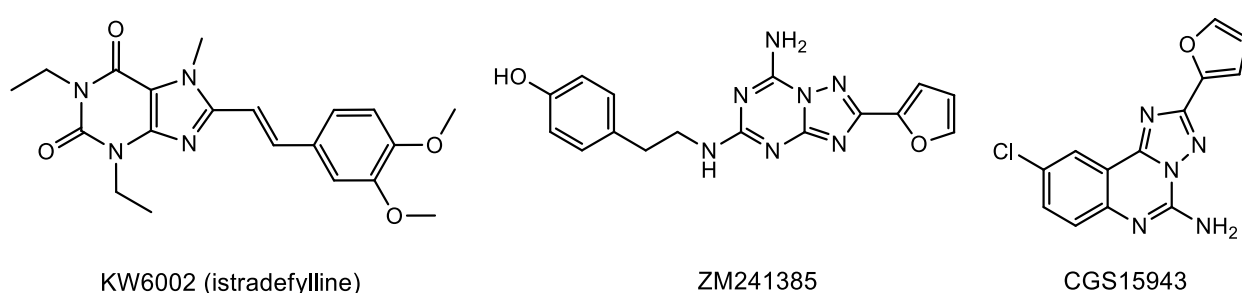


Figure 1–2: Selective A₁ and A_{2A} AR antagonists KW6002, ZM241385 and CGS15943.

Previously explored ligands (Ongini & Fredholm, 1996:369), such as ZM-241385 (A_{2A} K_i = 0.8 nM) (Poucher *et al.*, 1995:1096-1098; De Lera Ruiz *et al.*, 2013:3626) and CGS-15943 (A₁ K_i = 1.9 nM – 6.5 nM) (**Figure 1–2**) (Ongini *et al.*, 1999:9), with favourable results and binding affinities for the A₁ or A_{2A} ARs served as a foundation to build upon and improve novel scaffolds. The [1,2,4]triazolo[1,5-a][1,3,5]triazine backbone of ZM-241385 is planar and bicyclic and inspired by

structural similarity, it is envisaged that appropriately substituted 7-deazapurine analogues would exhibit good A_{2A} affinity, especially because modelling studies suggested that not all of the nitrogen atoms are involved in the interaction with the A_{2A} binding pocket (Deb *et al.*, 2018:967; Yuan & Jones., 2014:2).

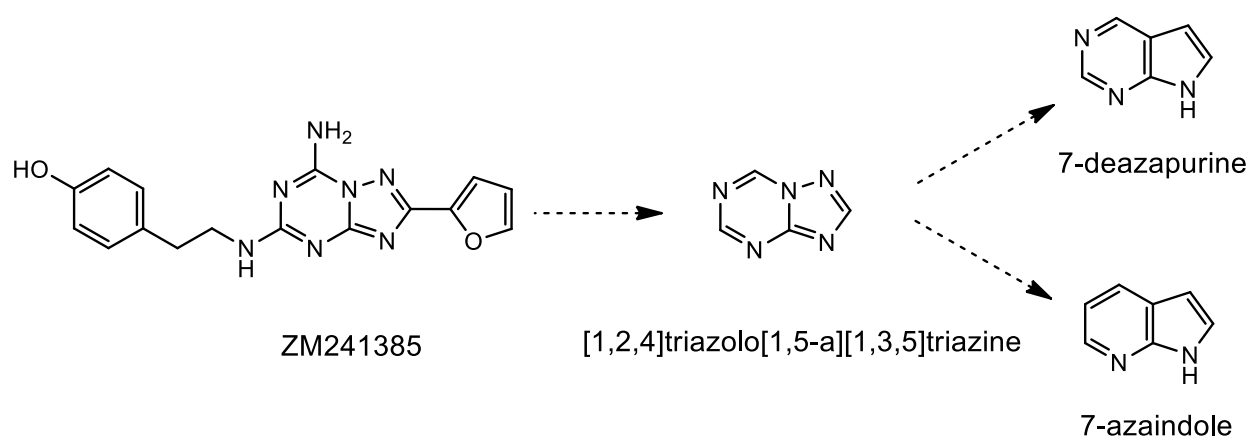


Figure 1–3: ZM-241385's [1,2,4]triazolo[1,5-a][1,3,5]triazine group simplified to the 7-deazapurine and 7-azaindole scaffolds.

Suitable substitution and addition with certain groups and heterocyclic rings, may be apt in the creation of compounds that have the necessary interactions in the binding pocket of the ARs, by having either allosteric modulatory (Kimatrai-Salvador *et al.*, 2013:292) or competitive inhibitory effects. The interactions observed between ZM-241385 and its binding site provided valuable insight into the essential interactions for optimisation of A_{2A} AR affinity (Yuan & Jones, 2014:2). The binding site of A_1 ARs is open and wide in comparison to the A_{2A} AR binding pocket, which is narrow and elongated (Glukhova *et al.*, 2017:871). Since the A_1 AR cavity is bigger, and thus more lenient and able to accommodate a larger variety of molecules, the structural focus will be on satisfying and meeting the A_{2A} AR binding site's requirements.

The following structures of biological compounds strengthened the rationale for using 7-deazapurine as a scaffold for design of A_1/A_{2A} AR analogues. Truncation of the adenosine structure to adenine, similar in structure to 7-deazapurine, makes it clear why 7-azaindole may serve as a building block for novel A_1/A_{2A} receptor ligands.

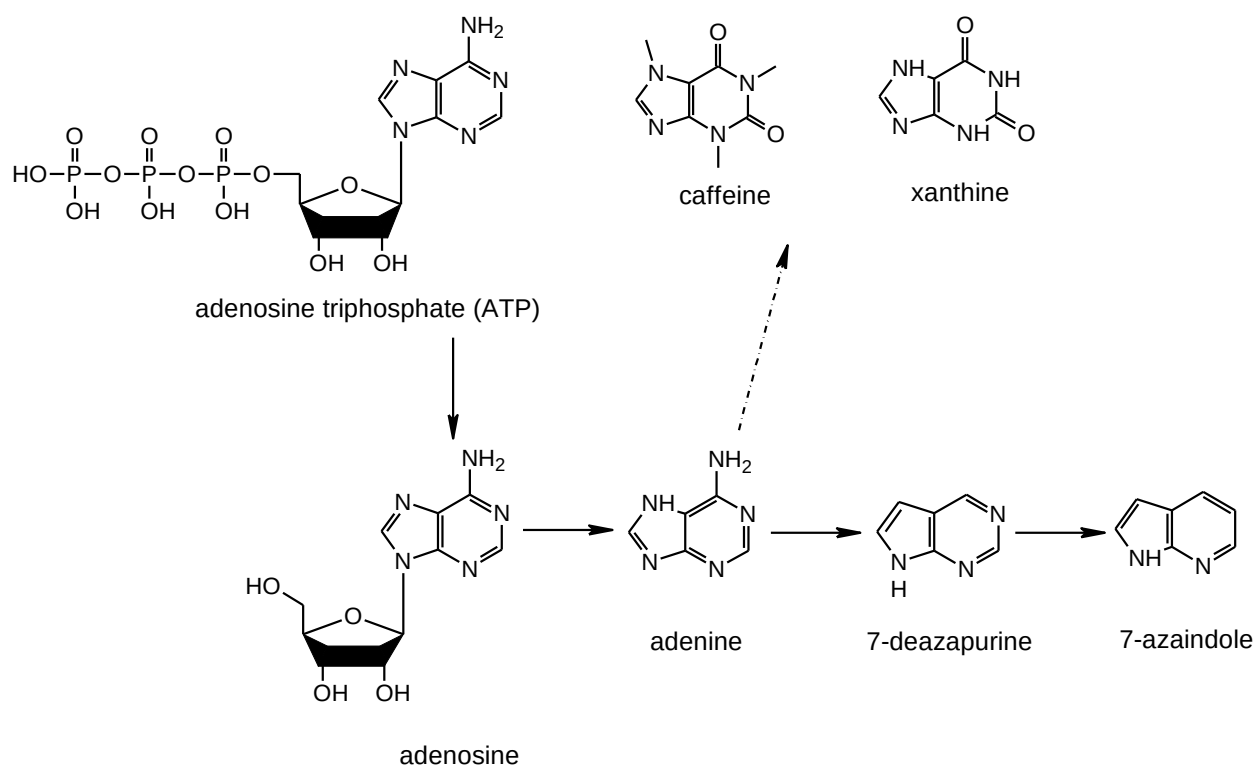


Figure 1-4: Truncation of ATP to 7-deazapurine and 7-azaindole

This study will focus on design, synthesis and evaluation of non-xanthine derivatives using the 7-azaindole and 7-deazapurine scaffolds to achieve better drug-like and physicochemical properties; less phenolic groups may equal less interactions with enzymes and thus improves bioavailability. Substituents will be stationed at the 4th (R₁) and 6th (R₂) positions of the scaffolds and synthesised using the Suzuki cross-coupling methodology as first described by Suzuki (1985:1749-1757).



Figure 1-5: Proposed 7-azaindole and 7-deazapurine scaffold for A₁ and/or A_{2A} binding

Furthermore, the investigation of potential ligands with dual targeting action for both A₁ and A_{2A} ARs must be explored. By integrating the design rationales for A₁ and A_{2A} AR antagonism, compounds with dual activity can be obtained. R₂ substitution can provide a planer surface for A_{2A} AR binding to allow compounds to fit into the narrow binding site of the A_{2A} AR by emulating

the biological structure of adenosine or other antagonists with planer tricyclic structures (Cacciari *et al.*, 2018:2). The addition of different cyclic structures at R₁ could be useful for A₁ AR binding.

The design of 7-azaindole derivatives that can fit into both A₁ and A_{2A} AR binding cavities with good affinity, would make this scaffold worthy of further attention. This study will focus on the design of 7-azaindole and 7-deazapurine based A₁ and A_{2A} AR antagonists for the betterment of cognitive decline (for A₁ antagonists) and locomotor deficits (for A_{2A} antagonists) related to PD. These ligands may have potential to treat neurodegenerative disorders and the comorbid conditions that sprout from these disorders, or solely relieve the motor symptoms and/or non-motor symptoms, such as cognitive deficits and depression.

This chapter provides the rationale and background to solve the research problem and lists the aims and objectives. Chapter 2 gives an overview of the role ARs play in neurological conditions. Chapter 3 contains an original article describing the synthesis and evaluation of 7-azaindole and 7-deazapurine derivatives. In Chapter 4 a conclusion is drawn of the work done.

1.2 Problem statement and hypothesis

The use of AR ligands for treatment of motor deficits and cognitive decline is not fully explored and extensive research, such as medicinal chemistry research – *in silico*, *in vitro* and *in vivo* evaluation - is required for validation. Motor dysfunction instigated by Levodopa-induced dyskinesia (LID) can be abetted with A₁ and A_{2A} AR antagonistic efforts. A_{2A} AR antagonism can be useful for treatment of PD without using traditional methods of dopamine replacement therapy (DRT) or monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) inhibition (Saki *et al.*, 2013:967). Depression and cognitive decline are associated with neurodegenerative disorders, *e.g.* PD. Patients diagnosed with AD or PD are usually older and more prone to experience adverse reactions and may already take medication that is contraindicated against the use of multitudes of drugs. Most existing pharmacotherapies indicated for AD, PD, cognitive impairment and depression have notorious side-effects that corrupt patient compliance. Only a select few have proven to be safe and effective. Creating selective compounds with favourable properties to cross the blood-brain barrier (BBB) will be cumbersome due to ubiquity of ARs.

Inspired by chemical similarity of adenine and its structural analogues, we hypothesise that appropriately substituted analogues of 7-deazapurine, that comply with the demands of the A₁ and A_{2A} AR's binding cavities, could potentially have AR affinity. In this current study, 7-deazapurine and 7-azaindole analogues have been designed, synthesised and evaluated via *in vitro* and *in silico* methodology as A₁ and/or A_{2A} AR ligands with probable pharmacotherapeutic use for CNS conditions. A₁ AR ligands are lucrative for cognitive dysfunction amelioration and A_{2A} receptor antagonists have promising antidepressant activity. Besides, A_{2A} AR ligands may

be useful for Parkinsonian motor symptom alleviation and provide neuroprotection against neurodegenerative disorders' pathogenesis.

1.3 Aims and objectives

This study aims to investigate 7-deazapurine, also known as pyrrolo[2,3-d]pyrimidine, and 7-azaindole, 1H-pyrrolo[2,3-b]-pyridine or 1,7-dideazapurine, scaffolds for the design of A₁ and A_{2A} AR antagonists for targeted treatment of neurodegenerative disorders and the respective symptoms.

Objectives:

- The A₁ and A_{2A} AR ligands will be designed and synthesised for the prospective treatment of cognitive impairment and motor dysfunction, respectively. These ligands could possibly lessen the burden of comorbid non-motor disorders, like depression and cognitive decline, or locomotor disorders that are apparent in patients diagnosed with neurodegenerative disorders, such as PD and AD. The Suzuki coupling method will bring these compounds into realisation.
- Molecular modelling studies can serve as a guide to determine whether the scaffolds and the derivatives have potential.
- Characterisation (via NMR, MS and melting points) and biological evaluation of the synthesised compounds as ligands for A₁ and A_{2A} ARs.
- Radioligand binding studies on male Sprague Dawley rat brains will be executed with [³H]DPCPX, also known as [³H]-8-cyclopentyl-1,3-dipropylxanthine, and [³H]NECA, 5'-N-[³H]-ethylcarboxamideadenosine, for A₁ and A_{2A} AR binding, respectively. Biological assays will either validate or nullify the results from the *in silico* evaluation and comparison with existing compounds can give an idea whether the synthesised compounds have potential as A₁ and/or A_{2A} antagonists.
- Guanosine triphosphate (GTP) shift assays to determine antagonism of ligands with affinity to the A₁ AR subtype.
- Identification of structural features that are indispensable for A₁ and A_{2A} AR affinity, and use them to design compounds with potential dual binding affinity for both the A₁ and A_{2A} ARs.

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CHAPTER 2 LITERATURE STUDY

2.1 Background to study

As life expectancy increases with the availability of modern technological- and biomedical advances and pharmaceutical solutions for age-old health problems and diseases, an exponential number of people are more susceptible to age-related neurodegeneration. Small changes in a person's homeostatic disposition can wreak havoc on a larger scale and prompt a pathological cascade – this can translate to the central nervous system (CNS) ([Frank-Cannon *et al.*, 2009:2](#)).

The brain is fastidious about the entrance of molecules into the CNS's domain. The blood-brain barrier (BBB) monitors the entry to the brain and denies access to malevolent and/or unfamiliar compounds. The problem is that harmful molecules can masquerade as intrinsic bodies, like inflammatory mediators, and cross the BBB, resulting in neuroinflammation. Numerous toxins and substances with favourable physicochemical properties can infiltrate the barrier, lead to neuronal cell death and intervene with physiological- and biochemical pathways. On the contrary, it can be a struggle to design compounds with favourable properties to cross the BBB, while having good affinity for target receptors without undergoing metabolic breakdown ([Beamer *et al.*, 2016:95](#)).

Acute- and chronic neuroinflammation, in most cases, cause neurodegenerative disorders, namely Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). Many of these diseases can be traced back to similar causes as the aetiology of these diseases is interconnected ([Ransohoff *et al.*, 2016:777](#)). The interrelations are complex and, sometimes, contradictory in nature – this is blatant in the amalgamation of literary sources that envelops neurodegenerative research.

Depression and cognitive decline are non-motor symptoms associated with neurodegenerative disorders and have crippling effects on a person's quality of life. Neurochemical changes in the brain can cause treatment resistance over time and the limited treatment options are not suitable for everyone. Most cases go undiagnosed and untreated since it is not always visible. Unlike the non-motor symptoms, the motor complications are noticeable in everyday life and pharmacotherapies can have disruptive side-effects; for instance, Levodopa causes dyskinesias ([Franco-Iborra *et al.*, 2016:269](#)). For future reference, a compilation of the hypotheses of the pathogeneses of the CNS conditions (particularly AD and PD), shortcomings of current treatments, clinical trial failures and/or victories, as well as a framework of evidential conclusions from past studies can provide insight on the target sites and compounds with therapeutic potential for neurodegeneration.

On surface level, oxidative stress, neuroinflammation and mitochondrial dysfunction can start off as minor changes that develop into full-blown deleterious CNS conditions. Over the years, researchers have devoted their lives to embellish on existing principles and discoveries of neurology to find cures for these diseases. Substantial progress has been made but the search for valid treatment options continues ([Borea et al., 2017:1947](#)).

The G-protein-coupled receptors (GPCRs) are a superfamily of transmembrane proteins that transduces a diverse array of signals. It had been difficult to obtain information about these receptors until recent years given that crystallisation of specific GPCRs brought about methodological hurdles ([Audet & Bouvier, 2012:14](#)). With the latest production, purification and engineering conquests, the structures and agonistic/antagonistic ligand binding modes are more accessible and molecular docking studies can give an indication of these binding modes ([Cooke et al., 2015:1363](#)).

The GPCRs of interest are the adenosine receptors (ARs) – these receptors are fascinating and of importance when discussing the mitochondrial dysfunction hypotheses and purinergic signalling. ATP, its metabolites, like adenosine, and other ligands modelled after the metabolites can bind in the AR cavities and elicit desirable and/or undesirable effects. Residual amounts of ATP or adenosine in tissues can have pathological consequences and the electron transport chain has multiple steps that can be targeted to correct defective signalling processes. There are four known AR subtypes - A₁, A_{2A}, A_{2B} and A₃. The A₁- and A_{2A} ARs have conspicuous effects on neurodegenerative disorders, the most notable being AD and PD – dual antagonism can be worthwhile for neuropathology; increased A_{2A} AR expression is imparted when pathology is present. In addition, the cognitive impairment, depression and motor dysfunction that accompany these disorders, can be targeted with A₁ and A_{2A} antagonistic efforts. It is much easier to prevent these disorders than to cure them in totality. Most AR ligands does not move past the stages of clinical testing. The main reason being the ubiquity of receptors and not being able to foresee all the ligands' interactions ([Jacobson & Gao, 2006:2](#)).

The ligands modelled after the adenosine metabolites include xanthine-, non-xanthine-, nucleotide-, non-nucleotide-, nucleoside- and non-nucleoside derivatives. A library of molecules has been tested for AR binding and these ligands have potential anti-inflammatory and anti-oxidative effects that is valuable for tackling neurodegeneration ([Yuzlenko et al., 2006:3611-3622](#)). Zeroing in on structure-based drug design of ligands with the 7-azaindole and 7-deazapurine scaffolds and comparing them with existing ligands, will indicate whether these scaffolds deserve further exploration to slow down or stop the progression of CNS conditions and the debilitating effects.

2.2 Adenosine triphosphate (ATP) and inflammation

Adenosine triphosphate (ATP) is often labelled as an energy source for living organisms; just as the mitochondria is known as the “powerhouse of the cell”. This oversimplification of the bioenergetic function undervalues the intricacies of ATP metabolism and -signalling. The mitochondria’s morphology is a portrayal of cell health. Mitochondrial dysfunction arises as the aftermath of stress or other destructive stimuli. The endoplasmic-reticulum (ER) and the mitochondria have synergistic responses to stress and reciprocally transmit danger signals. Ca^{2+} is transferred to the mitochondria and this results in apoptosis or other adaptive mechanisms, such as inflammation and autophagia. Inflammasomes, NLRP3 (NLR family pyrin domain containing 3), for example, detect irregularities on the ER’s surface, and the mitochondria and trigger inflammatory responses (Marchi *et al.*, 2014:464, 467). The implication is that elevated ATP and adenosine levels might signal cellular distress.

In 1984, Newby, AC. (1984:43) described adenosine as a “retaliatory metabolite” since it is unlike any other hormones or transmitters. It regulates a bewildering amount of cellular functions and has the capability to reduce its own sensitivity to external stimuli or the firing rate of its release. Adenosine is approved as a first-line agent in the treatment of narrow-QRS complex supraventricular tachycardia (Delacrétaz, 2006:1045). Adenosine is released as a product of metabolic processes and will not only have a purpose in the peripheral areas of the body but can also exert effects in the CNS, such as the glia and neurons. Besides, when the sugar moiety is omitted, the adenine structure remains which has previously been used as a scaffold for drug design; it is similar to hypoxanthine’s structure with an amine group instead of the hydroxyl group (refer to **Figure 2-1**).

ATP is a damage signal and Verkhratsky & Burnstock (2014:704) explored the evolution and diversification of purinergic signalling in a collection of organisms. This led to the discovery of manifold release pathways, enzymes and receptors. ATP’s conversion into adenosine and other nucleotides, signals to all kind of neuroglia and is responsible for the maintenance of cognition, several systemic functions and synaptic connectivity as a homeostatic response. ATP can be generated by autocrine pathways via self-induction which regenerates ATP release (Zimmermann, 2016:28). The astrocytes are the CNS’s main source of ATP and is integral for “neuron-glia networks” (Butt, 2011:211). ATP release can occur via several ways (Burnstock & Verkhratsky, 2009:416): exocytosis, plasmalemmal channels, transporters and cell damage. The exact mechanism of how these signals spread is merely speculative, but evidence persists that a central source transmits a wave in a multidirectional and circular formation which reaches the glial receptors (Butt., 2011:210).

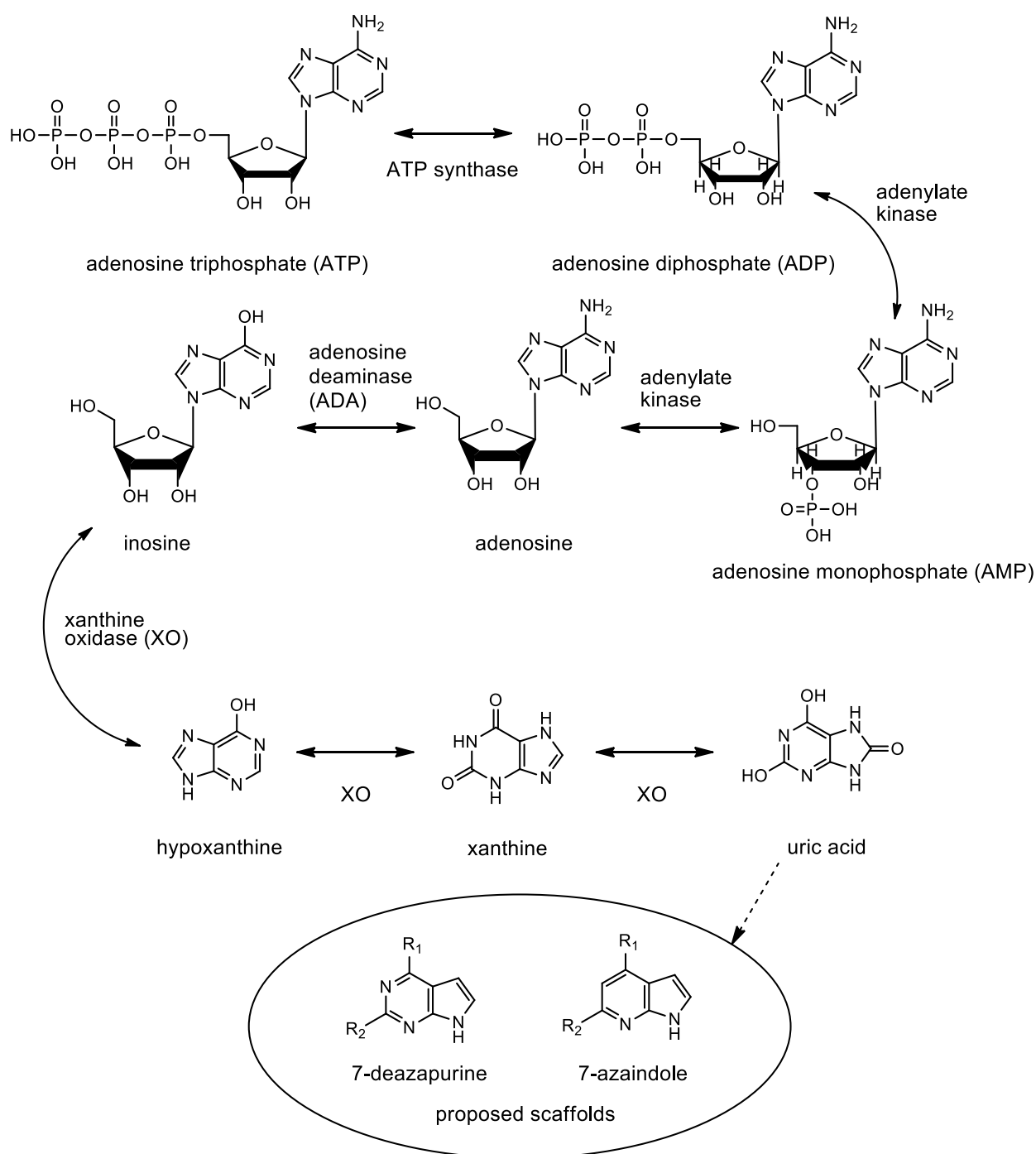


Figure 2-1: The thought process behind the selection of scaffolds, inspired by adenosine metabolism from [Bartoli et al. \(2020:5\)](#); chemical structures extracted from [ChemACX.Com](#)

Oxidative stress and inflammation are eminent in neurodegeneration. Cruz et al. (2007:2873) examined the macrophagic oxidative stress (OS) / inflammasome response that is dependent on “reactive oxygen species” (ROS) species provoked by extracellular ATP and previously, Mongin et al. (2002:577) showed that ATP induced swelling in astrocytes by stimulating “excitatory amino

acids" (EAAs) which increases the cell volume. Likewise, Rivera *et al.* (2016:8) summarised the pathological effects of ATP on glial networks as modulation of microglial activation, astrocyte reactivity and myelin-/oligodendrocyte loss which ultimately encourages neuroinflammation and neurodegeneration to emerge.

The dominant producers of free radicals originate from the first and third mitochondrial complexes. These free radicals attack proteins, deoxyribonucleic acid (DNA), carbohydrates and lipids which generate reactive carbonyl species (RCS). The composition of brain lipids is essential for functional and survival mechanisms. The lipid peroxidative cell damage and age-related deterioration of the brain occur when products of the RCS spread and damage components of other cells. ATP synthase has been identified as part of the electron transport chain and are targeted to oppose neurodegeneration. It is positioned within the inner membrane, which is made up of a lipid bilayer enriched with "polyunsaturated fatty acids" (PUFAs), facing the matrix of the mitochondria and this makes it a vulnerable target for free radicals (Jové *et al.*, 2019:11).

Rodrigues *et al.* (2015:4) provided an integrated approach to understand the pleiotropic effects of ATP with direct purinergic pathway activation, via purinoceptors, P2X7R's, or indirect pathways, via P2Y1R's and A_{2A} AR's, where it is broken down into ADP and thereafter, adenosine. The roles of signalling molecules that resemble the pyrimidine and purine structures have been researched extensively, although there remain many unanswered questions about the exact mechanisms by which it exerts its effects due to the ubiquity of the molecules. Two types of ATP receptors, sensitive to fluctuating ATP levels, are eminent: 1) metabotropic GCPR's, P2YR's, and 2) "ligand-gated ionotropic receptors", P2XR's (Florenzano *et al.*, 2008:41). The indirect pathways have been established to be of value in neuropathology as a result of its capabilities to increase the neurons' susceptibility to be damaged and integrate or promote the loss and/or dysfunction of synapses. The other effects are the reactive astrogliosis and neuroinflammation as formerly mentioned (Rodrigues *et al.*, 2015:4).

Furthermore, pro-inflammatory injuries can have pathological consequences and excessive ATP signalling in the extracellular environment can affect platelet aggregation and the transmigration of neutrophils across the endothelial. Certain stimuli, such as hypoxia, stress or depolarization, release ATP from endothelial cells and neutrophils' leading edge. This activates P2Y2 receptors and is dependent on neutrophils. A₃ ARs are also neutrophil-dependent and the resultant adenosine signalling promotes the transmigration in the event of inflammation. The endothelial barrier function is compromised during pathological inflammation and the P2Y6 receptors of the endothelial are incriminated. On the other hand, vascular function is encouraged during A_{2B}/A_{2A} AR activation which stifles inflammation. Platelet aggregation is elicited by the release of ADP

which ultimately propagates inflammatory injury by IL (interleukin 1) and the platelet adhesion to leukocytes (Dou *et al.*, 2018:70).

To better understand the undesirable effects elicited by surplus extracellular ATP and the influence on oxidative stress and neuropathology, further exploration is needed. Finding a sweet spot for therapeutic efficacy without interfering with normal inflammatory or oxidation responses is of great importance. Pathological states that originate from oxidative stress can be combated by reducing ATP or its metabolites, such as adenosine, by means of an antagonistic and/or antioxidative approach.

2.2.1 ATP and oxidative stress (OS)

In homeostatic conditions, mitochondria are involved in apoptosis, the breakdown of nutrients and cellular energy provision. Deviation from these physiological roles and changes in the morphology of mitochondria can create an environment where pathological conditions thrive. Changes can include protein denaturation, dysfunctional complex formation, a reduction in the number of mitochondria and inefficient coupling. This impairs normal functioning of cells and lead to metabolic disorders and neurodegeneration (Selvaraji *et al.*, 2019:4).

There are different therapeutic approaches to restore, prevent and improve mitochondrial dysfunction through negative conditioning (Selvaraji *et al.*, 2019:9-10): pharmacological- and biochemical interventions, phytochemicals and dietary restriction.

There is a clear link between oxidative stress and metabolic dysfunction. Free radicals have a deleterious effect on brain function and can result in neurodegeneration and cell death. It is still uncertain if oxidative stress causes neuropathology or is a result thereof. There are two types of free radicals: reactive oxidative species (ROS) and reactive nitrogen species (RNS). ROS include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radicals ($OH^\bullet$) which are highly reactive and RNS include nitric oxide (NO) (Singh *et al.*, 2019:2). Both ATP and NO are involved in inflammation, modulate neuronal functions, and are co-expressed after ischaemia and other damaging events in the CNS, like axotomy (Florenzano *et al.*, 2008:46).

Membrane lipids of neurons consist of PUFAs. The side chains of the PUFAs are highly susceptible to ROS and RNS. Oxidation of the neurons can lead to premature aging, bioenergetic dysfunction and damage to mitochondrial DNA. The brain has many oxidizable substrates. The therapeutic application of antioxidants in neuroinflammation has been a topic of interest where many polyphenol compounds have been tested in previous studies (Singh *et al.*, 2019:3-5).

Examples of polyphenol groups used as antioxidants include ([Losada-Barreir & Brav-Diaz, 2017: 388](#)): 1) phenolic acids, such as gallic acid, caffeic acid, tyrosol and hydroxytyrosol, 2) stilbenes, like resveratrol, 3) lignans, 4) anthocyanins, 5) chalcones and 6) flavonoids which can be extrapolated into flavonols and flavones. Dimethyl fumarate, idebenone and edaravone are a few examples of antioxidant drugs that have been approved for the use in neurological disorders. There are many more that are at the preclinical and clinical stages of development. There are several natural products and dietary supplements that have great antioxidative properties. Most of these products have limitations due to their large size; this hinders permeability over membranes. In blood circulation, these compounds usually have a short half-life and antigenic properties. A way to overcome this pitfall, is to reduce the molecular weights and synthesise “similar” compounds with better “drug-like” properties ([Patel, M. 2016:1-4](#)).

Patel ([2016:9](#)) asks some interesting questions regarding the targeting of oxidative stress for neuropathology treatment:

- Is oxidative stress a valid drug target?
- How and when should ROS be targeted for optimal therapeutic accuracy?
- How does the brain protect itself from oxidative damage?
- Do antioxidants affect the homeostasis of physiological processes?
- How can biomarkers be used in clinical settings to measure the drugs’ efficacy and safety?

To better understand the undesirable effects elicited by surplus extracellular ATP and the influence on oxidative stress and neuropathology, further exploration is needed. Achieving therapeutic efficacy without interfering with normal inflammatory or oxidation responses is of great importance. Pathological states that originate from oxidative stress can be combated by reducing ATP or its metabolites, such as adenosine, by means of an antagonistic and/or antioxidative approach.

2.3 Central nervous system (CNS) conditions and neuroinflammation

Inflammation is an innate response, local or systemic, that is in place to defend and protect the body from aggravators. The endothelial cells and leucocytes orchestrate responses and intracellular monocytes differentiate into macrophages which engulf threatening molecules and recruit, as well as mobilise the peripheral effector cells ([Lyman et al., 2014:2](#)).

During this past decade, revolutionary strides have been made involving neuroinflammation’s involvement in CNS conditions. Damaged neurons release immunogenic factors which prompts adaptive and innate immune responses. These inflammatory processes spiral out of control; this

is where neuropathological conditions start (Hirsch *et al.*, 2012:211). Peripheral immunity also affects the CNS. Even the brain's resident cells, microglia and astrocytes, are affected by the state of the brain parenchyma. Undoubtedly, neuroinflammation and mitochondrial dysfunction are the enemies in many of these conditions and the search for therapeutic solutions is ongoing. Minute changes in the homeostatic environment can cause destructive and even irreversible damage (Agostinho *et al.*, 2020:7).

Even though the BBB is selective, resilient and specialised in dealing with intruding molecules, a compromised BBB can be detrimental to neurological health and designing compounds that can cross the barrier at the correct dosage without being metabolised by all the peripheral and central processes is challenging. Besides, pro-inflammatory molecules can freely cross the BBB. Not even to mention the unforeseen adverse reactions, selectivity problems and competitive stimulation or inhibition of the receptors (Beamer *et al.*, 2016:95).

The microglia and astrocytes are targets for injuries and the lines are blurred between if mitochondrial dysfunction or neuroinflammation plays the bigger part in these injuries. The microglial modulation of plaque deposition is bi-directional and mediates neuroinflammation (Nichols *et al.*, 2019:571). Negative modulation includes phagocytosis or tight barrier formation around the plaques. Positive modulation involves seeding by the factors of microglial cells or cytokine secretion (Nichols *et al.*, 2019:571). **Figure 2-2** below illustrates how this phenomenon takes place and how the microglia influences A β aggregation.

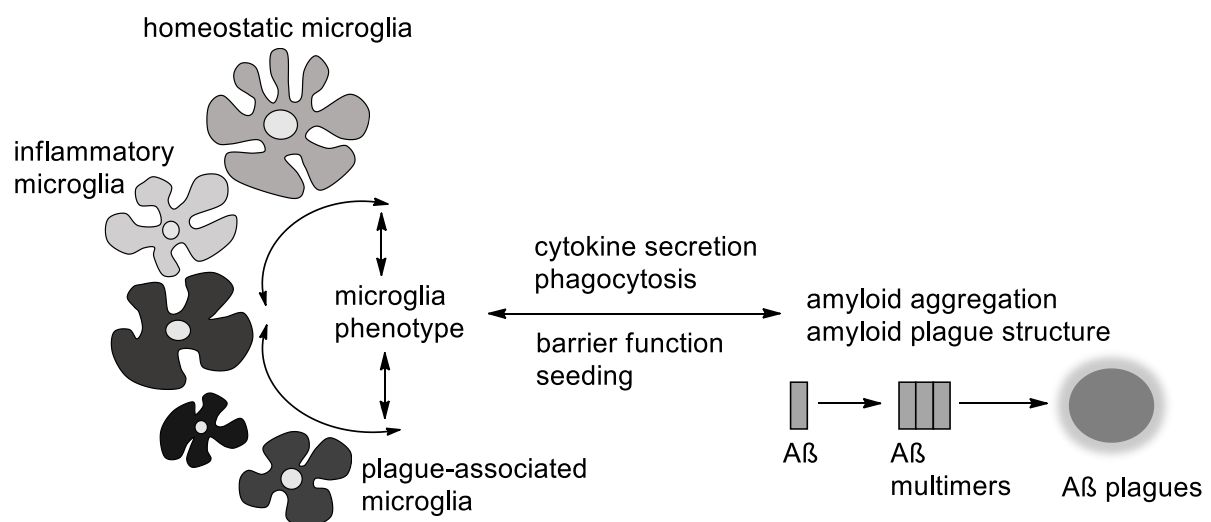


Figure 2-2: Neuroinflammation mediated by microglia - A β accumulation, -aggregation and plaque deposition cause changes in the microglia phenotype, adapted from Nichols *et al.* (2019:571)

Microglial activation secretes pro-inflammatory mediators - NF- κ B, IL-1 β , iNOS, NO, PGE₂, COX and ROS (Park *et al.*, 2011:1019). The microglia have a slow “turnover rate” and small changes in the phenotypes can have lasting effects. Improved comprehension of the ramifications of the altered maturation of resident and neighbouring CNS cells due to developmental neurotoxicology, should be obtained and the focus should be on how to prevent the cells from being compromised or how to restore the aftermath of neuronal damage, which is much more challenging as one can imagine (Harry & Kraft, 2012:202-203).

The drugs that act on the CNS are diverse and Holper *et al.* (2018:14) did a multivariant meta-analysis about the effects of psychiatric drugs and drugs that target neurodegenerative disorders and their effects on mitochondrial complexes (I & IV). Antidepressants demonstrate the greatest heterogeneity with a mixture of positive and negative effects on the mitochondrial complexes, whilst stimulants and antipsychotics generally increase complex IV and decrease complex I. In juxtaposition, the antiparkinsonian-, antidementia-, anxiolytic- and mood stabilising drugs exhibit positive effects on both complexes. As presented by Reddy & Beal (2005:628), mitochondrial defects lower neuronal ATP levels at nerve terminals that thwarts synaptic function and leads to the loss of synapses; this is a gateway for cognitive impairment in sporadic AD. Confirmation of the involvement of the mitochondria and oxidative stress as underlying mechanisms in AD is established by a recent article of Wang (2020:6-7). Investigation of how the crosstalk between organelles are influenced during the PD's pathogenesis emphasizes dysfunction of the lysosomes and endoplasmic reticulum (Sironi *et al.*, 2020:2).

Obstacles of targeting neuroinflammation and designing appropriate neuroprotective drug therapies (Frank-cannon *et al.*, 2009:9) are the following: design and synthesis of a molecule that can penetrate the BBB and reach the target site; identification of external and internal inflammatory triggers and pinpointing how “acute immune response” turns into chronic inflammation; constructing a framework of exclusion- and inclusion criteria for clinical trial candidates with thorough assessment of immune status; identification of precarious inflammatory mediators and finally, selective targeting of mediators without compromising immune function. Most of the existing drugs are ineffective, compromise immune function or have too many side-effects which impedes on patient compliance. The exploration of current therapies, new pharmacological targets and therapeutic failures might steer research in a new direction.

2.3.1 Depression

Major depressive disorder (MDD) is a serious debilitating mental illness. Relapse is not uncommon in the treatment of depression and many people do not respond to therapy or medication, also known as treatment-resistant depression (TRD) (Shelton *et al.*, 2010:149).

MDD can be set apart by impaired cognitive function, diminished interests, a depressed mood, weight fluctuations, disturbed appetite and/or sleeping patterns, also called “vegetative symptoms”. Depression can be hereditary and environmental factors, for example, emotional-, physical-, and sexual maltreatment and trauma, social determinants, such as socioeconomic status, low social support and childhood adversities, and the impact of stress or negative life events can cause depression (Li *et al.*, 2016:726-727). Depression may also present as part of a larger, more complex disorder. For example, it is a common non-motor feature of Parkinson’s disease (PD) (Lemke, M.R., 2008:9-11) that not only contributes to the long-term morbidity of the illness, but also reduces patients’ quality of life and life expectancy (Shulman *et al.*, 2002:196).

Since no known biological markers exist for depression, a controversy exists about it being a physical illness (Tobe & Rybakowski, 2013:3). Mitochondrial dysfunction and oxidative stress are thought to play an important role in abnormal brain function and mood disorders, including depression (Allen *et al.*, 2018:5-8; Wang *et al.*, 2019:248; Yu *et al.*, 2019:4-6). In a study done by Karabatsiakos *et al.* (2014:4-6), depressed individuals showed a significant reduction in ATP turnover-related respiration (resultantly decreasing cellular cAMP, cyclic adenosine monophosphate) and spare respiratory capacity. A more in-depth understanding of the role of mitochondrial dysfunction in depression may help to conceptualise the pathophysiology of this mood disorder better.

The study of Poleszak *et al.* (2019:349) selected various antidepressants from different therapeutic groups and the joint administration with the A_{2A} AR antagonist, DMPX, were observed to stimulate mice motility in either “tail suspension tests” (TSTs) or “forced swim tests” (FSTs) which is indicative of antidepressant-like activity. The concomitant use of DMPX with the antidepressants, imipramine, reboxetine and escitalopram, did bring about synergistic effects and increased the anti-depressive activity. To validate the reliability of the results and confirm that the observed results were not a false positive, it has been established that the results did not coincide with a rise in the “spontaneous locomotor activity” of the mice.

Furthermore, it is known that the enzyme, adenosine deaminase (ADA), breaks adenosine down into its metabolite, inosine. Inhibition of ADA has antidepressant-like activity in forced swim tests and subsequently augments the extracellular levels of endogenous adenosine. This nonselective AR stimulation also aids in antidepressant-like effects (Kaster *et al.*, 2013:485). The review article of van Calker *et al.* (2019:18) supports the hypothesis that up regulation of A₁ ARs and/or the concurrent down regulation of A_{2A} ARs, may contribute to the antidepressant activity in sleep deprivation, but more research should be conducted to explore these theories.

The current treatment options include a wide array of mono-, bi-, and tricyclic pharmacotherapies (**Figure 2-3** summarises some of the bicyclic therapies). The newer generations of antidepressants (ADs), selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), have higher selectivity for the serotonin receptors which is known to cause bleeding, hyponatremia and sexual dysfunction. It is debated whether the TCAs have worse side-effects or are less effective than the newer AD drugs and many studies have shown to have conflicting results ([Wang et al. 2018](#)).

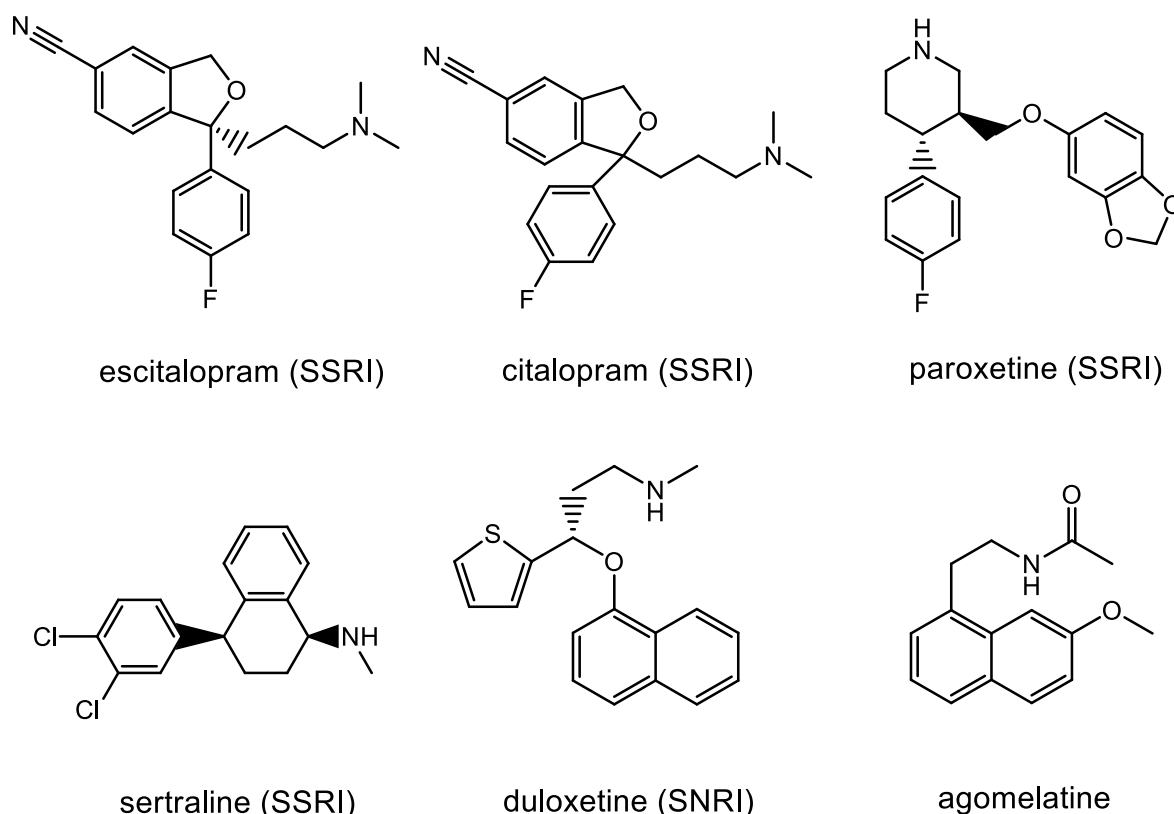


Figure 2-3: Current bicyclic treatment options, as adapted from Kok & Reynolds ([2017:2118](#)).

Abbreviations: selective serotonin reuptake inhibitor (SSRI), serotonin-norepinephrine reuptake inhibitor (SNRI)

Ormel *et al.* ([2019:349,352](#)) concurs that there is a gap in achieving successful treatment for patients dealing with the disabling reality of depression. Despite the increase in the number of treatment options since the 1970's, the prevalence has not declined. This gap is primarily due to the overestimation of the efficacy of current treatments, inadequate success rates in the long-term and the attribution fallacy that is apparent in psychotherapy.

2.3.2 Cognitive dysfunction

Cognitive disorder is defined as the “impairment of any of the mental processes of perception, memory, judgment and reasoning” (Black, 2008:79). Cognitive decline correlates with neurodegenerative disorders, like AD, dementia and PD, and causative factors might overlap and have mutual pathogeneses. This might complicate diagnostics and in recent years, blood-based biomarkers have become a popular field to resolve characterisation of the diseases. Nation *et al.* (2019:7) denotes that BBB breakdown is a sign of early onset loss of cognition, notwithstanding the tau and/or A β changes and the biomarkers that accompany AD and other forms of neurodegeneration. Capillary damage and injuries of the mural pericyte cells link to BBB breakdown.

The adenosine receptors have prevailed to be relevant in cognitive function preservation. Elmenhorst *et al.* (2018:8012) hypothesised that A₁ ARs may be involved in cognitive impairment induced by sleep deprivation and alcohol intake.

In a recent article, the A_{2A} AR agonist, CGS21680, has shown to restore memory and cognitive impairment in male Sprague Dawley rats that have been exposed to “noise induced hearing loss” (NIHL) which causes oxidative stress in the hippocampus. CGS21680 (see **figure 2-4**) is used as prophylaxis to noise exposure and promotes neurogenesis in the hippocampus (Shukla *et al.*, 2019:53).

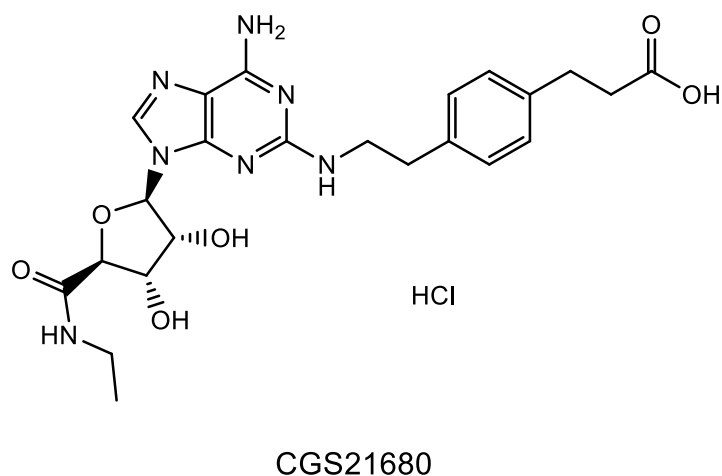
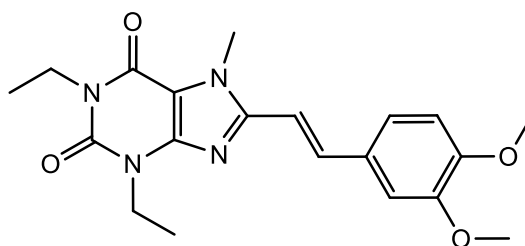


Figure 2-4: CGS21680

Prolonged and putative therapies involving cannabinoid activation have shown to disrupt memory and cognition processes. This can be resolved with the counter-administration of an A_{2A} AR antagonist, like istradefylline (refer to **Figure 2-5**), which has appeared to recover functional connectivity in recognition memory (Mouro *et al.*, 2019: 14-15, 20).



istradefylline

Figure 2-5: Istradefylline

2.3.3 Neurodegenerative disorders

2.3.3.1 Alzheimer's disease (AD)

Alzheimer's disease (AD) is named after the Bavarian physician, Alois Alzheimer (1864-1915), and was described in 1906 as “a disease where a person experiences progressive dementia due to nerve cell loss in specific brain areas, resulting in loss of mental faculties including memory”; whereas dementia is depicted as the “loss of mental ability and memory due to organic disease of the brain, causing disorientation a personality changes” ([Black, A.C., 2008:14,103](#)).

AD patients in the advanced stages of the disease are generally bed-bound and require full-time care. The neuronal and brain damage, such as neurofibrillary tangles (NFTs) and senile plaques (SPs), precede many complications where the patients lose control over swallowing, everyday activities and bodily functions. Moreover, it is defined by cognitive decline which impacts problem solving, completing typical tasks, language, judgement, planning, and memory. This instigates anxiety, apathy, sleep disturbances, depression, confusion and social and/or work withdrawal ([Alzheimer's Association, 2016:460-462](#)). Each case has a different degree of severity and debilitation. These complications contribute to the fatality of AD and dementia.

The societal and economic burden of Alzheimer's dementia is considerable and there are immense differences in incidence between developing and developed countries. AD is the main root of dementia, constituting about 50-70% of cases. An additional 15-20% of cases are attributed to vascular dementia. It is imperative to note that most cases are far more complex and consists of a mixture of neurodegenerative and vascular pathologies ([Xu & Qiu, 2018:3](#)).

Dementia has several causes besides AD ([Alzheimer's Association, 2016:460-462](#)) – “dementia with Lewy bodies (DLB)”, “frontotemporal lobar degeneration (FTLD)”, “normal pressure hydrocephalus”, dementia associated with PD, Down syndrome, Creutzfeldt-Jakob disease, mixed dementia and as previously mentioned, vascular dementia.

The exact pathogenesis of AD remains elusive and multifaceted. During the years many hypotheses about the aetiology of AD have become apparent ([Derakhshankhah et al., 2020:55](#)):

The cholinergic hypothesis is founded on the principle that acetylcholine (ACh), a critical neurotransmitter involved in memory, can cause dysfunction in neurons in the pathological state. The first acetyl cholinesterase inhibitor (AChEI) was introduced in 1997 and from thereon cholinergic drugs, AChEI's and cholinergic agonists like physostigmine, had been used in AD and dementia treatment ever since ([Birks, 2006:1-3](#)). Saify *et al.* ([2014:634](#)) modelled a 7-azaindole derivative after physostigmine and showcased how it can interact with the anionic locus, esteratic locus and the hydrophobic region of ACh's active site. Several of these 7-azaindole derivatives were assayed for AChE-, butyrylcholinesterase (BChE)- and glycation inhibition and the results were promising (**Figure 2-6**).

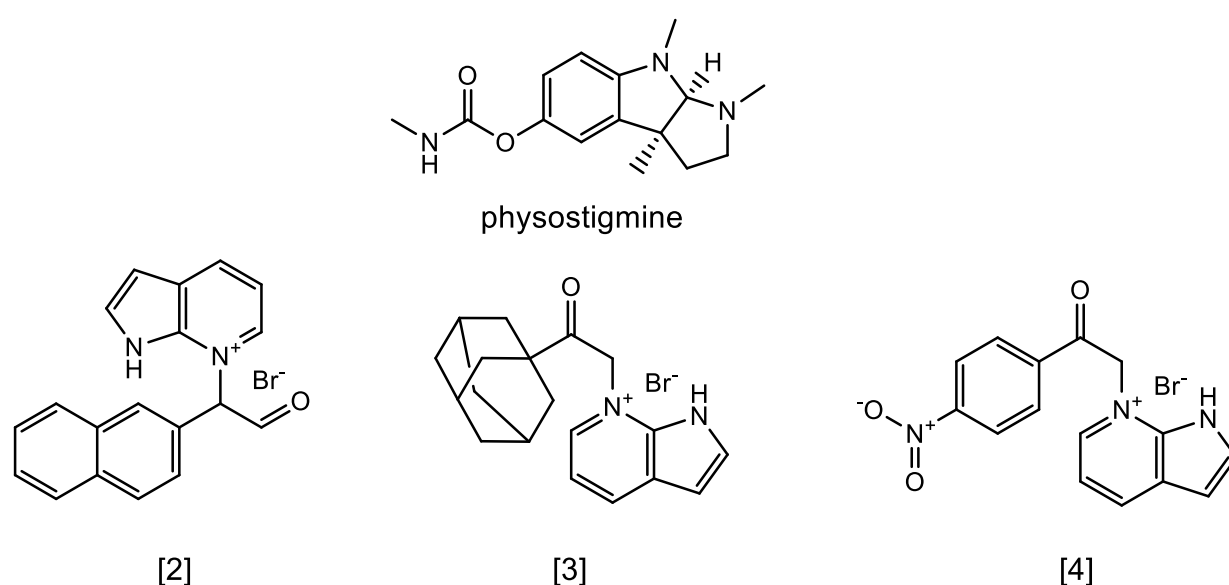


Figure 2-6: Compounds [2-4] with the 7-azaindole scaffold modelled after physostigmine for use in Alzheimer's disease (AD) (modified from Saify *et al.*, ([2014:629](#)); structures converted from name using [ChemDraw Ultra](#))

The amyloid beta (A β) cascade hypothesis refers to neurotoxicity as a consequence of amyloid beta protein (A β P), as a standalone, or A β P within the product of amyloid precursor protein (APP) cleavage. Two consecutive occurrences are mandatory for AD pathology. Firstly, is the generation of APP consisting of A β P or A β P as an entity of its own. An APP derivative with the COOH-terminal that contains A β P initiates the cascade. Secondly, the molecule will cause or facilitate the formation of neurofibrillary tangles and neuronal death. To refine the hypothesis, other genes or mutations of APP should be identified ([Hardy & Higgins, 1992:184-185](#)).

Next is the inflammation hypothesis. AD has a connotation to neuroinflammation that is unavoidable. Neuroinflammation is also a culprit in many other CNS conditions as well, and its mechanism is interwoven with stress and the accumulation of adenosine. Neuropsychiatric disorders are characterised by maternal inflammation and low-grade chronic inflammation which can lead to cell loss and toxicity (Beamer *et al.*, 2016:100). The CNS can be described as an “immune-privileged” environment. The BBB separates the brain and is highly specialised – especially in the activation of T-cell responses (Carson *et al.*, 2006:49, 61).

Another study reverberates the hypothesis that inflammation and the rise of pro-inflammatory cytokines, for instance tumor necrosis factor *alpha* (TNF- α) and interleukin *beta* (IL- β), may play a causative role in AD. Thus, the hypothesis exhibits the feasibility of the use of non-steroidal anti-inflammatory drugs (NSAIDs) and selective cyclo-oxygenase-2 (COX-2) inhibitors as instruments to delay the incidence of AD. However, randomised trials will be a requisite to prove this hypothesis (Zandi *et al.*, 2002:880-881).

The tau protein aggregation and phosphorylation hypothesis proposes that tau protein modification, which encompass methylation, acetylation, O-glycosylation, proteolytic cleavage, ubiquitination and phosphorylation, cause destructive cycles of NFT formation and oligomerisation of tau proteins (Derakhshankhah *et al.*, 2020:55; Bulic *et al.*, 2013:A).

The last 20 years has been instrumental in continuing research on the oxidative stress (OS) hypothesis in AD and the article of Smith *et al.* (2000:139) stated its concerns about a void in information about the pathogenesis of AD. There was uncertainty about how oxidation reactions that target sidechains, like lipid- and sugar-adduction reactions, and free radicals contribute to AD and neuropathology (Smith *et al.* 2000:142).

The “mitochondrial respiratory chain” is the main supplier of ROS in tissue. Repair enzymes and antioxidant defences keep oxidants in check to uphold a steady state in non-toxic concentrations. Inhibition of electron flow, antioxidant deficiency and xenobiotic exposure might throw this delicate system off-balance. Redox reactions and changes in transition metals, like copper and iron, cause tissue damage. Multicentre trials present evidence that the impediment of AD progression can possibly be achieved with antioxidative therapy (Huang *et al.*, 2016:521).

The sex hormone hypothesis emphasises that there are sex-specific differences in the brains of males and females and therefore gender biases exist in neurodegenerative disorders. It is important to note that sex involves a biological aspect, whereas various factors determine an individual's gender identification. The prevalence of AD is age-specific, and females are more prone to cognitive deficits and neuropathology of AD because of an increase in neurofibrillary

tangles and differences in sex hormones and neurotransmission. The ApoE- ϵ 4 allele is involved in AD in women (Ullah *et al.*, 2019:95-96).

Predominantly of interest to our research is the adenosinergic signalling hypothesis. In order to understand adenosine's connection to AD pathology, it is fundamental to understand how the adenosinergic pathway works (Cellai *et al.*, 2018:4). S-adenosyl-homocysteine (SAH) or AMP generates adenosine and intracellular uptake follows. Next, adenosine kinase (AK) phosphorylates adenosine to AMP or adenosine deaminase (ADA) converts the adenosine into inosine. Release of ATP into the extracellular cavity follows and CD73 and CD39, collaboratively, hydrolyse the ATP into adenosine. In cases of metabolic stress, adenosine levels rise and accumulate in the extracellular cavity. The adenosine can be transported back via the equilibrative nucleoside transporters (ENTs) into the cell or bind to the four GCPRs to elicit distinguished effects.

Factors that cause microglial disturbances, namely environmental factors, genetics and aging, may have a hand in faulty adenosinergic signalling. The environmental factors include “traumatic brain injury” (TBI), diabetes, high-fat diets, smoking and sleep disturbances; whilst examples of genetic factors that have been researched as of current are CD3, PS-1, PS-2, APP, TREM2 and ApoE- ϵ 4 (Katsumoto *et al.*, 2018:4).

Phosphorylated tau or aggregated A β , as explored in the aforementioned hypotheses, elicit neurotoxic mediator release from the microglia. This is significant since adenosine, ADP and ATP initiate NLRP3 inflammasomes and shortly after, IL-1 β . Neuronal damage can congruently be a result from gap junction release of glutamate from the hemichannels (Katsumoto *et al.*, 2018:4).

A novel functional and molecular observation of the underlying mechanisms by which A_{2A} ARs regulate glutamate uptake has been achieved by Matos *et al.* (2013:18500). The sodium gradient is a key player in ion homeostasis and establishes metabolic control with substantial energetic supplies. ATP is the source of energy and consummation thereof satisfies the energetic needs of the metabolic processes. Adenosine levels rise owing to ATP breakdown. Curtailment of metabolic imbalance caused by Na⁺/K⁺ ATPase (NKA), specifically NKA- α 2's undertakings to control ion homeostasis is attempted with A_{2A} AR activation in the astrocytes where the receptors are expressed.

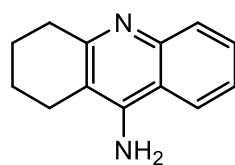
According to Matos *et al.* (2013:18495), the activities of gliosomes' glutamate and NKA receptors selectively decrease with A_{2A} AR activation. The striatum or cerebral cortex's synaptosomes are not affected. Synaptosome and gliosome incubation ensued with or without SCH58261 (50 nM), the A_{2A} AR antagonist, and /or CGS21680 (30-100 nM), the selective A_{2A} AR agonist. CGS21680

hippocampus, and 9 months, in the cortex, were identified. A_{2A} AR increase has a connotation with amyloid pathophysiology. The lucrative effects of chronic blockade of A_{2A} ARs can be translated to spatial learning, anxiety and memory in AD opposition. Prophylactic administration of A_{2A} AR antagonistic pharmacotherapies prevents changes in spatial memory and lessens the burden of cortical amyloid pathology.

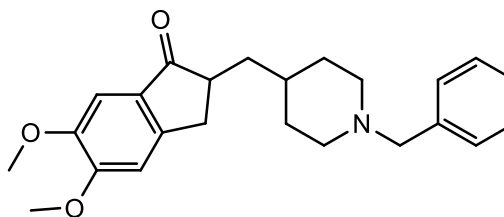
The study of Silva *et al.* (2018:74-79), performed on 3×Tg AD mice, supports the notion that over-functioning of A_{2A} ARs may bring about pathologies and has a quintessential role in AD's onset. The study revealed that the *in vivo* treatment with a A_{2A} AR antagonist recovers deficits of hippocampal long-term potentiation (LTP), memory dysfunction and synaptic markers. It also alters glutamatergic synapses and normalizes the synaptic up-regulation of A_{2A} AR in the hippocampus.

A₁- and A_{2A} ARs' involvement in AD has been affirmed with numerous studies and Angulo *et al.* (2003:448-450) stated that there is a definitive raise in hippocampal A₁ ARs associated with AD, even though the receptor's transcription gene levels remain the same in pathological- and control states. Promotion of tau translocation and -phosphorylation, mediated by extracellular signal-regulated kinase (ERK), and the release of APP's nonamyloidogenic forms will hinder AD's deterioration. Cieślak *et al.* (2018:340) added that purinergic signalling and the ARs, as listed above, can prevent neurotoxicity induced by Aβ and affect synaptic plasticity by supporting brain-derived neurotrophic factor (BDNF) release and glutamatergic- and dopaminergic signalling.

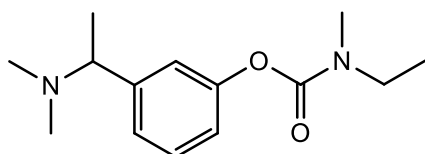
The existing therapeutic strategies to combat AD include the following classes of drugs: cholinesterase inhibitors (ChEI's) (**Figure 2-8**) and the uncompetitive *N*-methyl-*D*-aspartate (NMDA) receptor inhibitor, memantine (**Figure 2-9**), which is not as popular for the use in AD (Wang *et al.*, 2016:1528; Simunkova *et al.*, 2019:2492).



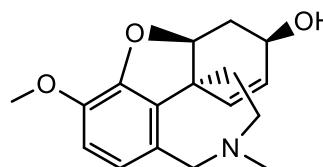
tacrine



donepezil

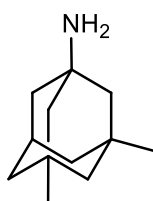


rivastigmine



galantamine

Figure 2-8: Cholinesterase inhibitors used in AD (ChemACX.Com)



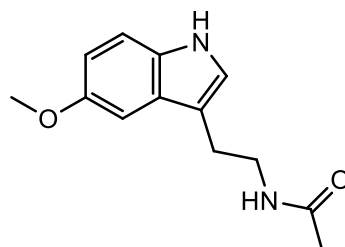
memantine

Figure 2-9: *N*-methyl-*D*-aspartate receptor inhibitor used in AD (ChemACX.Com)

There are several setbacks with the current therapies and the search for an effective treatment option for AD continues. There are no available therapies that provide lasting effects and the symptoms can only be temporarily relieved with the abovementioned options. It is not yet possible to reverse or inhibit the progression of AD and since the current drug therapies have grave side-effects, such as bradycardia, diarrhoea and nausea, patient compliance is low. Even though the amyloid theory and the use of A β as a biomarker are still viable, several clinical trials have failed as the anti-amyloid β administration of solanezumab and bapineuzumab, which are both monoclonal antibodies, failed to produce promising results and did not improve the progression of AD and cognitive decline ([Tayeb et al., 2012:19-20](#); [Wang, 2014: 1-2](#)).

Several lessons can be learnt from the immunotherapeutic failures of AD treatment at the clinical stages of development. As discussed, neuroinflammation is infamous as the cause or fuel of neuropathology; microglial dysfunction and all the hypotheses should be considered when

designing compounds. The targeting of different stages of the progression of AD may provide an answer. There are other compounds and natural derivatives that may cover some of the bases and further exploration is needed.

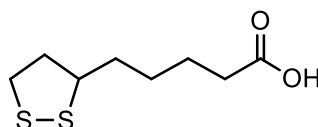


melatonin

Figure 2-10: Melatonin structure ([ChemACX.Com](https://chemacx.com))

Melatonin (**Figure-2.10**) is an indole moiety that is endogenously secreted by the pineal gland in mammals. It plays an intricate role in the regulation of the circadian rhythm and is a powerful antioxidant. It exerts its antioxidative effects by scavenging free radicals. Normal melatonin function diminishes neuronal cell death, neuroinflammation, proinflammatory cytokines expression and the damaging effects of oxidative stress. It prevents mitochondrial dysfunction, neurotoxicity, tau hyperphosphorylation and A β aggregation. Melatonin also stabilises the structure and plasticity of the synapses which aids in functioning cognition and memory processes ([Hossain et al., 2019:8268](#)).

Patients with AD have lower melatonin levels. The route of pathology can occur in the case of nocturnal light exposure in the retinas which interferes with melatonin formation in the pineal gland by degrading *N*-acetyltransferase. As a result, the disruption of the circadian rhythm leads to amyloid plaque build-up and tau hyperphosphorylation. The *N*-acetyltransferase degradation can also impact the suppression of melatonin synthesis which initiates oxidative stress ([Hossain et al., 2019:8258](#)). Hence, the negation of melatonin function may be a contributing factor in AD.



alpha-lipoic acid

Figure 2-11: Alpha-lipoic acid structure (α -LA) ([ChemACX.Com](https://chemacx.com))

Alpha-lipoic acid (α -LA) is a cofactor that can be derived from various food sources and is an important antioxidant in nutritional studies. In oxidative phosphorylation, α -LA has a physiological role in enzymatic complexes (refer to **Figure 2-11**). The complexes involved are pyruvate dehydrogenase (PDH), α -ketoglutarate dehydrogenase (KGDH) and “branched chain α -ketoglutarate dehydrogenase” (BCKDH) ([dos Santos et al., 2019:8](#)). This is significant since α -LA is essential in normal mitochondrial function and this could mean that it may be advantageous as an antioxidant in the mitigation of AD which potentially targets the mitochondria ([dos Santos et al., 2019:11](#)).

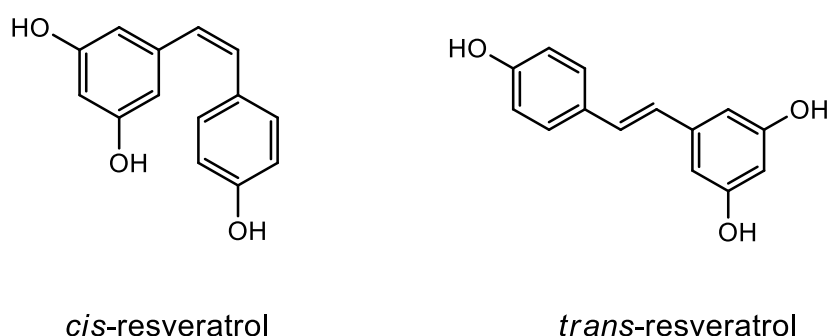


Figure 2-12: Isomeric structures of resveratrol as adapted from [Rege et al.\(2014:2\)](#)

Resveratrol is a natural-occurring phenolic compound with antioxidative properties that can be found in grapes and wine (**Figure 2-12**). It reduces neuroinflammation and prevents apoptosis with diverse mechanisms. It upregulates nitric oxide, AMPK, NRF2, PPAR γ co-activator, G6PD, catalase, SOD, HO1, glutathione, glutathione peroxidase and -reductase. It also downregulates iNOS, lipid peroxides, myeloperoxidase, COX1, PAR (Poly ADP-ribose) and NADPH oxidase ([Rege et al., 2014:5](#)). It is still unknown how the stereochemistry of the different isomers affects bioavailability in humans. Although resveratrol has proven antioxidative properties that may be valuable in AD treatment; it is not yet an effective treatment option in humans.

The different stages that can be pursued in future studies are as follow: protect synaptic dysfunction and neurons, remove A β plaques, prevent the formation of A β plaques, preclinical stage inhibition of the hyperphosphorylation of tau proteins and reduction of tau protein hyperphosphorylation at the mild cognitive decline phase. At the dementia stage, the collective targeting of synaptic dysfunction, oxidative stress, neuroinflammation, A β accumulation, tau hyperphosphorylation and the provision of cognitive training and neuronal protection may provide a solution ([Wang, 2014:2](#)). A $_1$ AR accumulation is prevalent during AD and several A $_{2A}$ AR antagonists are also putative in neurocognitive processing ([Angulo et al., 2003:449-450](#)); this can be a promising multitarget approach since the scaffold, 7-azaindole, has also been used as a design for anticholinergic therapy against AD. It seems that an arsenal of therapies is needed

to treat AD, but it may be possible to design a pharmacotherapy that addresses multiple stages of the disease at a given time.

2.3.3.2 Parkinson's disease (PD)

The manifestations of PD have been ostensible for centuries aside from it being anomalous and unnamed at the time. During the formative years of medical writing, the causation of diseases was difficult to ascertain as the focus remained on the symptoms. A shift could be seen from the end of the 19th Century as PD reached clinical recognition ([Kempster et al., 2007:485](#)).

In 1817, James Parkinson wrote “an essay on the shaking palsy” ([Parkinson, J., 1817:441](#)) which described in prolific detail the first clinical observations of a disorder that is now known as PD. Parkinson defined the “paralysis agitans” as “involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to running pace: the senses and intellects being uninjured”. Years later, in 1888, Jean-Martin Charcot praised James’s work and identified gaps in his observations: the involvement of facial motor complications and weakness causing bradykinesia. Charcot labelled the disorder as “maladie de Parkinson”, a French eponym meaning “Parkinson’s disease” ([Kempster et al., 2007:485](#)).

Now, two centuries since the first clinical observations, progress has been made on the PD’s pathogenesis and the search for drug therapies continues. Multitudes of studies added on James Parkinson’s work but there is still room for resolving incongruities in research and bridging the gap to find a cure ([Bello et al., 2019:25](#)).

The longitudinal study of [Locascio et al. \(2003:107\)](#) concluded that the cognitive capacities of PD patients are impaired in the following aspects: language, frontal lobe and visuospatial functions and long-term memory.

In healthy brains, voluntary movements are processed by gathering sensory-motor information. The basal ganglia are interjected between the thalamus and the cortex. PD is defined as the gradual loss of control of voluntary movement due to neurodegeneration that occurs because of functional changes of the substantia nigra pars compacta that manages the “dopaminergic nigrostriatal system” ([Jellinger, 1991:154](#)); these changes lead to faulty circuitry and the disorganisation of the basal ganglia’s nuclei. Dopamine depletion and Lewy bodies are hallmarks of PD’s histological features. After AD, PD is the second-leading neurodegenerative disorder and in the United States, PD affect around 1% of people over the age of 65 years and 50 000 new cases are diagnosed each year ([Lee et al., 2016: 1](#); [Shastry, 2001:6](#)).

The differentiation between AD and PD is clear since the cortico-subcortical degeneration is exclusive to AD, although there are several parallels between the two neurodegenerative disorders. Additional output changes in PD affect the medial globus pallidus and causes hyperactivity of the substantia nigra pars reticulata. Other mechanisms involved in the pathophysiology of PD are responsible for subthalamic hyperactivity ([Blandini et al., 2000:64-65](#)).

The clinical manifestations of PD are separated into two categories known as 1) motor- and 2) non-motor symptoms. The motor symptoms comprise of postural instability, rigidity, tremors and bradykinesia. Usually, the non-motor symptoms are less obvious and not always documented. This include fatigue, sleeplessness, faecal incontinence, unexplained pain, difficulty or disinterest with sexual activity, delusions, weight change, delusions, autonomic decline and neuropsychiatric disorders, like anxiety, depression, cognitive dysfunction and hallucinations. Most of these non-motor symptoms negatively affect the quality of life ([Gallagher et al., 2010:2498-2499](#)).

Males are twice as susceptible to develop PD during their lifespan as females. Females experience a delayed onset in PD and can be distinguished as tremor dominant. There is a greater prevalence in the following symptoms in females: depression, postural instability and the loss of visuospatial cognition. In contrast, males experience rigidity, a lack in verbal fluency and facial emotions, sleep disturbances and “rapid eye movement (REM) behaviour disorder” ([Ullah et al., 2019:96](#)).

Several hypotheses about the aetiology of PD exist that either have been disproved or persisted to be feasible ([Franco-Iborra et al., 2016:266](#)): neuroinflammation hypothesis, mitochondrial hypothesis, prion hypothesis, modification in protein degradation processes and the aggregation and misfolding of proteins, like α -synuclein, covered by the Braak hypothesis ([McCann et al., 2016:152](#)). The prion hypothesis is controversial, and studies show that infective diseases are a probable cause of PD but not applicable in most cases ([Warner, A., 2019:17](#)).

The hypothetical reasoning behind the development of PD neurodegeneration is attributed to multifactorial genetic and/or environmental factors mentioned by [Shastry, B.S. \(2001:9\)](#):

- Genetic – molecular chaperones convert and unfold α -synuclein. Hence, unfolded proteins undergo degradation via ubiquitin-mediated pathways. Note that the presence of intermediates and mutations in UCHL1 and parkin form aggregates that can be converted to Lewy bodies by dint of molecular chaperones.
- Environmental - inhibition of the first mitochondrial complex and MAO-B later oxidises N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to MPP⁺ causing superoxide production. “Cu/Zn superoxide dismutase” (SOD) can inhibit superoxide.

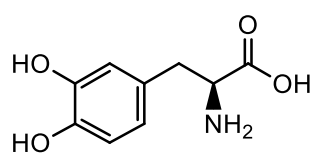
- Environmental - nitric oxide synthase (NOS) produces NO from arginine and 7-nitroindazole (7-NI) can inhibit NOS. NO and superoxide interactions may generate peroxynitrite which is a reactive species that oxidises lipids and proteins and breaks strands of DNA.

In 1983 (Langston *et al.*, 1983:980), the mitochondrial hypothesis was recognised after humans had been exposed to MPTP and developed acute cases of Parkinsonian syndrome that is essentially the same as idiopathic PD – the only difference being the “motor vagus nucleus” and locus coeruleus were virtually unchanged. Cell damage occurred in the substantia nigra and the microglial cells displayed a neuro-melanin pigment, focal glial scarring due to the astrocytic response plus a Lewy body-like structure. Betarbet *et al.* (2000:1301) noticed a gap to develop an *in vivo* model for PD using the synthetic insecticide, rotenone. Rotenone has a high affinity for complex I of the “inner mitochondrial membrane” and, unlike MPTP, yields systemic inhibitory results for complex 1 of the mitochondria. Rotenone is exceptionally hydrophobic and does not rely on a dopamine transporter to reach the cytoplasm.

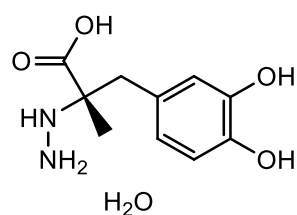
The approved therapies for PD are divided into dopaminergic drugs and non-dopaminergic drugs and are summarised on the www.drugs.com website (Mao *et al.*, 2019:3-4):

1) Dopaminergic drugs (Mao *et al.*, 2019:3):

- 1.1) The dopaminergic drugs comprise of the dopamine precursor, levodopa, which is the mainstay drug used to treat the symptoms of PD: spasms, tremors, weak muscle control and muscle stiffness. Carbidopa, a dopamine decarboxylase inhibitor, is used in combination with levodopa.



levodopa



carbidopa

Figure 2-13: Levodopa and carbidopa

Despite L-dopa's effectivity, it reaches a therapeutic plateau which encumbers any further improvement. The major side-effect of L-dopa therapy is unsolicited, involuntary movements, also known as “L-DOPA-induced-dyskinesia” or LID for short. The word dyskinesia stems from “dys” which means difficult and “kinesis” which means movement (Manson *et al.*, 2012:189).

The mechanism that instigates LID's repercussions on synaptic plasticity is described in the article of Wang & Zhang (2016:4) as the “maladaptive corticostriatal synaptic plasticity mechanism”. After the long-term administration of L-dopa, the cholinergic interneurons demonstrate elevated firing rates and ACh release, raised levels of immunoreactivity of phospho-ERK (extracellular signal-related kinase), as well as lowered signalling of striatal cGMP and long-term depression (LTD) – this is stringently dependent on PKG's (protein kinase G) NO activation which is lacking in LID.

Many clinicians prefer to postpone L-dopa therapy to delay LID, but contradictory studies show otherwise. Cilia *et al.* (2014:2740) conducted a study with a large cohort group of PD patients from Ghana in sub-Saharan Africa. The study was executed over the span of four years and tracked the chronic use of antiparkinsonian drugs. The findings suggest that it is not necessary to delay L-dopa therapy, since the dyskinesias and motor fluctuations are linked to the progression of PD itself. These findings call for similar studies to support or invalidate these claims. In contrary, Schrag & Quinn (2000:2303) reported statistics that an approximate 40% of patients develop motor fluctuations and 30% develop dyskinesias with L-dopa treatment. The motor dysfunctions will show up due to natural disease progression, but the severity is dependent on the dose and duration of L-dopa treatment.

All the other approved therapies are only adjunctive and the needs for a suitable replacement/ successor for L-dopa or a cure for PD have remained unmet. Most investigational drugs at the phase I and -II clinical stages of testing never reach phase III.

1.2) Monoamine oxidase B (MAO-B) inhibitors, rasagiline and selegiline, are used in the early stages of PD and in combination with levodopa. Whereas safinamide is utilised for the “wearing-off” in PD patients in combination with levodopa therapy.

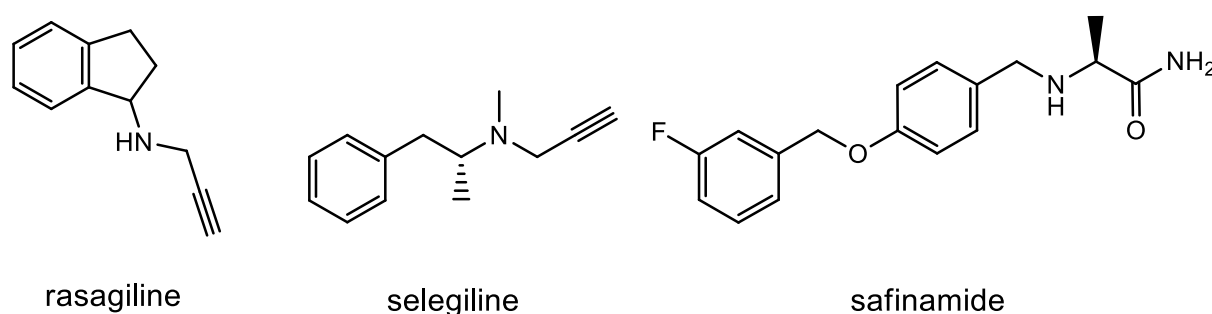


Figure 2-14: MAO-B inhibitors

1.3) COMT inhibitors can be used in combination with either levodopa or carbidopa.

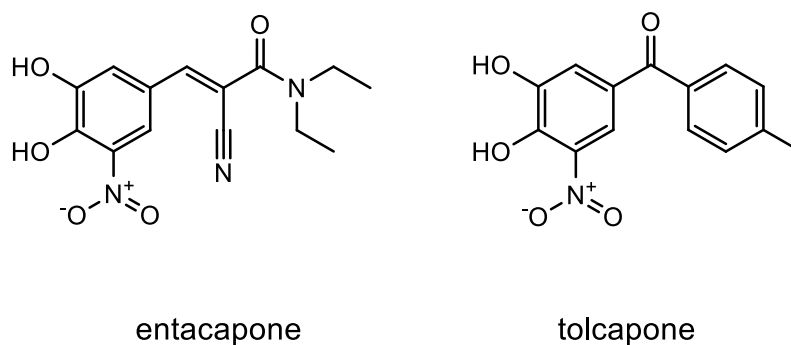


Figure 2-15: COMT inhibitors

- 1.4) The last group of drugs that are approved in the dopaminergic category, are the dopamine agonists: apomorphine, bromocriptine, pramipexole, ropinirole and rotigotine. Apomorphine is indicated for patients with advanced PD who experience “wearing-off” episodes with Levodopa. Bromocriptine is specifically used to treat acromegaly, as well as PD. Pramipexole, ropinirole and rotigotine are all used for PD treatment and restless legs syndrome (RLS).

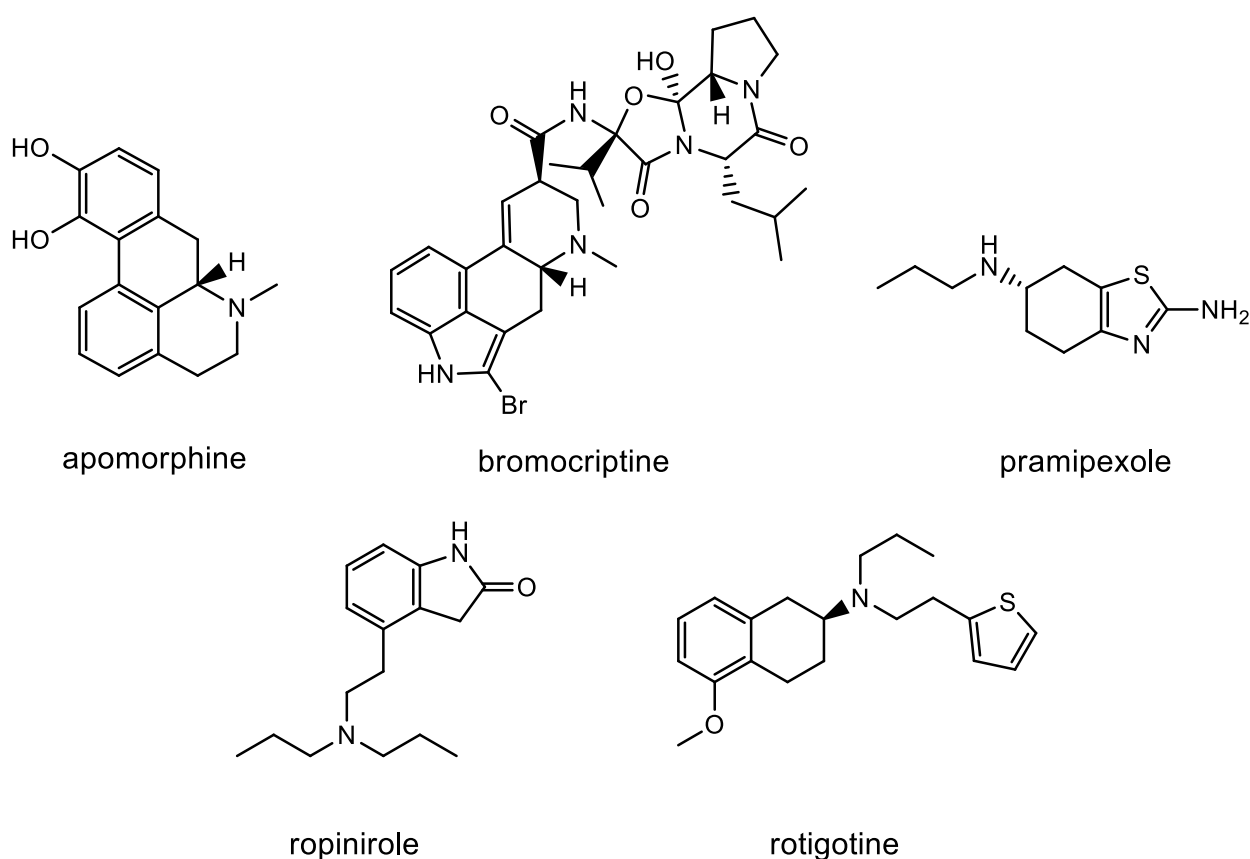


Figure 2-16: Dopamine agonists

- 2) Non-dopaminergic drugs ([Mao et al., 2019:4](#)):

2.1) Muscarinic receptor antagonists

For centuries, the use of anticholinergic drugs has been customary. During the 1800's, atropine was purified from the belladonna plants and was found to be useful in PD besides its established effects on the heart and gastrointestinal tract (GIT) (Behere et al., 2019:151).

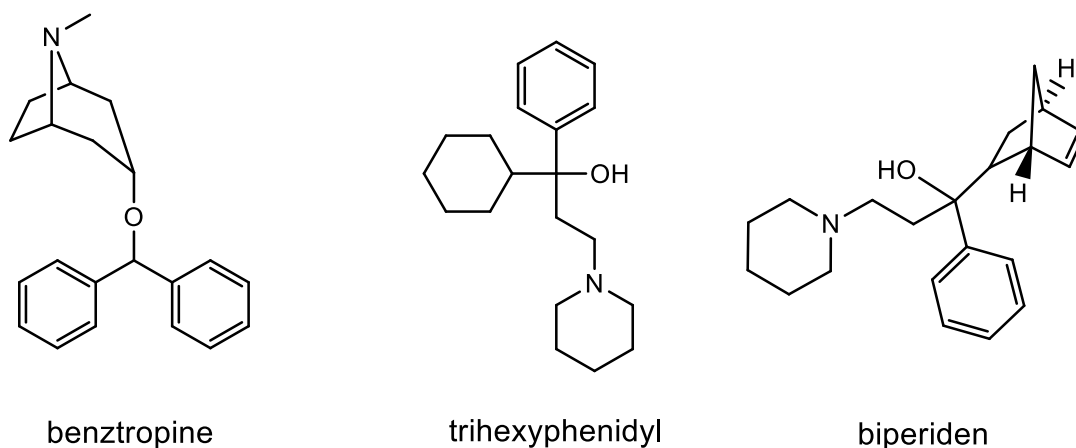


Figure 2-17: Muscarinic receptor antagonists

Compounds containing tertiary amine groups, like benztropine, biperiden, procyclidine and trihexyphenidyl, can readily cross the BBB (Behere et al., 2019:151).

2.2) Cholinesterase inhibitors

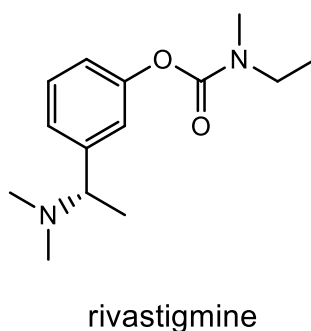
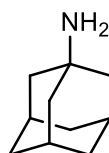


Figure 2-18: Cholinesterase inhibitor

Rivastigmine is approved to treat dementia and the mild and/or moderate cases of PD or AD.

2.3) *N*-methyl-*D*-aspartate (NMDA) receptor antagonists



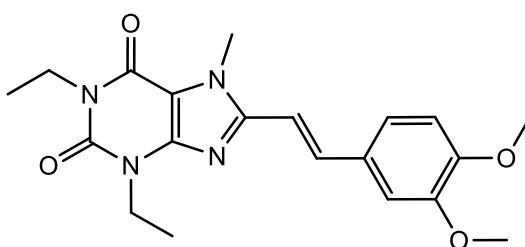
amantadine

Figure 2-19: NMDA receptor antagonist used in PD

The anticholinergic drugs act on the muscarinic receptors and the drug, amantadine, can decrease the symptoms of the influenza viruses ([Behere et al., 2019:151](#)). Amantadine is also used for treatment of the symptoms of PD or similar side-effects associated with the use of specific drugs. The symptoms include tremors, stiffness, shaking and uncontrolled muscular movements.

2.4) A_{2A} AR antagonists

Istradefylline has recently been approved (2019) as an add-on therapy in patients experiencing “off episodes” in PD. It is indicated to be used with levodopa or carbidopa in adults.



istradefylline

Figure 2-20: The A_{2A} AR antagonist, istradefylline

The “central dopaminergic system” is directly impacted by adenosine levels and the A_{2A} ARs can curb dopamine function. This may accelerate neuropathological conditions ([Ferré et al., 2018:2](#)).

According to [Morelli et al. \(2009:595-596\)](#), the A_{2A} ARs have a prominent role in the basal ganglia where adenosine excites the striatopallidal neurons by binding to these receptors, whilst dopamine exerts an inhibitory effect. In neuropathological conditions, specifically PD, there exists an imbalance in the output of the striatal pathways – striatopallidal neurons become hyperactive and the striatonigral neurons is left in a hypoactive state. The reasons for the striatopallidal neurons’ hyperactivity are the simultaneous undergoing of adenosine stimulation and loss of dopamine inhibition. Overexertion of Ach is also prevalent in the caudate putamen. Hypokinetic symptoms manifest as a result of the SNr/GPI’s (substantia nigra pars reticulata/ internal segment

of the globus pallidus) increased inhibitory output to the thalamocortical neurons. A_{2A} AR blockade restores the hyperactivity and the thalamocortical and SNr is rendered normoactive and akinesia is relieved.

The review of Mally & Stone (1996:248) predicted that a combinational therapeutic approach with a molecule that can serve as both a dopamine agonist and A_{2A} AR antagonist, might be a promising bifunctional tactic to target surfaces rich in both receptor cell types, like the striatopallidum. This may be the golden key to treatment of tremor disorders and/or motor dysfunction escorting PD.

The findings of the meta-analysis of Wang *et al.* (2017:4) reinforced notions and warrant further investigation of the A_{2A} AR antagonists' protective benefits on L-dopa-induced dyskinesia (LID) in vivo on 152 animal models. According to the study, A_{2A} AR antagonism alleviates the motor symptoms and reduces the "abnormal involuntary movements" (AIM) in LID of animal models. Caffeine improved the total AIM scores significantly when compared to SCH-412348 and KW-6002 (Wang *et al.*, 2017:4-5).

Haddad *et al.* (2018:3) explored various prodrugs for levodopa: carrier-mediated, dimeric amides, amides, esters and cyclic prodrugs, as well as different enzyme model delivery systems. This might be a solution to overcome bioavailability issues and related problems.

Another consideration when designing new drug therapies, is light. Previous research called attention to the photochemical drawbacks of how light affects stereochemistry and the drug, MRS5543, was deemed unsuitable *in vivo*. The cis configurations, a result of photosensitivity, led to short lifetimes and low selectivity which ended up hampering pharmacological efficacy. The study of Taura *et al.* (2018:136-141) achieved photocontrol and developed a light-dependent drug known as MRS7145, a caged A_{2A} AR antagonist, which has shown to effectively improve locomotor activity and have prominent antiparkinsonian effects.

One of the deciding factors of whether a drug will be safe and effective, is the side-effect profile. Nakanishi & Takahashi (2019: 2-3) provides an overview of the side-effects correlated with antiparkinsonian drug use. Neuropsychiatric effects are mainly associated with amantadine and anticholinergic use; these side-effects include delusion, confusion, hallucination and agitation. To avoid these side-effects, levodopa is the preferred therapy although it may still cause LID, dizziness and somnolence. Next up, hypotension and other cardiovascular effects, like orthostatic hypotension, are common with antiparkinsonian drug use, as well as gastrointestinal discomfort, like vomiting and nausea. The concurrent use of domperidone can alleviate the GIT discomfort. Chronic use of "dopamine replacement therapy" (DRT) or dopamine agonists can instigate

“dopamine dysregulation syndrome” (DDS) or be the root of “impulse control disorders” (ICDs). A solution might be to taper the use of these therapies, but this creates other problems, such as “dopamine agonist withdrawal syndrome” (DAWS). For this reason, a non-dopaminergic approach, for example A_{2A} AR antagonists, can evade the side-effects associated with dopaminergic drugs.

2.4 Adenosine receptors (ARs)

G-protein-coupled receptors (GPCRs) are a branch of transmembrane receptors. The engineering field of GPCRs, with both computational and empiric method designs, is vast and reprogramming is possible. Different outcomes can be achieved through programming: modification of the conformational stability of the inactive or active states, alteration of protein function and redesign of the monomeric interface (Keri & Barth, 2018:191). Heterotrimeric G proteins, such as G_o , facilitate the signalling of the GPCRs. As the progression of natural aging takes its toll, the protein density of the GPCRs decrease and the expression and/or function of the receptors are affected. The factors influenced by normal aging are (de Oliveira *et al.*, 2019:9):

- Modification of the fluidity and structure of the plasma membranes by processes, such as lipid peroxidation which leads to instability of the membranes.
- Obstruction of protein synthesis increases the variability of mRNA, adaptation of transcription factor and the accumulation of defective signalling proteins, like the GPCRs. In aging brains, autophagy and less effective proteasomes decrease the number of functional GPCRs.
- Lastly, neuronal death is a result of physical trauma associated with aging, neurodegeneration and toxin exposure.

The adenosine receptors have many similarities; however, they differ in distinctive ways which include the distribution of the receptors and the transduction of signals. These signals cause different physiological effects, and this implicates that the ARs can have diverse therapeutic applications and functions in the human body (Kashfi *et al.*, 2017:8128-8129). The adenosine receptors' affinity to adenosine varies. A_1 ARs have the highest affinity to adenosine, ± 70 nM, and A_{2A} ARs have poorer affinity of ± 150 nM (Dunwiddie & Masino, 2001:33 ;Stockwell *et al.*, 2017:2). According to the Peleli *et al.* (2017:6) there are many obstacles that are faced when targeting the ARs since some of the ligands targeting the receptors do not perform well in clinical settings; there are a few AR ligands that show promising results and have progressed to the next stages in clinical trials. In April 2019, the FDA had approved the use of istradefylline as an adjunctive therapy in PD (Hoffman, 2019:1).

Some of the challenges when designing AR ligands that should be taken into consideration ([Peleli et al., 2017:4-5](#)):

- 1) The ubiquity of ARs in biological systems. This means that the ligands may interfere with essential physiological processes. This can be surmounted by designing selective ligands or increasing the selectivity of existing compounds. Partial agonists can also be used in cases where there are a surplus number of receptors available for binding ([Chen et al., 2013:274](#)). Partial agonists, unlike full agonists, can simulate the actions of antagonists by means of inverse agonism and may have less side-effects than full agonists ([Müller, 2001:80](#)).
- 2) Receptor desensitisation, tolerance, can be a consequence of the repeated stimulation of AR agonists. This problem can be evaded by the use receptor antagonists. Desensitisation rates differ and according to the article of [Mundell & Kelly \(2011:1321\)](#), the A₁ ARs experience a slower rate of tolerance build-up than the other ARs.
- 3) The effectivity of the ligands is dependent on the stage and/or development of the diseases and the age of the patient should also be deemed as an important factor. Sometimes the ligands can elucidate an undesired response because of the complexity of adenosine signalling ([Chen et al., 2013:276-277](#)).
- 4) ARs have marked effects on different cells, especially regarding metabolic dysfunction ([Peleli et al., 2017:5](#)), and adverse responses that may not have been foreseen, can disrupt the homeostasis of biological systems.
- 5) Caffeine consumption can impede on the effectivity of ligands or therapy because of its non-selective antagonistic effects. However, caffeine has neuroprotective benefits of its own – the study of [Garcez et al. \(2019:791\)](#) suggests that low-dose caffeine consumption in aged mice can reduce cellular damage in the hippocampus by reducing the A_{2A} adenosine content. This may be helpful in the prevention of AD and PD. Caffeine and its metabolites – methylxanthines, paraxanthines, theobromine and theophylline – are found in the human body and have non-selective AR antagonistic effects. The blockade of A_{2A} ARs enhances cognition and synaptic plasticity, whilst A₁ AR antagonism promotes neurotransmitter release, vasoconstriction and alertness ([Camandola et al., 2019:217-218](#)).

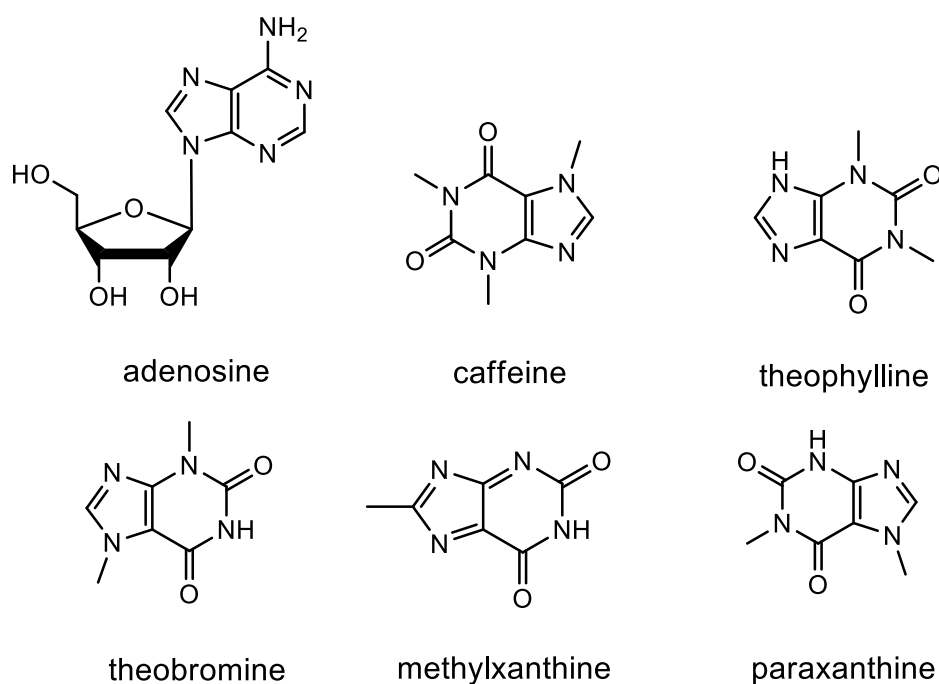


Figure 2-21: Non-specific AR ligands (adenosine and several methylxanthines)

The results of [Fredholm, Irenius *et al.* \(2001:446\)](#) compared the binding affinities of the non-selective makeshift adenosine antagonists – caffeine, theophylline and paraxanthine – and found that the micromolar K_b values for the A_1 ARs are 33,8 μM , 8.9 μM and 15.8 μM , respectively, and the affinities to the A_{2A} ARs are 12.3 μM , 7.9 μM and 5.3 μM , in that order. Other compounds that inhibit adenosine's uptake are dipyridamole and methotrexate (**Figure 2-22**). There are ways to manipulate endogenous adenosine levels: adenosine agonists, adenosine deaminase (ADA), adenosine kinase (ADK), enzymes (CD73 & CD39), and inhibition of transporters via dipyridamole ([Jammes *et al.*, 2015:673-674](#); [Liu *et al.*, 2019:908](#)).

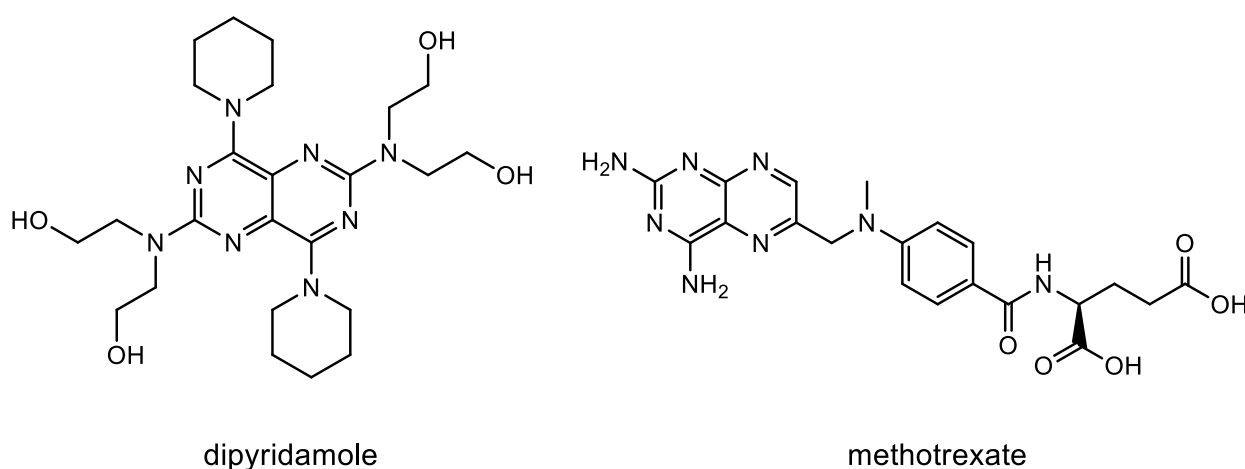


Figure 2-22: Adenosine uptake inhibitors: dipyridamole & methotrexate

- 6) The accurate *in vivo* measurement of adenosine levels and ARs are tricky and more reliable analytical methods are needed for assessment. The study of [Zsuga et al. \(2017:7-8\)](#) may provide a solution despite the rapid metabolism and endogenous accumulation of adenosine.

In agreement with [Takahashi et al. \(2008:2624\)](#), it is too complicated to assign behavioural effects to one specific AR, since the memory and learning processes, plasticity and/or interactions take place in numerous brain regions. In a homeostatic environment, the expression of A_{2A} ARs are trivial and A₁ ARs have more noteworthy mnemonic functions in healthy brains. Therefore, A_{2A} AR antagonists are signified for having neuroprotective benefits and dual antagonism can have indispensable therapeutic application.

There are numerous unanswered questions concerning the chemical diversity of the GCPRS ([Vass et al., 2018:509](#)): how many ligands have cross-sectional poly-pharmacological interactions, in other words $K_D/K_i/EC_{50}/IC_{50} \leq 1 \mu M$, with no less than two receptors from the GCPR subfamily? Do the scaffolds of GCPR ligands have similarities or are the existing structures resultant of tried-and-true synthetic approaches in medicinal chemistry? Will structure-based drug design reign supreme in realising novel heteroaromatic rings and macrocycles for use as novel scaffolds? Can the current ligands be repurposed for use to target other GCPRs?

2.4.1 A₁ adenosine receptors (A₁ ARs)

The A₁ ARs are distributed in both the peripheral- and central nervous systems. The role of these receptors when stimulated include the mediation of anticonvulsant-, depressant- and sedative effects. The receptors modulate the conductance of Ca²⁺ and K⁺ ions and function through close coupling with the G_{i/o} proteins. When coupled with the TRKs (receptor tyrosine kinases) and G_s proteins, essential functions are operative – muscle contraction, nerve stimulation, gene expression and release phenomena. In regions of the brain, the Ca²⁺ influx inhibition is mediated by the A₁ ARs via voltage-gated channels; the consequent limited Ca²⁺ availability reduces excitatory transmitter release and counteracts the postsynaptic excitatory effects ([Abbracchio et al., 1999:80](#); [Fredholm et al., 1996:265](#)).

[Chen, Lee et al. \(2014:11-12\)](#) describes the canonical pathway of the A₁ ARs. The A₁ ARs' transduction mechanism inhibits adenylyl cyclase (AC) and Ca²⁺ channels but activates phospholipase C (PLC) by means of G- protein βγ dimer subunits in an array of cell types ([Biber et al., 1997:4962-4963](#)). This translates to physiological effects of hyperpolarisation of neurons and the inhibition of synaptic transmission ([Dunwiddie & Masino, 2001:33](#)). AC inhibition is confirmed by [Jeong et al. \(2002:1219\)](#) and consequently, cAMP production is reduced.

GABAergic inhibition is mediated by A₁ ARs and it is expected to depend on PKA (cAMP-dependent kinase) activity.

A₁- and A₃ ARs have the highest affinity to adenosine, values ranging from 0.18 - 0.53 μ M (Fredholm, Irenius *et al.*, 2001:446), and are widely distributed in different cells. In vertebrates, the expression of the A₁ ARs is conserved, and this has called attention to these receptors specifically (Kashfi *et al.*, 2017:8129). The A₁ AR's carboxyl terminus is condensed as it lacks the third, extended extracellular loop (Pankevych *et al.*, 2003:30290).

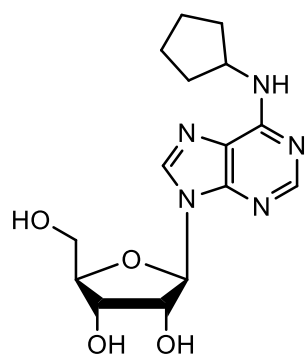
The hippocampus has four subfields: CA1, CA2, CA3, and CA4. Muñoz & Solís (2019:17) provides data that presynaptic A₁ AR antagonism potentiates a stronger synaptic response in the CA2 area of the hippocampus in comparison with the CA1 area. This is attributed to enhanced coupling and signalling mediated by cAMP towards the downstream effectors.

As previously discussed, adenosine has a retaliatory metabolic tendency and as stated by Kusek *et al.* (2015:48), the A₁ AR is recognised as the first GPCR that responds to “pharmacochaperoning” by adenosine – the endogenous agonist. “Pharmaco-chaperoning” is a concept that is still poorly understood. It involves the restoration of misfolded amino acid chains or protein aggregates. The chaperones enter the cells and assist with the assembly of aberrant proteins to fix the functionality and prevent a pathogenic cascade (Muchowski & Wacker, 2005:11-12). Martin *et al.* (2019:12) illuminates the biochemical, structural and functional mechanisms that dictate K_{ATP} complex formation and gating/ channel trafficking with a selection of compounds that act as pharmacochaperones with cryo-electron microscopy analyses. The K_{ATP} channels are hetero-multimeric proteins that can fold on its own or disturb assembly partners indirectly.

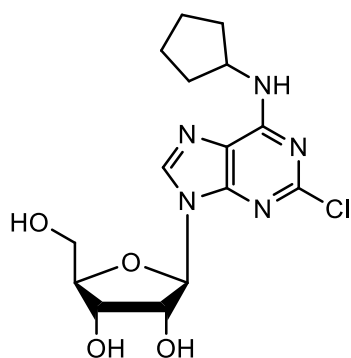
The jarring effects of ischaemia can be prevented by adenosine, according to Rudolphi *et al.* (1992:444). Ischaemia precedes escalated neurotransmitter release, glutamate, ATP catabolism, Ca²⁺ loading and unnecessary depolarization causing cell death. The proposed mechanism of the neuroprotective benefit of adenosine is as follow: it stabilizes the membrane potential, regulates Ca²⁺ homeostasis, restrains postsynaptic depolarization, limits the release of presynaptic glutamate, diminishes the congregation of free radicals and augments “cerebral blood flow”.

The benefits of A₁ AR antagonism, in this study, have been indicated for alleviation of cognitive decline by improving synaptic responses.

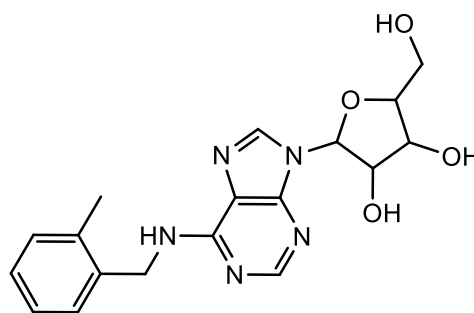
2.4.1.1 A₁ AR ligands



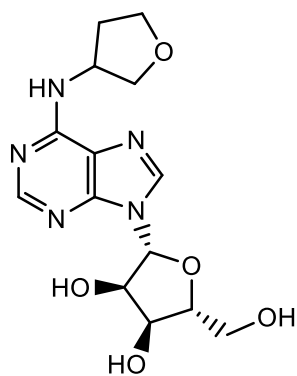
CPA



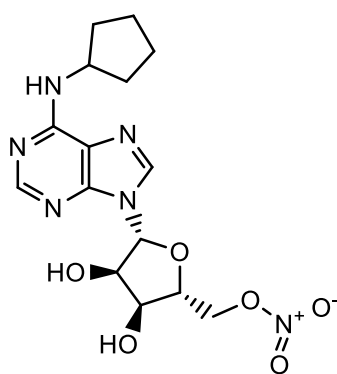
CCPA



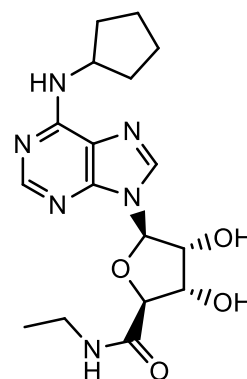
metrifudil



tecadenoson



trabodenoson
(INO 8875)



selodenoson

Figure 2-23: A₁ AR agonists – nucleoside derivatives adapted from [Jacobson et al. \(2019:3\)](#)

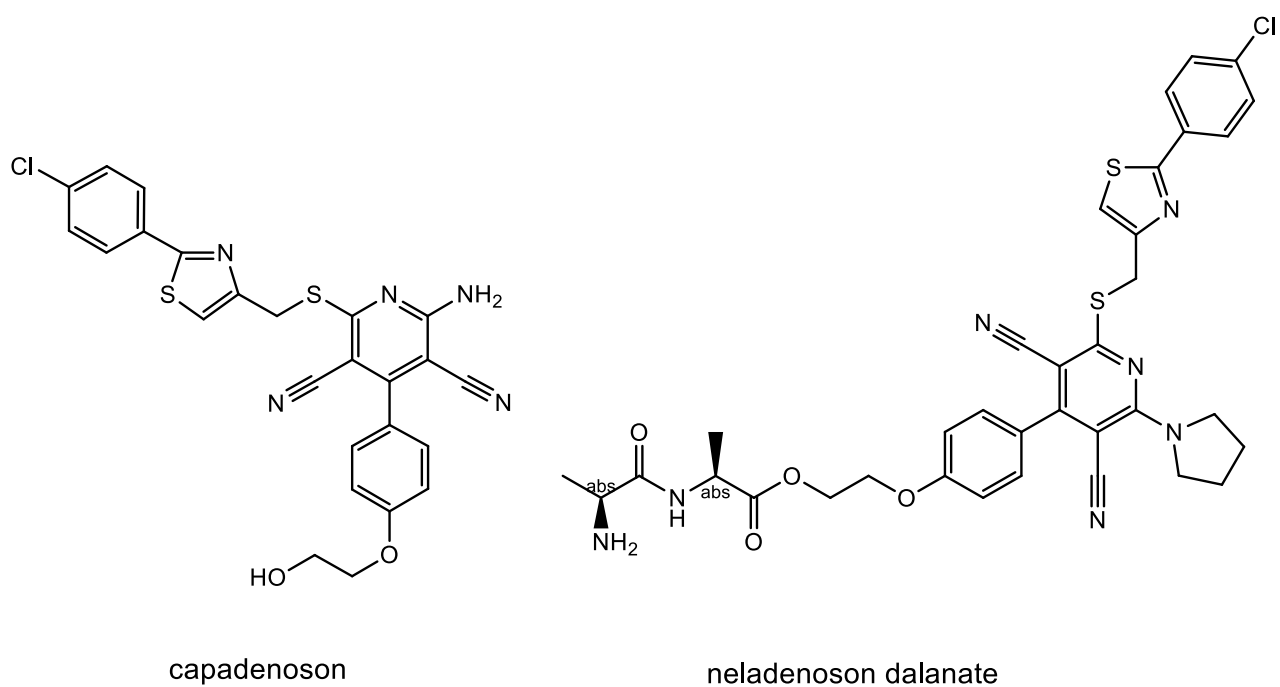


Figure 2-24: A₁ AR agonists – nonnucleoside derivatives

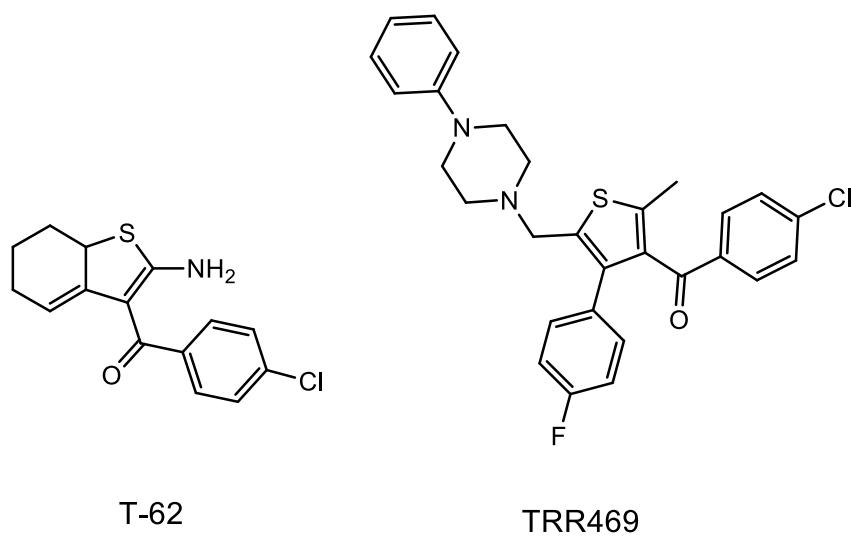


Figure 2-25: Allosteric enhancers of the A₁ ARs

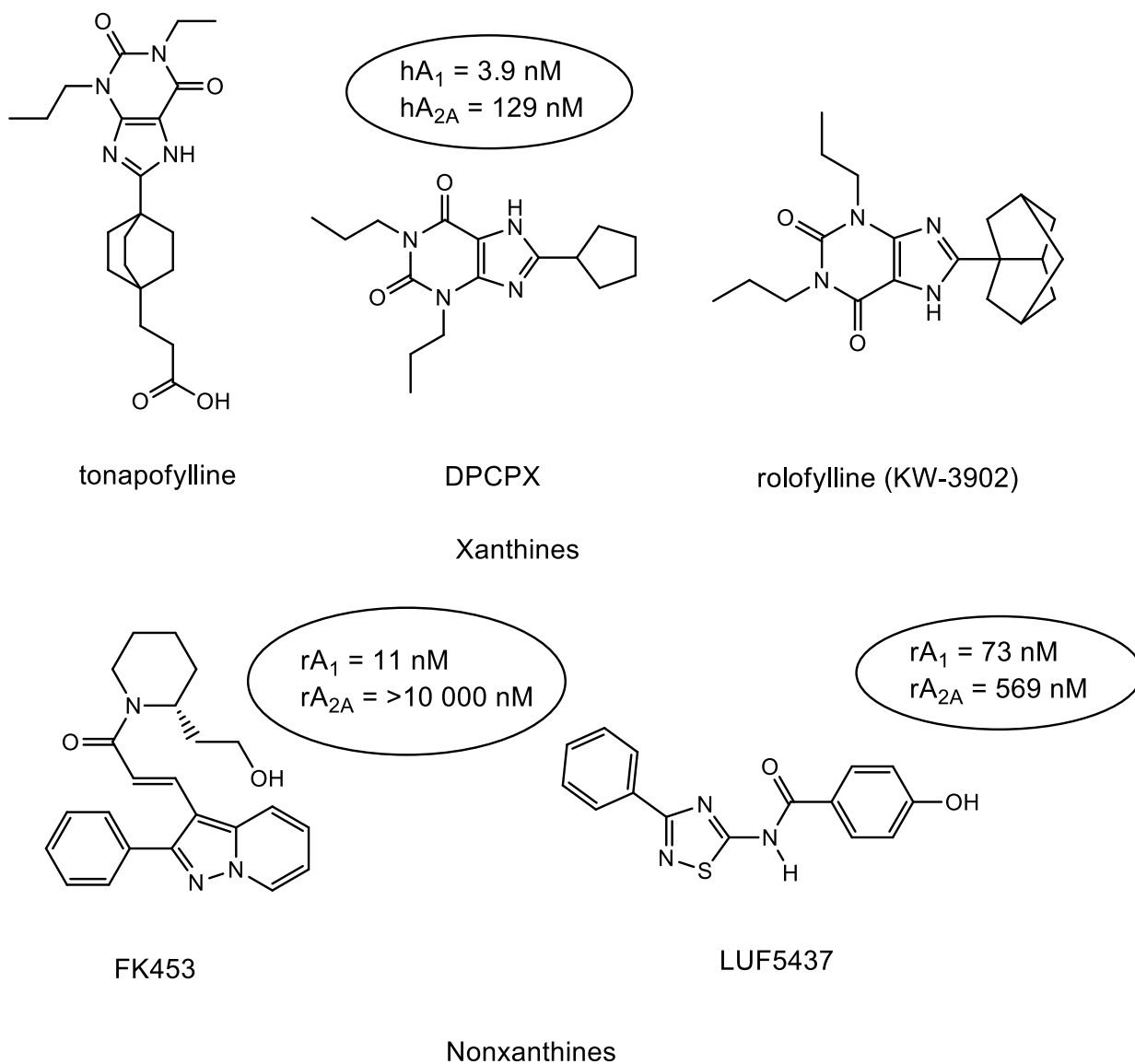


Figure 2-26: A₁ AR antagonists (xanthines and nonxanthines), r-values indicate rat assays and h-values are indicative of assays on humans (values retrieved from [Moro et al., 2006:138-150](#))

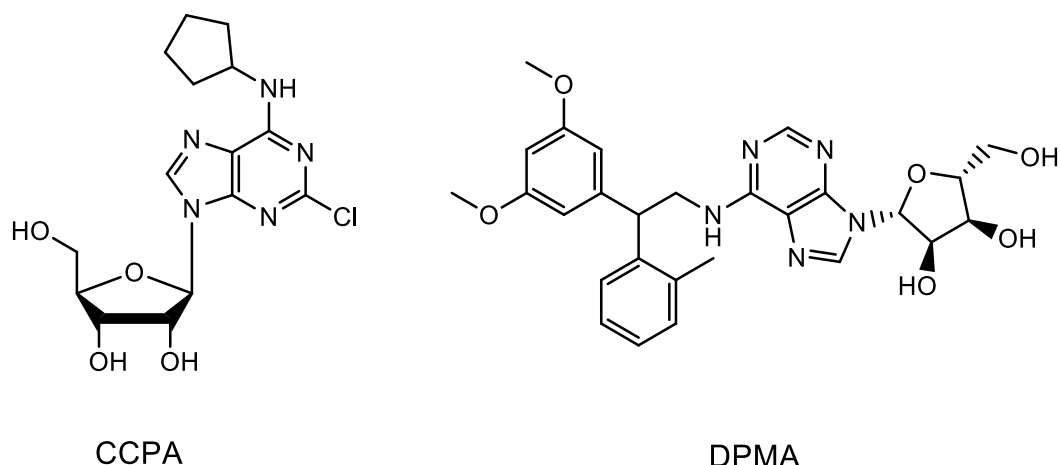


Figure 2-27: CCPA & DPMA

CCPA & DPMA are full agonists at A_1 and A_{2A} ARs, respectively, but antagonists at the A_3 ARs.

2.4.2 A_{2A} adenosine receptors (A_{2A} ARs)

The A_{2A} ARs elicit various effects in the CNS-, digestive-, respiratory-, renal-, eye-, and reproductive systems as summarised by [Diniz et al. \(2008:1712-1713\)](#). A_{2A} AR-mediated activation increase the release of the following transmitters in the striatum alone: the cAMP formation, ACh release, glutamate release, as well as the conductance of the NMDA receptors ([Diniz et al., 2008:1712](#)). These receptors have limited distribution in tissue in comparison to the A_1 ARs and is co-localised alongside the G_{olf} proteins ([Fredholm et al., 1996:265](#)). In contrast to A_1 ARs, the A_{2A} ARs' transduction mechanism activates adenylyl cyclase (AC) but can inhibit or activate the Ca^{2+} channels in nerve endings which physiologically facilitates or inhibits neurotransmitter release ([Dunwiddie & Mansino, 2001:33](#)).

[Stockwell et al. \(2017:9\)](#) provided discourse on the plausibility of the “cross-talk” between the A_1 - and A_{2A} ARs set off by traumatic events, like a stroke. A pathological state may desensitize the A_1 ARs and downregulate the “serine/threonine protein kinase CK2” (synonymous as casein kinase 2) which, in turn, increases the expression of the A_{2A} ARs.

Adenosine stimulation exerts different effects in the A_1 and A_{2A} ARs. A_1 AR activation by adenosine decreases the activity of adenylyl cyclase (AC) and cyclic adenosine monophosphate (cAMP) levels with the consequent blockade of c-AMP-responsive element binding protein (CREB) and protein kinase A (PKA) and coinciding stimulation of Ca^{2+} and PLC- β . Moreover, K^+ channels are activated and the N, P, Q and Ca^{2+} channels are inhibited. A_1 AR stimulation determines extracellular signal-related protein kinase (ERK1/2), c-Jun N-terminal kinase (JNK1/2) and p38 phosphorylation. Contrariwise, the signalling cascade of A_{2A} ARs initiated by adenosine

stimulation increases CREB phosphorylation, PKA, and cAMP. A_{2A} ARs activity also conducts the employment of ERK1/2, JNK1/2, p38 and protein kinase B (AKT) (Merighi *et al.*, 2018:42,44).

The A_{2A} AR antagonists are a lucrative therapeutic option to alleviate the locomotor dysfunction that is evident with patients that are struggling with neurodegeneration with added neuroprotective benefits.

2.4.2.1 A_{2A} AR ligands

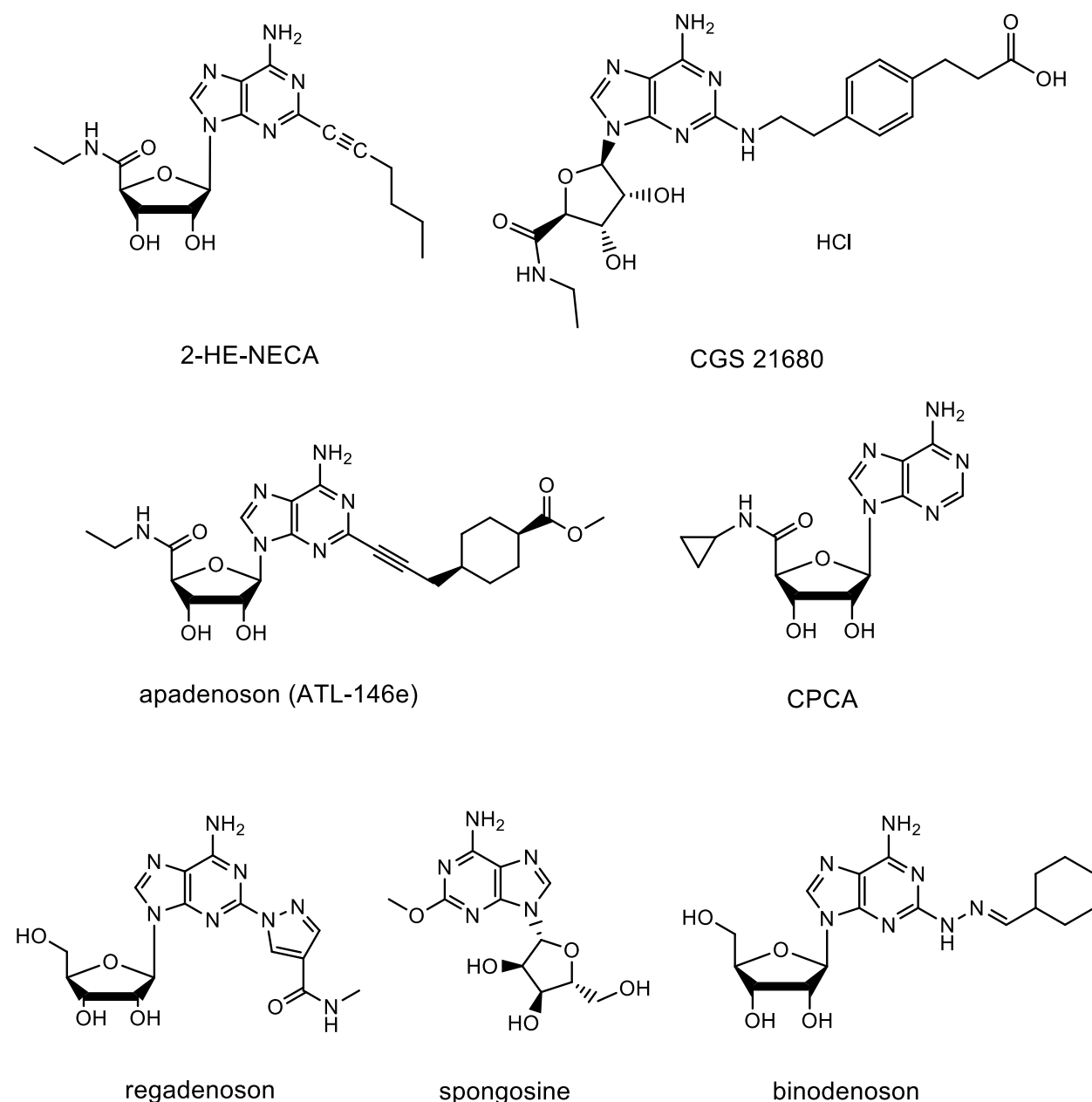


Figure 2-28: A_{2A} AR agonists (structures extracted from [ChemACX.Com](https://chemacx.com))

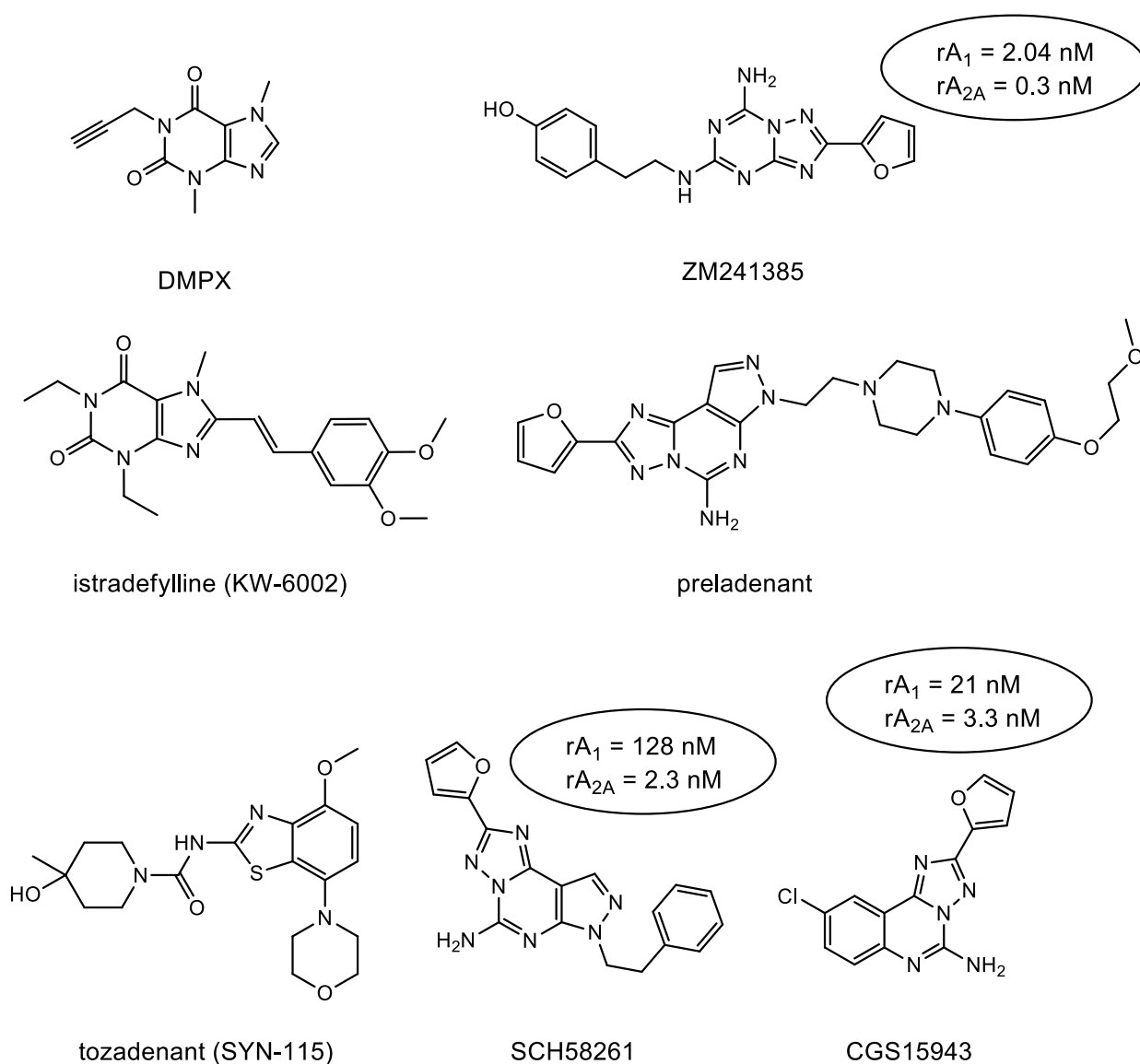


Figure 2-29: A_{2A} AR antagonists (r-values, assays performed on rat brains, obtained from [Moro et al., 2006:142-143](#); structures generated from [ChemACX.Com](#) from [ChemDraw Ultra](#))

2.5 Previous molecular docking studies

To increase the chances of designing auspicious compounds for neurodegenerative disorders, it is always beneficial to have a target receptor in mind. Instead of haphazardly designing many compounds and testing them, it is better to visualise the receptor cavity and how the binding will take place. To grasp what the individual ARs look like and which residues are indispensable for optimal binding, molecular docking studies can be a tool to customise or predict outcomes without wasting resources.

Even though this study will be gravitating towards a structure-based design, it is advantageous to determine if the drug candidates, or at least the scaffold, will have structural characteristics and functional groups that can interact with the moieties that line the receptor cavities. Molecular docking is not an end-all be-all solution to designing compounds. Compounds may have great binding affinity *in silico* but can fall short when tested *in vitro* and *in vivo*. Stability and favourable properties that will allow the compound to reach the target site might not harmonise with the *in-silico* results. Similarly, adverse reactions and contra-indications cannot always be predicted which purports that clinical testing is mandatory for safety and efficacy (Figueira *et al.*, 2017:8-10).

Numerous studies have explored the A₁ ARs and Draper-Joyce *et al.* (2019:563) achieved molecular insights, using class A cryo-electron microscopy, into the first known active state conformation of the human A₁ AR in complex with G₁₂ protein and adenosine (ADO). Using an endogenous agonist, the A₁ AR activation mechanism is better understood by means of the heterotrimeric structure, ADO-A₁R-G₁₂ (Draper-Joyce *et al.*, 2019:560). Glycosylation has a pronounced role in agonist binding and is indicated with N159's added density. The adenosine molecule resides in the orthosteric site and substitution with alanine decreases the affinity of NECA, an adenosine derivative.

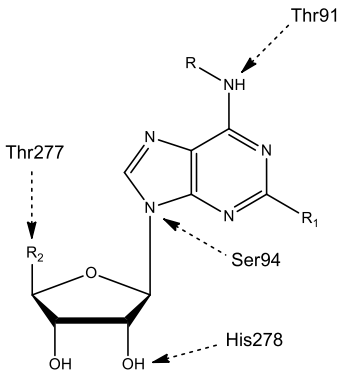
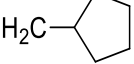
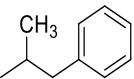
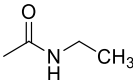
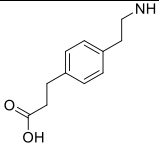
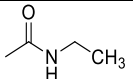
Former molecular docking studies recognised that the C8 position of xanthine rings have a bulky substituent. It was also ascertained that the alkyl chains support selectivity and affinity for A₁ ARs at N3- and N1 positions. The ligands' xanthine core is sandwiched between the protein's hydrophobic patches and these patches are influential in the binding to these receptors. The H-bonds with amino acids have less significance in subtype selectivity but is also noteworthy; Asn254^{6.55} creates an H-bond in A₁ ARs, whereas Asn253^{6.55} is significant in A_{2A} ARs (Cheng *et al.*, 2017:1281).

Seven of the A₁ ARs' "transmembrane (TM) helices" are pertinent (Cheng *et al.*, 2017:1276): A43^{2.38} – G72^{2.67}, K224^{6.25} – F259^{6.60}, H78^{3.23} – V109^{3.54}, M177^{5.35} – Q210^{5.68}, P121^{4.39} – P142^{4.60}, S267^{7.32} – K294^{7.59} and S6^{1.29} – V36^{1.59}. A₁ AR's general structure is remarkably similar to the A_{2A} AR due to adoption of a TM trajectory and counterparts that are akin to the A_{2A} ARs. There are similar kinks in the 4th, 6th and 7th TM helices of both receptors. Minor distinctions disturb the helical trajectories in A_{2A} ARs' non-proline counterparts - P863.31, P251.48, and P1915.49.

The transition of A₁ AR activation is indicated by a sizable outward movement of 10.5 Å of TM6 (transmembrane helix 6); more specifically, the intracellular side of TM6. This is done to accommodate the Gα_i protein and the α5-helix (Draper-Joyce *et al.*, 2019:560). Gutiérrez-de-Terán *et al.* (2004:712) explored the molecular dynamics of the hA₁ AR by running various

simulations of adenosine in the receptor cavity. Generally, adenosine exhibits stable polar interactions. Three hydroxyl groups of the ribose structure interact with the following residues of the A₁ AR by way of H-bond interactions: His7.43, Asp2.50, Ser7.46 and Ser1.46. Val3.32 also has a useful hydrophobic interaction with the heterocyclic system of adenine. **Table 2-1** illustrates the shared interactions for a dual targeting approach intended for both the A₁- and A_{2A} ARs.

Table 2-1: The key interactions of certain ligands in both the A₁- and A_{2A} AR cavities, modified from [Tuccinardi *et al.* \(2006:325\)](#) (*CGS21680 has an added interaction between Asn181 and the carboxylic group substituent at R₁)

		R	R ₁	R ₂
	CPA		H	CH ₂ -OH
	RPIA		H	CH ₂ -OH
	CADO	H	Cl	CH ₂ -OH
	NECA	H	H	
	CGS21680*	H		

The inactive conformation of the A₁ AR was defined by [Glukhova *et al.* \(2017:869\)](#). Reconciliation of the fact that the orthosteric binding pocket conserves most residues between the two AR subtypes, A₁ and A_{2A}, is difficult to comprehend due to the assortment of selective antagonists and agonists of each subtype that are available. The A₁ AR's cavity is open and wide. This explains how the binding pocket can accommodate allosteric and orthosteric ligands, whereas the compound ZM241385 occupies most of the A_{2A} AR site. It is speculated that the residues that line the orthosteric site, are not as influential in subtype selectivity as the spatial discrepancies between the subtypes. These variations are mostly caused by ECL3, TMs 1,2,3 and 7 and lastly, the substitution of one amino acid at the 270^{7.35} position that acts as a “gatekeeper” ([Glukhova *et al.*, 2017:868](#)).

Mutagenetic experiments were performed as a confirmation that the amino acid, position 270^{7.35}, contributes to subtype selectivity (Glukhova *et al.*, 2017:871). The residues at the same positions of the A₁ and A_{2A} ARs were swapped and as anticipated, DPCPX experienced a decreased affinity when T270M was substituted in the A₁ AR. In contrast, DPCPX's affinity increased during the reverse substitution of M270T into the A_{2A} AR.

In accordance with the abovementioned resources, both the A₁- and A₃ ARs receptors have one cysteine amino acid which creates a disulphide bridge at EL2 and another one that is situated at TM3. Margiotta & Moro (2019:2) ponders whether the human A₁ AR can be used as a template to uncover the homology model of the human A₃ AR subtype owing to fact that the receptors share a higher sequence identity. The A_{2A} AR lacks the disulphide bridge at EL2 and that is responsible for the difference in shape of the orthosteric binding cavity. Moreover, Margiotta & Moro (2019:2) explains that the A₁ and A_{2A} ARs have a disulphide bridge at C260-C263 which constructs EL3.

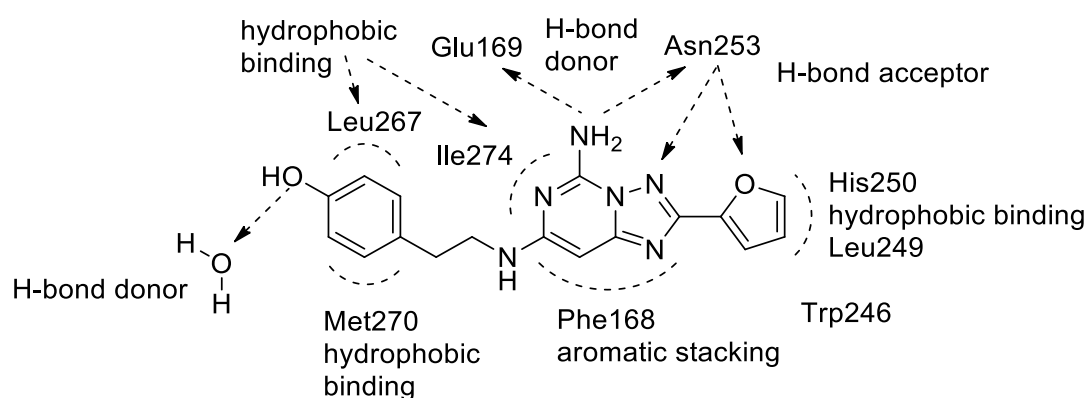


Figure 2-30: ZM241385 and its interactions in the A_{2A} AR binding site, as adapted from Yuan & Jones (2014:2)

Docking results of Varano *et al.* (2019:567) explores the 2-, 5- and 7-positions of novel 7-amino-thiazolo[5,4-d]pyrimidines. When the thiazolo[5,4-d]pyrimidine scaffold is inserted into the human A_{2A} AR binding site, it closely relates to the interactions of ZM241385, which is a co-crystallized analogue with a triazolo-triazine group, refer to **Figure 2.30**. The side chain of Asn253 engages in double H-bond interactions and in addition, there is polar contact with Glu169, non-polar interactions with Leu249 and π - π interactions with Phe168. The 5-substituent faces the extracellular environment and encounters the following residues: Ile66, Ser67, Leu167 (EL2), Glu169 (EL2), Leu267 (EL3) and Tyr271.

Sun *et al.* (2017:2068, 2070) discovered an obscure allosteric pocket in the A_{2A} AR which has not been documented before with a different ligand-binding mode that necessitates a new crystal

packing. Analogues of the ligand, compd-1, were used for the docking study. The differences are as follow: stabilisation of TM6's inactive conformation with an ionic bond between Arg107^{3.55} and Glu228^{6.30}, the location of Met4^{1.30}'s α -carbon facing TM1's extracellular end is further away and unlike A_{2A} R-BRIL-ZM241385's straight helix, helix 8 has a kink from Gln297.

By identifying interactions that are necessary for optimal binding, it will be easier to decide on the scaffolds that have potential and shift the focus towards what will most likely work.

2.6 7-Deazapurine- and 7-Azaindole scaffolds

Purines, such as guanine and adenine, and pyrimidines, like cytosine, thymine and uracil, are heteroaromatic molecules that have pervasive biological activity. Biopolymers of these molecules form deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) which play an integral part in the normal function of cells and genetics. The fusion of pyrimidine to a pyrrole core presents a pyrrolopyrimidine group, also known as "deazapurine" (De Coen *et al.*, 2016:80-81).

According to the review of De Coen *et al.* (2016:86-98), the deazapurine scaffold has been used to design and synthesise ligands that have been utilised as biocides, such as bactericides (Mohamed *et al.*, 2009:155) and protozoicides (Srinivasan *et al.*, 2013:3), and various receptor antagonists, such as corticotropin-releasing factor (CRF) receptors (Zorilla & Koob, 2010:5), adrenergic receptors (Pittalà *et al.*, 2011:5261-5262), histamine H₄ receptors (Gao *et al.*, 2013:132-133) and ARs (Gillespie *et al.*, 2008:2924-2925), which is the focus of this study. It has also been used as kinase inhibitors and enzyme inhibitors which is especially applicable to this study since it has shown to hydrolyse cholinesterase, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), for the potential use in AD because of the scaffold's antioxidative properties (Parnetti *et al.*, 1997:753). This can be a privileged scaffold in drug design due to its inherent affinity to biological receptors.

To sidestep the problems that accompany polyphenol use and brain bioavailability, other scaffolds have been explored – this include nucleoside and non-nucleoside analogues. In this study, the 7-azaindole and 7-deazapurine structures are in the forefront. Hall *et al.* (1997:900-902) discusses the potential antioxidative properties of the pyrrolopyrimidine, deazapurine, structure and explain the ways it can delocalise and transfer electrons. The radical species that cause oxidation are quenched by the nitrogen and this can lend to its inherent antioxidative nature. These properties are significant for neuroprotection since the characteristics of pyrrolopyrimidines include outstanding brain penetration and oral bioavailability which makes it a valued contender for drug design in neuropathology. Refer to the mechanism in **Figure 2-31** down below.

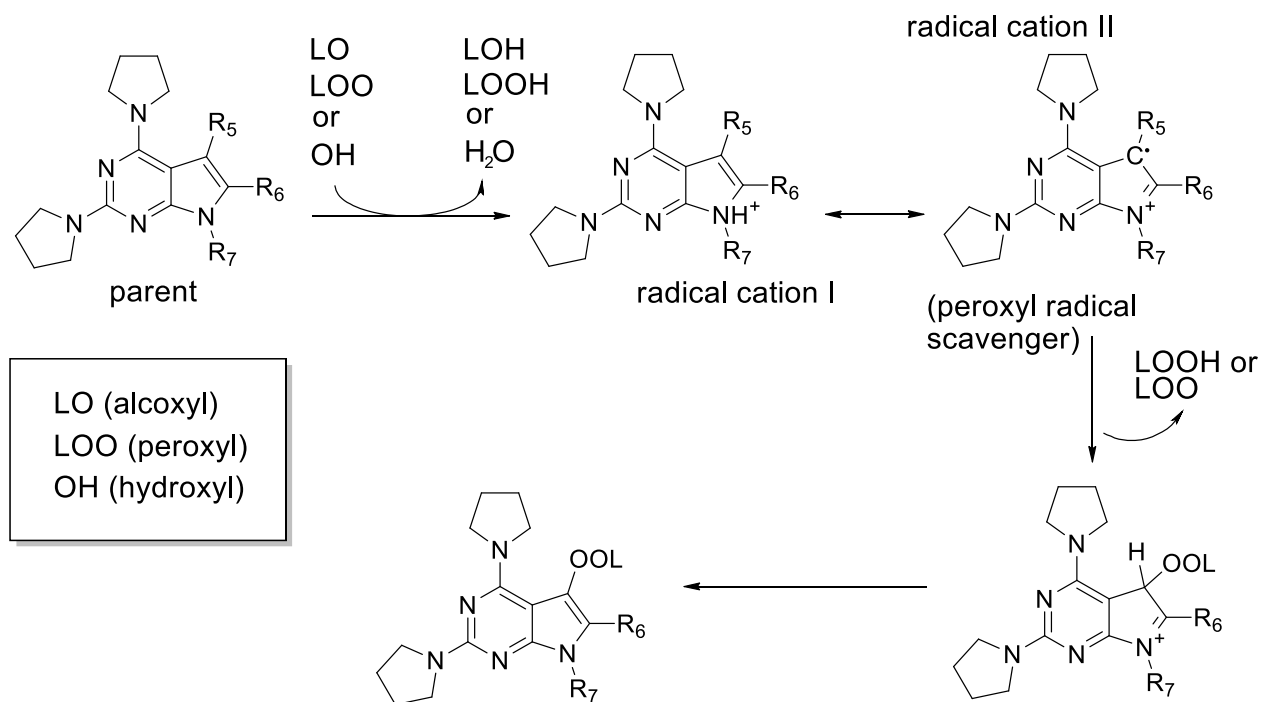


Figure 2-31: The likely antioxidative mechanisms of pyrrolopyrimidines, modified from [Hall et al. \(1997:900\)](#)

The indole moiety is a favoured scaffold in the pharmaceutical industry and has also been isolated from natural sources, for example the plant growth hormone, indole-3-acetic acid, and other marine sources ([Thanikachalam et al., 2019:563](#)). The review of [Thanikachalam et al. \(2019:563\)](#) discusses the structural activity relationships (SAR) of indole-based derivatives and how it is used in the mitigation of various diseases. A few examples of known pharmaceutical drugs that contain the indole moiety is: roxindole, fluvastatin, delavirdine, ondansetron, tadalafil, indomethacin, indalpine, perindopril and sumatriptan. Furthermore, several drugs that include the 7-deazapurine as a nucleus have been approved by the FDA – pemetrexed, ruxolitinib, tofacitinib, ribociclib and baricitinib ([Pathania & Rawal, 2018:504](#)).

There are five sites on the 7-azaindole scaffold where alterations can be made. The article of [Irie & Sawa \(2018:29-30\)](#) exhibits the incorporation of the 7-azaindole scaffold as an element for hinge- and non-hinge binding in the development of kinase inhibitors by means of X-rays of the cocrystal structures in the binding site of ATP. Previous studies researched the photo-physics of 7-azaindole and depicted its interactions with the environment and different solvents. In alcohols, double-proton transfer in the excited state or tautomerization is mediated by one molecule of the solvent and the solute creates a circular complex with the solvent ([Smirnov et al., 1997:2761](#)). This works relatively different in water and the idealized interactions for proton transfer are portrayed in the article where the solute is sufficiently solvated ([Smirnov et al., 1997:2759](#)).

The 7-deazapurine and 7-azaindole scaffolds have distinctive properties that make the scaffolds propitious for drug design, particularly since countless derivatives have been studied in recent years for a myriad of pathologies (De Coen *et al.*, 2016:80-81).

2.6.1 Synthetic approaches

Cross-coupling synthetic methods will be applied in this study. These cross-coupling methods are generally effective and require a one-step reaction mechanism that eliminates chances of losing product yield or risk contamination that is evident in multi-step reactions.

Reactions employing cross-coupling mechanisms and transition metal catalysts, such as tetrakis(triphenylphosphine)palladium, have increased in popularity in the passing years. In 1972, Grignard reagents and organic halides were used in cross-coupling mechanisms catalysed by complexes of Nickel-phosphine (Tamao *et al.*, 1972:4374). Two of the most revered cross-coupling synthetic methods are the Stille- (Stille, JK., 1986:508) and Suzuki coupling reactions (Suzuki, A., 1985:1750). The Stille reaction couples organostannanes with sulfonates or unsaturated halides, but this method is less desirable than the Suzuki method given that it requires stoichiometric quantities of tin which is not preferred for large-scale pharmaceutical applications. The Suzuki reaction couples organoboranes, such as boronic acids or esters, with the sulfonates or unsaturated halides (Smith *et al.*, 1994:8151).

The exact mechanism of the Suzuki cross-coupling method is unknown, but Fitton & Rick (1971:290) inferred that it starts off with oxidative addition followed by the trans-metalation process. The final step is reductive elimination and it is conjectured that electron withdrawing groups, like organic halides, tend to the reduction elimination mechanism.

There have been several adaptations of the synthesis route of the Suzuki coupling method during the years. Larhed *et al.* (1996:8220) noted a general microwave-assisted procedure in 1996. The reaction was sealed properly and took place under inert conditions. This was achieved by charging nitrogen into Pyrex tubes and the reagents were as follow: 100 mg of functionalised 4-bromo or 4-iodo resin (0.23 mmol), 1.15 mg of Pd(PPh₃)₄ (0.0010 mmol), 0.2 mmol “aryl boronic acid”, 0.1 ml 2M Na₂CO₃ (0.2 mmol), 0.3 ml H₂O, 0.1 ml EtOH and 0.75 ml DME. Since then, many solvent system variations and other changes, such as reaction conditions, have been implemented and the reaction is tailored to fit the desired outcome and reagents used.

Many observations have been made on how to optimise yields and acknowledge the limitations of the Suzuki method. Maddaford & Keay (1994:6501-6502) recognised that when water was added after the B(OMe)₃, it increased the yields by increasing the solution’s basicity, and the “*in situ* hydrolysis” of phenyldihydroxyborane or phenyldimethoxyborane may contribute as well. In

contrast, Suzuki (1999:166) eluded that alkynyl-boranes and other reagents that are considered strong Lewis acids, will hydrolyse easily in basic solutions and should be steered away from. It was also disclosed that aryl boranes have increased yields when cross-coupled with aryl halides (Maddaford & Keay, 1994:6501-6502).

Steric hindrance is a negligible problem and does not affect bi-aryl substitution too much, but it has been reported that aryl-boronic acids that are disubstituted on the *ortho*-position, may produce low yields (Suzuki, A., 2004:363; Chi *et al.*, 2000:921).

Experimental evidence exists that allows for the possibility of homo-coupled product formation, also known as “aryl scrambling” (Wallow & Novak, 1994:5036), where the boronic acid reagent binds to “itself” instead of the scaffold to form a symmetrical bi-aryl compound that is not anticipated but may still be of use to serve other purposes. This is common when using Palladium catalysts. The problem is that it may dramatically reduce the yields obtained from the reactions. The mechanisms that are most likely involved are 1) the “halogen-metal exchange” of the transmetalating agent and the organic halide, 2) reduction elimination of Palladium (IV), 3) reduction elimination of Palladium(II) complex, 4) radical mechanism reactions and 5) “disproportionation reactions” (Van Asselt & Elsevier, 1994:1978).

It is therefore of primary importance to achieve ideal reaction conditions and stoichiometric quantities of the reagents to diminish the possibility of homo-coupled compounds, although the formation of the by-products is inevitable. Another consideration in trying to achieve disubstituted compounds, is the likelihood of synthesising monosubstituted products when the boronic acid only binds to one spot. This may not be detrimental since the products may have good affinity on their own or be used as intermediates in future studies, but this may complicate the purification process.

Wolfe *et al.* (1999:9556) owes the reaction’s success to the electron-richness of the ligands. This boosts the oxidative addition rate whilst maintaining the palladium catalyst in solution. The other reason for its effectivity, is the steric bulk of the ligands which amplifies the reductive elimination

rate and increases the trans-metalation rate by augmenting the amount of L_1Pd complexes.

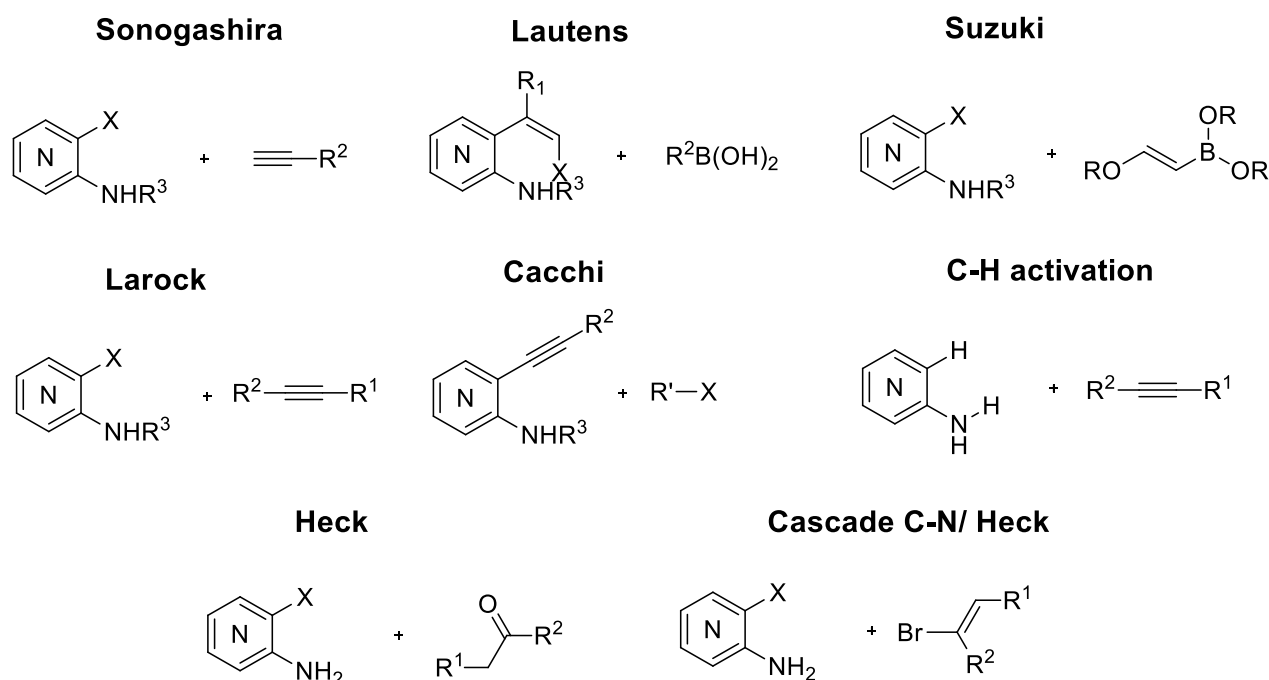


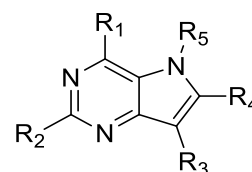
Figure 2-32: The reported methods used to construct an azaindole core by means of metal-catalysation, as adapted from Santos *et al.* (2018:2)

The synthesis of the azaindole presents an extraordinary challenge. A strategy that is often used, is to employ substituted pyridines as a starting point to derive pyrrole rings from. Pyridine rings have electron-deficient attributes which changes the π -system's electronics. Therefore, conventional methods utilised for the formation of indole moieties are insufficient for azaindole derivative formation (Song *et al.*, 2007:1120-1121). There are several methods to construct the azaindole core – see **Figure 2-32**. Usually, functionalisation of the azaindole scaffold with conventional methods is convoluted, but Chi *et al.* (2000:921-922) used the Stille-, Suzuki-, and Heck coupling reactions to successfully functionalise the 7-azaindole on the 2nd position. Allegretti *et al.* (2001:611) also used a palladium-catalysed method to obtain a functionalised 7-azaindole on the 4th and 6th positions according to their placement. There are also numerous ways to construct the 7-deazapurine scaffold - refer to **Table 2-2**.

Table 2-2: Compilation of various synthesis routes to produce the 7-deazapurine scaffold, as adapted from Pathania & Rawal (2018:505)

1)		
2)		
3)		
4)		
5)		
6)		
7)		
8)		

9)		
10)		
11)		
12)		
13)		
14)		
15)		
16)		
17)		



The scaffolds that are of interest in this study - 7-azaindole and 7-deazapurine – will be dichlorinated on the 4th- and 6th positions and used as starting material to undergo the Suzuki coupling with various boronic acid substituents. Chlorinated compounds are targeted drug candidates in the pharmaceutical industry. Heteroaromatic or olefinic moieties benefit greatly from non-reactive chlorine substitution. A chlorine substituent improves the lipophilicity of a compound; which, in return, increases the partitioning in proteins' lipid domains and cell membranes' lipophilic phases. As a result, there will be an increase of the drug concentration at the biological active site. This is not applicable to all compounds but is significant in unsaturated and heteroaromatic structures as mentioned above (Fang *et al.*, 2019:118).

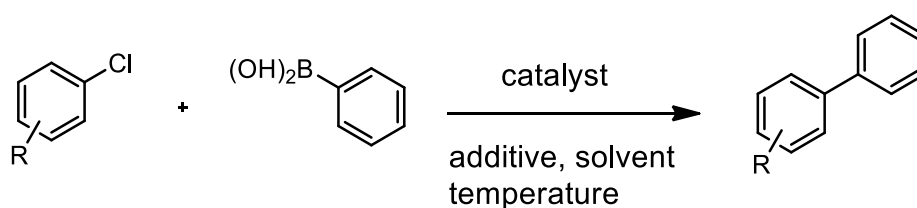


Figure 2-33: Suzuki coupling with aryl chloride and boronic acid reagents, as portrayed in [Littke & Fu \(2002:4183\)](#)

Functionalisation of these scaffolds will follow the basic synthetic methodology as described in [Littke & Fu \(2002:4183\)](#) where organic aryl chlorides undergo Suzuki coupling with boronic acids in the presence of a palladium catalyst, an additive, ideal temperature/reaction conditions and a suitable solvent system (refer to **Figure 2-33**).

The investigation of the variety of reported synthetic methods hints that the Suzuki cross-coupling method is suitable to bring the proposed compounds into fruition and will be explored in this study.

2.7 Summary

This chapter explores how a multitargeted approach with antagonistic adenosine A₁ and/or A_{2A} receptor signalling can serve to halt the progression of neurodegenerative disorders. A₁ AR antagonism being promising for enhancing cognition and A_{2A} AR inhibition is lucrative for the improvement and neuroprotection of the locomotor degeneration. The main malefactors of the aetiology of neuropathological diseases are neuroinflammation, oxidative stress and mitochondrial dysfunction. Clarification needs to be provided on whether any one of these play a bigger part and how the pathophysiology overlap with one another. Design of novel and selective ligands will be challenging since similar binding bodes to the various ARs and the ubiquity of ligands have proved difficult to overcome in the past, especially since only a handful of compounds have progressed in clinical stages of development. Raised levels of ATP correlates

with CNS conditions, as well as an increase in A_{2A} AR stimulation. The 7-deazapurine and 7-azaindole scaffolds have proven to be versatile and have many drug discovery applications, displaying inherent antioxidative properties for neuroprotection and favourable properties for permeation across the BBB. Investigation of the hypotheses and contributing factors for depression, cognitive decline, neurodegenerative diseases, like AD and PD, and the motor dysfunction that is associated with these disorders will provide insight to confirm or deny adenosine's involvement in these diseases and determine if purinergic signalling is a worthy target for design and synthesis of potential pharmacotherapies that can mitigate these disorders. Potential ligands can be synthesised using Suzuki coupling synthetic methods and compared with existing compounds by means of radioligand binding assays. Identifying trends in structure activity relationships (SARs) obtained from molecular modelling will be valuable for future research.

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CHAPTER 3 ARTICLE

Chapter 3 consists of the article entitled **“Pyrrolopyrimidine analogues with adenosine rA_1 and/or rA_{2A} receptor affinity in the low micromolar range”** which will be submitted to the journal, Molecular Diversity. The article presents and evaluates the biological and molecular modelling findings of this study.

The article was prepared in compliance with the Author Guidelines of the journal, Molecular Diversity (refer to **ANNEXURE F**), and can be accessed via the following link:

<https://www.springer.com/journal/11030/submission-guidelines>

Permission Letter

Subject: Permission Letter

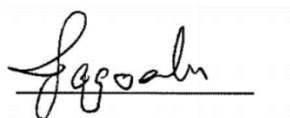
December 17, 2020

To Whom It May Concern:

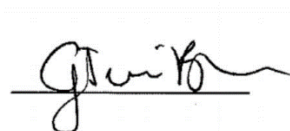
I, Nadia Janse van Vuuren, obtained permission from the co-authors to include this article, **"Pyrrolopyrimidine analogues with adenosine rA_1 and/or rA_{2A} receptor affinity in the low micromolar range"**, in Chapter 3 of the dissertation entitled **"Synthesis and evaluation of 7-deazapurine derivatives as ligands of adenosine A1 and A2A receptors"**.

Sincerely,

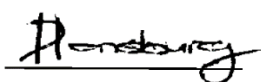
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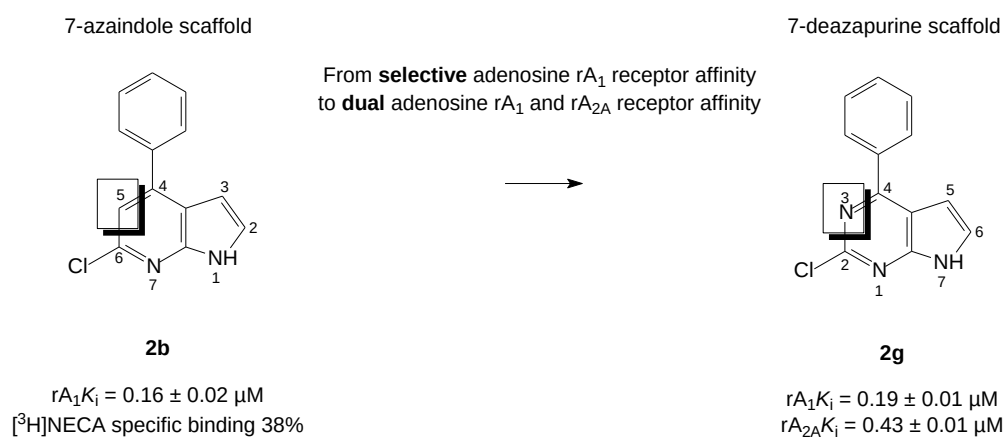
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Prof. G. Terre'Blanche

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Ms. H.D. Janse van Rensburg

Graphical abstract



Pyrrolopyrimidine analogues with adenosine rA_1 and/or rA_{2A} receptor affinity in the low micromolar range

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Declarations

Conflict of interest

The authors declare no conflict of interest.

Abstract

In a pilot study, eleven (11) pyrrolopyridine and pyrrolopyrimidine derivatives (specifically, 7-azaindoles (**2a–e**) and 7-deazapurines (**2f–k**)) were synthesised by Suzuki cross-coupling reactions and evaluated via radioligand binding assays as potential adenosine receptor (AR) antagonists in order to investigate the structure-activity relationships of these compounds further. Compound **2b**, with a 7-azaindole scaffold, was identified as a selective A₁ AR antagonist with a rA_1K_i value of $0.16 \pm 0.02 \mu\text{M}$, and interestingly, the addition of a N-atom to the aforementioned fused heterocyclic ring system, creating corresponding 7-deazapurines, led to a dual A₁/A_{2A} AR ligand (**2g**: rA_1K_i : $0.19 \pm 0.02 \mu\text{M}$; $rA_{2A}K_i$: $0.43 \pm 0.01 \mu\text{M}$). Introducing an additional N-atom into the heterocyclic ring system was tolerable for rA_1 AR affinity and led to rA_{2A} AR affinity. A molecular docking study revealed that the interactions between test compounds (**2b** & **2g**) and the A₁ and/or A_{2A} ARs most likely involve pi-pi stacking of the bicyclic ring systems and residues, such as Phe171 at the A₁ ARs and Asn253 at the A_{2A} ARs as well as conventional hydrogen bond interactions with the N-H group of the said scaffolds. This pilot study concluded that 7-azaindole and 7-deazapurine derivatives represent interesting scaffolds to investigate further structure-activity relationships in the design of dual A₁/A_{2A} AR antagonists for the treatment of neurodegenerative disorders.

Keywords

7-deazapurine derivatives

7-azaindole derivatives

Suzuki cross-coupling reactions

Adenosine A₁ and/or A_{2A} receptor antagonists

Parkinson's disease

Alzheimer's disease

Introduction

The structures of adenosine receptor (AR) antagonists are varied, but the heteroaromatic feature is consistent throughout the library of mono-, bi-, and tricyclic derivatives. Compounds with affinity against the ARs are generally based on xanthine- or nucleoside structures. However, some compounds do not conform to these structures and several non-xanthine- and non-nucleoside derivatives exist [1]. Synthetic strategies involve the alteration of nucleoside-, purine- and other

heterocyclic scaffolds [2]. The nucleotide- and nucleoside derivatives originate from the adenosine triphosphate (ATP) structure, which can be simplified to adenosine and adenine, a nucleobase, by metabolic processes. These compounds naturally occur in the human body and exert vital functions and the term “purinergic signalling” embodies these activities. G-coupled protein receptors (GPCRs) are a large family of transmembrane receptors and include ARs [3].

ARs’ potential as a drug target for pharmacotherapy development is still not exhausted and research to utilise these receptors for neurodegenerative treatment purposes remains a hot topic. There is a need for strategies to increase bioavailability, streamline synthetic methodology, and improve the potential AR ligands’ physicochemical properties.

Even though the ARs predominantly share the same genetic makeup, the structural characteristics, localisation in the brain, shape of the receptors and residues that line the receptor cavities, vary. It is therefore possible to design compounds that are selective for a specific subtype [4]. Analyses of the results of various studies have justified the use of A_1 AR antagonists for the improvement of cognitive decline and the A_{2A} AR antagonists for use in neurodegenerative diseases, such as PD, and the mitigation of the motor symptoms and non-motor symptoms, like depression, that accompany these disorders [5]. Some neuropathophysiological conditions are associated with elevated adenosine levels. Several studies showed that the A_{2A} ARs are upregulated during neuropathological states and, thus the administration of A_{2A} AR antagonists might provide a solution. It is suggested that surplus adenosine, often associated with neurodegeneration, activates the A_{2A} ARs in the signalling pathways, which further offsets dopamine stimulation at the dopamine D_2 receptors [4]. The inception of acute neurodegeneration during stressful events, such as ischaemia or traumatic brain injury, overstimulates A_1 ARs and leads to desensitization of the receptors [5]. A multitarget approach might be the solution in neurodegenerative disorders like Parkinson’s disease (PD), since dual A_1/A_{2A} AR antagonism can thus provide added neuroprotective benefits.

In 2019, the xanthine-based A_{2A} AR antagonist, istradefylline, was approved as an add-on therapy to improve motor dysfunction associated with PD. The disadvantages of istradefylline are light sensitivity, low water solubility and its efficacy as monotherapy for PD is under question. ZM-241385 (**Figure 1**), another compound popular for its [1,2,4]triazolo[1,5-a][1,3,5]triazine core, experience handicaps in oral bioavailability and metabolic stability due to the metabolic reactivity of the furan ring. Previously explored ligands such as ZM-241385 ($A_{2A}K_i = 0.8$ nM) [6,7,8] and CGS-15943 ($A_1K_i = 1.9\text{--}6.5$ nM) (**Figure 1**) [9], with favourable results for binding affinity for the A_1 or A_{2A} ARs have served as foundation to build upon and improve AR affinity. The [1,2,4]triazolo[1,5-a][1,3,5]triazine, which is the central scaffold for ZM-241385, is planer and bicyclic like most A_{2A} AR ligands.

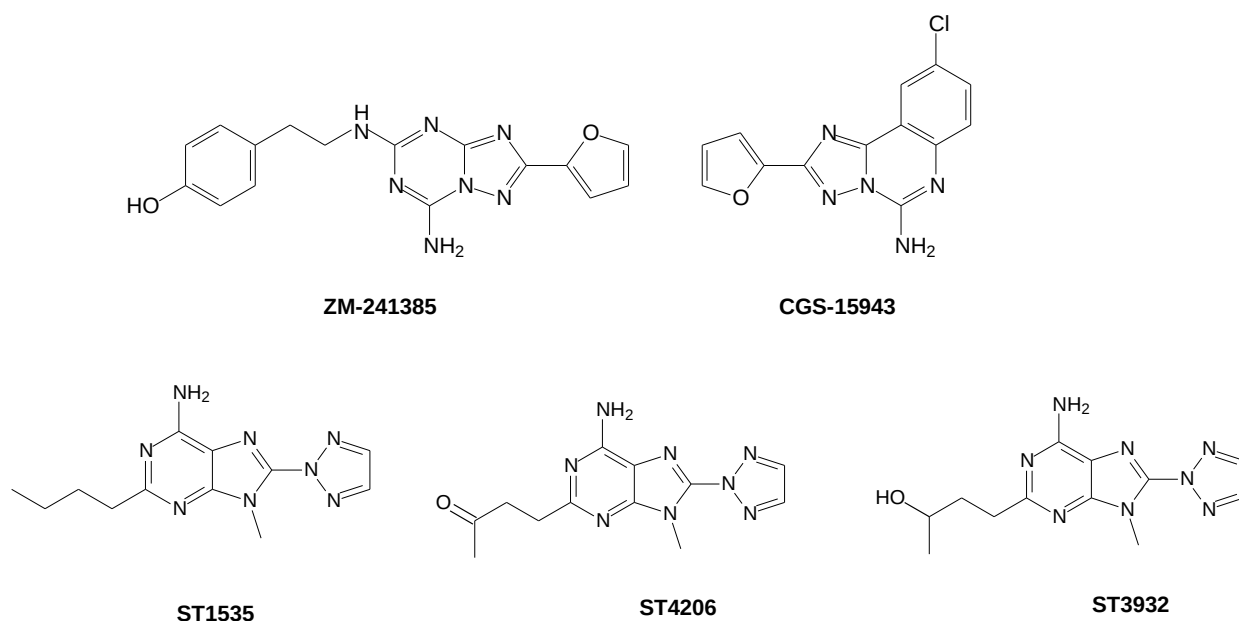


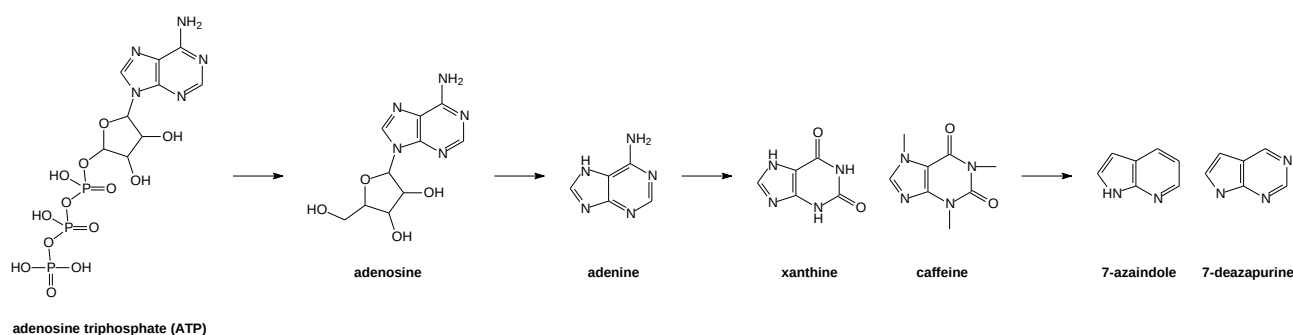
Figure 1: The chemical structures of ZM-241385, CGS-15943, ST1535, ST4206 and ST3932.

More recently, an adenine derivative, ST1535 (**Figure 1**), categorized as a non-xanthine dual antagonist against both the A_1 and A_{2A} ARs, received attention due to its safety and tolerability profiles in humans. Active metabolites of ST1535, namely ST3932 and ST4206, showed lasting pharmacological activities, including amelioration of cognitive deficits in PD and preclinical efficacy in animal studies [4,10]. In 2015, clinical trial testing still had to be done on ST1535 and its metabolites, but to date, progressed to Phase I clinical testing together with the following A_{2A} AR antagonists: PBF-509 (undisclosed structure) and V81444. These drugs are speedily absorbed, and dosages are indicated for twice-daily use with minimal excretion in the urine [11]. ST1535 has substituents on the 4th and 6th positions on the scaffold and has an affinity of 6.6 nM for A_{2A} ARs and 71.8 nM affinity for the A_1 ARs [10].

As mentioned above, most xanthine derivatives are associated with bioavailability issues, inadequate selectivity and poor water solubility, except for caffeine due to methylation of the nitrogen atoms [12, 13]. Therefore, the non-xanthine scaffolds are widely explored to design AR ligands with better physicochemical properties. Indole moieties are popular in the pharmaceutical industry and can be used as a scaffold for many targeted therapies. The azaindole is favoured for drug design; it has a fused bicyclic ring system with unique properties, it is commercially available, has numerous binding modes (normal, flipped and non-hinge binding) and the nitrogen heteroatoms facilitate bidentate hydrogen bonds [14]. Pyrrolopyrimidine-based drugs can have extensive pharmacological profiles with anticancer, antiviral, antibacterial, anti-hypertensive, anti-inflammatory and anti-hyperglycaemic, activities. The FDA approved the following

pyrrolopyrimidine derivatives: pemetrexed, ruxolitinib, tofacitinib, baricitib and ribociclib [15]. Not all the heteroaromatic groups may be useful for A₁ and/or A_{2A} AR binding, and removal of unnecessary groups can improve or diminish the selectivity and drug-like properties.

Inspired by chemical similarity to [1,2,4]triazolo[1,5-a][1,3,5]triazine core (**Figure 2**), it is envisaged that appropriately substituted 7-azaindole and 7-deazapurine analogues will exhibit good A_{2A} affinity, especially because modelling studies suggested that not all of the nitrogen atoms are involved in the interaction with the A_{2A} binding pocket [16, 17]. Suitable substitution and addition with certain groups and heterocyclic rings, may be apt in the creation of compounds that have the necessary interactions in the binding pocket of the ARs, by having either allosteric modulatory [18] or competitive inhibitory effects.



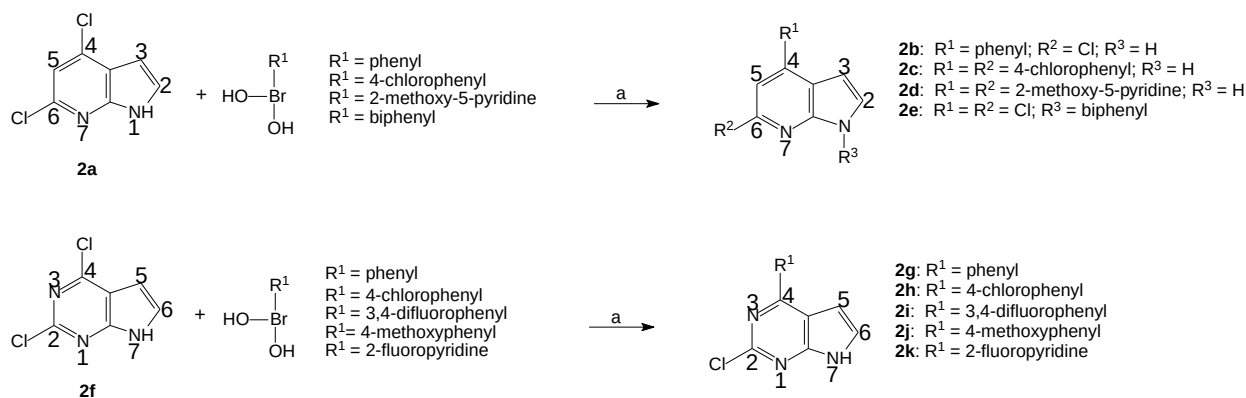
Scheme 1: Truncation of adenosine triphosphate (ATP) to 7-azaindole and 7-deazapurine.

Extensive research on the four AR subtypes (A₁, A_{2A}, A_{2B} and A₃) has been done during the last 30 years and in continuation of these efforts, the current study focused on the design, synthesis and evaluation of 7-deazapurine and 7-azaindole derivatives for dual antagonistic binding at the A₁/A_{2A} ARs via radioligand binding assays and molecular modelling.

Results and discussion

Chemistry

As depicted in **Scheme 1**, compounds **2b–e** & **2g–k** were synthesised by a cross-coupling reaction (specifically, a Suzuki reaction) utilising a transition metal catalyst (namely tetrakis(triphenylphosphine)palladium, abbreviated either Pd(PPh₃)₄ or PdP₄). The appropriate boronic acids were coupled with either 4,6-dichloro-1H-pyrrolo[2,3-b]pyridine (denoted compound **2a**) to obtain compounds **2b–e** or 2,4-dichloro-7H-pyrrolo[2,3-d]pyrimidine (**2f**) to yield compounds **2g–k**.



Scheme 2: Synthesis of 2b–e & 2g–k. Reagents and conditions:

a) $\text{Pd}(\text{PPh}_3)_4$, 2 M K_2CO_3 , toluene/ethanol (3:1), 80–100°C.

Notably, Stille [19] and Suzuki [20] methods are two of the most revered cross-coupling reactions. The Stille reaction is a less desirable method than Suzuki; due that stoichiometric quantities of tin are required which is not ideal for large-scale pharmaceutical applications.

The exact mechanism of the Suzuki cross-coupling reaction is unknown, but Fitton & Rick [21] inferred that it starts with oxidative addition followed by the trans-metalation process, and finally, reductive elimination. It is speculated that electron withdrawing groups, like the organochlorides **2a** and **2f**, lean toward the aforementioned reductive elimination.

Wolfe *et al.* [22] attribute the reaction's success to the electron-richness of the ligands. This boosts the oxidative addition rate while maintaining the palladium catalyst in solution. The other reason for its effectivity, is the steric bulk of the ligands which amplifies the reductive elimination rate and increases the trans-metalation rate by augmenting the amount of L_1Pd complexes.

Experimental evidence exists that suggests the possibility of homo-coupled product formation, also known as “aryl scrambling” [23], where the boronic acid reagent binds to “itself” instead of the scaffold to form a symmetrical bi-aryl compound that is not anticipated but may still be of use to serve other purposes. In the present study, this homo-coupled product was observed when monitoring reaction progress. Homo-coupling is common when using Palladium catalysts. The problem is that it may dramatically reduce the yields obtained from the reactions; indeed, this was the case with poor yields obtained for compounds **2d** and **2g–h** ranging from 26–42%. The mechanisms that are most likely involved are (1) the “halogen-metal exchange” of the trans-metalating agent and the organic halide, (2) reduction elimination of Palladium (IV), (3) reduction elimination of Palladium(II) complex, (4) radical mechanism reactions and (5) “disproportionation reactions” [24]. Therefore it is of primary importance to achieve ideal reaction conditions and stoichiometric quantities of the reagents to diminish the possibility of homo-coupled compounds.

It seems that the formation of by-products is inevitable. Another consideration in trying to achieve disubstituted compounds (such as **2c–d**), is the likelihood of synthesising monosubstituted products as was the case for compounds **2b** and **2g–k**. Monosubstituted product formation may not be disadvantageous since these products may have good affinity for a specific drug target.

Test compound **2e** has a biphenyl group bound to the N-H of the 7-azaindole scaffold, unexpectedly synthesised via Buchwald-Hartwig amination described in Sunesson *et al.* [25]. Buchwald-Hartwig amination was first reported with the use of nickel catalysts. In this case, the palladium catalyst instigated the amination most probably due to the basic reaction environment [25].

Biology

The affinities of the test compounds **2a–k** against rat (r) A₁ and A_{2A} ARs were determined by radioligand binding assays and are expressed as inhibition constant (K_i, μM) values (**Table 1**). Affinity against the rA₁ and rA_{2A} ARs were determined using 1,3-[³H]-dipropyl-8-cyclopentylxanthine ([³H]DPCPX) and [³H]5'-N-ethylcarboxamide-adenosine ([³H]NECA), respectively.

The unsubstituted parent compound of the 7-azaindole scaffold, namely **2a**, exhibited A₁ AR affinity (rA₁K_i = 3.8 μM) but no affinity for A_{2A} ARs ([³H]NECA = 29%), whereas **2f** (the unsubstituted parent compound of the 7-deazapurine scaffold) was devoid of affinity for either the A₁ or A_{2A} ARs (**2f**: [³H]DPCPX = 43%; [³H]NECA = 31%).

The test compounds **2b**, **2d**, **2g** and **2k** exhibited affinity against the rA₁ and/or rA_{2A} ARs below 1 μM. Generally, the test compounds showed better affinity against the rA₁ AR than the rA_{2A} AR; **2b** showed the best selective rA₁ AR affinity (**2b**: 0.16 ± 0.02 μM [³H]NECA = 38%). The incorporation of a phenyl ring at position C4 of the 7-azaindole scaffold, as seen with **2b**, increased selective rA₁ AR affinity almost 25 fold upon comparison to the 4-Cl substituted **2a** (**2a**: 3.8 μM; [³H]NECA = 29%). Notably, neither of these compounds (**2a** & **2b**) possess A_{2A} AR affinity. Bi-substitution of the 7-azaindole scaffold at positions C4 and C6 with *p*-Cl phenyl rings (exemplified by test compound **2c**) decreased A₁ AR affinity upon comparison to its unsubstituted counterpart **2a**. Interestingly, A₁ AR affinity below 0.5 μM was obtained when the *p*-Cl phenyl rings (**2c**: [³H]DPCPX = 75%) were replaced with 2-methoxypyridine rings (**2d**: rA₁K_i = 0.44 ± 0.04 μM) on positions C4 and C7 of the said scaffold. Test compound **2e** substituted at the NH-group showed neither A₁ nor A_{2A} AR affinity. Apparently replacing the hydrogen atom on the pyridine ring with a biphenyl (**2a** versus **2e**) diminished A₁ AR affinity (**2e**: [³H]DPCPX = 130%; [³H]NECA = 128%). Specific binding greater than 100% indicates saturated binding. Perhaps suggesting

that the NH-group of the pyrrole ring is essential to selective A₁ AR affinity and that larger groups may not be well tolerated.

As stated, changing the unsubstituted 7-azaindole **2a** to the corresponding 7-deazapurine **2f** diminished A₁ affinity; however, changing the 7-azaindole moiety (as in **2b**) to the corresponding 7-deazapurine moiety (as in **2g**) led to a dual A₁/A_{2A} AR ligand with the highest A₁/A_{2A} AR affinity in the low micromolar range (**2g**: rA₁K_i = 0.19 ± 0.01 μM; rA_{2A}K_i = 0.43 ± 0.01 μM) with a selectivity index for the rA₁ isoform of 2.3. This finding suggested that 7-deazapurine is a better scaffold for compounds with dual A₁/A_{2A} AR affinity 7-azaindole (**2b** versus **2g**). Furthermore, the addition of a 4-chloro substituent on the phenyl ring of compound **2g**, yield compound **2h** which was devoid of both A₁ and A_{2A} AR affinity (**2h**: [³H]DPCPX = 36%; [³H]NECA = 25%). A 3,4-difluoro substituent on the phenyl ring of test compound **2f** remarkably led to another dual A₁/A_{2A} antagonist with relatively good affinity (rA₁K_i = 1.4 ± 0.16 μM; rA_{2A} K_i = 0.33 ± 0.03). Replacing the 4-chloro group on the phenyl ring present in **2h** with a 4-methoxy group (**2j**) led to a loss in A₁ and A_{2A} AR affinity (**2j**: [³H]DPCPX = 35%; [³H]NECA = 57%). The 2-fluoropyridine substituted test compound, **2k**, in contrast to the 4-chlorophenyl (**2h**) and 4-methoxyphenyl (**2j**) substituted compounds, showcased dual compound with micromolar A₁/A_{2A} AR affinity (rA₁K_i = 0.72 ± 0.10 μM; rA_{2A} K_i = 0.52 ± 0.08 μM).

The 7-azaindole scaffold was more suited for A₁ AR binding while 7-deazapurine scaffold would be more suited for dual A₁ and A_{2A} affinity; demonstrated by comparison of test compounds **2b** and **2g**. These scaffolds may be a good starting point to further explore 7-deazapurine and 7-azaindole derivatives (**Figure 3**). These findings corroborate the literature since Gillespie and co-workers [26] found that two nitrogen atoms in a heterocyclic ring were ideal to enhance both the A₁ and A_{2A} AR binding upon comparison of the binding affinities of pyridine, pyrimidine and triazine derivatives. The latter statement was based on their finding that the pyridine scaffold's binding affinity was seven-fold less potent than the triazine and 45-fold less potent than the corresponding aminopyrimidine. As a limited number of derivatives were synthesised and evaluated, these structure-activity relationships should be seen as preliminary.

Table 1: Inhibition constant (K_i) values of pyrrolopyrimidine derivatives against rA_1 and rA_{2A} ARs.

^a All inhibition constant (K_i) values were determined in triplicate and expressed as mean $\pm$ standard error of the mean (SEM) in μ M.

^b Specific binding of the radioligand at a maximum tested concentration of 100 μ M was determined in duplicate and expressed in percentage (%).

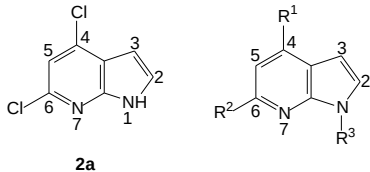
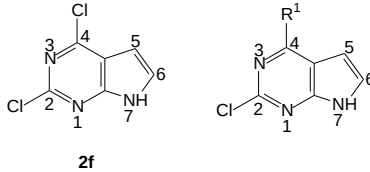
^c Rat (r) whole brain membrane proteins expressing the A_1 AR were used.

^d Rat (r) striatal membrane proteins expressing the A_{2A} AR were used.

^e 0.1 nM [³H]DPCPX

^f 4 nM [³H]NECA

^g Selectivity index (SI) for the rA_1 AR isoform calculated as a ratio of $rA_{2A}K_i/rA_1K_i$.

#	$K_i \pm \text{SEM } (\mu\text{M})^a$ (Specific binding (%)) ^b		SI ^g
	rA_1^c vs [³ H]DPCPX ^e	rA_{2A}^d vs [³ H]NECA ^f	rA_{2A}/rA_1
 2a 2b: R^1 = phenyl; R^2 = Cl; R^3 = H 2c: R^1 = R^2 = 4-chlorophenyl; R^3 = H 2d: R^1 = R^2 = 2-methoxypyridine; R^3 = H 2e: R^1 = R^2 = Cl; R^3 = biphenyl			
7-azaindole derivatives			
2a	3.8 ± 0.23^a	(29%) ^b	-
2b	0.16 ± 0.02^a	(38%) ^b	-
2c	(75%) ^b	(235%) ^b	-
2d	0.44 ± 0.04^a	(57%) ^b	-
2e	130% ^b	(128%) ^b	-
 2f 2g: R^1 = phenyl 2h: R^1 = 4-chlorophenyl 2i: R^1 = 3,4-difluorophenyl 2j: R^1 = 4-methoxyphenyl 2k: R^1 = 2-fluoropyridine			
7-deazapurine derivatives			
2f	(43%) ^b	(31%) ^b	-
2g	0.19 ± 0.01^a	0.43 ± 0.01^a	2.3
2h	(36%) ^b	(25%) ^b	-
2i	1.4 ± 0.16^a	0.33 ± 0.03^a	0.24
2j	(35%) ^b	(57%) ^b	-
2k	0.72 ± 0.10^a	0.52 ± 0.08^a	0.72

7-azaindole scaffold versus 7-deazapurine scaffold

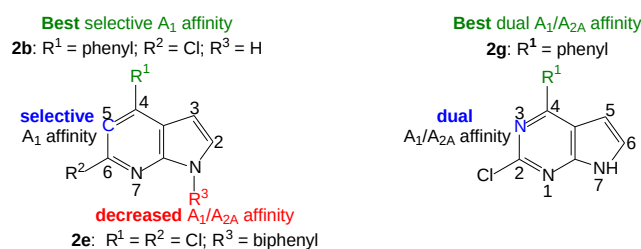


Figure 2: The structure-activity relationships (SAR) of the 7-azaindole and 7-deazapurine scaffolds.

Test compound **2b** with the best A₁ AR affinity out of all the test compounds was selected as representative and subjected to a GTP shift assay. The results suggested that compound **2b** acted as an A₁ AR antagonist based on the binding curve in the presence of GTP that was almost unaffected and the calculated GTP shift of approximately 1 (**Table 2** and **Fig. 4**) [29, 30]. Based on the likeness of the test compounds, it may be concluded that all test compounds (**2a–k**) were A₁ AR antagonists.

Table 2: A₁K_i values (in the absence and presence of GTP) of **2b** and calculated GTP shifts

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

^b Rat (r) receptors were used (rA₁: rat whole brain membranes).

^c 0.1 nM [³H]DPCPX

^d Addition of 100 μM GTP to rA₁ AR radioligand binding assay.

^e GTP shift calculated by dividing K_i value in the presence of 100 μM GTP by K_i value in the absence of 100 μM GTP.

#	K _i ± SEM (μM) ^a		GTP shift ^e
	rA ₁ ^b vs [³ H]DPCPX ^c	rA ₁ ^b + GTP ^d vs [³ H]DPCPX ^c	
2b	0.16 ± 0.02 ^a	0.15 ± 0.01 ^a	0.94

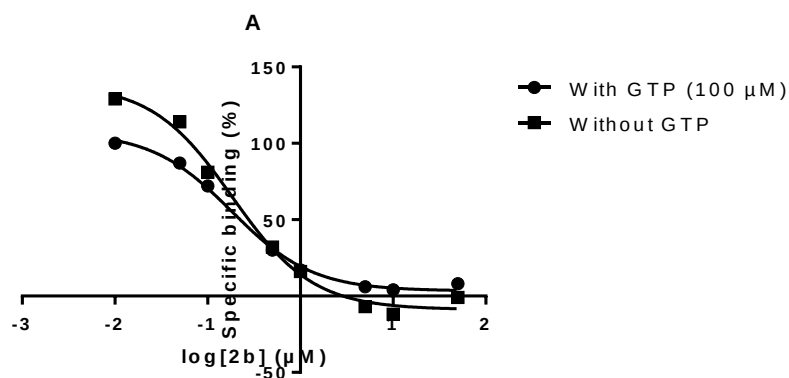


Figure 3: The binding curve of compound **2b** (A) is an example of A₁ AR antagonistic action determined via a GTP shift assay (with and without 100 μM GTP) in rat whole brain membranes expressing rA₁ ARs with [³H]DPCPX as radioligand determined in triplicate. With a calculated GTP shift of 0.94 (**2b**).

The radioligand binding assays were validated with DPCPX (A₁ antagonist), istradefylline (A_{2A} antagonist) and caffeine (dual A₁/A_{2A} antagonist) as reference compounds and results were in accordance with literature (**Table 3**).

Table 3: Inhibition constant (K_i) values against rA_1 and rA_{2A} ARs as well as GTP shifts of DPCPX, istradefylline and caffeine.

^a All inhibition constant (K_i) values were determined in triplicate and expressed as mean $\pm$ standard error of the mean (SEM) in μ M.

^b Rat (r) receptors were used (rA_1 : rat whole brain membranes).

^c Rat (r) receptors were used (rA_{2A} : rat striatal membranes).

^d 0.1 nM [3 H]DPCPX

^e 4 nM [3 H]NECA

^f Addition of 100 μ M GTP to rA_1 AR radioligand binding assay.

^g Selectivity index (SI) for the A_1 AR isoform calculated as a ratio of $A_{2A}K_i/A_1K_i$.

^h GTP shift calculated by dividing K_i value in the presence of 100 μ M GTP by K_i value in the absence of 100 μ M GTP.

ⁱ Literature values obtained from Alnouri et al. [28]

#	$K_i \pm \text{SEM} (\mu\text{M})^a$			SI ^g	GTP shift ^h
	rA_1^b vs [3 H]DPCPX ^d	rA_{2A}^c vs [3 H]NECA ^e	$rA_1^c + \text{GTP}^f$ vs [3 H]DPCPX ^d		
DPCPX (A_1 antagonist)	0.0005 ± 0.00003^a (0.0005) ⁱ	0.23 ± 0.03^a (0.16) ⁱ	0.0006 ± 0.00003^a	460	1.2
Istradefylline (A_{2A} antagonist)	0.19 ± 0.01^a (0.23) ⁱ (0.15) ⁱ	0.0014 ± 0.0003^a (0.0022) ⁱ (0.0052) ⁱ	0.15 ± 0.02^a	0.007	0.8
Caffeine (A_1/A_{2A} antagonist)	47.1 ± 1.9^a (18.8) ⁱ (41.0) ⁱ	41.0 ± 1.2^a (43.0) ⁱ (32.5) ⁱ	48.8 ± 2.0^a	0.87	1.04

In order to gain more insight on structure activity relationship, based on *in vitro* evaluation of the test compounds **2a–k**, *in silico* evaluation of the best selective A_1 (**2b**: rA_1K_i = 0.16 μ M) and dual A_1/A_{2A} (**2g**: rA_1K_i = 0.19 μ M; $rA_{2A}K_i$ = 0.43 μ M) AR ligands were performed using AutoDock Vina. The selected test compounds **2b** and **2g** were each docked into a model of the binding site of the hA_1 (PDB ID: 6D9H) and hA_{2A} (PDB ID: 3PWH) ARs. Visual inspection of the docked poses with the most favourable AutoDock Vina interaction energy revealed that the fused 6- and 5-membered rings present in the 7-azaindole scaffold of **2b** and the 7-deazapurine scaffold of **2g** were both pi-pi stacked with Phe171 at the A_1 AR (**Figure 5**). The presence of an additional N-atom in the 7-deazapurine scaffold (**2g**), when compared to **2b**, does not seem to influence *in silico* binding at the A_{2A} AR, however, this was not the case with *in vitro* results. An additional N-atom led to dual A_1/A_{2A} AR affinity *in vitro* (**2b** versus **2g**). In general, the docking results gave no clear explanation for the observed selective A_1 and dual A_1/A_{2A} AR affinity of **2b** and **2g**. Future studies can compare rat and human docking results to identify similarities and differences.

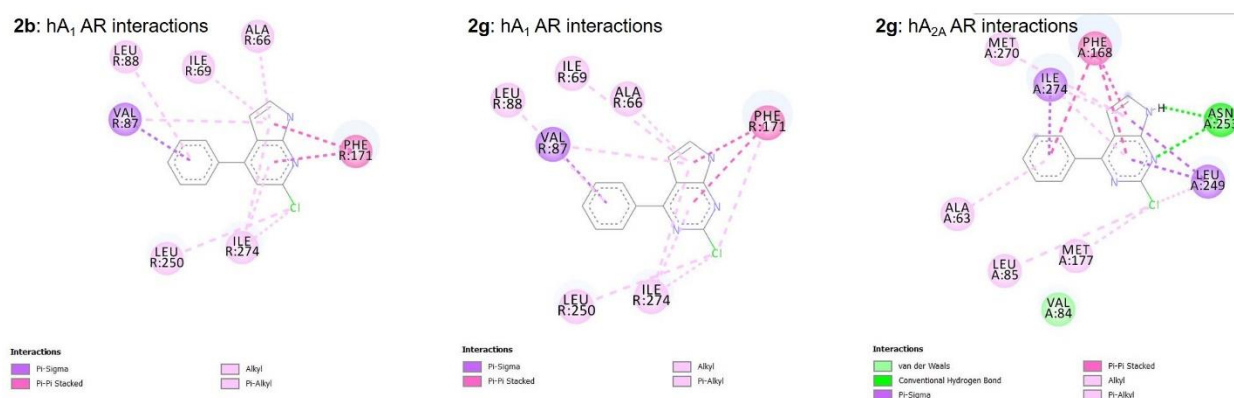


Figure 4: Molecular docking interactions of test compounds **2b** and **2g** with A₁ and/or A_{2A} ARs.

There are some discrepancies when comparing the *in vitro* results obtained from the radioligand binding assays with the *in silico* binding affinities. Molecular docking studies should only be taken at face value as it cannot simulate *in vivo* interactions given that the receptors are crystallised and isolated. Biological evaluation was performed on male Sprague Dawley rats, whereas the ARs, retrieved from the protein data bank (PDB), were crystallised from human ARs. The protein data bank has an orthologue of rodent AR structures although human ARs were chosen since the goal is to explore these ligands' potential for further testing on humans.

Conclusion

The test compound **2b** proved to be a selective rA₁ AR antagonist with the best rA₁K_i value ($0.16 \pm 0.02 \mu\text{M}$) of the 7-azaindole derivatives and **2g** was a dual rA₁/A_{2A} AR ligand with affinities below $0.5 \mu\text{M}$. Thus, the 7-azaindole and 7-deazapurine scaffolds of **2b** and **2g** containing an unsubstituted phenyl ring may be optimised for the design of either selective A₁ AR or dual A₁/A_{2A} AR antagonists. These results reiterated a finding of Gillespie and co-workers [26] that two N-atoms in heterocyclic ring system seem to be ideal for increased affinity against A₁ and A_{2A} ARs. While investigating purine compounds. Koch [27] found that a simple conversion from an amino to a trifluoromethyl group influences the ARs' selectivity. Notably, Koch stated that nitrated 1-deazapurine bases have modest affinity against ARs and that the introduction of substituents at position C2 1-deazapurine scaffold resulted in slightly A_{2A} AR affinity. These findings may be taken into consideration for the future design of pyrrolopyrimidine analogues. A molecular docking study revealed that the interactions between test compounds (**2b** & **2g**) and the A₁ and/or A_{2A} ARs most likely involve pi-pi stacking of the bicyclic ring systems and select residues, such as Phe171 at the A₁ ARs and Asn253 at the A_{2A} ARs as well as conventional hydrogen bond interactions with the N-H group of the said scaffolds. This pilot study concludes that 7-azaindole and 7-deazapurine analogues represent an attractive scaffold to investigate further structure-

activity relationships in the design of dual A₁/A_{2A} AR antagonists for the treatment of neurodegenerative disorders.

Experimental section

Chemistry

Materials and reagents

All materials and reagents were commercially available and purchased from various manufacturers. Thin layer chromatography on TLC silica gel 60 F₂₅₄ aluminium sheets from Merck was used to monitor reaction progress. Melting points were determined on a Buchi M-545 melting point apparatus. Proton (¹H) and carbon (¹³C) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 600 spectrometer at frequencies of 600 MHz and 151 MHz, respectively, using either CDCl₃ or DMSO-d₆ as solvent and TMS as reference. Chemical shifts were reported in parts per million (ppm) in relation to the solvent peak (CDCl₃: residual CH at 7.26 ppm and DMSO-d₆: residual CH₃ at 2.50 ppm for ¹H NMR). Spin multiplicities were indicated as follow: singlet (s), doublet (d), triplet (t), multiplet (m), doublet of doublets (dd), doublet of triplets (dt), triplet of doublets (td). Coupling constant (*J*) values were reported in Hertz (Hz). High resolution mass spectra (HRMS) were recorded on a Bruker micrOTOF-Q II mass spectrometer in atmospheric chemical ionisation (APCI) mode.

Synthesis

6-chloro-4-phenyl-1H-pyrrolo[2,3-b]pyridine (**2b**)

4,6-dichloro-1H-pyrrolo[2,3-b]pyridine (**2a**) (0.296 g, 1.6 mmol) and 0.084 g tetrakis(triphenylphosphine)palladium(0) were added to a round bottomed flask. Thereafter, 25 mL of a toluene and ethanol mixture (3:1) was added, as well as 5 mL of a 2 M K₂CO₃ solution. The reaction mixture was refluxed, and the temperature was monitored until it reached 80°C and the reaction mixture was homogenous and only then 1.574 g (12.9 mmol) of the phenylboronic acid was added. The reaction mixture was mechanically stirred at 80–100°C under reflux while being continuously monitored by TLC. Upon completion, the reaction mixture was cooled down to room temperature and the volume reduced. The subsequent solid was rinsed with distilled water and filtered to yield the crude product which was then dried overnight (60°C) and recrystallised to yield the title compound **2b** (0.273 g, 75.2%) as brown rust powder: R_f: 0.60 (2.5PE:1EtOAc); mp: 208.4–209.4°C (EtOAc/PE); ¹H NMR (600 MHz, DMSO) δ 12.10 (s, 1H), 8.12 (d, *J* = 7.5 Hz, 2H), 7.78 (s, 1H), 7.61 (s, 1H), 7.49 (t, *J* = 7.4 Hz, 2H), 7.41 (m, *J* = 7.1 Hz, 1H), 6.52 (s, 1H); ¹³C NMR (151 MHz, DMSO) δ 150.73, 149.06,

138.65, 134.98, 128.77, 128.70, 127.89, 126.65, 117.65, 112.43, 98.13. APCI-HRMS m/z calculated for $C_{13}H_9ClN_2$ $[M+H]^+$: 229.052702, found: 229.0521.

6-chloro-4-(4-chlorophenyl)-1H-pyrrolo[2,3-b]pyridine (**2c**)

Test compound **2c** was prepared as for **2b** with **2a** (0.300 g, 1.6 mmol) and (4-chlorophenyl)boronic acid (1.254 g, 6.4 mmol) to yield **2c** (0.345 g, 63.6%) as auburn powder: Rf: 0.67 (5PE:2EtOAc:1DCM); mp:192.3-194.3 °C (ACN); 1H NMR (600 MHz, DMSO) δ 11.91 (s, 1H), 8.20 (d, J = 8.5 Hz, 2H), 7.89 (d, J = 8.4 Hz, 2H), 7.76 (s, 1H), 7.62 (d, J = 8.4 Hz, 3H), 7.54 (d, J = 8.5 Hz, 4H), 6.62 (s, 1H); ^{13}C NMR (151 MHz, DMSO) δ 149.34, 149.00, 140.08, 138.62, 137.35, 133.46, 133.19, 130.34, 129.14, 128.79, 128.52, 128.04, 116.81, 111.52, 99.28. APCI-HRMS m/z calculated for $C_{19}H_{12}Cl_2N_2$ $[M+H]^+$: 339.04503, found: 339.0465.

4,6-bis(6-methoxypyridin-3-yl)-1H-pyrrolo[2,3-b]pyridine (**2d**)

Test compound **2d** was prepared as for **2b** with **2a** (0.297 g, 1.6 mmol) and (6-methoxypyridin-3-yl)boronic acid, also known as 2-methoxy-5-pyridineboronic acid, (0.990 g; 6.5 mmol) to yield **2d** (0.107 g, 32.3%) as slate grey powder with a pink tinge: Rf: 0.67 (5PE:2EtOAc:1DCM); mp:196.6-198.7 °C (Chlf wash and ACN recrystallisation); 1H NMR (600 MHz, DMSO) δ 11.87 (s, 5H), 8.95 (s, 4H), 8.68 (s, 3H), 8.46 (d, J = 7.8 Hz, 3H), 8.22 (d, J = 7.3 Hz, 3H), 7.84 (s, 1H), 7.65 (d, J = 118.3 Hz, 5H), 7.17 (s, 1H), 6.98 (dd, J = 48.9, 8.0 Hz, 5H), 6.62 (s, 3H), 3.93 (d, J = 16.5 Hz, 12H); ^{13}C NMR (151 MHz, DMSO) δ 163.73, 163.69, 149.34, 148.05, 146.46, 145.36, 139.23, 138.14, 137.60, 129.29, 127.84, 127.38, 116.37, 110.91, 110.82, 110.49, 99.18, 53.60, 53.52. APCI-HRMS m/z calculated for $C_{19}H_{16}N_4O_2$ $[M+H]^+$: 333.134602, found: 333.1360.

1-[[1,1'-biphenyl]-4-yl]-4,6-dichloro-1H-pyrrolo[2,3-b]pyridine (**2e**)

Test compound **2e** was prepared as for **2b** with **2a** (0.300 g, 1.6 mmol) and {[1,1'-biphenyl]-4-yl}boronic acid (1.273 g, 6.4 mmol) to yield **2e** (0.180 g, 33.2%) as greyish yellow-green powder; Rf: 0.53 (2.5 PE:1EtOAc); mp:201.3-204.3 °C (Chlf wash and ACN recrystallisation); 1H NMR (600 MHz, DMSO) δ 12.43 (d, J = 1.9 Hz, 1H), 8.24 (d, J = 8.4 Hz, 2H), 7.90 (s, 1H), 7.81 (dd, J = 6.7, 2.0 Hz, 3H), 7.80-7.73 (m, 2H), 7.50 (t, J = 7.7 Hz, 2H), 7.46-7.37 (m, 1H); ^{13}C NMR (151 MHz, DMSO) δ 151.24, 147.62, 140.65, 139.38, 136.94, 134.79, 129.01, 127.75, 127.28, 127.01, 126.62, 125.66, 114.06, 112.78, 101.36. APCI-HRMS m/z calculated for $C_{19}H_{12}Cl_2N_2$ $[M+H]^+$: 339.04503, found: 339.0471.

2-chloro-4-phenyl-7H-pyrrolo[2,3-d]pyrimidine (**2g**)

Test compound **2g** was prepared as for **2b** with 2,4-dichloro-7H-pyrrolo[2,3-d]pyrimidine (**2f**) (0.300 g, 1.6 mmol) and phenylboronic acid (0.780 g, 6.4 mmol) under an inert (nitrogen)

environment to yield **2g** as yellow crystals (0.156 g, 42.6%); Rf: 0.54 (5PE:2EtOAc:1DCM); mp:236.1-240.2°C (EtOH); ¹H NMR (600 MHz, DMSO) δ 12.48 (s, 1H), 8.14 (dd, *J* = 7.6, 2.0 Hz, 2H), 7.69 (d, *J* = 3.5 Hz, 1H), 7.61 – 7.58 (m, 3H), 6.94 (d, *J* = 3.6 Hz, 1H); ¹³C NMR (151 MHz, DMSO) δ 157.65, 154.04, 152.21, 136.51, 130.79, 129.01, 128.81, 128.71, 113.73, 100.60. APCI-HRMS *m/z* calculated for C₁₂H₈ClN₃ [M+H]⁺: 230.047951, found: 230.0477.

2-chloro-4-(4-chlorophenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2h**)

Test compound **2h** was prepared as for **2b** with **2f** (0.325 g, 1.7 mmol) and (4-chlorophenyl)boronic acid (1.495 g, 9.6 mmol) under an inert (nitrogen) environment to yield **2h** (0.121 g, 26.614873%) as lemon-yellow coloured crystals; Rf: 0.67 (5PE:2EtOAc:1DCM) mp:246.9-247.4°C (ACN); ¹H NMR (600 MHz, DMSO) δ 12.53 (s, 1H), 8.18 (d, *J* = 8.5 Hz, 2H), 7.73 (d, *J* = 3.6 Hz, 1H), 7.65 (d, *J* = 8.6 Hz, 2H), 6.95 (d, *J* = 3.6 Hz, 1H); ¹³C NMR (151 MHz, DMSO) δ 156.15, 154.13, 152.08, 135.58, 135.25, 130.45, 129.11, 129.07, 113.62, 100.44. APCI-HRMS *m/z* calculated for C₁₂H₇Cl₂N₃ [M+H]⁺: 264.008979, found: 264.0083.

2-chloro-4-(3,4-difluorophenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2i**)

Test compound **2i** was prepared as for **2b** with **2f** (0.305 g, 1.6 mmol) and (3,4-difluorophenyl)boronic acid (1.022 g, 6.5 mmol) under an inert (nitrogen) environment to yield **2i** (0.369 g, 78%) as flaxen yellow crystals; Rf: 0.55 (5PE:2EtOAc:1DCM + 2 drops of ammonia solution); mp:250.1-250.5°C (ACN); ¹H NMR (600 MHz, DMSO) δ 12.57 (s, 1H), 8.11 (ddd, *J* = 11.4, 7.8, 2.0 Hz, 1H), 8.06-8.02 (m, 1H), 7.74 (d, *J* = 3.6 Hz, 1H), 7.64 (dt, *J* = 17.3, 8.5 Hz, 1H), 6.99 (d, *J* = 3.6 Hz, 1H); ¹³C NMR (151 MHz, DMSO) δ 154.97 (s), 154.17 (s), 151.94 (s), 151.81 (d, *J* = 12.7 Hz), 150.49 (d, *J* = 12.6 Hz), 150.15 (d, *J* = 12.6 Hz), 148.85 (d, *J* = 13.0 Hz), 133.95 (dd, *J* = 5.9, 3.6 Hz), 126.01 (dd, *J* = 7.1, 3.2 Hz), 118.18 (d, *J* = 17.5 Hz), 117.51 (d, *J* = 18.5 Hz), 113.59 (s). APCI-HRMS *m/z* calculated for C₁₂H₆ClF₂N₃ [M+H]⁺: 266.029108, found: 266.0281.

2-chloro-4-(4-methoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2j**)

Test compound **2j** was prepared as for **2b** with **2f** (0.302 g, 1.6 mmol) and (4-methoxyphenyl)boronic acid (0.970 g, 6.4 mmol) under an inert (nitrogen) environment to yield **2j** 0.283 g (68%) as mustard-yellow crystals; Rf: 0.52 (5PE:2EtOAc:1DCM + 2 drops of ammonia solution); mp:246.5-247°C (ACN); ¹H NMR (600 MHz, DMSO) δ 12.32 (s, 1H), 8.16 (d, *J* = 8.9 Hz, 2H), 7.63 (d, *J* = 3.6 Hz, 1H), 7.14 (d, *J* = 8.9 Hz, 2H), 6.92 (d, *J* = 3.6 Hz, 1H), 3.87 (s, 3H); ¹³C NMR (151 MHz, DMSO) δ 161.29, 157.15, 153.80, 152.06, 130.19, 128.83, 127.98, 114.26,

112.79, 100.49, 55.26. APCI-HRMS m/z calculated for $C_{13}H_{10}ClN_3O$ $[M+H]^+$: 260.058516, found:260.0569.

5-{2-chloro-7H-pyrrolo[2,3-d]pyrimidin-4-yl}-2-fluoropyridine (**2k**)

Test compound **2k** was prepared as for **2b** with **2f** (0.320 g, 1.7 mmol) and (6-fluoropyridin-3-yl)boronic acid (1.2 g, 8.5 mmol) under an inert (nitrogen) environment to yield **2k** as amber crystals (0.285 g, 67.4%); Rf: 0.43 (5PE:2EtOAc:1DCM + 2 drops of ammonia); mp:260.1-261.7°C (ACN); 1H NMR (600 MHz, DMSO) δ 12.51 (s, 1H), 8.98 (d, J = 2.5 Hz, 1H), 8.69 (td, J = 8.3, 2.5 Hz, 1H), 7.73 (dd, J = 3.6, 2.4 Hz, 1H), 7.39 (dd, J = 8.6, 2.7 Hz, 1H), 6.99 (dd, J = 3.6, 1.7 Hz, 1H); ^{13}C NMR (151 MHz, DMSO) δ 164.57, 162.98, 153.82 (d, J = 26.9 Hz), 151.94, 147.69 (d, J = 16.1 Hz), 142.06 (d, J = 8.8 Hz), 130.80 (d, J = 4.4 Hz), 129.21, 113.66, 109.92 (d, J = 37.7 Hz), 100.14. APCI-HRMS m/z calculated for $C_{11}H_6ClFN_4$ $[M+H]^+$: 249.033779 , found: 249.0328.

Biology

In vitro evaluation

Materials and reagents

All materials and reagents were commercially available and purchased from various manufacturers. Adenosine deaminase from bovine spleen (182 units/mg, 7.6 mg/mL) was obtained from Sigma-Aldrich and radioligands [3H]DPCPX (120 Ci/mmol) and [3H]NECA (27.1 Ci/mmol) from PerkinElmer. Radio activity was counted by a Packard Tri-CARB 2810 liquid scintillation counter.

Ethics

The collection of tissue samples for the A_1 and A_{2A} AR radioligand binding assays were approved by the Health Sciences Ethics Office for Research, Training and Support, North-West University (application number NWU-00592-19-A5) and were performed in accordance with the guidelines of the South African National Standard (SANS) document (The care and use of animals for scientific purposes).

Tissue samples

Male Sprague-Dawley rats were dissected to attain rat whole brain membranes (excluding cerebellum and brain stem) and rat striatal membranes for the A_1 and A_{2A} AR radioligand binding

assays, respectively. Tissue samples were prepared and stored as described in literature [31]. Protein concentrations were determined according to the Bradford assay, using bovine serum albumin as reference standard [32].

Adenosine A₁ and A_{2A} receptor radioligand binding assays

Radioligand binding assays were performed as described previously [31, 33, 34]. For the A₁ AR radioligand binding assay each sample comprised of: membrane suspension equivalent to 120 µg rat whole brain membrane proteins, 0,1 units/mL adenosine deaminase, 0,1 nM 1,3-[³H]-dipropyl-8-cyclopentylxanthine ([³H]DPCPX) and the test compound (at a chosen concentration) [31, 34]. For the A_{2A} AR radioligand binding assay each sample comprised of: membrane suspension equivalent to 120 µg rat striatal protein, 0,2 units/mL adenosine deaminase, 4 nM 5'-N-ethylcarboxamido[³H]adenosine ([³H]NECA), 50 nM CPA, 10 mM MgCl₂ and the test compound (at a chosen concentration). The final volume of all samples contained 1 mL 50 mM Tris/HCl buffer and 1% DMSO. Non-specific binding of [³H]DPCPX and [³H]NECA for the radioligand binding assays were defined as binding in the presence of 10 µM DPCPX. Specific binding was defined as the total binding minus the non-specific binding.

Guanosine triphosphate (GTP) shift assays

The type of binding affinity test compounds exhibited at the rat A₁ AR was determined via a guanosine triphosphate (GTP) shift assay, as described previously [29, 31, 35]. A GTP shift assay resembles the A₁ AR radioligand binding assay; however, it requires the addition of 100 µM GTP.

Statistical data analyses

In short, all statistical data analyses were done using Microsoft Excel and GraphPad Prism Software based on non-linear curve fitting procedures. From the half maximal inhibitory concentration (IC₅₀) values, the inhibition constant (K_i) values for the competitive inhibition of [³H]DPCPX (K_d = 0.36 nM) against rat whole brain membranes and [³H]NECA (K_d = 15.3 nM) against rat striatal membranes by the test compounds were calculated by means of the Cheng-Prusoff equation. All calculated K_i values were determined in triplicate and given as mean ± standard error of the mean (SEM). Specific binding (%) of the radioligand at a maximum tested concentration of 100 µM were determined in duplicate and expressed as the mean in %. SI index values were calculated as a ratio of the A₁ and A_{2A} AR K_i values of test compounds. GTP shifts were calculated by dividing the K_i values of compounds reported in the presence of GTP by the K_i values obtained in the absence of GTP [31, 33, 34].

***In silico* evaluation**

Molecular docking studies were carried out using AutoDock Vina [36] and a Python script collection (AutoDock Tools) [37] in order to predict the bound conformations and the binding affinity between the A₁ and A_{2A} ARs and selected test compounds.

The X-ray crystallographic structure of the cryogenic electron microscopy derived structure of the human adenosine A₁ receptor-Gi2-protein complex (Protein Data Bank (PDB) ID: 6D9H) and the thermostabilised human adenosine A_{2A} receptor (PDB ID: 3PWH) were obtained from the RCSB PDB repository. The co-crystallized ligands were removed from the structures with Discovery Studio [38]. Discovery Studio was then also used to draw the investigated compounds and to then modify their coordinates to that of each receptor's active binding sites.

Autodock Vina requires receptor and ligand representations in a pdbqt file format (modified protein data bank format) which contains atomic charges, atom type definitions and, for ligands, topological information (bond rotability). These preparations were carried out using the Python script package, Autodock Tools 1.5.6. Autodock Vina was launched via a command prompt line, with a search zone of 14 Å in the x, y and z axes and an exhaustiveness value of 8. The results were written to a log text file for later reference and the conformations were split from the output file using the Autodock Vina split subroutine.

The top conformations of each compound were converted to pdb files using Autodock Tools 1.5.6 and were imported into Discovery Studio, where they were transposed over the corresponding receptor structures. Discovery Studio was then used to define and visualise the noncovalent interactions between the compounds and the relevant amino acid residues.

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CHAPTER 4 CONCLUSION

4.1 Introduction

The past decades have been instrumental in the advancement of neurodegenerative research that focuses on the adenosine receptors (ARs). ARs form part of the G-protein-coupled receptor (GPCR) family, which are widely distributed in biological systems; the ubiquity of the receptors has made it challenging to design selective AR ligands. This study aimed to add upon existing research of neurodegeneration by means of structure-based design of A₁- and A_{2A} AR antagonists and bringing the compounds into fruition with the Suzuki cross-coupling methodology (Suzuki, A., 1985:1749-1758; Suzuki, A., 1999:148-166). This methodology was chosen since it is straightforward and requires a one-pot reaction. The pyrrolopyrimidine and -pyridine scaffolds highlighted in this study, 7-deazapurine and 7-azaindole, have several sites where functionalisation and optimisation can occur. These scaffolds are privileged with inherent antioxidative properties, as discussed in Chapter 2, where the nitrogen heteroatoms quench radical species of oxidative stress (Hall *et al.*, 1997:900).

There is still some ambiguity that surrounds the validity of targeting neuroinflammation for neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD), seeing as neuroinflammation is seen as a 'double-edged sword' that has an objective to respond to acute inflammatory triggers to heal and sort out pathogenic intrusion. However, sustained inflammation levels caused by chronic stressors can have deleterious effects and cause neuronal damage (Bonifati & Kishore, 2007:1007). The hypotheses of the neurodegenerative disorders' pathogeneses all involve mitochondrial dysfunction, oxidative stress and neuroinflammation. The exact interrelations are still unclear, which calls for more in-depth biochemical inspection and identification of biomarkers. Nonetheless, it is known that raised levels of adenosine triphosphate (ATP) (Rodrigues *et al.*, 2015:3,6), adenosine (Borea *et al.*, 2017:1947) and A_{2A} AR expression (Zygmunt *et al.*, 2015:77) accompany neuropathology and inhibition thereof, can be helpful to counteract or delay these pathological states and the non-motor symptoms, like depression (Dziubina *et al.*, 2017:228-229) and cognitive decline (Chen *et al.*, 2018:103), and motor symptoms (Marcellino *et al.*, 2010:465) that are resultant of these states.

4.2 Specific findings and conclusions

The 4th and 6th positions on both the 7-deazapurine and 7-azaindole scaffolds are promising for the design of A₁- and A_{2A} AR antagonists and the hypothesis is confirmed since the derivatives revealed *in vitro* affinity for rat A₁ and A_{2A} ARs. Most of the test compounds were monosubstituted on the 4th position or "R₁" as indicated in **Figure 1-5** in Chapter 1. Disubstituted compounds have

indicated to fit better into the A₁ AR cavity, which can be a means to improve selectivity, whereas the A_{2A} AR pocket is narrower and more elongated, which limits the number of binding conformations. The 7-deazapurine derivatives, with an extra nitrogen heteroatom, have improved dual A₁ and A_{2A} AR binding affinity and inhibition constant (*K_i*) values *in vitro*; while 7-azaindole derivatives showed selectivity for A₁ ARs.

Test compounds with 7-azaindole scaffolds, **2b** and **2d**, displayed the best *in vitro* inhibition results at the A₁ ARs with low micromolar affinity: $0.16 \pm 0.02 \mu\text{M}$ and $0.44 \pm 0.04 \mu\text{M}$, respectively. Disubstituted test compound, **2d**, showcased four binding conformations in the A₁ AR cavity with unique amino acid interactions (refer to **ANNEXURE D**). The 7-deazapurine derivatives, **2g** ($0.19 \pm 0.01 \mu\text{M}$ at the A₁ ARs and $0.43 \pm 0.01 \mu\text{M}$ at the A_{2A} ARs) and **2i** ($1.4 \pm 0.16 \mu\text{M}$ at the A₁ ARs and $0.33 \pm 0.03 \mu\text{M}$ at the A_{2A} ARs), have the best dual A₁ and A_{2A} AR affinity. Overall, test compounds **2b** and **2g** have the best dual affinity *in vitro* and *in silico*. Notably, the boronic acid substituents with groups on the *para*-position have better yields than the *meta*-position. The NH on the scaffolds facilitate crucial conventional hydrogen bonds, but in some instances, it can repel selected amino acid (AA) residues. These hydrogen bonds and π - π stacking due to the bicyclic ring structures, these interactions serve as anchors for the ligands in the AR cavities.

As discussed in Chapter 3, test compound **2b** displayed the best A₁ AR affinity and was selected as representative to be subjected to the guanosine triphosphate (GTP) shift assay. Evaluation of the results indicated that compound **2b** acted as an A₁ AR antagonist based on the binding curve in the presence of GTP that was almost unaffected; the GTP shift of was calculated as approximately 1. The likeness of the test compounds coincides with the other test compounds (**2a–k**), it can be concluded that all test compounds are A₁ AR antagonists.

The synthesised test compounds appear to have drug-like properties and are predicted to cross the blood-brain barrier (BBB) due to the reduction in phenolic groups, which improves on some existing polyphenol compounds and naturally derived sources ([Figueira et al., 2017:9-10](#); [Patil et al., 2017:179](#)). The physicochemical properties should be tested in the future to verify these claims.

4.3 Concluding remarks and future perspectives

In conclusion, the structural features of the 7-azaindole and 7-deazapurine structural features are favourable for antagonistic A₁ and A_{2A} AR binding. In the future, the synthetic methodology can be optimised to improve yields (impeded by the homo-coupled by-products) and the advancement of biological evaluation on human ARs can be explored. The one-pot reaction of the Suzuki coupling methodology is effective, although the reaction mechanism should be adapted for

manufacturing on a larger scale. Testing of multi-target drug activity, like acetylcholine esterase (AChE) inhibition, gastro-intestinal (GI) absorption after oral administration, preclinical- and clinical safety and efficacy *in vivo* and identification of valid biomarkers to monitor neurodegeneration, are all necessitated to ensure the applicability of these drugs.

4.4 References

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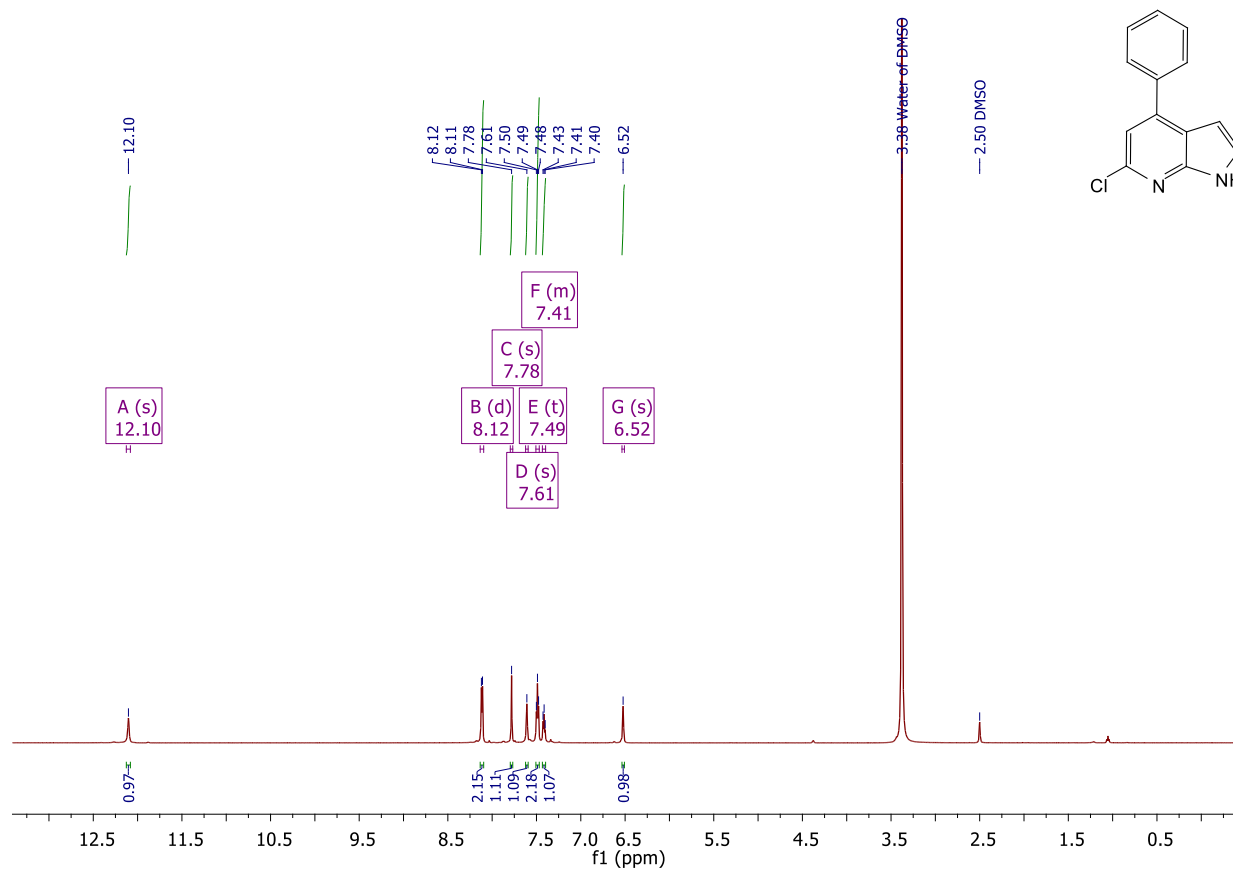
Tang, Q., Wang, L., Duan, Y., Wang, W., Huang, S., Zhi, J., Jia, S., Zhu, W., Wang, P. & Luo, R. 2017. Discovery of novel 7-azaindole derivatives bearing dihydropyridazine moiety as c-Met kinase inhibitors. *European Journal of Medicinal Chemistry*, 133:97-106. DOI: 10.1016/j.ejmech.2017.03.045.

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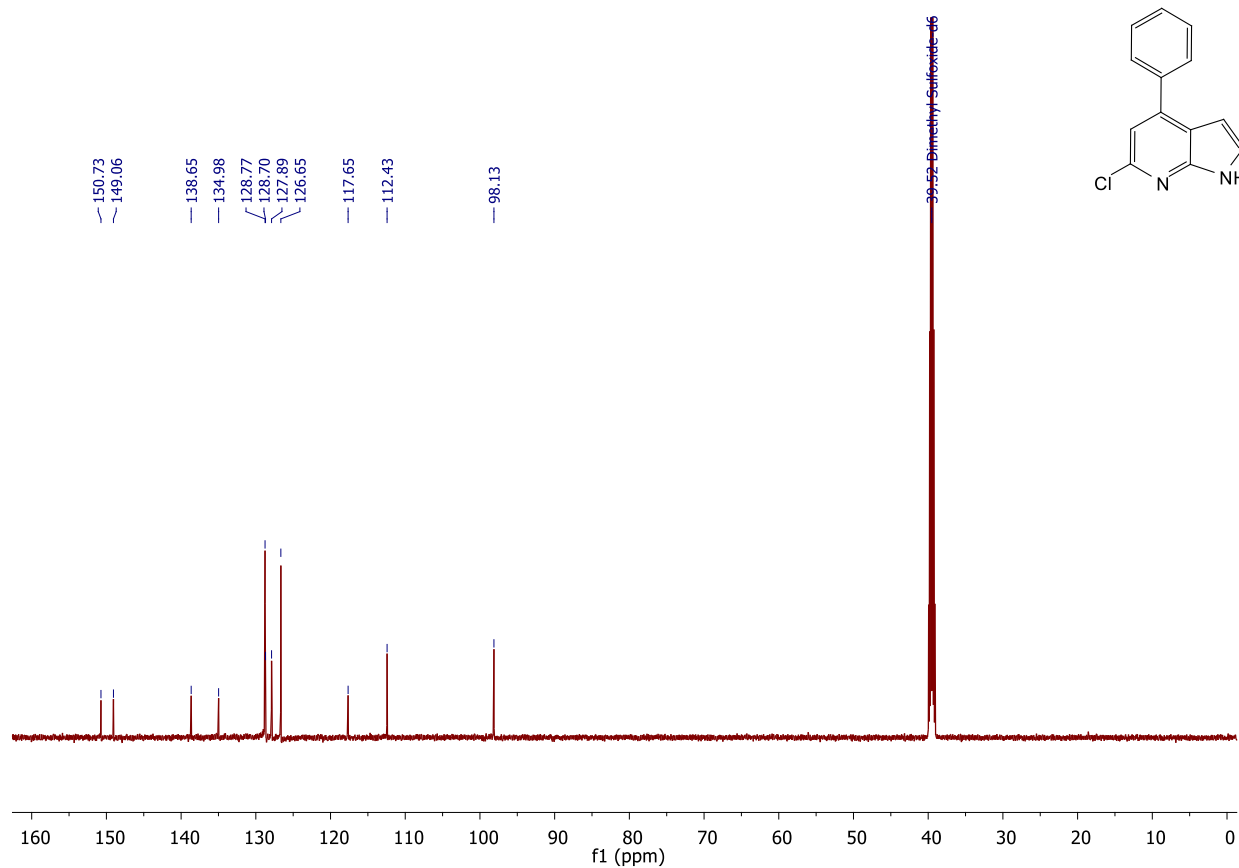
ANNEXURES

ANNEXURE A NUCLEAR MAGNETIC RESONANCE (NMR) SPECTRA OF TEST COMPOUNDS

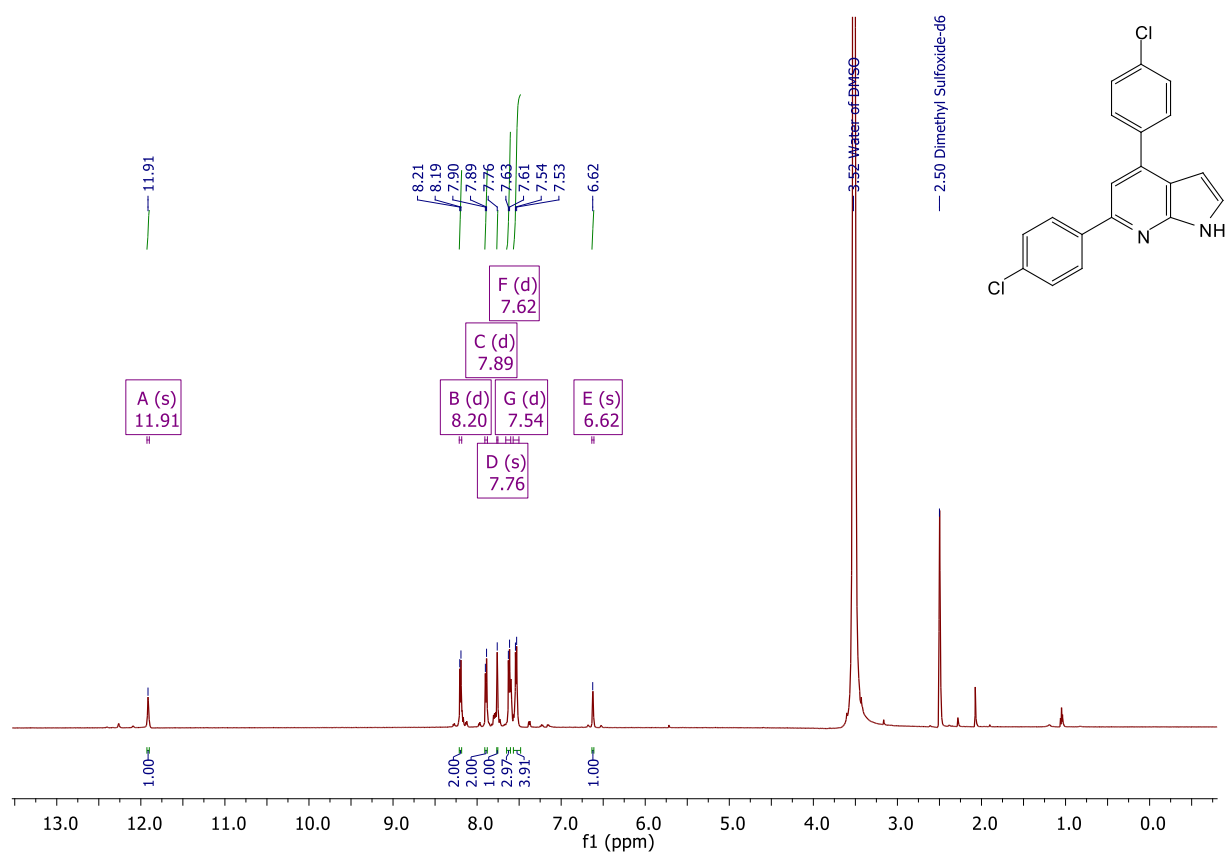
^1H NMR (DMSO-d_6): 6-chloro-4-phenyl-1H-pyrrolo[2,3-b]pyridine (**2b**):



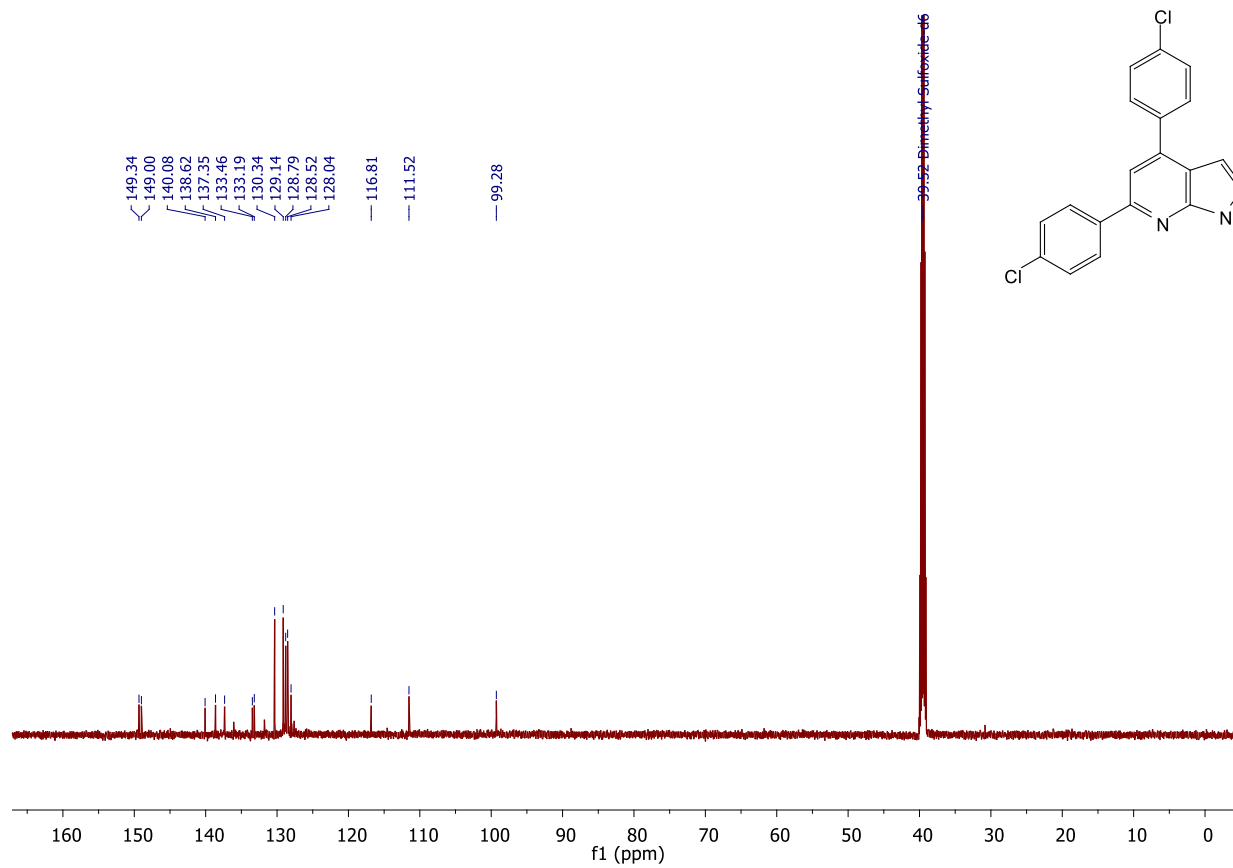
^{13}C NMR (DMSO-d_6): 6-chloro-4-phenyl-1H-pyrrolo[2,3-b]pyridine (**2b**):



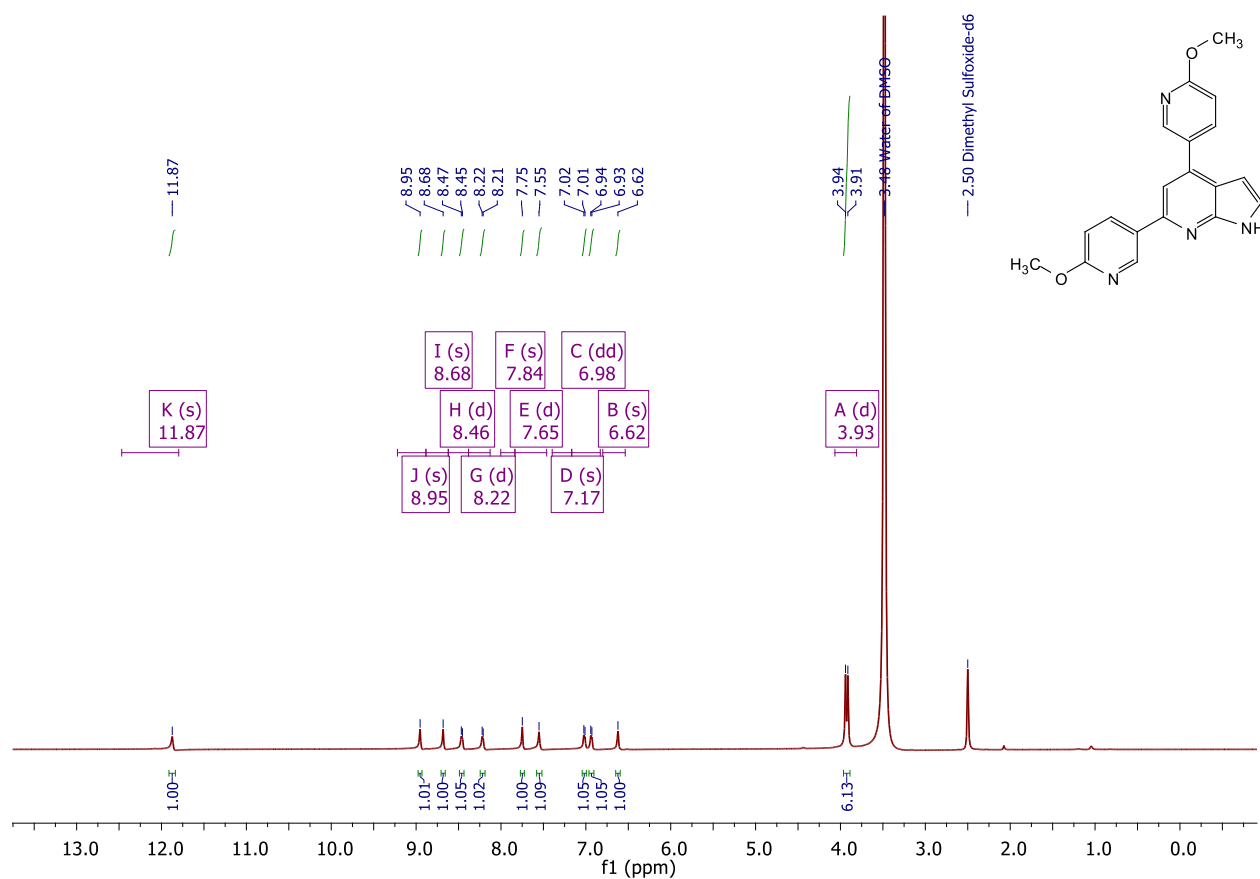
¹H NMR (DMSO-d₆): 6-chloro-4-(4-chlorophenyl)-1H-pyrrolo[2,3-b]pyridine (**2c**):



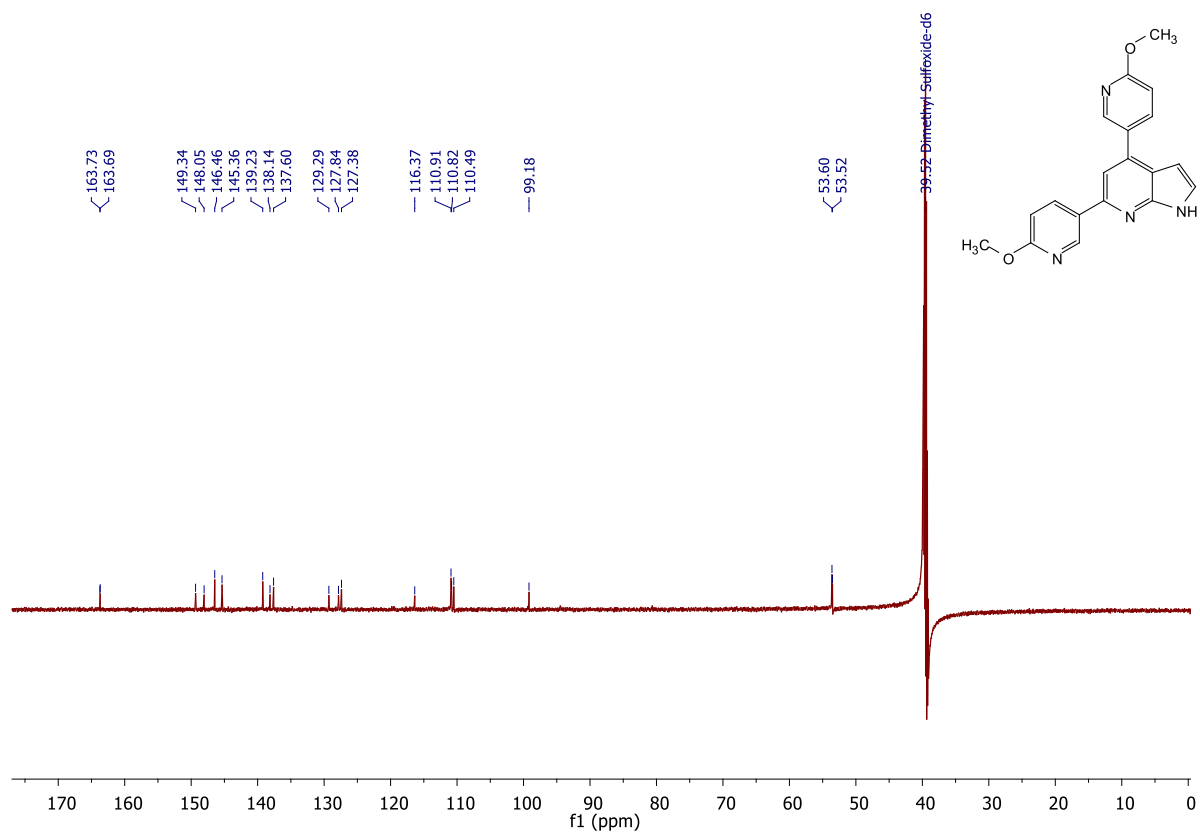
^{13}C NMR (DMSO- d_6): 6-chloro-4-(4-chlorophenyl)-1H-pyrrolo[2,3-*b*]pyridine (**2c**):



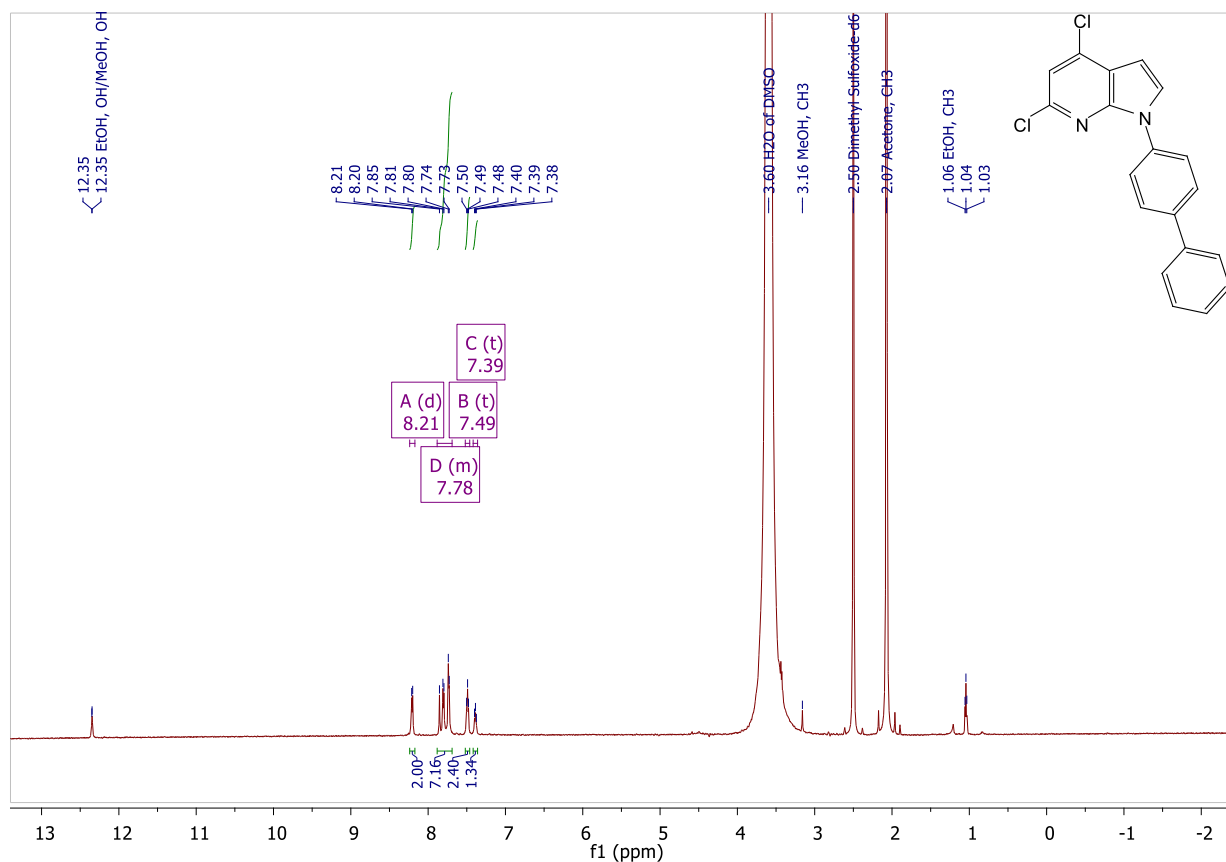
^1H NMR (DMSO- d_6): 4,6-bis(6-methoxypyridin-3-yl)-1H-pyrrolo[2,3-*b*]pyridine (**2d**):



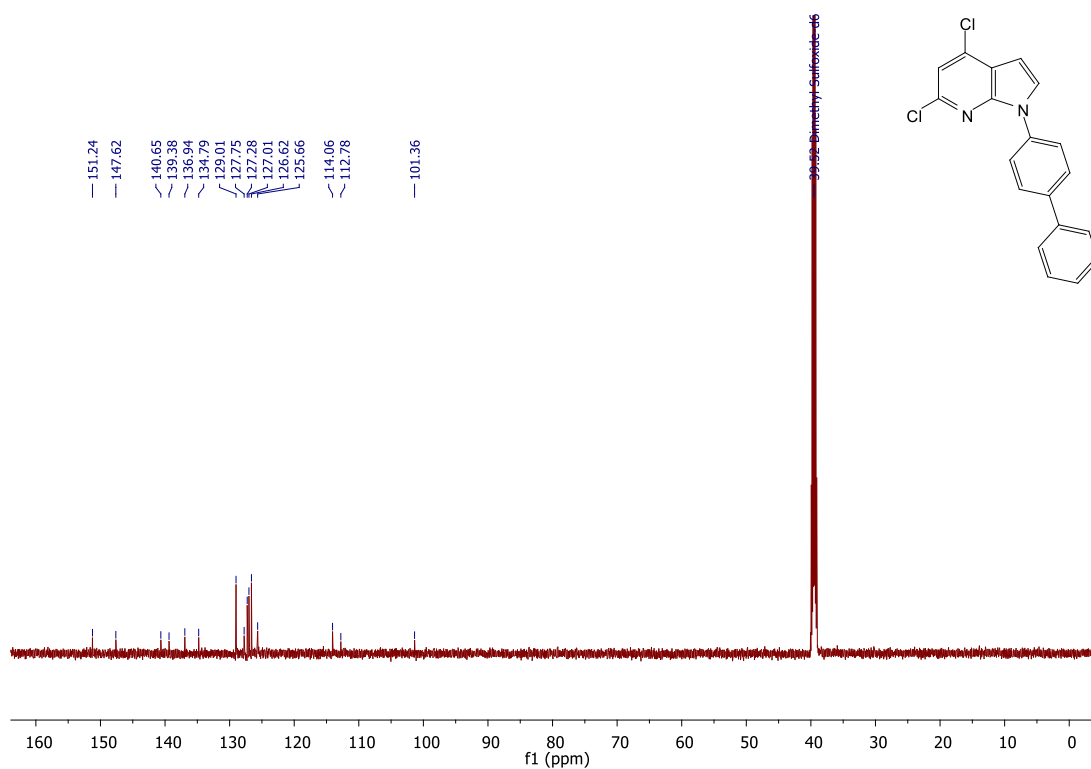
¹³C NMR (DMSO-d₆): 4,6-bis(6-methoxypyridin-3-yl)-1H-pyrrolo[2,3-b]pyridine (2d**):**



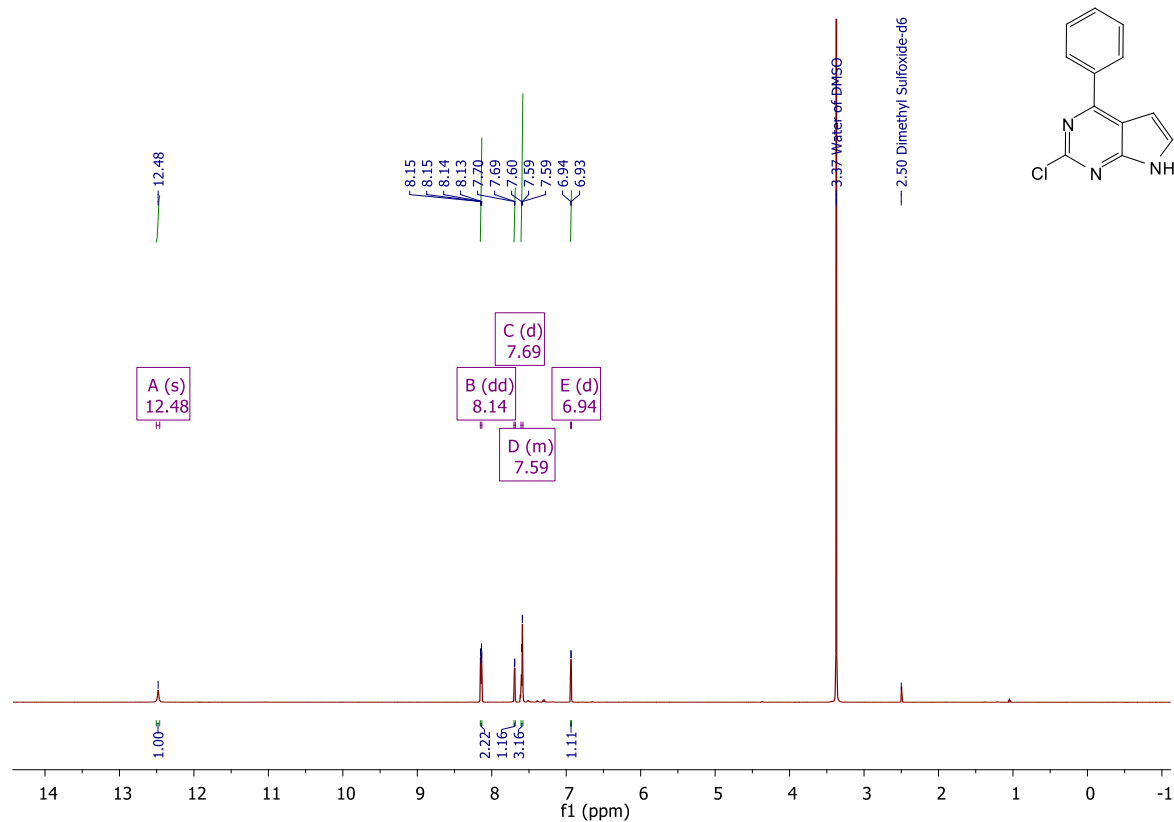
^1H NMR (DMSO- d_6): 1-[[1,1'-biphenyl]-4-yl]-4,6-dichloro-1H-pyrrolo[2,3-b]pyridine (**2e**):



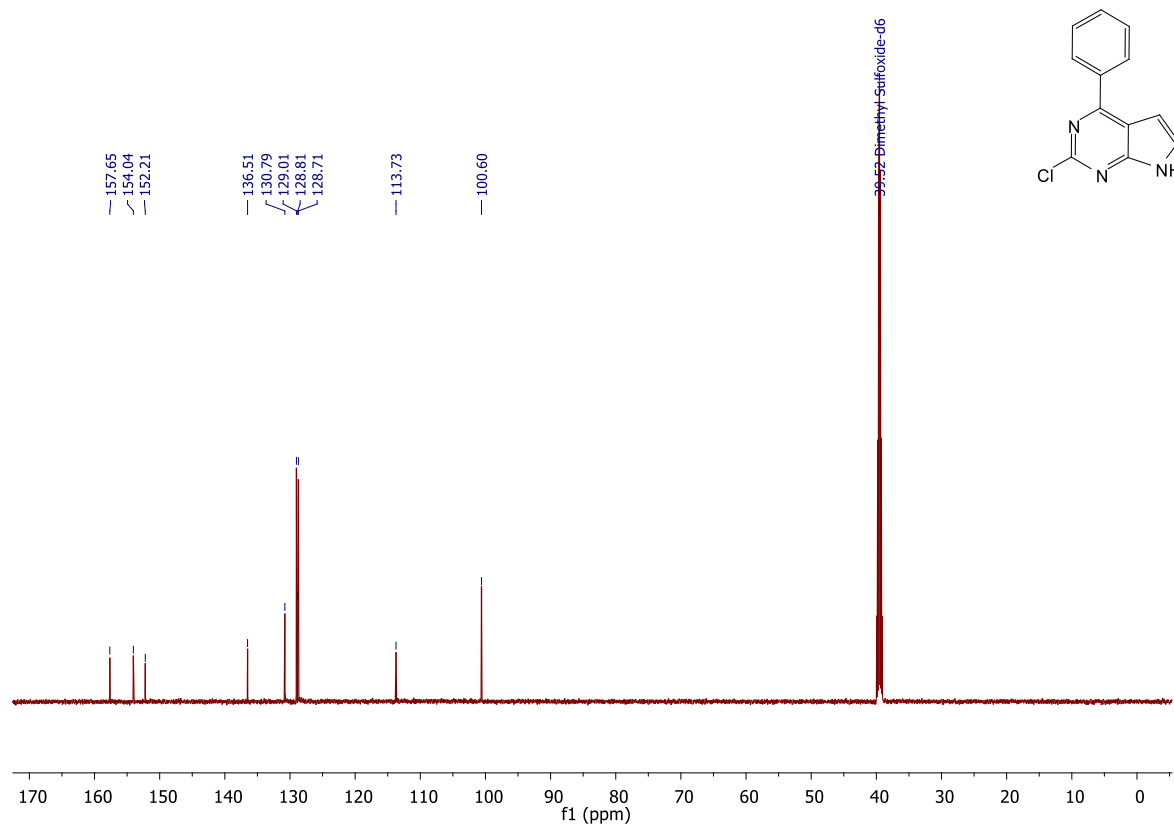
^{13}C NMR (DMSO- d_6): 1-[[1,1'-biphenyl]-4-yl]-4,6-dichloro-1H-pyrrolo[2,3-b]pyridine (**2e**):



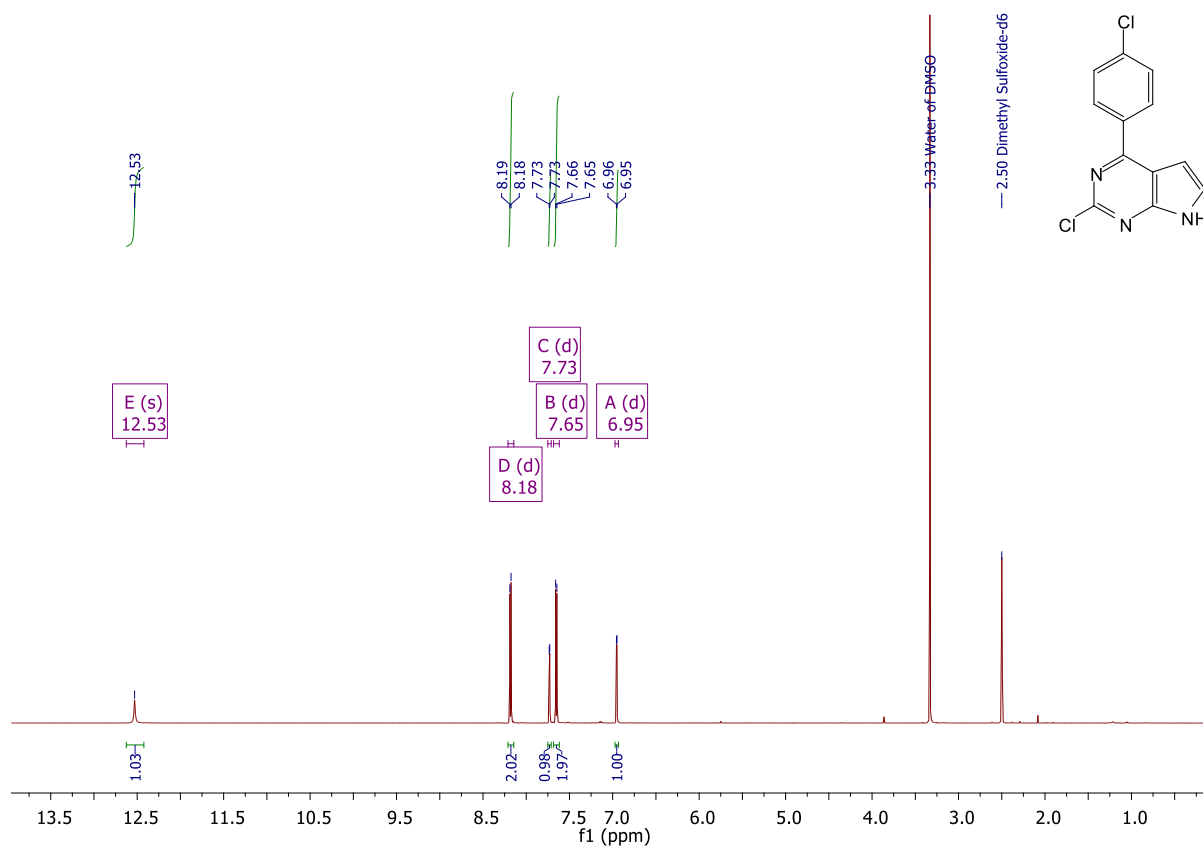
^1H NMR (DMSO- d_6): 2-chloro-4-phenyl-7H-pyrrolo[2,3-d]pyrimidine (**2g**):



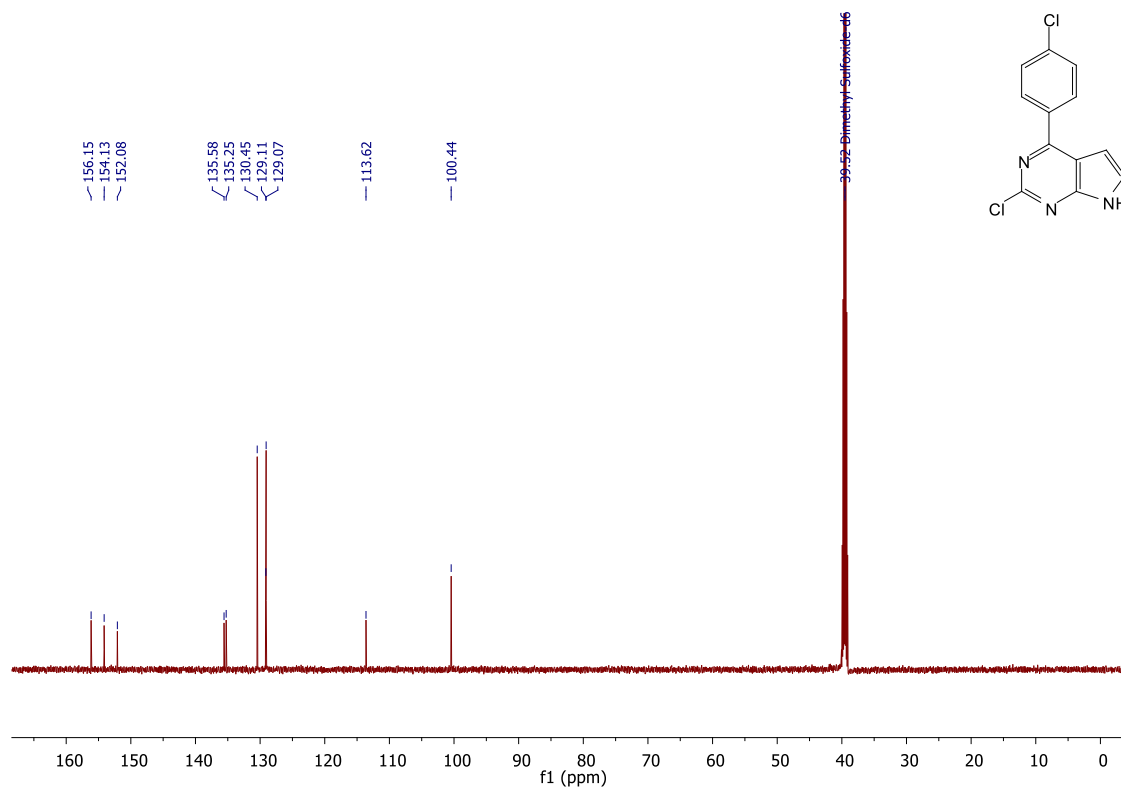
^{13}C NMR (DMSO- d_6): 2-chloro-4-phenyl-7H-pyrrolo[2,3-d]pyrimidine (**2g**):



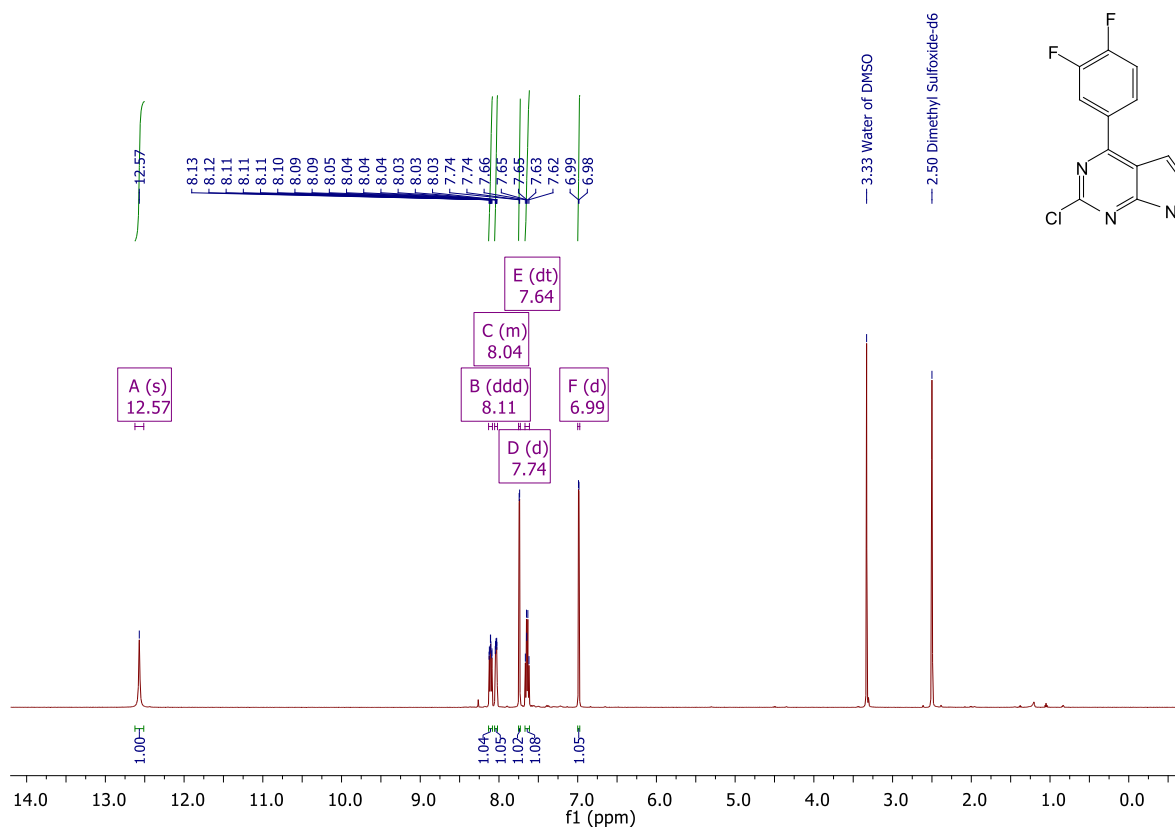
^1H NMR (DMSO-d_6): 2-chloro-4-(4-chlorophenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2h**):



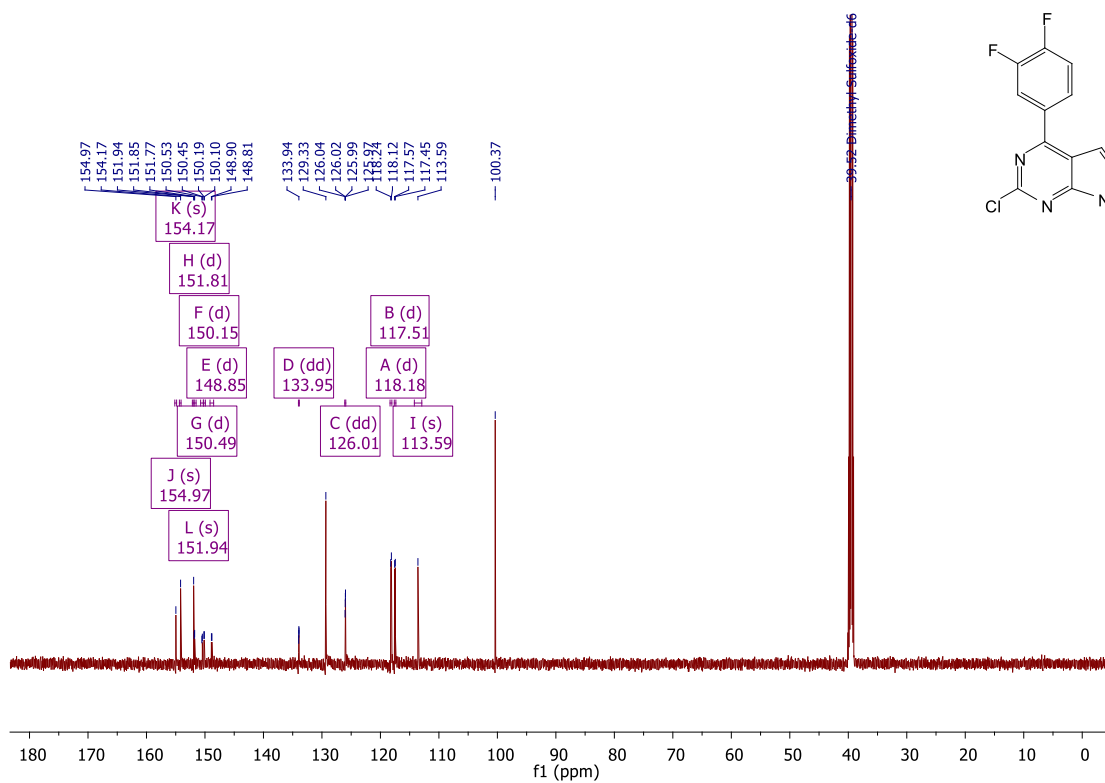
^{13}C NMR (DMSO-d_6): 2-chloro-4-(4-chlorophenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2h**):



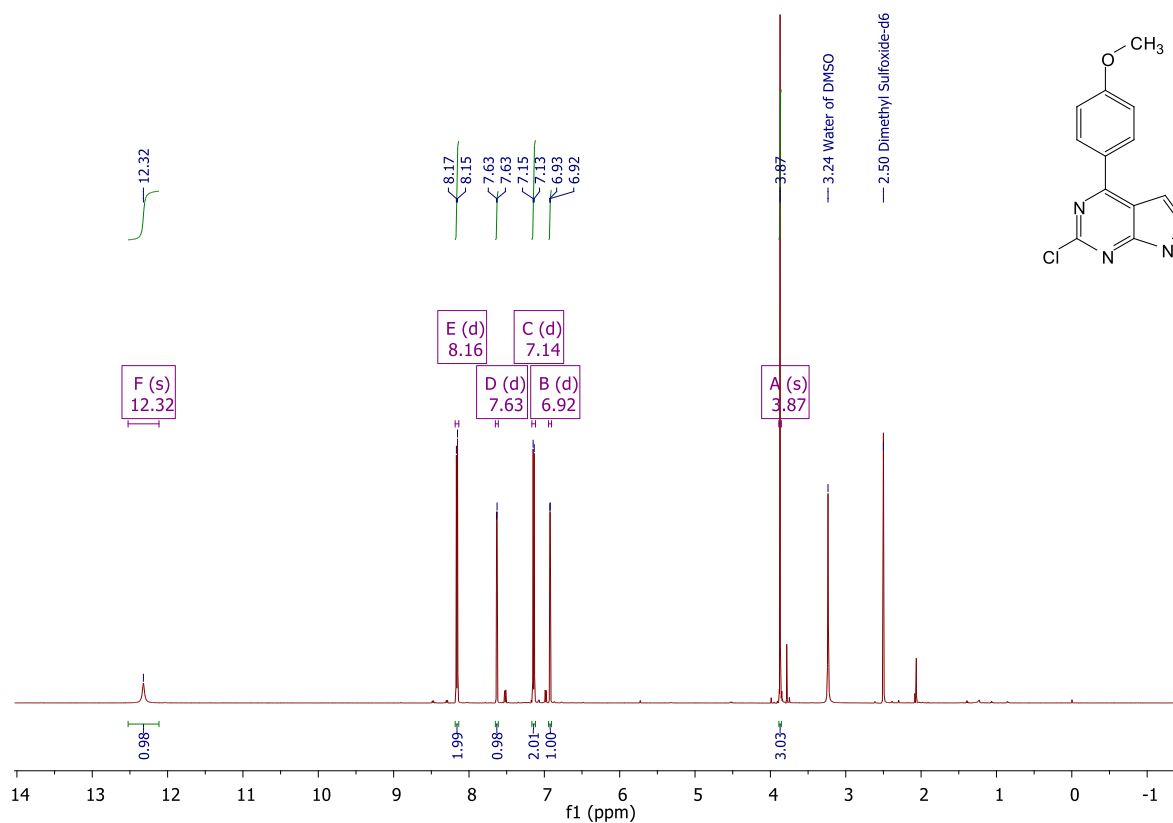
^1H NMR (DMSO-d_6): 2-chloro-4-(3,4-difluorophenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2i**):



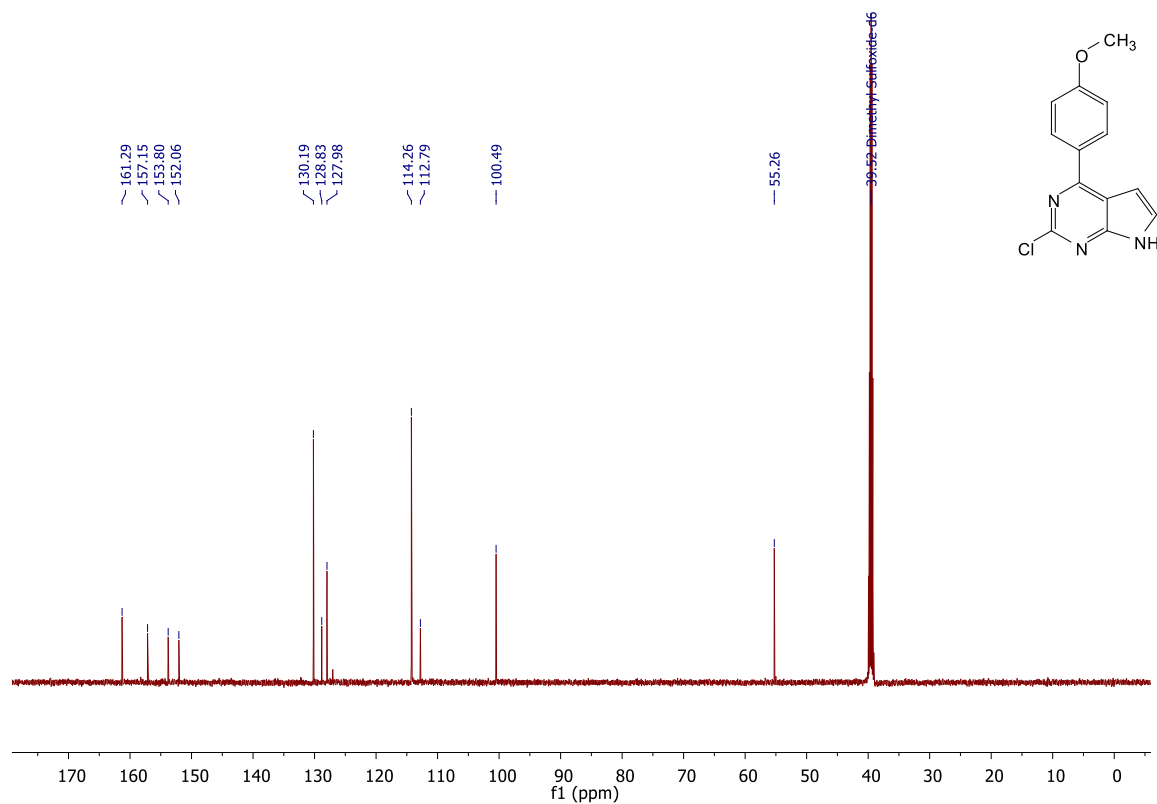
^{13}C NMR (DMSO-d_6): 2-chloro-4-(3,4-difluorophenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2i**):



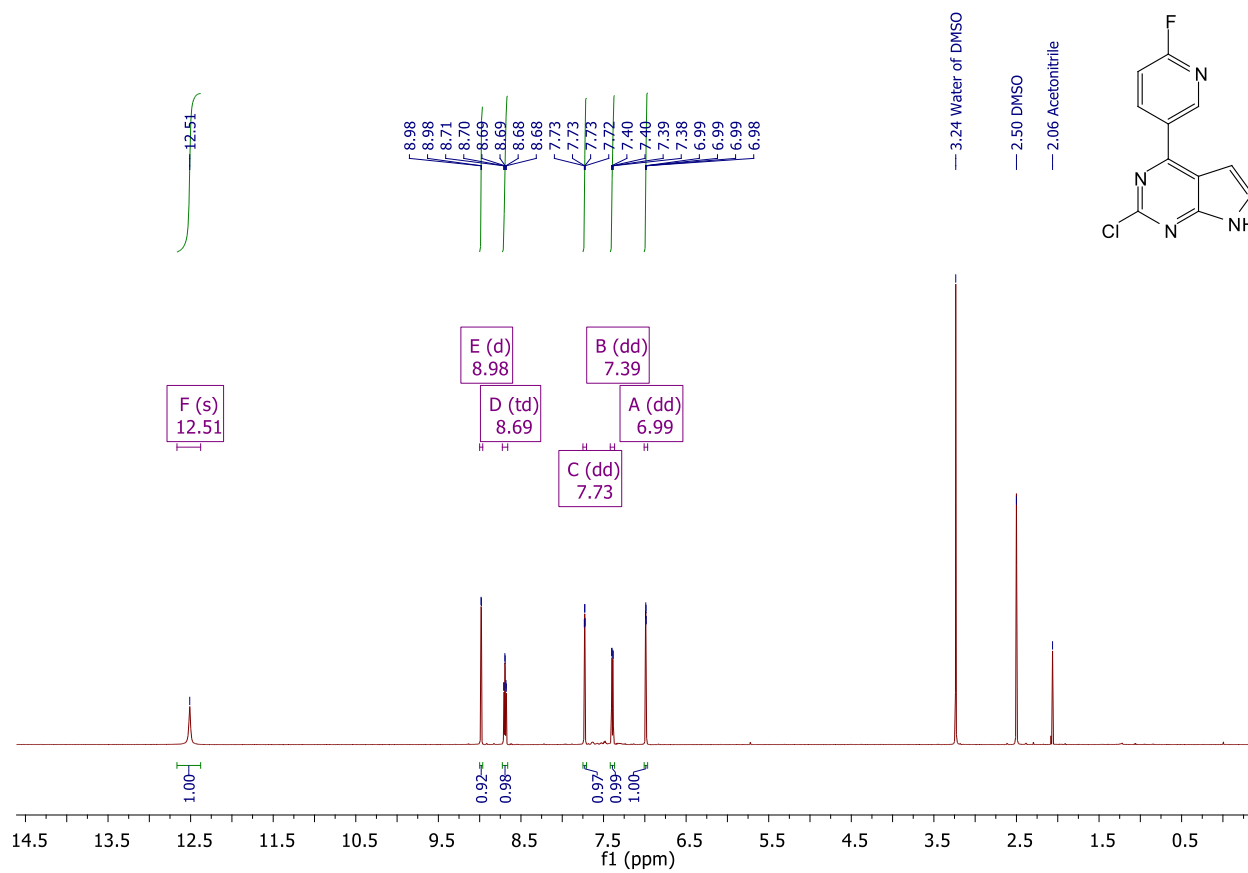
^1H NMR (DMSO-d_6): 2-chloro-4-(4-methoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2j**):



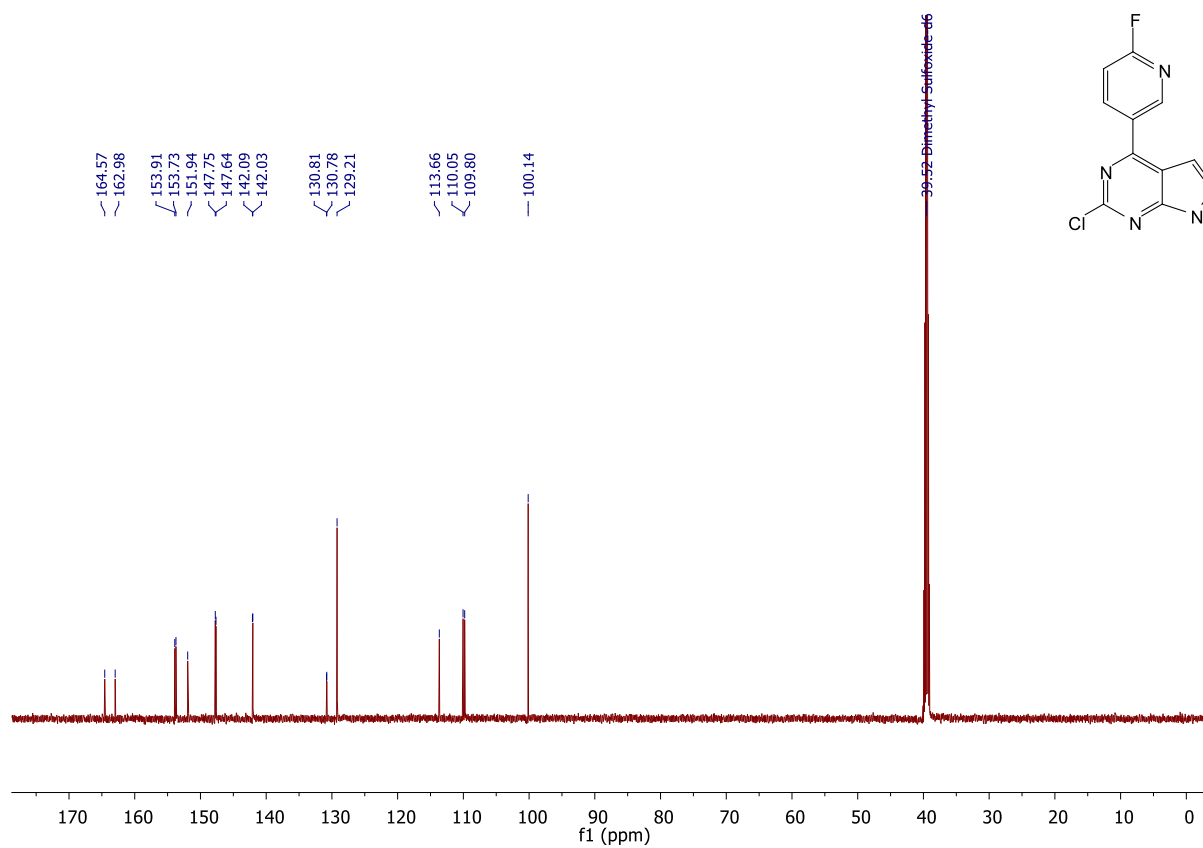
^{13}C NMR (DMSO-d_6): 2-chloro-4-(4-methoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2j**):



^1H NMR (DMSO-d_6): 5-{2-chloro-7H-pyrrolo[2,3-d]pyrimidin-4-yl}-2-fluoropyridine (**2k**):

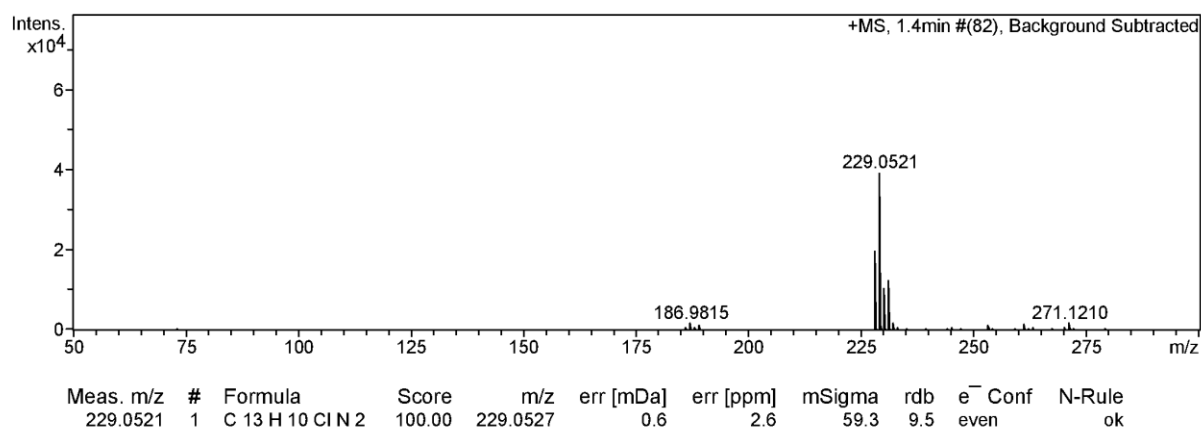


^{13}C NMR (DMSO-d_6): 5-{2-chloro-7H-pyrrolo[2,3-d]pyrimidin-4-yl}-2-fluoropyridine (**2k**):

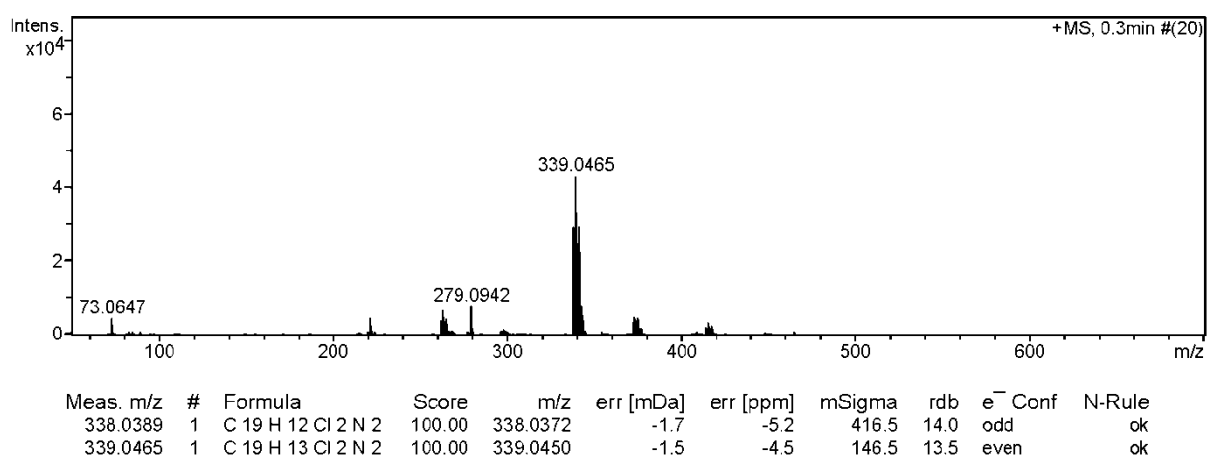


ANNEXURE B MASS SPECTROMETRY (MS) DATA OF TEST COMPOUNDS

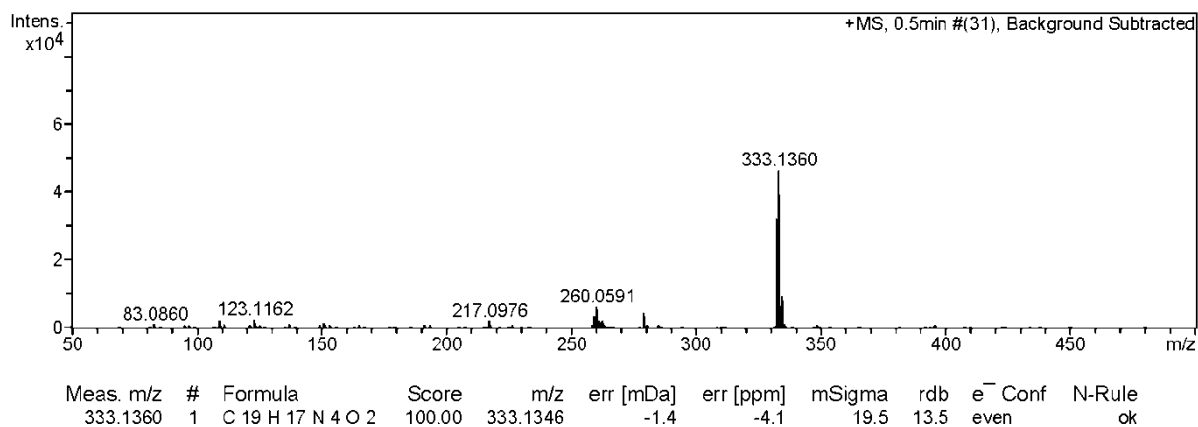
6-chloro-4-phenyl-1H-pyrrolo[2,3-b]pyridine (**2b**)



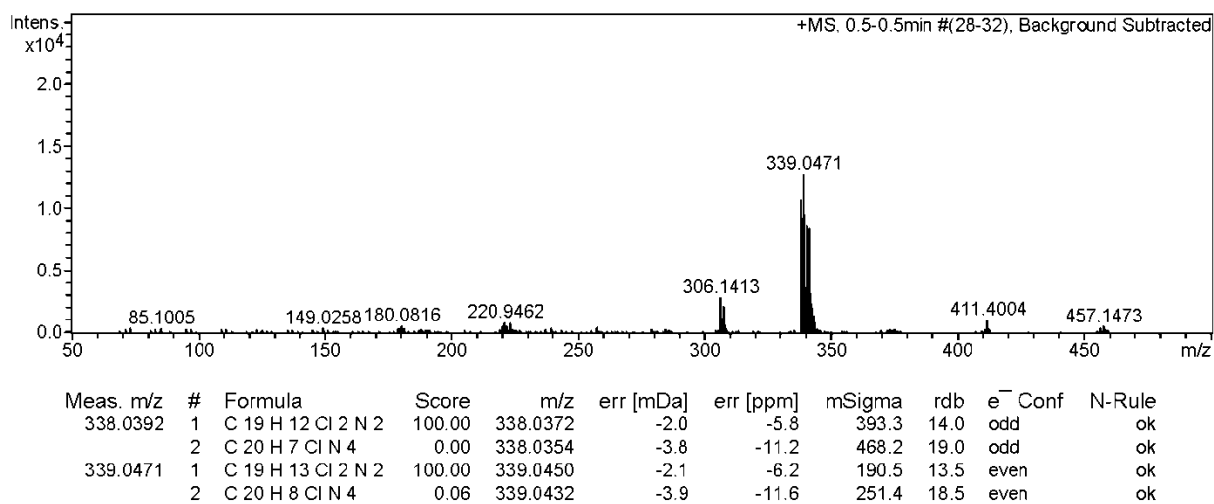
6-chloro-4-(4-chlorophenyl)-1H-pyrrolo[2,3-b]pyridine (**2c**)



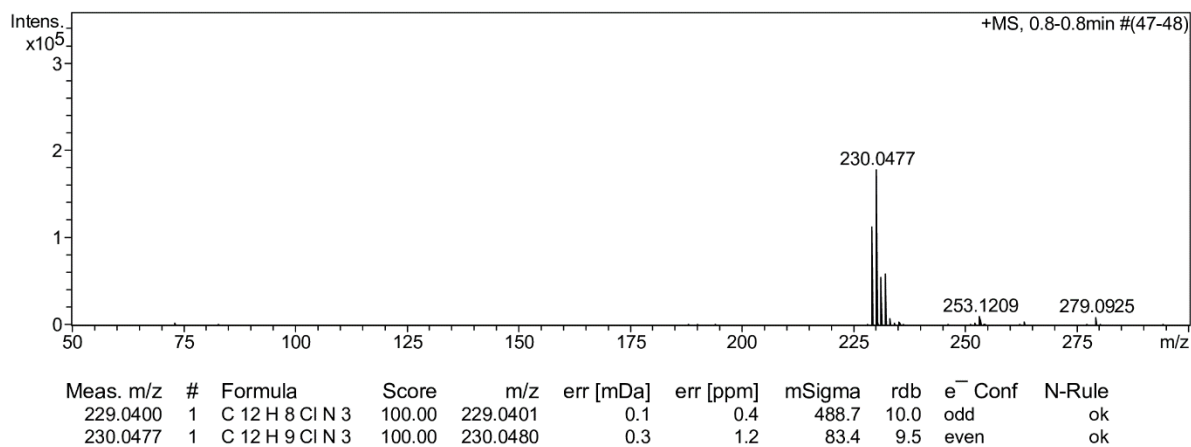
2-methoxy-5-[2-(6-methoxypyridin-3-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]pyridine (**2d**)



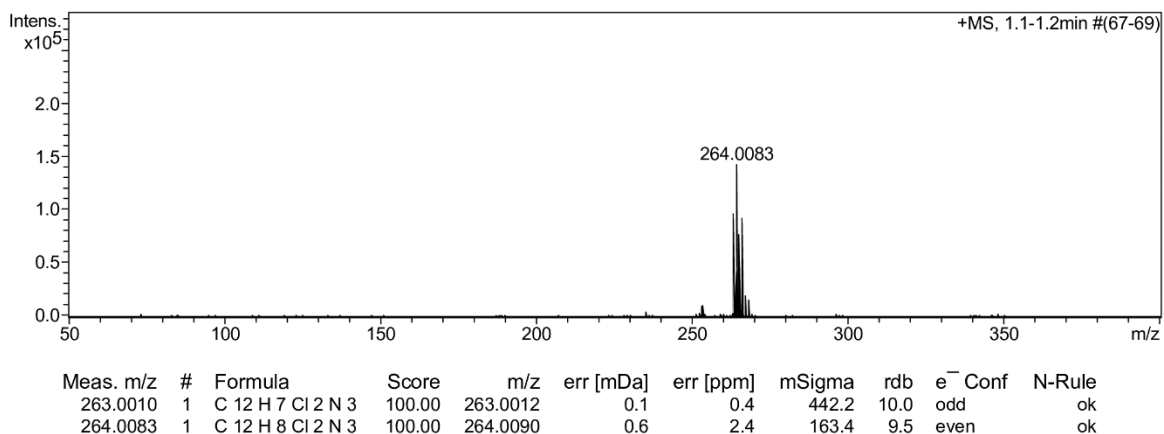
1-[[1,1'-biphenyl]-4-yl]-4,6-dichloro-1H-pyrrolo[2,3-b]pyridine (**2e**)



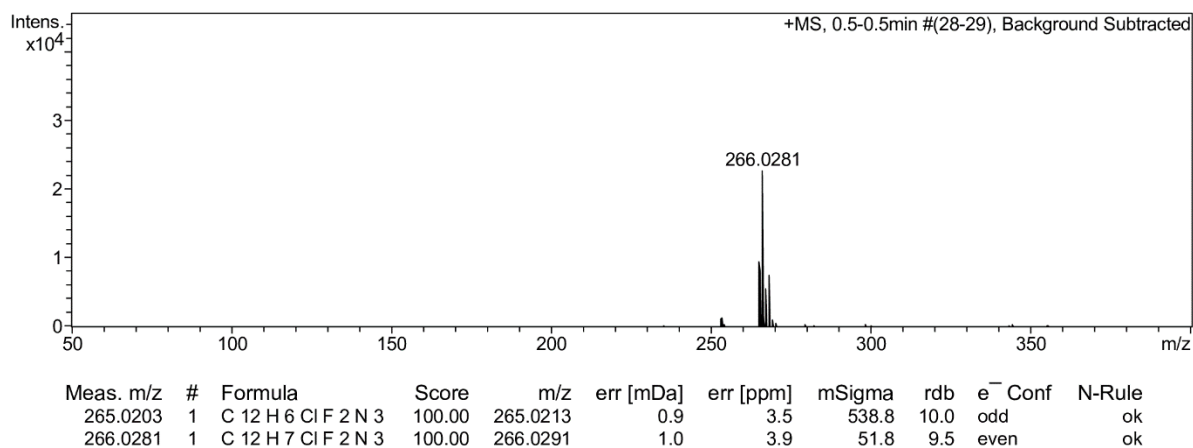
2-chloro-4-phenyl-7H-pyrrolo[2,3-d]pyrimidine (**2g**)



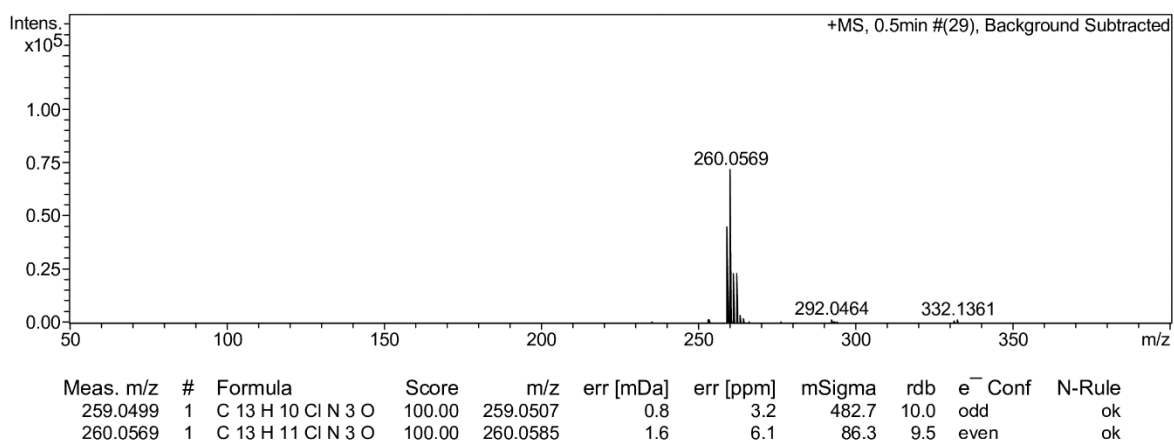
2-chloro-4-(4-chlorophenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2h**)



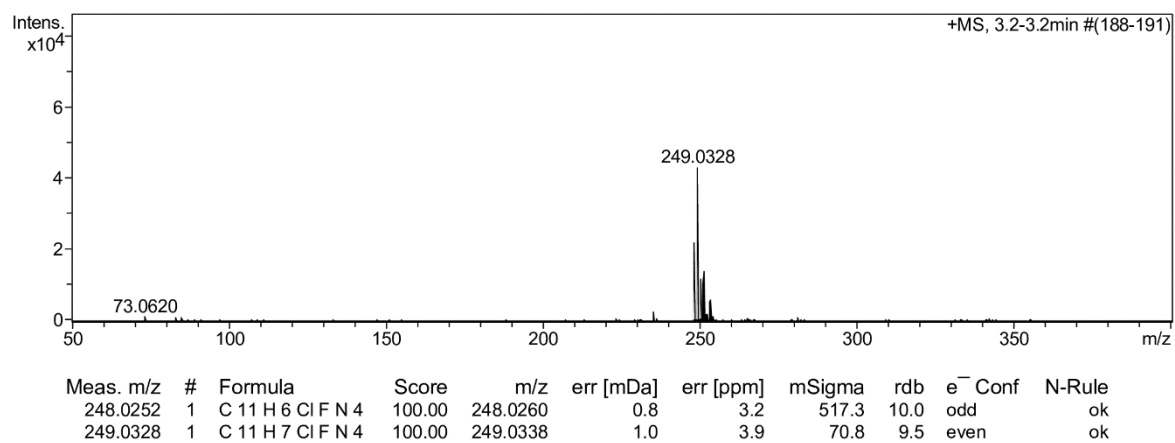
2-chloro-4-(3,4-difluorophenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2i**)



2-chloro-4-(4-methoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidine (**2j**)



5-{2-chloro-7H-pyrrolo[2,3-d]pyrimidin-4-yl}-2-fluoropyridine (**2k**)



ANNEXURE C ETHICAL APPROVAL



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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)

6 October 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 06/10/2019, 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: Synthesis and evaluation of 7-deazapurine derivatives as ligands of adenosine A1 and A2A receptors																														
Principal Investigator/Study Supervisor/Researcher: Prof L Legoabe																														
Student: N van Vuuren - 26239434																														
Ethics number:	<table><tr><td>N</td><td>W</td><td>U</td><td>-</td><td>0</td><td>0</td><td>5</td><td>9</td><td>2</td><td>-</td><td>1</td><td>9</td><td>-</td><td>A</td><td>5</td></tr><tr><td colspan="3">Institution</td><td></td><td colspan="5">Study Number</td><td></td><td>Year</td><td colspan="3">Status</td></tr></table>	N	W	U	-	0	0	5	9	2	-	1	9	-	A	5	Institution				Study Number						Year	Status		
N	W	U	-	0	0	5	9	2	-	1	9	-	A	5																
Institution				Study Number						Year	Status																			
<u>Status:</u> S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation																														
Application Type: Category 0 study	Risk: <table><tr><td>Category 0</td></tr></table>	Category 0																												
Category 0																														
Commencement date: 06/10/2019																														
Expiry date: 31/10/2020																														
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 October annually until completion.																														

General conditions:
<i>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:</i>
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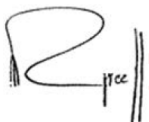
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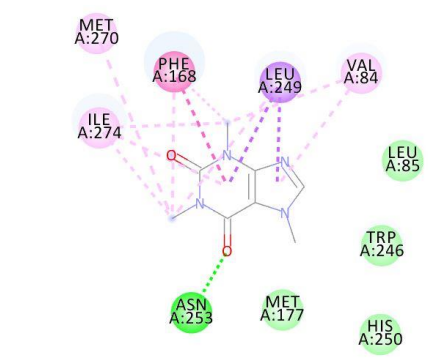
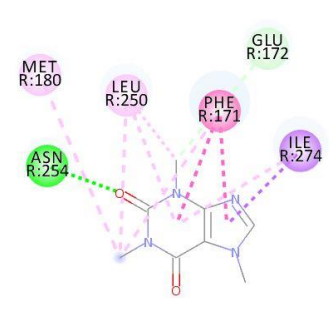
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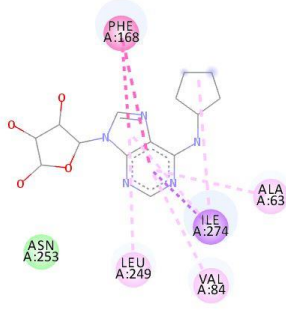
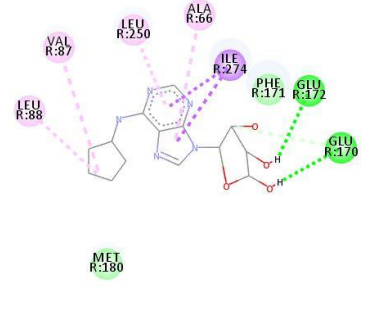
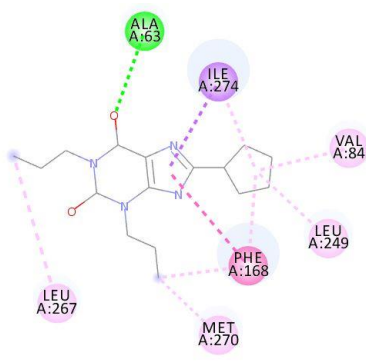
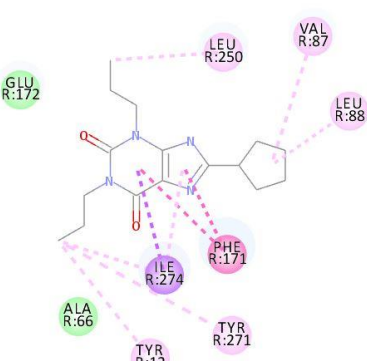
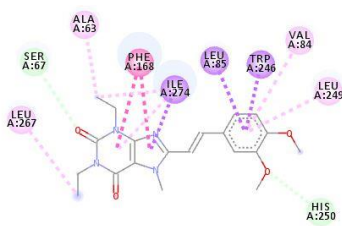
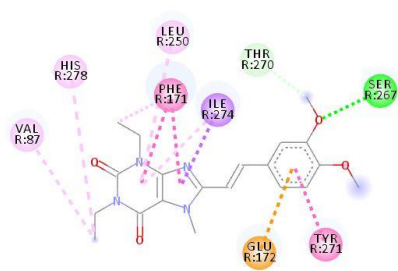
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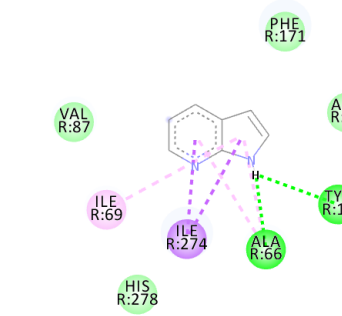
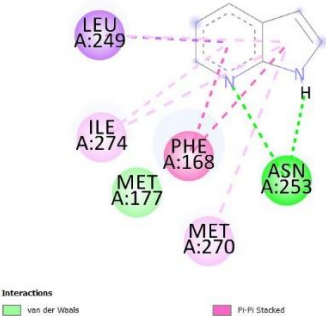
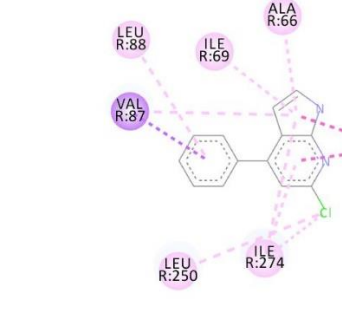
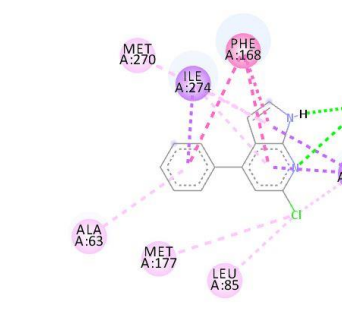
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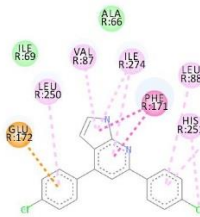
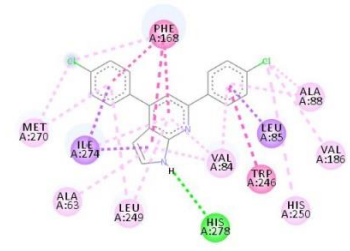
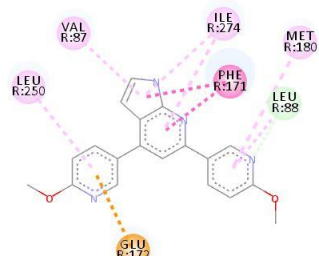
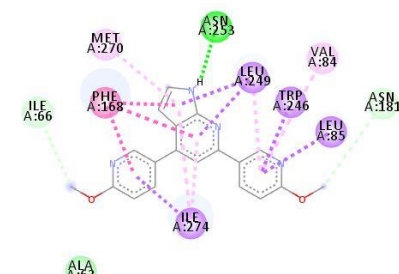
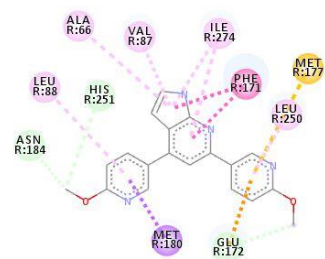
ANNEXURE D TWO-DIMENSIONAL MOLECULAR MODELLING SCHEMATICS

Table of Annexure D: Two-dimensional schematics of the molecular modelling configurations of reference- and test compounds at the human A₁ and A_{2A} ARs (binding affinity measured in -kcal)

Reference compounds		
Reference compound	Binding affinity (-kcal) and configurations at human A ₁ adenosine receptor (AR) (PDB ID: 6D9H)	Binding affinity (-kcal) and configurations at human A _{2A} adenosine receptor (AR) (PDB ID: 3PWH)
Caffeine	-6.0  Interactions - van der Waals - Conventional Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl	-5.0  Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl
CPA	-7.5	-6.2

	 Interactions - van der Waals - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl	 Interactions - van der Waals - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Pi-Sigma - Alkyl - Pi-Alkyl
DPCPX	-7.7  Interactions - Conventional Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl	-7.1  Interactions - van der Waals - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl
Istradefylline	-5.6  Interactions - Carbon Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl	-6.4  Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Pi-Anion - Pi-Sigma - Pi-Pi Stacked - Pi-Pi T-shaped - Alkyl - Pi-Alkyl
Test compounds		

Test compound	Binding affinities, first two configurations (-kcal), and interactions at human A ₁ adenosine receptor (AR) (PDB ID: 6D9H)	Binding affinities, first two configurations (-kcal), and interactions at human A _{2A} adenosine receptor (AR) (PDB ID: 3PWH)
2a	-5.5 & -5.2  Interactions - van der Waals - Conventional Hydrogen Bond - Pi-Sigma - Pi-Alkyl	-4.9 & -4.8  Interactions - van der Waals - Conventional Hydrogen Bond - Pi-Sigma - Pi-Alkyl
2b	-8.4 & -8.4  Interactions - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl	-8.3 & -8.1  Interactions - Conventional Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl

2c	-9.2 & -8.1  Interactions - van der Waals - Pi-Pi Stacked - Pi-Alkyl - Pi-Anion	-8.4 & -7.5  Interactions - Conventional Hydrogen Bond - Pi-Sigma - Pi-Pi T-shaped - Pi-Pi Stacked - Alkyl - Pi-Alkyl
2d	Config 1: -8.8  Interactions - Carbon Hydrogen Bond - Pi-Pi Stacked - Pi-Alkyl - Pi-Anion	-7.6 & -7.5  Interactions - van der Waals - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Pi-Alkyl
	Config 2: -8.7  Interactions - Carbon Hydrogen Bond - Pi-Anion - Pi-Sigma - Pi-Sulfur - Pi-Pi Stacked - Pi-Alkyl	

	Config 3: -6.6 Interactions - van der Waals - Carbon Hydrogen Bond - Pi-Sigma - Pi-Sulfur - Pi-Pi Stacked - Pi-Alkyl	
	Config 4: -5.8 Interactions - van der Waals - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Pi-Anion - Pi-Sigma - Pi-Lone Pair - Pi-Pi Stacked - Pi-Alkyl	
2e	-8.8 & -7.1 Interactions - Pi-Sigma - Pi-Pi Stacked - Pi-Pi T-shaped - Alkyl - Pi-Alkyl	-8.4 & -8.3 Interactions - van der Waals - Carbon Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl
2f	-4.2 & -4.2	-5.0 & -4.9

	Interactions - van der Waals - Conventional Hydrogen Bond - Pi-Sigma - Pi-Alkyl	Interactions - van der Waals - Pi-Sigma - Pi-Pi Stacked - Pi-Alkyl
2g	-8.0 & -8.0 Interactions - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl	-7.7 & -7.6 Interactions - van der Waals - Conventional Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl
2h	-7.9 & -7.8 Interactions - van der Waals - Conventional Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Pi-Pi T-shaped - Alkyl - Pi-Alkyl	-7.6 & -7.4 Interactions - van der Waals - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl
2i	-8.3 & -8.1	-8.0 & -7.9

	Interactions - van der Waals - Unfavorable Bump - Conventional Hydrogen Bond - Halogen (Fluorine) - Pi-Alkyl	Interactions - van der Waals - Unfavorable Bump - Carbon Hydrogen Bond - Pi-Donor Hydrogen Bond - Alkyl - Pi-Alkyl
2j	-7.9 & -7.9 Interactions - van der Waals - Conventional Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Pi-Pi T-shaped - Alkyl - Pi-Alkyl	-7.6 & -7.4 Interactions - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl
2k	-7.8 & -7.2 Interactions - van der Waals - Conventional Hydrogen Bond - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl	-7.6 & -7.5 Interactions - van der Waals - Conventional Hydrogen Bond - Halogen (Fluorine) - Pi-Sigma - Pi-Pi Stacked - Alkyl - Pi-Alkyl

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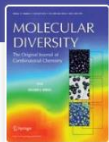
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Submission guidelines

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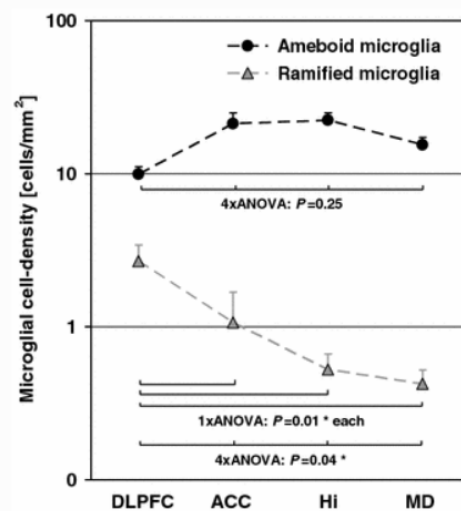
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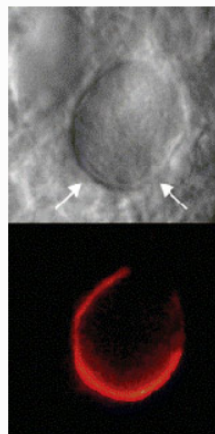
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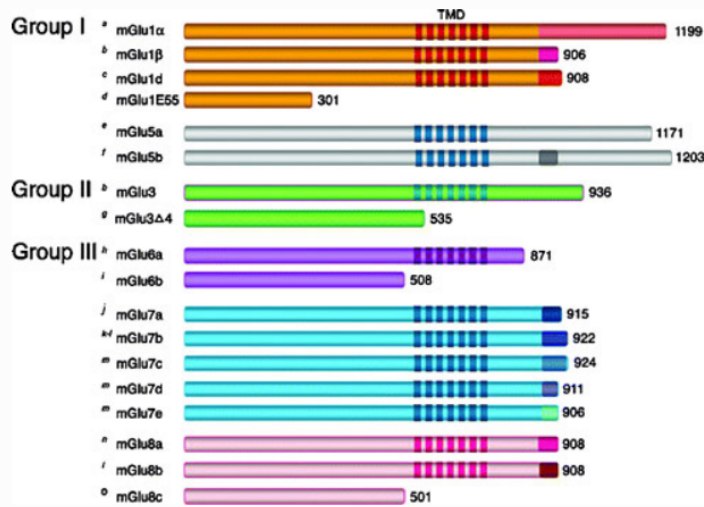
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