Adenosine A1/A2A receptor antagonistic properties of selected 2-substituted benzoxazinone and quinazolinone derivatives

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Dedicated to my grandfather, Daniel Jacobus Smith

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ABSTRACT

The A_1 and A_{2A} adenosine receptor antagonists are the subject of extensive research based on their aptitude for ameliorating Parkinson's disease related cognitive deficits (A_1 adenosine receptor subtype) and motor dysfunction (A_{2A} adenosine receptor subtype), while also exhibiting neuroprotective properties (A_{2A} adenosine receptor subtype). A benzo-α-pyrone based derivative, 3-phenyl-1H-2-benzopyran-1-one, was previously reported to display both A_1 and A_{2A} adenosine receptor affinity in the low micromolar range, thereby, prompting the current investigation of structurally related benzoxazinone and quinazolinone homologues afforded by structural modifications to the benzo-α-pyrone core. Benzoxazinone and quinazolinone derivatives were hitherto not known to be adenosine receptor antagonists. Although the C2-substituted quinazolinone derivatives displayed varying degrees of affinity (low micromolar range), overall they exhibited superior A_1 and A_{2A} adenosine receptor affinity (in the low micromolar range) over their C2-substituted benzoxazinone counterparts. The C2-substituted quinazolinone derivative bearing a methyl *para*-substitution of the phenyl ring B, was documented with the highest affinity and selectivity toward the A_1 adenosine receptor (A_1K_i = 2.50 μM). In turn, the 3,4-dimethoxy substitution of the phenyl ring B resulted in the best A_{2A} adenosine receptor binding ($A_{2A}K_i$ = 2.81 μM). However, amongst the benzoxazinone derivatives only two compounds possessed A_1 adenosine receptor activity and displayed a complete lack of A_{2A} adenosine receptor affinity. Therefore, it may be concluded that the quinazolinones are ideal lead compounds for further structural optimization to gain improved adenosine receptor affinity, which may prove to be of value in Parkinson's disease with regards to neuroprotection and amelioration of the motor dysfunction and cognitive deficits associated with Parkinson's disease.

Keywords: benzoxazinone; quinazolinone; benzo-α-pyrone; A_1 receptor adenosine; A_{2A} receptor adenosine; antagonist; Parkinson's disease.

OPSOMMING

Die A_1-en A_{2A}-adenosienreseptorantagoniste is die onderwerp van uitgebreide navorsing met betrekking tot Parkinson se siekte. Hierdie navorsing is gebaseer op adenosienreseptorantagoniste se vermoë om die kognitiewe (A_1-adenosienreseptorsubtipe), sowel as die motoriese gebreke (A_{2A}-adenosienreseptorsubtipe) van Parkinson se siekte te verbeter en terselfde tyd ook neurobeskermend (A_{2A}-adenosienreseptorsubtipe) op te tree. Tydens 'n vorige studie is bewys dat 'n benso-α-piroongebaseerde derivaat, 3-feniel-1H-2-bensopiran-1-oon, oor beide A_1-en A_{2A}-adenosienreseptor-aktiwiteit beskik en met die oog op potensiële A_1-en A_{2A}-adenosienreseptor-aktiwiteit, is verwante strukture, naamlik die bensoksasinoon- en kinasolinoonderivate, ondersoek. Hierdie strukture is bekom deur sekere voorgestelde strukturele veranderinge aan die benso-α-piroonskelet te maak en is tot op hede nog nie as moontlike adenosienreseptorantagoniste ondersoek nie. Alhoewel die C2-gesubstitueerde kinasolinoonderivate in variërende mates A_1-en A_{2A}-adenosienreseptor-affiniteit (lae mikromolaar hoeveelhede) getoon het, het hulle in die geheel beter affiniteit as die ooreenstemmende C2-gesubstitueerde bensoksasinoonderivate getoon. Die C2-gesubstitueerde kinasolinoon wat die beste A_1-adenosienreseptor-aktiwiteit en -selektiwiteit getoon het, is 'n kinasolinoon met 'n metoksigroep in die *para*-posisie van fenielring B (A_1K_i = 2.50 µM), terwyl die C2-gesubstitueerde kinasolinoon wat die beste A_{2A}-adenosienreseptor-aktiwiteit getoon het, 'n kinasolinoon met 'n 3,4-dimetoksie-substitusie aan die fenielring B is ($A_{2A}K_i$ = 2.81 µM). Die reeks C2-gesubstitueerde bensoksasinoonderivate het slegs twee verbindings gelewer wat A_1-adenosienreseptor-aktiwiteit getoon het en geen van die verbindings in hierdie reeks het A_{2A}-adenosienreseptor-aktiwiteit getoon nie. Daar is dus tot die gevolgtrekking gekom dat die kinasolinoonderivate ideale leidraadverbindings is vir verdere ondersoek, om te bepaal watter alternatiewe strukturele verbeteringe optimale adenosienreseptor-affiniteit tot gevolg sal hê. Sodoende kan die verligting van die kognitiewe en motoriese gebreke, verwant aan Parkinson se siekte, sowel as die moontlike neurobeskermende effekte van die kinasolinoonderivate, geöptimaliseer word.

Sleutelterme: bensoksasinoon; kinasolinoon; benso-α-piroon; A_1-adenosienreseptor; A_{2A}-adenosienreseptor; antagonis; Parkinson se siekte.

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LIST OF ABBREVIATIONS

3-OMD	-	3-O-methyldopa
AR	-	Adenosine receptor
ATP	-	Adenosine triphosphate
AUC	-	Area under the curve
b	-	Bovine
cAMP	-	Cyclic adenosine monophosphate
$CDCL_3$	-	Deuterated chloroform
CNS	-	Central nervous system
COMT	-	Catechol-O-methyltransferase
CPA	-	N^6-cyclopentyladenosine
CSC	-	Chlorostyrylcaffeine
d	-	Doublet
dd	-	Doublet of doublets
DDC	-	Dopa decarboxylase
DMPX	-	3,7-Dimethyl-1-propagylxanthine
DMSO-d_6	-	Deuterated dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
DPCPX	-	1,3-Dipropyl-8-cyclopentylxanthine
GABA	-	γ-amino-butyric acid
GTP	-	[^{35}S]guasine-5'-O-(3-thio)triphosphate
h	-	Human
H_2O_2	-	Hydrogen peroxide
[^{3}H]DPCPX	-	1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine

[^{3}H]NECA	-	5'-N-ethylcarboxamido[^{3}H]adenosine
LAMB	-	Laboratory for analytical and molecular biology
MAO	-	Monoamine oxidase
m	-	Multiplet
$MgCl_2$	-	Magnesium chloride
mp	-	Melting point
MPP^+	-	1-Methyl-4-phenylpyridine
MS	-	Mass spectrometry
MPTP	-	1,2,3,6-Methyl-phenyl-tetrahydropyridine
NECA	-	5'-N-ethylcarboxamidoadenosine
NMDA	-	N-methyl-D-aspartate
NMR	-	Nuclear magnetic resonance
PCP	-	Phencyclidine
PD	-	Parkinson's disease
r	-	Rat
ROS	-	Reactive oxygen species
s	-	singlet
SAR	-	Structure activity relationship
SEM	-	Standard error of the mean
SI	-	Selectivity index
SNpc	-	Substantia nigra pars compacta
t	-	Triplet
TLC	-	Thin layer chromatography

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Parkinson's disease (PD) is a well-known neurodegenerative disease. It is defined by the formation of Lewy bodies concurrent with the loss of nigrostriatal dopaminergic neurons, resulting in diminished dopamine in the corpus striatum (Kalia & Lang, 2015; Erhringer & Hornykiewicz, 1998). PD is estimated to be prevalent in more than 1% of people aged above 60, and by 2030 the prevalence is expected to have doubled (Dorsey *et al.*, 2007). Individuals suffering from PD generally present with a series of motor dysfunctions and was first documented by James Parkinson in 1861 (Jankovic, 2008). The four clinical features, tremor, rigidity, bradykinesia and postural instability, outline the characteristic motor dysfunctions usually present in PD (Jankovic, 2008). Although PD is predominantly associated with motor symptoms, it is important to note that some non-motor symptoms are prone to develop with progression of the disease. The non-motor symptoms that arise are typically related to cognitive impairment (Lees & Smith, 1983).

Presently, treatment of PD consists largely of symptomatic relief due to the fact that PD treatment still lacks a cure (Calne, 1993). The classic treatment of PD seeks to halt the degradation of endogenous dopamine, whilst replenishing the depleted dopamine levels in the corpus striatum (Goodarzi *et al.,* 2015). Treatment options available for the motor symptoms associated with PD include, but is not limited to, dopamine agonists, monoamine oxidase inhibitors, dopa-decarboxylase inhibitors and catechol-O-methyltransferase inhibitors (Chen & Swope, 2007). Furthermore, nearly all cases of PD are treated with L-3,4-dihydroxyphenylalanine (levodopa) at one stage or another. This may be ascribed to levodopa's standing as the gold standard in PD therapy (Calne, 1993). Unfortunately, the symptomatic relief gained by the administration of levodopa is overshadowed by the risk of developing incapacitating dyskinesia associated with long term use (Cotzias *et al.,* 1969).

Considering the rising prevalence of PD, the lack of a cure and the risk of developing dyskinesia, it can be said that there exists an escalating need for novel treatment options in PD. Drug development may prove to be beneficial and can give rise to a novel generation of neuroprotective and disease modifying agents in PD.

The quest for novel treatment options in PD revealed that adenosine receptor (AR) antagonists hold promise for future pharmacological treatment in PD (Fredholm, 2010). Adenosine functions as a neuromodulator in the brain (Snyder, 1985), where it fulfils the physiological role opposite

that of dopamine by binding at the ARs (Ferre *et al.,* 2001). Four AR subtypes (A_1, A_{2A}, A_{2B} and A_3) have been identified and of the four subtypes, the A_1 and A_{2A} ARs are predominant in the brain (Gomes *et al.,* 2011). The A_1 ARs are spread diffusely throughout the brain, whereas the distribution of the A_{2A} ARs is mostly restricted to the dorsal striatum, nucleus accumbens and olfactory tubercle (Cunha, 2005). Consequently, both the A_1 and A_{2A} ARs are believed to be possible targets for drug development in neurological disorders (Fredholm, 2010), and as such, are currently being investigated for their potential benefit in PD (Xu *et al.,* 2005).

In terms of the potential benefit that the A_1 and A_{2A} AR antagonists may possess, it is the A_{2A} AR antagonists that boast likely relief of motor symptoms. In addition to their propensity to alleviate motor symptoms, it has been found that the A_{2A} AR antagonists exhibit a reduced risk of developing the dyskinesia universally associated with long term use of levodopa. (Bara-Jiminez *et al.,* 2003). Furthermore, existing preclinical data suggest that the A_{2A} AR antagonists could be prospective neuroprotective agents (Armentero *et al.,* 2011). All of the abovementioned properties indicate that the A_{2A} AR antagonists are viable candidates for the treatment of PD. On the other hand, the A_1 AR antagonists have demonstrated their worth in improving the cognitive impairment linked to neurodegenerative diseases, such as PD and Alzheimer's disease (Ribeiro & Sebastiao, 2010). Moreover, according to Shook and Jackson (2011), the simultaneous blockade of both the A_1 and A_{2A} ARs is speculated to evoke a synergistic positive motor response, which might be attributed to the release of dopamine (prompted by antagonism of the A_1 AR) with concurrent enhancement of the postsynaptic response to dopamine (potentiated by the A_{2A} AR). Bearing in mind all possible advantages to be elicited by simultaneous blockade of both the A_1 and A_{2A} ARs, it stands to reason that developing dual A_1/A_{2A} AR antagonists would be ideal for novel PD treatment.

1.2 RATIONALE

To reiterate, the problem of PD treatment lacking disease modifying and neuroprotective agents remains to be solved (Shook & Jackson, 2011). The exploration of the A_1 and A_{2A} AR antagonists may present a possible solution (Fredholm, 2010; Xu *et al.,* 2005). Previously, the xanthine derivatives received a considerable amount of attention and served as the prototype which afforded a vast quantity of AR antagonists. However, the focus has now shifted to various non-xanthine scaffolds. Among the known non-xanthine scaffolds, that possess activity as AR antagonists, are the triazoloquinazolines, triazolotriazines, dihydropyridines and adenine derivatives, to name but a few (Klotz, 2000).

Certain benzopyrone classes, have also been examined as AR antagonists. More specifically the flavone (benzo-γ-pyrone) (Moro *et al.,* 1998; Alexander, 2006; Karton *et al.,* 1996; Jacobson *et al.,* 2002; Ji *et al.,* 1996) and coumarin (benzo-α-pyrone) classes (Matos *et al.,* 2015;

Vazquez-Rodriguez *et al.,* 2013; Matos *et al.,* 2013). Formerly, antibacterial, antifungal and antiviral properties (Cushnie & Lamb, 2005), as well as anti-inflammatory, antioxidant and hepatoprotective properties (Tapas *et al.,* 2008) have been associated with the flavones. In turn, the coumarins have, at present, also been linked to antimicrobial, antiviral, anti-inflammatory and antioxidant properties, as well as anticancer properties (Borges *et al.,* 2009).

Based on the above, the isocoumarin class (benzo-α-pyrone), which is structurally related to the flavone and coumarin classes, was evaluated as non-xanthine AR antagonists (Van der Walt & Terre'Blanche, article accepted). It was established that the benzo-α-pyrone compound 3-phenyl-1H-2-benzopyran-1-one (**1**) possessed both A_1 and A_{2A} AR affinity in the low micromolar range (A_1K_i = 7.41 µM; $A_{2A}K_i$ = 3.35 µM) and could prove to be a good candidate for further studies (**Figure 1-1;Table 1-1**). Therefore, 3-phenyl-1H-2-benzopyran-1-one (**1**) will serve as the lead compound in this pilot study.

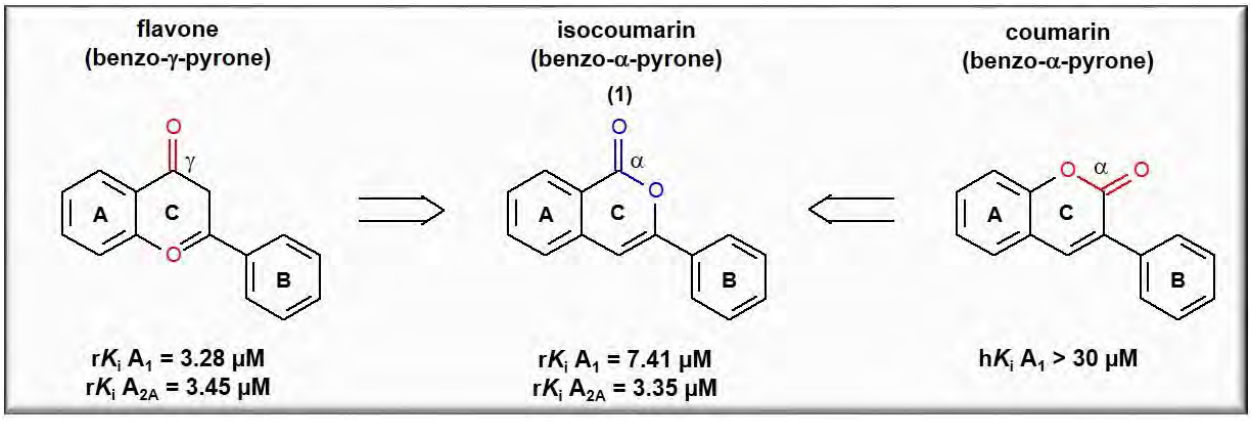
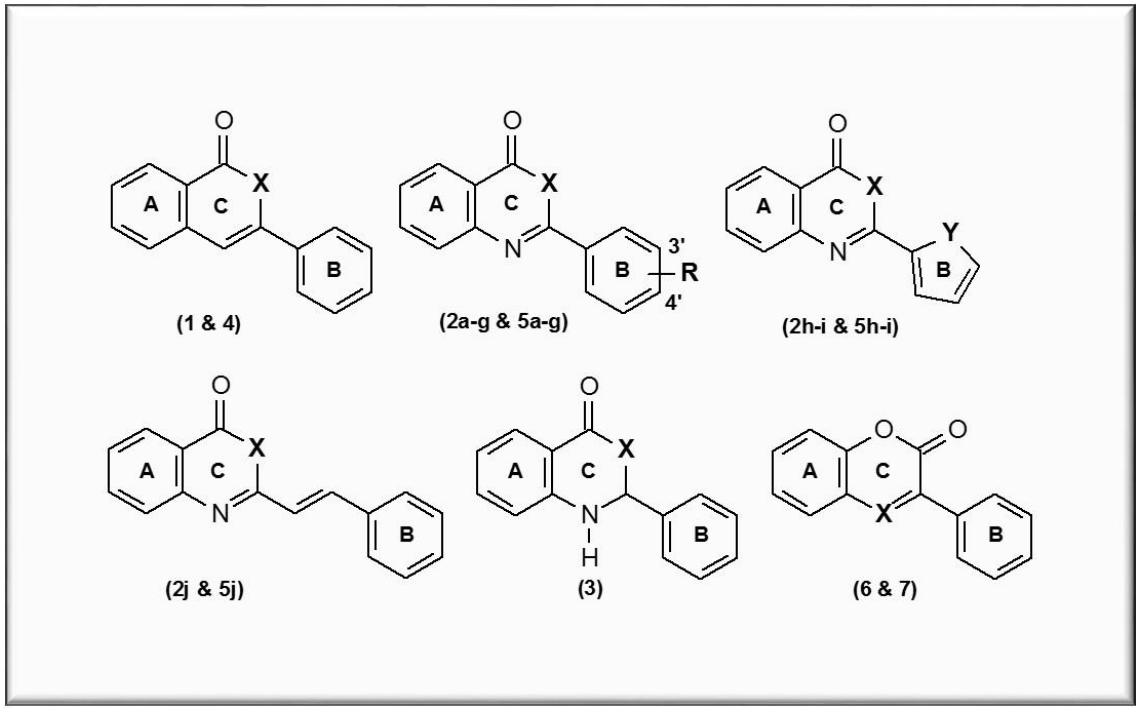


Figure 1-1: **An illustration of the structurally related flavone, isocoumarin and coumarin benzopyrone classes (Van der Walt & Terre'Blanche, article accepted).**

3-Phenyl-1H-2-benzopyran-1-one (**1**) consists of two fused rings (A and C), which forms the basic benzo-α-pyrone skeleton, and a C3-phenyl side-chain on ring C, that serves as ring B. A previous study (Van der Walt & Terre'Blanche, article accepted)(**Figure 1-2, A**) recognised that the double bond between C3 and C4, on ring C, was imperative for AR binding and that the arrangement of the ketone and hetero oxygen in ring C was optimal for A_{2A} AR binding. That being said, the present study will examine the A_1 and A_{2A} AR binding properties of proposed structurally related C2-substituted benzoxazinones (**2a-j, 3**), 3-phenyl-isoquinolinone (**4**) and C2-substituted quinazolinones (**5a-j**) in analogy to the structure of 3-phenyl-1H-2-benzopyran-1-one (**1**)(**Figure 1-2**). The A_1 and A_{2A} AR binding properties for these compounds have not been evaluated prior to this study. The projected compounds are listed in **Table 1-1**.

Table 1-1: Proposed compounds for the current pilot study



Compound	X	R	Y	Compound	X	R	Y
1	-O	-	-	**4**	-NH	-	-
2a	-O	-H	-	**5a**	-NH	-H	-
2b	-O	-4-F	-	**5b**	-NH	-4-F	-
2c	-O	-4-Cl	-	**5c**	-NH	-4-Cl	-
2d	-O	-4-Br	-	**5d**	-NH	-4-Br	-
2e	-O	-4-CH_3	-	**5e**	-NH	-4-CH_3	-
2f	-O	-4-OCH_3	-	**5f**	-NH	-4-OCH_3	-
2g	-O	-4-OCH_2CH_3	-	**5g**	-NH	-3,4-OCH_3	-
2h	-O	-	-O	**5h**	-NH	-	-O
2i	-O	-	-S	**5i**	-NH	-	-S
2j	-O	-	-	**5j**	-NH	-	-
3	-O	-	-	**6**	-CH	-	-
				7	-N	-	-

The proposed C2-substituted benzoxazinones (**2a-j**) will retain the basic scaffold of compound **1** with the addition of a hetero nitrogen to ring C (**Figure 1-2, B**). This structural modification will allow for determination of the structure activity relationship (SAR) of the additional nitrogen on

ring C, whilst preserving compound **1**'s abovementioned double bond (ring C), which is considered essential for AR affinity. Supplementary assessment of the proposed benzoxazinones will entail several substitutions on the phenyl ring B (**2b-g**). The inclusion of compound **3** will provide confirmation on whether the double bond of the benzoxazinone scaffold (ring C) will possess the ability to govern AR binding (**Figure 1-2, B**). The incorporation of the isoquinolinone derivative (**4**) into the study is set to highlight whether the hetero oxygen on ring C of the benzo-α-pyrone backbone of compound **1** is favoured for AR affinity, when compound **4** is compared with compound **1** (**Figure 1-2, C**).

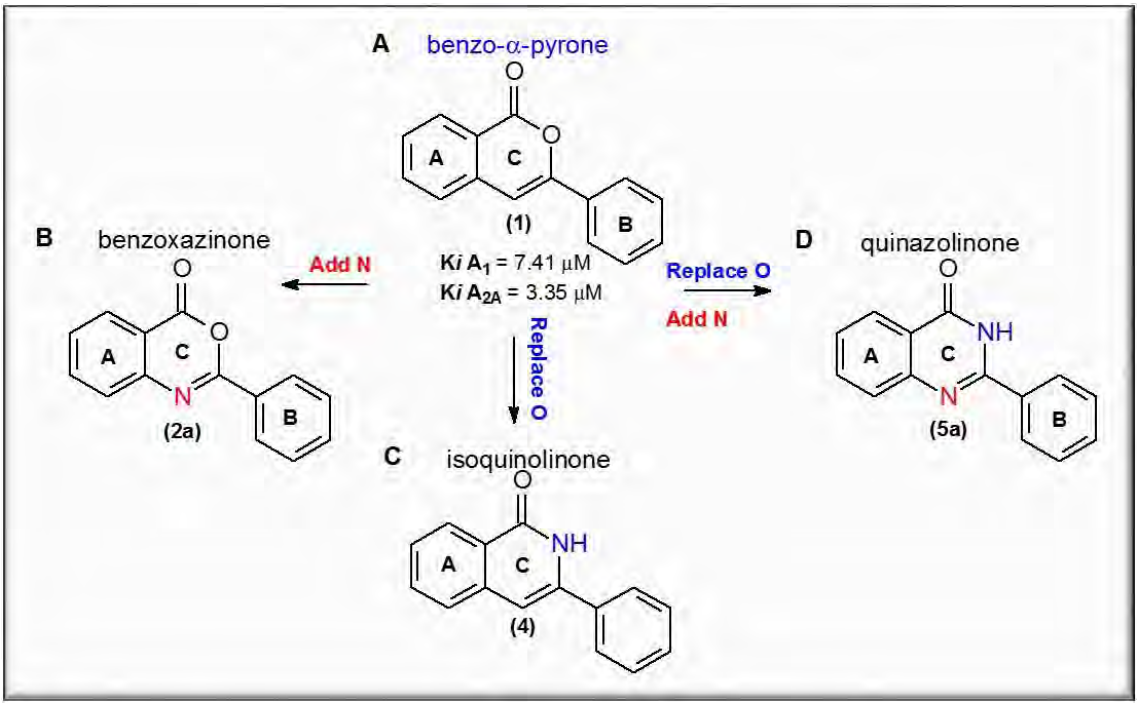


Figure 1-2: **General structures of the various scaffolds to be explored in the current investigative study: (A) 3-phenyl-1H-2-benzopyran-1-one (1), (B) 2-phenyl-4H-3,1-benzoxazin-4-one (2a), (C) 3-phenylisoquinolin-1(2H)-one (4) and (D) 2-phenylquinazolin-4(3H)-one (5a).**

Alongside the SAR of proposed benzoxazinones (**2a-j**), the exploration of the effect elicited by two hetero nitrogen atoms of ring C on the SAR, and thereby AR affinity, will be assessed by means of the C2-substituted quinazolinones (**5a-j**) (**Figure 1-2, D**). Structural modifications of the phenyl ring B to be evaluated, will be implemented in the following fashion: the phenyl ring B will be replaced with other heterocyclic ring systems (e.g. furyl and thiophene) to yield the desired benzoxazinone (**2h-i**) and quinazolinones derivatives (**5h-i**); a styryl side chain between ring B and C will be incorporated to provide the intended benzoxazinone (**2j**) and quinazolinone (**5j**). Lastly, the rearrangement of the ketone and hetero oxygen configuration (compound **1**) is

to be studied by including compounds **6** and **7**, where the ketone and oxygen configuration is inverted (**Figure 1-3**).

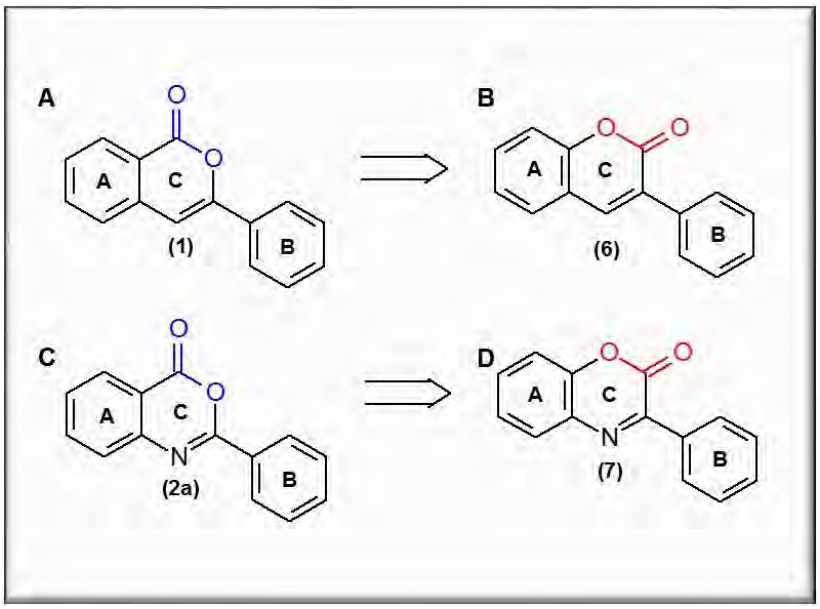


Figure 1-3: **Depicting the proposed rearrangement of the ketone and hetero oxygen configuration of ring C on the benzo-α-pyrone (1 vs 6) and the benzoxazinone scaffolds (2a vs 7).**

1.3 HYPOTHESIS

Based on the affinity exhibited by 3-phenyl-1H-2-benzopyran-1-one (**1**), the proposed structural modifications (see **section 1.2**) to the SAR, in comparison to compound **1**, will lend insight into which structural components are fundamental in retaining or improving A_1 and A_{2A} AR affinity. This study is also expected to illustrate whether the proposed drug classes hold any promise as prospective AR antagonists.

1.3.1 Aims and objectives

The principal aim of this pilot study is to explore if the selected known benzoxazinone, quinazolinone and isoquinolinone structures are structurally ideal to govern optimal AR affinity. Hereby, novel and potent AR antagonists for the treatment of PD may be identified. The objectives of this study are as follow:

- Selected C2-substituted benzoxazinones (**2a**, **2d**, **2f**, **2h** & **2i**) will be synthesised according to an adapted method described by Khan and co-workers (2014) (**Table 1-1**).

- Selected C2-substituted benzoxazinones (**2b**, **2c**, **2e**, **2g**, **2j**, **3**, **6** & **7**) will be purchased commercially.
- The desired C2-substituted quinazolinones (**5a-j**) will be synthesised according to a modified method described by Rao and co-workers (2015).
- Some of the C2-substituted quinazolinones (**5a**, **5d**, **5f**, **5h** & **5i**) and the isoquinolinone derivative (**4**) will be synthesised according to a method described by Asundaria and co-workers (2012).
- The synthesised benzoxazinones (**2a**, **2d**, **2f**, **2h** & **2i**), quinazolinones (**5a-j**) and isoquinolinone derivatives (**4**) will be verified with proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) and melting points (mp).
- Affinity toward the A_1 and A_{2A} ARs of all proposed compounds, both synthesised and commercially procured, will be evaluated by means of *in vitro* radioligand binding studies as defined in literature (Van der Walt & Terre'Blanche, 2015).
- Selected test compounds exhibiting superior affinity for the A_1 AR will be subjected to a GTP shift assay in order to establish whether a compound functions as an agonist or antagonist of the A_1 AR.

CHAPTER 2

PARKINSON'S DISEASE AND EXISTING TREATMENT

2.1 GENERAL BACKGROUND

The neurodegenerative disorder known as Parkinson's disease (PD) was first described by James Parkinson in his 1817 essay titled: "An essay on the shaking palsy" (Parkinson, 2002). Until 1861 PD was more commonly known as "paralysis agitans" before being dubbed as "maladie de Parkinson" by Charcot (Jankovic, 2008). Presently, it is generally accepted that individuals suffering from this malady usually present with a set of principal motor symptoms and may be simultaneously plagued by a series of non-motor symptoms. The principal motor symptoms include tremors, rigidity, bradykinesia and postural instability, while the non-motor symptoms commonly present as a form of cognitive impairment (Jankovic, 2008; Lees & Smith, 1983).

In terms of prevalence, PD is the second most prevalent age-related neurodegenerative disorder, surpassed only by Alzheimer's disease. Statistics for the year 2005 documented the prevalence of PD in the United States at 95 per every 1000 persons, aged above 65. Furthermore, Dorsey and co-workers (2007) estimate that by 2030 the prevalence will have doubled. As the second most prevalent neurodegenerative disorder, PD boasts an arsenal of treatment options. Nevertheless, all known treatment options are of a symptomatic nature rather than curative. Thus the expected increase in prevalence coupled with the lack of a cure, as of yet, indicates the urgency of developing of novel therapies with regards to PD (Dauer & Przedborski, 2003).

2.2 NEUROPATHOLOGY

The pathological archetype of PD is characterised by the loss of the nigrostriatal dopaminergic neurons accompanied by the presence of Lewy bodies. (Dauer & Przedborski, 2003). Where Lewy bodies, according to Gibb and Lees (1988), are distinct neuronal inclusions that are always present in the substantia nigra and other specific regions of the brain in PD. These inclusions are essentially composed of structurally altered neurofilament and occur where there is excessive loss of neurons. Although in some cases elderly individuals may present with LBs, they are rarely documented in other degenerative diseases (Gibb & Lees, 1988). However, the pattern of neurodegeneration in PD was shown to differ from that of normal aging. In PD, the ventrolateral and caudal portions of the substantia nigra pars compacta (SNpc) suffers significant cell loss, whereas during the normal aging process the dorsomedial region of the SNpc is affected instead (Fearnley & Lees, 1991). Furthermore, the abovementioned neuronal

loss results in the notorious depigmentation of SNpc, which is elicited by the coinciding loss of remarkable amounts of neuromelanin (Gibb & Lees, 1991).

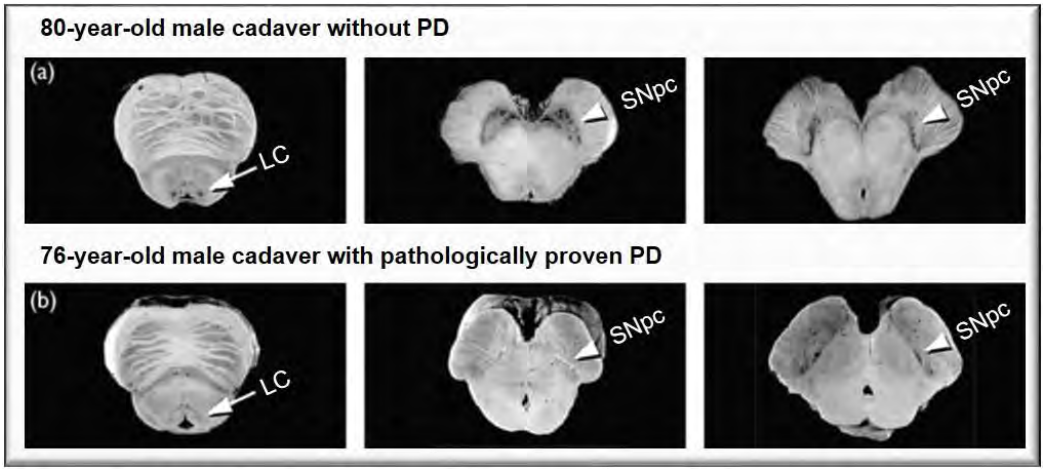


Figure 2-1: **An illustration of the characteristic neuropathology in PD. The difference between the locus coeruleus (LC) and the substantia nigra (SNpc) in a healthy brain (a) vs a brain with pathologically proven PD (b) (Sasaki *et al.*, 2006) reproduced with permission from Wolters Kluwer.**

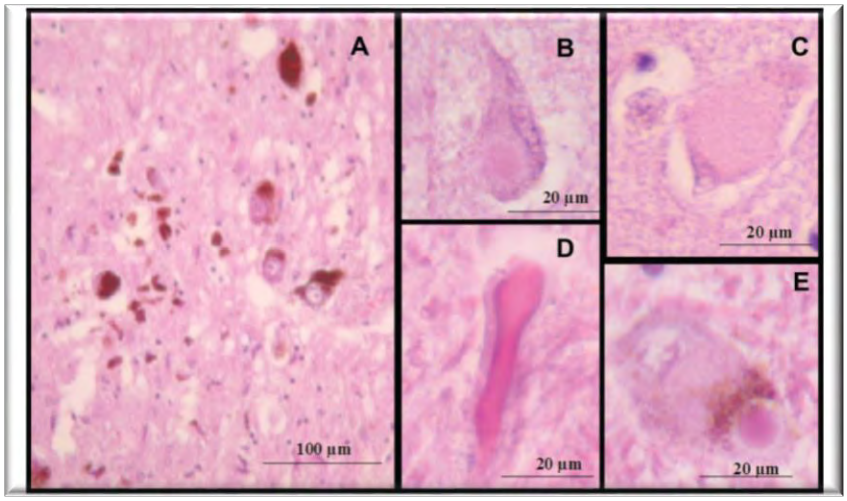
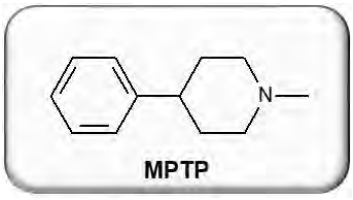


Figure 2-2: **An illustration of PD associated neuropathology. Severe neuronal loss, secondary spongiosis and pigment-laden macrophages present in the SN (A). A concentric Lewy body (B) and a non-concentric Lewy body (C) present in the Cingular cortex. An atypical elongated rod-like Lewy body (D) in the Pontine tegmentum. A classic Lewy body (E) in the LC (Zarranz *et al.*, 2004) reproduced with permission from Wiley.**

Even though the neuropathology of PD is greatly characterized by loss of dopaminergic neurons, the neurodegeneration may transcend dopaminergic neuron loss and could also be found in the noradrenergic, serotonergic and cholinergic systems, as well as in the cerebral cortex, olfactory bulbs and the autonomic nervous system (Dauer & Przedborski, 2003).

2.3 ETIOLOGY

To date, there is no known specific etiology for PD. However, based on an assortment of epidemiological studies various environmental risk factors, ranging from exposure to pesticides to poisoning with an exogenous toxin, have been identified. The toxin 1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP) can be used to demonstrate the validity of an argument for environmental risk factors. MPTP being a by-product of illegally synthesised meperidine, a commonly abused substance, resulted in a syndrome closely resembling that of PD. The latter syndrome was first observed in drug addicts (Langston *et al.,* 1983).



Opposed to environmental factors, genetic factors or positive family history have been described as the most significant risk factor for the development of the disease, alongside age (Polymeropoulos, 2000). A study on familial PD revealed the gene that encodes for the protein α-synuclein was of some interest (Polymeropoulos *et al.,* 1997). It was found that 85% of the patients who expressed a mutation on this gene presented with clinical features of PD. Although this mutation of the α-synuclein gene is not present in cases of sporadic PD, it was discovered that Lewy bodies contain an abundance of the α-synuclein protein, regardless of familial or sporadic PD (Spillantini *et al*., 1997). This indicates that the accumulation of α-synuclein may indeed play a role in the development of PD (Olanow & Tatton 1999).

Aside from the environmental and genetic factors, endogenous toxins is another plausible cause of PD neurodegeneration (Dauer & Przedborski, 2003). Toxic substances may be formed during defective metabolism, which in turn may be caused by environmental factors or inherited mutations of the metabolic pathways. The reactive oxygen species (ROS) are an example of endogenous toxins and are formed during the process of normal dopamine metabolism (Cohen, 1984).

Although all the aforementioned factors are considered as plausible explanations as to the etiology of PD, it is highly unlikely that a single cause can be ascribed to the majority of PD cases (Olanow & Tatton, 1999).

2.4 PATHOGENESIS

In terms of the pathogenesis of PD, oxidative stress is a widely renowned topic, due to the potential of the oxidative metabolism of dopamine to yield hydrogen peroxide (H_2O_2) and other ROS (Jenner, 2003; Spina & Cohen, 1988). Under the following circumstances oxidative stress could result in cell death in the SNpc: (1) increased dopamine metabolism, resulting in increased peroxide formation; (2) a glutathione deficiency (GSH), causing inefficient H_2O_2 clearance; or (3) an increase in reactive iron, which in turn may increase formation of hydroxyl radicals. These markers of oxidative stress were confirmed by post-mortem studies in PD brains (Jenner & Olanow, 1996).

Another main factor considered in the pathogenesis of PD is that of mitochondrial dysfunction (Olanow & Tatton, 1999). The SNpc of PD patients has been shown to suffer a selective decrease (30-40%) in complex I activity of the mitochondrial respiratory chain (Schapira *et al*., 1990). Cell degeneration in PD is a likely consequence of decreased adenosine triphosphate (ATP) synthesis and bioenergetics defects rendered by a defective mitochondrial complex I (Scotcher *et al*., 1990). Moreover, defects at this site may also result in increased free radical formation, subsequently resulting in cell death (Di Monte *et al.,* 1986).

Other factors such as excitotoxicity, neurotrophic factors and glia immune modulators, as well as misfolding and aggregation of proteins, have also been implicated in the pathogenesis of PD (Olanow & Tatton, 1999; Dauer & Przedborksi, 2003).

2.5 MECHANISM OF NEURODEGENERATION

In recent years the focus shifted from necrosis to apoptosis as a probable mode of cell death in PD (Olanow & Tatton, 1999). As stated by Olanow and Tatton (1999), necrosis transpires rapidly and is characterized by: 1) massive ionic fluxes across the plasma membrane (especially Ca^{2+}), 2) activation of Ca^{2+}-dependent proteases, 3) disruption of mitochondrial functions accompanied by complete loss of ATP production, 4) immense cellular swelling and rupture of plasma membrane, 5) secondary inflammatory response and 6) the relative preservation of nuclear deoxyribonucleic acid (DNA). Opposed to necrosis, apoptosis occurs gradually and is recognized by: 1) marked cell shrinkage, 2) preserved plasma membranes, 3) absent inflammatory responses, 4) cytoskeletal depolymerisation, 5) nuclear DNA fragmentation and 6) the formation of apoptotic bodies as a result of chromatin condensation (Olanow & Tatton, 1999).

Excess cell replication is normally countermanded by apoptosis, but as mature nerve cells rarely undergo replication they were initially disregarded as a site for apoptosis. Now it is believed that

neuronal apoptosis may be the product of various injuries sustained, thus linking apoptosis to the pathogenesis of PD (Tatton & Kish, 1997).

2.6 TREATMENT OF PARKINSON'S DISEASE

PD boasts the broadest collection of pharmacological and surgical treatment options when compared to other neurodegenerative diseases (Tarsy, 2006). Therefore, as stated by Tarsy (2006), each patient needs to be managed according to individual merit (signs and symptoms, age, stage of disease, degree of functional disability and the level of physical activity and productivity). The focus of PD management rests on improving both motor and non-motor deficits in order to maintain the best quality of life possible (Chen & Swope, 2007).

Treatment of PD is commonly divided into three categories, namely pharmacological, non-pharmacological and surgical treatment and is initiated upon a diagnosis made by means of a clinical evaluation supported by laboratory studies and brain imaging (Tarsy, 2006). Initial treatment often entails monotherapy, where the use of a singular agent is optimised until increased dosages are no longer tolerated or have reached the maximum prescribable dose (Chen & Swope, 2007). **Figure 2-1** depicts initial treatment possibilities. The disease progression that follows should be accompanied by the meticulous addition of the necessary adjunctive agents in order to maintain symptomatic relief and control motor complications. Polytherapy is only sustained whilst intolerance and comorbidities remain absent, thereafter it becomes advisable to revert back to monotherapy (Chen & Swope, 2007).

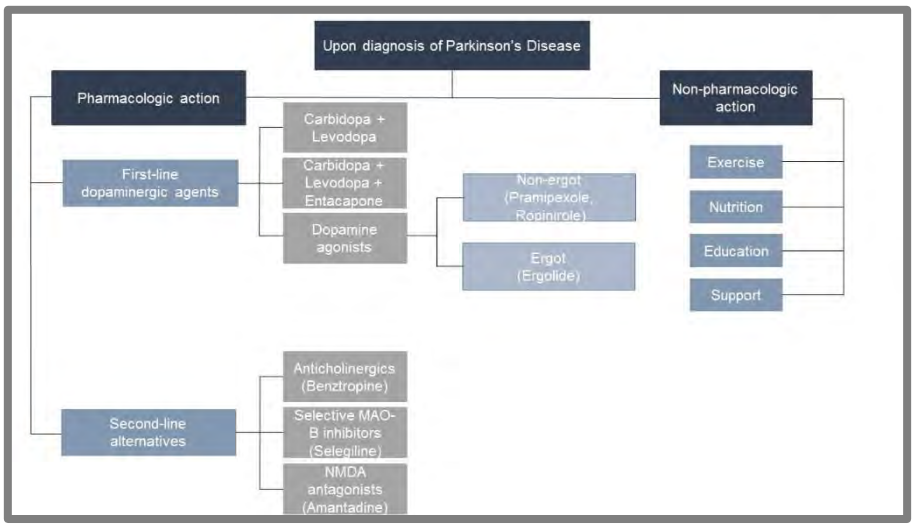
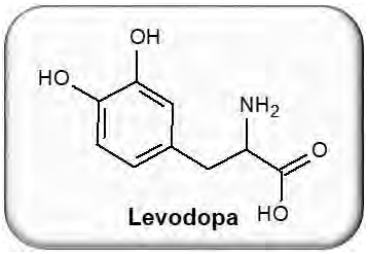


Figure 2-3: Initial treatment of PD adapted from Chen & Swope (2007) reproduced with permission from Wiley.

2.6.1 Levodopa

According to Katzenschlager and Lees (2002), the treatment of PD took a revolutionary turn in the 1960s when levodopa, a dopamine precursor, was introduced and since then it has been deemed the most effective symptomatic treatment option. Although various alternative treatment options in early PD do exist, Fahn (2006) states that all patients will inevitably require levodopa therapy as the disease progresses. Furthermore, levodopa therapy may also be combined with dopa-decarboxylase inhibitors (see **6.2.3**), catechol-O-methyltransferase inhibitors (see **6.2.4**) and monoamine oxidase B inhibitors (see **6.2.5**) to obtain maximum levodopa levels at target receptors and increase half-life (Fahn, 2006).



Levodopa

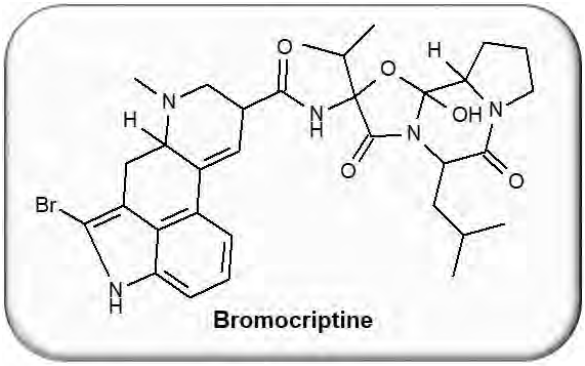
Levodopa provides symptomatic relief for a number of PD related symptoms, but not all symptoms of PD respond in equal measure. For example, bradykinesia and rigidity are known to display the best response to dopaminergic therapy, but symptoms like tremors tend to be fickle. Also, symptoms like postural instability, micrographia and speech impairments are more often than not unresponsive to dopaminergic therapy and in all likelihood point to deficits in other neurotransmitter systems (Fahn, 2006). Despite the symptomatic relief to be gained by administration of levodopa, the risk of developing debilitating dyskinesia associated with long term use and the possibility of hastening neurodegeneration, give cause for concern (Parkinson Study Group, 2004; Cotzias *et al.,* 1969)

2.6.2 Dopamine agonist

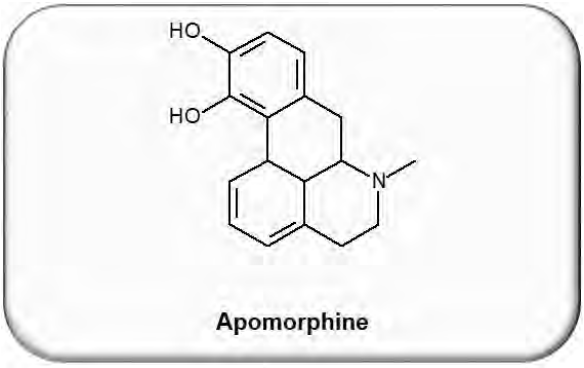
Given the reservations concerning levodopa therapy, dopamine receptor agonists were presented as possible treatment options for PD in the early 1970s. The dopamine receptor agonists display a diverse set of physical and chemical properties but find common ground in their aptitude for stimulating dopamine receptors to elicit an antiparkinsonian effect (Stocchi, 1998). These agents were originally introduced as adjunctive therapy to levodopa in patients with an advanced stage of PD (Calne *et al*., 1974) and studies have found that they exhibit the capacity to ameliorate motor fluctuations and reduce dyskinesia in PD patients treated with levodopa (Rinne, 1985). Additionally, a comparison of levodopa and dopamine agonist therapy highlighted that the dopamine agonists may have some theoretical advantages over levodopa. The first being that dopamine agonists exhibit a levodopa-sparing effect. Secondly, the

consequence of levodopa-sparing, concurrent with stimulation of presynaptic autoreceptors, is a diminished dopamine turnover. The diminished dopamine turnover also results in a diminished amount of toxic metabolites, which may be indicative of a neuroprotective effect (Münchau & Bhatia, 2000). Thirdly, dopamine agonists may act as free radical scavengers and therefore, potent antioxidants (Yoshikawa *et al.,* 1994).

However, dopamine receptor agonist monotherapy is more likely to cause nausea, vomiting, postural hypotension, gastralgia and hallucinations, particularly in geriatric patients, than levodopa monotherapy (Stocchi, 1998). In a review on simultaneous use of a dopamine receptor agonist and levodopa in early PD, Factor and Weiner (1993) have concluded that the literature may be misleading and that the trials do not support the efficacy of such a combination in early PD. Nonetheless, there are two general classes of dopamine agonists, namely the ergot and non-ergot derivatives (Stocchi, 1998). The ergot derivatives consist of drugs like bromocriptine, pergolide, lisuride and cabergoline, whilst apomorphine, pramipexole and ropinirole represent the non-ergot derivative category (Münchau & Bhatia, 2000).



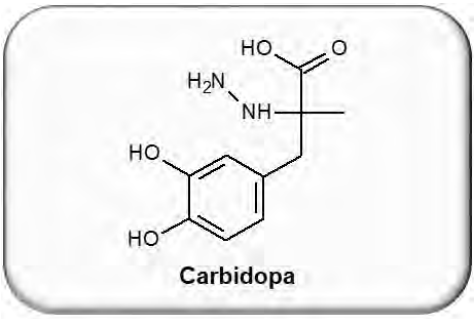
Bromocriptine



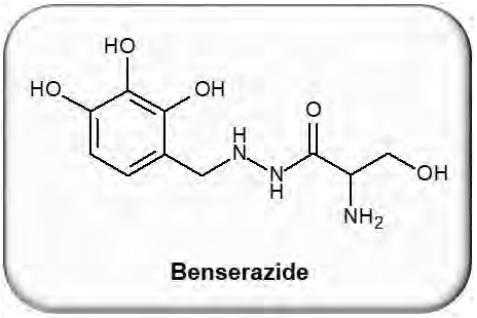
Apomorphine

2.6.3 Dopa-decarboxylase inhibitors

Once administered, levodopa is subjected to peripheral decarboxylation by the enzyme dopa decarboxylase (DDC), which considered as the most important metabolic pathway for levodopa. Consequently, lower concentrations of levodopa reach the brain (less than 1%) (Nutt *et al*. 2005; Kaakkola, 2000). In order to lessen the effect of peripheral decarboxylase, levodopa is typically co-administered with a decarboxylase inhibitor such as carbidopa and benserazide, thereby, allowing the effective dose administered to be reduced by 75% and not only increasing the concentration of levodopa that crosses the blood-brain barrier, but also diminishing nausea, vomiting and orthostatic hypotension caused by increased peripheral dopamine (Tarsy, 2006; Kaakkola, 2000).



Carbidopa

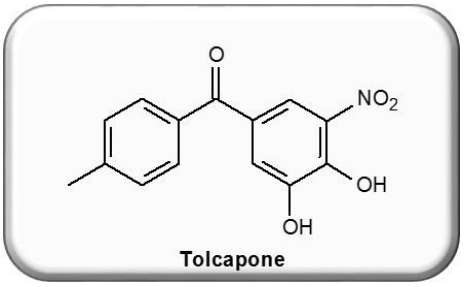


Benserazide

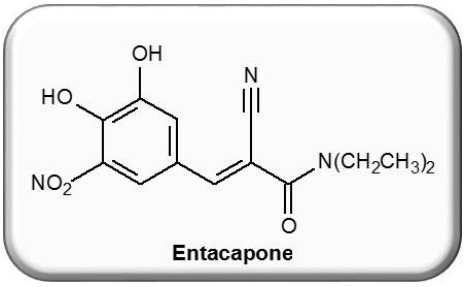
2.6.4 Catechol-O-methyltransferase inhibitors

Catechol-O-methyltransferase (COMT) is an intracellular enzyme that is found extensively throughout the entire body. COMT acts as the catalyst during the transfer of the methyl group of the S-adenosyl-L-methionine to one of the hydroxyl groups of the catechol substrate (Axelrod, 1957). The notable physiological substrates of COMT include the following catechols: dopamine, adrenaline, noradrenaline and their hydroxylated metabolites and catecholestrogens (Guldberg & Marsden, 1975). In addition to the endogenous physiological substrates, numerous medicinal substances with a catechol structure have been confirmed as substrates. Apomorphine, benserazide, carbidopa, dobutamine, isoprenaline, methyldopa and rimiterol are all examples of the aforementioned medicinal substances (Kaakkola, 2000). Accordingly, the function of COMT can be outlined as the elimination of biologically active or toxic catechols, as well as the elimination of a few other hydroxylated metabolites (Kaakkola, 2000).

COMT-inhibitors were introduced in the 1960s for the first time (Guldberg & Marsden, 1975), but it soon became apparent that they were unsuited for clinical purposes due to the fact that they are unselective, non-potent and toxic (Kaakkola, 2000). Conversely, the new COMT-inhibitors that were developed in the 1980s were both potent and selective. This reignited the initial interest vested in the COMT-inhibitors (Männistö & Kaakkola, 1999). The latter COMT-inhibitors are all equipped with a nitrocatechol structure, with the exception of CGP-28014, which is a pyridine derivative. Two of these structures, tolcapone and entacapone, have endured intense scrutiny and is presently in use in many countries (Kaakkola, 2000).



Tolcapone



Entacapone

The aforementioned COMT-inhibitors are essentially applied as adjuncts in levodopa therapy, as levodopa is metabolised peripherally to a great extent. As discussed in the preceding section

(see **section 2.6.3**), DDC, along with L-aromatic acid decarboxylase, forms the most important metabolic pathway through which levodopa is converted to dopamine (Kaakkola, 2000). Benserazide and carbidopa make it possible to eliminate this pathway and allows for the effective dose of levodopa to be reduced by 75%. Hence, the O-methylation of levodopa to 3-O-methyldopa (3-OMD) becomes the principal pathway for by which levodopa is eliminated in the absence of DDC. Unfortunately, 3-OMD is of no therapeutic value in PD (Kaakkola, 2000). However, Kaakkola (2000) states that when a COMT-inhibitor is co-administered in combination with levodopa, the following benefits may be anticipated:

- Decreased levodopa elimination or prolonged half-life
- Increased area under the concentration time curve (AUC) of levodopa
- Reduced formation of the metabolite, 3-OMD
- Enhanced distribution of levodopa to the brain
- Levodopa dose and administration frequency reduction
- Enhanced and prolonged clinical response to levodopa

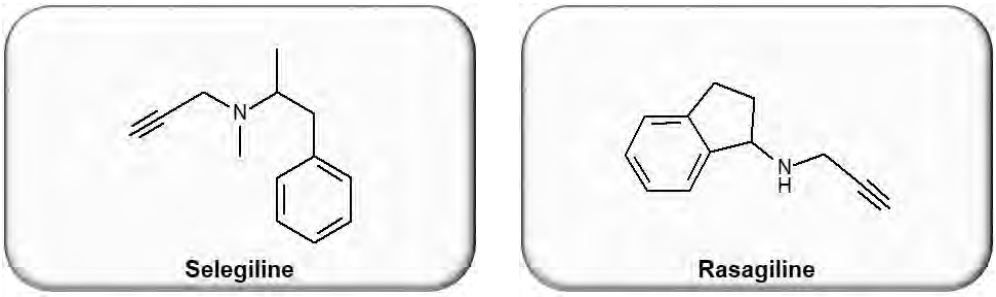
2.6.5 Monoamine oxidase inhibitors

Similar to COMT, monoamine oxidase (MAO) is an enzyme found in the body and is subdivided into MAO-A and MAO-B (Johnston, 1968). MAO is responsible for the metabolism of both endogenous and dietary biogenic amines by means of oxidative deamination (Riederer & Laux, 2011). The predominant substrates for MAO are noradrenaline, adrenaline, dopamine, β-phenylethylamine (PEA) and serotonin. Deficiencies in these substrates are implicated in the biochemical pathology of depression and PD (Riederer & Laux, 2011).

The early 1960s announced the arrival of the first MAO inhibitors. This class of drugs are psycho-pharmacologically active compounds with the ability to inhibit the degradation of the biogenic amine neurotransmitters and thereby increasing the respective concentrations of the previously mentioned substrates in the synaptic cleft and at the relevant postsynaptic receptor sites (Riederer & Laux, 2011). Although the early non-selective MAO inhibitors have the capacity to potentiate the antiparkinsonian effect of levodopa, Bernheimer and co-workers (1962) established that they cause a severe hypertensive crisis.

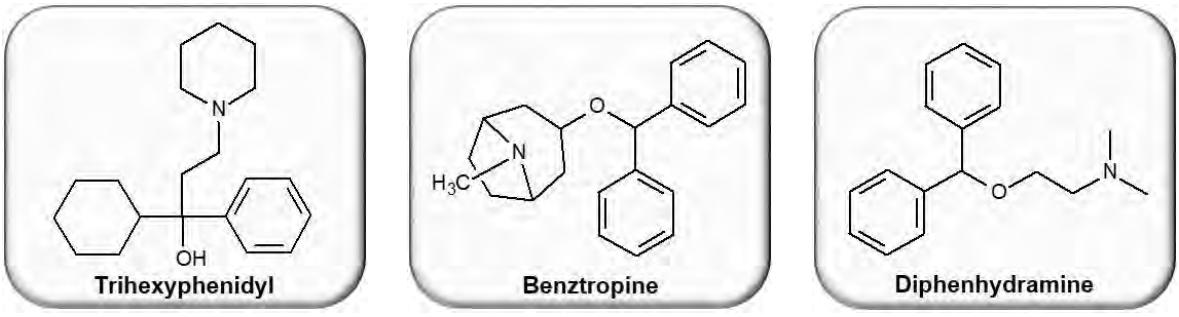
At present the selective MAO type B (MAO-B) inhibitors are preferred and have been in use in PD for nearly two decades (Victor & Waters, 2003). MAO-B has been connected to the conversion of the synthetic dopaminergic pro-neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), to its toxic metabolite 1-methyl-4-phenylpyridine (MPP^+), which is responsible for selective damage to the nigrostriatal neurons (Gerlach *et al.,* 1996). This results in diminished striatal dopamine and elicits almost all of the clinical features relevant in PD

(Langston *et al.,* 1999). Selegiline, a selective MAO-B inhibitor, has been found to prevent the degeneration of striatal dopaminergic neurons caused by MPTP and thus promotes the evidence for MAO-B inhibitors being neuroprotective (Birkmayer *et al.,* 1985*)*. Furthermore, selegiline has generated a significant amount of scientific interest and recent studies have been investigating the selective irreversible MAO-B inhibitor, rasagiline, and the selective, competitive MAO-B inhibitor, lazabemide (Victor & Waters, 2003). Selegiline and rasagiline have both been acknowledged as viable treatment options for the motor symptoms in PD, either as monotherapy or combined with levodopa and a decarboxylase inhibitor (Riederer & Laux, 2011).



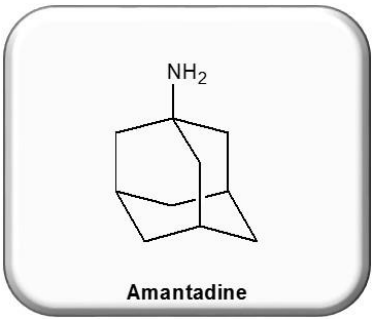
2.6.6 Anticholinergics

Before the discovery of levodopa, the treatment of PD rested mainly on the use of anticholinergic agents. The basis for their therapeutic activity in PD is not fully understood, but it could be postulated that they act within the neostriatum through the receptors that customarily mediate the response to the intrinsic cholinergic innervation of this structure, which primarily originate from the cholinergic striatal interneurons. (Standaert & Roberson, 2011). These agents have only moderate antiparkinsonian activity and fit into the modern-day treatment of PD as either monotherapy in early PD or as adjunctive therapy to the dopaminergic agents. The adverse effects commonly associated with anticholinergic administration range from confusion and sedation to constipation, urinary retention and cycloplegia. Presently, the anticholinergic agents most widely used in PD include trihexyphenidyl, benztropine, and diphenhydramine (Standaert & Roberson, 2011).



2.6.7 Amantadine

Amantadine forms part of a drug class known as the adamantanamines. It was originally indicated as an antiviral drug for the treatment of influenza, but was discovered to alleviate the symptoms of PD perchance (Crosby *et al,* 2003). The exact mechanism for amantadine is still unclear, however, amantadine is known to act as a non-competitive antagonist at the phencyclidine (PCP) site within the N-methyl-D-aspartate receptor (NMDA-receptor) at therapeutic concentrations (Kornhuber *et al,* 1994). Moreover, administration of amantadine is known to enhance the release of dopamine from nerve terminals and to halt the re-uptake thereof (Takahashi *et al,* 1996). Even though amantadine has been widely used in PD in the 1970s, not all patients experience amelioration of symptoms and along with the high probability of developing tolerance to its beneficial effects, amantadine now finds itself infrequently used in PD (Zeldowicz *et al,* 1973; Crosby *et al,* 2003).



Amantadine

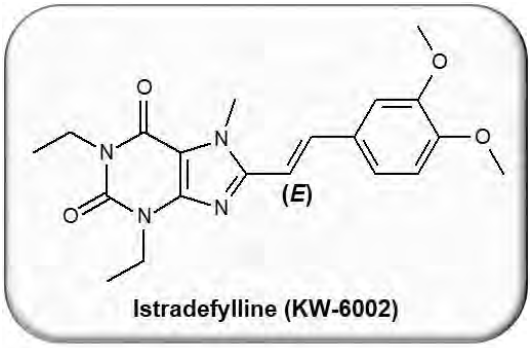
2.6.8 Surgery

The current available pharmacological treatment for PD lack the means to unremittingly fulfil all of the needs typically associated with PD patients. A vast amount of patients who receive levodopa and other dopaminergic drugs chronically develop motor complications (fluctuations and dyskinesia), as well as psychiatric complications (Obeso *et al.,* 1989). In addition to the possible complications, certain symptoms such as gait, balance, speech and deglutition become less responsive to treatment with disease progression and the longer the duration of treatment endures (Obeso *et al.,* 1997*)*. Consequently, the quest for alternative treatment options in PD continues. Surgical treatment is one such alternative under exploration. The current surgical techniques for PD is comprised of techniques such as pallidotomy, thalamotomy, deep brain stimulation and striatal grafting of dopaminergic fetal tissue (Obeso *et al.,* 1997*)*.

2.6.9 Adenosine receptor antagonists

In recent years, another neuromodulator other than dopamine, namely adenosine, has been shown to influence striatal function (Richardson *et al.,* 1997). According to Ferre and co-workers (2001) it fulfils a physiological role opposite that of dopamine by binding to the ARs. The known

ARs include A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 2001). It is postulated that A_{2A} AR receptor modulation may have a profound effect on motor fluctuations (Shiozaki *et al.,* 1999). For example, when the AR agonist, 5'-*N*-ethylcarboxamidoadenosine (NECA), is injected intraperitoneally into mice, catalepsy is induced as a consequence (Zarrindast *et al.,* 1993). In turn, when the selective A_{2A} AR antagonist, SCH-58261, is injected into rats with a unilateral 6-hydroxydopamine lesion of the dopaminergic nigrostriatal pathway an increase in the contralateral turning behaviour induced by levodopa is observed (Fenu *et al.,* 1997). These findings suggest that stimulation of the A_{2A} ARs elicits a negative effect on motor function and that the selective antagonism of the A_{2A} ARs could improve the motor dysfunction associated with PD (Richardson *et al.,* 1997). Furthermore, a study by Ikeda and co-workers (2002) concluded that A_{2A} AR antagonist can prevent dopaminergic neurodegeneration based upon experimental animal models and may thus possess neuroprotective qualities. Although the A_{2A} AR antagonists are more prone toward amelioration of motor symptoms, the A_1 AR antagonists exhibit the valuable advantage of improving the cognitive impairment often associated with neurodegenerative diseases, such as PD and Alzheimer's disease (Ribeiro & Sebastiao, 2010). In combination, A_1 and A_{2A} AR antagonists may exhibit a synergistic positive motor effect, where the release of dopamine is prompted by antagonism of the A_1 AR and is accompanied by a simultaneous enhancement of the postsynaptic response to dopamine by the A_{2A} AR antagonism (Shook & Jackson, 2011). To date, the A_{2A} antagonists that have been subjected to clinical trials, according to Pinna (2014), are as follow: istradefylline (KW-6002), PBS-509, ST-1535 and its metabolite ST-4206, tozadenant, V-81444, prelandenant (recently discontinued) and vipandenant (discontinued). Istradefylline, however, completed phase III clinical trials and is currently recognized as adjunctive therapy for PD in Japan (Dungo & Deeks, 2013). Thus, it stands to reason that AR antagonists, especially the A_{2A} AR antagonists, hold promise as potential pharmacological treatment in PD (Fredholm, 2010). The ARs as potential drug targets in PD will be discussed in more detail in Chapter 3.



Istradefylline (KW-6002)

2.7 CONCLUSION

This chapter depicts various characteristics of neurodegeneration in PD. It also illustrates the symptoms experienced by patients suffering from PD, as well as the current treatment options that are available to them. The shortfalls concerning the treatment of PD are briefly mentioned and it is important to note that current treatment focuses on symptomatic alleviation rather than curative solutions. Henceforth, in addition to symptomatic alleviation, future research ought to be concerned with addressing the disease progression and neurodegeneration of PD through avenues such as neuroprotection.

AR antagonists have been acknowledged as potential pharmacological treatment options in PD (Fredholm, 2010). The alleviation of motor symptoms and display of simultaneous neuroprotective properties are potential properties of A_{2A} AR antagonist treatment. It is also expected that the A_{2A} AR antagonists will exhibit a reduced risk of developing dyskinesia as both monotherapy and as adjunctive therapy with the gold standard levodopa (Fenu *et al.,* 1997; Shiozaki *et al.,* 1999). In turn, the A_1 AR antagonists are recognised as prospective treatment for the cognitive impairment often found in PD and Alzheimer's disease patients (Takahashi *et al.,* 2008), as will be discussed in more detail in Chapter 3.

CHAPTER 3

ADENOSINE RECEPTORS AND ADENOSINE RECEPTOR ANTAGONISTS

3.1 GENERAL BACKGROUND

The current symptomatic treatment regimens of PD, although highly effective in the early stages of therapy, are notorious for their risk of developing complications with prolonged administration, especially levodopa and the dopamine agonists. Some of the more severe complications include motor fluctuations and levodopa-induced dyskinesia (Fahn, 2000) (see **Chapter 2**). Calon and co-workers (2004) describe these motor complications as equally or more debilitating than the symptoms of PD itself, thereby limiting the safe use of pharmaceutical care in PD at all stages of the disease. Attempts to resolve the present treatment dilemma, has resulted in a search for novel non-dopaminergic modulators of the basal ganglia motor circuit that may have worth as alternative or adjunctive therapy, provided they exhibit a reduced adverse effect profile (Xu *et al.,* 2005).

Adenosine is described as a nucleoside that consists of a purine base, adenine, and ribose (Jenner *et al.,* 2009). It functions as a neuromodulator in the brain (Snyder, 1985), with a physiological role opposite to that of dopamine (Ferre *et al.,* 2001). This neuromodulator acts on four G-protein coupled receptors, namely: A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 2001). In the case of the adenosine receptors (ARs), the coupling is to either G_i or G_s and signals mainly through means of activation (A_{2A} and A_{2B}) or inhibition (A_1 and A_3) of cyclic adenosine monophosphate (cAMP) (Ham & Evans, 2012) (see **Figure 3-1**). According to Svenningsson and co-workers (1999), in order for adenosine to function optimally, a copious amount of ARs must be present. Therefore, it is important to note that the AR subtypes with the highest density in the brain are the A_1 and A_{2A} AR subtypes (Gomes *et al.,* 2001). The A_1 ARs are found to a diffuse extent throughout the brain (Cunha, 2005), whereas their A_{2A} AR counterparts are essentially encountered along the dorsal striatum, nucleus accumbens and the olfactory tubercle (Sachdeva & Gupta, 2013).

The ARs present a possible target for a multitude of diseases, including PD, and has long been of interest to the scientific and medical community. Even though there are currently a limited amount of commercially available therapeutic drugs that act on the ARs (such as adenosine in the form of Adenocard® or Adenoscan®), it is still generally maintained that drugs acting on the ARs will be of therapeutic value (Armentero *et al.,* 2011). In fact, Armentero and co-workers report that at least five clinical trials (ranging from phase I to III) are being conducted to evaluate

the feasibility of A_{2A} AR antagonists in the treatment of PD. The prospect of these antagonists as a potential treatment option in PD derives from a substantial amount of investigation on the fundamental interactions between the dopamine receptors and ARs in the basal ganglia (Armentero *et al.,* 2001). Therefore, this chapter aims to elucidate some of the properties generally associated with AR antagonists that may be useful in the treatment of PD.

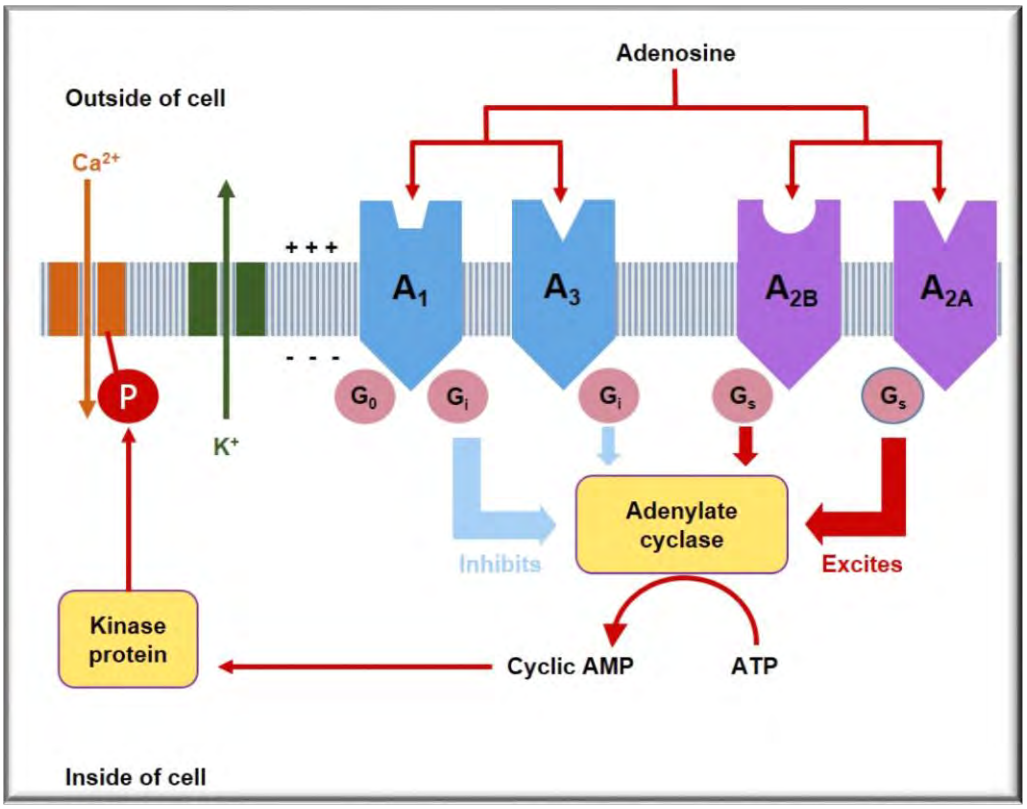


Figure 3-1: **Depicts AR signaling (subtypes: A_1, A_{2A}, A_{2B} and A_3). ARs are G-protein coupled and act through activation or inhibition of cAMP, adapted from Gemignani & Abbott (2010) reproduced with permission from Springer.**

3.2 ADENOSINE RECEPTOR ANTAGONIST PROPERTIES OF POTENTIAL BENEFIT IN THE TREATMENT OF PARKINSON'S DISEASE

3.2.1 Reduction of motor symptoms

The implementation of selective ligands in behavioural studies has unlocked knowledge as to the part that the A_{2A} ARs play in the modulation of motor activity (Armentero *et al.,* 2011). A number of animal models exist that illustrate the advantageous effects on motor dysfunction that may be elicited upon A_{2A} AR inhibition. These effects include: the regression of haloperidol-induced catalepsy or reserpine-induced hypomobility, the modulation of turning behaviour in

unilateral 6-hydroxydopamine lesioned rats and the diminution of motor impairment in MPTP-treated non-human primates (Xu *et al.,* 2005).

Various A_{2A} ARs antagonists, such as KW-6002 and ST-1535, have been shown to successfully oppose catalepsy in rodents by diminishing the severity and duration thereof, thus improving the PD-like motor dysfunctions (Shiozaki *et al.,* 1999; Villanueva-Toledo *et al.*, 2003; Pinna *et al.,* 2005; Stasi *et al.,* 2006). Furthermore, the A_{2A} AR antagonists (such as KW-6002) may possess the capacity to potentiate levodopa's anti-cataleptic effect during combined administration, thereby signifying that a certain synergy between levodopa and the A_{2A} AR antagonists might exist (Shiozaki *et al.,* 1999). The supposed synergistic effect, as demonstrated in the experimental animal catalepsy model, is supported by the potentiating effect observed with the acute administration of a variety of A_{2A} AR antagonists in combination with either levodopa or a dopaminergic drug. In this case, the effect was observed as a marked strengthening of the levodopa or dopaminergic-induced turning behaviour in unilateral 6-hydroxydopamine lesioned rats (Fenu *et al.,* 1997; Koga *et al.,* 2000). Additionally, A_{2A} AR inhibition by the A_{2A} AR antagonist, SCH-58261, exerts a positive effect in rat models of parkinsonian rigidity and resting tremor. Moreover, the co-administration of levodopa and SCH-58261 is also responsible for stimulating a synergistic effect, which results in substantial alleviation of the latter symptoms (Wardas *et al.,* 2001).

Forelimb akinesia, gait impairment and sensory-motor integration deficits are some of the finer features of PD and result from neuron degeneration. Specific tests (initiation of stepping time, adjusting step counting and vibrissae forelimb placing tests) have been evaluated in unilateral 6-hydroxydopamine lesioned rats on the basis that these symptoms are comparable to the PD-linked symptoms in humans (Olsson *et al.,* 1995; Schallert *et al.,* 2000). A_{2A} AR antagonists have been found to reverse the impairments associated with the abovementioned tests (Pinna *et al.,* 2007). A_{2A} AR antagonists are also responsible for the reversal of jaw tremor induced by tacrine, haloperidol or pimozide in rats (Correa *et al.,* 2004).

Concerning the role of A_1 AR antagonists in motor function, it is important to note that a general synergistic effect is experienced with dual A_1 and A_{2A} AR inhibition: dopamine release is prompted by antagonism of the A_1 AR, while A_{2A} AR antagonism exacts an increased sensitivity for postsynaptic dopamine (Shook & Jackson, 2011).

3.2.2 Neuroprotection

An existing hypothesis states that caffeine, a nonselective A_1 and A_{2A} AR antagonist, may protect humans from the dopaminergic neurodegeneration underlying in PD. Although this hypothesis has not been proven, it is strongly supported by evidence suggesting that caffeine

and more specifically selective A_{2A} AR antagonists can exert protective effects against dopaminergic neuron toxicity in rodent models of PD (Xu *et al.,* 2005). According to Gerlach and Riederer (1996), neuron toxicity is simulated in some animal models by exposing the animals to MPTP. MPTP is a dopamine neuron-specific toxin that causes biochemical and anatomical lesions in the dopaminergic nigrostriatal system that mimic a series of symptoms that are of clinical importance in PD. Caffeine, exhibits the ability to, dose-dependently, reverse the loss of striatal dopamine prompted by MPTP when administered to mice at doses equivalent to that of human consumption (5–30 mg/kg) (Chen *et al.,* 2001). However, caffeine is not the only non-specific AR antagonist that, at low micromolar concentrations, neutralises MPTP toxicity. Theophylline and paraxanthine were also reported to have a diminishing effect on MPTP toxicity in a preliminary study on mice (Xu *et al.,* 2010).

Furthermore, by exploring the protective effect that caffeine provides, a revelation concerning the pathophysiology and epidemiology of PD may be discovered. The mechanism of action through which caffeine acts to preserve dopaminergic neurons could be key in the development of novel PD therapeutics with an aptitude for hindering the underlying neurodegenerative process (Armentero *et al.,* 2011). Fredholm and co-workers (1999) notes that the central nervous system effects of caffeine seem to be facilitated predominantly by means of A_1 and A_{2A} AR blockade. Consequently, A_1 and A_{2A} AR antagonists of relative selectivity were tested for their ability to mimic caffeine's attenuation of MPTP toxicity in mice (Armentero *et al.,* 2011). It was found that by pretreating the mice with the A_{2A} AR antagonists relevant to the study, MPTP-induced nigrostriatal lesions could indeed be attenuated (Armentero *et al.,* 2011). These A_{2A} AR antagonists included both xanthine-based compounds (such as DMPX and KW-6002) and non-xanthine structures (such as SCH-58261), alike (Chen *et al.,* 2001). Conversely, an A_1 AR antagonist, at a series of concentrations, exhibited no evidence of neuroprotection against the neuron toxicity stimulated by varying concentrations of MPTP in mice (Chen *et al.,* 2001). More recently, another species and model of PD was examined in order to assess the validity of the neuroprotection hypothesis of A_{2A} AR antagonists, where KW-6002 (A_{2A} AR antagonist), demonstrated the ability to inhibit nigral dopaminergic neuron loss induced by 6-hydroxydopamine in rats (Ikeda *et al.,* 2002).

3.2.3 Antidepressant effects

Depression in PD is a fairly common non-motor symptom, occurring in approximately half of patients with PD and has a marked impact on quality of life (Dooneief *et al.,* 1992). Depression in PD has mostly been attributed to the toll that the prognosis and disability take on PD patients (Schrag *et al.,* 2001). However, depression has been found to precede the onset of motor-symptoms and subsequent diagnosis of PD in an increasing amount of patients, thereby diminishing the validity of reactive depression as the main cause (Leentjens *et al.,* 2003). Irrespective of the etiology of depression in PD, the impact it has on the lives of patients suffering from PD should not be taken lightly.

The A_{2A} ARs have been implicated in PD associated mood modulation and depression as a result of preclinical evidence, thus the effects of A_{2A} AR antagonists on depression in PD during PD trials, should be carefully considered (Xu *et al.,* 2005). During two standard preclinical mouse models, El Yacoubi and co-workers (2001; 2003) studied the effects of certain A_{2A} AR antagonists (SCH-58261, KW-6002 and ZM-241385) and A_{2A} AR depletion, in A_{2A} knockout mice, on depression. The antidepressant activity of the A_{2A} AR antagonists in the aforementioned pharmacological and genetic mouse models, was determined according to recognized predictors of clinical antidepressant activity. The A_{2A} AR antagonists invariably reduced immobility scores in the tail suspension and forced swim tests, thereby demonstrating the possibility of antidepressant activity (El Yacoubi *et al.,* 2001; 2003). Furthermore, although Kaster and co-workers (2004) did not replicate the antidepressant activity of the A_{2A} antagonist ZM-241385, they found that A_{2A} AR antagonism did stimulate the modulation of escape behaviour in the tail suspension and forced swim tests.

3.2.4 Effects on cognition

Elucidation of the relationship between ARs in the central nervous system and the modulation of cognitive function became the objective of various studies over the past decade (Takahashi *et al.,* 2008). The reasoning that adenosine, in its capacity as a neuromodulator, can influence the cognitive processes, likely originated from the general belief that caffeine, by means of nonselective AR blockade, can enhance cognition in humans (Takahashi *et al.,* 2008). A diverse range of pharmacological tools, including knockout mice strains, can be implemented to investigate the relation of ARs to cognitive function (Takahashi *et al.,* 2008).

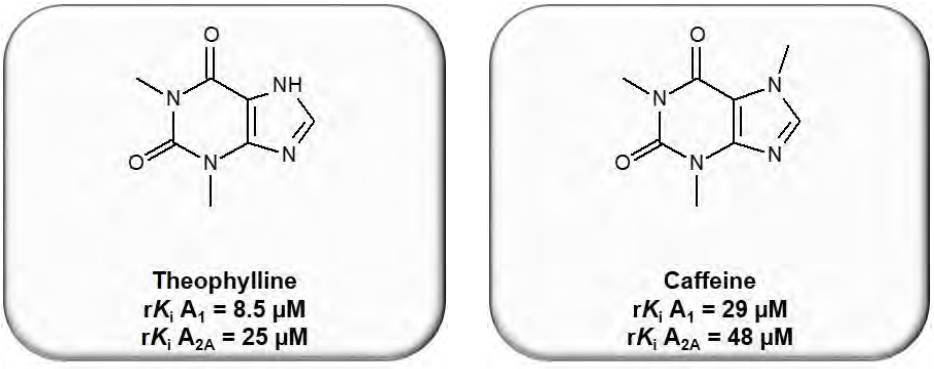
Memory and learning (in rodents) has been found to be disrupted by the stimulation of ARs, mainly by A_1 AR agonists (Corodimas *et al.,* 2001; Normile & Barraco, 1991; Ohno & Watanabe, 1996). In turn, inhibition of the A_1 AR is followed by an increased release of acetylcholine and glutamate (Carter *et al.,* 1995; Solinas *et al.,* 2002) in the higher brain areas associated with the

integration of cognitive and emotional functions (Maemoto *et al.,* 2004). Thus, administration of nonselective ARs antagonists (such as caffeine and theophylline) and selective A_1 AR antagonists, is expected to facilitate learning and diverse memory and behavioural tasks (Hauber & Bareiss, 2001; Kopf *et al.,* 1999; Perreira *et al.,* 2002; Prediger & Takahashi, 2005; Suzuki *et al.,* 1993). Maemoto and co-workers (2004) state that although further examination is needed to fully understand the role of A_1 ARs in memory formation, the success of the selective A_1 AR antagonist, FR-194921, to stimulate a positive effect on memory modulation demonstrates the worth of A_1 AR antagonists in development of cognitive enhancers.

3.3 A_{2A} ADENOSINE RECEPTOR ANTAGONISTS

A_{2A} AR antagonists have generally been accepted amongst the more promising non-dopaminergic agents in the treatment of PD (Feigin, 2003). The growing renown associated with this drug class may greatly be attributed to the success of the A_{2A} AR antagonists in improving the motor deficits in the various animals models (Xu *et al.,* 2005) and certain preliminary preclinical studies of PD (Hauser *et al.,* 2003). Moreover, the distinct pattern of A_{2A} AR expression in the striatum generates additional interest in the A_{2A} AR antagonists. The abovementioned expression encompasses a subset of striatal γ-amino-butyric acid (GABA) output neurons that co-express a vast amount of dopamine D_2 receptors, which project to the globus pallidus (Xu *et al.,* 2005). It is also believed that the limited pattern of A_{2A} AR expression may be an important factor behind the low adverse effect profile associated with A_{2A} AR antagonist administration in PD patients, up to date (Hauser, 2003). All of these features have considerable worth in PD, but according to Xu and co-workers (2005), neuroprotection remains the "holy grail of PD therapeutics". Needless to say, the A_{2A} AR antagonists' potential for neuroprotection renders them exceptionally valuable in the treatment of PD.

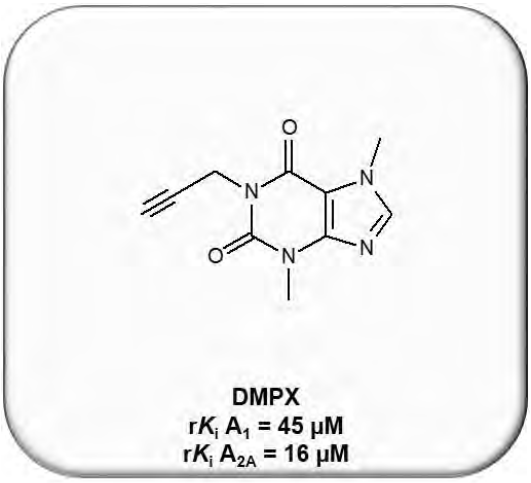
3.3.1 Xanthine A_{2A} adenosine receptor antagonists



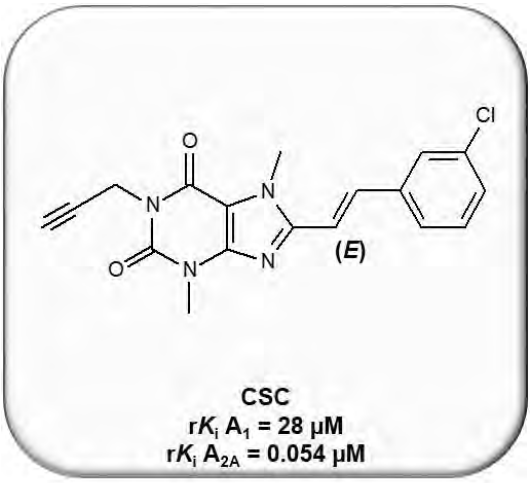
The original interest generated by the A_{2A} AR antagonists' ability to ameliorate the motor symptoms associated with PD, is well associated with early methylxanthines, such as

theophylline and caffeine. Unfortunately, theophylline and caffeine was not only found to be nonselective, but also to possess poor affinity for the A_{2A} ARs (Yuzlenko & Kieć-Kononowicz, 2006). This prompted the pursuit for A_{2A} AR antagonists that are both more potent and selective towards the A_{2A} AR.

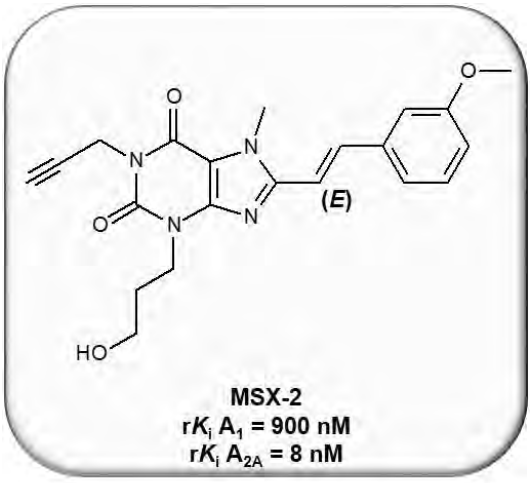
The first xanthine derivative to be acknowledged for its A_{2A} AR affinity, however, was 3,7-dimethyl-1-propargylxanthine or DMPX. This triumph turned out to be short-lived as DMPX too was proven to be nonselective and of rather poor affinity towards the A_{2A} ARs (Yuzlenko & Kieć-Kononowicz, 2006). Nevertheless, numerous 8-styrylxanthines followed the appearance of DMPX, joining the ranks of xanthine derivatives. One such 8-styrylxanthine, 3-chlorostyrylcaffeine (CSC), was documented to exhibit a 520-fold affinity in favour of the A_{2A} ARs over the A_1 ARs, thereby demonstrating a greater selectivity towards the A_{2A} ARs (Jacobson *et al.,* 1993). MSX-2 is another example of an 8-styrylxanthine derivative that possesses a high affinity for the A_{2A} ARs and by structurally modifying it to MSX-3, the disodium phosphate prodrug of MSX-2, high water-solubility is gained along with the apparent affinity for both A_{2A} and A_1 ARs (Sauer *et al.,* 2000). Yet another xanthine-based discovery lead to the xanthine derivative, (*E*) 1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (KW-6002). KW-6002 not only exhibits a potency comparable to that of MSX-2, but is also currently used as adjunctive therapy in PD in Japan (Cacciari *et al.,* 2003; Dungo & Deeks, 2013). Alas, the 8-styrylxanthines are plagued by photosensitivity, where the exposure of a dilute solution of the (*E*)-isomer to normal daylight causes rapid isomerisation to the (*Z*)-isomer. This phenomenon would not be problematic, had the (*Z*)-isomer not been less potent at the A_{2A} ARs than its (*E*)-isomer counterpart (Cacciari *et al.,* 2003). Additionally, the highly lipophilic nature of most xanthines causes them to be poorly water soluble, possibly limiting their *in vivo* capability (Müller *et al.,* 2002).



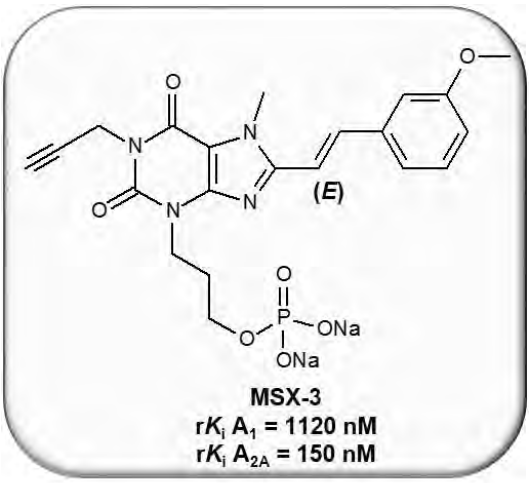
DMPX
rK_i A_1 = 45 µM
rK_i A_{2A} = 16 µM



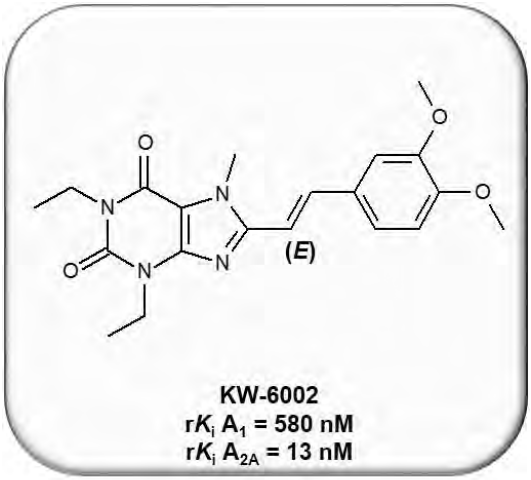
CSC
rK_i A_1 = 28 µM
rK_i A_{2A} = 0.054 µM



MSX-2
rK_i A_1 = 900 nM
rK_i A_{2A} = 8 nM



MSX-3
rK_i A_1 = 1120 nM
rK_i A_{2A} = 150 nM



KW-6002
rK_i A_1 = 580 nM
rK_i A_{2A} = 13 nM

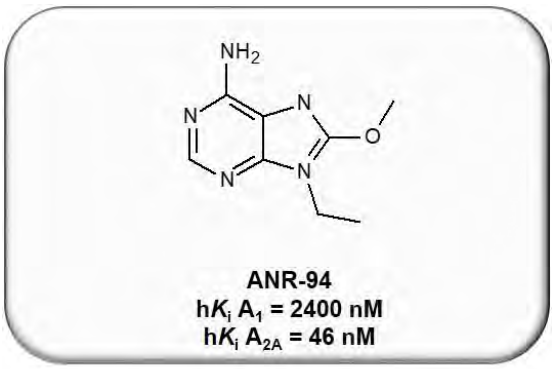
3.3.2 Non-xanthine A_{2A} adenosine receptor antagonists

Prompted by the poor water solubility and the probability of photoisomerization associated with the xanthine derivatives, various other structures were investigated to conquer these undesirable attributes (Yuzlenko & Kieć-Kononowicz, 2006).

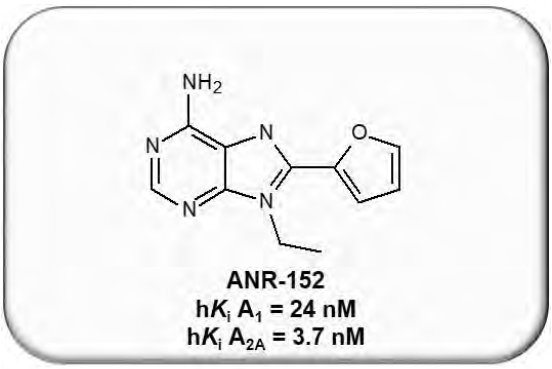
3.3.2.1 Bicyclic fused heteroaromatic systems

The 8-substituted 9-ethyladenine derivatives display promise as A_{2A} AR antagonists by exhibiting high affinity and selectivity towards the A_{2A} ARs. One such compound, ANR-94, possesses an 8-ethoxy group and is deemed a potent A_{2A} AR antagonist. The introduction of a furanyl group into the 8-position resulted in ANR-152 that exhibited a welcome gain in affinity, but unfortunately also resulted in the concurrent loss of selectivity towards the A_{2A} ARs. Nevertheless, both the former (8-ethoxy) and latter (8-furanyl) derivatives have displayed the capacity to reduce haloperidol-induced catalepsy (0.2 mg/kg) in rats after administration of 5 mg/kg doses during a 90 minute testing period (Klotz *et al.,* 2003; Pinna *et al.,* 2005). Furthermore, the 2- or 8-substituted alkynyl derivatives exhibited A_1 and/or A_{2A} AR binding

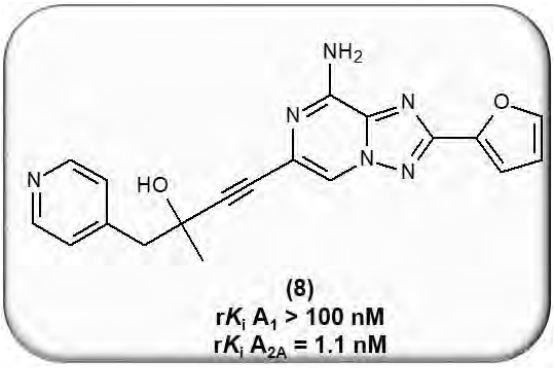
affinity akin to that of the 8-ethoxy and 8-furanyl derivatives (Volpini *et al.,* 2005), and of the alkynyl substituted derivatives 6-alkynyl-2-furan-yl-[1,2,4]-triazolo[1,5-α]pyrazine-8-ylamine (**8**) was deemed the most potent and selective A_{2A} AR antagonist (Yao *et al.,* 2005).



ANR-94
hK_i A_1 = 2400 nM
hK_i A_{2A} = 46 nM

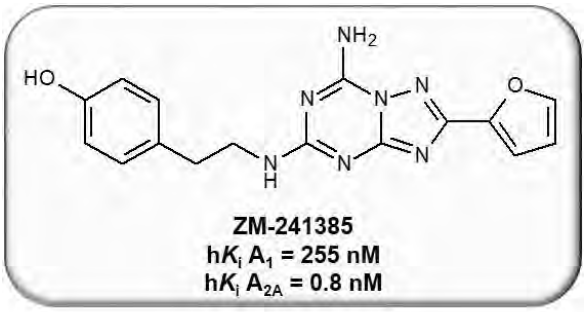


ANR-152
hK_i A_1 = 24 nM
hK_i A_{2A} = 3.7 nM



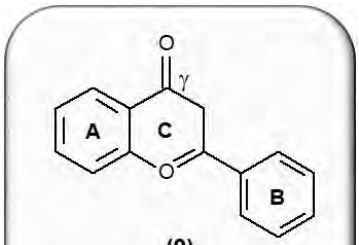
(8)
rK_i A_1 > 100 nM
rK_i A_{2A} = 1.1 nM

The drug discovery process yielded another potent and selective A_{2A} AR, ZM-241385, which is presently used as a radioligand, rather than a drug, due to its poor oral bioavailability (Ongini *et al.,* 1999; Sihver *et al.,* 2004). In an effort to improve the poor bioavailability associated with ZM-241385, the [1,2,4]triazolo[2,3-α][1,3,5]triazine skeleton was implemented to create various new 6:5 fused heteroaromatic compounds. The 5-piperazinyl-2-furanyl derivatives were among the new 6:5 fused heteroaromatic compounds that followed the discovery of ZM-341385 and of these derivatives the fluorine-containing compounds proved to be the most potent compounds, in terms of A_{2A} AR affinity (Vu *et al.,* 2004). Vu and co-workers (2004) noted that the abovementioned compounds displayed good bioavailability and oral efficacy at 3 mg/kg in rodent models of PD.



ZM-241385
hK_i A_1 = 255 nM
hK_i A_{2A} = 0.8 nM

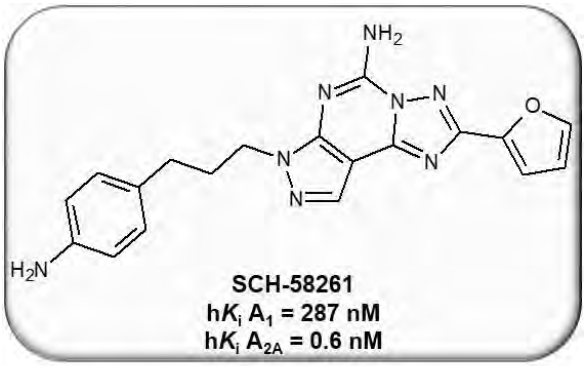
6:6 Fused heteroaromatic ring systems may be represented by the flavone class. Flavones are benzo-γ-pyrone derivatives that have been found to exhibit activity as A_{2A} AR antagonists (see **Chapter 1**). 2-Phenyl-4*H*-1-benzopyran-4-one (**9**), part of the flavone class, is an example of a non-selective A_{2A} AR antagonist (Ji *et al.,* 1996).



(9)
rK_i A_1 = 3.28 µM
rK_i A_{2A} = 3.45 µM

3.3.2.2 Tricyclic fused heteroaromatic systems

SCH-58261, a tricyclic 5:6:5 pyrazolotriazolopyrimidine, is deemed as the first potent and selective tricyclic heteroaromatic A_{2A} AR antagonist (Ongini *et al.,* 1999). Prompted by the potency of SCH-58261 as A_{2A} AR antagonist, various studies aimed to investigate the influence of substitutions on the pyrazole or imidazole ring on activity and water-solubility of the pyrazolotriazolopyrimidines and imidazotriazolopyrimidines, respectively. This resulted in an extended series of tricyclic heteroaromatic compounds with varying affinity towards the A_{2A} ARs (Yuzlenko & Kieć-Kononowicz, 2006). However, a study performed by Baraldi and co-workers (2002), showed promise, where the most active 7-substituted 5-amino-2-furylo-pyrazolotriazolopyrimidines synthesised in this study exhibited both good affinity and selectivity towards the A_{2A} AR. Unfortunately, these compounds also presented with poor water solubility (Baraldi *et al.,* 2002).



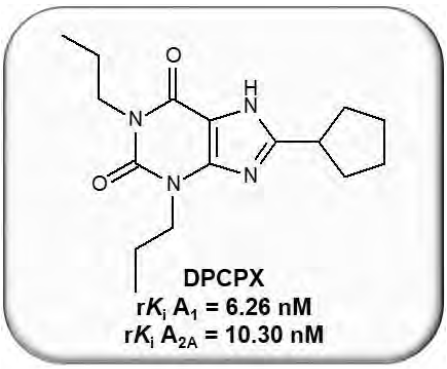
SCH-58261
hK_i A_1 = 287 nM
hK_i A_{2A} = 0.6 nM

3.4 A_1 ADENOSINE RECEPTOR ANTAGONISTS

The A_1 AR antagonists are currently being explored for a multitude of indications. In the heart they hold promise as possible treatment of cardiac arrhythmias, oedemas, as positive inotropes and cardiac protectants, in the kidneys for oedemas and nephritis and, important to this study, in the central nervous system (CNS), where the selective A_1 AR antagonists are essentially valued for their ability to stimulate the CNS. Therefore, the development of novel selective A_1 AR antagonists as cognitive enhancers may prove beneficial in neurodegenerative diseases such as PD and Alzheimer's disease (Yuzlenko & Kieć-Kononowicz, 2006).

3.4.1 Xanthine A_1 adenosine receptor antagonists

The xanthines, theophylline and caffeine, represent the prototypical A_1 AR antagonists. However, these natural xanthines are nonspecific AR antagonists and they exhibit low affinity toward the A_1 ARs (Yuzlenko & Kieć-Kononowicz, 2006). Currently, the xanthine A_1 AR antagonists are mostly investigated for their applications in heart conditions. However, DPCPX is an example of a potent xanthine A_1 AR antagonist of value in PD. It is especially valuable to this study, where its labelled form serves as the radioligand in the A_1 AR radioligand binding assay (see **Chapter 5**) (Bruns *et al.,* 1987).

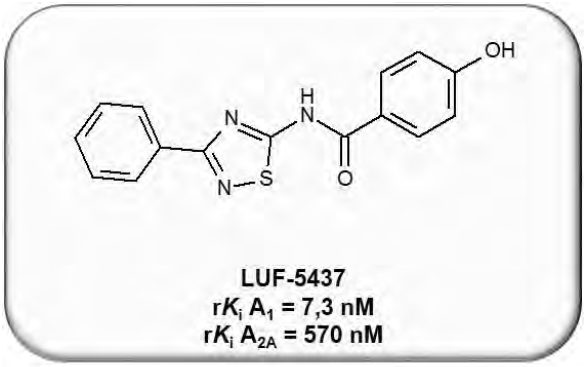


DPCPX
rK_i A_1 = 6.26 nM
rK_i A_{2A} = 10.30 nM

3.4.2 Non-xanthine A_1 adenosine receptor antagonists

3.4.2.1 Non-fused rings

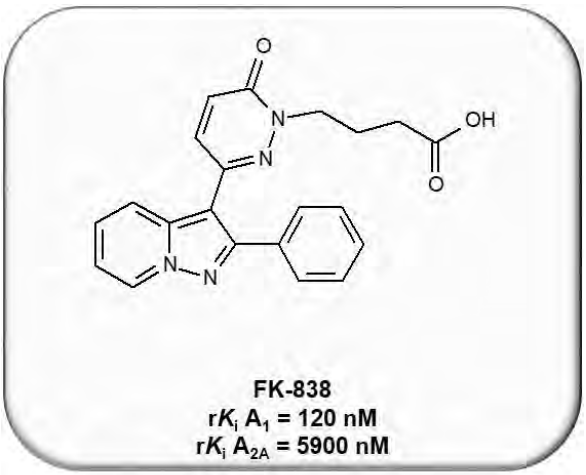
A_1 AR antagonists are rarely found amongst compounds consisting of a monocyclic heteroaromatic ring and if found they generally exhibit low affinity towards the A_1 AR. However, the five-membered heterocycles, thiazole and thiadiazole derivatives, are the exception, with the most potent compound in this series, N-(3-phenyl-1,2,4-thiadiazol-5-yl)-4-hydroxybenzamide (LUF-5437) (Van Muijlwijk-Koezen *et al.,* 2001).



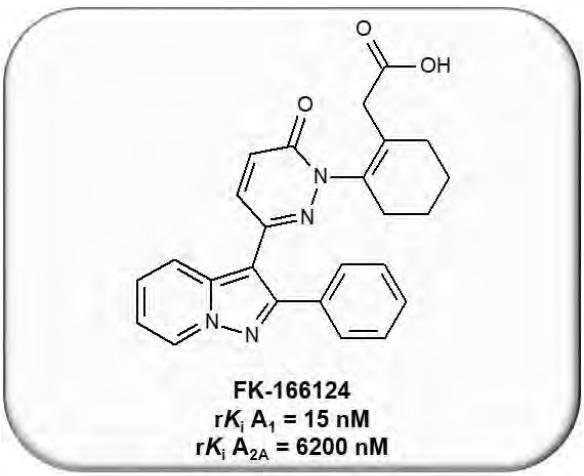
LUF-5437
rK_i A_1 = 7,3 nM
rK_i A_{2A} = 570 nM

3.4.2.2 Bicyclic fused heteroaromatic systems

The non-xanthine A_1 AR antagonist class is presently dominated by 6:5 fused heteroaromatic compounds (Yuzlenko & Kieć-Kononowicz, 2006). Amongst the 6:5 fused heteroaromatic compounds, the pyrazolo[1,5-α]pyridines are the most investigated compounds for A_1 AR affinity. FK-838 and FR-166124 belong to this class and both compounds possess high selectivity towards the A_1 AR, as well as adequate water solubility (Kuroda *et al.,* 1999).



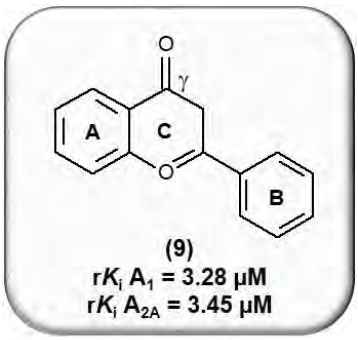
FK-838
rK_i A_1 = 120 nM
rK_i A_{2A} = 5900 nM



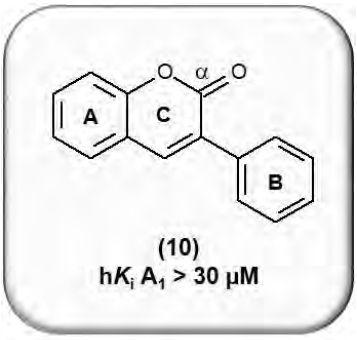
FK-166124
rK_i A_1 = 15 nM
rK_i A_{2A} = 6200 nM

Relative to the 6:5 fused heteroaromatic compounds, the 6:6 fused heteroaromatic compounds are investigated a great deal less (Girreser *et al.,* 2004). Compound **9**, a flavone, and 3-phenyl-

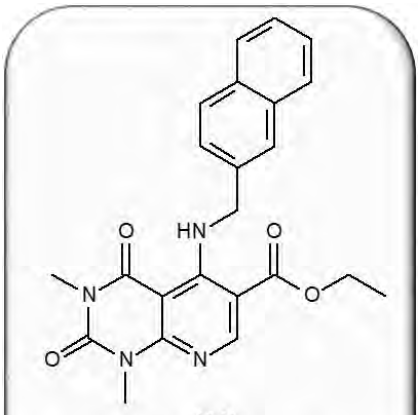
2*H*-chromen-2-one (**10**), a coumarin, are both benzopyrone derivatives that may serve as examples of 6:6 fused heteroaromatic compounds possessing activity as A_1 AR antagonists (Ji *et al.,* 1996; Matos *et al.,* 2013). The substituted pyrido[2,3-*d*]pyridimines also belong to the 6:6 fused heteroaromatic class and the most promising compound in this class, 6-carbethoxy-1,2,3,4-tetrahydro-1,3-dimethyl-5-(2-naphthyl)methylaminopyrido[2,3-*d*]pyrimidine-2,4-dione (**11**), with a naphthyl group at the 5-position, was reported to not only possess nanomolar affinity towards the A_1 AR, but also prominent selectivity over the A_{2A} AR (Bulicz *et al.,* 2006).



(9)
rK_i A_1 = 3.28 µM
rK_i A_{2A} = 3.45 µM

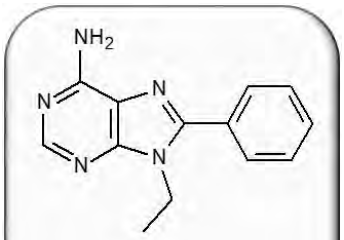


(10)
hK_i A_1 > 30 µM



(11)
rK_i A_1 = 4.97 nM
rK_i A_{2A} ≥ 10000 nM

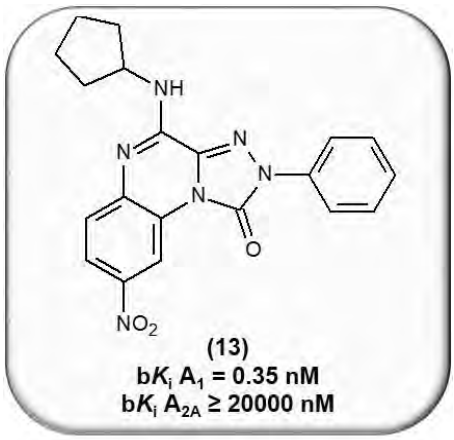
The adenine derivatives represent yet another bicyclic heteroaromatic class that is extensively investigated for activity as A_1 AR antagonists. 9-Ethyl-8-phenyl-9H-adenine (**12**) serves as an example of a potent compound of this class, possessing nanomolar affinity towards the A_1 AR, as well as high selectivity for the A_1 ARs over all other AR subtypes (Klotz *et al.,* 2003).



(12)
hK_i A_1 = 27 nM
hK_i A_{2A} = 360 nM

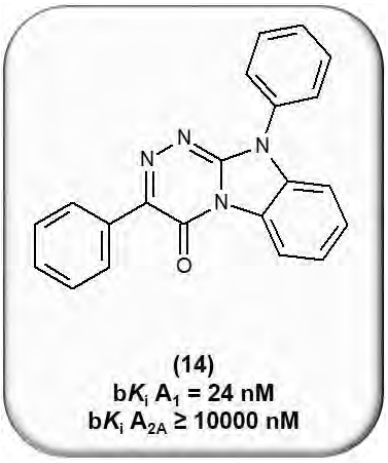
3.4.2.3 Tricyclic fused heteroaromatic systems

Exploration of the tricyclic fused heteroaromatic compounds as A_1 AR antagonists, largely revolves around the 6:6:5 fused heteroaromatic compounds containing nitrogen atoms (Yuzlenko & Kieć-Kononowicz, 2006). 4-Cyclopentylamino-1,2-dihydro-8-nitro-2-phenyl-1,2,4-triazolo[4,3-α]quinoxalin-1-one (**13**) is an example of a potent 6:6:5 fused heteroaromatic compound, it possesses a cyclopentyl as the 4-aminosubstituent and an NO_2 group at C8. It belongs to the 1,2,4-triazolo[4,3-α]quinoxalin-1-one class and is likely the most effective A_1 AR antagonist in this class (Colotta *et al.,* 2004).



(13)
bK_i A_1 = 0.35 nM
bK_i A_{2A} ≥ 20000 nM

A group of 3-aryl[1,2,4]triazino[4,3-α]benzimidazol-4-(10H)-one derivatives are known 6:5:6 fused heteroaromatic compounds proved interesting as they were originally developed as benzodiazepine receptor ligands, but acted as A_1 AR antagonists instead. The most active compound in this class, compound **14**, displays selectivity for the A_1 AR over the A_{2A} and A_3 ARs (Da Settimo *et al.,* 2004).



(14)
bK_i A_1 = 24 nM
bK_i A_{2A} ≥ 10000 nM

3.5 CONCLUSION

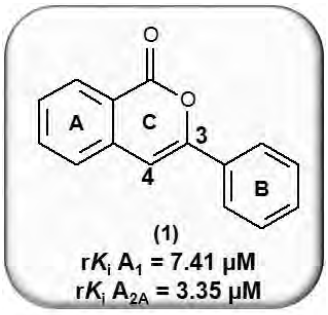
This chapter aimed to outline the physiological role that the ARs play in the brain and which of the AR antagonism-related effects may be of benefit in the treatment of PD. Regarding the latter, this chapter focussed on enhanced motor function, neuroprotection, antidepressant effects and cognitive enhancement as beneficial effects in the treatment of PD. Furthermore, an overview of the various xanthine and non-xanthine structures investigated for A_1 and A_{2A} AR antagonist activity was provided. The following chapter will discuss the synthesis of a series of C2-substituted benzoxazinones and quinazolinones to be evaluated as A_1 and A_{2A} AR antagonists.

CHAPTER 4

SYNTHESIS

4.1 INTRODUCTION

During the continued search for novel treatment options in PD, it was found that compounds that exhibit a high potency antagonism towards the A_1 and A_{2A} adenosine receptor (AR) subtypes were deemed as therapeutically beneficial (see **Chapter 1** and **3**). Therefore, the purpose of this pilot study was to synthesise compounds with a high likelihood of exhibiting the aforementioned antagonist activity at the A_1 and/or A_{2A} AR subtypes. The benzo-α-pyrone, 3-phenyl-1H-2-benzopyran-1-one **(1)**, was previously found to possess both A_1 and A_{2A} AR affinity in the low micromolar range (A_1K_i = 7.41 µM; $A_{2A}K_i$ = 3.35 µM) (Van der Walt & Terre'Blanche, article accepted). Hence, 3-phenyl-1H-2-benzopyran-1-one **(1)** served as the lead compound for this pilot study. In analogy to the structure of 3-phenyl-1H-2-benzopyran-1-one (**1**) the present study aims to investigates the A_1 and A_{2A} AR binding properties of structurally related C2-substituted benzoxazinones (**2a**–**j**, **3**), 3-phenyl-isoquinolinone (**4**) and C2-substituted quinazolinones (**5a**–**j**). In order to evaluate all the proposed structural modifications, compounds were either synthesised (**2a**, **2d**, **2f**, **2h**, **2i** & **5a-j**) or obtained through commercial means (**1**, **2b**, **2c**, **2e**, **2g**, **2j**, **3**, **7** & **8**) from Sigma-Aldrich® and used without further purification.



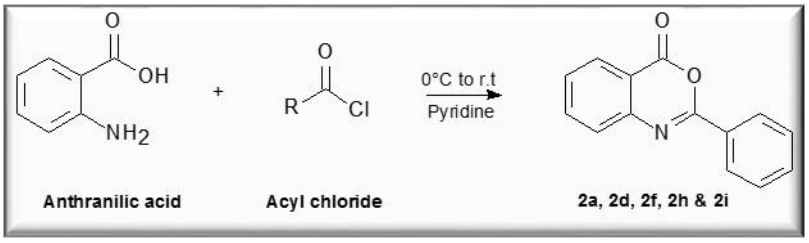
(1)
rK_i A_1 = 7.41 µM
rK_i A_{2A} = 3.35 µM

4.2 GENERAL SYNTHETIC APPROACH

The benzoxazinone derivatives that were designated for synthesis (**2a**, **2d**, **2f**, **2h**, **2i**), were synthesised within an alkaline reaction environment with commercially available acyl chlorides and anthranillic acid as starting material (**Scheme 1**) (Kahn *et al.*, 2014). However, the synthesis of the proposed quinazolinone compounds (**5a**–**j**) comprised of method A and/or B: (A) a one pot synthesis with the appropriate aldehyde, formamide and isatoic anhydride (**Scheme 2**) (Rao *et al.,* 2015) or (B) where the corresponding benzoxazinone was treated with an ammonium hydroxide solution (**Scheme 3**) (Asundaria *et al.,* 2012). The isoquinolinone

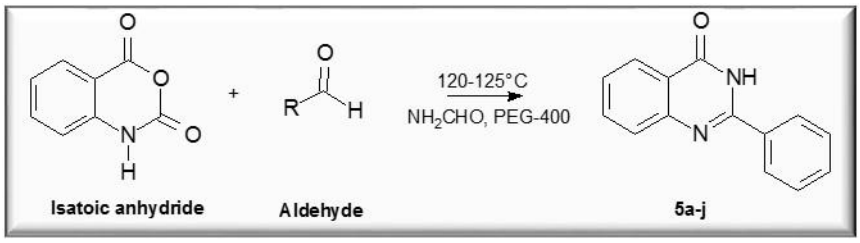
derivative **4** was synthesized by a similar synthetic approach than outlined in method B (**Scheme 3**) (Asundaria *et al.*, 2012). The reaction conditions and compound characterizations are described below. **Table 4-1** depicts the proposed series of compound derivatives that include 3-phenyl-1H-2-benzopyran-1-one **(1)**, 3-phenyl-2H-chromen-2-one **(6)** and selected benzoxazinones. **Table 4-2** depicts the proposed series of compound derivatives that include 3-phenylisoquinolin-1(2H)-one **(4)** and the selected quinazolinones.

Approach for the synthesis of the benzoxazinones derivatives: An adapted version of the method described by Khan and co-workers (2014) was used for the synthesis of the desired C2-substituted benzoxazinone derivatives (**2a**, **2d**, **2f**, **2h** & **2i**). A solution of anthranilic acid (0.01 mol) in pyridine (30 mL) was obtained and cooled to a temperature of 0°C on an ice bath. Gradual addition of the appropriate acyl chloride (0.02 mol) followed and the reaction mixture was stirred for 5 min. At this point the ice bath was removed in order for the reaction mixture to warm to room temperature. Upon reaching room temperature the reaction mixture was continuously stirred for an additional 30 minutes before being poured into a prepared ice-and-water mixture (200 mL) to remove pyridine. The precipitate was then filtered and washed with an additional 200 mL ice-cold water and recrystallized from the appropriate solvent. Throughout the reaction, the reaction progress was monitored by TLC.



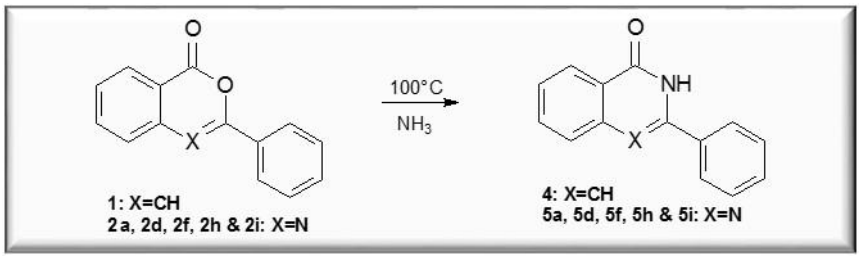
Scheme 4-1: Synthetic pathway to obtain the C2-substituted benzoxazinone derivatives (2a, 2d, 2f, 2h & 2i).

Approach for the synthesis of the quinazolinones via method A: A modified version of the method described by Rao and co-workers (2015) formed the basis of method A to obtain the desired quinazolinone test compounds. Equal parts of the appropriate aldehyde (0.01 mol), formamide (0.01 mol) and isatoic anhydride (0.01 mol) were added to PEG-400 (4 mL) and mechanically stirred at 120–125°C until completion. The reaction progress was monitored by TLC and upon completion was left to cool to room temperature. Hereafter, a measure of ethyl acetate (10 mL) was added and left overnight. Precipitate was filtered and recrystallized from methanol.



Scheme 4-2: Synthetic pathway to obtain the synthesized C2-substituted quinazolinone derivatives (5a–j), method A.

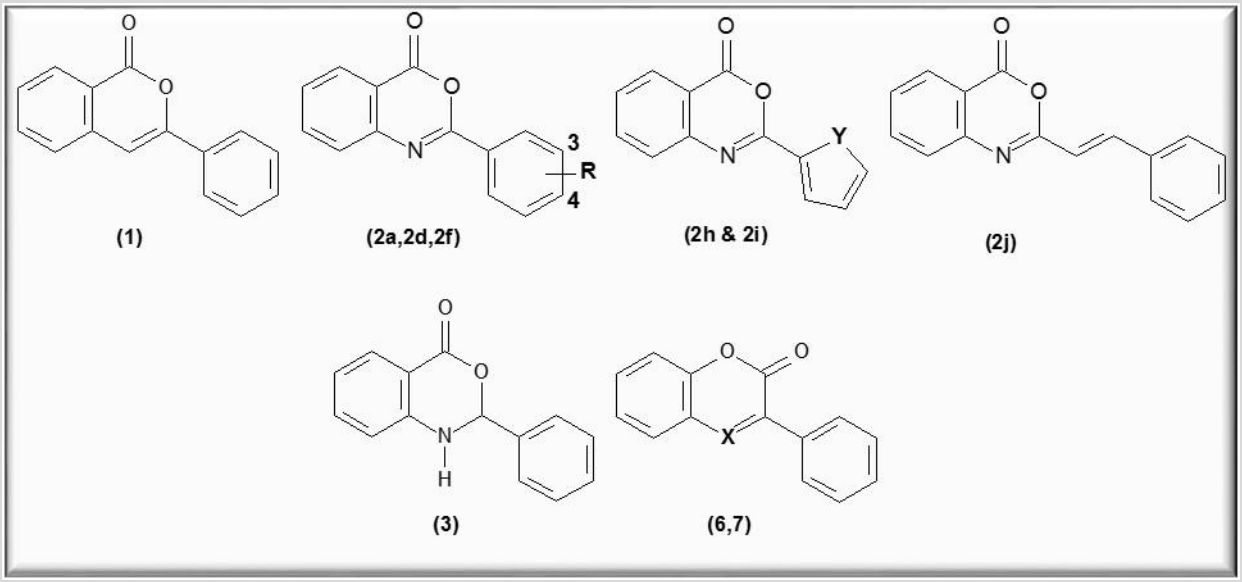
Approach for the synthesis of 3-phenylisoquinalin-1(2H)-one (4) and the quinazolinones via method B: Method B was utilized to synthesise the proposed isoquinolinone and quinazolinone derivatives and was based on protocols described by Asundaria and co-workers (2012). The appropriate benzoxazinone (0.01 mol) was suspended in 30 mL of an ammonium hydroxide solution (30-33% NH_3 in H_2O) and heated to a temperature of 100°C for 4h, hereafter the resulting precipitate was filtered and dried. Recrystallization was performed with methanol. The reaction progress was monitored by TLC.



Scheme 4-3: Synthetic pathway to obtain the isoquinolinone (4, X=CH) and C2-substituted quinazolinone derivatives (5a, 5d, 5f, 5h and 5i, X=N), method B.

Synthesis of the desired benzoxazinone derivatives (**2a**, **2d**, **2f**, **2h** & **2i**) resulted in fair yields (32–49%) (**Table 4-1**). The synthesized quinazolinones (**5a**–**j**) were originally prepared either via method A, with the exception of 2-(furan-2-yl)quinazolin-4(3H)-one (**5h**) which could not be obtained via this method. Although method A resulted in the desired quinazolinones, method B was employed in an attempt to synthesize 2-(furan-2-yl)quinazolin-4(3H)-one (**5h**). An improvement in the yields documented for method A was noted. Method B made use of the previously synthesized benzoxazinones (**2a**, **2d**, **2f**, **2h** and **2i**) as starting material to successfully deliver the corresponding quinazolinones (**5a**, **5d**, **5f**, **5h** and **5i**) in fair to excellent yields (24–95%). Furthermore, compound **4** was successfully obtained by means of method B with a yield of 95% (**Table 4-2**).

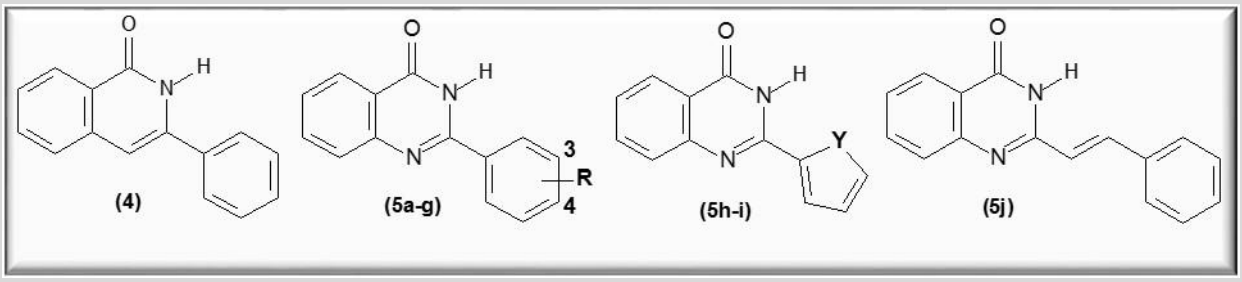
Table 4-1: 3-phenyl-1H-2-benzopyran-1-one, 3-phenyl-2H-chromen-2-one and selected benzoxazinones investigated in the current study



Compound	R	X	Y	Yield %[a]	Obtained Commercially or Synthesised
1	-	-	-	-	Commercially obtained[bc]
2a	-H	-	-	49%[a]	Synthesised[b]
2b	-4-F	-	-	-	Commercially obtained[bc]
2c	-4-Cl	-	-	-	Commercially obtained[bc]
2d	-4-Br	-	-	32%[a]	Synthesised[b]
2e	-4-CH_3	-	-	-	Commercially obtained[bc]
2f	-4-OCH_3	-	-	48%[a]	Synthesised[b]
2g	-4-OCH_2CH_3	-	-	-	Commercially obtained[bc]
2h	-	-	-O	47%[a]	Synthesised[b]
2i	-	-	-S	37%[a]	Synthesised[b]
2j	-	-	-	-	Commercially obtained[bc]
3	-	-	-	-	Commercially obtained[bc]
6		-CH	-	-	Commercially obtained[bc]
7	-	-N	-	-	Commercially obtained[bc]

[a] Isolated yields after purification; [b] Test compound used for biological evaluation (radioligand binding assays). [c] Commercially available compounds were used without further purification

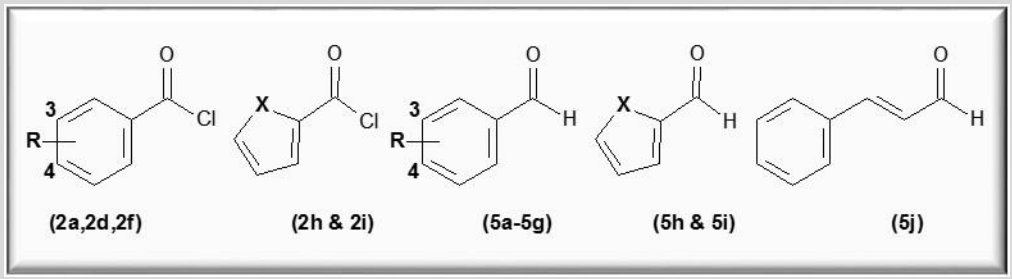
Table 4-2: 3-phenylisoquinalin-1(2H)-one and selected quinazolinones investigated in the current study



Compound	R	Y	Method A Scheme 2 Yield %[a]	Method B Scheme 3 Yield %[a]
4	-H		-[ab]	95%[ab]
5a	-H	-	35%[ab]	28%[ab]
5b	-4-F	-	3%[ab]	-
5c	-4-Cl	-	5%[ab]	-
5d	-4-Br	-	3%[ab]	22%[a]
5e	-4-CH_3	-	4%[ab]	-
5f	-4-OCH_3	-	11%[ab]	27%[a]
5g	-3,4-OCH_3	-	9%[ab]	-
5h	-	-O	n/a[c]	24%[ab]
5i	-	-S	14%[a]	95%[ab]
5j	-	-	6%[ab]	-

[a] Isolated yields after purification; [b] Test compound used for biological evaluation (radioligand binding assays); [c] The indicated method did not yield the desired compound.

Table 4-3: Commercially available acyl chlorides and aldehydes used as starting material.



Compound[a]	R	X
2a	-H	-
2d	-4-Br	-
2f	-4-OCH_3	-
2h	-	-O
2i	-	-S
5a	-H	-
5b	-4-F	-
5c	-4-Cl	-
5d	-4-Br	-
5e	-4-CH_3	-
5f	-4-OCH_3	-
5g	-3,4-OCH_3	-
5h	-	-O
5i	-	-S
5j	-	-

[a] Compounds used without further purification.

4.3 MATERIALS AND INSTRUMENTATION

Reagents and materials: Sigma-Aldrich® was the supplier of choice for all commercially procured compounds and starting materials, which were used without further purification.

Thin layer chromatography (TLC): TLC was used to monitor the progress of all the reactions. The TLC's were performed with Silica gel 60 TLC plates with UV_{254} fluorescent indicator supplied by Merck. The mobile phase consisted of 80% dichloromethane, 10% ethyl acetate and 10% petroleum ether. By exposing the TLC plates to UV light, at a wavelength of 254 nm, the desired chromatograms were visualised.

Melting point (mp): A Büchi melting point apparatus model B-545 was used to obtain the melting points (mp) of the various synthesized compounds (**2a**, **2d**, **2f**, **2h**, **2i** & **5a-5j**).

Mass spectrometry (MS): Characterisation of the synthesised compounds was accomplished by mass spectra, alongside nuclear magnetic resonance. A Bruker micrOTOF-Q II mass spectrometer in atmospheric pressure chemical ionisation (APCI), positive mode, was used to record high resolution mass spectra (HRMS) and nominal mass spectra (MS).

Nuclear magnetic resonance (NMR): Characterization of the synthesised test compounds was achieved by means of proton (^{1}H) and carbon (^{13}C) NMR. A Bruker Avance III 600 spectrometer instrument was implemented at frequencies of 600 MHz and 150 MHz, for ^{1}H and ^{13}C NMR, respectively. The samples for NMR were dissolved in either deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO-d_6) depending on compound solubility. The chemical shifts were noted in parts per million (δ) and given in reference to the residual solvent signal. In the case of $CDCl_3$ the residual solvent signals were positioned at 7.26 and 77.2 ppm for ^{1}H and ^{13}C spectra, respectively. Whereas the DMSO-d_6 residual solvent signals were detected at 2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C spectra. Spin multiplicities were given as singlet (s), doublet (d), doublet of doublets (dd), triplet (t) or multiplet (m). The coupling constants (J) are given Hz.

Structure confirmation was based on clarification of ^{1}H and ^{13}C NMR (Annexure A), in combination with the supplementary evidence provided by the melting points and mass spectrometry (Annexure B).

4.4 PHYSICAL CHARACTERIZATION

2-phenyl-4H-3,1-benzoxazin-4-one (2a)

Title compound was synthesised from anthranilic acid and benzoyl chloride in a yield of 49%, mp 122.7–123.4°C (**Lit: 123–125°C** (Asundaria *et al.*, 2012)), recrystallized from ethanol, white crystals. ^{1}H NMR ($CDCl_3$) δ 7.43 (dd, *J* = 10.6, 4.6 Hz, 3H), 7.50 (t, *J* = 7.3 Hz, 1H), 7.61 (d, *J* =

8.0 Hz, 1H), 7.74 (t, *J* = 7.7 Hz, 1H), 8.16 (d, *J* = 7.8 Hz, 1H), 8.23 (d, *J* = 7.6 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 117.04, 127.25, 128.28, 128.34, 128.62, 128.77, 130.25, 132.65, 136.59, 147.00, 157.13, 159.59. APCI-HRMS *m/z*: calc. for $C_{14}H_9NO_2$ (MH^+), 223.0633, found 224.0708.

2-(4-bromophenyl)-4H-3,1-benzoxazin-4-one (2d)

Title compound was synthesised from anthranilic acid and 4-bromobenzoyl chloride in a yield of 32%, mp 172.3–179.8°C (**Lit: 190–191°C** (Yamashita & Iida, 2014)), recrystallized from ethanol, white crystals. ^{1}H NMR ($CDCl_3$) δ 7.46 (t, *J* = 7.8 Hz, 1H), 7.59 (dd, *J* = 19.0, 8.3 Hz, 3H), 7.74 – 7.78 (m, 1H), 8.10 (d, *J* = 8.6 Hz, 2H), 8.16 (dd, *J* = 7.8, 1.0 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 117.00, 127.28, 127.71, 128.52, 128.71, 129.20, 129.75, 132.11, 136.70, 146.78, 156.35, 159.28. APCI-HRMS *m/z*: calc. for $C_{14}H_8BrNO_2$ (MH^+), 300.9738, found 301.9812.

2-(4-methoxyphenyl)-4H-3,1-benzoxazin-4-one (2f)

Title compound was synthesised from anthranilic acid and 4-methoxybenzoyl chloride in a yield of 48%, mp 151.4–151.8°C (**Lit: 150–151°C** (Yamashita & Iida, 2014)) recrystallized from diethyl ether, white crystals. ^{1}H NMR ($CDCl_3$) δ 3.81 (s, 3H), 6.92 (d, *J* = 9.0 Hz, 2H), 7.39 (t, *J* = 7.6 Hz, 1H), 7.56 (d, *J* = 7.8 Hz, 1H), 7.69 – 7.74 (m, 1H), 8.14 (d, *J* = 9.1 Hz, 1H), 8.18 (d, *J* = 9.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 55.53, 114.16, 116.74, 122.57, 126.94, 127.71, 128.56, 130.29, 136.50, 147.37, 157.13, 159.80, 163.29. APCI-HRMS *m/z*: calc. for $C_{15}H_{11}NO_3$ (MH^+), 253.0739, found 254.0789.

2-(furan-2-yl)-4H-3,1-benzoxazin-4-one (2h)

Title compound was synthesised from anthranilic acid and 2-furoyl chloride in a yield of 47%, mp 108.4–112.4°C (**Lit: 161–163°C** (Noolvi *et al.,* 2011)), recrystallized from ethanol, white crystals. ^{1}H NMR ($CDCl_3$) δ 6.55 (dd, *J* = 3.5, 1.7 Hz, 1H), 7.30 (d, *J* = 3.5 Hz, 1H), 7.41 – 7.46 (m, 1H), 7.64 (d, *J* = 9.0 Hz, 2H), 7.72 – 7.78 (m, 1H), 8.14 (dd, *J* = 7.9, 1.2 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 112.60, 116.94, 117.24, 127.18, 128.30, 128.79, 136.83, 144.43, 146.70, 147.10, 149.80, 158.62. APCI-HRMS *m/z*: calc. for $C_{12}H_7NO_3$ (MH^+), 213.0426, found 214.0487.

2-(thiophen-2-yl)-4H-3,1-benzoxazin-4-one (2i)

Title compound was synthesised from anthranilic acid and 2-thiophenecarbonyl chloride in a yield of 37%, mp 137.8–138.5°C (**Lit: 131–132°C** (Yamashita & Iida, 2014)), recrystallized from ethanol, white crystals. ^{1}H NMR ($CDCl_3$) δ 7.07 – 7.12 (m, 1H), 7.41 (t, *J* = 7.5 Hz, 1H), 7.51 – 7.59 (m, 2H), 7.68 – 7.76 (m, 1H), 7.89 (d, *J* = 3.1 Hz, 1H), 8.13 (d, *J* = 7.8 Hz, 1H).^{13}C NMR ($CDCl_3$) δ 116.74, 126.88, 127.99, 128.38, 128.76, 131.80, 132.43, 134.23, 136.67, 147.09, 153.73, 159.09. APCI-HRMS *m/z*: calc. for $C_{12}H_7NO_2S$ (MH^+), 229.0197, found 230.0258.

3-phenylisoquinolin-1(2H)-one (4)

Title compound was synthesised from 3-phenyl-1H-2-benzopyran-1-one **(1)** and ammonia in a yield of 95%, mp 146.6–181.6°C (**Lit: 198–200°C** (Khadka *et al.,* 2012)), white crystals (no further purification). ^{1}H NMR (DMSO-d_6) δ 6.93 (s, 1H), 7.45 – 7.52 (m, 4H), 7.72 (d, *J* = 3.7 Hz, 2H), 7.79 – 7.82 (m, 2H), 8.22 (d, *J* = 7.9 Hz, 1H), 11.55 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 103.71, 125.32, 126.88, 127.11, 127.15, 127.19, 129.25, 129.73, 133.10, 134.35, 138.39, 140.51, 163.26. APCI-HRMS *m/z*: calc. for $C_{15}H_{11}NO$ (MH^+), 221.0840, found 222.0916.

2-phenylquinazolin-4(3H)-one (5a)

Title compound was synthesised from 2-phenyl-4*H*-3,1-benzoxazin-4-one **(2a)** and ammonia in a yield of 28%, mp 241.0–241.3°C (**Lit: 250–252°C** (Asundaria *et al.*, 2012)), recrystallized from methanol, white crystals. ^{1}H NMR (DMSO-d_6) δ 7.51– 7.61 (m, 4H), 7.75 (d, *J* = 8.1 Hz, 1H), 7.84 (t, *J* = 7.5 Hz, 1H), 8.15 – 8.18 (m, 1H), 8.19 (d, *J* = 7.2 Hz, 2H), 12.56 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 121.45, 126.34, 127.05, 127.91, 128.25, 129.08, 131.86, 133.21, 135.06, 149.16, 152.84, 162.79. APCI-HRMS *m/z*: calc. for $C_{14}H_{10}N_2O$ (MH^+), 222.0793, found 223.0845.

2-(4-fluorophenyl)quinazolin-4(3H)-one (5b)

Title compound was synthesised from isatoic anhydride and 4-fluorobenzaldehyde in a yield of 3%, mp 280.8–281.4°C (**Lit: 284–286°C** (Hu *et al.,* 2016)), recrystallized from methanol, white crystals. ^{1}H NMR (DMSO-d_6) δ 7.40 (t, *J* = 8.8 Hz, 2H), 7.50 – 7.55 (m, 1H), 7.74 (d, *J* = 8.0 Hz, 1H), 7.82 – 7.86 (m, 1H), 8.16 (dd, *J* = 7.9, 1.1 Hz, 1H), 8.23 – 8.29 (m, 2H), 12.57 (s, 1H).^{13}C NMR (DMSO-d_6) δ 116.04, 116.18, 121.35, 126.33, 127.08, 127.88, 129.72, 130.82, 130.88, 135.11, 151.90, 162.74, 163.69, 165.34. APCI-HRMS *m/z*: calc. for $C_{14}H_9FN_2O$ (MH^+), 240.0699, found 241.0764.

2-(4-chlorophenyl)quinazolin-4(3H)-one (5c)

Title compound was synthesised from isatoic anhydride and 4-chlorobenzaldehyde in a yield of 5%, mp 299.3–299.9°C (**Lit: 298–300°C** (Hu *et al.,* 2016)), recrystallized from methanol, white crystals ^{1}H NMR (DMSO-d_6) δ 7.52 – 7.56 (m, 1H), 7.65 – 7.65 (m, 2H), 7.75 (d, *J* = 8.0 Hz, 1H), 7.83 – 7.87 (m, 1H), 8.16 (dd, *J* = 7.9, 1.3 Hz, 1H), 8.21 (d, *J* = 8.6 Hz, 2H), 12.60 (s, 1H).^{13}C NMR (DMSO-d_6) δ 121.48, 126.36, 127.23, 127.93, 129.16, 130.11, 132.11, 135.12, 136.75, 149.02, 151.93, 162.77. APCI-HRMS *m/z*: calc. for $C_{14}H_9ClN_2O$ (MH^+), 256.0403, found 257.0452.

2-(4-bromophenyl)quinazolin-4(3H)-one (5d)

Title compound was synthesised from isatoic anhydride and 4-bromobenzaldehyde in a yield of 3%, mp 244.1–244.9°C (**Lit: 293–295°C** (Hu *et al.,* 2016)), recrystallized from methanol, white crystals. ^{1}H NMR (DMSO-d_6) δ 7.54 (t, *J* = 7.5 Hz, 1H), 7.76 (dd, *J* = 13.1, 8.3 Hz, 3H), 7.85 (t, *J* = 7.6 Hz, 1H), 8.15 (dd, *J* = 14.4, 8.3 Hz, 3H), 12.61 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 121.49, 125.70, 126.37, 127.25, 127.93, 127.94, 130.29, 132.10, 132.46, 135.14, 138.41, 152.05. APCI-HRMS *m/z*: calc. for $C_{14}H_9BrN_2O$ (MH^+), 299.9898, found 300.9971.

2-(4-methylphenyl)quinazolin-4(3H)-one (5e)

Title compound was synthesised from isatoic anhydride and p-tolualdehyde in a yield of 4%, mp 241.7–243.3°C (**Lit: 241–243°C** (Hu *et al.,* 2016)), recrystallized from ethanol, white crystals. ^{1}H NMR (DMSO-d_6) δ 2.39 (s, 3H), 7.36 (d, *J* = 8.1 Hz, 2H), 7.51 (t, *J* = 7.8 Hz, 1H), 7.73 (d, *J* = 8.0 Hz, 1H), 7.83 (t, *J* = 7.6 Hz, 1H), 8.10 (d, *J* = 8.2 Hz, 2H), 8.15 (d, *J* = 7.8 Hz, 1H), 12.40 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 21.48, 121.36, 126.32, 126.87, 127.27, 127.84, 128.16, 129.67, 130.37, 135.04, 141.93, 152.74, 162.79. APCI-HRMS *m/z*: calc. for $C_{15}H_{12}N_2O$ (MH^+), 236.0950, found 237.1043.

2-(4-methoxyphenyl)quinazolin-4(3H)-one (5f)

Title compound was synthesised from isatoic anhydride and 4-anisaldehyde in a yield of 11%, mp 248.8–249.2°C (**Lit: 248–251°C** (Hu *et al.,* 2016)), recrystallized from diethyl ether, white crystals. ^{1}H NMR (DMSO-d_6) δ 3.85 (s, 3H), 7.09 (d, *J* = 8.9 Hz, 2H), 7.46 – 7.51 (m, 1H), 7.71 (d, *J* = 8.1 Hz, 1H), 7.79 – 7.84 (m, 1H), 8.14 (dd, *J* = 7.9, 1.4 Hz, 1H), 8.20 (d, *J* = 8.9 Hz, 2H), 12.38 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 55.94, 114.47, 121.15, 125.30, 126.31, 126.59, 127.67, 129.94, 135.00, 149.34, 152.41, 162.34, 162.87. APCI-HRMS *m/z*: calc. for $C_{15}H_{12}N_2O_2$ (MH^+), 252.0899, found 253.0980.

2-(3,4-dimethoxyphenyl)quinazolin-4(3H)-one (5g)

Title compound was synthesised from isatoic anhydride and 3,4-dimethoxybenzaldehyde in a yield of 9%, mp 247.7–249.7°C (**Lit: 240–242°C** (Hour *et al.,* 2000)), recrystallized from methanol, white crystals. ^{1}H NMR (DMSO-d_6) δ 3.85 (s, 3H), 3.88 (d, *J* = 10.4 Hz, 3H), 7.12 (d, *J* = 8.5 Hz, 1H), 7.49 (t, *J* = 7.4 Hz, 1H), 7.72 (d, *J* = 8.1 Hz, 1H), 7.80 – 7.83 (m, 2H), 7.88 (d, *J* = 8.4 Hz, 1H), 8.14 (d, *J* = 7.7 Hz, 1H), 12.45 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 56.15, 56.15, 111.11, 111.82, 121.16, 121.61, 125.20, 126.32, 126.62, 127.79, 135.04, 149.00, 149.37, 152.04, 152.31, 162.84. APCI-HRMS *m/z*: calc. for $C_{16}H_{14}N_2O_3$ (MH^+), 282.1004, found 283.1077.

2-(furan-2-yl)quinazolin-4(3H)-one (5h)

Title compound was synthesised from 2-(furan-2-yl)-4*H*-3,1-benzoxazin-4-one **(2h)** and ammonia in a yield of 24%, mp 221.3–222.6°C (**Lit: 218–220°C** (Hu *et al.,* 2016)), recrystallized from methanol, white crystals. ^{1}H NMR (DMSO-d_6) δ 6.76 (dd, *J* = 3.5, 1.7 Hz, 1H), 7.47 – 7.52 (m, 1H), 7.64 (d, *J* = 3.4 Hz, 1H), 7.79 – 7.84 (m, 1H), 7.70 (d, *J* = 8.0 Hz, 1H), 8.01 (d, *J* = 0.9 Hz, 1H), 8.13 (dd, *J* = 7.9, 1.0 Hz, 1H), 12.51 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 113.01, 114.99, 121.63, 126.42, 126.96, 127.73, 135.13, 144.51, 146.57, 147.08, 149.15, 162.06. APCI-HRMS *m/z*: calc. for $C_{12}H_8N_2O_2$ (MH^+), 212.0586, found 213.0641.

2-(thiophen-2-yl)quinazolin-4(3H)-one (5i)

Title compound was synthesised from isatoic anhydride and 2-thiophenecarboxaldehyde in a yield of 14%, mp 284.0–284.2°C **(Lit: 275–276°C** (Hu *et al.,* 2016)), recrystallized from methanol, brown crystals. ^{1}H NMR (DMSO-d_6) δ 7.21 – 7.25 (m, 1H), 7.49 (t, *J* = 7.5 Hz, 1H), 7.66 (d, *J* = 8.0 Hz, 1H), 7.81 (t, *J* = 7.6 Hz, 1H), 7.88 (d, *J* = 5.0 Hz, 1H), 8.13 (d, *J* = 7.9 Hz, 1H), 8.24 (d, *J* = 3.7 Hz, 1H), 12.67 (s, 1H). ^{13}C NMR (DMSO) δ 121.35, 126.47, 126.81, 127.38, 128.99, 129.88, 132.65, 135.17, 137.86, 148.35, 149.07, 162.32. APCI-HRMS *m/z*: calc. for $C_{12}H_8N_2OS$ (MH^+), 228.0357, found 229.0409

2-[(E)-2-phenylethenyl]quinazolin-4(3H)-one (5j)

Title compound was synthesised from isatoic anhydride and *trans*-cinnamaldehyde in a yield of 6%, mp 237.5–237.7°C (**Lit: 238–240°C** (Trashakhova *et al.,* 2011)), recrystallized from methanol, white crystals. ^{1}H NMR (DMSO-d_6) δ 7.02 (d, *J* = 16.2 Hz, 1H), 7.41 − 7.49 (m, *J* = 7.2 Hz, 4H), 7.65 – 7.70 (m, 3H), 7.79 − 7.83 (m, 1H), 7.96 (d, *J* = 16.2 Hz, 1H), 8.12 (dd, *J* = 7.9, 1.2 Hz, 1H), 12.35 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 121.58, 126.35, 126.71, 127.58, 128.11, 129.56, 130.26, 134.99, 135.48, 138.75, 149.45, 151.92, 162.25. APCI-HRMS *m/z*: calc. for $C_{16}H_{12}N_2O$ (MH^+), 248.0950, found 249.1010.

4.5 CONCLUSION

This chapter aimed to describe the synthetic procedures used to obtain the desired test compounds (**2a**, **2d**, **2f**, **2h, 2i** and **5a-j**) as listed in **Table 4-1** and **Table 4-2**. However compounds **1**, **2b**, **2c**, **2e**, **2g**, **2j**, **3**, **7** & **8** were obtained commercially. The syntheses relevant to this study were successfully performed in accordance to synthetic practices previously described by relevant literature. Test compounds obtained by the aforementioned syntheses included five C2-substituted benzoxazinones (**2a**, **2d**, **2f**, **2h** & **2i**), ten C2-substituted quinazolinones (**5a-j**) and one isoquinolinone (**4**). Characterization of all the synthesised structures were achieved by means of ^{1}H-NMR, ^{13}C-NMR and ms. The characterization results corresponded to available literature data, where applicable.

CHAPTER 5

RADIOLIGAND BINDING STUDIES

5.1 INTRODUCTION

As previously mentioned, this study was guided by the aim to deliver compounds that possess activity as A_1 and A_{2A} adenosine receptor (AR) antagonists. Both the commercially obtained (**1**, **2b**, **2c**, **2e**, **2g**, **2j**, **3**, **6** & **7**) and synthesised compounds (**2a**, **2d**, **2f**, **2h**, **2i**, **4** & **5a-j**) underwent *in vitro* A_1 and A_{2A} AR radioligand binding studies to determine whether they exhibit affinity for the A_1 and/or A_{2A} ARs. The abovementioned radioligand binding studies were performed according to methods detailed in literature (Bruns *et al.,* 1986; Bruns *et al.,* 1987; Van der Walt & Terre'Blanche., 2015). This chapter entails the methods used in the *in vitro* biological evaluation and the results obtained for the test compounds of this pilot study.

5.2 A_1 AND A_{2A} ADENOSINE RECEPTOR RADIOLIGAND BINDING ASSAY

5.2.1 PRINCIPAL

The A_1 and A_{2A} AR radioligand binding assays provide a means for assessing the degree of binding affinity that selected test compounds may possess toward the A_1 and/or A_{2A} ARs. Typically, a radioligand binding study is conducted using proteins that express the desired receptor in the presence of a radioligand known to exhibit a high affinity towards the receptor in question.

For the current study, receptor expression was achieved by using the proteins described in literature (Bruns *et al.,* 1986; Bruns *et al.,* 1987). Therefore, the A_1 ARs were expressed by using rat whole brain membranes and the A_{2A} ARs were expressed by using rat striatal membranes. 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX), known to exhibit affinity toward the A_1 AR, and 5'-N-ethylcarboxamido[^{3}H]adenosine ([^{3}H]NECA), known to exhibit affinity toward the A_{2A} AR, served as radioligands (Bruns *et al.,* 1986; Bruns *et al.,* 1987). Unlabelled DPCPX and NECA's documented K_i values were reported as: 0.55 nM (A_1 AR) and 530 nM (A_{2A} AR) for DPCPX versus 6.26 nM (A_1 AR) and 10.30 nM (A_{2A} AR) for NECA (Van der Walt & Terre'Blanche, 2015; Bruns *et al.,* 1986; Bruns *et al.,* 1987). To deter the radioligand, [^{3}H]NECA, from compromising the validity of the A_{2A} AR binding assay by binding to the A_1 ARs, N^6-cyclopentyladenosine (CPA) was also added to the assay. CPA is a potent A_1 AR agonist with a higher affinity toward the A_1 ARs than [^{3}H]NECA. Consequently, the presence of CPA increases [^{3}H]NECA's selectivity for the A_{2A} ARs (Van der Walt & Terre'Blanche, 2015).

Therefore, a test compound's A_1 or A_{2A} AR affinity can be successfully measured in the presence of [^{3}H]DPCPX or [^{3}H]NECA and CPA, respectively.

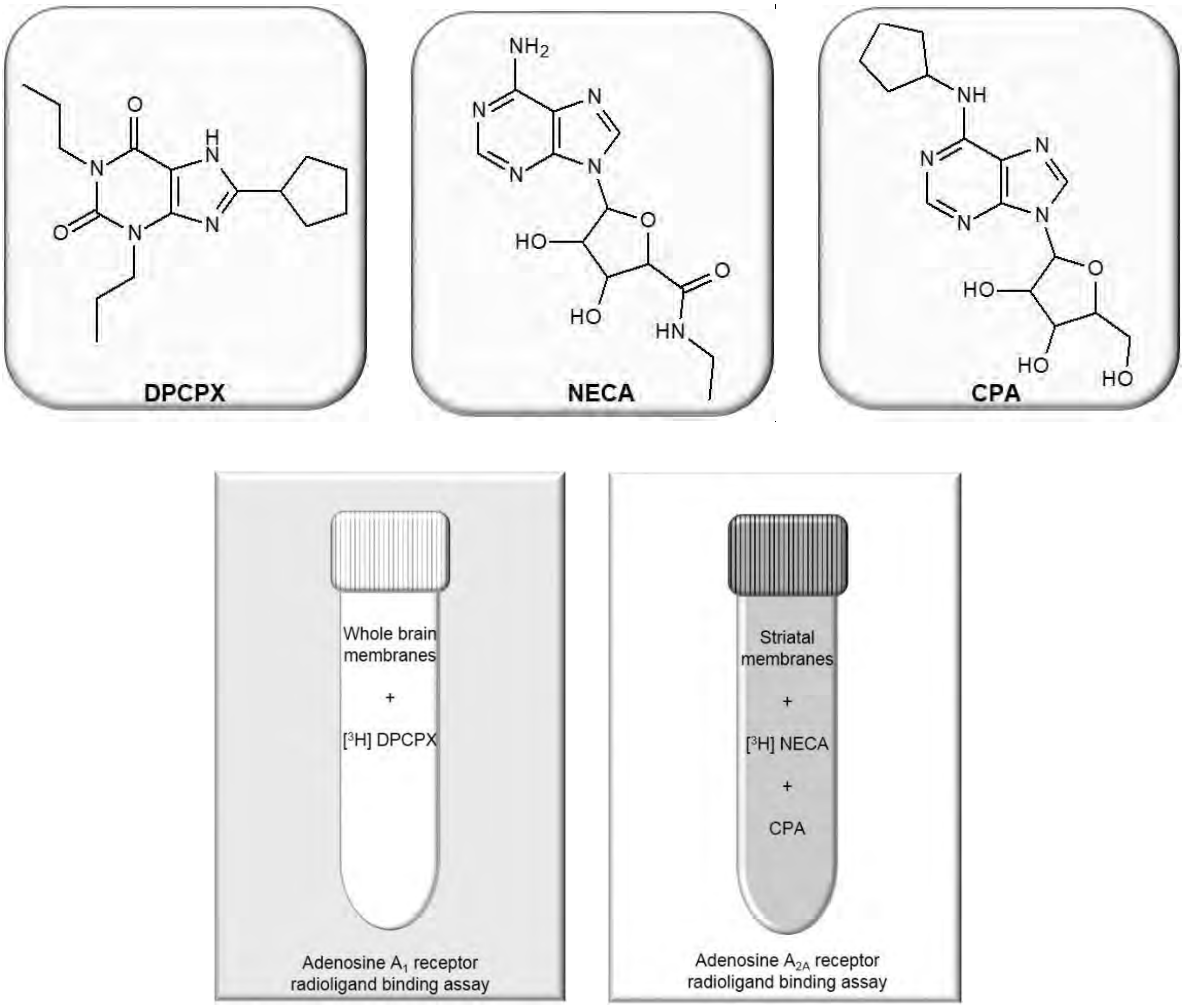


Figure 5-1: Illustrates the various rat membranes and appropriate radioligands used within the A_1 and A_{2A} AR radioligand binding assays.

Concurrent radioligand binding assays assessing a test compound's affinity for more than one receptor at a time, as with the current study, also allow for the calculation of the degree of selectivity toward either receptor (either the A_1 or A_{2A} AR in this case). In this study, this was accomplished by calculating a selectivity index for each compound. The selectivity index is calculated as a ratio of the applicable A_1 and A_{2A} AR K_i values of each test compound. For instance, unlabelled DPCPX has a selectivity index (SI) of 958 favouring the A_1 AR (K_i (A_{2A})/ K_i (A_1)) (Van der Walt & Terre'Blanche, 2015).

5.2.2 PRE-ANALYTICAL

5.2.2.1 Membrane preparation (prepare at least a day in advance)

It is important to note that ethical approval from the North-West University's (NWU) Animal Research Ethics Committee (**NWU-00261-17-A5**) was obtained prior to all membrane preparations. Male Sprague-Dawley rats, weighing between 250g and 500g, were purchased from the NWU Preclinical Drug Development Platform's Vivarium for this study.

Chemicals and Reagents: 50 mM Tris.HCl buffer (pH 7.7 at 25°C) prepared a day in advance and Bradford reagent procured from Sigma Aldrich®

Instrumentation: Polytron PT 10-35 GT

Method: Membrane preparation was performed in accordance with literature methods (Bruns *et al.,* 1986; Van der Walt *et al.*, 2015). Dissection of the male Sprague-Dawley rats provided rat whole brain tissue (excluding brainstem and cerebellum) and the rat striatal tissue used in the A_1 and A_{2A} AR radioligand binding assays, respectively. Upon completion of the tissue harvest, all brain tissues were immediately snap frozen and stored at -70°C until the membrane preparation commenced. Once needed, the tissues were thawed on ice and weighed before being suspended in 10 volumes of ice-cold 50 mM Tris.HCl buffer (pH 7.7 at 25°C). Hereafter, the tissue suspensions were disrupted (90s for whole brain or 30s for striatal tissue) with the aid of a Polytron homogenizer to provide the desired homogenates. The homogenates were then centrifuged at 20000 *g* for 10 minutes at 4°C and the resulting pellets were resuspended in 10 volumes of ice-cold 50 mM Tris.HCl buffer (pH 7.7 at 25°C) using a Polytron homogenizer, as above. The resulting suspensions were centrifuged, yet again, for 10 minutes at 4°C. After decanting the supernatant, the residual pellets were resuspended in ice-cold Tris.HCl (pH 7.7 at 25°C) to yield tissue suspensions with a final volume of 5 mL/g original tissue weight. The protein content of the rat whole brain and striatal membrane preparations were determined with Bradford reagent as described by Bradford (1979) and the final tissue suspensions were stored at -70°C until needed.

5.2.2.2 Stock solution preparation (prepared a day in advance and refrigerated until assay)

Stock solutions for each test compound was prepared with DMSO as solvent. In order to prepare stock solutions with accurate concentrations (10 mM), the amount weighed and molecular weight of each test compound was taken into account. These stock solutions were used to produce a series of additional dilutions to provide a final concentration range, between 0 and 100 µM (or 300 µM where applicable), for each test compound.

5.2.2.3 50 mM Tris.HCl buffer preparation (prepared at least a day in advance and refrigerated until assay)

The Trizma-hydrochloride and Trizma-base used in preparation of the 50 mM Tris.HCl buffer (pH 7.7 at 25°C) was procured from Sigma Aldrich® and the 50 mM Tris.HCl buffer (pH 7.7 at 25°C) was prepared by first preparing two solutions: 1400 mL of a 0.008 g/mL Trizma-hydrochloride solution and 700 mL of a 0.006 g/mL Trizma-base solution. The final solution was prepared by adding 350 mL of the Trizma-base solution to the 1400 mL Trizma-hydrochloride solution and measuring the pH at room temperature. Thereafter, the pH of the resulting solution was adjusted to 7.7 by gradual addition of small volumes of the remaining Trizma-base solution until the desired pH was achieved. The resulting 50 mM Tris.HCl buffer was then refrigerated until needed for the assay.

5.2.2.4 Coating of consumables with Sigma-cote® (prepared at least a day in advance)

The Sigma-cote® used in preparation for the assays was procured from Sigma Aldrich®. All polypropylene vials, caps and tips to be used in the A_1 and A_{2A} AR radioligand binding assays were first coated with Sigma-cote® to prevent adhesion of any of the substances used in the assays and then dried overnight at room temperature.

5.2.3 ANALYTICAL

Reagents and materials: Several manufacturers were involved in procuring the necessary reagents and materials for the A_1 and A_{2A} AR radioligand binding assays. The A_1 AR radioligand, [^{3}H]DPCPX (specific activity 120 Ci/mmol), along with the scintillation vials and Filter-count scintillation fluid were obtained from PerkinElmer, while the A_{2A} AR radioligand, [^{3}H]NECA (specific activity 25 Ci/mmol), was purchased from Amersham Biosciences. The adenosine deaminase (8,3 mg protein/mL, 141 units/mg protein), anhydrous magnesium chloride ($MgCl_2$) and CPA was purchased from Sigma-Aldrich®. Lastly, Merck provided the Whatman GF/B 25 mm diameter filters and dimethyl sulfoxide (DMSO).

Instrumentation: This study makes use of a Hoffeler vacuum system and a Packard Tri-CARB 2810 TR liquid scintillation counter.

5.2.3.1 Procedure for the A_1 adenosine receptor radioligand binding assay

The A_1 AR radioligand binding assay was routinely performed at the NWU's Laboratory for Analytical and Molecular Biology (LAMB). The A_1 AR radioligand binding assay was based on the methods described by Bruns and co-workers (1987) and Van der Walt and co-workers (2015). On the day of assay, the previously prepared rat whole brain membranes, expressing

the A_1 ARs (see **section 5.2.2**), were thawed on ice. The stock solutions were removed from the refrigerator and allowed to warm to room temperature. In the meantime, the rat whole brain membranes, together with the adenosine deaminase, were suspended in 50 mM Tris.HCl buffer to provide the desired membrane suspension. The radioligand solution was prepared using [^{3}H]DPCPX and 50 mM Tris.HCl buffer. Each incubation consisted of: membrane suspension equivalent to 120 µg whole brain protein (expressing the A_1 ARs), 0.1 units/mL adenosine deaminase, 0.1 nM [^{3}H]DPCPX (radioligand) and the test compound (at the desired concentration). The final volume of all incubations also contained 1mL 50 mM Tris.HCl buffer and 1% DMSO. The order of additions were as follow: 1) test compound (10 µL) 2) radioligand (100 µL) 3) membrane suspension (890 µL). Noteworthy, nonspecific binding was defined by the addition of a final volume of CPA (100 µM) (Van der Walt *et al.,* 2015) and control incubations consisting of 1% DMSO served to prove that specific binding is not influenced by this concentration of DMSO (Bruns *et al.,* 1986).

Upon completion of all the additions mentioned above, each vial was vortexed before being transferred into a shaking water bath to be incubated at 25°C for 60 minutes. 30 Minutes post commencement of incubation, each sample was vortexed once again and returned to the shaking water bath where incubation resumed. Before termination of incubation, the 25 mm GF/B filters were prepared by soaking them in 50 mM Tris.HCl buffer. At the appropriate time (60 minutes after incubation commenced), incubation was terminated by depositing each sample onto a separate 25 mm GF/B filter and the pertaining vial was rinsed twice with 4 mL 50 mM Tris.HCl buffer. Filtration occurred under reduced pressure using a Hoffeler vacuum system and upon completion each filter was placed into a scintillation vial, along with 4 mL Filter-count scintillation fluid. The vials were subjected to agitation before being left in a stationary position for two hours. At that time, the residual radioactivity of each filter was measured with a Packard Tri-CARB 2810 TR liquid scintillation counter (Bruns *et al.,* 1987; Van der Walt *et al.,* 2015).

5.2.3.2 Procedure for the A_{2A} adenosine receptor radioligand binding assay

The A_{2A} AR radioligand binding assay was routinely performed at the NWU's LAMB. The protocol utilized to determine the A_{2A} AR affinity is based on methods described in the literature (Bruns *et al.,* 1986; Van der Walt *et al.,* 2015). On the day of assay, initial preparation entailed the thawing of previously prepared rat striatal membranes, expressing the A_{2A} ARs (see **section 5.2.2**), on ice and removing the test compound dilutions, prepared from the corresponding stock solutions, from the refrigerator, allowing them to thaw. The membrane suspension for the A_{2A} AR radioligand binding assay was prepared by suspending the rat striatal membranes, $MgCl_2$ and adenosine deaminase in a volume of 50 mM Tris.HCl buffer. In turn, the radioligand solution was prepared using [^{3}H]NECA and 50 mM Tris.HCl buffer. Each incubation consisted of: membrane suspension equivalent to120 µg rat striatal protein (expressing the A_{2A} ARs), 0,2

units/mL adenosine deaminase, 4 nM [^{3}H]NECA (radioligand), 50 nM CPA, 10 mM $MgCl_2$ and the test compound (at the desired concentration). The final volume of all incubations also contained 1mL 50 mM Tris.HCl buffer and 1% DMSO. The order of additions were as follow: 1) membrane suspension (790 µL) 2) test compound (10 µL) 3) CPA (100 µL) 4) radioligand (100 µL). Similar to the A_1 AR radioligand binding assay, the nonspecific binding was defined by the addition of a final volume volume of CPA (100 uM) (Van der Walt *et al.,* 2015) and control incubations consisting of 1% DMSO served to prove that specific binding is not influenced by this concentration of DMSO (Bruns *et al.,* 1986).

Following the additions mentioned above, the resulting incubations were vortexed before being transferred into a shaking water bath to be incubated at 25°C for 60 minutes. Each incubation was vortexed once again after 30 minutes and returned to the shaking water bath where incubation resumed. Similar to the A_1 AR radioligand binding assay, the 25 mm GF/B filters were prepared by soaking them in 50 mM Tris.HCl buffer before termination of incubation. At the appropriate time, incubation was terminated by depositing each sample onto a separate 25 mm GF/B filter and the pertaining vial was rinsed twice with 4 mL 50 mM Tris.HCl buffer. Filtration occurred under reduced pressure using a Hoffeler vacuum system and upon completion each filter was placed into a scintillation vial, along with 4 mL Filter-count scintillation fluid. The vials were subjected to agitation before being left in a stationary position for two hours. At that time, the residual radioactivity of each filter was measured with a Packard Tri-CARB 2810 TR liquid scintillation counter (Bruns *et al.,* 1987; Van der Walt *et al.,* 2015).

Table 5-1: A table depicting the relevant components of the A_1 and/ or A_{2A} AR radioligand binding assays and their function.

Component	Function
Sigma-Cote®	Prevents adhesion of membranes and radioligand to the materials used
DMSO	Solvent for the A_1 and A_{2A} AR radioligand binding assays
50 mM Tris.HCl	Serves as buffer
A_1 AR membrane suspension	Rat whole brain membranes express the A_1 ARs (Bruns *et al.,* 1987)
A_{2A} AR membrane suspension	Rat striatal membranes express the A_{2A} ARs (Bruns *et al.,* 1986)
[^{3}H]DPCPX	Radioligand for A_1 AR binding studies (Van de Walt *et al.,* 2015; Bruns *et al.,* 1987)
[^{3}H]NECA	Radioligand for A_{2A} AR binding studies (Bruns *et al.,* 1986)
Adenosine deaminase	Prevents binding of endogenous adenosine (Bruns *et al.,* 1987)
CPA	Non-specific binding and saturation of A_1 AR during the A_{2A} AR radioligand binding studies (Bruns *et al.,* 1986)
$MgCl_2$	Increases specific A_{2A} AR binding during the A_{2A} AR radioligand binding studies (Bruns *et al.,* 1986)

5.2.4 POST-ANALYTICAL

5.2.4.1 Data analysis

All results obtained are documented in **Table 5-2**. For the A_1 and A_{2A} AR radioligand binding assays, initial screenings were performed in duplicate. Subsequently, full assays (assessment at a final concentration series, ranging between 0 and 100 µM) were performed in triplicate for the test compounds that warranted full assays based on the results of their initial screenings. These initial screenings were performed at final concentrations of 1 µM, 10 µM and 100 µM. From the results of the screenings the percentage displacement of the control was calculated. The equation pertaining the calculation of the abovementioned percentage is given below. The percentage displacement of the control lends insight into whether a compound displays a tendency of affinity toward the A_1 and/or A_{2A} ARs. A high percentage is indicative of poor affinity toward the A_1 and A_{2A} ARs and vice versa. Therefore, only test compounds that exhibited less than 30% at a final concentration of 100 µM, in the preliminary screening assays, were considered for further evaluation by means of full assay.

$$\frac{\textit{Specific Binding (Test Compound)}}{\textit{Specific Binding (Control)}} \textit{ x 100 = Percentage Displacement of the Control}$$

Where full assays were merited, the resulting data analyses of the test compounds were executed as follow: IC_{50} values were determined by plotting the specific binding (nine different concentrations between 0 and 100 µM) against the logarithm of the test compounds' concentrations to attain a sigmoidal dose-response curve (produced by GraphPad Software Inc.'s Prism Software package) (Van der Walt *et al.,* 2015). The IC_{50} values were used to calculate the dissociation constant (K_i) values for the competitive inhibition of [^{3}H]DPCPX, K_d = 0.36 nM (Bruns *et al.,* 1987), and [^{3}H]NECA, K_d = 15.3 nM (Bruns *et al.,* 1986), by the test compounds. The calculated K_i values are given as mean ± standard error of the mean (SEM) upon triplicate evaluation of the chosen concentrations (see **Table 5-2**). For this study, CPA and ZM-241385 were chosen as reference compounds and exhibited K_i values that correspond with former literature values (**Table 5-2)**, thus serving as the quality control for this study.

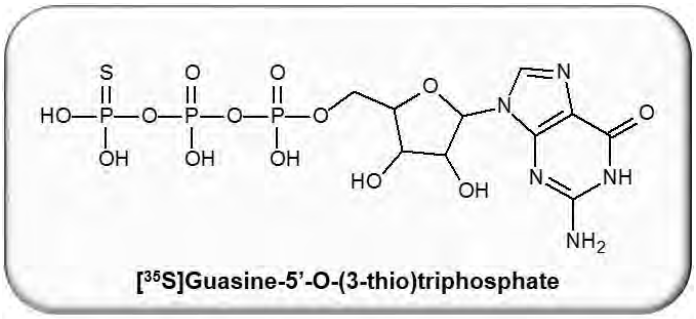
5.3 GTP SHIFT ASSAY

5.3.1 PRINCIPAL

A GTP shift assay provides a means for assessing the type of binding activity (such as agonist, antagonist or inverse agonist activity) that a test compound exhibits at the target receptor(s) (DeLapp *et al.,* 2012). The typical GTP shift assay resembles the A_1 AR radioligand binding

assay (see **section 5.2.1**) in the sense that this assay is also performed using proteins that express the desired receptor in the presence of a radioligand known to exhibit a high affinity towards the receptor in question. However, a GTP shift assay requires the addition of [^{35}S]guasine-5'-O-(3-thio)triphosphate (GTP) to the assay. GTP is thought to act by uncoupling the receptors from the G-proteins. This causes agonists of the receptor to exhibit a diminished affinity for the receptor in question in the presence of GTP (Kull *et al.,* 2000). Thus, by comparing a selected compound's competition curves in the presence and the absence of GTP it is possible to determine whether a compound will act as an agonist or antagonist (Van der Walt & Terre'Blanche, 2015). Gütschow and co-workers (2012) state that the competition curve of an antagonist, in the presence of GTP, will be unaffected. Therefore, a compound where the calculated GTP shift is approximately 1 is considered an antagonist (Van der Wenden *et al.,* 1995). Noteworthy, Kull and co-workers (2000) state that rat and human proteins exhibit similar regulation of GTP and endogenous adenosine binding interactions in the A_1 and A_{2A} AR binding studies.

In this study, the GTP shift assay was performed to assess the type of binding activity the test compounds exhibited toward the A_1 ARs. The A_1 ARs were expressed by using rat whole brain membranes and [^{3}H]DPCPX (documented K_i value of 0.55 nM for the A_1 AR) served as radioligand, as described in literature (Van der Walt & Terre'Blanche, 2015)



[^{35}S]Guasine-5'-O-(3-thio)triphosphate

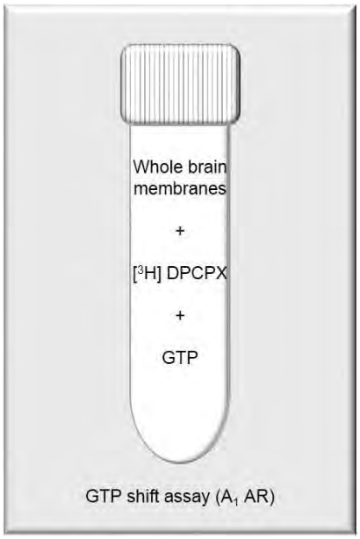


Figure 5-2: Illustrates the rat membranes and appropriate radioligand used within the GTP shift assay.

5.3.2 PRE-ANALYTICAL

All the pre-analytical preparations, such as membrane preparation, stock solution preparation, 50 mM Tris.HCl buffer preparation and coating of consumables, for the GTP shift assay were performed according to the procedures previously described in **section 5.2.2**. However, in the case of the membrane preparations, only rat whole brain membrane suspensions, expressing A_1 ARs, were prepared.

5.3.3 ANALYTICAL

Reagents and materials: Several manufacturers were involved in procuring the necessary reagents and materials for the GTP shift assay. The A_1 AR radioligand, [^{3}H]DPCPX (specific activity 120 Ci/mmol), along with the scintillation vials and Filter-count scintillation fluid were obtained from PerkinElmer. The adenosine deaminase (8,3 mg protein/mL, 141 units/mg protein), GTP and unlabelled DPCPX was purchased from Sigma-Aldrich®. Lastly, Merck provided the Whatman GF/B 25 mm diameter filters and dimethyl sulfoxide (DMSO).

Instrumentation: This study makes use of a Hoffeler vacuum system and a Packard Tri-CARB 2810 TR liquid scintillation counter.

5.3.3.1 Procedure for the GTP shift assay

The GTP shift assay was routinely performed at the NWU's LAMB and according to a method described in literature (Van der Walt & Terre'Blanche, 2015). The GTP shift assay follows a protocol similar to that of the A_1 AR radioligand assay. On the day of assay, the previously

prepared rat whole brain membranes, expressing the A_1 ARs (see **section 5.2.2**), were thawed on ice. The test compound dilutions, prepared from corresponding stock solutions, were removed from the refrigerator and allowed to warm to room temperature. The rat whole brain membranes, together with the adenosine deaminase, were suspended in a volume of 50 mM Tris.HCl buffer to provide the desired membrane suspension. The radioligand solution was prepared using [^{3}H]DPCPX and 50 mM Tris.HCl buffer, while a GTP solution was prepared using GTP and 50 mM Tris.HCl buffer. Each incubation consisted of: membrane suspension equivalent to 120 µg whole brain protein (expressing the A_1 ARs), 0.1 units/mL adenosine deaminase, 0.1 nM [^{3}H]DPCPX (radioligand), 0.1 mM GTP and the test compound (at the desired concentration). The final volume of all incubations also contained 1 mL 50 mM Tris.HCl buffer and 1% DMSO. The order of additions were as follow: 1) test compound (10 µL) 2) radioligand (100 µL) 3) GTP 100 µL 4) membrane suspension (790 µL). Similar to the A_1 and A_{2A} AR radioligand binding assays, nonspecific binding was defined by the addition of a final volume unlabelled DPCPX (10 µM) (Van der Walt & Terre'Blanche, 2015) and control incubations consisting of 1% DMSO served to prove that specific binding is not influenced by this concentration of DMSO (Bruns *et al.,* 1986).

After performing all of the additions mentioned above, each vial was vortexed before being transferred into a shaking water bath to be incubated at 25°C for 60 minutes. 30 Minutes post commencement of incubation, each sample was vortexed once again and returned to the shaking water bath where incubation resumed. Before termination of incubation, the 25 mm GF/B filters were prepared by soaking them in 50 mM Tris.HCl buffer. At the appropriate time, incubation was terminated by depositing each sample onto a separate 25 mm GF/B filter and the pertaining vial was rinsed twice with 4 mL 50 mM Tris.HCl buffer. Filtration occurred under reduced pressure using a Hoffeler vacuum system and upon completion each filter was placed into a scintillation vial, along with 4 mL Filter-count scintillation fluid. The vials were subjected to agitation before being left in a stationary position for two hours. At that time, the residual radioactivity of each filter was measured with a Packard Tri-CARB 2810 TR liquid scintillation counter (Bruns *et al.,* 1987; Van der Walt *et al.,* 2015).

5.3.4 POST-ANALYTICAL

5.3.4.1 Data analysis

All results obtained are documented in **Table 5-2**. The GTP shift assay was performed in triplicate for the test compounds that warranted GTP assays. Subsequently, assessment at a final concentration series, ranging between 0 and 100 or 300 µM, where appropriate, were performed in both the absence and presence of GTP. Similar to the A_1 and A_{2A} AR radioligand binding assays, the resulting data analyses of the test compounds were executed as follow: IC_{50}

values were determined by plotting the specific binding (nine different concentrations between 0 and 300 µM) against the logarithm of the test compounds' concentrations to attain a sigmoidal dose-response curve (produced by GraphPad Software Inc.'s Prism Software package) (Van der Walt *et al.,* 2015). The IC_{50} values were used to calculate the dissociation constant (K_i) values for the competitive inhibition of [^{3}H]DPCPX, K_d = 0.36 nM (Bruns *et al.,* 1987), by the test compounds. The calculated K_i values are given as mean ± standard error of the mean (SEM) upon triplicate evaluation of the chosen concentrations both in the presence and absence of GTP (see **Table 5-2**). The resulting K_i values are used to calculate the GTP shift. For this study, CPA was chosen as reference compounds and exhibited K_i values that correspond with former literature values (**Table 5-2)**, thus serving as the quality control for this study.

$$\frac{K_i \text{ value in the presence of GTP}}{K_i \text{ value in the absence of GTP}} = \textit{GTP shift}$$

5.4 RESULTS AND DISCUSSION

The results of the A_1 and A_{2A} AR radioligand binding assays along with the results of the GTP shift assay are compiled in **Table 5-2**. The first of the C2-substituted benzoxazinone derivatives, compound **2a**, was the result of the introduction of a hetero nitrogen (ring C) to the α-pyrone core of compound **1**. This structural change afforded a similar A_1 AR affinity as reported for compound **1** ((**1**) A_1K_i = 7.41 µM vs (**2a**) A_1K_i = 6.38 µM), accompanied by a diminished affinity for the A_{2A} AR ((**1**) $A_{2A}K_i$ = 3.35 µM vs (**2a**) $A_{2A}K_i$ = >100 µM).

Certain structural modifications at ring B allowed for further analysis of the relevant structure activity relationships (SARs) pertaining to the benzoxazinone scaffold. A phenyl ring, in combination with various *para*-substituents as ring B, afforded compounds **2b–g**. In turn, ring B as either a furyl (**2h**) or a thiophene (**2i**) ring, allowed for the effect of other heterocyclic ring systems on the SAR to be examined. Moreover, the insertion of a styryl side chain (**2j**) between ring C and a phenyl ring B was also explored. In comparison to **2a,** *para*-substitution (**2b–g**) of the phenyl ring B with either F (**2b**), Cl (**2c**), Br (**2d**), CH_3 (**2e**), OCH_3 (**2f**) and OCH_2CH_3 (**2g**), resulted in a diminished A_1 AR activity and a continued lack of A_{2A} AR affinity. Neither the furyl compound (**2h**), nor the compound with the styryl side chain (**2j**) exhibited any A_1 or A_{2A} AR affinity. Consequently, the C2-substituted benzoxazinone derivatives were deemed to be generally devoid of A_1 and A_{2A} AR affinity, with the exception being the unsubstituted phenyl ring B (**2a**) and the thiophene ring B (**2i**), exhibiting K_i values for the A_1 AR of 6.38 µM and 32.4 µM, respectively.

Further structural modification to ring C included: a) Saturation (elimination of the double bond) of ring C of compound **2a** to afford compound **3**. This structural modification was equal to a slight improvement in A_1 AR affinity when to compared to **2a** ((**2a**) A_1K_i = 6.38 μM vs (**3**) A_1K_i = 5.06 μM). Regrettably, A_{2A} AR affinity remained elusive. b) Replacement of the hetero oxygen of compound **1** with a hetero nitrogen to yield compound **4**, which resulted in a reduced A_1 and A_{2A} AR affinity. Thus, emphasising a preference for the benzo-α-pyrone scaffold (**1**) over the analogous isoquinolinone moiety (**4**) with regards to A_1 and A_{2A} AR affinity.

The abovementioned finding that the hetero oxygen (ring C) of the benzo-α-pyrone derivative **1** is integral to AR activity, prompted the comparison of the structurally related homologues **2a** (benzoxazinone) and **5a** (quinazolinone). The quinazolinone derivative (**5a**) bore a hetero nitrogen (ring C) in lieu of the hetero oxygen of its benzoxazinone counterpart (**2a**), in addition to the primary hetero nitrogen inherent to the quinazolinone structure, resulting in superior affinity for the A_1 and A_{2A} ARs when compared to that of **2a** ((**5a**) A_1K_i = 3.67 μM; $A_{2A}K_i$ = 18.7 μM vs (**2a**) A_1K_i = 6.38 μM; $A_{2A}K_i$ = >100 μM). The latter observation (**2a** vs **5a**) contradicts the former finding (**1** vs **4**) that stated that the hetero oxygen on ring C was imperative to AR affinity, but could be partially attributed to ring C containing two hetero nitrogen atoms (**5a**) rather than one (**2a**). This observation is supported by the research of Gillespie and co-workers (2009 a, b), where the comparison of the binding affinities of pyridine, pyrimidine and triazine revealed that two nitrogen atoms in the heterocyclic ring are optimum for enhancement of both A_1 and A_{2A} AR binding. The latter conclusion was reached upon the pyridine scaffold exhibiting a potency of seven-fold less than the triazine and forty five-fold less than the corresponding aminopyrimidine (Gillespie *et al.,* 2009a; Gillespie *et al.,* 2009b).

In analogy to compound **1**, compound **5a**'s two hetero nitrogen atoms, resulting from the hetero oxygen (on ring C of the benzo-α-pyrone core) being replaced by a second hetero nitrogen, in addition to the primary hetero nitrogen, occasioned an improved A_1 AR affinity, but diminished A_{2A} AR binding (**5a** vs **1**). Unlike compounds **1** and **5a**, the benzoxazinone derivative **2a** only exhibited affinity for the A_1 AR. Furthermore, it is worth mentioning that an approximate two-fold improvement in A_1 AR affinity was documented for compound **5a**, when compared to compounds **1** and **2a**, while compound **1** displayed a six-fold higher A_{2A} AR than **5a**.

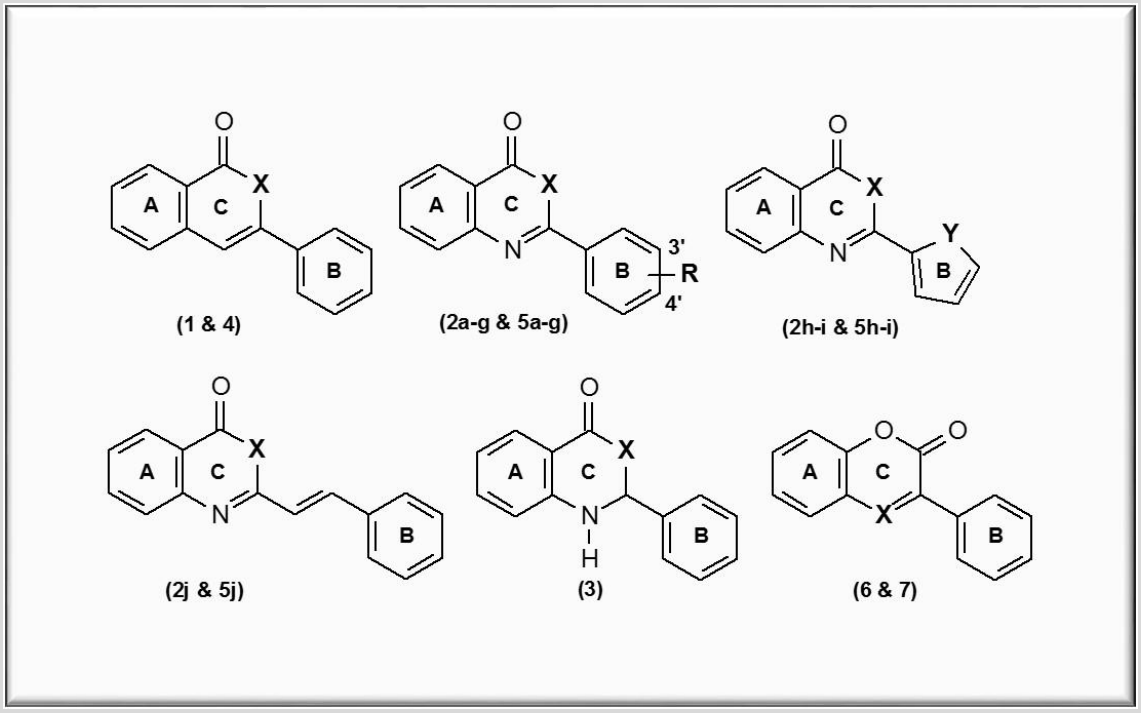
Since the quinazolinone derivative **5a** boasted encouraging results regarding affinity for both the A_1 and A_{2A} ARs (A_1K_i = 3.67 μM; $A_{2A}K_i$ = 18.7 μM), further investigation of the SAR at ring B of the quinazolinones (**5a–j**) was warranted. Similar to the benzoxazinones, several *para*-substituents on the phenyl ring (**5b–g**) and alternate heterocyclic ring systems, including a furyl (**5h**) and a thiophene ring (**5i**), were explored for the quinazolinones. Additionally, the insertion of a styryl side chain (**5j**) between ring C and the phenyl ring B was also explored. Halogen

substitution proved in vain as none of these substitutions (**5b–d**) exhibited affinity for either the A_1 or A_{2A} ARs. However, *para*-substitution with a CH_3 group (**5e**) exerted a favourable effect on A_1 AR affinity (A_1K_i = 2.50 µM). This structural modification yielded the highest affinity for the A_1 AR for this study. Remarkably, simultaneous *meta*- and *para*-substitution with an OCH_3 group (ring B) resulted in compound **5g**, with gained A_{2A} AR affinity ($A_{2A}K_i$ = 2.81 µM), which was the best recorded A_{2A} AR affinity amongst the test compounds of this study. Conversely, mono-substitution with an OCH_3 in the *para*-position (**5f**) led to diminished A_{2A} AR affinity. Compound **5h**, with a furyl ring as ring B, exhibited both A_1 and A_{2A} AR affinity in the low micromolar range (A_1K_i = 4.62 µM; $A_{2A}K_i$ = 8.11 µM)

Furthermore, the comparison of compound **7** to compound **2a** was merited upon the finding by Van der Walt and Terre'Blanche (in press) that the rearrangement of the ketone and hetero oxygen in ring C of compound **1** to afford compound **6**, diminished the affinity for both the A_1 and A_{2A} AR subtypes. Employment of this structural modification, compound **2a** to compound **7**, resulted in a three-fold decrease in A_1 AR affinity ((**2a**) A_1K_i = 6.38 µM vs (**7**) A_1K_i = 17.3 µM), while A_{2A} AR affinity remained absent. Thus, it was surmised that the optimal arrangement of the ketone and hetero oxygen (ring C) for A_1 and A_{2A} AR affinity, was the one equal to the arrangement illustrated by both the benzo-α-pyrone derivative **1** and benzoxazinone derivative **2a**.

Regarding the GTP shift assay, the benzo-α-pyrone derivative **1** was previously found to act as an A_1 AR antagonist (Van der Walt & Terre'Blanche, article accepted). Compounds **2a**, **3**, **5a**, **5e**, and **5h** were selected to determine whether agonist or antagonist activity for the A_1 AR subtype is exhibited, by means of a GTP shift assay performed in accordance to literature (Van der Walt & Terre'Blanche, article accepted). A_1 AR agonist activity was defined by a rightward shift of the binding curve in the presence of GTP (due to the uncoupling of the A_1 AR from its G_i protein). In turn, A_1 AR antagonism was expected to generate no significant shift in the presence of GTP (Gütschow *et al.,* 2012; Van der Walt & Terre'Blanche, 2015). Compounds **2a**, **3**, **5a**, **5e**, and **5h** were reported to possess insignificant shifts of the binding curve in the presence GTP and was, therefore, deemed as A_1 AR antagonists, as anticipated.

Table 5-2: Dissociation constant values (K_i values) for the binding of the test compounds to rat A_1 and A_{2A} ARs.



Compd		R	Y	K_i ± SEM (μM)[a] (% displacement)[b] A_1[c] vs [^{3}H]DPCPX	A_{2A}[c] vs [^{3}H]NECA	A_1[c] + GTP[d] vs [^{3}H]DPCPX	GTP shift[e]	SI[f] A_{2A}/A_1
Benzo-α-pyrone scaffold								
1	-O	-	-	7.41 ± 0.4[a,g]	3.35 ± 0.80[a,g]	6.49 ± 0.82[a,g]	0.9[g]	0.5[g]
Benzoxazinone scaffold								
2a	-O	-H	-	6.38 ± 0.40[a]	> 100 (49%) [b]	6.92 ± 1.16[a]	1.09	-
2b	-O	-4-F	-	> 100 (100%)[b]	> 100 (70%)[b]			-
2c	-O	-4-Cl	-	> 100 (86%)[b]	> 100 (81%)[b]			-
2d	-O	-4-Br	-	> 100 (95%)[b]	> 100 (92%)[b]			-
2e	-O	-4-CH_3	-	> 100 (84%)[b]	> 100 (77%)[b]			-
2f	-O	-4-OCH_3	-	> 100 (54%)[b]	> 100 (37%)[b]			-
2g	-O	-4-OCH_2CH_3	-	> 100 (99%)[b]	> 100 (77%)[b]			-
2h	-O	-	O	> 100 (42%)[b]	> 100 (66%)[b]			-
2i	-O	-	S	32.4 ± 0.58[a]	> 100 (59%)[b]			-
2j	-O	-	-	> 100 (56%)[b]	> 100 (85%)[b]			-
3	-O	-	-	5.06 ± 0.46[a]	> 100 (69%)[b]	5.72 ± 0.56[a]	1.12	-

Isoquinolinone scaffold								
4	-NH	-	-	>100 (100%)[b]	> 100 (100%)[b]			-
Quinazolinone scaffold								
5a	-NH	-H	-	3.67 ± 0.02[a]	18.7 ± 1.99[a]	3.77 ± 0.28[a]	1.02	5.09
5b	-NH	-4-F	-	> 100 (50%)[b]	> 100 (62%)[b]			-
5c	-NH	-4-Cl	-	> 100 (54%)[b]	> 100 (82%)[b]			-
5d	-NH	-4-Br	-	> 100 (98%)[b]	> 100 (84%)[b]			-
5e	-NH	-4-CH_3	-	2.50 ± 0.47[a]	> 100 (55%)[b]	2.95 ± 0.18[a]	1.18	-
5f	-NH	-4-OCH_3	-	> 100 (44%)[b]	> 100 (79%)[b]			-
5g	-NH	-3,4-OCH_3		> 100 (67%)[b]	2.81 ± 0.40[a]			-
5h	-NH	-	O	4.62 ± 0.63[a]	8.11 ± 0.03[a]	5.89 ± 0.42	1.23	1.76
5i	-NH	-	S	> 100 (39%)[b]	> 100 (30%)[b]			-
5j	-NH	-	-	> 100 (100%)[b]	> 100 (56%)[b]			-
Coumarin scaffold								
6	-CH	-	-	> 100 (51%)[b,g]	> 100 (77%)[b,g]			-
Benzoxazinone scaffold								
7	-N	-	-	17.3 ± 1.9[a]	> 100 (91%)[b]			-
Reference compounds								
CPA (A_1 agonist)				0.0051 ± 0.0002 (0.0079)[h]; (0.0059)[i]	0.557 ± 0.024 (0.460)[h]	29.5 ± 1.14 (35.2)[i]	5.8 (6)[i]	5784
ZM-241385 (A_{2A} antagonist)				0.420 ± 0.01 (0.225)[j]	0.0013 ± 0.00002 (0.002)[j]	0.510 ± 0.03	1.2	0.003

[a] All K_i values determined in triplicate and expressed as mean ± SEM; [b] Percentage displacement of the radioligand at a maximum tested concentration (100 µM); [c] Rat receptors were used (A_1: rat whole brain membranes; A_{2A}: rat striatal membranes); [d] GTP shift assay, where the 100 µM GTP was added to the A_1 AR radioligand binding assay; [e] GTP shifts calculated by dividing the K_i in the presence of GTP by the K_i in the absence of GTP; [f] Selectivity index (SI) for the A_1 AR isoform calculated as the ratio of K_i (A_{2A})/K_i (A_1); [g] Literature value obtained from reference (Van der Walt et al., 2017); [h] Literature value obtained from reference (Bruns et al., 1987); [i] Literature value obtained from reference (Van der Wenden, 1995); [j] Literature value obtained from reference (Müller & Ferre, 2007).

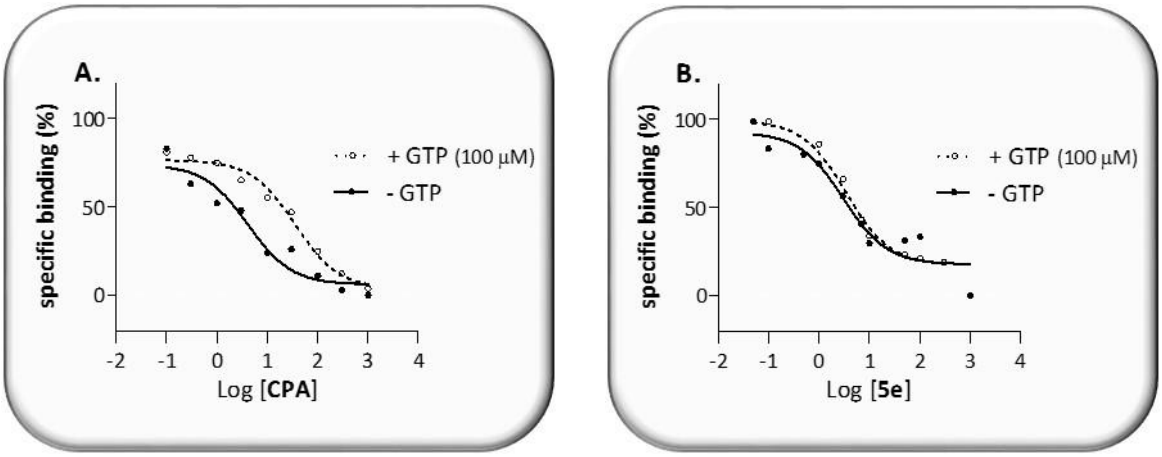


Figure 5-3: **The binding curves of the reference compound CPA and 5e are examples of A_1 AR agonistic and antagonistic action, respectively. The functionality was determined via GTP shift assays (with and without 100 µM GTP) in rat whole brain membranes expressing A_1 ARs with [^{3}H]DPCPX as radioligand. (A) A GTP shift of 5.8 was calculated for CPA and (B) a GTP shift of 1.18 was calculated for compound 5e.**

5.5 CONCLUSION

All the commercially obtained and synthesised compounds in **Chapter 4** were assessed for possible A_1 and A_{2A} AR antagonist activity. The assessment entailed radioligand binding studies to determine the affinity towards the A_1 and A_{2A} ARs. [^{3}H]DPCPX and [^{3}H]NECA served as the radioligands in the A_1 and A_{2A} AR binding studies, respectively. In this study, the C2-substituted benzoxazinones proved to be devoid of A_{2A} AR activity and only two compounds exhibited A_1 AR affinity. Whereas, the C2-substituted quinazolinones presented with varying degrees of affinity toward both A_1 and A_{2A} ARs. The results obtained during the course of the radioligand binding studies are presented in **Table 5-2**. The final conclusion will follow in **Chapter 6**.

CHAPTER 6

CONCLUSION

In this pilot study, as per the aims stated in **Chapter 1**, a series of C2-substituted benzoxazinone derivatives (**2a**, **2d**, **2f**, **2h** & **2i**), a series of C2-substituted quinazolinone derivatives (**5a-j**) and an isoquinolinone derivative (**4**) were synthesised (see **Chapter 4**) and assessed in terms of A_1 and A_{2A} adenosine receptor (AR) antagonist activity (see **Chapter 5**). The series of synthesised C2-substituted benzoxazinone derivatives was augmented by the addition of several commercially obtained C2-substituted benzoxazinone derivatives (**2b**, **2c**, **2e**, **2g**, **2j**, **3**, **6** & **7**) and they too were assessed in terms of A_1 and A_{2A} AR antagonist activity. Evaluation of all test compounds in terms of A_1 and A_{2A} AR antagonist activity was performed with the expectation of discovering potent A_1 and/or A_{2A} AR antagonists that may be of future benefit in treatment of Parkinson's disease (PD), based on the existing literature depicting the potential benefit of AR antagonists in PD treatment (see **Chapter 2** and **3**).

The benzo-α-pyrone derivative, 3-phenyl-1H-2-benzopyran-1-one (**1**) served as the lead compound for this pilot study (A_1K_i = 7.41 µM and $A_{2A}K_i$ = 3.35 µM) and the proposed structural modifications thereof resulted in diverse degrees of selectivity and affinity towards the A_1 and A_{2A} ARs. Improved A_1 AR binding (two-fold) was observed upon replacing the α-pyrone core (ring C of compound **1**) with a pyrimidone ring to afford the quinazolinone derivative **5a** ((**1**) A_1K_i = 7.41 µM vs (**5a**) A_1K_i = 3.67 µM), whilst replacing the α-pyrone core (ring C of compound **1**) with an oxazinone to afford the benzoxazinone derivative **2a**, yielded only a slight improvement of A_1 AR affinity ((**1**) A_1K_i = 7.41 µM vs (**2a**) A_1K_i = 6.38 µM). In general, the A_{2A} AR binding was best governed by the α-pyrone as ring C ((**1**) $A_{2A}K_i$ = 3.35 µM) compared to the oxazinone ((**2a**) $A_{2A}K_i$ = >100 µM) and pyrimidone ((**5a**) $A_{2A}K_i$ = 18.7 µM) (see **figure 6-1**).

It is important to note that the benzoxazinone and quinazolinone based scaffolds were previously unknown to exhibit AR affinity and that analogues of these moieties may well be of worth as A_1 AR antagonists. Overall, the C2-substituted quinazolinone derivatives of the current study claimed superiority over their C2- benzoxazinone counterparts where affinity, toward both the A_1 and A_{2A} AR subtypes, was concerned. Among the compounds tested in this study, the quinazolinone derivative **5a** exhibited the second-highest A_1 AR affinity (A_1K_i = 3.67 µM) and introduction of a CH_3 group (*para*-position) to ring B of **5a** afforded the compound with the highest A_1 AR affinity, **5e** (A_1K_i = 2.50 µM). Compound **5e** also displayed selectivity towards the A_1 AR. The 3,4-dimethoxy substituted quinazolinone **5g** possessed the highest A_{2A} AR affinity, as well as selectivity ($A_{2A}K_i$ = 2.81 µM). Therefore, the most promising compounds in this study, **5a**, **5e** and **5g**, proved to be both more selective and potent than caffeine ((**5a**) A_1K_i = 3.67 µM

and $A_{2A}K_i$ = 18.7 μM; (**5e**) A_1K_i = 2.50 μM and $A_{2A}K_i$ > 100 μM; (**5g**) A_1K_i > 100 μM and $A_{2A}K_i$ = 2.81 μM vs (**caffeine**) A_1K_i = 29 μM and $A_{2A}K_i$ = 48 μM).

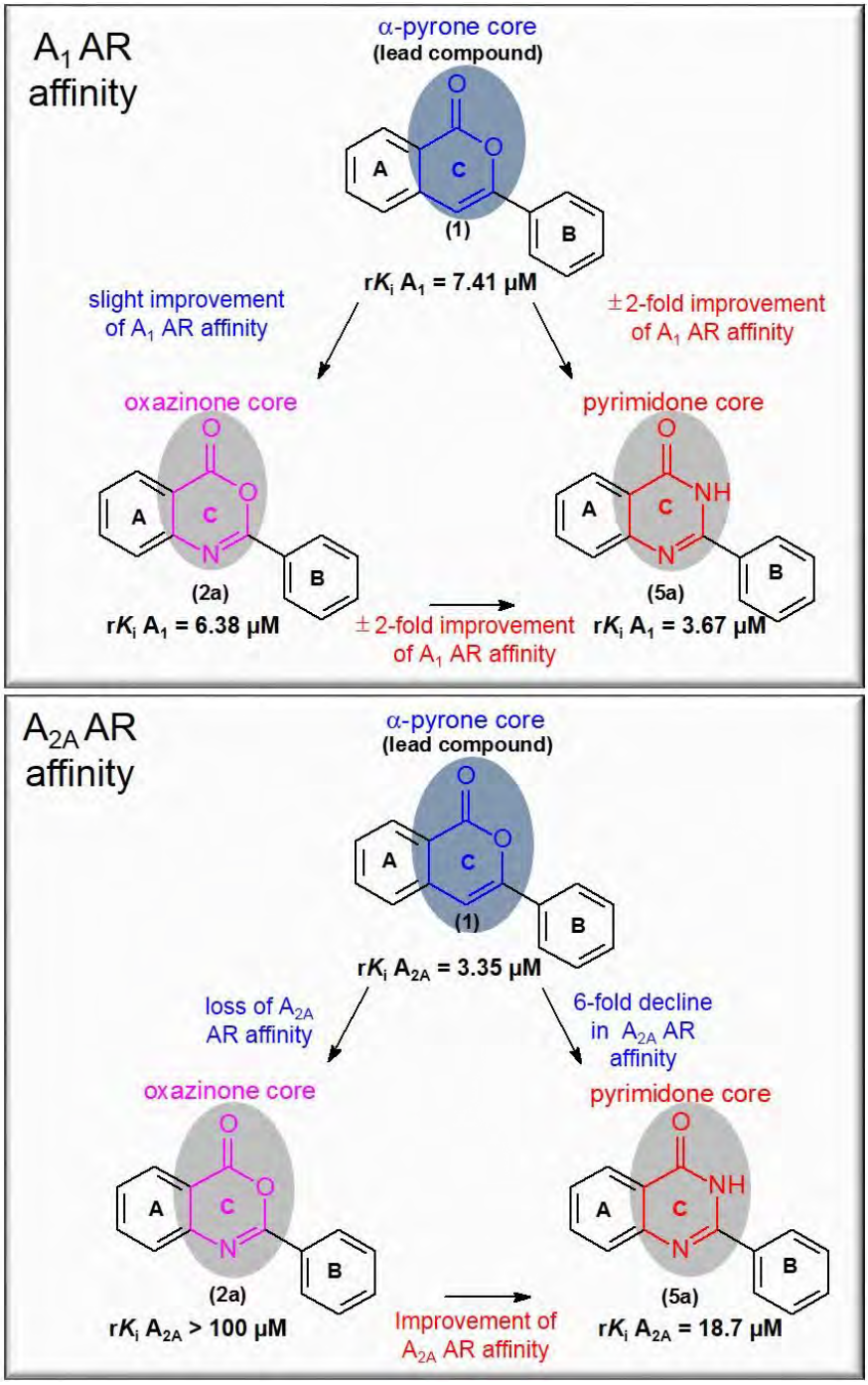


Figure 6-1: **Structural changes to the α-pyrone core resulted in the pyrimidone core and oxazinone core. The pyrimidone core (5a) exhibited improved A_1 AR and A_{2A} AR affinity in analogy to the oxazinone core (2a).**

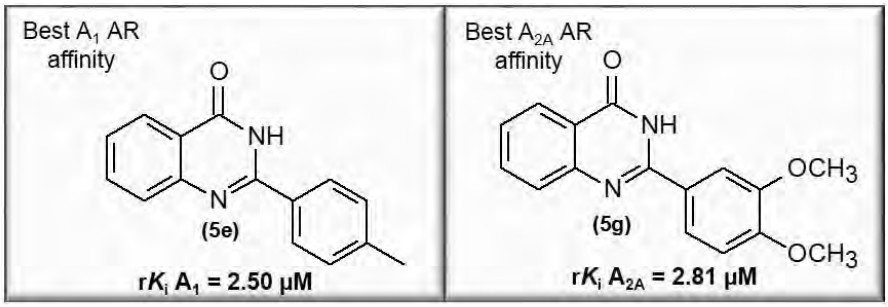


Figure 6-2: Compound 5e exhibited the best A_1 AR affinity and compound 5g exhibited the best A_{2A} AR affinity among the test compounds.

In conclusion, PD remains a disease not wholly understood and continued research is of utmost importance to lend insight into the underlying pathology and deliver novel treatment options. Thus, bearing in mind the possible properties that the A_{2A} AR antagonists possess with regards to PD-related motor dysfunction and neuroprotection, it may be concluded that **5g** may be of therapeutic relevance as a selective A_{2A} AR drug in PD. Alongside compound **5g**'s possible amelioration of motor dysfunction and neuroprotection, compound **5e**, being a selective A_1 AR antagonist, may enhance the cognitive dysfunction associated with PD. As such, the compounds, **5a**, **5e** and **5g**, were identified as possible drug candidates during this study and are deemed as ideal candidates for future *in vivo* examinations as selective A_1 (**5a**, **5e**) and A_{2A} (**5g**) AR antagonists in the treatment of neurological disorders.

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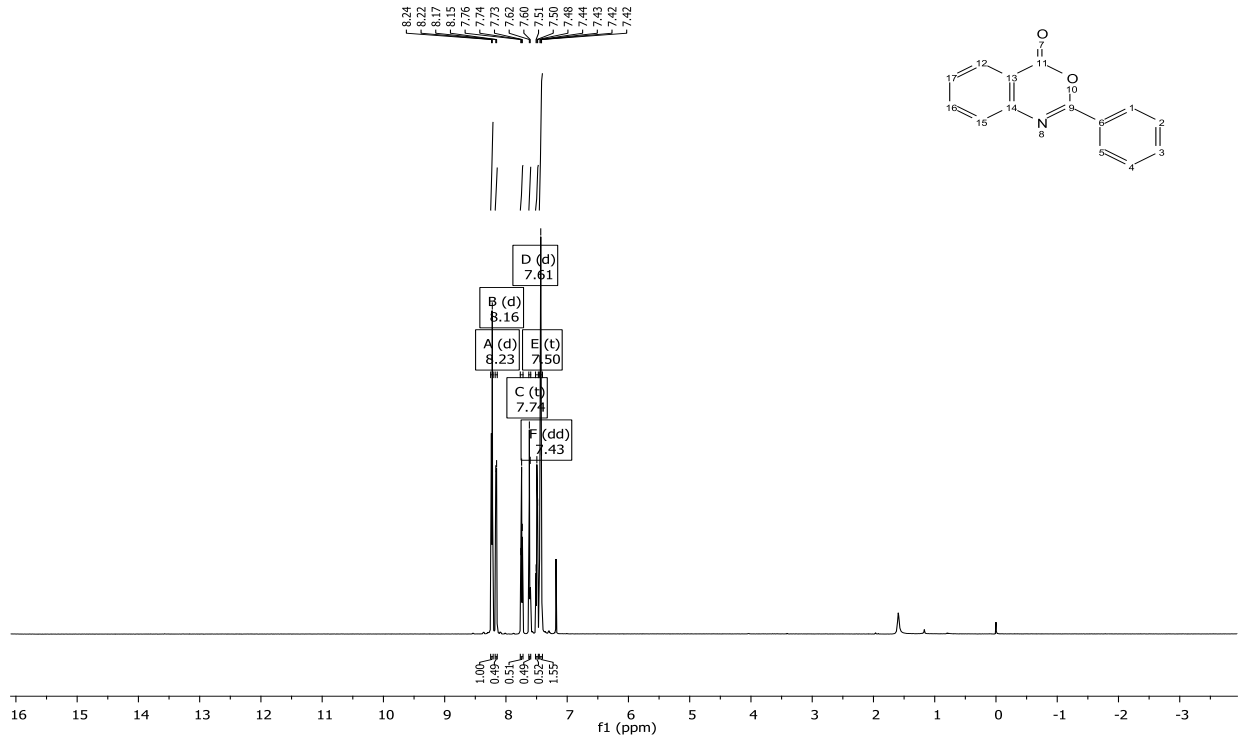
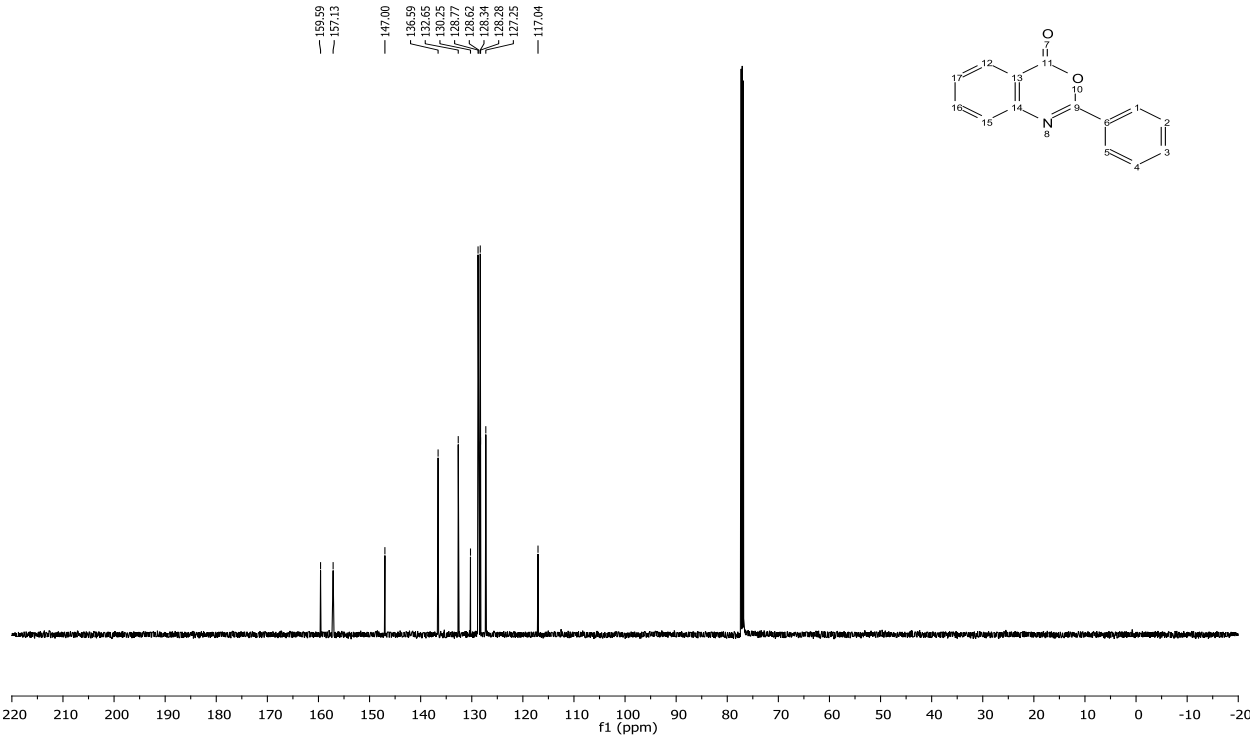
ANNEXURE A-NMR SPECTRA OF THE TEST COMPOUNDS (2a, 2d, 2f, 2h 2i, 4 & 5a-j)

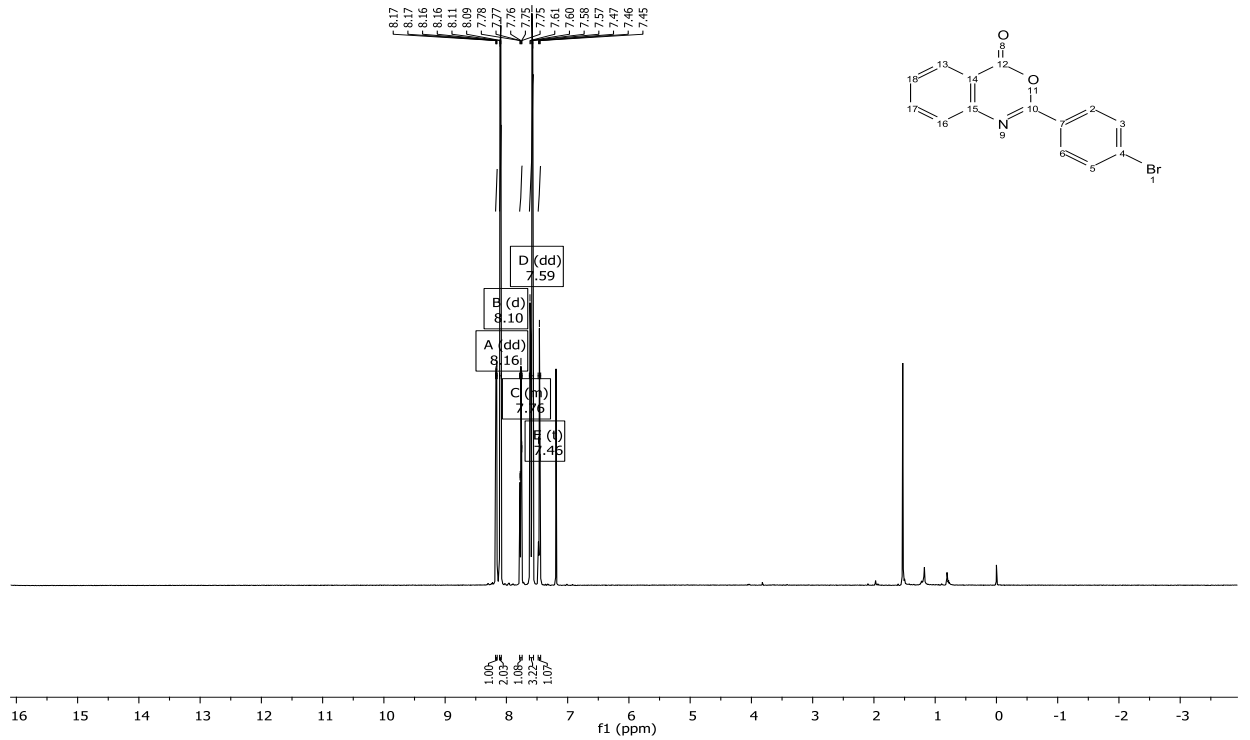
^{1}H NMR ($CDCl_3$): ***2-phenyl-4H-3,1-benzoxazin-4-one (2a)***:



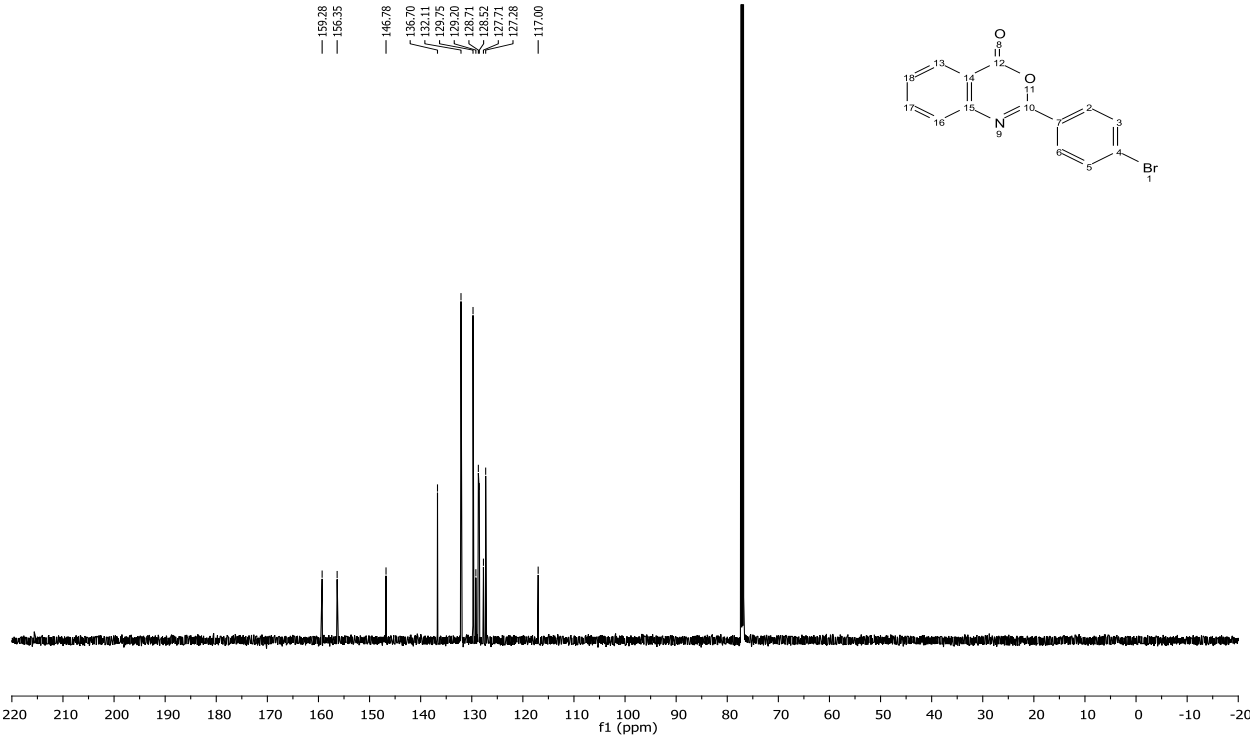
^{13}C NMR ($CDCl_3$): ***2-phenyl-4H-3,1-benzoxazin-4-one (2a)***:



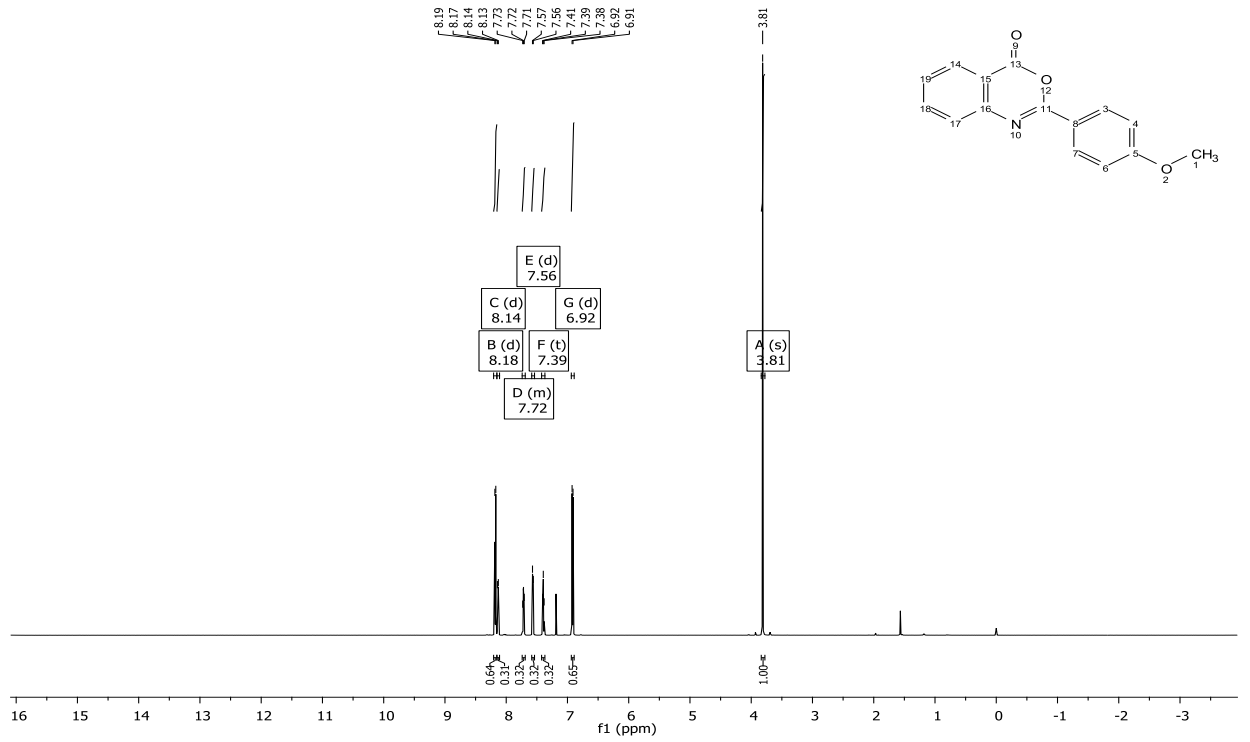
H NMR ($CDCl_3$): ***2-(4-bromophenyl)-4H-3,1-benzoxazin-4-one (2d):***



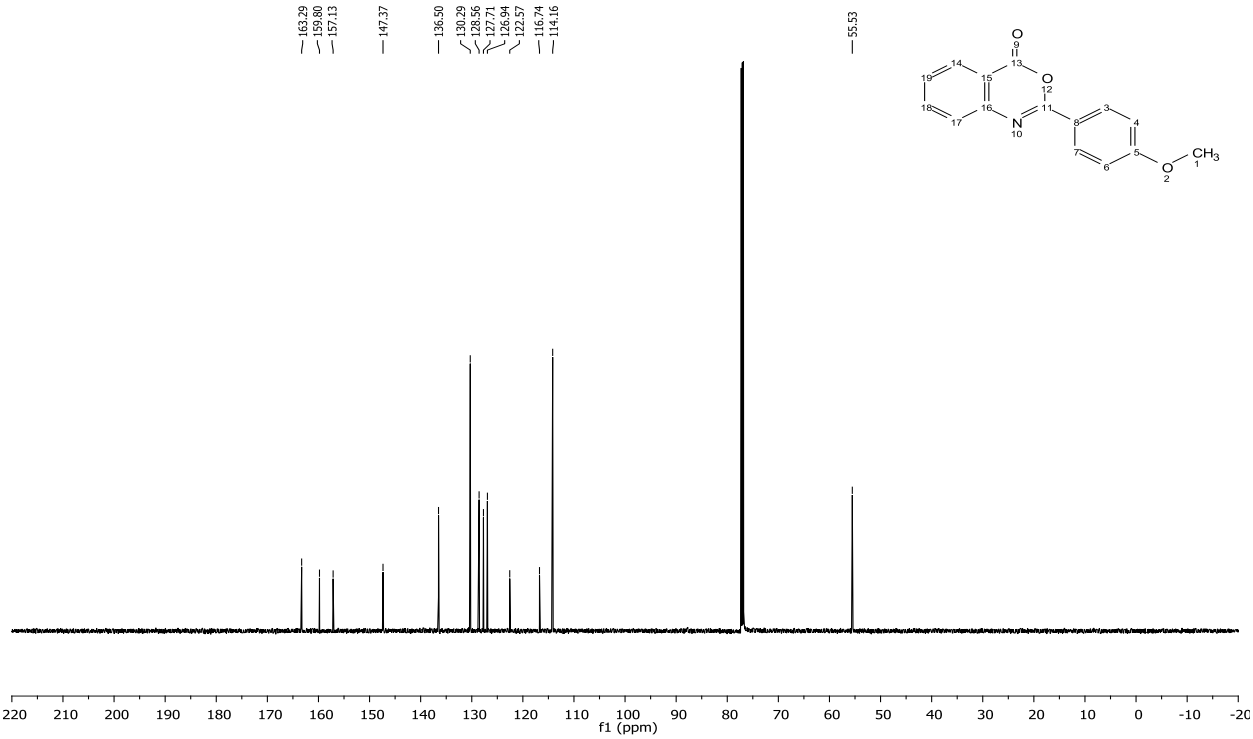
^{13}C NMR ($CDCl_3$): ***2-(4-bromophenyl)-4H-3,1-benzoxazin-4-one (2d):***



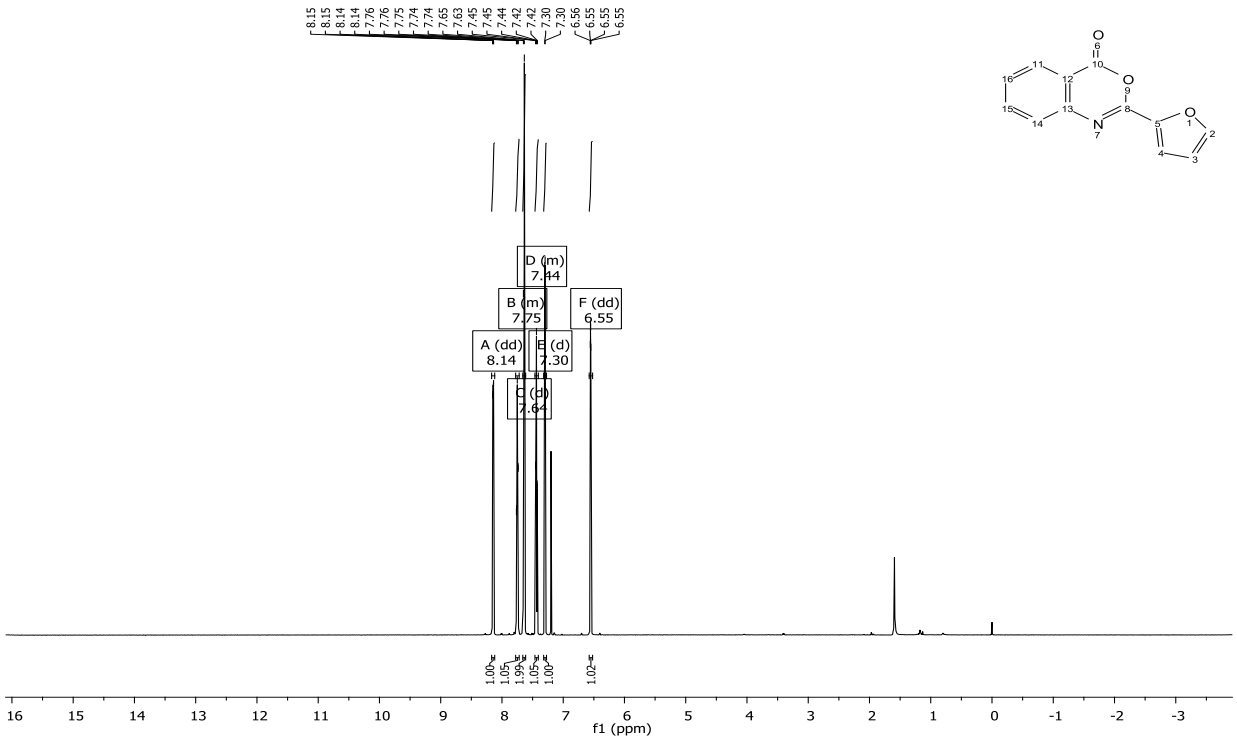
^{1}H NMR ($CDCl_3$): ***2-(4-methoxyphenyl)-4H-3,1-benzoxazin-4-one (2f)***



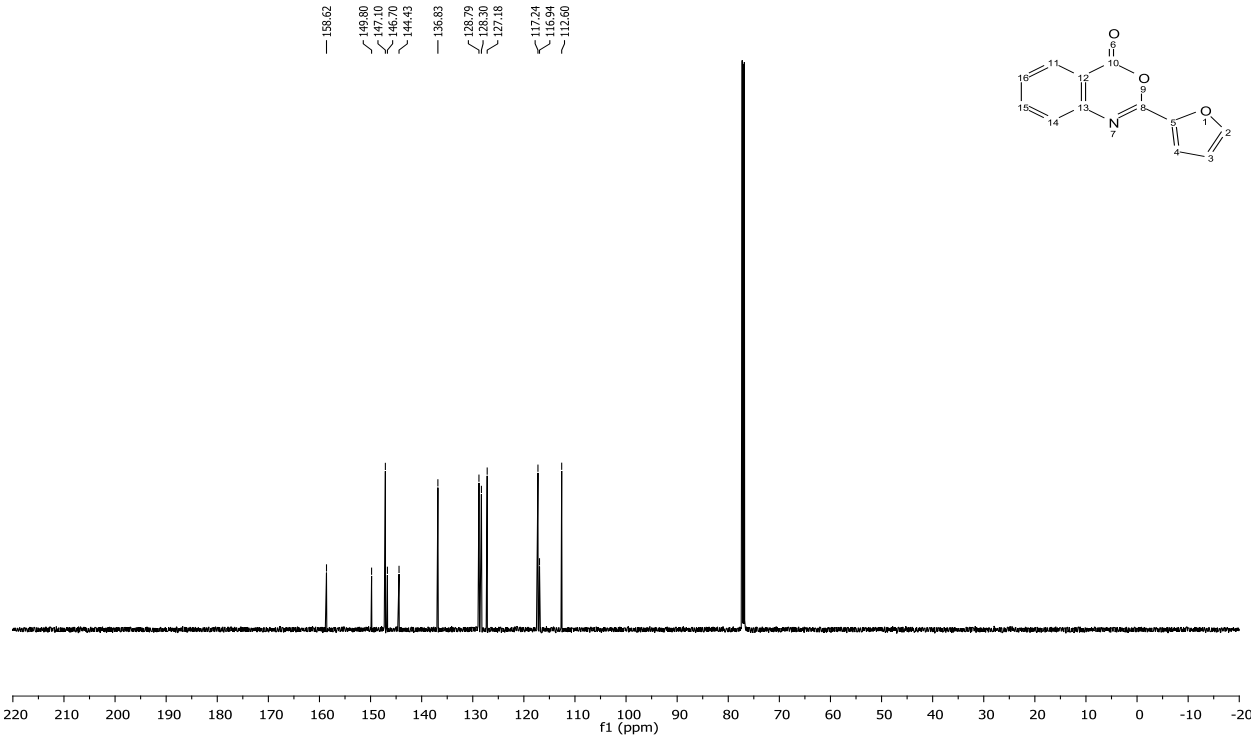
^{13}C NMR ($CDCl_3$): ***2-(4-methoxyphenyl)-4H-3,1-benzoxazin-4-one (2f):***



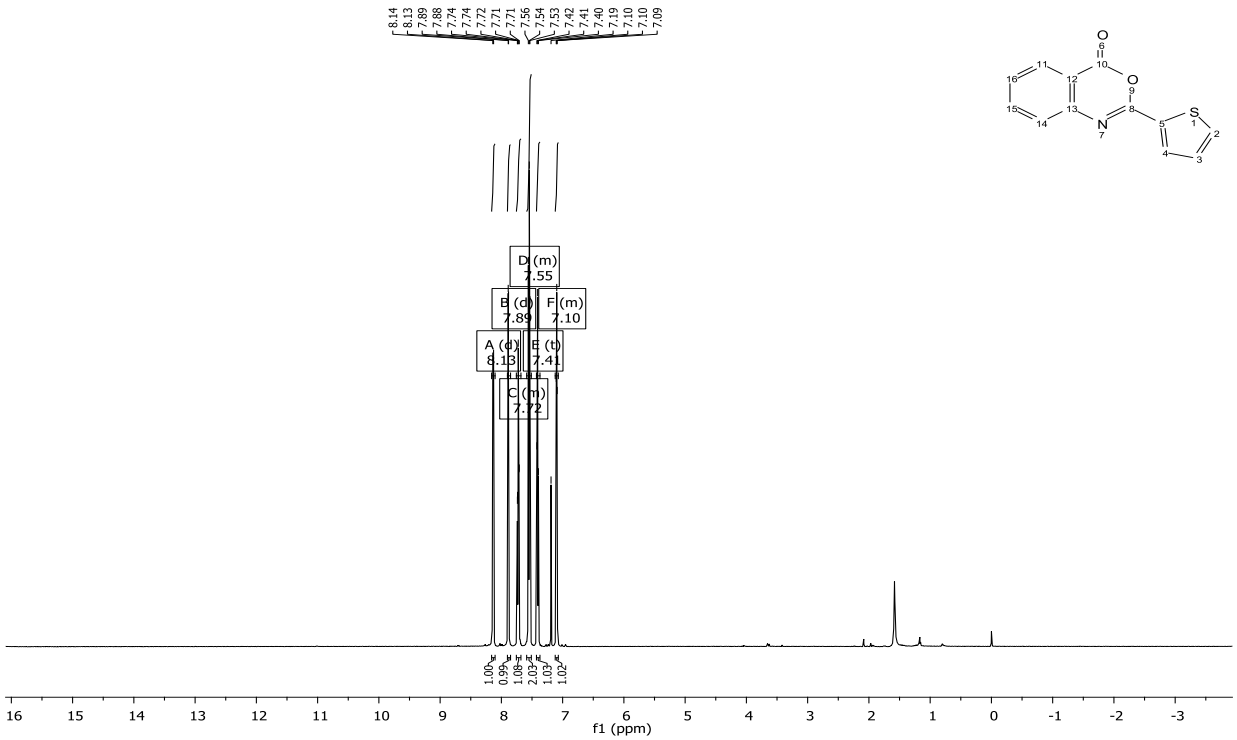
^{1}H NMR ($CDCl_3$): ***2-(furan-2-yl)-4H-3,1-benzoxazin-4-one (2h):***



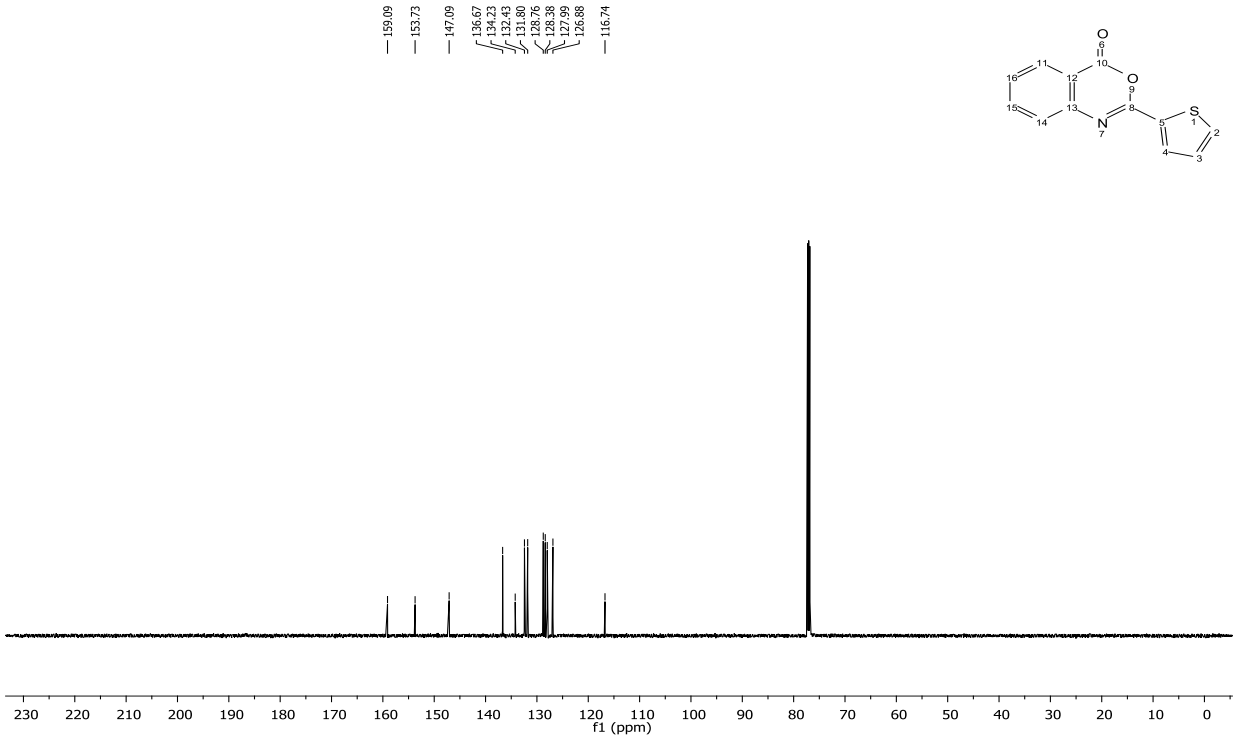
^{13}C NMR ($CDCl_3$): ***2-(furan-2-yl)-4H-3,1-benzoxazin-4-one (2h)***



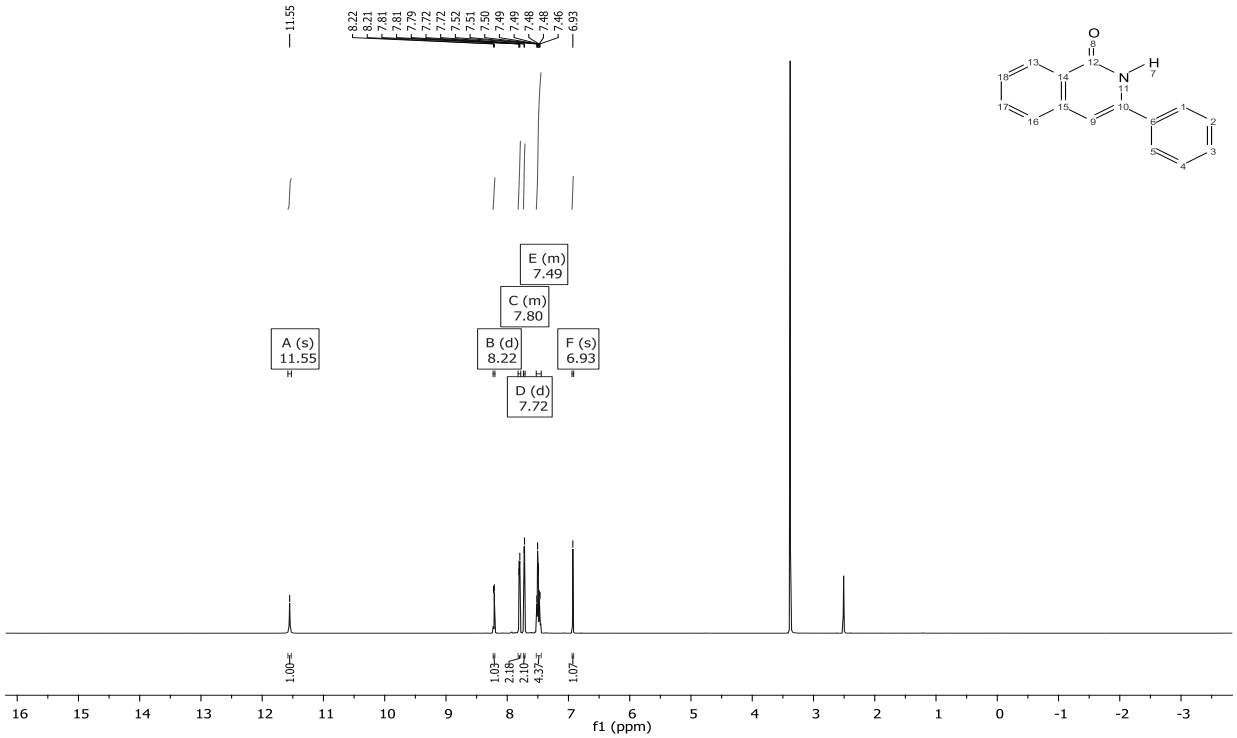
^{1}H NMR ($CDCl_3$): ***2-(thiophen-2-yl)-4H-3,1-benzoxazin-4-one (2i):***



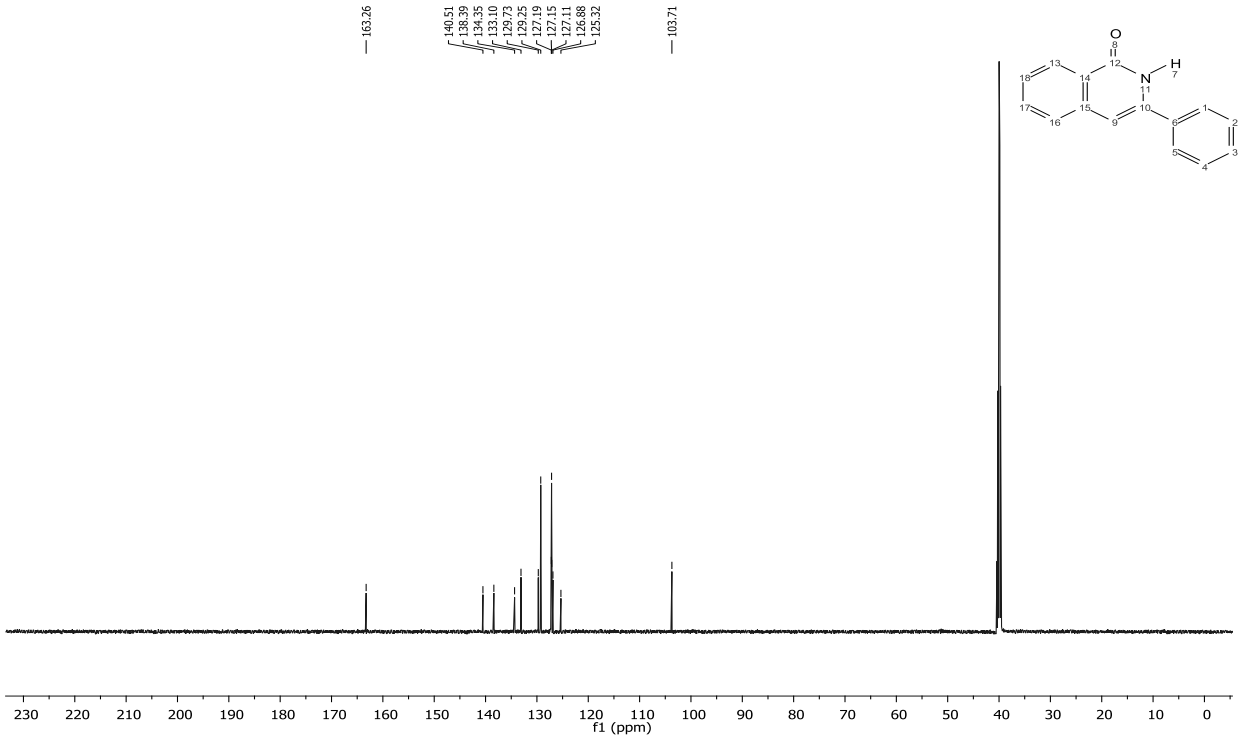
^{13}C NMR ($CDCl_3$): ***2-(thiophen-2-yl)-4H-3,1-benzoxazin-4-one (2i):***



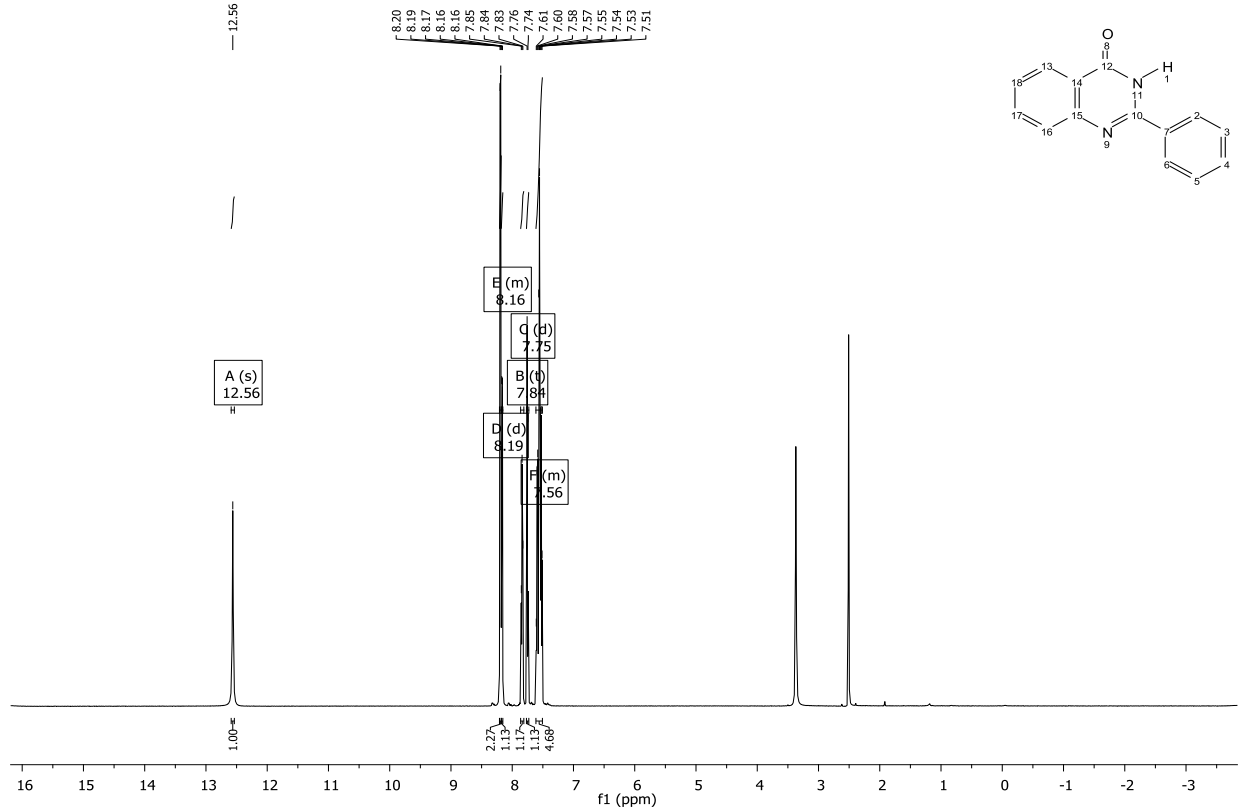
^{1}H NMR (DMSO-d_6): ***3-phenylisoquinolin-1(2H)-one (4):***



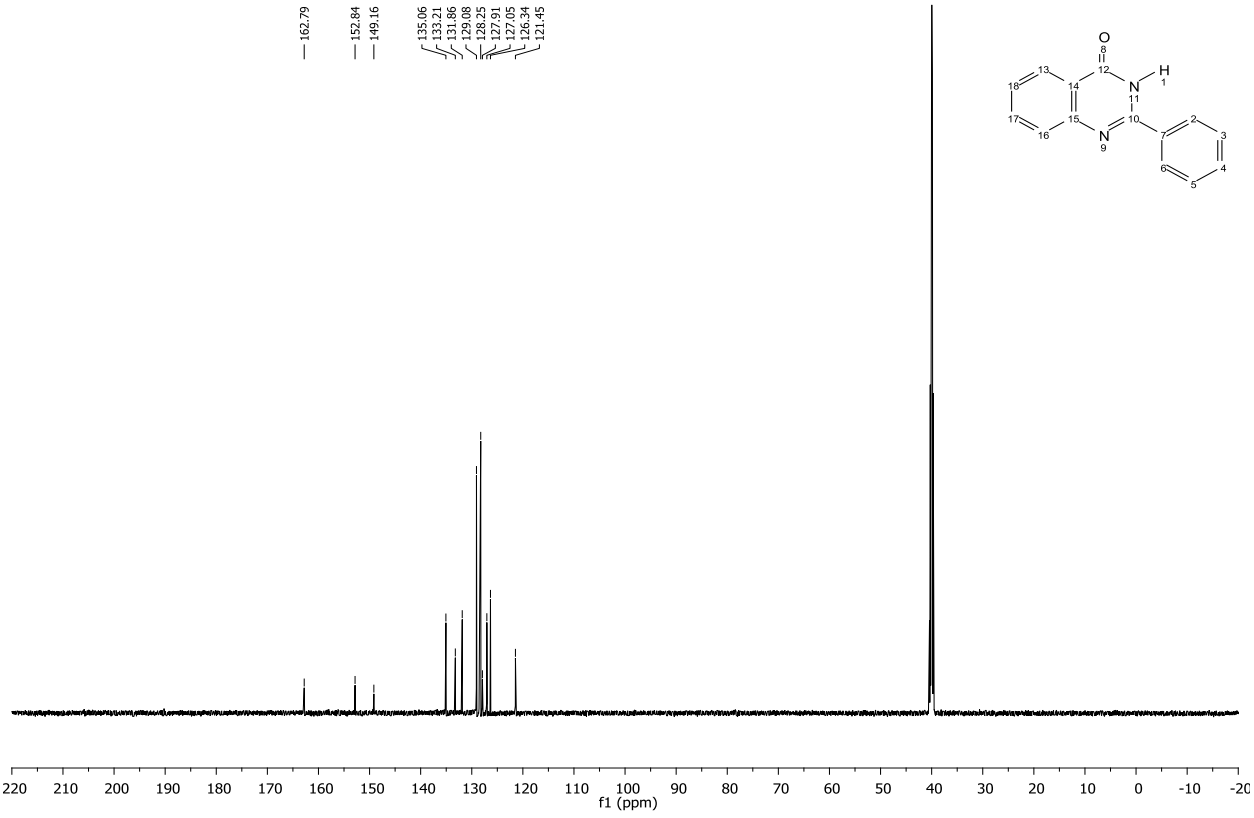
^{13}C NMR (DMSO-d_6): ***3-phenylisoquinolin-1(2H)-one (4):***



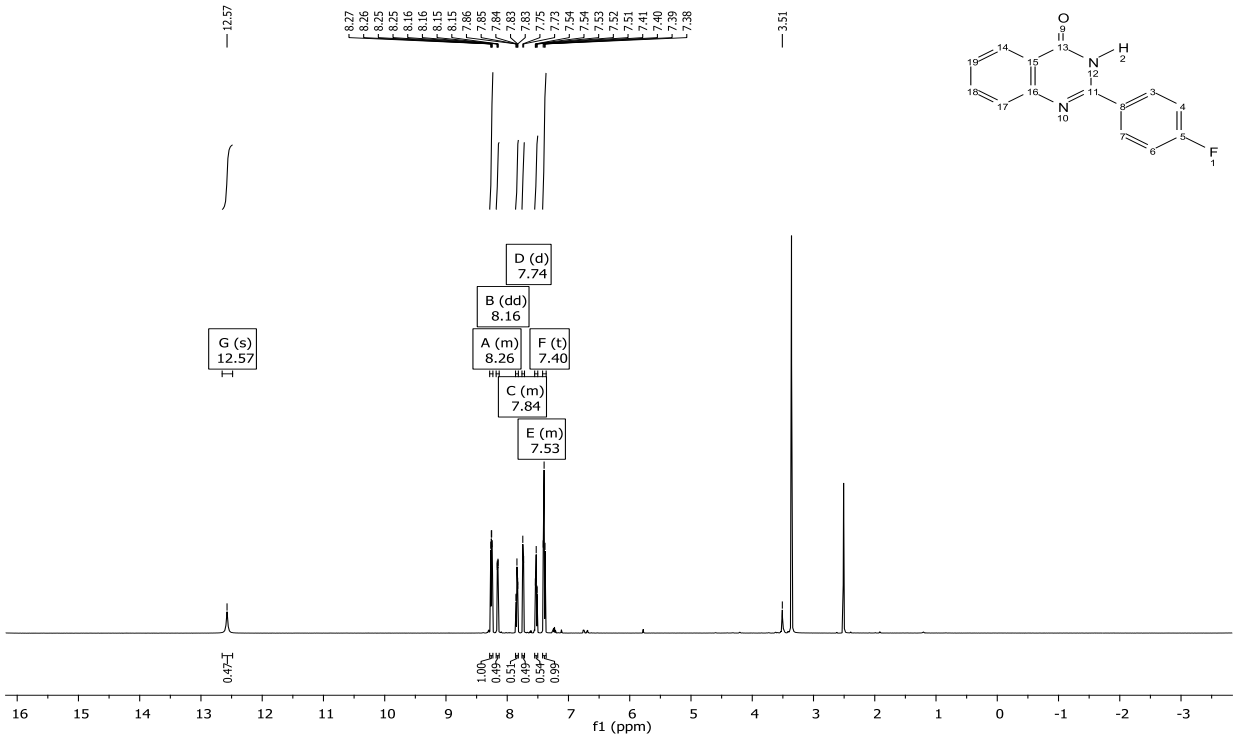
^{1}H NMR (DMSO-d_6): ***2-phenylquinazolin-4(3H)-one (5a):***



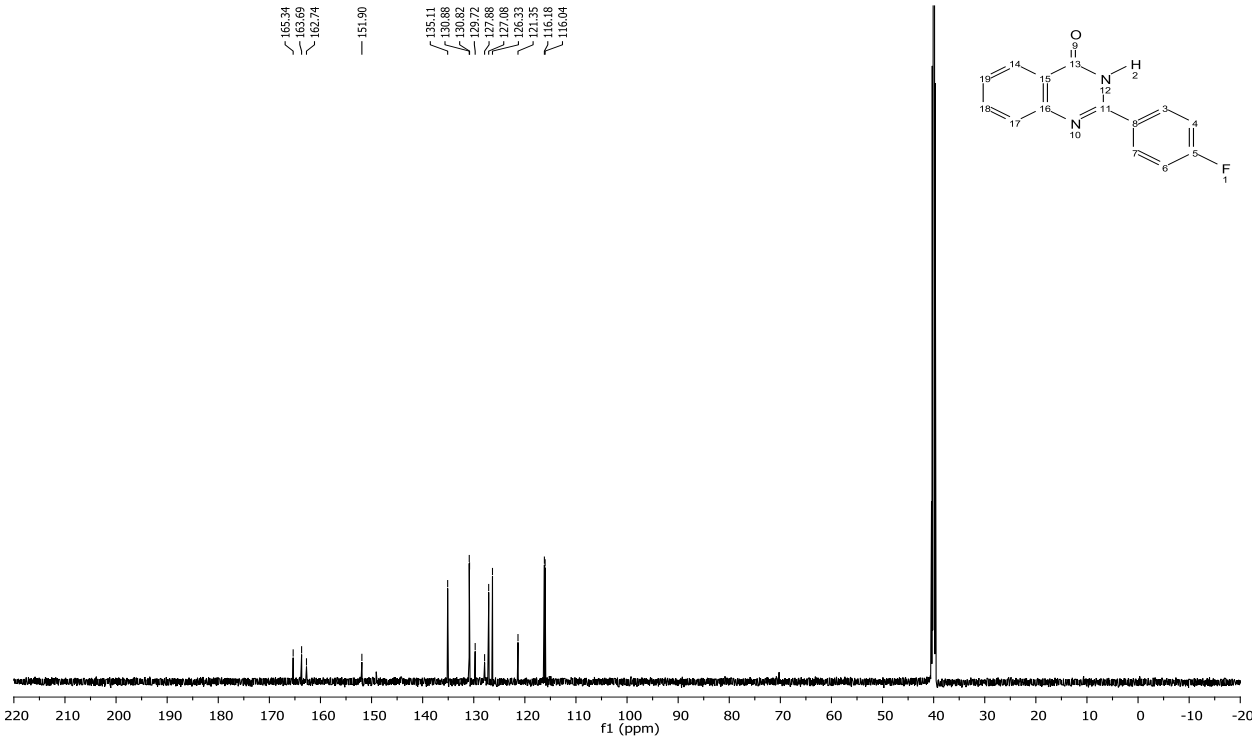
^{13}C NMR (DMSO-d_6): ***2-phenylquinazolin-4(3H)-one (5a):***



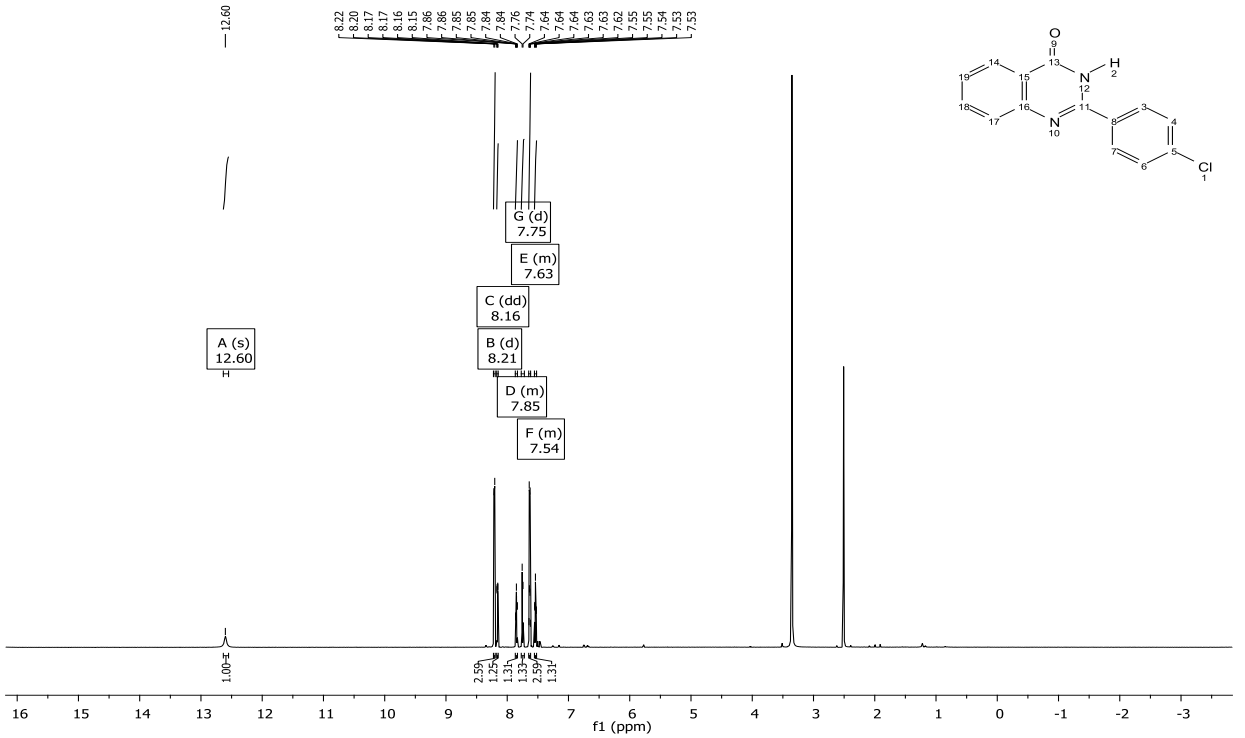
^{1}H NMR (DMSO-d_6): ***2-(4-fluorophenyl)quinazolin-4(3H)-one (5b):***



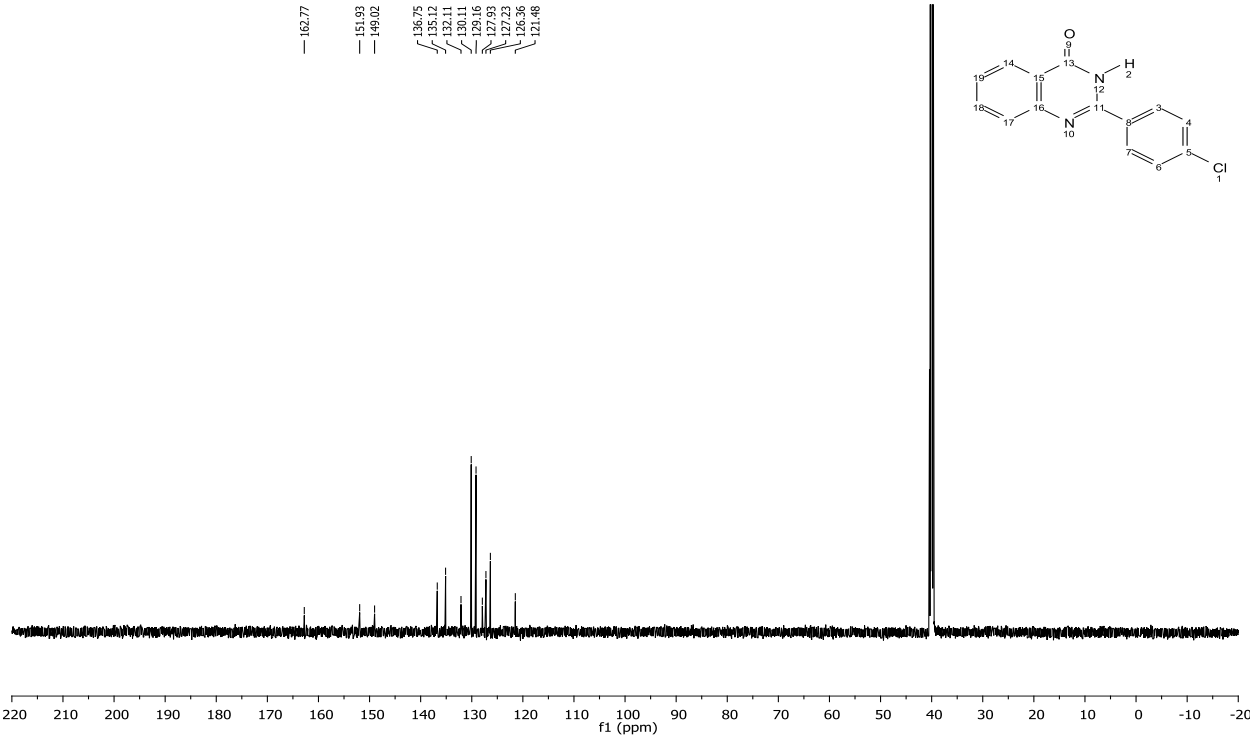
^{13}C NMR (DMSO-d_6): ***2-(4-fluorophenyl)quinazolin-4(3H)-one (5b):***



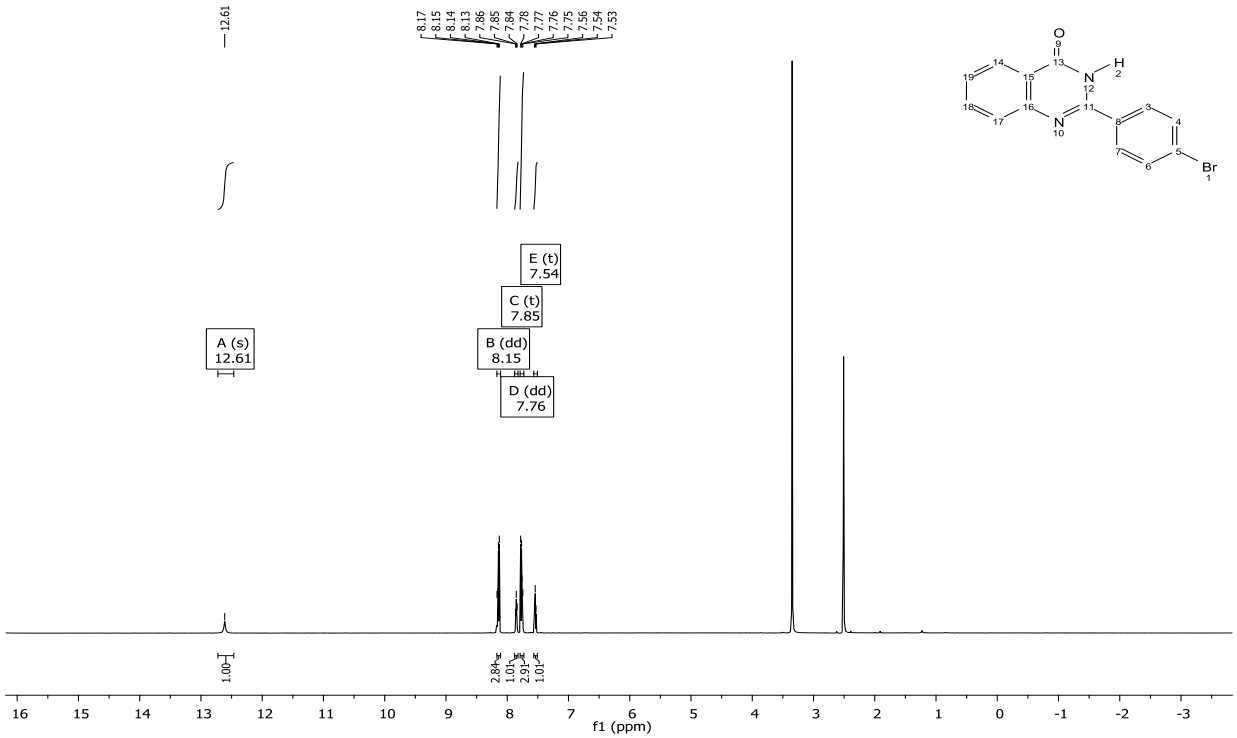
^{1}H NMR (DMSO-d_6): ***2-(4-chlorophenyl)quinazolin-4(3H)-one (5c):***



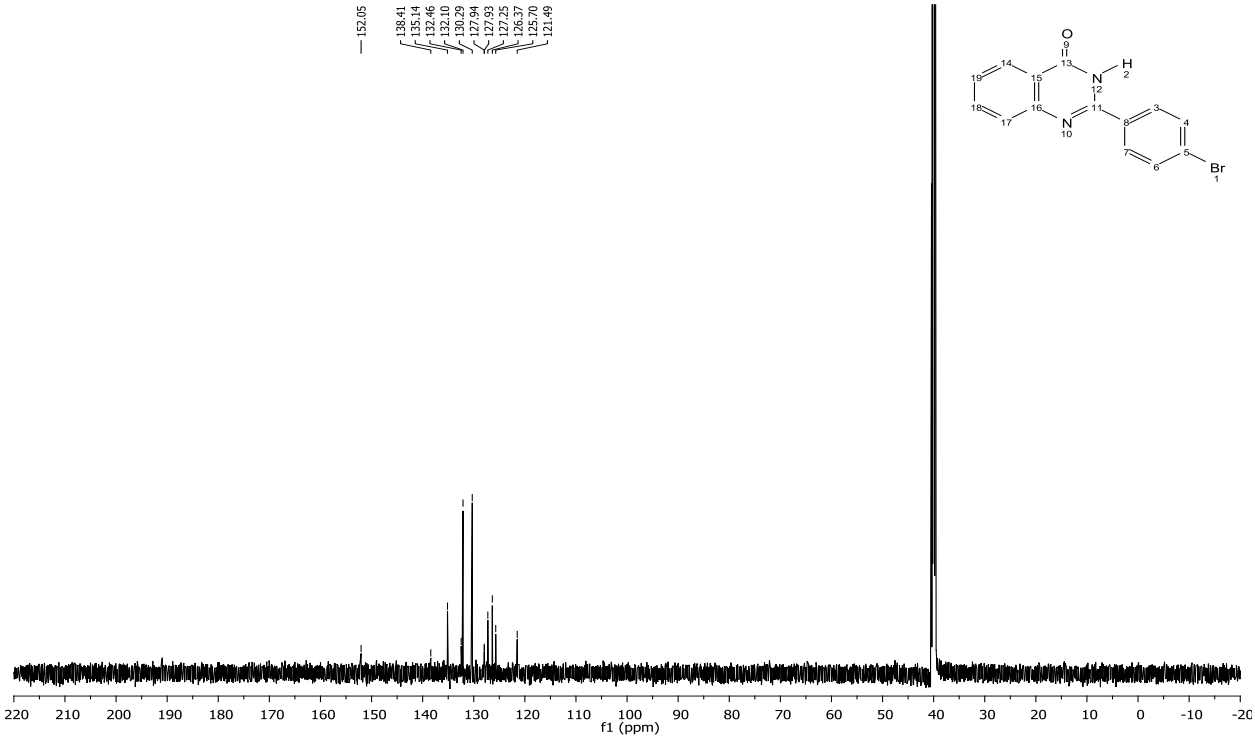
^{13}C NMR (DMSO-d_6): ***2-(4-chlorophenyl)quinazolin-4(3H)-one (5c)*:**



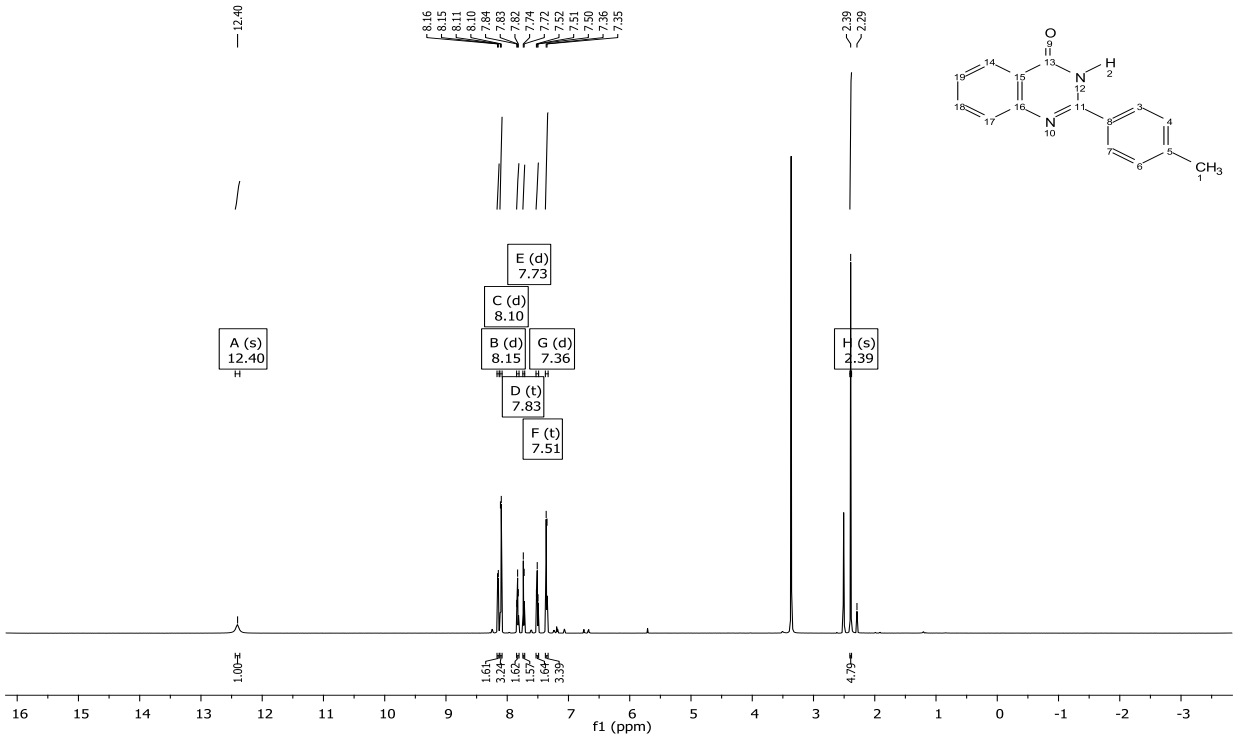
^{1}H NMR (DMSO-d_6): ***2-(4-bromophenyl)quinazolin-4(3H)-one (5d):***



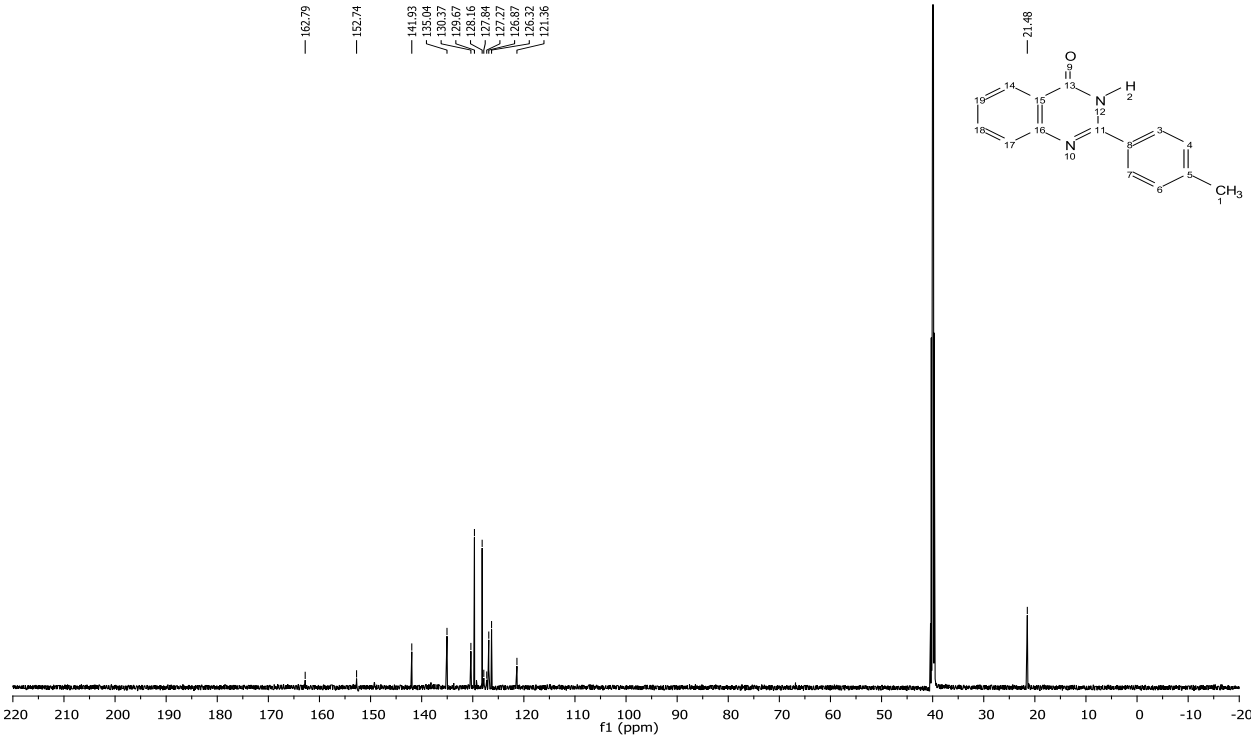
^{13}C NMR (DMSO-d_6): ***2-(4-bromophenyl)quinazolin-4(3H)-one (5d):***



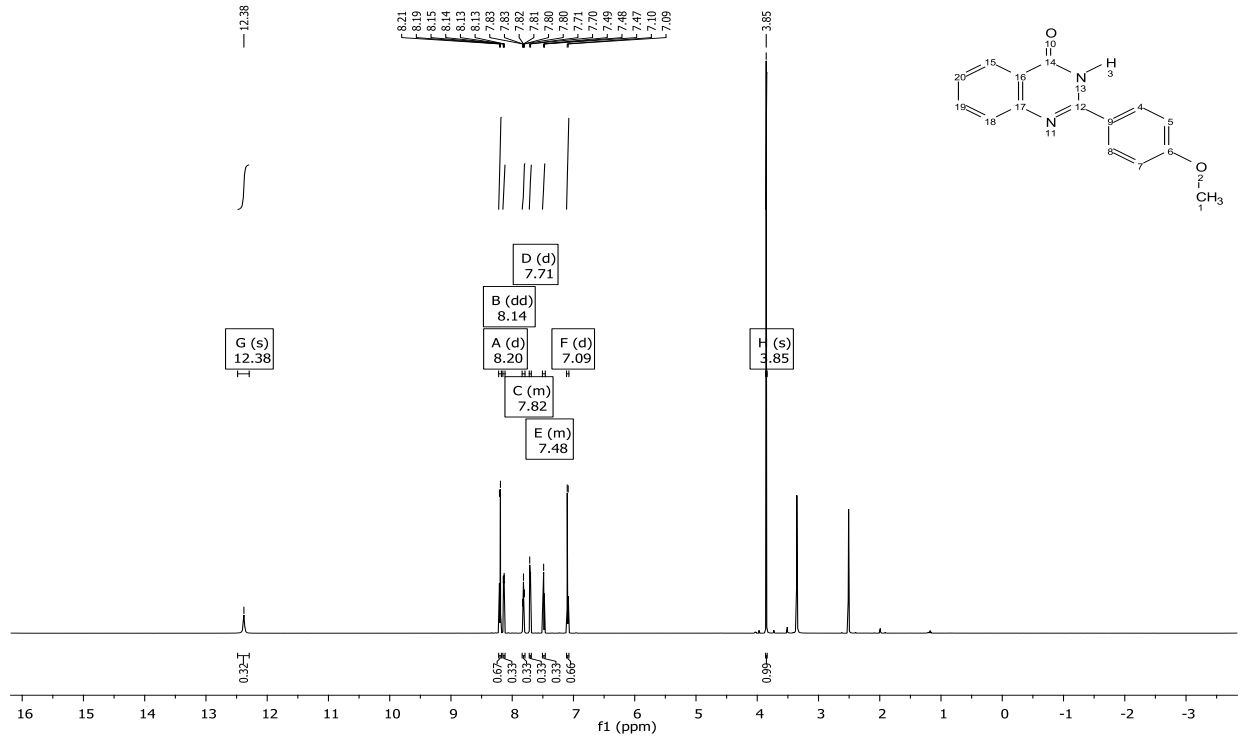
^{1}H NMR (DMSO-d_6): ***2-(4-methylphenyl)quinazolin-4(3H)-one (5e):***



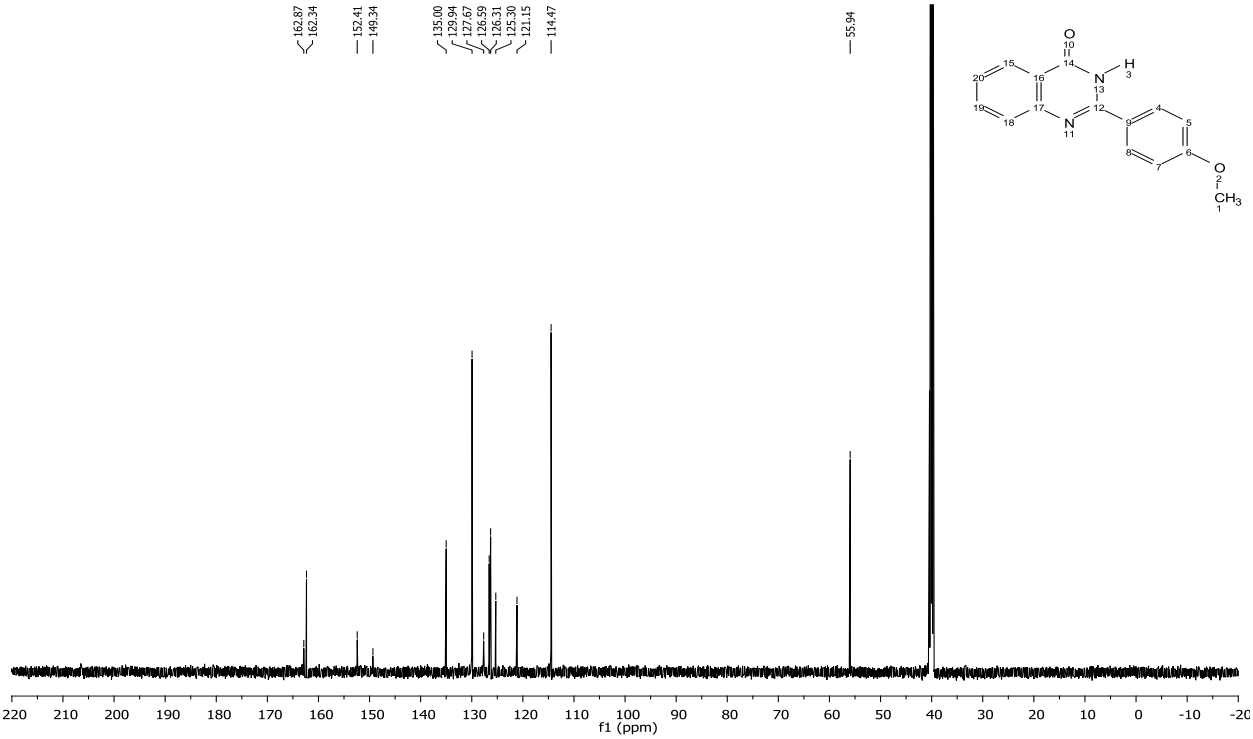
^{13}C NMR (DMSO-d_6): ***2-(4-methylphenyl)quinazolin-4(3H)-one (5e):***



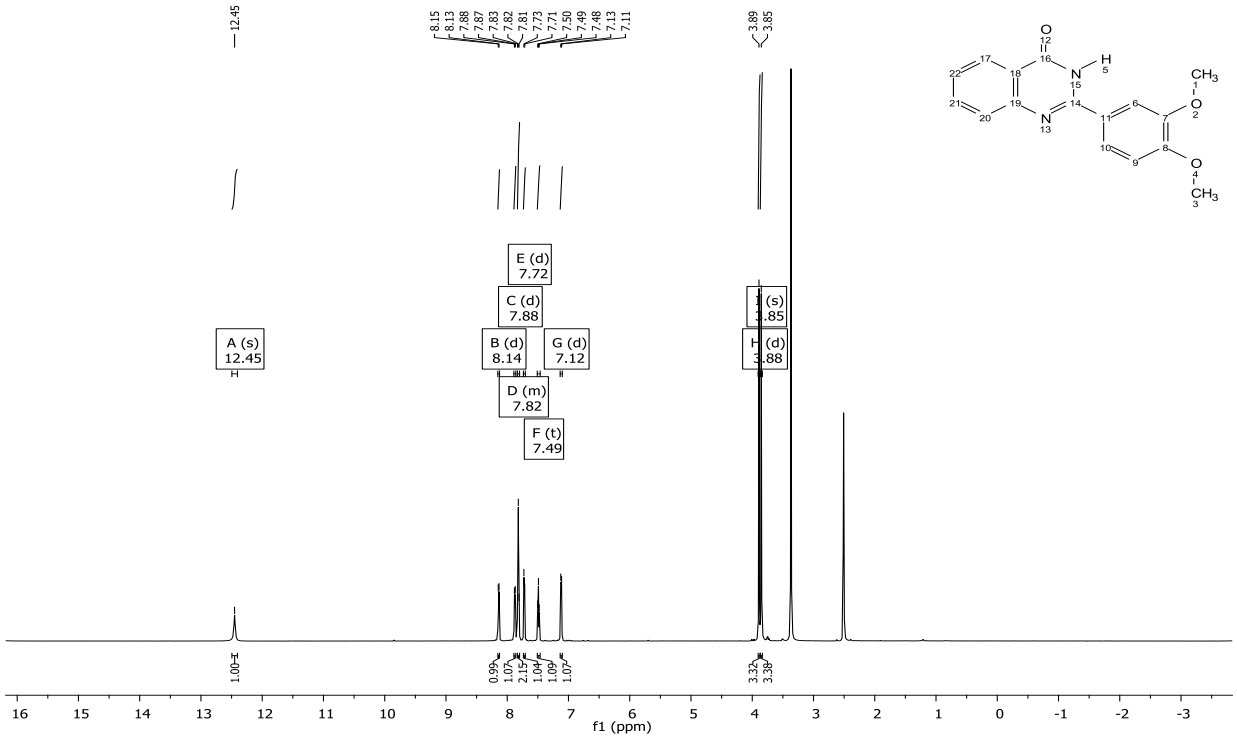
^{1}H NMR (DMSO-d_6): ***2-(4-methoxyphenyl)quinazolin-4(3H)-one (5f):***



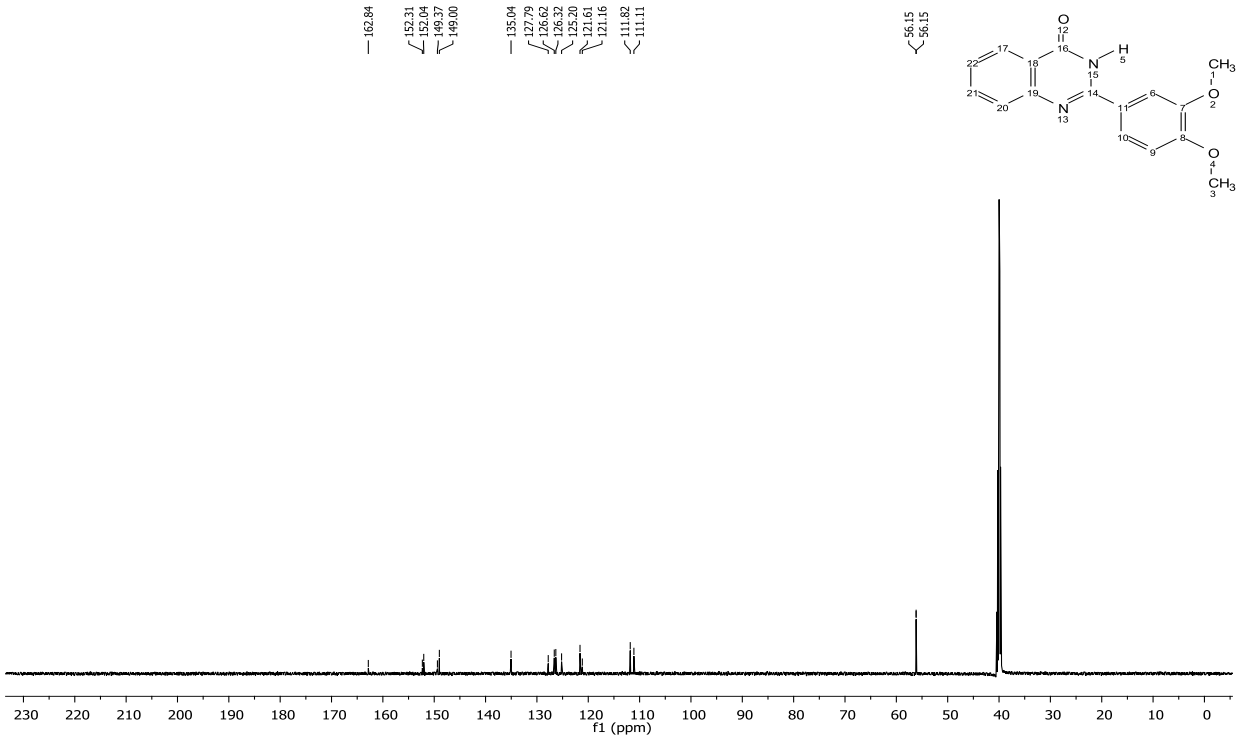
^{13}C NMR (DMSO-d_6): ***2-(4-methoxyphenyl)quinazolin-4(3H)-one (5f):***



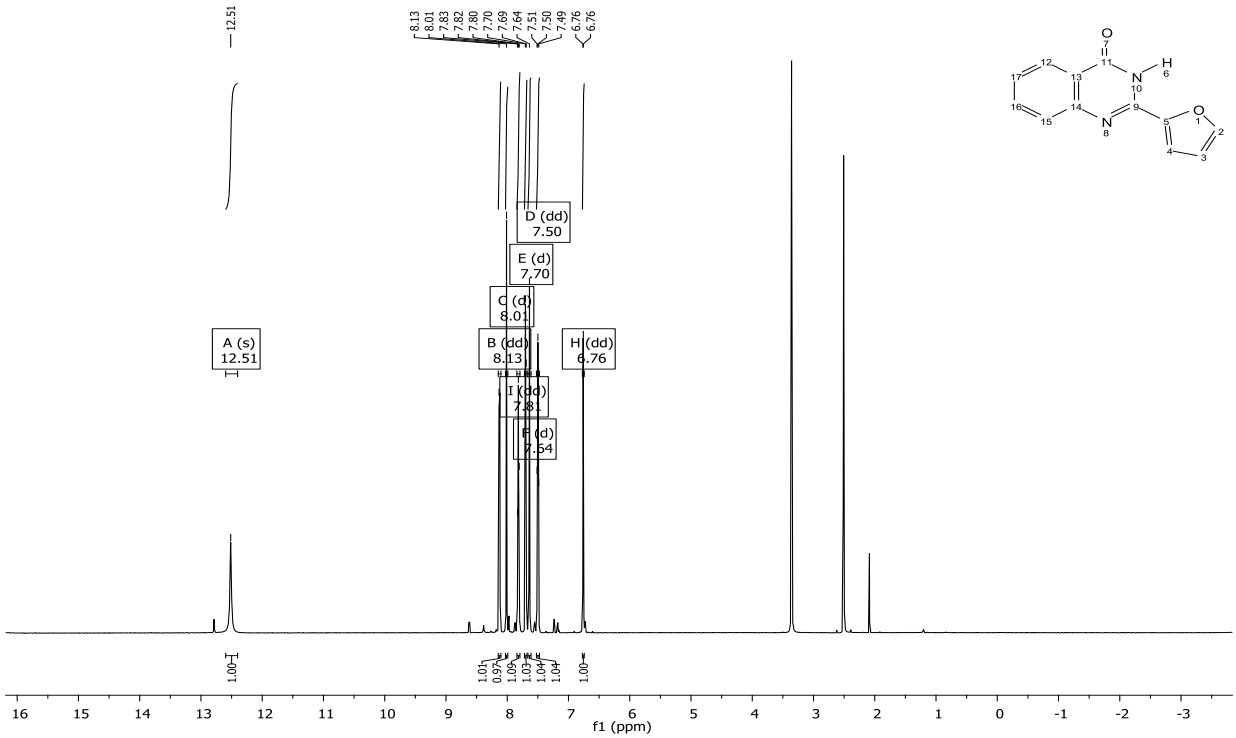
^{1}H NMR (DMSO-d_6): ***2-(3,4-dimethoxyphenyl)quinazolin-4(3H)-one (5g):***



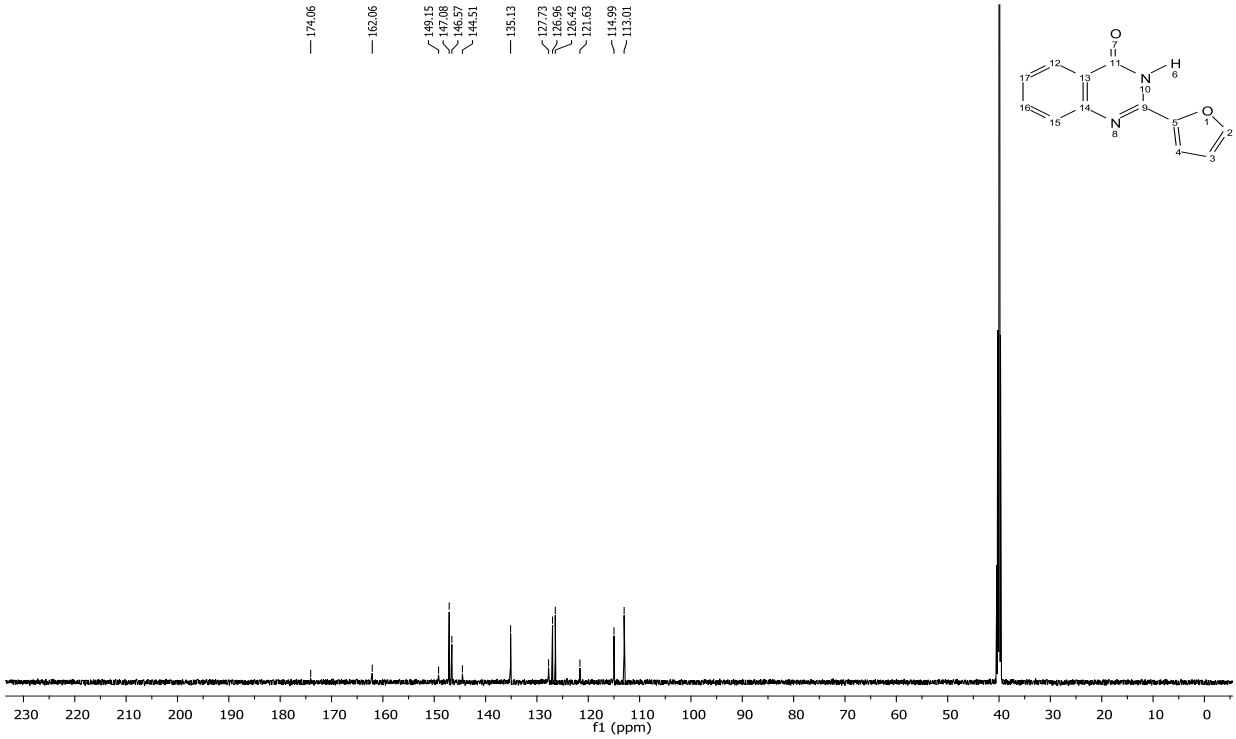
^{13}C NMR (DMSO-d_6): ***2-(3,4-dimethoxyphenyl)quinazolin-4(3H)-one (5g):***



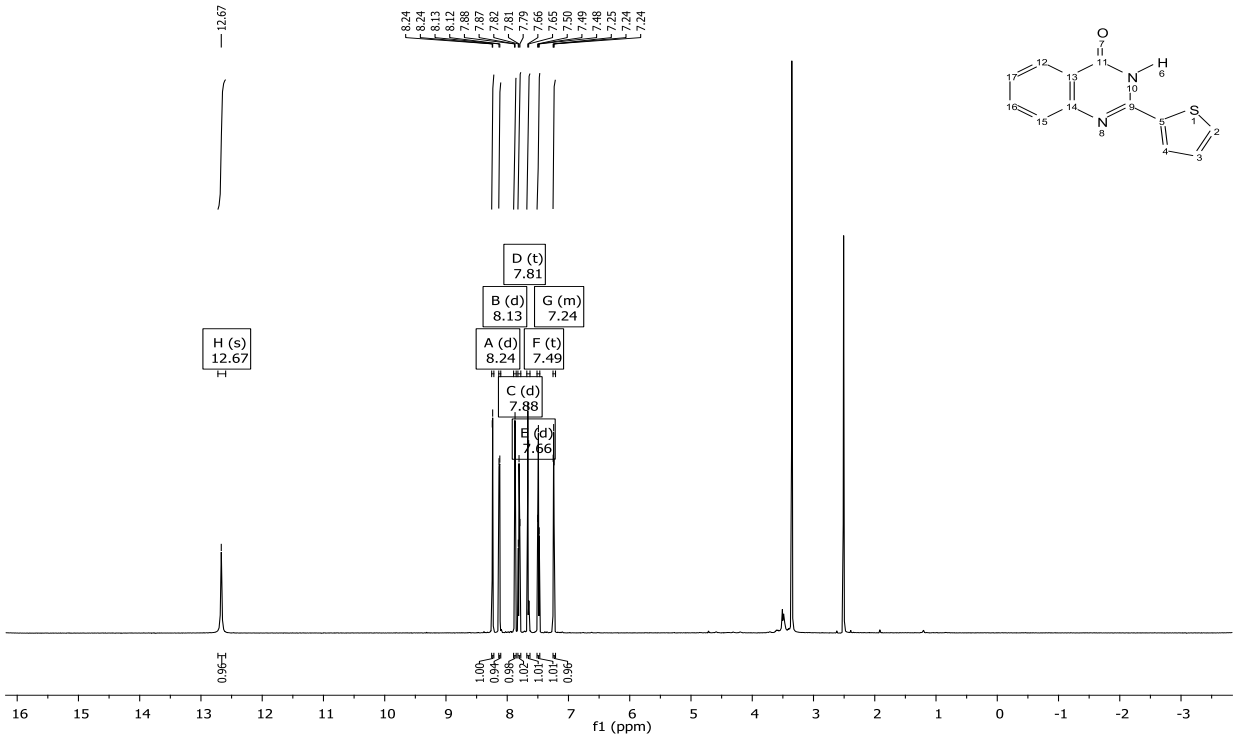
^{1}H NMR (DMSO-d_6): ***2-(furan-2-yl)quinazolin-4(3H)-one (5h):***



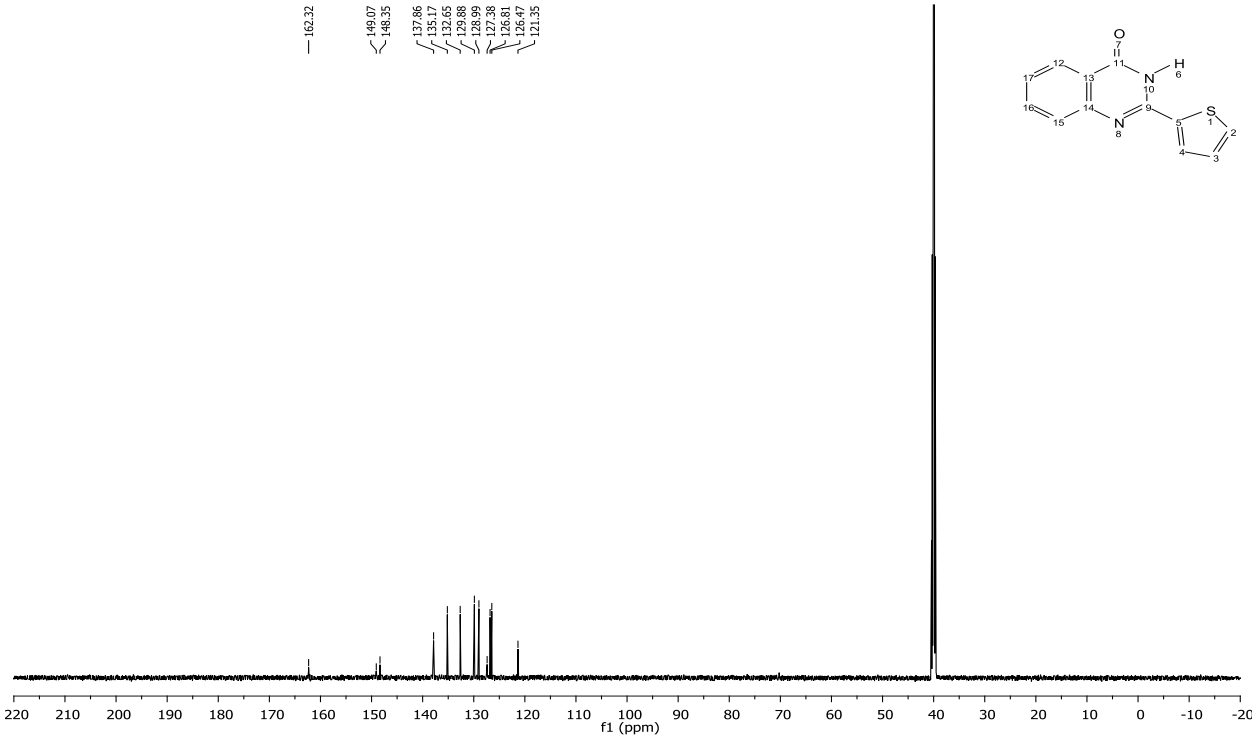
^{13}C NMR (DMSO-d_6): ***2-(furan-2-yl)quinazolin-4(3H)-one (5h)*:**



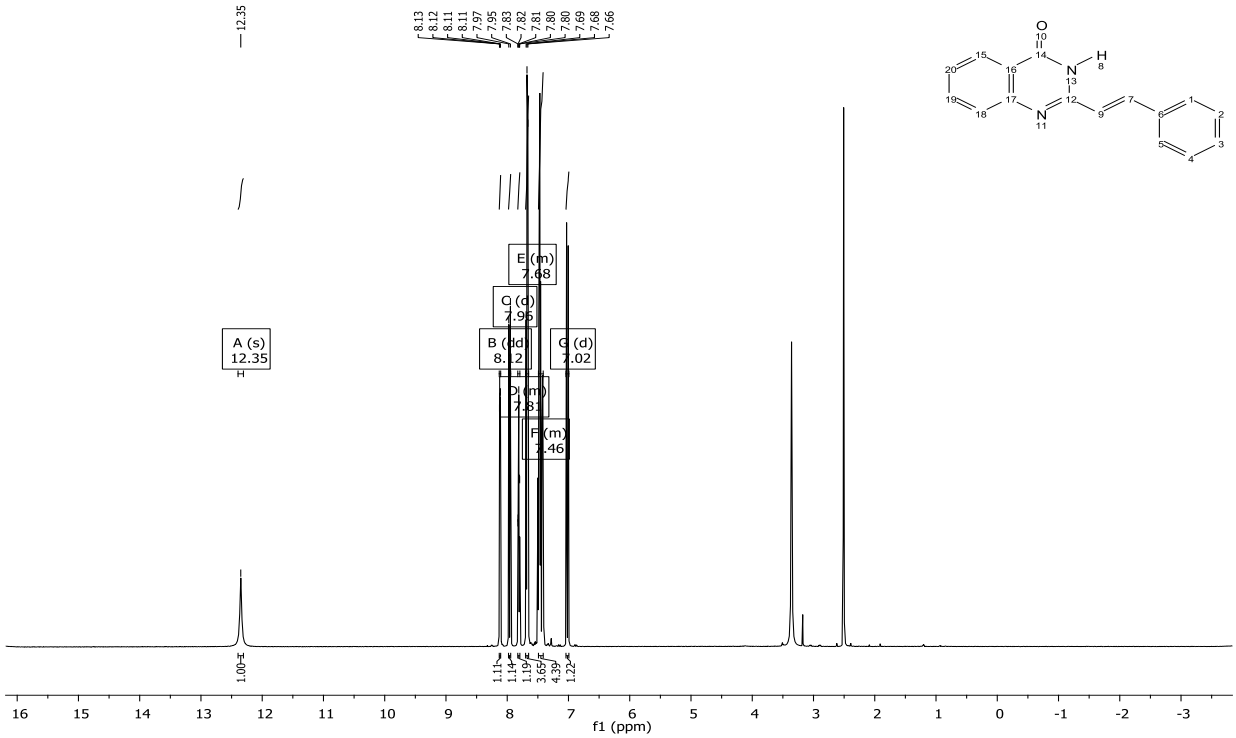
^{1}H NMR (DMSO-d_6): ***2-(thiophen-2-yl)quinazolin-4(3H)-one (5i)*:**



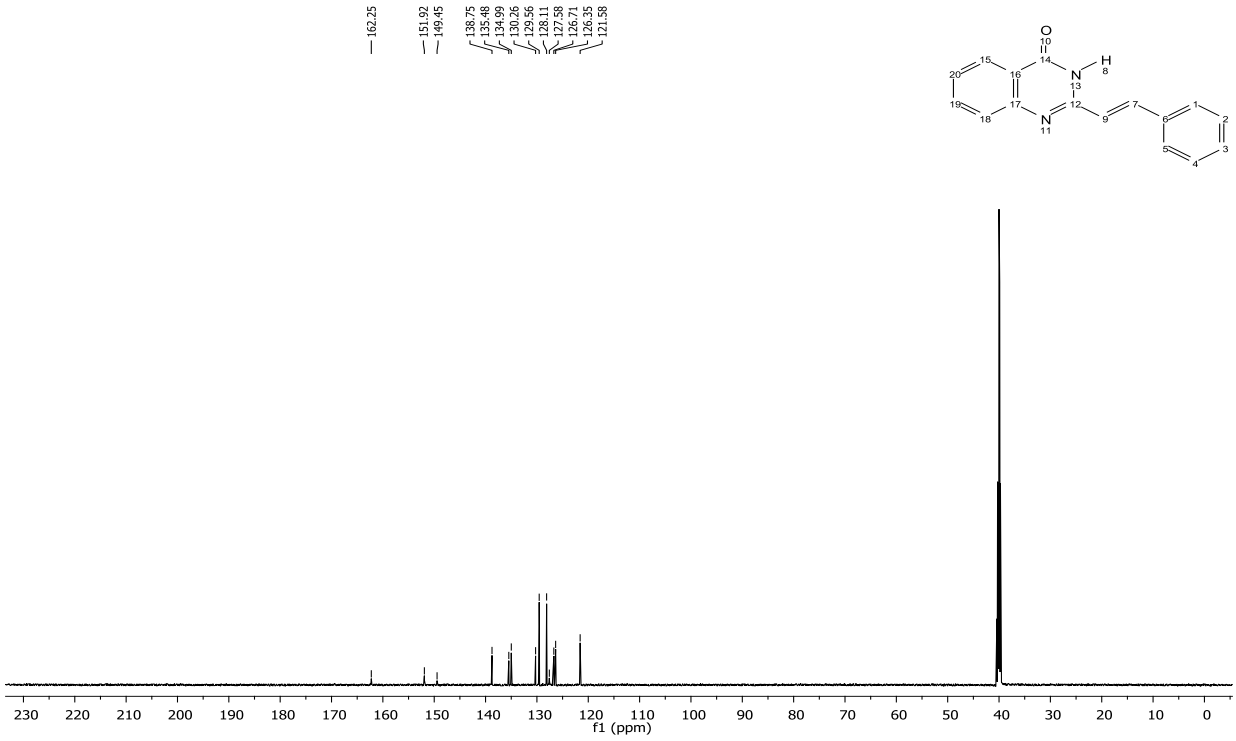
^{13}C NMR (DMSO-d_6): ***2-(thiophen-2-yl)quinazolin-4(3H)-one (5i):***



^{1}H NMR (DMSO-d_6): ***2-[(E)-2-phenylethenyl]quinazolin-4(3H)-one (5j):***

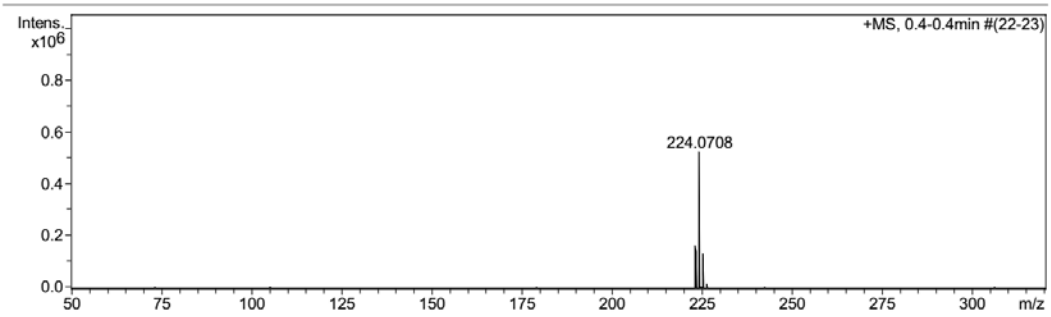


^{13}C NMR (DMSO-d_6): ***2-[(E)-2-phenylethenyl]quinazolin-4(3H)-one (5j):***



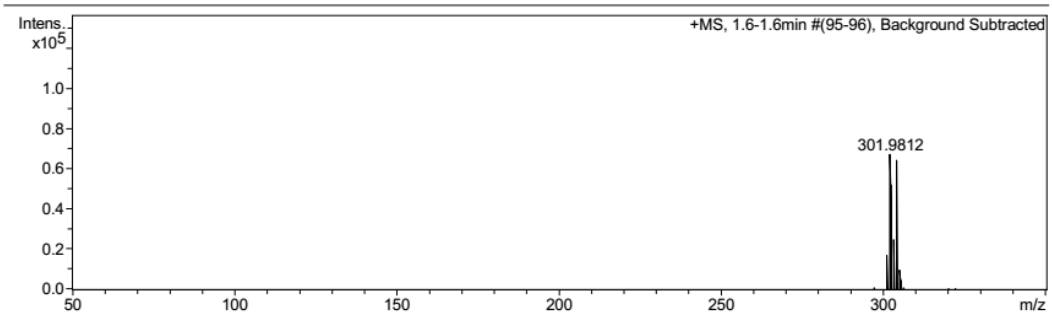
ANNEXURE B- MS DATA OF THE TEST COMPOUNDS (2a, 2d, 2f, 2h 2i, 4 & 5a-j):

2-phenyl-4H-3,1-benzoxazin-4-one (2a):



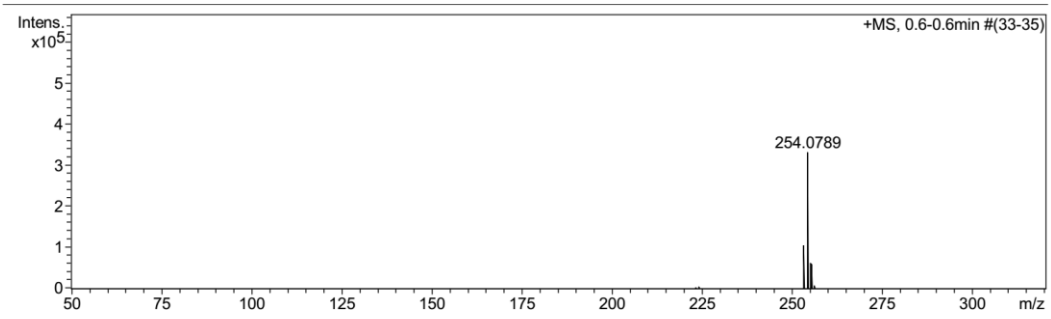
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
224.0708	1	C 14 H 10 N O 2	100.00	224.0706	-0.2	-1.1	55.4	10.5	ok	even
	2	C 12 H 8 N 4 O	38.22	224.0693	-1.6	-7.1	61.9	11.0	ok	odd

2-(4-bromophenyl)-4H-3,1-benzoxazin-4-one (2d):



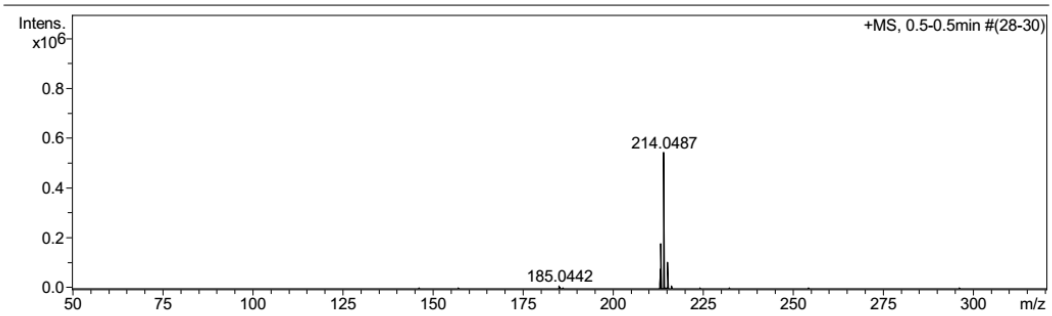
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
301.9812	1	C 14 H 9 Br N O 2	100.00	301.9811	-0.1	-0.3	97.1	10.5	ok	even
	2	C 17 H 2 O 6	0.00	301.9846	3.4	11.2	545.4	17.0	ok	odd

2-(4-methoxyphenyl)-4H-3,1-benzoxazin-4-one (2f)



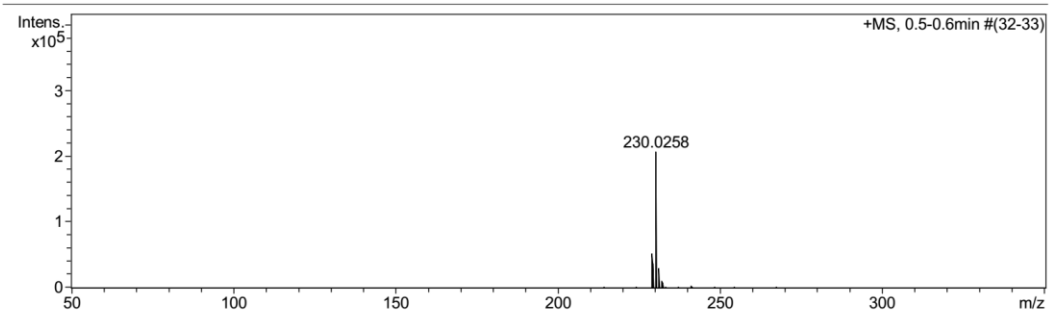
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
254.0789	1	C 15 H 12 N O 3	100.00	254.0812	2.3	8.9	12.7	10.5	ok	even

2-(furan-2-yl)-4H-3,1-benzoxazin-4-one (2h):



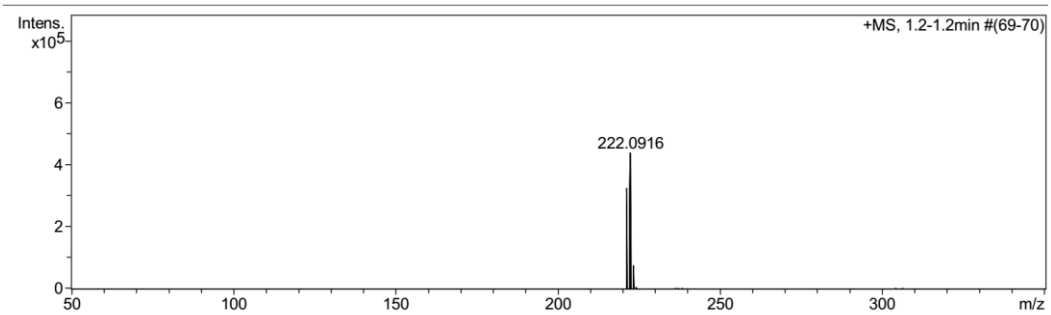
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
214.0487	1	C 12 H 8 N O 3	100.00	214.0499	1.2	5.7	34.3	9.5	ok	even

2-(thiophen-2-yl)-4H-3,1-benzoxazin-4-one (2i):



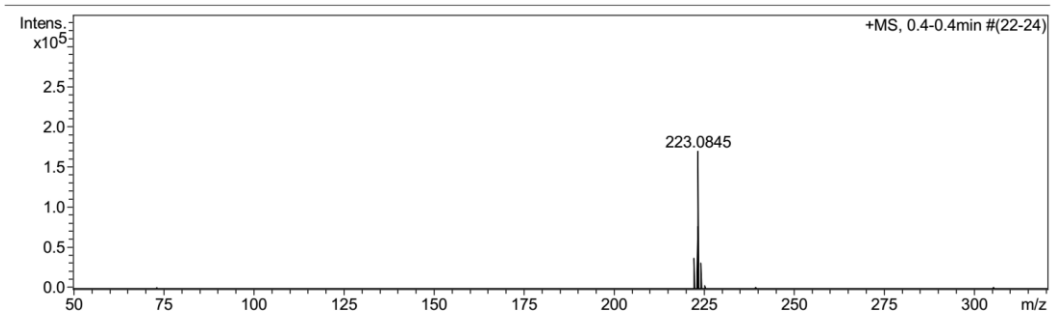
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
230.0258	1	C 12 H 8 N O 2 S	100.00	230.0270	1.3	5.5	3.2	9.5	ok	even
	2	C 15 H 4 N O 2	36.72	230.0237	-2.1	-9.1	24.3	14.5	ok	even

3-phenylisoquinolin-1(2H)-one (4):



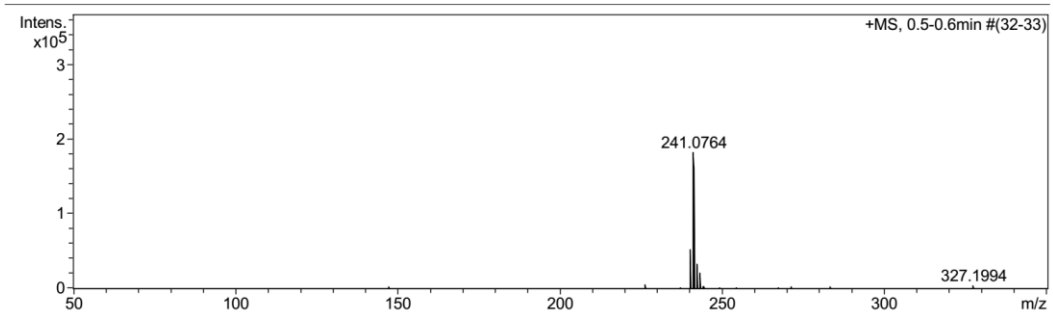
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
222.0916	1	C 15 H 12 N O	100.00	222.0913	-0.3	-1.2	6.1	10.5	ok	even

2-phenylquinazolin-4(3H)-one (5a):



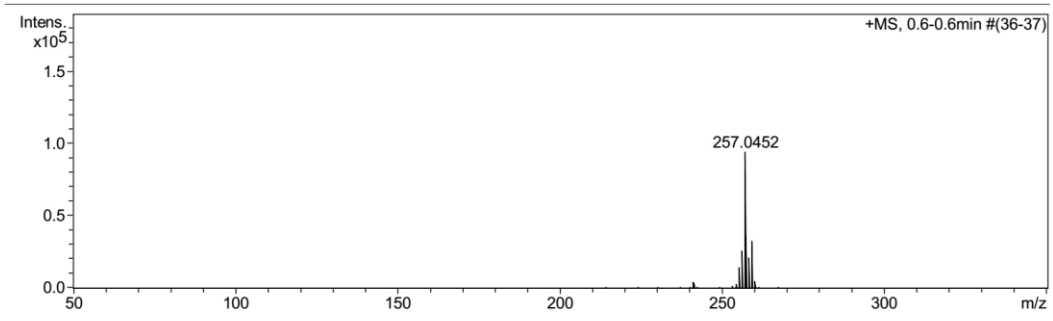
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
223.0845	1	C 14 H 11 N 2 O	46.87	223.0866	2.1	9.6	14.3	10.5	ok	even
	2	C 12 H 9 N 5	100.00	223.0852	0.8	3.5	20.4	11.0	ok	odd
	3	C 11 H 13 N O 4	80.98	223.0839	-0.5	-2.4	34.7	6.0	ok	odd

2-(4-fluorophenyl)quinazolin-4(3H)-one (5b):



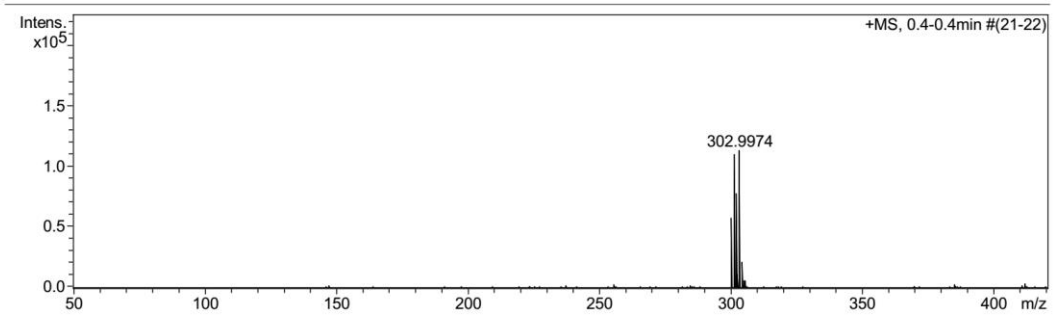
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
241.0764	1	C 17 H 9 N 2	100.00	241.0760	-0.4	-1.7	58.4	14.5	ok	even
	2	C 14 H 10 F N 2 O	74.56	241.0772	0.7	3.0	62.6	10.5	ok	even

2-(4-chlorophenyl)quinazolin-4(3H)-one (5c):



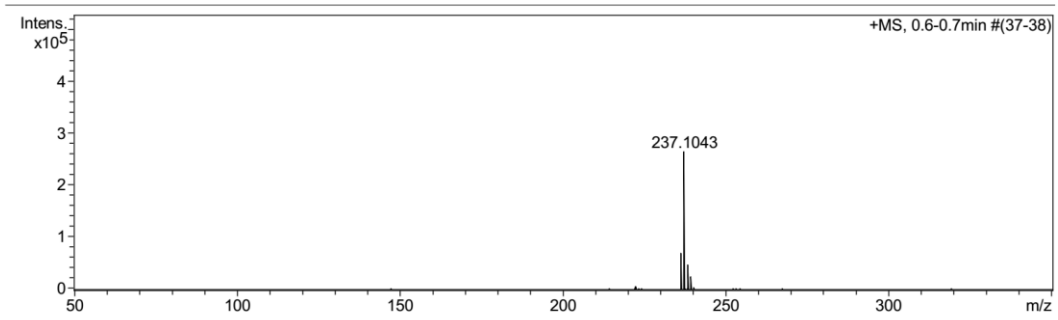
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
257.0452	1	C 14 H 10 Cl N 2 O	100.00	257.0476	2.4	9.5	33.2	10.5	ok	even

2-(4-bromophenyl)quinazolin-4(3H)-one (5d):



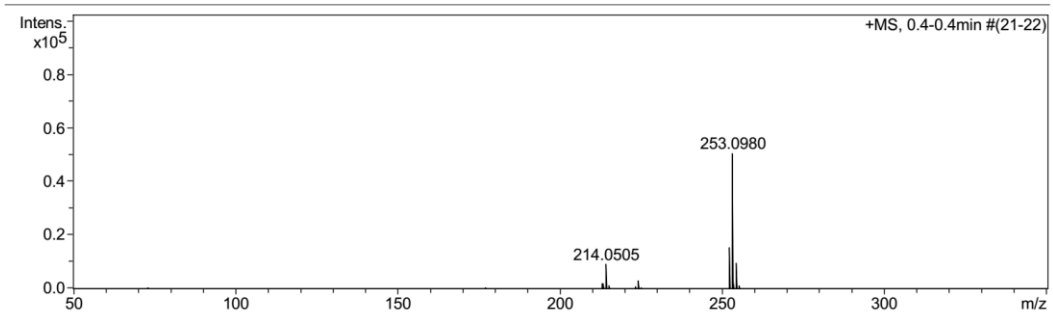
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
299.9917	1	C 14 H 9 Br N 2 O	100.00	299.9893	-2.4	-7.9	590.6	11.0	ok	odd
	2	C 20 N 2 O 2	0.00	299.9954	3.8	12.6	655.7	22.0	ok	odd
300.9987	1	C 14 H 10 Br N 2 O	100.00	300.9971	-1.6	-5.3	236.9	10.5	ok	even

2-(4-methylphenyl)quinazolin-4(3H)-one (5e):



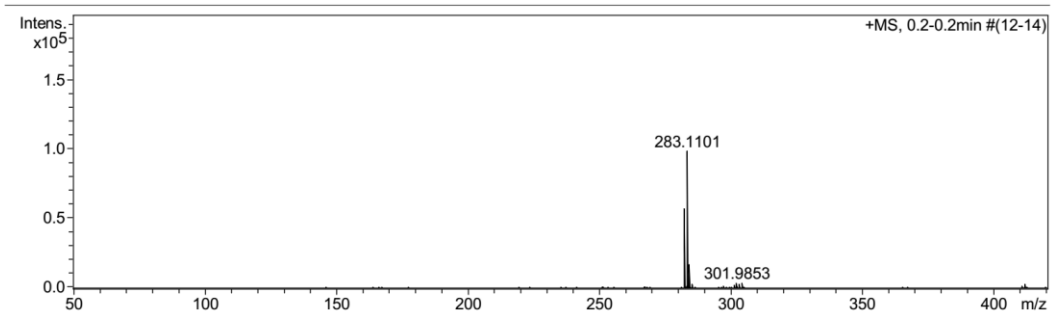
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
237.1043	1	C 15 H 13 N 2 O	100.00	237.1022	-2.1	-8.8	45.0	10.5	ok	even

2-(4-methoxyphenyl)quinazolin-4(3H)-one (5f):



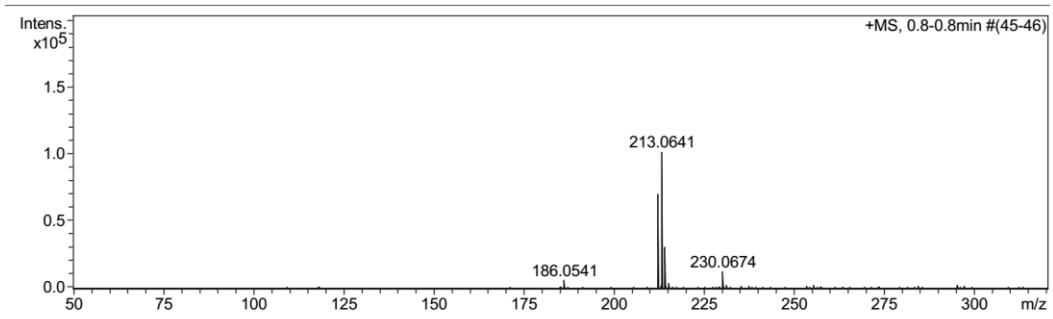
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
253.0980	1	C 15 H 13 N 2 O 2	100.00	253.0972	-0.8	-3.3	12.6	10.5	ok	even
	2	C 20 H 13	16.10	253.1012	3.2	12.5	14.5	14.5	ok	even

2-(3,4-dimethoxyphenyl)quinazolin-4(3H)-one (5g):



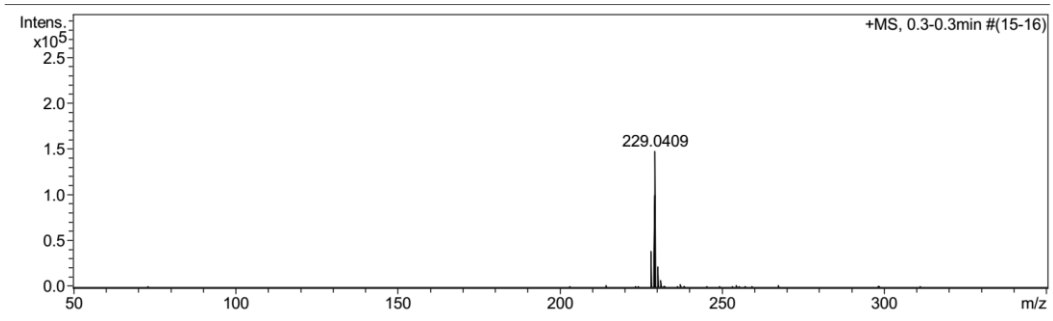
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
282.1028	1	C 21 H 14 O	100.00	282.1039	1.1	4.1	515.7	15.0	ok	odd
	2	C 19 H 12 N 3	51.39	282.1026	-0.2	-0.7	521.2	15.5	ok	even
	3	C 16 H 14 N 2 O 3	0.17	282.0999	-2.9	-10.2	539.5	11.0	ok	odd
283.1101	1	C 16 H 15 N 2 O 3	42.38	283.1077	-2.3	-8.2	6.9	10.5	ok	even
	2	C 19 H 13 N 3	100.00	283.1104	0.3	1.2	26.8	15.0	ok	odd
	3	C 21 H 15 O	40.00	283.1117	1.7	6.0	32.8	14.5	ok	even

***2-(furan-2-yl)quinazolin-4(3H)-one (5h)*:**



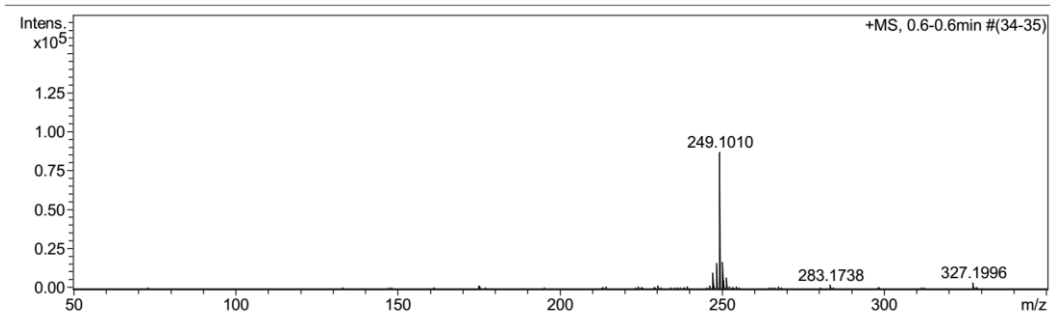
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
213.0641	1	C 12 H 9 N 2 O 2	100.00	213.0659	1.8	8.5	95.6	9.5	ok	even

2-(thiophen-2-yl)quinazolin-4(3H)-one (5i):



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
229.0409	1	C 12 H 9 N 2 O S	86.26	229.0430	2.1	9.2	2.7	9.5	ok	even
	2	C 15 H 5 N 2 O	100.00	229.0396	-1.3	-5.5	24.8	14.5	ok	even

2-[(E)-2-phenylethenyl]quinazolin-4(3H)-one (5j):



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
249.1010	1	C 16 H 13 N 2 O	100.00	249.1022	1.2	5.0	36.4	11.5	ok	even

ANNEXURE C: PROPOSED DRAFT ARTICLE FOR SUBMISSION

C2-substituted quinazolinone derivatives exhibit A_1 and/or A_{2A} adenosine receptor affinities in the low micromolar range

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Abstract

Antagonists of the adenosine receptors (A_1 and A_{2A} subtypes) are widely researched as potential drug candidates for their role in Parkinson's disease-related cognitive deficits (A_1 subtype), motor dysfunction (A_{2A} subtype) and to exhibit neuroprotective properties (A_{2A} subtype). Previously the benzo-α-pyrone based derivative, 3-phenyl-1H-2-benzopyran-1-one, was found to display both A_1 and A_{2A} adenosine receptor affinity in the low micromolar range. Prompted by this, the α-pyrone core was structurally modified to explore related benzoxazinone and quinazolinone homologues previously unknown as adenosine receptor antagonists. Overall, the C2-substituted quinazolinone analogues displayed superior A_1 and A_{2A} adenosine receptor affinity over their C2-substituted benzoxazinone homologues. The benzoxazinones were devoid of A_{2A} adenosine receptor binding, with only two compounds displaying A_1 adenosine receptor affinity. In turn, the quinazolinones displayed varying degrees of affinity (low micromolar range) towards the A_1 and A_{2A} adenosine receptor subtypes. The highest A_1 adenosine receptor affinity and selectivity were favored by methyl *para*-substitution of phenyl ring B (A_1K_i = 2.50 μM). On the other hand, 3,4-dimethoxy substitution of phenyl ring B afforded the best A_{2A} adenosine receptor binding ($A_{2A}K_i$ = 2.81 μM) among the quinazolinones investigated. In conclusion, the quinazolinones are ideal lead compounds for further structural optimization to gain improved adenosine receptor affinity, which may find therapeutic relevance in Parkinson's disease associated cognitive deficits and motor dysfunctions as well as exerting neuroprotective properties.

Keywords

Keywords: benzoxazinone; quinazolinone; benzo-α-pyrone; Adenosine A_{2A} receptor; Adenosine A_1 receptor; antagonist; GTP shift; radioligand binding assay; Parkinson's disease

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Abbreviations: PD; Parkinson's Disease; AR, adenosine receptor; GTP, guanosine triphosphate; [^{3}H]DPCPX, [^{3}H]-dipropyl-8-cyclopentylxanthine; [^{3}H]NECA, N-[^{3}H]-ethyladenosin-5'-uronamide; CPA, N^6-cyclopentyladenosine.

1. Introduction

Parkinson's disease (PD) is a complex neurodegenerative disorder and is characterized by the formation of Lewy bodies and the death of dopaminergic neurons along the nigrostriatal pathway.[1,2] The loss of dopaminergic neurons results in a diminished amount of dopamine present in the corpus striatum.[3] In 2005 the prevalence for PD among individuals living in the United States, aged above 65 to 70 years, was documented as 9.5 per 1000 individuals [4] and it is estimated that between 2005 and 2030 the prevalence will have doubled.[5] Individuals suffering from PD have been found to present with a range of mainly motor features as first described by James Parkinson in 1817.[6,7] Four principal motor features were identified, namely tremors, rigidity, bradykinesia and postural instability.[6] Over time some non-motor symptoms may develop which are typically defined by cognitive impairment.[8]

At present, the treatment of PD still follows a more traditional approach in the sense that current treatment is more symptomatic rather than curative.[9] Symptomatic treatment seeks to replenish dopamine stores or to disrupt the degradation of endogenous dopamine by inhibition of dopamine degrading enzymes.[10] The current treatment with regards to the motor symptoms of PD entails oral preparations of levodopa (L-3,4-dihydroxyphenylalanine) and dopamine receptor agonists as well as deep brain stimulation and apomorphine in severe cases.[11] Although levodopa remains the cornerstone treatment of PD-related motor symptoms,[9] the development of dyskinesia with long-term use,[12] along with the alarming prevalence and the need for neuroprotective and disease-modifying agents, demonstrate the necessity for novel drug development.[1]

In the brain adenosine functions as a neuromodulator and fulfils a physiological role opposite to that of dopamine.[13,14] Four adenosine receptor (AR) subtypes (A_1, A_{2A}, A_{2B} and A_3) exist and of the four subtypes, A_1 and A_{2A} ARs have the highest density in the brain.[15] The A_1 ARs are diffusely spread throughout the brain,[16] and in turn, the A_{2A} ARs are generally more restricted to the dorsal striatum, nucleus accumbens, and olfactory tubercle.[17] Both the A_1 and A_{2A} ARs have been identified as possible targets for drug development in neurological disorders, such as PD,[18] and are currently being examined for their potential benefit in PD.[19] The A_{2A} AR antagonists have been found to enhance motor activity with a reduced risk of developing dyskinesia and other associated side effects, making them viable candidates for the treatment of PD.[20] Furthermore, preclinical data exists demonstrating that A_{2A} AR antagonists may possess neuroprotective potential,[21] where neuroprotective qualities have been displayed by selective A_{2A} AR antagonists, KW-6002, in an MPTP induced dopaminergic toxicity study.[22]

A_1 ARs are important for cognitive function and antagonism of these receptors may improve cognition.[23] A selective A_1 AR antagonist, such as FR-194921, have been examined in animal models and appear to positively modulate memory processes while having a stimulating effect on the central nervous system.[24] Therefore, A_1 AR antagonists are being examined for use as cognitive enhancers in neurological disorders such as dementia, Alzheimer's disease and PD.[25] It was also observed that the blockade of both the A_1 and A_{2A} AR subtypes lead to a synergistic positive motor response, which could be ascribed to the release of dopamine triggered by the antagonism of the A_1 AR with the simultaneous enhancement of the postsynaptic response to dopamine potentiated by antagonism of the A_{2A} AR.[26]

Thus, antagonists of the A_1 and/or A_{2A} AR are thought to exert neuroprotective properties and enhance motor function via A_{2A} AR antagonism, while A_1 AR antagonism improves PD-associated cognitive dysfunction. To date, an ideal drug candidate has not been identified for PD treatment. Therefore, considering the aforementioned potential of AR antagonists as potential PD treatment and the lack of an ideal drug candidate, the need exists to discover high affinity A_1 and/or A_{2A} AR antagonists.

Recently a series of benzopyrone derivatives were evaluated as AR antagonists.[27] The benzo-α-pyrone compound 3-phenyl-1H-2-benzopyran-1-one (**1**), possessed both A_1 and A_{2A} AR affinity (A_1K_i = 7.41 μM; $A_{2A}K_i$ = 3.35 μM) with a selectivity index (SI) of 2 towards the A_{2A} AR subtype (Figure 1; Table 1). Compound **1** consists of a basic benzo-α-pyrone skeleton (ring A and C is fused) with ring C bearing a C3-phenyl substituted side-chain (ring B) (Figure 1, A). The double bond between position C3 and C4 of ring C was found to be imperative for AR binding and the arrangement of the ketone and hetero oxygen in ring C was optimal for A_{2A} AR binding. In analogy to the structure of 3-phenyl-1H-2-benzopyran-1-one (**1**) the present study investigates the A_1 and A_{2A} AR binding properties of structurally related C2-substituted benzoxazinones (**2a**–**j**, **3**), 3-phenyl-isoquinolinone (**4**) and C2-substituted quinazolinones (**5a**–**j**) (Figure 1; Table 1).

The investigated C2-substituted benzoxazinones (**2a**–**j**) possess the basic scaffold of compound **1** with the addition of a hetero nitrogen to ring C (Figure 1, B). This structural modification allows the structure-activity relationship (SAR) exploration of an additional nitrogen to ring C, while retaining compound **1**'s aforementioned double bond (ring C) deemed essential for AR affinity. The benzoxazinones are further assessed by various substitutions on phenyl ring B (**2b**–**g**). The inclusion of compound **3** will provide insight into the necessity of the double bond in ring C of the benzoxazinone scaffold to govern AR binding (Figure 1, B). The comparison of the isoquinolinone derivative (**4**) to compound **1** will

highlight the importance of the hetero oxygen in ring C of the benzo-α-pyrone backbone to favour AR affinity (Figure 1, C). Furthermore, the C2-substituted quinazolinones (**5a–j**) is included to afford further SAR exploration of the AR affinity if ring C bears two hetero nitrogen atoms (Figure 1, D). The effect of other heterocyclic ring systems (e.g. furyl and thiophene) replacing phenyl ring B of the benzoxazinones (**2h–i**) and quinazolinones (**5h–i**), as well as the insertion of a styryl side chain between ring C and B of the benzoxazinones (**2j**) and quinazolinones (**5j**) are additionally evaluated *in vitro*. Lastly, the rearrangement of the ketone and hetero oxygen of compound **1** is investigated by including compounds **6**, and **7**, where this rearrangement was switched/flipped (Figure 2). Table 1 document the A_1 and A_{2A} AR dissociation constant (K_i) values determined via radioligand binding assays and Table 2, in the experimental section, summarize the commercial availability or synthetic approach to obtain the test compounds (**1**, **2a–j**, **3**, **4**, **5a–j**, **6** and **7**).

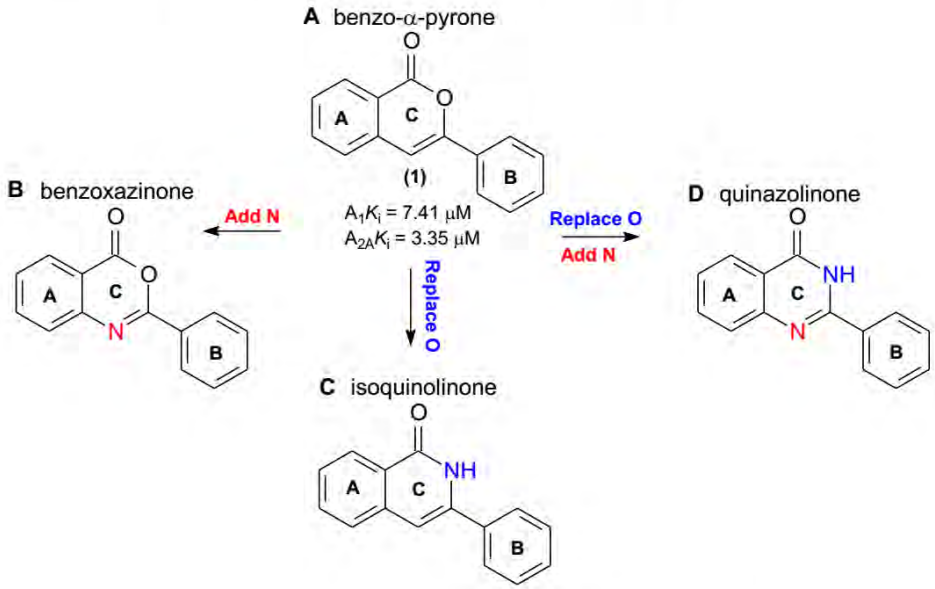


Figure 1: General structures of the various scaffolds explored in the current investigative study: (**A**) benzo-α-pyrone (**1**), (**B**) benzoxazinone (**2**), (**C**) isoquinolinone (**4**) and (**D**) quinazolinones (**5**).

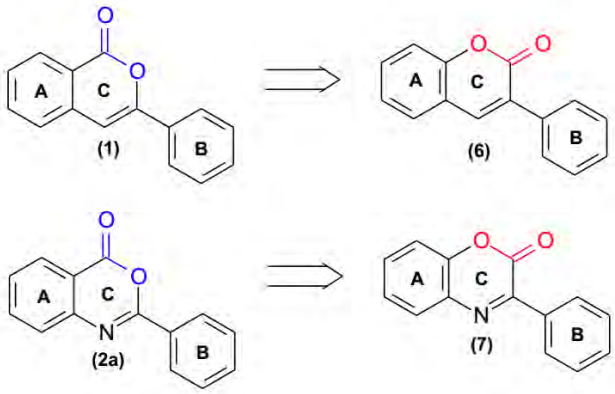


Figure 2: Depicting the rearrangement of the ketone and hetero oxygen of ring C by the benzo-α-pyrone (**1** vs **6**) and the benzoxazinones (**2a** vs **7**).

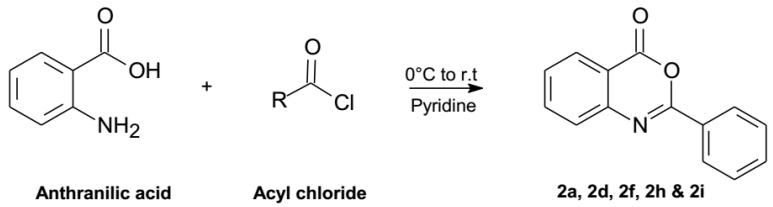
2. Results and discussion

2.1. Chemistry

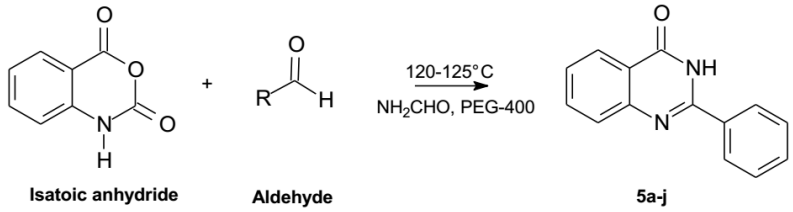
The test compounds were either obtained commercially (**1**, **2b**, **2c**, **2e**, **2g**, **2j**, **3**, **6** and **7**) or synthesized (**2a**, **2d**, **2f**, **2h**, **2i**, **4** and **5a–j**) according to literature procedures. The test compounds obtained from standard commercial sources (Sigma Aldrich) were used without further purification.

The benzoxazinone analogues (**2a**, **2d**, **2f**, **2h** and **2i**) that were not commercially obtained, were synthesized in an alkaline environment with commercially available acyl chlorides and anthranillic acid as key starting materials (Scheme 1).[28]

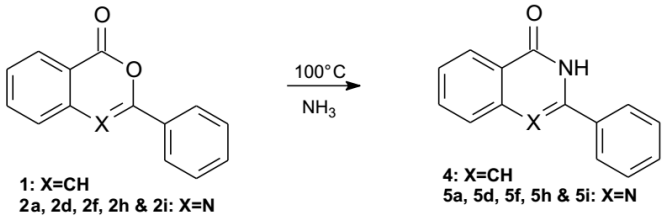
The synthesis of the proposed quinazolinone derivatives (**5a–j**) were obtained by either method A and/or B: (A) a one-pot synthesis with the appropriate aldehyde, formamide and isatoic anhydride (Scheme 2) [29] or (B) where the corresponding benzoxazinone was treated with ammonia (Scheme 3) [30]. The isoquinolinone derivative **4** was synthesized by treating 3-phenyl-1H-2-benzopyran-1-one (**1**) with ammonia — a similar synthetic approach than outlined in method B (Scheme 3). [30] The reaction conditions and compound characterizations are described in the experimental section. Table 2, in section 4.2., provides a summary of the proposed synthesized methods used and the percentage yields obtained. In each instance, the structures of the proposed synthesized compounds (**2a**, **2d**, **2f**, **2h**, **2i**, **4** and **5a–j**) were verified by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry, as cited in the supplementary material.



Scheme 1: A synthetic pathway for C2-substituted benzoxazinone derivatives (**2a**, **2d**, **2f**, **2h** and **2i**).



Scheme 2: A synthetic pathway to the synthesized C2-substituted quinazolinone derivatives (**5a**–**j**), method A.



Scheme 3: A synthetic pathway for the isoquinolinone (**4**, X=CH) and C2-substituted quinazolinone derivatives (**5a**, **5d**, **5f**, **5h** and **5i**, X=N), method B.

The synthesized benzoxazinones (**2a**, **2d**, **2f**, **2h** and **2i**) were obtained in fair yields (32–49%) and purified via recrystallization from ethanol. The synthesized quinazolinones (**5a**–**j**) were prepared either via method A and/or B. Overall, method A resulted in the desired quinazolinones, except for compound **5h** which could not be obtained via method A. In an attempt to synthesize the aforementioned compound (**5h**), method B was employed. Method B also resulted in improved yields compared to method A. For method B the synthesized benzoxazinones (**2a**, **2d**, **2f**, **2h** and **2i**) were utilized as key starting material and the corresponding quinazolinones (**5a**, **5d**, **5f**, **5h** and **5i**) were successfully synthesized in fair to excellent yields (24–95%). In addition, compound **4** was successfully obtained via method B with a yield of 95% (Table 2).

2.2 Radioligand binding assays

The A_1 and A_{2A} AR binding affinities of the test compounds (**1**, **2a**–**j**, **3**, **4**, **5a**–**j**, **6** and **7**) were evaluated *in vitro* with radioligand binding assays described previously.[31] The AR binding

affinities are expressed as the mean K_i values of triplicate determinations and are documented in Table 1.

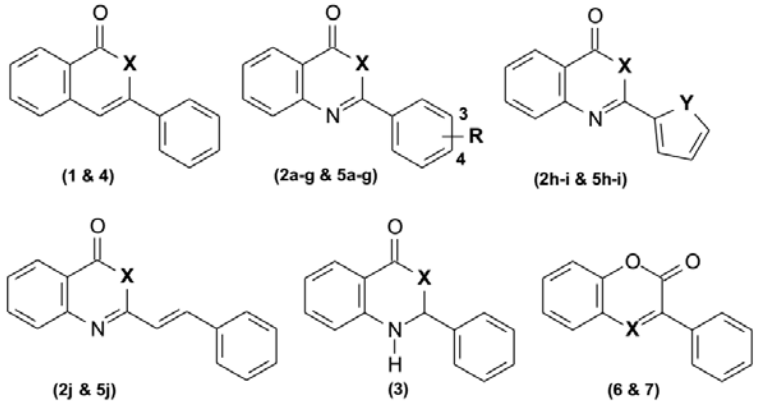
Introducing hetero nitrogen (ring C) to the α-pyrone core of compound **1** afforded the benzoxazinone derivative **2a** (Figure 1, B). The latter compound exhibited a similar A_1 AR affinity as documented for compound **1** (**1** A_1K_i = 7.41 μM vs **2a** A_1K_i = 6.38 μM), whereas the A_{2A} AR binding affinity was diminished (**1** $A_{2A}K_i$ = 3.35 μM vs **2a** $A_{2A}K_i$ = > 100 μM).

The SARs of the benzoxazinone scaffold were further explored at ring B by investigating several *para*-substituents on the phenyl ring (**2b**–**g**) and other heterocyclic ring systems which included a furyl (**2h**) and a thiophene (**2i**) ring. In addition, the insertion of a styryl side chain (**2j**) between ring C and phenyl ring B was also investigated. Compared to **2a**, p*ara*-substitution (**2b**–**g**) of phenyl ring B with F (**2b**), Cl (**2c**), Br (**2d**), CH_3 (**2e**), OCH_3 (**2f**) and OCH_2CH_3 (**2g**) diminished A_1 AR affinity, while A_{2A} AR activity remained elusive. The furyl compound (**2h**) and insertion of a styryl side chain (**2j**) showed no A_1 or A_{2A} AR affinity. Thus, the C2-substituted benzoxazinones (**2a**–**j**) were generally found to be devoid of A_1 and A_{2A} AR affinity. However, the unsubstituted phenyl ring B (**2a**) and the thiophene ring B (**2i**) were the only benzoxazinones investigated to exhibited A_1 AR affinity with K_i values of 6.38 μM and 32.4 μM, respectively.

Upon further investigation, ring C of compound **2a** was saturated (absence of the double bond) to yield compound **3**. This structural modification led to a slight improvement of A_1 AR affinity compared to **2a** (**2a** A_1K_i = 6.38 μM vs **3** A_1K_i = 5.06 μM), but affinity at the A_{2A} AR subtype remained absent (Figure 1, B; Table 1).

Further structural modification to ring C included the replacement of the hetero oxygen of compound **1** with hetero nitrogen to yield compound **4** (Figure 1, C). Both the A_1 and A_{2A} AR affinity were diminished, emphasizing the preference of the benzo-α-pyrone scaffold (**1**) over the isoquinolinone moiety (**4**) for enhanced A_1 and A_{2A} AR activity.

Table 1 Dissociation constant values (K_i values) for the binding of the test compounds to rat A_1 and A_{2A} adenosine receptors.



Compd		R	Y	K_i ± SEM (μM)[a] (% displacement)[b] A_1[c] vs [^{3}H]DPCPX	A_{2A}[c] vs [^{3}H]NECA	A_1[c] + GTP[d] vs [^{3}H]DPCPX	GTP shift[e]	SI[f] (A_{2A}/A_1)
Benzo-α-pyrone scaffold								
1	-O	-	-	7.41 ± 0.4[a,g]	3.35 ± 0.80[a,g]	6.49 ± 0.82[a,g]	0.9[g]	0.5[g]
Benzoxazinone scaffold								
2a	-O	-H	-	6.38 ± 0.40[a]	> 100 (49%)[b]	6.92 ± 1.16[a]	1.09	-
2b	-O	-4-F	-	> 100 (100%)[b]	> 100 (70%)[b]			-
2c	-O	-4-Cl	-	> 100 (86%)[b]	> 100 (81%)[b]			-
2d	-O	-4-Br	-	> 100 (95%)[b]	> 100 (92%)[b]			-
2e	-O	-4-CH_3	-	> 100 (84%)[b]	> 100 (77%)[b]			-
2f	-O	-4-OCH_3	-	> 100 (54%)[b]	> 100 (37%)[b]			-
2g	-O	-4-OCH_2CH_3	-	> 100 (99%)[b]	> 100 (77%)[b]			-
2h	-O	-	-O	> 100 (42%)[b]	> 100 (66%)[b]			-
2i	-O	-	-S	32.4 ± 0.58[a]	> 100 (59%)[b]			-
2j	-O	-	-	> 100 (56%)[b]	> 100 (85%)[b]			-
3	-O	-	-	5.06 ± 0.46[a]	> 100 (69%)[b]	5.72 ± 0.56[a]	1.12	-
Isoquinolinone scaffold								
4	-NH	-	-	>100 (100%)[b]	> 100 (100%)[b]			-
Quinazolinone scaffold								
5a	-NH	-H	-	3.67 ± 0.02[a]	18.7 ± 1.99[a]	3.77 ± 0.28[a]	1.02	5.09
5b	-NH	-4-F	-	> 100 (50%)[b]	> 100 (62%)[b]			-
5c	-NH	-4-Cl	-	> 100 (54%)[b]	> 100 (82%)[b]			-
5d	-NH	-4-Br	-	> 100 (98%)[b]	> 100 (84%)[b]			-
5e	-NH	-4-CH_3	-	2.50 ± 0.47[a]	> 100 (55%)[b]	2.95 ± 0.18[a]	1.18	-
5f	-NH	-4-OCH_3	-	> 100 (44%)[b]	> 100 (79%)[b]			-
5g	-NH	-3,4-OCH_3		> 100 (67%)[b]	2.81 ± 0.40[a]			-
5h	-NH	-	-O	4.62 ± 0.63[a]	8.11 ± 0.03[a]	5.89 ± 0.42	1.23	1.76
5i	-NH	-	-S	> 100 (39%)[b]	> 100 (30%)[b]			-
5j	-NH	-	-	> 100 (100%)[b]	> 100 (56%)[b]			-
Coumarin scaffold								
6	-CH	-	-	> 100 (51%)[b,g]	> 100 (77%)[b,g]			-
Benzoxazinone scaffold								
7	-N	-	-	17.3 ± 1.9[a]	> 100 (91%)[b]			-
Reference compounds								
CPA (A_1 agonist)				0.0051 ± 0.0002 (0.0079)[h]; (0.0059)[i]	0.557 ± 0.024 (0.460)[h]	29.5 ± 1.14 (35.2)[i]	5.8 (6)[i]	5784
ZM-241385 (A_{2A} antagonist)				0.420 ± 0.01 (0.225)[j]	0.0013 ± 0.00002 (0.002)[j]	0.510 ± 0.03	1.2	0.003

[a] All K_i values determined in triplicate and expressed as mean ± SEM; [b] Percentage displacement of the radioligand at a maximum tested concentration (100 μM); [c] Rat receptors were used (A_1: rat whole brain membranes; A_{2A}: rat striatal membranes); [d] GTP shift assay, where the 100 μM GTP was added to the A_1 AR radioligand binding assay; [e] GTP shifts calculated by dividing the K_i in the presence of GTP by the K_i in the absence of GTP; [f] Selectivity index (SI) for the A_1 AR isoform calculated as the ratio of K_i (A_{2A})/K_i (A_1); [g]Literature value obtained from reference.[31];[h] Literature value obtained from reference.[32]; [i] Literature value obtained from reference.[33]; [j] Literature value obtained from reference.[34]

Based on the aforementioned finding that the hetero oxygen (ring C) of the benzo-α-pyrone derivative **1** is essential for AR activity, the structurally related benzoxazinone (**2a**) and quinazolinone (**5a**) homologues were compared. It was found that replacing the hetero oxygen (ring C) of **2a** with hetero nitrogen resulted in **5a** (A_1K_i = 3.67 μM; $A_{2A}K_i$ = 18.7 μM) which exhibited superior A_1 and A_{2A} AR affinity over its benzoxazinone counterpart **2a** (A_1K_i = 6.38 μM; $A_{2A}K_i$ >100 μM). The latter observation (**2a** vs **5a**) is contrary to the above finding (**1** vs **4**). Thus, replacement of compound **1**'s hetero oxygen of ring C (benzo-α-pyrone) with hetero nitrogen (**5a**) in addition to a second hetero nitrogen led to improved A_1 AR affinity and reduced A_{2A} AR binding (Figure 1, D). The improved AR affinity of **5a** compared to **2a** may be ascribed, in part, to ring C containing two hetero nitrogens (**5a**) compared to one (**2a**). The latter observation is supported by the research of Gillespie and colleagues.[35,36] In the study by Gillespie and co-workers, they compared the binding affinities of pyridine, pyrimidine and triazine to each other and found that two nitrogens in the heterocyclic ring are optimum to enhance both the A_1 and A_{2A} AR binding.[35,36] The latter statement was based on their finding that the binding affinity of the pyridine scaffold was seven-fold less potent than the triazine and 45-fold less potent than the corresponding aminopyrimidine. [35,36]

Furthermore, unlike compounds **5a** and **1,** the benzoxazinone derivative **2a** only possessed affinity toward the A_1 AR. Noteworthy, an approximate two-fold A_1 AR affinity improvement was documented for **5a** compared to compounds **1** and **2a**. On the other hand, compound **1** displayed a six-fold higher A_{2A} AR binding than **5a**.

Since compound **5a** favored both A_1 and A_{2A} AR affinity (A_1K_i = 3.67 μM; $A_{2A}K_i$ = 18.7 μM), further SAR of the quinazolinones was explored (**5a**–**j**) at ring B. Several *para*-substituents on the phenyl ring (**5b**–**g**) and other heterocyclic ring systems which included a furyl (**5h**) and a thiophene (**5i**) ring were investigated. In addition, the insertion of a styryl side chain (**5j**) between ring C and phenyl ring B was also explored. Halogen substitution (**5b**–**d**) exhibited no affinity towards either the A_1 or A_{2A} AR subtypes. In contrast, *para*-substitution with a CH_3 (**5e**) group exerted a favorable effect on A_1 AR affinity with a K_i value of 2.50 μM. This structural modification resulted in the highest A_1 AR activity documented for the current study. Interestingly, simultaneous *meta*- and *para*-substitution of ring B with a methoxy group led to compound **5g** with gained A_{2A} AR affinity ($A_{2A}K_i$ = 2.81 μM), whereas mono-substitution with an OCH_3 in the *para*-position (**5f**) led to diminished A_{2A} AR affinity. Noteworthy, compound **5g** displayed the best A_{2A} AR affinity amongst the test compounds. Surprisingly the C2-substituted furyl ring (**5h**) exhibited both A_1 and A_{2A} AR affinity in the low micromolar range (A_1K_i = 4.62 μM; $A_{2A}K_i$ = 8.11 μM).

In a previous study rearrangement of the ketone and hetero oxygen in ring C of compound **1** afforded compound **6** with diminished AR affinity of both AR subtypes.[27] Based on the latter finding, compound **2a** was directly compared to compound **7**. This structural modification resulted in a three-fold decrease in A_1 AR activity (**2a** A_1K_i = 6.38 μM vs **7** A_1K_i = 17.3 μM), while A_{2A} AR affinity remained elusive. Generally, the optimal arrangement of the ketone and hetero oxygen of ring C to favor A_1 AR affinity is exhibited by the benzo-α-pyrone (**1**) and benzoxazinone (**2a**) backbones.

2.3 GTP shift assay

Previously the benzo-α-pyrone based derivative **1** was found to act as an A_1 AR antagonist.[27] In analogy to compound **1**, the agonistic or antagonistic activity at the A_1 AR subtype for compounds **2a**, **3**, **5a**, **5e** and **5h** were explored by performing GTP shift experiments as described in the literature.[37] In the case of an A_1 AR agonist, a rightward shift of the binding curve in the presence of GTP (due to an uncoupling of the A_1 AR from its G_i protein) is expected. On the other hand, an A_1 AR antagonist is anticipated not to shift the binding curve significantly in the presence of GTP.[31,38] As expected, compounds **2a**, **3**, **5a**, **5e** and **5h** was not documented with a significant shift of the binding curve in the presence of GTP, thus, these compounds may be considered antagonists of the A_1 AR (Table 1; Figure 3).

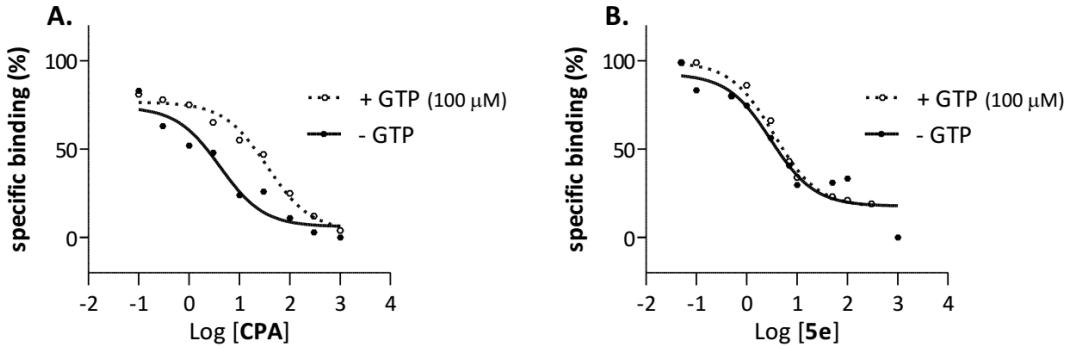


Figure 3: The binding curves of the reference compound CPA and **5e** are examples of A_1 AR agonistic and antagonistic action, respectively. The functionality was determined via GTP shift assays (with and without 100 μM GTP) in rat whole brain membranes expressing A_1 ARs with [^{3}H]DPCPX as radioligand. (**A**) A GTP shift of 5.8 was calculated for CPA and (**B**) a GTP shift of 1.18 was calculated for compound **5e**.

3. Conclusion

In conclusion, the newly proposed structural modifications of the benzo-α-pyrone based derivative 3-phenyl-1H-2-benzopyran-1-one (**1**) resulted in varying degrees of affinity and selectivity towards the A_1 and A_{2A} AR subtypes. The A_1 AR binding was improved two-fold after replacing the α-pyrone core (ring C) of compound **1** with a pyrimidone ring to afford the quinazolinone derivative **5a** (**1** A_1K_i = 7.41 μM vs **5a** A_1K_i = 3.67 μM). However, replacement of the α-pyrone core (ring C) with an oxazinone afforded the benzoxazinone derivative **2a** which only showed a slight A_1 AR affinity improvement (**1** A_1K_i = 7.41 μM vs **2a** A_1K_i = 6.38 μM). Generally the A_{2A} AR binding was best governed by ring C as the α-pyrone (**1** $A_{2A}K_i$ = 3.35 μM) compared to oxazinone (**2a** $A_{2A}K_i$ = > 100 μM) and pyrimidone (**5a** $A_{2A}K_i$ = 18.7 μM).

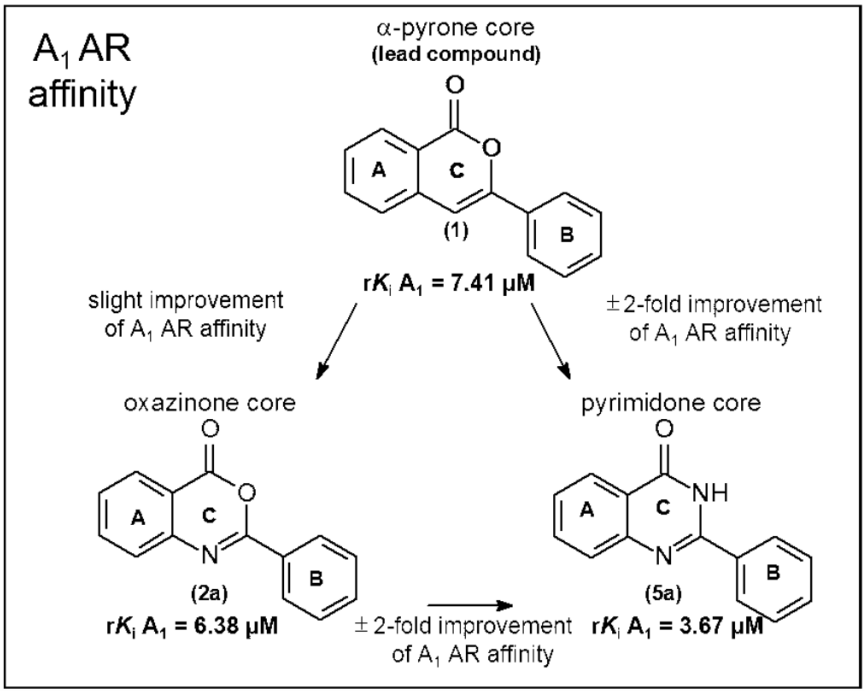


Figure 4: Structural changes to the α-pyrone core resulted in the pyrimidone core and oxazinone core. The pyrimidone core (**5a**) exhibited improved A_1 AR affinity in analogy to the oxazinone core (**2a**).

Noteworthy, the benzoxazinone and quinazolinone based scaffolds were previously unknown to exhibit AR affinity and that analogues of these moieties may act as A_1 AR antagonists. Generally, the C2-substituted quinazolinone derivatives displayed superior A_1 and A_{2A} AR affinity over the C2-substituted benzoxazinone derivatives. Among the test compounds, the quinazolinone analogue **5a** exhibited the second-highest A_1 AR binding (A_1K_i = 3.67 μM). The introduction of a CH_3 group (*para*-position) to ring B of **5a** afforded

compound **5e** (A_1K_i = 2.50 µM)) with the highest A_1 AR affinity among the compounds investigated. Furthermore, **5e** was found to be selective for the A_1 AR subtype. The 3,4-dimethoxy substituted quinazolinone **5g** exhibited the highest A_{2A} AR affinity and selectivity ($A_{2A}K_i$ = 2.81 µM). Thus, **5g** is an example of a selective A_{2A} AR drug and may find therapeutic relevance to enhance PD-related motor dysfunction and exert neuroprotective properties, while **5e** may enhance cognitive dysfunction associated with PD. The identified drug candidates (**5a**, **5e** and **5g**) are ideal for future *in vivo* examinations as selective A_1 (**5a**, **5e**) and A_{2A} (**5g**) AR antagonists in the treatment of neurological disorders.

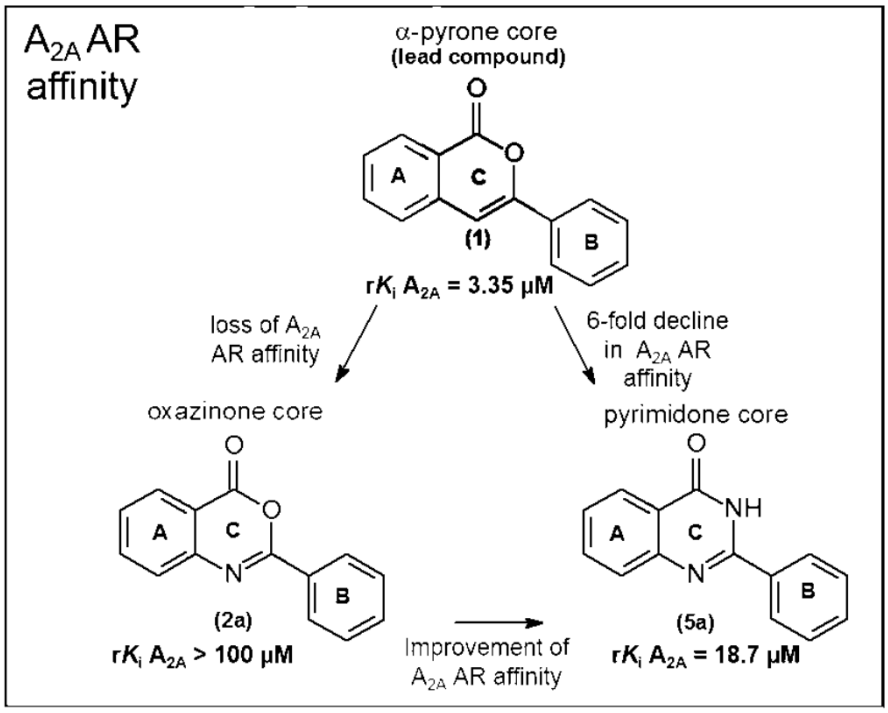


Figure 5: Structural changes to the α-pyrone core resulted in the pyrimidone core and oxazinone core. The pyrimidone core (**5a**) exhibited improved A_{2A} AR affinity in analogy to the oxazinone core (**2a**).

4. Experimental section

4.1. Chemistry

4.1.1. Materials and reagents: All starting materials, reagents and solvents were purchased from Sigma-Aldrich and were used without further purification. The structures of the synthesised test compounds (**2a**, **2d**, **2f**, **2h**, **2i**, **4** and **5a**–**j**) were elucidated by proton (^{1}H) and carbon (^{13}C)-NMR spectra and recorded on a A Bruker Avance III 600 spectrometer instrument at frequencies of 600 MHz and 150 MHz, for ^{1}H and ^{13}C NMR, respectively. The samples for NMR were dissolved in either deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO-d_6) depending on compound solubility. The chemical shifts are expressed

in parts per million (δ) relative to the signal of tetramethylsilane and coupling constants J in hertz (Hz). The residual solvent signal of $CDCl_3$ was documented at 7.26 and 77.2 ppm for ^1H and ^{13}C spectra, respectively. In turn, the DMSO-d_6 residual solvent signal was found at 2.50 ppm for ^1H and 39.5 ppm for ^{13}C NMR spectra. Spin multiplicities were given as singlet (s), doublet (d), doublet of doublets (dd), triplet (t) or multiplet (m). Furthermore, high-resolution mass spectra (HRMS) were recorded with a BrukermicrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionization (APCI) mode. Melting points (mp) were measured with a Buchi M-545 melting point apparatus and are uncorrected.

4.2. Synthetic approach

*4.2.1. Approach for the synthesis of the benzoxazinones derivatives (***2a**, **2d**, **2f**, **2h** and **2i***)*: An adapted version of the method described by Khan and co-workers (2014) was used for the synthesis of the desired benzoxazinones. A solution of anthranilic acid (10 mmol) in pyridine (30 mL) was obtained and cooled to a temperature of 0°C in an ice bath. The appropriate acyl chloride (20 mmol) was gradually added to the above reaction mixture and was stirred for 5 min. At this point the ice bath was removed in order for the reaction mixture to warm to room temperature. Upon reaching room temperature the reaction mixture was continuously stirred for an additional 30 minutes and poured onto ice (200 mL) to remove pyridine. The precipitate was filtered and flushed with 200 mL ice water and recrystallized from ethanol. Completion of the reaction was monitored by TLC (Scheme 1; Table 2).

*4.2.2. Approach for the synthesis of the quinazolinones (***5a**–**j***) via method A*: A modified method described by Rao and co-workers [29] (method A) was used to synthesize the desired quinazolinone analogues. Equal parts of the appropriate aldehyde (10 mmol), formamide (10 mmol) and isatoic anhydride (10 mmol) were added to PEG-400 (4mL) and stirred at 120-125°C until completion. The reaction progress was monitored by TLC and upon completion left to cool to room temperature. Subsequently, ethyl acetate (10mL) was added and left overnight. The resulting precipitate was filtered and recrystallized from methanol (Scheme 2; Table 2).

*4.2.3. Approach for the synthesis of the isoquinolinone (***4***) and quinazolinones via method B*: Method B was utilized to synthesize the quinazolinone derivatives (**5a**, **5d**, **5f**, **5h** and **5i**). Method B is a modified procedure described by Asundaria and co-workers [30] The appropriate benzoxazinones synthesized at 4.2.1 (10 mmol) was suspended in liquid ammonia (30mL) and heated to a temperature of 100°C for 4h. The ammonia was reduced and the resulting precipitate filtered and dried. The crude products were subsequently recrystallized from methanol. The reaction progress was monitored by TLC (Scheme 3; Table 2).

Table 2 Test compounds that were obtained either commercially or via organic synthesis.

Comp	X	R	Y	Benzoxazinones	Quinazolinones	
				Method Scheme 1 Yield %[a]	Method A Scheme 2 Yield %[a,]	Method B Scheme 3 Yield %[a]
1	-O	-	-	-	-	-
2a	-O	-H	-	49%[a,b] [c]	-	-
2b	-O	-4-F	-	-	-	-
2c	-O	-4-Cl	-	-	-	-
2d	-O	-4-Br	-	32%[a,b]	-	-
2e	-O	-4-CH_3	-	-	-	-
2f	-O	-4-OCH_3	-	48%[a,b]	-	-
2g	-O	-4-OCH_2CH_3	-	-	-	-
2h	-O	-	-O	47%[a,b]	-	-
2i	-O	-	-S	37%[a,b]	-	-
2j	-O	-	-	-	-	-
3	-O	-	-	-	-	-
4	-NH	-	-	-	-	95%[a,b]
5a	-NH	-H	-	-	35%[a,b]	28%[a,b]
5b	-NH	-4-F	-	-	3%[a,b]	-
5c	-NH	-4-Cl	-	-	5%[a,b]	-
5d	-NH	-4-Br	-	-	3%[a,b]	22%[a]
5e	-NH	-4-CH_3	-	-	4%[a,b]	-
5f	-NH	-4-OCH_3	-	-	11%[a,b]	27%[a]
5g	-NH	-3,4-OCH_3	-	-	9%[a,b]	-
5h	-NH	-	-O	-	n a[c]	24%[a,b]
5i	-NH	-	-S	-	14%[a]	95%[a,b]
5j	-NH	-	-	-	6%[a,b]	-
6	-CH	-	-	-	-	-
7	-N	-	-	-	-	-

[a] Isolated yields after purification. Commercially available compounds were used without further purification; [b] Test compound used for biological evaluation (radioligand binding assays).; [c] The indicated method did not yield the desired compound.

4.3. Radioligand binding assay

4.3.1. Materials and reagents: The commercially available reagents were obtained from various manufacturers. The radioligand [^{3}H]NECA (specific activity 25 Ci/mmol) and filter-count were procured from PerkinElmer, while the [^{3}H]DPCPX (specific activity 120 Ci/mmol) was obtained from Amersham Biosciences and Whatman GF/B 25 mm diameter filters from Merck. Radio activity was calculated by a Packard Tri-CARB 2810 TR liquid scintillation counter.

4.3.2 A_1 and A_{2A} AR radioligand binding assays: The Research Ethics Committee of North-West University approved the collection of tissue samples for the A_1 and A_{2A} AR radioligand binding assays (application number NWU-0035-10-A5). The previously reported A_1 and A_{2A} AR radioligand binding assay protocols were used to determine the A_1 and A_{2A} AR binding affinities.[37] Briefly, the A_1 AR radioligand binding assay was performed with rat whole brain membranes in the presence of the radioligand [^{3}H]-8-cylcopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX).[37] In turn, the A_{2A} AR affinity was measured at rat striatal membranes with 5'-N-[^{3}H]-ethylcarboxamideadenosine ([^{3}H]NECA) as radioligand.[37] The A_1 AR agonist, N^6-cyclopentyladenosine (CPA), was added to the A_{2A} AR competition experiments to minimize the binding of the radioligand [^{3}H]NECA to the A_1 ARs.[37,39] Non-specific binding of [^{3}H]DPCPX and [^{3}H]NECA was defined as binding in the presence of 100 µM CPA.[37,39] On the other hand, specific binding was defined as the total binding minus the non-specific binding [37,39]. The protein content of all membrane preparations was determined with Bradford reagent according to the method described by Bradford.[40]

4.*3.3. GTP shift assay:* Compounds **2a**, **3**, **5a**, **5e** and **5h** were explored via GTP ([^{35}S]guasine-5'-O-(3-thio)triphosphate) shift assays to determine these compounds' agonistic or antagonistic functionality towards the A_1 AR subtype by the compounds investigated. The GTP shift assays were performed as described in the literature with rat whole brain membranes and [^{3}H]DPCPX (0.1 nM; K_d = 0.36 nM) in the absence and presence of 100 µM GTP (Table 1; Figure 3).[37] The non-specific binding was defined by the addition of 10 µM DPCPX (unlabelled).[37]

4.3.4 Data analysis: The data analysis of the test and reference compounds was performed as follow: the IC_{50} values were determined by plotting the specific binding vs. the logarithm of the test compounds' concentrations to obtain a sigmoidal dose-response curve via the Prism software package (GraphPad Software Inc.).[37] The K_i values for the competitive inhibition of [^{3}H]DPCPX (0.1 nM; K_d = 0.36 nM) [32] or [^{3}H]NECA (4 nM; K_d = 15.3 nM) [39] by

the test compounds were calculated from the IC_{50} values. Furthermore, the calculated K_i values are expressed as a mean ± standard error of the mean (SEM) after triplicate determinations.[37] GTP shifts were calculated by dividing the K_i value of a compound reported in the presence of GTP by the corresponding value obtained in the absence of GTP. [37] A compound with a calculated GTP shift of approximately 1 is considered an antagonist; the presence of GTP affects the competition curves of an agonist and shifts the curve to the right.[33,37]

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ANNEXURE F- ETHICAL APPROVAL DOCUMENTS:

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

The Applicant / Primary Investigator

Faculty of Health Sciences
Ethics Office for Research, Training and Support
Animal Care, Health and Safety in Research
Ethics Committee (AnimCare)

Tel: 018 299 2234
Fax: 018 299 2225
Email: Tiaan.Brink@nwu.ac.za

28 April 2017

Dear Prof Terre'Blanche

APPROVAL OF YOUR APPLICATION BY THE ANIMAL CARE, HEALTH AND SAFETY IN RESEARCH ETHICS COMMITTEE (ANIMCARE) OF THE FACULTY OF HEALTH SCIENCES

Ethics Number: NWU-00261-17-A5

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the AnimCare secretariat.

Study Title:	Evaluation of selected 2-substituted benzoxazinone and quinazolinone derivatives as adenosine A1/A2A receptor antagonists
Study leader/Supervisor:	Prof G Terre'Blanche
Student:	L Pieterse
Application type:	New Application - Category 0

Project Category *(impact on animal wellbeing)*	**Not applicable**	**0**	**1**	**2**	**3**	**4**	**5**
		X					

The abovementioned application has been through the expedited review process and discussed by the AnimCare, Animal Research Ethics Committee Potchefstroom Campus, North-West University, Potchefstroom. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years when extension will be facilitated during the monitoring process

The commencement date for this study is **28th of April 2017** dependent on fulfilling the conditions indicated below. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years when extension will be facilitated during the monitoring process.

After ethical review

The AnimCare, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the AnimCare, Faculty of Health Sciences prior to implementing these

changes. Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form sent to Ethics-AnimCareIncident-SAE@nwu.ac.za

A monitoring report should be submitted within one year of approval of this study (or as otherwise stipulated) and before the year has expired, to ensure timely renewal of the study. A final report must be provided at completion of the study or the AnimCare committee, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-Monitoring@nwu.ac.za.

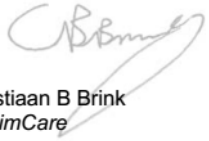
The AnimCare, Faculty of Health Sciences has the authority and responsibility to initially approve and subsequently monitor animal activities to confirm on-going compliance with and adherence to the approved protocol in terms of section 5.2.7 of the SANS 10386:2008.The AnimCare, Faculty of Health Sciences reserves the right to visit sites where approved protocols will be conducted and any animal housing facility under the authority of NWU as often as it deem necessary either announced or unannounced.

Please note that for any permits/permission must still be obtained from relevant authorities and provided to the AnimCare, Faculty of Health Sciences. Ethics approval is required BEFORE approval can be obtained from these authorities.

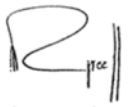
The AnimCare Committee, Faculty of Health Sciences complies with the South African National Health Act 61 (2003), the Regulations on Research with Human Participants (2014), the Ethics in Health Research: Principles, Structures and Processes (2015), the SANS 10386:2008 document, the Belmont Report and the Declaration of Helsinki (2013).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimCare@nwu.ac.za.

Yours sincerely



Prof Christiaan B Brink
Chair: AnimCare



Prof Minrie Greeff
Head: Ethics Office

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Institutional Research Ethics Regulatory Committee
Tel: +27 18 299 4849
Email : Ethics@nwu.ac.za

ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by **AnimCare Animal Research Ethics Committee (AREC-130913-015)** on **28/04/2017,** the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby **approves** your study as indicated below. This implies that the NWU-IRERC grants its permission that provided the special 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: Evaluation of selected 2-substituted benzoxazinone and quinazolinone derivatives as adenosine A1/A2A receptor antagonists.

Study Leader/Supervisor: **Prof G Terre'Blanche**
Student: **L Pieterse**

Ethics number: N W U - 0 0 2 6 1 - 1 7 - A 5

Application Type: New Application - Category 0

Commencement date: 2017-04-28 **Category:** 0

Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years.

Special conditions of the approval (if applicable):

- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the AnimCare. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via AnimCare:*
  - *annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *Annually a number of studies may be randomly selected for an external audit.*
- *The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the AnimCare, prior to implementation. Would there be deviated from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility the NWU-IRERC and AnimCare retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the AnimCare or that information has been false or misrepresented,*
    - *the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *AnimCare can be contacted for further information or any report templates via Ethics-AnimCare@nwu.ac.za or 018 299 2197.*

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the IRERC or AnimCare for any further enquiries or requests for assistance.

Yours sincerely

Prof LA Du Plessis

Digitally signed by Prof LA Du Plessis
Date: 2017.05.03 07:49:22 +02'00'

Prof Linda du Plessis
Chair NWU Institutional Research Ethics Regulatory Committee (IRERC)

ACKNOWLEDGEMENTS:

I would like to express my heartfelt gratitude towards the following persons and organisations for making this dissertation possible:

- My heavenly Father for the passion and every other blessing and endowment that made this dissertation possible.
- Prof. Gisella Terre'Blance and Dr. Dalene van der Walt for the diligent guidance, patience, encouragement and kind nurture of their students.
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- Miss. Sharlene Lowe for the help provided during the assays and Mrs. Anriëtte Pretorius for her assistance in locating necessary reference articles.
- My fellow MSc students, who made the road a little easier and certainly a whole lot more fun.
- Lastly, to my family, especially my parents. Without their support and encouragement throughout my life, I never would have made it this far.