

Chapter 4: Biological evaluation

4.1. Introduction

In this chapter the biological screening of the synthesised 2-aminopyrimidines will be discussed. Biological evaluation included determination of affinities for both the adenosine A₁ and A_{2A} receptor subtypes, determination of cell viability and *in vivo* screening of selected compounds. Rationalisation of results, using a QSAR and a molecular docking study is also included.

4.2. Results and Discussion

4.2.1. Radioligand binding study

The affinities of the synthesised compounds for adenosine A₁ and A_{2A} receptors were determined using standard radioligand binding assays (Bruns *et al.*, 1986, Bruns *et al.*, 1987, Van der Walt *et al.*, 2013). It is important to note that while tissue from male Sprague Dawley rats were used as receptor source in the A₁ assays, tissue from female Sprague Dawley rats were used during A_{2A} assays (this was due to the unavailability of male rats during certain stages of this project). This was not expected to be a problem, since the use of mixed sex tissue is indicated in the published method (Bruns *et al.*, 1986). However, to verify that the use of female rat tissue would give comparable results to male rat tissue, a control study was undertaken. The test compounds used were the known adenosine A_{2A} receptor antagonist ZM 241385 and N⁶-cyclopentyladenosine (CPA), a known adenosine A₁ receptor agonist.

As indicated in Figure 4.1 and Figure 4.2, similar results were obtained with female and male tissue. For the adenosine A_{2A} assays, a K_i value of 2.31 nM was obtained using male striata, while a similar K_i of 2.78 nM was obtained using female striata (Figure 4.1). In the adenosine A₁ assays, similar K_i values of 10.4 and 11.2 nM were also obtained using male and female whole brains, respectively (Figure 4.2).

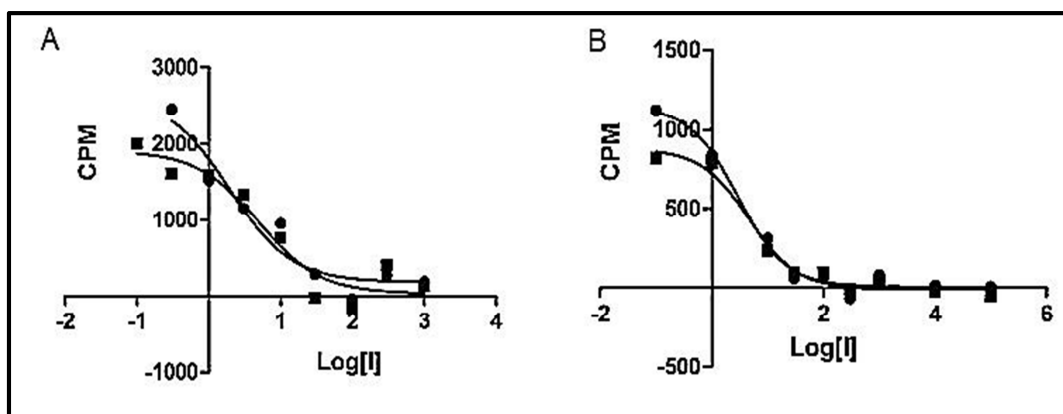


Figure 4.1: **A)** The sigmoidal dose-response curve illustrating adenosine A_{2A} affinity of ZM 241385 using striata from male Sprague Dawley rats with a K_i value of 2.31 ± 1.30 nM and **B)** The sigmoidal dose-response curve illustrating adenosine A_{2A} affinity of ZM 241385 using striata from female Sprague Dawley rats with a K_i value of 2.78 ± 0.73 nM

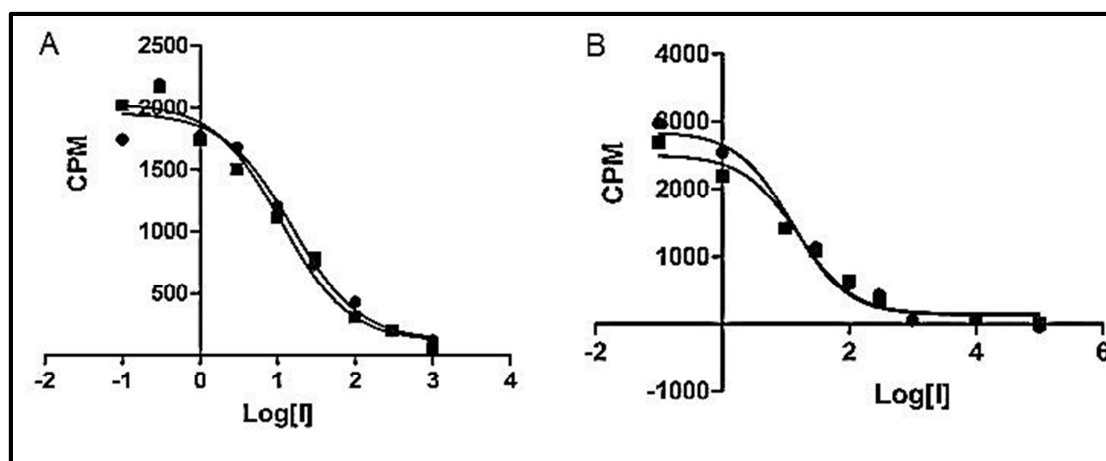
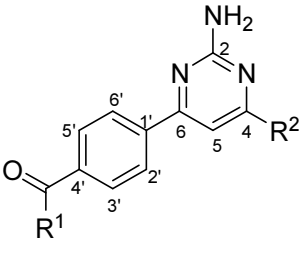
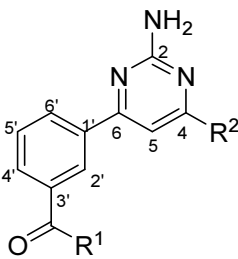


Figure 4.2: **A)** The sigmoidal dose-response curve illustrating adenosine A_1 affinity of CPA using whole brains from male Sprague Dawley rats with a K_i value of 10.4 ± 1.57 nM and **B)** The sigmoidal dose-response curve illustrating adenosine A_1 affinity of CPA using whole brains from female Sprague Dawley rats with a K_i value of 11.2 ± 1.29 nM.

These values also correlated well with the literature K_i values reported for ZM 245381 of 2 nM for the adenosine A_{2A} receptor (Müller and Ferré, 2007) and CPA of 7.9nM for the adenosine A_1 receptor (Bruns *et al.*, 1987).

The results obtained in the radioligand binding assays for the synthesised compounds are presented as K_i values in Table 4.1.

Table 4.1: Affinities of the synthesised 2-aminopyrimidines and reference compounds (CPA and ZM 241385) for the adenosine A_{2A} and A₁ receptor subtypes:

 39a, 39l  39b - 39k, 39m - 39n					
	R ¹	R ²	^{a,b} A _{2A} K _i (nM)	^{a,c} A ₁ K _i (nM)	^d SI (A ₁ /A _{2A})
39a	1-piperidiny	2-(5-methylfuranyl)	15479 ± 2033	2035 ± 30.1	0.13
39l	1,3-benzothiazol-2-aminyl	2-(5-methylfuranyl)	143460 ± 12076	61.1 ± 2.53	0.0004
39b	1-piperidiny	2-pyridiny	7.70 ± 0.65	16.5 ± 0.92	2.04
39c	1-piperidiny	phenyl	4.03 ± 0.45	1.03 ± 0.08	0.25
39d	1-piperidiny	2-furanyl	4.20 ± 0.64	8.39 ± 1.23	1.91
39e	1-piperidiny	4-fluorophenyl	15.0 ± 0.78	2.76 ± 0.27	0.18
39f	1-piperidiny	2-(5-methylthienyl)	0.49 ± 0.21	2.34 ± 0.26	4.57
39g	1-piperidiny	2-benzofuranyl	51.9 ± 1.29	11.8 ± 2.71	0.22
39h	4-ethyl-1-piperaziny	2-(5-methylthienyl)	28.1 ± 0.92	17.9 ± 1.14	0.61
39i	morpholiny	2-(5-methylthienyl)	20.4 ± 8.07	7.09 ± 1.23	0.33
39j	pyrrolidiny	2-(5-methylthienyl)	110 ± 0.52	17.5 ± 3.24	0.15
39k	4-ethyl-1-piperaziny	2-furanyl	10.2 ± 0.07	133 ± 6.14	12.5
39m	1,3-benzothiazol-2-aminyl	2-(5-methylfuranyl)	50217 ± 4685	435 ± 36.4	0.0008
39n	6-chloro-1,3-benzothiazol-2-aminyl	2-(5-methylfuranyl)	28811 ± 48.3	298 ± 20.9	0.01
	ZM 241385		2.78 ± 0.73	-	-
	CPA		-	10.4 ± 1.57	-

^a All values expressed as the mean ± SD of duplicate determinations.

^b Affinities determined using female rat tissue

^c Affinities determined using male rat tissue

^d SI = selectivity index, calculated as follows: A_{2A}K_i/ A₁K_i

Firstly, when considering the results as presented in Table 4.1, it was clear that substitution in the 4' position (e.g. **39a**) with simple cyclic amines, such as piperidine, resulted in a loss of both adenosine A₁ and A_{2A} affinity. These results were not completely unexpected, as similar results were obtained by Shook and co-workers (2010b) in a series of analogous arylindenopyrimidines, where affinities decreased for derivatives substituted on the “equivalent” 7 position (Figure 4.3). In contrast, although the 4' thiazole substituted derivative (**39l**) had negligible A_{2A} affinity, it surprisingly, exhibited good A₁ affinity and selectivity.

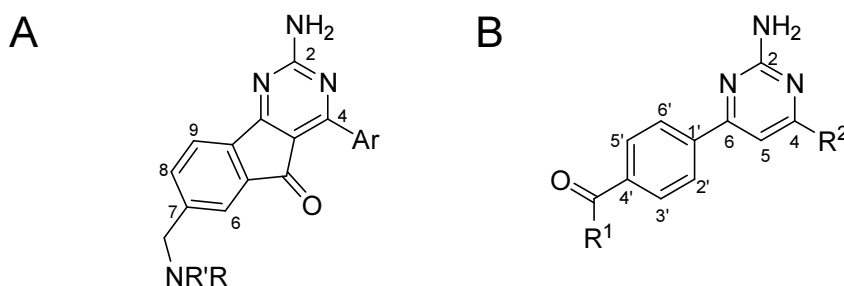


Figure 4.3: **A)** General structure of the series of arylindenopyrimidines substituted on position 7, displaying poor affinity for the adenosine A₁ and A_{2A} receptors. **B)** General structure of the 2-aminopyrimidines substituted in position 4' that demonstrated poor affinity for the adenosine receptors.

Variation of the aromatic moiety on the 4 position (**39b** - **39g**) resulted in several high affinity derivatives, with the 5-methylthiophene derivative (**39f**) in particular, exhibiting excellent dual affinity. Compound (**39f**) also showed the highest adenosine A_{2A} affinity, which is in contrast to literature where furan substitution generally results in optimal A_{2A} affinity (Shook *et al.*, 2010b; Müller and Ferré, 2007; Matasi *et al.*, 2005). Substitution with a benzofuran group in particular was detrimental to A_{2A} binding. The effect of the heteroaryl/aryl substituent on A_{2A} affinity can be summarised in declining order as follows: 5-methylthiophene > phenyl > furan > pyridine > *p*-fluorophenyl > benzofuran while the effect on A₁ receptor affinity can be summarised (in declining order) as follows: phenyl > 5-methylthiophene > *p*-fluorophenyl > furan > benzofuran > pyridine.

The affinities as exhibited by the methylthiophene derivatives **39f**, **39h** – **39j**, further showed that while piperidine substitution (**39f**) resulted in optimal A_{2A} and A₁ affinity, pyrrolidine substitution (**39j**) was less favourable. The order of decreasing affinity (for both the adenosine A_{2A} and A₁ receptors) for the amine substituent can be summarised as follows: piperidine > morpholine > 4-ethyl-1-piperazine > pyrrolidine. This same trend was observed in a previous study (Robinson, 2013) and also holds true when comparing the furan derivatives **39d** and **39k**. Interestingly, the combination of a furan ring (R²) with the

ethylpiperazine (R¹) amine substituent resulted in the most selective compound, **39k**, with a selectivity index (SI) of 12.5.

Incorporation of a thiazole moiety in the phenylamide side chain (**39l** – **39m**) generally resulted in poor adenosine A₁ and A_{2A} receptor affinity. Affinity for the adenosine A₁ receptor was generally higher, and these compounds are thus selective for the adenosine A₁ receptor. As previously mentioned, compound **39l** was the highest affinity derivative identified in the thiazole series, showing moderate affinity (61.1 nM) for the adenosine A₁ receptor. This compound is also 2348 times more selective for the A₁ receptor than for the A_{2A} receptor. The high affinity of this derivative for adenosine A₁ receptors requires further investigation, as these results were rather unexpected. According to literature, an increase in planarity could increase affinity for the A₁ receptor and the high degree of planarity observed in **39l** could contribute to this unexpected result (Daly *et al.*, 1990; Jiang *et al.*, 1996).

4.2.2 QSAR and Molecular modelling

In order to rationalise results obtained in the radioligand binding assays both QSAR and preliminary molecular docking studies were done. Since there are currently no adenosine A₁ receptor crystal structures available, QSAR and docking studies were only performed using adenosine A_{2A} receptor affinity data.

QSAR

The physicochemical parameters selected for the QSAR study and the calculated values are presented in Table 4.2.

Table 4.2: Calculated physicochemical parameters of synthesised aminopyrimidines:

Compound	Log $K_i(A_{2A})$	Log P	Polarizability ($\text{\AA}^2 \cdot \text{s}^4 \cdot \text{kg}^{-1}$)	Molecular mass (amu)	Molecular surface area ($\text{\AA}^2$)	Polar surface area ($\text{\AA}^2$)
39a	4.19	3.37	41.55	362.17	530.81	85.25
39b	0.89	3.28	42.04	359.18	518.73	85.00
39c	0.61	4.11	42.97	358.18	526.07	72.11
39d	0.62	3.17	39.78	348.16	498.67	85.25
39e	1.18	4.26	42.53	376.17	533.19	72.11
39f	-0.31	4.54	43.33	378.49	538.41	100.35
39g	1.72	4.19	47.36	398.17	562.21	85.25
39h	1.45	3.89	46.70	407.18	593.45	103.59
39i	1.83	3.47	42.16	380.13	523.09	109.58
39j	2.04	4.09	41.49	364.14	509.63	100.35
39k	1.01	2.53	43.07	377.19	556.56	88.49
39l	5.16	5.14	48.16	427.11	542.69	135.17
39m	4.70	5.14	48.16	427.11	543.21	135.17
39n	4.46	5.75	50.06	461.07	559.59	85.25
SJR1*	1.43	2.31	40.38	364.15	516.69	94.48
SJR2*	1.17	2.37	43.07	377.19	556.56	88.49
SJR3*	1.45	2.73	44.91	391.20	586.27	88.49
SJR4*	0.76	3.37	41.55	362.17	531.28	85.25
SJR5*	1.72	2.93	39.70	348.16	502.85	85.25

*Aminopyrimidines synthesised in a previous study (Robinson, 2013) (Structures provided in Appendix, p. 190).

A significant correlation was only observed between one of the five selected physicochemical parameters (LogP, polar surface area, molecular weight, polarizability, molecular surface area) and the affinity of the aminopyrimidines for the adenosine A_{2A} receptor (log K_i). This was the polar surface area, which showed a correlation with log K_i with a R^2 value of 0.71 (Figure 4.4). The statistical F value for this correlation was found to be 41.96, which is higher than the $F_{\max}$ value (9.68), required for 95% significance (a higher F value indicates a better fit). The addition of a second parameter did not result in a better fit and therefore polar surface area was the only identifiable factor contributing to the affinity of

test compounds in the selected group of physiochemical properties. The equation describing this correlation is:

$$\text{Log } K_i = m(\pm\text{sd})\text{PSA} + c(\pm\text{sd})$$

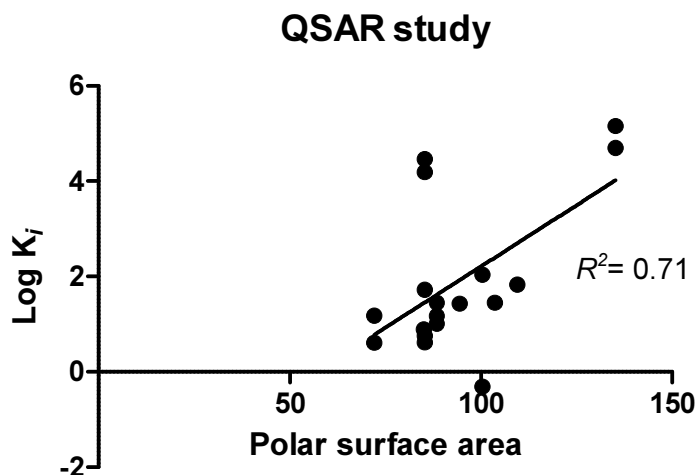


Figure 4.4: The correlation between the $\log K_i$ of the synthesised 2-aminopyrimidines and their calculated polar surface area. $R^2 = 0.71$

According to the equation, it is apparent that an increase in polar surface area of the aminopyrimidines leads to a decreased affinity for the A_{2A} receptor. The large polar surface area of the thiazoles, can thus at least in part, be used to explain the loss of affinity observed for these derivatives.

Molecular modelling

The fourteen 2-aminopyrimidines synthesised during this study were all docked successfully into the adenosine A_{2A} receptor active site. Compounds **39f** and **39c**, docked in the active site, are given as examples in Figure 4.5. This suggests that all the compounds fit into the active site and provides some validation for the selection of these compounds as potential adenosine receptor antagonists.

For all docked compounds, except **39a** and **39i** (which are substituted at position 4'), the exocyclic amino group of the aminopyrimidine ring is orientated towards the amino acid residues Asn 253 and Glu 169 and hydrogen bonds to either or both of these (Figure 4.5, Figure 4.6). Additional hydrogen bonding interactions between the carbonyl oxygen and Tyr 271 were also observed for some derivatives (Figure 4.6B). The tricyclic ring system of the 2-aminopyrimidines exhibited aromatic pi-stacking interactions with Phe 168, similar to those observed for the bicyclic triazolotriazine core of the reference compound, ZM 241385, in the crystal structure (See paragraph 2.3.3). The heteroaryl or aryl substituents of all compounds

(R^2) were less than 3 Å away from the highly conserved Trp 246 residue (Figure 4.6), and it is thus hypothesised that the receptor is forced into an inactive state, since movement of this residue will be restricted (Jaakola *et al.*, 2008a; Piirainen *et al.*, 2011). Similar interactions with residues in the adenosine A_{2A} receptor active site were thus observed with the synthesised aminopyrimidines with A_{2A} affinity and ZM 241385, as obtained during crystallisation.

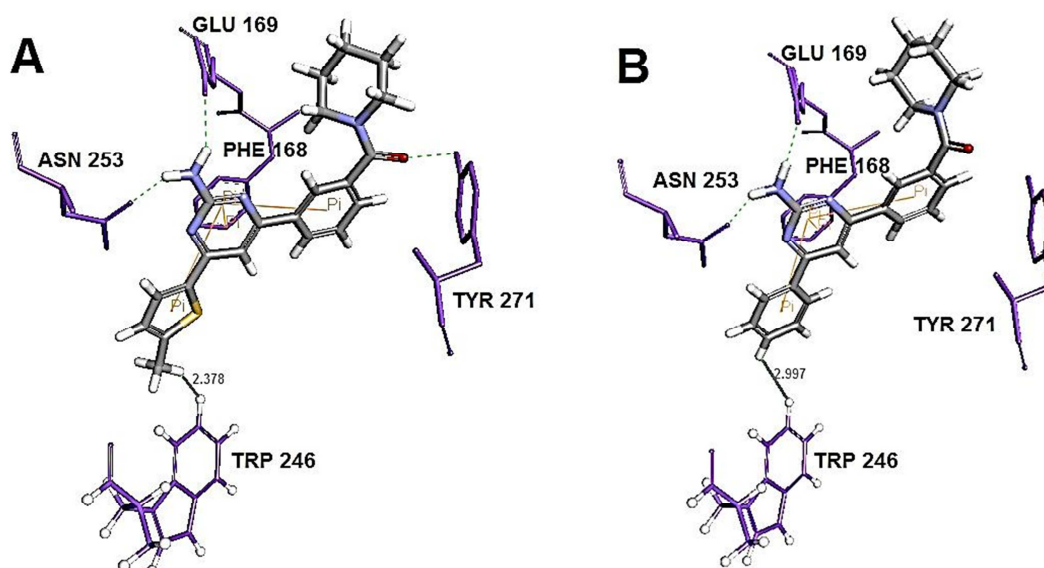


Figure 4.5: The docked compounds **39f** (A) and **39c** (B) in the adenosine A_{2A} receptor active site (docked with water of crystallisation present).

A) Compound **39f** forms three intermolecular hydrogen bonds, indicated in green (note the additional bond between the amide carbonyl and Tyr 271) and three pi-stacking receptor interactions, shown in orange. The distance of the thiophene ring from Trp 246 is 2.378 Å.

B) Compound **39c** forms at least two hydrogen bonds as well as three pi-stacking interactions and the phenyl ring is 2.997 Å from Trp 246.

The interactions observed for the low affinity thiazole derivatives, **39m** and **39n**, were similar to those observed for the high affinity derivatives **39b** - **39k**, and in these instances, the loss of affinity cannot be explained by the docking results. The high polar surface area of these compounds is probably in part, responsible for their low affinity, as discussed previously.

Interestingly, for the low affinity 4'-amide derivatives **39a** and **39l**, the orientation in the receptor site was of such a nature that no hydrogen bonding was possible with either Asn 253 or Glu 196 (the exocyclic amino group of **39l** hydrogen bonds with Tyr 271), even though π -stacking interactions between the benzothiazole group and Phe 168 are observed (Figure 4.6). It is hypothesised that the absence of these hydrogen bonds is, at least in part,

responsible for the low affinity observed for these derivatives. The fact that mutation of Asn 253 and Glu 169 disrupts (reduces or destroys) antagonist and agonist function, clearly indicates the importance of these residues for ligand recognition and further lends credibility to this hypothesis (Jaakola *et al.*, 2008).

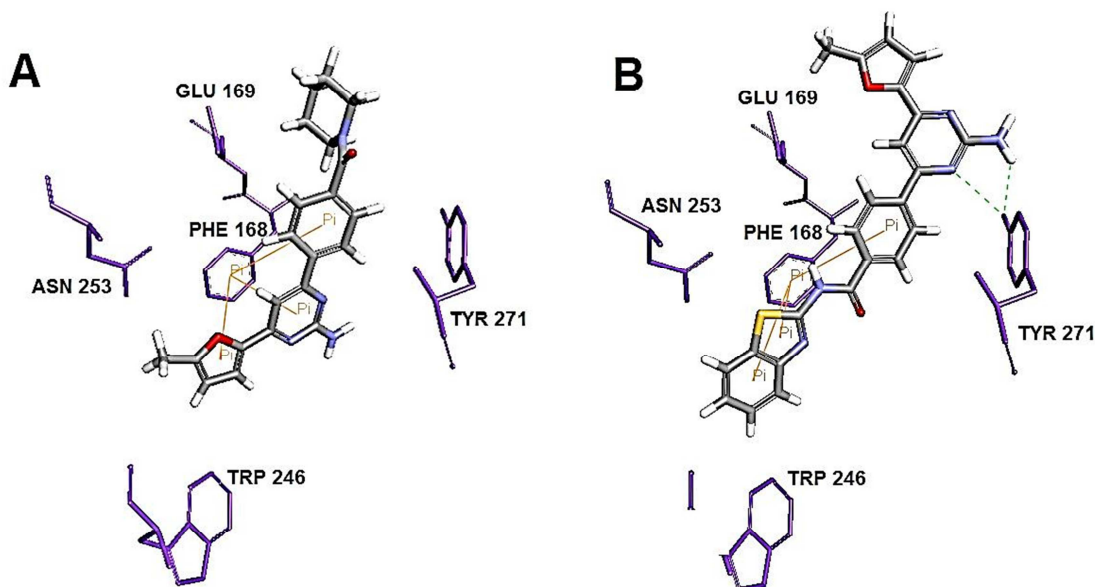


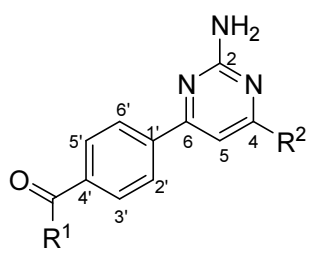
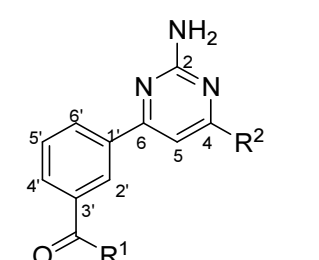
Figure 4.6: A) Compound **39a** docked in active site with observed pi-stacking interactions with Phe 168 indicated in orange.

B) Compound **39l** docked in the adenosine A_{2A} active site. Note the pi-stacking interactions between the benzothiazole and Phe 168, instead of pi-stacking interactions with the pyrimidine and methylfuran rings.

4.2.3. MTT cell viability assay

The effect of the synthesised 2-aminopyrimidines (except derivatives **39n** and **39m**) on cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay and these results are presented in Table 4.3. When metabolically active cells are exposed to MTT, metabolism of MTT by mitochondrial reductase enzymes results in the formation of a coloured formazan product which can be quantified spectrophotometrically. Since this reduction of MTT to formazan only occurs in living cells, the conversion can be directly correlated with the number of living cells.

Table 4.3: Cell viability (%) after exposure to the synthesised aminopyrimidines*, as obtained with the MTT assay

 39a, 39l  39b - 39k				
	R ¹	R ²	% Cell viability	
			1 µM	10 µM
39a	1-piperidinyl	2-(5-methylfuranyl)	108	106
39l	1,3-benzothiazol-2-amine	2-(5-methylfuranyl)	97	86
39b	1-piperidinyl	2-pyridinyl	96	90
39c	1-piperidinyl	phenyl	96	73
39d	1-piperidinyl	2-furanyl	79	60
39e	1-piperidinyl	4-fluorophenyl	90	62
39f	1-piperidinyl	2-(5-methylthienyl)	98	85
39g	1-piperidinyl	2-benzofuranyl	57	51
39h	4-ethyl-1-piperazinyl	2-(5-methylthienyl)	101	101
39i	morpholinyl	2-(5-methylthienyl)	119	88
39j	pyrrolidinyl	2-(5-methylthienyl)	69	47
39k	4-ethyl-1-piperazinyl	2-furanyl	99	86

Cell viability of more than 90% was obtained in the presence of most compounds, with the exception of **39d**, **39g** and **39j**, at a concentration of 1 µM. Although cell viability for most compounds was decreased at the higher concentration of 10 µM, the toxicity data of the most promising derivatives (e.g. **39c** and **39f**) are quite acceptable, since the concentrations at which cytotoxicity were observed, are several fold higher than the K_i values obtained for these derivatives.

When the cytotoxicity of derivatives **39b** – **39g** (with the same amide substituent) is considered, it appears that furan, benzofuran and *p*-fluorophenyl substitution present liabilities, especially at high concentrations. The amine substituent (R¹) further plays a significant role (when **39f**, **39h** – **39j** are compared) and pyrrolidine substitution (**39j**) in particular, was detrimental (the cell viability observed for 4-ethyl-1-piperazine, morpholine and piperidine substitution were acceptable). Interestingly, when furan derivatives **39d** and **39k** are considered, cell viability was noticeably improved when the piperidine was replaced

with the 4-ethyl-1-piperazine group (R^1). This highlights the importance of considering both these factors during design, e.g. if a particular heteroaryl/aryl substituent (R^2) is required, cytotoxic properties could be improved by changing the amine substituent (R^1).

4.2.4. *In vivo* assay: Haloperidol catalepsy assay

Compounds **39f** ($A_{2A}K_i = 0.49$ nM and $A_1K_i = 2.34$ nM) and **39c** ($A_{2A}K_i = 4.03$ nM and $A_1K_i = 1.03$ nM) demonstrated promising affinities for the adenosine A_{2A} and A_1 receptors and these compounds were therefore selected for further *in vivo* studies by means of the haloperidol induced catalepsy assay in rats. This assay is often performed (e.g. Shook *et al.*, 2012; Hodgson *et al.*, 2009; Trevitt *et al.*, 2009; Shiozaki *et al.*, 1999; Tomoyuki *et al.*, 1994) to assess *in vivo* activity of adenosine receptor antagonists. In addition to providing proof of *in vivo* efficacy, this assay also gives an indication of adenosine receptor antagonism or agonism, as only adenosine receptor antagonists would reverse catalepsy (the underlying principle of the haloperidol catalepsy is discussed in paragraph 2.7 in chapter 2).

Firstly, istradefylline (KW 6002), a known adenosine A_{2A} antagonist, (Figure 4.7) was assayed to verify the method. These results are presented in Figure 4.7 and clearly show a statistically significant difference between the control group (that received only haloperidol and DMSO) and the groups receiving 0.4 mg/kg and 2 mg/kg of istradefylline. Catalepsy was thus clearly reduced by this compound.

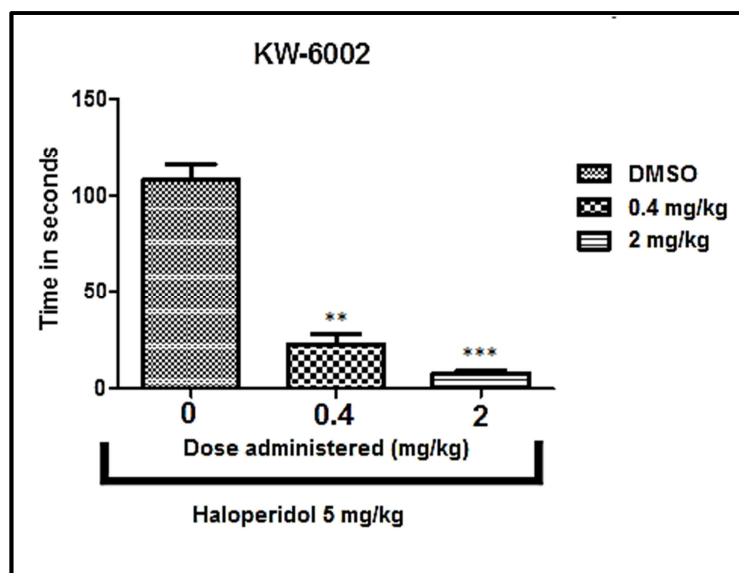


Figure 4.7: The results obtained during the catalepsy assay of known adenosine A_{2A} receptor antagonist istradefylline (KW 6002).

Unfortunately, when the results of the catalepsy assay of the highest affinity derivative **39f** (Figure 4.8) were considered, it was clear that no statistically significant difference was

observed between the control and treated groups after the standard 90 minutes (Figure 4.8A). However, after 180 minutes a statistically significant reduction of catalepsy was observed at the higher dose of 2 mg/kg (Figure 4.8B), providing preliminary evidence of adenosine receptor antagonism. It is postulated that this compound suffers from poor brain bioavailability due to unfavourable physiochemical properties. For example, it has a polar surface area of 100 Å², compared to the mean value of marketed CNS drugs of 40.5 Å² (Pajouhesh and Lenz, 2005). Interestingly, Shook and co-workers (2010b) also reported a lack of *in vivo* activity for the thiophene substituted derivatives in a related series of arylindenopyrimidines.

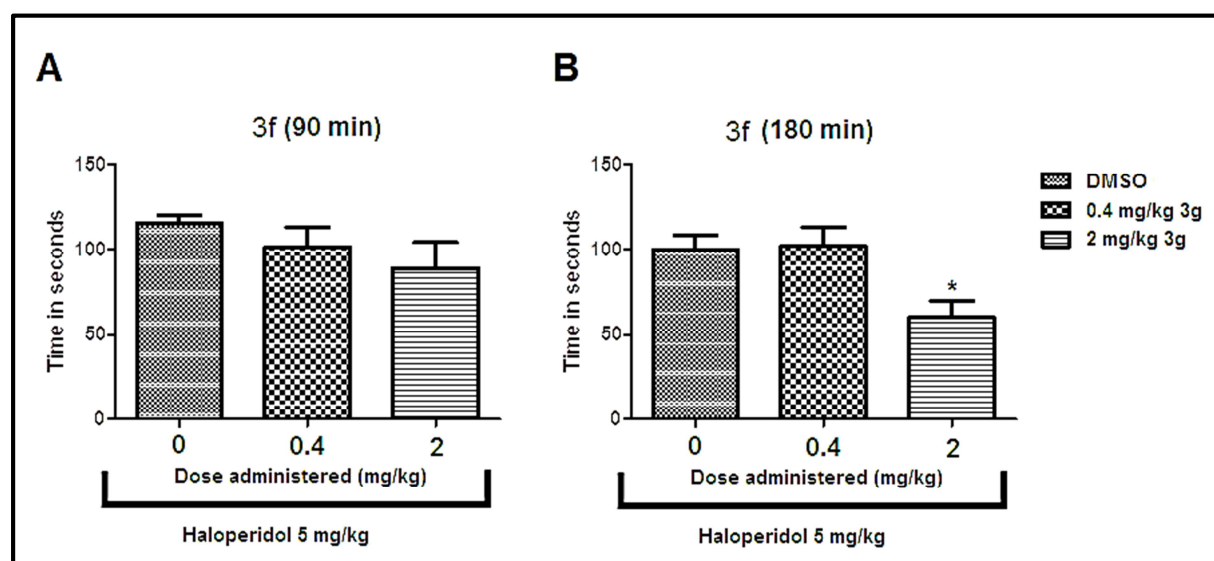


Figure 4.8: The results obtained during the catalepsy assay of compound **39f** at 90 min and 180 min after administration of haloperidol.

In vivo activity of compound **39c** was subsequently determined, and pleasingly, catalepsy was reduced in this case (Figure 4.9). A statistically significant difference was obtained between the control and treated groups at both doses (0.4 and 2 mg/kg), also indicating adenosine receptor antagonism. This compound was therefore the most promising derivative identified during this study, exhibiting dual affinity for the adenosine A₁ and A_{2A} receptors, an acceptable toxicity profile and *in vivo* activity.

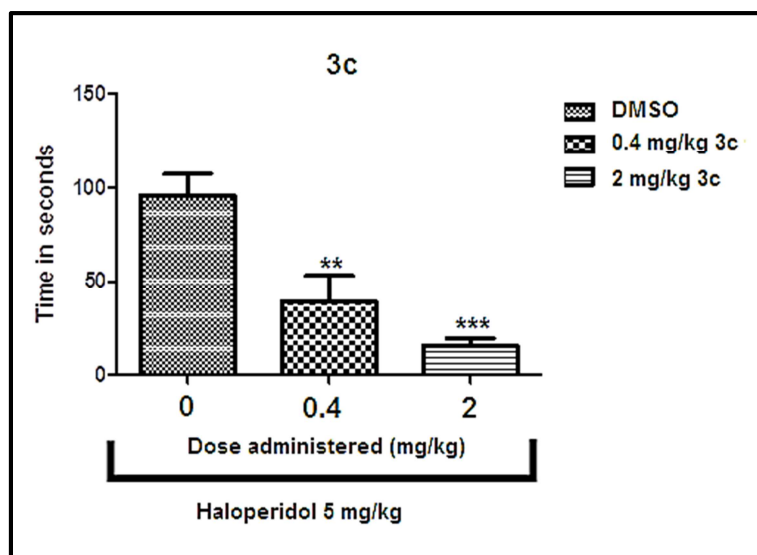


Figure 4.9: The results obtained during the catalepsy assay of compound **39c**.

4.2.5. Summary

In this chapter the *in vitro* and *in vivo* biological evaluation of the synthesised 2-aminopyrimidines were discussed. The affinities of the synthesised 2-aminopyrimidines for the adenosine A_{2A} and A₁ receptors were evaluated with a radioligand binding assay. Highly potent, dual affinity aminopyrimidine derivatives were identified e.g. compound **39f** with K_i values of 0.49 nM and 2.34 nM for the adenosine A_{2A} and A₁ receptors, respectively. Interesting structure activity relationships could be derived from the current series. These relationships (for adenosine A_{2A} affinity) were further rationalised by a QSAR study and molecular docking experiments.

Cell viability was determined with the MTT cell viability assay. Cell viability in this series is affected by both the choice of the amine and aromatic substituents. None of the most active compounds demonstrated significant toxicity at 1 µM (a concentration several fold higher than obtained K_i values).

The two most potent derivatives, **39f** and **39c**, were evaluated in the haloperidol induced catalepsy assay. Compound **39f** failed to reduce catalepsy when compared to the control group, probably due to a lack of brain bioavailability. However, compound **39c** reduced catalepsy in a statistically significant manner, compared to the control group and this compound was therefore the most promising candidate identified during this study.

4.3.Experimental

4.3.1. Radioligand binding studies

For the evaluation of the affinities of compounds for the adenosine A₁ receptor, the selective adenosine A₁ antagonist, 1,3-[³H]-dipropyl-8-cyclopentylxanthine ([³H]DPCPX), was selected as radioligand. On the other hand, the radioligand [³H]5'-N-ethylcarboxamide-adenosine ([³H]NECA), used in the determination of adenosine A_{2A} receptor affinity, was non-selective (with affinity for both A₁ and A_{2A} receptors). Addition of N⁶-cyclopentyladenosine (CPA), which is a high affinity selective adenosine A₁ receptor agonist, was thus required during A_{2A} assays, in order to selectively eliminate binding to the A₁ receptors. Magnesium chloride is added to reduce nonspecific binding of the radioligand and to increase binding to the adenosine receptor (Bruns *et al.*, 1986).

Materials

Adenosine deaminase (250 units, type X from calf spleen), CPA, anhydrous magnesium chloride, silicone solution (Sigma-cote), Trizma® base and Trizma® hydrochloride were obtained from Sigma-Aldrich. Dimethylsulfoxide and Whatman® GF/B 25 mm diameter filters were acquired from Merck. The radioligands, namely [³H]NECA, 25*Ci/mmol and [³H]DPCPX, 120*Ci/mmol were obtained from Amersham Biosciences and Filtercount scintillation fluid from Perkin Elmer.

Tissue preparation for binding studies

Ethics approval for the collection of animal tissue (NWU-0035-10-A5) was obtained from the Animal Research Ethics Committee of the North-West University. Both male (weighing between 250 – 400g) and female Sprague Dawley rats (weighing between 250 – 400 g) were obtained from the Vivarium of the North-West University, Potchefstroom campus. The striata were dissected from female rat brains, whereas the male brains were kept intact. All tissue was kept on ice during dissection and immediately snap frozen with liquid nitrogen after removal. Tissue was then stored at -70 °C until required.

The striata were disrupted for 30 seconds in 10 ml of ice-cold 50 nM Tris HCl buffer (pH 7.7 at 25 °C) with a Polytron PT-10 homogenizer (setting 5). The suspension was then centrifuged at 50 000 x g for 10 minutes, after which the supernatant was discarded. The pellet that formed during centrifugation was resuspended in 10 ml of ice-cold Tris buffer, again centrifuged and the pellet finally resuspended at a concentration 1g/5ml. The suspension was then aliquoted and stored at -70 °C until the day of the assay. Whole brain

tissue was prepared in a similar manner, but homogenised for 90 seconds to ensure complete homogenisation.

Preparation of membrane suspension

On the day of the assay the membrane suspension was prepared as follows for the adenosine A_{2A} assay: To 35 ml of Tris buffer was added adenosine deaminase (6.09 µl), MgCl₂ (48.21 mg) and prepared membrane tissue, (2.53 ml, containing 506 mg of original tissue weight of rat striatal membranes in Tris buffer). This suspension was then made up to 40 ml in Tris buffer.

On the day of the assay, the membrane suspension was prepared as follows for the adenosine A₁ assay: To 45 ml of Tris buffer was added adenosine deaminase (3.38 µl) and prepared membrane tissue, (1.4 ml, containing 280.9 mg of original tissue weight of rat whole brain in Tris buffer). This suspension was then made up to 50 ml in Tris buffer.

CPA

CPA was dissolved at 10 mM in DMSO and diluted to 500 nM in Tris buffer on the day of the experiment.

[³H]NECA and [³H]DPCPX

[³H]NECA was diluted to 40 nM in Tris, while [³H]DPCPX was diluted to 1 nM in Tris buffer.

Test compounds

Stock solutions of test compounds were dissolved at 10 mM in DMSO and further diluted to 100 times the required final incubation concentration (which typically was: 0 nM, 0.1 nM, 1 nM, 10 nM, 30 nM, 100 nM, 300 nM and 1000 nM).

Binding study

An adapted version (Van der Walt *et al.*, 2013) of the method used by Bruns and co-workers (1986) was used. Tris buffer at a pH of 7.7 (at 25 °C) was used for all incubations which was done in 4 ml polypropylene tubes (precoated with Sigmacote®). For the adenosine A_{2A} assay, each 1 ml incubation volume contained the following: Membrane suspension (yielding a final concentration of 10 mg of the original tissue weight of female rat striatal tissue, 10 mM MgCl₂ and 0.2 units/ml of adenosine deaminase), 4 nM [³H]NECA, 50 nM CPA, test compound and 1% DMSO. The order of addition was: test compound (10 µl), CPA (100 µl), [³H]NECA (100 µl) and membranes (790 µl).

For the adenosine A₁ assay, each 1 ml incubation volume contained the following: membrane suspension, (yielding a final concentration of 5 mg of the original tissue weight of male whole brain tissue and 0.1 units/ml of adenosine deaminase), 0.1 nM [³H]DPCPX, test compound and 1% DMSO. The order of addition was: test compound (10 µl), [³H]DPCPX (100 µl) and membranes (890 µl).

All incubations were vortexed before incubation in a shaking water bath at 25 °C for 60 minutes and again after the first half hour of incubation. After completion of incubation, filtration with prewetted Whatman glass microfiber filters which were connected to a Hoffeler vacuum system commenced. The contents of each tube were poured onto a filter, the tubes rinsed with 4 ml of ice cold Tris buffer and this was also poured onto the filter. The filters (which now contained the tritiated membrane tissue) were then placed in scintillation vials together with 4 ml of scintillation fluid (Filter-Count) and radioactivity (counts per minute, CPM) recorded after an incubation period of 2 hours, by utilizing the Packard Tri-CARB 2100 TR scintillation counter. The assay was performed in duplicate for every compound tested.

Data analysis

Specific binding was determined by total binding minus nonspecific binding (defined as binding in the presence of 100 µM of CPA). Data were interpreted by means of the one site competition model of the Prism 5 software package (GraphPad). A sigmoidal dose-response curve (used to calculate the IC₅₀ value) was generated by plotting the CPM value obtained against logarithm of inhibitor concentration.

The calculation of the IC₅₀ value with non-linear regression was done by the following equation:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{x - \log IC_{50}}}$$

The Y value is the CPM calculated and X is the concentration of unlabelled ligand. The top and bottom refers to the top and bottom of the sigmoidal curve. The IC₅₀ values were further used to calculate the K_i (inhibition constant) by utilizing the Cheng-Prusoff equation (Cheng and Prusoff, 1973) that can be implemented in radioligand binding assays (shown below).

$$K_i = \frac{IC_{50}}{\left(1 + \frac{[L]}{K_d}\right)}$$

With L being the concentration of radioligand (0.1 nM [³H]DPCPX in the A₁ assays) and K_d (0.36 nM for [³H]DPCPX in the A₁ assays), the dissociation constant for the radioligand (at

equilibrium). Since the A_{2A} assays were performed in the presence of CPA, an adapted version of this equation was used:

$$K_i = \frac{IC_{50}}{\left(1 + \frac{[L]}{K_d} + \frac{C}{K_c}\right)}$$

Where L = 4 nM of [³H]NECA with a K_d of 15.3 nM, and C is the concentration of CPA, (50 nM) with a K_c value of 685 nM (for CPA).

K_i values were expressed in concentrations of nM and the lower the K_i value, the higher the affinity of the test compound for the receptor. The binding affinities for the known adenosine A_{2A} antagonist ZM 245381 and A₁ agonist CPA were determined for comparative purposes, as well as to determine the influence of female as opposed to male tissue as receptor sources.

4.3.2 QSAR and molecular modelling

QSAR

A quantitative structure activity relationship (QSAR) study was carried out by correlating the affinity values obtained for the rat adenosine A_{2A} receptor with selected physiochemical properties (LogP, polar surface area, molecular weight, polarizability, molecular surface area). The values of these physiochemical parameters were generated by using MarvinSketch 5.2.3 and multiple regression analysis of data were done on STATISTICA (data analysis software system version 11) by StatSoft, Inc. (2012).

Molecular docking

Windows based Accelrys® Discovery Studio (DS 3.1) was used for molecular docking studies. The crystal structure of the adenosine A_{2A} receptor, co-crystallised with the known adenosine A_{2A} antagonist ZM 241385, was obtained from the Protein Data Bank (PDB code 3EML). The protein was imported into Discovery Studio and prepared for docking by using the 'Clean protein' functionality where after it was typed with the CHARm force field. After the application of a fixed atom constraint to the backbone of the protein, minimization of the protein was carried out using the Generalised Born approximation with Molecular Volume model. The existing ligand (ZM 241385) was selected and a radius of 5 Å was used to identify the binding sphere, where after it was removed from the crystal structure. The selected ligands were drawn in ChemWindow® 6.0 (Bio-Rad Laboratories) and imported into Discovery Studio and prepared for docking by employing the 'Prepare ligand' function. The ligands were docked by using CDOCKER function. The best conformation of each ligand (of the ten obtained) was chosen (based on visual inspection, C-Docker and C-Docker

interaction energies) and *in situ* ligand minimisation was then performed on the selected conformers. These data were then visually inspected in order to obtain some rationalisation of A_{2A} affinity results.

To validate the method of docking, the ZM 241385 (the known adenosine A_{2A} antagonist that is co-crystallised in the available crystal structure) was docked back into the active site and the RMSD (root square mean deviation) was calculated for the most suitable pose of the ligand produced during the docking process (Figure 4.10). The misalignment of the two structures was due to the flexible side chain and the RMSD of 1.3671 Å was therefore deemed acceptable, as this area is not critical for ligand binding.

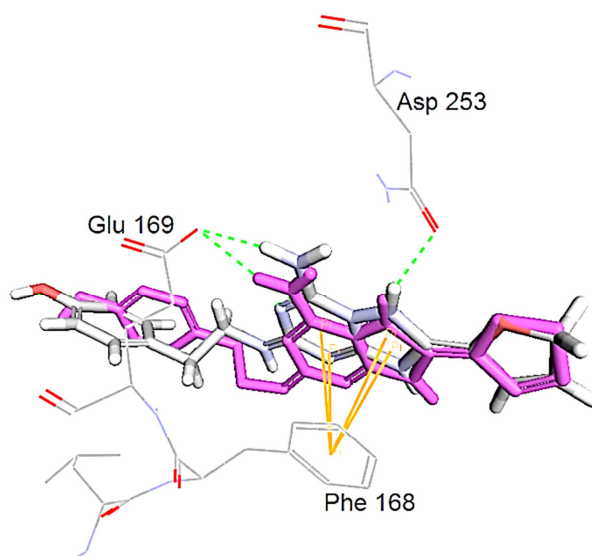


Figure 4.10: The original structure of ZM 241385 (violet), co-crystallised with the adenosine A_{2A} receptor superimposed on the structure of ZM 241385 that was docked during the validation study after preparation of the human adenosine A_{2A} receptor for docking. The green lines indicate hydrogen bonding interactions with the receptor and the orange lines indicates π stacking interactions.

4.3.3. Cell viability assay

The method used for the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was adapted from literature standardised methods (Mosmann, 1983; Brink *et al.*, 2008). The MTT assay is a standard method which is used to determine the percentage of metabolically active cells left after exposure to the test compound.

Materials

MTT, phosphate-buffered saline (PBS) and isopropanol were obtained from Sigma Aldrich. Cell culture media DMEM (Dulbecco's Modified Eagle Medium), fungizone, trypsin/EDTA, streptomycin, fetal bovine serum and penicillin were obtained from Gibco and formic acid from Merck. Well-plates (24 and 96) were obtained from Corning. Sterile syringe filters (0.22 µM) were bought from Pall Corporation Life Sciences. All incubations took place at 37 °C.

Cell culture

HeLa cells were cultured in DMEM media (500 ml) containing fungizone (250 µg/ml), trypsin/EDTA (0.25% / 0.02%), streptomycin (10 mg/ml), fetal bovine serum (50 ml) and penicillin (10 000 units/ml). The media was replaced once a week and cells were allowed to reach confluency before use in assays.

Preparation of compounds

Stock solutions at 10 mM in DMSO of test compounds were prepared and further diluted in DMSO to concentrations of 1 µM and 10 µM.

MTT Assay

Cells were counted and seeded in 24-well plates (to obtain approximately 500 000 cells per well) in cell culture media. After 24 h of incubation, test compounds (10 µl, final concentrations of 1 µM and 10 µM) were added to the HeLa cells to obtain a final volume of 1000 µl in each well. Control groups included a negative control, DMSO only, and a positive control of formic acid (33%). The plates were then incubated for a further 24 h where after media was aspirated from each well. The cells were then washed twice with phosphate-buffered saline and MTT 200 µl (0.5%) added to each well (in dark conditions due to light sensitivity of MTT). After a 2 h incubation period, the residual MTT was aspirated and 250 µl isopropanol was added to dissolve the formed formazan crystals. The formazan-containing isopropanol solution of each well plate was transferred to a corresponding well in a 96-well plate. The absorbance was read at 560 nM by a Labsystems Multiscan RC UV/V spectrophotometer. The following equation was employed to determine the viable cells as a percentage of the negative control (100% viable cells):

$$\% \text{ Viable cells} = 100 \times \frac{\text{Abs (sample)} - \text{Abs (positive control)}}{\text{Abs (negative control)} - \text{Abs (positive control)}}$$

Abs = absorbance

The number of viable cells is provided as a percentage of the control. The assay was performed in triplicate and two control groups were included namely the negative control (100% cell viability) and the positive control (100% cell death). The assay was performed under aseptic conditions.

4.3.4 In vivo: Haloperidol induced catalepsy assay

Animals

Male Sprague-Dawley rats (n = 30), weights ranging from 218 – 300 g were obtained from the Vivarium of the North-West University's Potchefstroom campus. The animals were housed in groups of six per cage and a 12 hour day and night cycle kept, under controlled temperature conditions. Free access to food and water was provided. The experimental method was approved by the North-West University Ethics committee (NWU-00035-10-S5). The National Institute of Health's guidelines for the Care and Use of Laboratory Animals were followed in all aspects to minimise animal discomfort as far as possible.

Catalepsy assay

Haloperidol (Serenace® injection, 5 mg/ml) was injected at a dose of 5 mg/kg as described by Trevitt and co-workers (2009) for the induction of catalepsy. Istradefylline (KW 6002) was used as control drug to validate the experimental protocol and dissolved in DMSO to yield concentrations of 0.4 and 2 mg/ml. The dose administered to the test animals depended on the weight of the animal in order to obtain final dose concentrations of 0.4 and 2 mg/kg. The test compounds (**39f** and **39c**) were also dissolved in DMSO to yield concentrations of 0.4 and 2 mg/ml which were also administered to the test animals according to weight to obtain final dose concentrations of 0.4 and 2 mg/kg. Injections of all agents were administered by intraperitoneal injection.

The assay was done during daytime (10:00 - 13:00) in a temperature and light controlled area. The animals used were drug naive and used only once where after they were euthanized. Firstly, 5.0 mg/kg haloperidol was injected intraperitoneally to induce catalepsy. After thirty minutes, the test compound or DMSO (for the control group) was injected intraperitoneally. Finally, 60 minutes after the injection of the test compound, catalepsy was measured.

The standard bar test was employed to measure catalepsy, where catalepsy is defined as the loss of voluntary motion, in other words remaining in a placed position. The apparatus consists of a Perspex chamber (23 cm in length, 10.5 cm in width and 9 cm in height) with a horizontal bar fixed 9 cm above floor level and 7 cm from the back of the box (Figure 4.11A).

The rats were placed in the box with their front paws placed in position on the bar. The time that the animal maintained the externally induced position was then recorded, and stopped after 120 seconds or when the rat removed one or both front paws from the bar (Figure 4.11B).

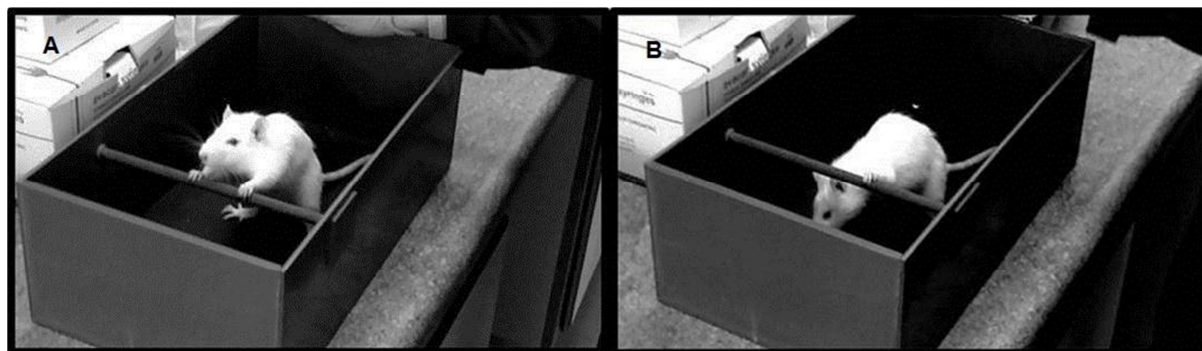


Figure 4.11: **A)** The cataleptic rat with front paws on the horizontal bar in a fixed position.
B) Rat removes feet from fixed position and catalepsy is therefore overturned.

The groups employed in the assay of compounds KW 6002, **39f** and **39c** was:

- Group 1: Haloperidol and vehicle (DMSO) as the control group (n = 10)
- Group 2: Haloperidol + test compound 0.4 mg/kg (n = 10)
- Group 3: Haloperidol + test compound 2 mg/kg (n = 10)

The data were statistically analysed using a 1 way ANOVA test followed by Dunnett's multiple comparison's test. A probability of $p < 0.05$ was deemed statistically significant.