

CHAPTER 2

Literature review

2.1 Parkinson's disease

2.1.1 Symptoms and incidence

Parkinson's disease (PD) is a chronic neurodegenerative disorder, characterised by typical motor symptoms such as bradykinesia (slowness), muscle rigidity, resting tremor and an impairment of postural balance (Schwarzschild *et al.*, 2006). While PD is mainly regarded as a movement disorder, patients suffer not only from motor symptoms, but also non-motor symptoms, which are also common and can significantly impair patients' activities as well as their quality of life. These complications include: cognitive, psychiatric (depression, anxiety and apathy), autonomic (orthostatic hypotension, bladder disturbances and sexual dysfunction), sleep (restless legs and periodic limb movements) and sensory disorders (pain, fatigue and weight changes). The non-motor symptoms are more troublesome in the advanced stages of PD when they can often pose a challenge to the treating physicians and become a major problem for patients. With multiple medications often being used to treat all these symptoms, the side effects of the drugs may further exacerbate problems (Chaudhuri *et al.*, 2006; Adler, 2005).

PD is not related to creed or race, although it's incidence steeply rises with an increase in age, from 20/100,000 overall (Dauer & Przedborski, 2003) to 160/100,000 at the age of 65 (Dorsey *et al.*, 2007; Von Campenhausen *et al.*, 2005), making it the second most common age related neurodegenerative disease after Alzheimer's disease (Dauer & Przedborski, 2003). It is expected that the number of individuals afflicted by PD will double by 2030 in line with the ageing world population and increase in life expectancy (Dorsey *et al.*, 2007). Therefore, more effective antiparkinsonian therapies need to be developed if quality of life is to be maintained and the socio-economic burden is to be reduced (Jenner *et al.*, 2009).

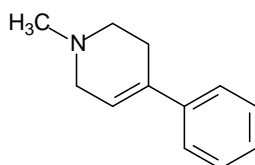
2.1.2 Etiology and pathogenesis

The etiology of Parkinson's disease is still unknown (Cieślak *et al.*, 2008) although it is believed that ageing, genetic and various environmental factors, such as exposure to pesticides and herbicides may contribute to the cause (Talpade *et al.*, 2000).

The potential role of ageing in the etiology of PD is suggested by the usual occurrence of the disease in late middle age, and by a marked increase in its prevalence at older ages (Dorsey *et al.*, 2007; Von Campenhausen *et al.*, 2005). The possible contribution of age to the expression of the disease is further supported by studies confirming a loss of dopamine in the substantia nigra and striatum with age (Kish *et al.*, 1992). The level of biochemical, motor and cognitive degeneration is, however more severe in Parkinson's disease, both at qualitative and quantitative level. For example, several imaging studies have indicated that although dopaminergic degeneration and the loss of dopamine transporters is associated with normal aging, the rate of the degeneration is faster in patients with Parkinson's disease (Nurmi *et al.*, 2000). This indicates that aging itself is not directly involved in the degenerative process of the disease. Nevertheless there is no question that increased age is a risk factor for PD, although the precise role that aging plays in the pathogenesis remains unclear.

As mentioned, genetics is also suggested to play a part in the etiology of Parkinson's disease. In 1997, it was discovered that mutations in the gene for α -synuclein cause an inherited form of PD, leading to renewed interest in genetic susceptibility to the disease. Mutations in four different proteins can lead to genetically determined forms of the disease: DJ-1, a protein thought to be involved in the neural response to stress; parkin, a ubiquitin hydrolase; UCHL1, which also participates in ubiquitin-mediated degeneration of proteins in the brain; and α -synuclein, an abundant synaptic protein (Standaert & Young, 2006).

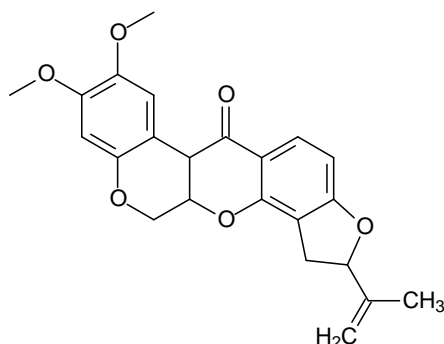
The environmental toxin hypothesis gained credibility with the observation that the administration of the "designer drug" 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (**18**), a toxin that kills dopaminergic neurons in the brain, results in a parkinsonian syndrome strikingly similar to the idiopathic disorder in humans.



MPTP **18**

More recently, the widely used agricultural pesticide rotenone (**19**) has been shown to induce a parkinsonian condition in rodents, but sustained parenteral treatment was required. Early onset of Parkinson's disease appears to be associated with rural residence in North America and

Europe. Factors associated with this include paper and steel industries, wood pulp, well water drinking and vegetable farming (Betarbet *et al.*, 2002).



Rotenone **19**

The possible role of environmental factors has been addressed by a number of epidemiological studies that have been well reviewed, however, the precise role played by any specific compound remains elusive (Tanner, 1999; Langston *et al.*, 1992; Corrigan *et al.*, 2000; Elbaz *et al.*, 2004).

The cardinal pathophysiological event in PD is the progressive damage of the dopaminergic neurons in the substantia nigra, which leads to a substantial reduction in the dopamine concentration in the striatum. The disease is also typified by the presence of intracellular inclusions known as Lewy bodies (abnormal aggregates of protein that develop inside nerve cells during Parkinson's disease) (Cieślak *et al.*, 2008; Dauer & Przedborski, 2003).

The specific molecular events responsible for the neurodegeneration seen in PD are still unknown, although several hypotheses exist. One hypothesis suggests that the death of dopaminergic neurons in the substantia nigra pars compacta (SNpc) is due to the aggregation and misfolding of proteins (Ueda *et al.*, 1993). Another hypothesis suggests that mitochondrial dysfunction and oxidative stress, including the production of toxic oxidised dopamine species, may be important in the pathogenesis of the disease (Olanow, 1992; Fahn & Cohen, 1992).

The abnormal deposition of proteins in brain tissue is a feature of several age-related neurodegenerative diseases (including Parkinson's disease) and it has been suggested that this common feature or some related event may be toxic to neurons. It remains unclear whether misfolded proteins directly cause toxicity or damage cells via the formation of protein aggregates (Lewy bodies) (Dauer & Przedborski, 2003). According to the mitochondrial

dysfunction and oxidative stress theories, nearly 100% of molecular oxygen is consumed by mitochondrial respiration, and powerful oxidants are normally produced as byproducts, including hydrogen peroxide and superoxide radicals. These byproducts may cause cellular damage by reacting with lipids, nucleic acids and proteins in patients with mitochondrial dysfunction (Schapira *et al.*, 1992; Dauer & Przedborski, 2003).

Excitotoxicity and apoptosis are also factors which may contribute to the development of the disease and should be considered when examining the pathogenesis of Parkinson's disease. Excitotoxicity describes the neural injury that results from the presence of an excess of excitatory neurotransmitters in the brain. Glutamate, for example, is an excitatory neurotransmitter and is required for normal brain function. However, when excessive amounts of glutamate are present it can lead to excitotoxic cell death (Lipton & Rosenberg, 1994). The role of excitotoxicity in neurodegenerative disorders such as Parkinson's disease is not clear, but it's believed that the increased release of stimulating transmitters like glutamate might play a role in the death of neurons (Popoli *et al.*, 2003; Chen *et al.*, 2001).

Apoptosis can be described as programmed cell death, and increasing evidence support the presence of apoptosis in neurodegeneration. It appears to play a key role in regulating cell death in traumatic brain injury (Zhang, *et al.*, 2002), oxidative stress (Fonfria, *et al.*, 2002), cerebral hypoxia–ischemia (Zhu, *et al.*, 2003), epilepsy (Cheung, *et al.*, 2005) and Alzheimer's disease (Reix, *et al.*, 2007). Several other studies have also shown apoptotic-like features in Parkinson's disease patients (Anglade, *et al.*, 1997; Mochizuki, *et al.*, 1996).

One of the key aims of current Parkinson's disease research is to elucidate the sequence in which these pathogenic factors act and whether points of interaction between these pathways are key to the termination of SNpc dopaminergic neurons (Dauer & Przedborski, 2003).

2.1.3 Animal models

Although the specific etiology of Parkinson's disease is still unknown, a better understanding of the pathogenesis of the disease as well as the development of novel therapeutic strategies has been provided by the use of animal models (Porras *et al.*, 2010).

Animal models have been critical to the study of Parkinson's disease. Models have historically been developed to replicate aspects of idiopathic Parkinson's disease pathology (Soderstrom *et al.*, 2009). During the last decade, promising opportunities for research in the field of Parkinson's disease has been raised by the gene-based models (or etiological models) of

Parkinson's disease, which are models based on genetic mutations that cause rare familial forms of the disease (Porras *et al.*, 2010). Discoveries of genetic mutations have led to a number of different genetic models of Parkinson's disease; however none of these shows the typical degeneration of dopaminergic neurons. Thus, among various accepted animal models of Parkinson's disease, neurotoxins have remained the most popular tools to produce selective neural death in both *in vitro* and *in vivo* systems (Bové *et al.*, 2005).

Traditional neurotoxin models include the reserpine model, which replicates striatal dopamine depletion seen in Parkinson's disease and the 6-hydroxydopamine (6-OHDA) and MPTP environmental toxin models that replicate Parkinson's disease nigral neurohistopathology. While very useful to researchers, these models have limited ability to produce many important features of Parkinson's disease such as Lewy bodies (Soderstrom *et al.*, 2009; Bové *et al.*, 2005). Thus, none of these models should be regarded as suitable to represent all aspects or to address all questions that pertain to PD, since they all produce specific clinical or neuropathological abnormalities that make them different from each other (Bové *et al.*, 2005).

2.2 Brain structures and pathways involved in movement and Parkinson's disease

The basal ganglia, which can be subdivided into the brainstem, cerebellum, thalamus, and striatum, guide all aspects of gross muscular activity and when injured, movement may become labored, stiff, clumsy, or even frozen as well as marked by uncontrollable tremors as is the case with Parkinson's disease. The striatum (composed of the putamen, caudate nucleus and nucleus accumbens) is the major input structure of the basal ganglia and is functionally subdivided in a dorsal and ventral striatum (Figure 2.1) (David, 2009).

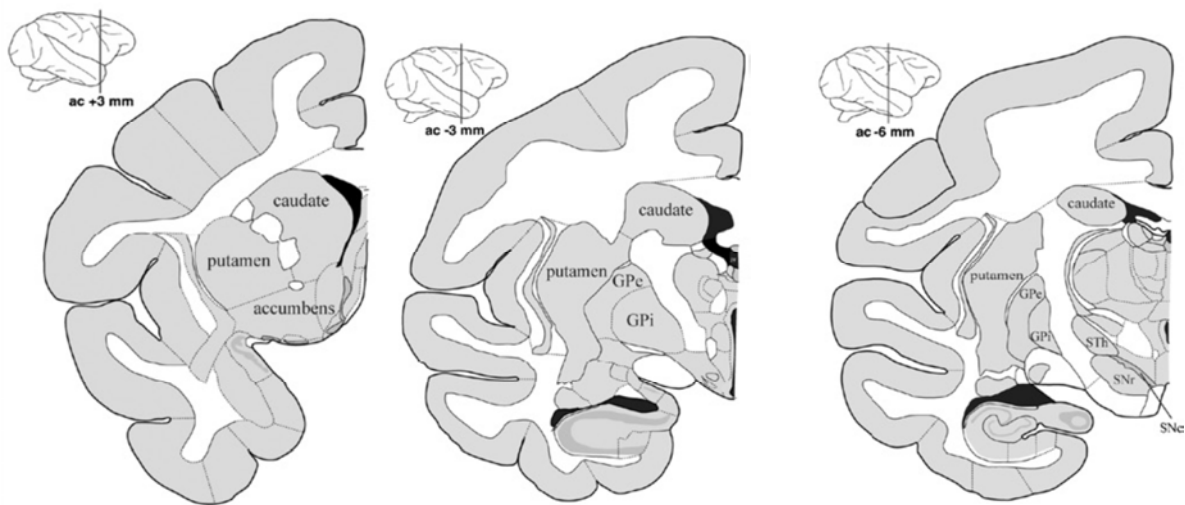


Figure 2.1: Representative examples of non-human primate and three rostro-caudal stereotaxic coordinates of the basal ganglia including the caudate, putamen, nucleus accumbens, external (GPe) and internal (GPi) segments of the globus pallidus, subthalamic nucleus (STN) and substantia nigra (SNc & SNr) (Morelli *et al.*, 2007).

The ventral striatum is mostly represented by the nucleus accumbens and forms part of brain circuits involved in goal-directed behavior, in the conversion of motivation into action and into the selection of appropriate behavioral responses (Gerfen, 2004; Parent & Hazrati, 1995). The dorsal striatum which is mostly represented by the putamen, is involved in the learning and performance of complex motor acts (Gerfen., 2004; Parent & Hazrati, 1995) (Figure 2.1). Two subtypes of striatal GABAergic efferent neurons in the dorsal part of the striatum give rise to two dorsal striatal efferent systems, which connects the output structures of the basal ganglia, the substantia nigra and the globus pallidus, with the dorsal striatum (Figure 2.2). These efferent neurons are classified into two major classes according to their peptide expression, namely the GABAergic enkephalinergic neurons of the “Indirect” and the GABAergic dynorphinergic neurons of the “Direct” pathway (Figure 2.2) (Gerfen, 2004).

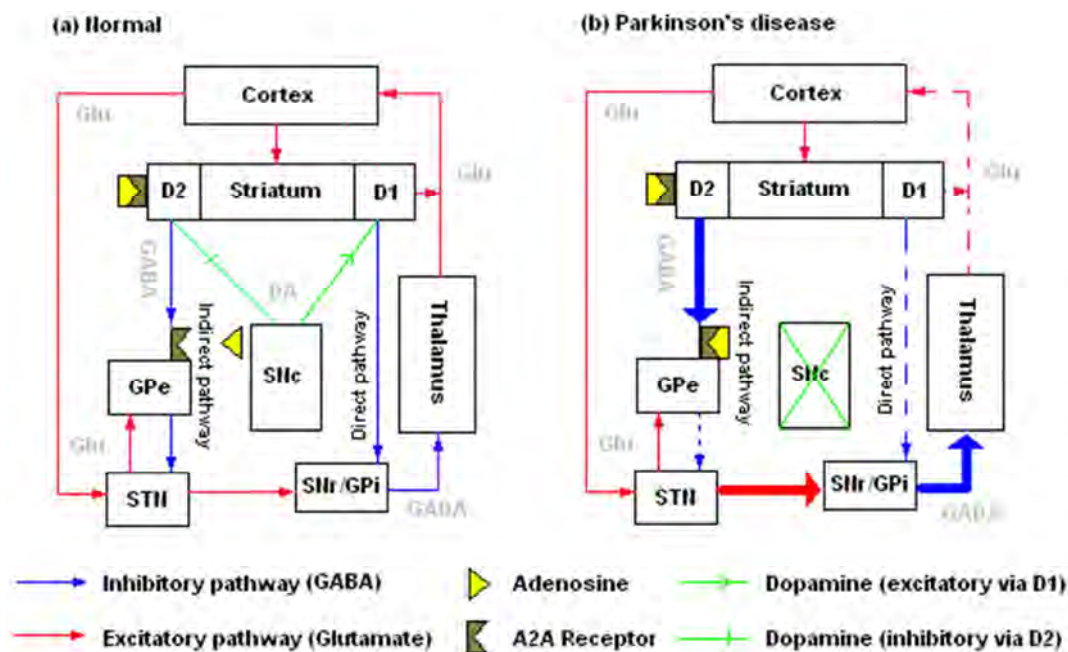


Figure 2.2: Schematic diagram of the anatomical relationship between various basal ganglia nuclei, responsible for motor dysfunction in Parkinson's disease. (a): normal state; (b): degeneration of SNc in Parkinson's disease; Thickness of arrows indicate the degree of activation of the pathway; STN: subthalamic nucleus; SNc: substantia nigra pars compacta; SNr: substantia nigra pars-reticula; GPi: globus pallidus interna; GPe: globus pallidus externa; Glu: glutamate; DA: dopamine (adapted from Morelli *et al.*, 2007; Schwarzschild *et al.*, 2006)

The direct pathway [from the striatum to the globus pallidus interna (GPi) and the substantia nigra pars-reticula (SNr)] uses the inhibitory transmitter gamma-aminobutyric acid (GABA) and expresses primarily the excitatory D₁ dopamine and adenosine A₁ receptors (Figure 2.2). The indirect pathway (from the striatum through the globus pallidus externa (GPe) and the subthalamic nucleus (STN) to the SNr and the GPi) expresses the inhibitory D₂ dopamine receptor as well as the opposing adenosine A_{2A} receptor and consists of two inhibitory GABAergic links and one excitatory glutamatergic projection (Glu) (Standaert & Young, 2006) (Figure 2.2). With Parkinson's disease, the loss of dopaminergic input in the striatum has a differential effect on the two outflow pathways; the direct pathway is less active whereas the activity in the indirect pathway is increased (Figure 2.2b) This leads to reduced excitatory input in the cortex and increased inhibition of the thalamus. Additionally, the increased activity of GABA efferent from the GPi/SNr finally induces abnormal behavior of spinal neurons, participating in the development of symptoms which is associated with hypokinesia (Morelli *et*

al., 2007). Stimulation of the direct pathway will thus result in motor activation while motor inhibition will be the result of indirect pathway stimulation. Dopamine or dopamine agonists used in Parkinson's treatment, induce motor activation by re-activating the direct pathway and depressing the indirect pathway (Müller & Ferré, 2007).

The selective localisation of adenosine A_{2A} receptors in the striatopallidal system (the indirect pathway) as well as their co-localisation with dopamine D_2 receptors, provides motivation for studying these receptors as alternative targets in the management of Parkinson's disease (Mori & Shindou, 2003). As previously mentioned, the inhibitory dopamine D_2 receptors and stimulatory adenosine A_{2A} receptors, act in an opposing manner to provide a balanced output of activity from the indirect pathway. Therefore, A_{2A} receptor antagonists have, like D_2 receptor agonists, (which are commonly used to treat Parkinson's disease), an impact on the downstream activity of this pathway (Figure 2.3) (Hodgson *et al.*, 2010). The blockade of A_{2A} receptors should therefore lead to a reversal of parkinsonian motor deficits as some balance between the direct and indirect pathways is restored (Figure 2.3) (Mori *et al.*, 2003).

(c) A_{2A} receptor blockade

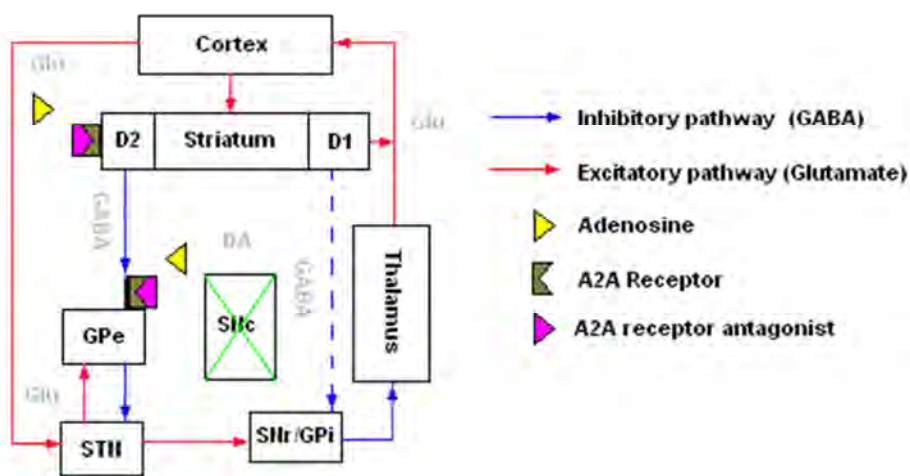


Figure 2.3: Proposed mechanism of symptomatic anti-parkinsonian activity of A_{2A} receptor antagonists. STN: subthalamic nucleus; SNc: substantia nigra pars compacta; SNr: substantia nigra pars-reticula; GPi: globus pallidus interna; GPe: globus pallidus externa; Glu: glutamate; DA: dopamine (adapted from Morelli *et al.*, 2007; Schwarzschild *et al.*, 2006).

2.3 Therapy

None of the currently approved antiparkinsonian agents alters the underlying degeneration of dopaminergic neurons (Schwarzschild *et al.*, 2006) and thus at present there exists no cure for Parkinson's disease. The primary aim of current therapy is to control the motor symptoms of the disease (Jenner *et al.*, 2009). Dopamine receptor agonists, levodopa, anticholinergic compounds and catechol-*o*-methyltransferase (COMT) inhibitors that increase dopamine levels as well as MAO-B inhibitors are drugs that currently play a role in Parkinson's disease therapy.

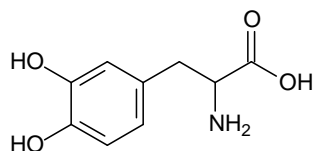
2.3.1 Levodopa

Administration of the dopamine precursor levodopa (**15**) with benserazide or carbidopa (**20**) is at present the mainstay of treatment. Levodopa is the metabolic precursor of dopamine and the single most effective agent in the treatment of Parkinson's disease (Standaert & Young, 2006). It markedly improves the quality of life of patients with Parkinson's disease and reduces mortality, at least in the early years of treatment (Hely *et al.*, 2000). Levodopa is itself largely inert; both its adverse and therapeutic effects result from its decarboxylation to dopamine (Standaert & Young, 2006). When administered orally, levodopa is absorbed rapidly and crosses the blood-brain barrier into the brain where it's converted to dopamine (primarily by decarboxylation) within the presynaptic terminals of dopaminergic neurons in the striatum.

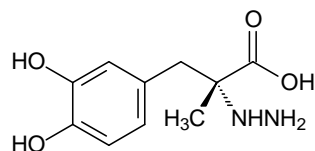
As previously mentioned, in practice, levodopa (**15**) is almost always administered in combination with carbidopa (**20**) or benserazide which are inhibitors of peripheral aromatic L-amino acid decarboxylase. If levodopa is administered alone, the drug is largely decarboxylated by peripheral enzymes so that little unchanged drug reaches the cerebral circulation. Thus by adding these decarboxylase inhibitors (carbidopa and benserazide) the fraction of administered levodopa that remains unmetabolized and available to cross the blood-brain barrier is markedly improved (Standaert & Young, 2006).

Although this therapy may initially be effective, two groups of problems arise as the disease progresses (Hely *et al.*, 2000). The first is related to the long term use of levodopa and includes side-effects such as dyskinesia (excessive and abnormal involuntary movements) and end of dose failure. The development of the "wearing off" phenomenon is a common problem, where each dose of levodopa effectively improves mobility for a period of time (1-2 hours), but akinesia and rigidity returns rapidly at the end of dose interval. This situation can be improved by increasing the dose and frequency of administration, but this is often limited by the development of dyskinesias (Standaert & Young, 2006). Eventually most patients will also suffer from non-

motor complications such as sleep disturbance, depression, dementia, and psychosis from both the disease and the dopaminergic treatments (Schwarzschild *et al.*, 2006).



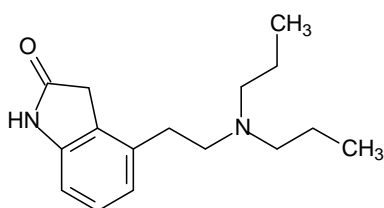
15 Levodopa



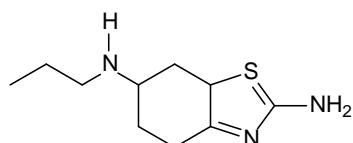
20 Carbidopa

2.3.2 Dopamine receptor agonists

All currently available oral dopamine agonists are less effective, more poorly tolerated, and more expensive than levodopa. However, dopamine agonists have a number of advantages over levodopa, including: lack of competition with dietary protein absorption from the gut and competition with transfer across the blood–brain barrier; a much longer half life allowing more physiological stimulation of receptors; reduced dyskinesia; reduced need for levodopa and direct stimulation of post synaptic dopamine receptors in the striatum, thus bypassing the need for metabolism in the degenerating substantia nigra (Factor, 1999). The most important role of dopamine agonists is as early add-on therapy in combination with a low dose of levodopa/decarboxylase inhibitor. This approach has been shown to reduce the incidence of motor fluctuations and dyskinesia (Hely *et al.*, 1994). Examples of dopamine agonists are ergot derivatives like bromocriptine and pergolide and two newer, more selective compounds, ropinirole (**21**) and pramipexole (**22**).



21 Ropinirole



22 Pramipexole

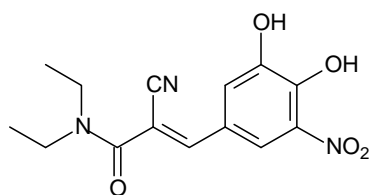
2.3.3 Anticholinergic compounds

Anticholinergics such as benztropine and trihexyphenidyl that blocks the function of the neurotransmitter acetylcholine in the central and the peripheral nervous system are specifically effective against tremor. On the other hand, these agents have little influence on reducing

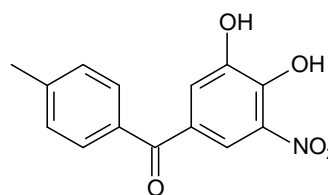
bradykinesia or akinesia (Comella & Tanner, 1995). The usable dosage of anticholinergics is a limiting factor as side effects often occur, especially at higher doses. Common side effects are drowsiness, confusion, agitation and hallucinations. Effects on memory have also been documented and use of anticholinergics is also associated with an increased sensitivity to dementia. Furthermore, abrupt withdrawal leads to precipitation of acute parkinsonian symptoms (Comella & Tanner, 1995).

2.3.4 Catechol-O-methyltransferase (COMT) inhibitors

COMT is responsible for the catabolism of dopamine as well as levodopa. The principle therapeutic action of COMT inhibitors is to block the peripheral conversion of levodopa to 3-O-methyl DOPA, and thus increase both the plasma half-life of levodopa as well as the fraction of each dose that reaches the central nervous system (Standaert & Young, 2006). COMT inhibitors are used mainly in combination with levodopa. Examples of COMT inhibitors include entacapone (**23**) and tolcapone (**24**). The incidence of orthostatic hypotension, dyskinesias, sleep disturbances, insomnia and confusion are common with these agents (Singh *et al.*, 2007).



23 Entacapone



24 Tolcapone

2.4 Monoamine oxidase type B inhibitors

2.4.1 Introduction

MAO consists of two isoforms, namely MAO-A and MAO-B. MAO is similar to COMT as it is also responsible for the catabolism of dopamine and levodopa. Over the past 50 years monoamine oxidases served as target enzymes for antidepressant drugs, and since then has received extensive attention from the pharmaceutical and biochemical communities.

Before molecular characterisation, the two types of MAO (MAO-A and MAO-B) were characterised on the basis of substrate and inhibitor sensitivity (Bortolato *et al.*, 2008). Only recently the crystal structures of the two MAO isoenzymes have been elucidated which clarified some long-held ideas about these enzymes and how they interact with inhibitors and substrates.

Inhibitors of the enzyme have therapeutic value not only as antidepressant drugs, but also in a number of common neurodegenerative conditions such as Parkinson's disease (Youdim *et al.*, 2006). The activity of both endogenously and exogenously derived dopamine can be prolonged by administering inhibitors of MAO with specificity and selectivity for MAO type B. Therefore, MAO-B inhibitors can either be used as adjunctive therapy in Parkinson's disease patients treated with levodopa or as monotherapy in early Parkinson's disease (Fernandez & Chen, 2007). Byproducts of MAO-mediated reactions further include several chemical species with neurotoxic potential that may cause neurodegenerative disorders like Parkinson's disease (Bortolato *et al.*, 2008) and MAO inhibitors may therefore also be neuroprotective. As MAO's are of particular importance to this study, it will be discussed in further detail.

2.4.2 General background

MAO's are flavin adenine dinucleotide (FAD)-containing enzymes which are bound to the mitochondria and catalyzes the oxidative deamination of monoamine neurotransmitters, hormones and dietary amines (Shih *et al.*, 1999; Bortolato *et al.*, 2008). This includes several biogenic molecules such as dopamine, noradrenalin and adrenaline, indolamines such as serotonin and tryptamine as well as trace amines such as β -phenylethylamine, tyramine and octopamine. These brain monoamines such as dopamine, serotonin and norepinephrine are crucial for the correct functioning of synaptic neurotransmission and thus without these amines the control of motor, perceptual and cognitive functions as well as modulation of mood and emotion will be impaired (Bortolato *et al.*, 2008).

The two isoforms of MAO are encoded by different genes on the X chromosome and share 70% sequence identity as well as the same type of covalent flavin: 8 α -S-cysteinyl flavin adenine dinucleotide (FAD) (Figure 2.4) (Edmondson *et al.*, 2004). Nonetheless, these two isoforms differ from each other. MAO-B has a higher affinity for β -phenylethylamine and is inhibited by low concentrations of clorgyline while MAO-A is inhibited by low concentrations of selegiline and has an affinity for serotonin and noradrenalin. Both isoforms contribute to the metabolism of dopamine and other monoamines such as tryptamine and tyramine. However, in humans, MAO-B plays a substantial role in the degradation of dopamine while MAO-A mainly degrades dopamine in the rodent brain (Bortolato *et al.*, 2008).

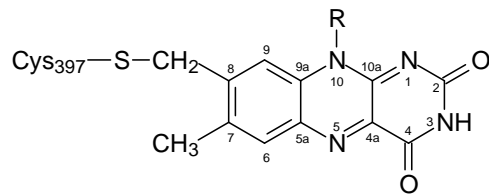


Figure 2.4: Chemical structure of the FAD co-factor

Both isoforms are present in the periphery and are involved in the inactivation of monoamines of the intestinal origin (Standaert & Young, 2006). The highest levels of these isoforms can be found in the hypothalamus as well as the basal ganglia (striatum). However in the striatum, MAO-B is the predominant isoform (Youdim *et al.*, 2006) which make its role in neurodegenerative disorders like Parkinson's disease very important since this is the region of the brain affected (dopamine depletion) during the disease.

2.4.3 Role in Parkinson's disease

As previously mentioned, in Parkinson's disease, a substantial reduction in dopamine concentration in the striatum occurs due to the death of dopaminergic neurons in this region of the brain (Cieślak *et al.*, 2008). It has also been established that the activity of MAO-B in the brain is increased in patients with Parkinson's disease resulting in increased dopamine metabolism (Adolfsson *et al.*, 1980; Alexopoulous *et al.*, 1987). Since MAO-B is the most predominant isoform responsible for dopamine degradation in the striatum, inhibition of the MAO-B enzyme should compensate for the deficits in dopamine (Knoll, 2000).

Furthermore, the oxidation of amines by MAO can produce harmful products (Figure 2.5). Monoamines are degraded into corresponding aldehydes, which are then oxidised by aldehyde dehydrogenase into acids or converted by aldehyde reductase into alcohols or glycols. Neurotoxic species such as hydrogen peroxide and ammonia are the byproducts of these reactions. Hydrogen peroxide in particular can induce mitochondrial damage and neural apoptosis by reacting with iron to form OH^\bullet and thus triggering the production of reactive oxygen species (ROS) (Bortolato *et al.*, 2008; Riederer *et al.*, 2004). The damage caused by ROS may lead to the development of neurological disorders like Parkinson's disease. Patients suffering from neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease have high levels of ROS because of increased activities of MAO-B in the brain and blood platelets (Adolfsson *et al.*, 1980; Alexopoulous *et al.*, 1987). The amount of ammonia and hydrogen peroxide formed by amine oxidation can therefore be decreased by the inhibition of MAO-B,

(Youdim *et al.*, 2006) making MAO-B inhibitors possible neuroprotective agents in the treatment of Parkinson's disease.

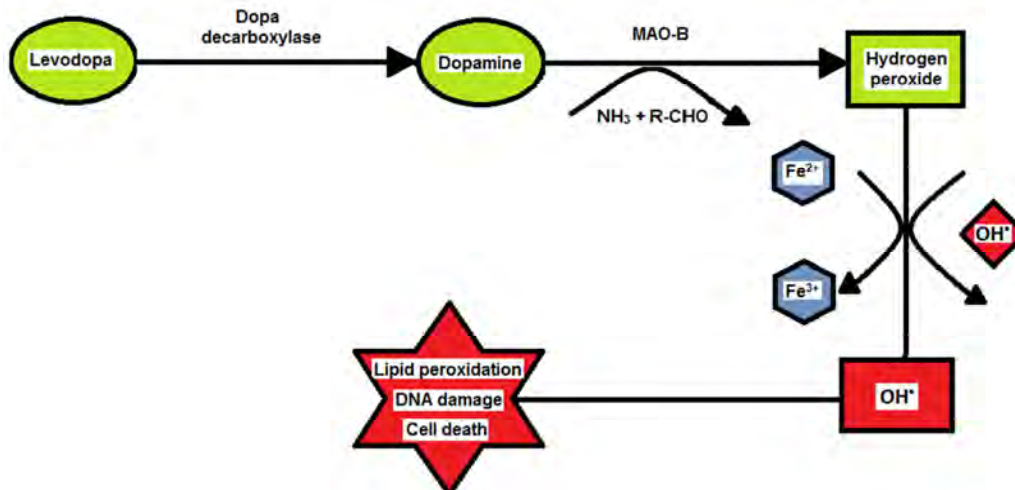


Figure: 2.5: The oxidative deamination of dopamine via MAO-B with the formation of free radicals.

In addition to the above, Parkinson's disease may also develop due to the exposure to environmental toxins like MPTP. MPTP is metabolised by MAO-B to the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) (Figure 2.6) which induces Parkinsonism in humans. Administration of MAO-B inhibitors will inhibit the formation of MPP^+ , thus giving further credibility to the neuroprotective effects of MAO-B inhibitors (Dauer & Przedborski, 2003; Youdim *et al.*, 2006).

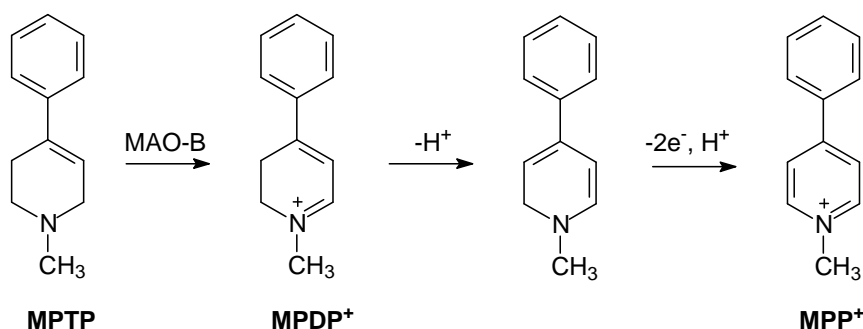
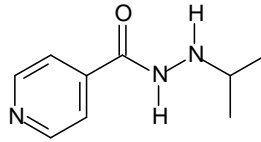


Figure: 2.6: The oxidation of MPTP to the intermediate MPDP⁺ and MPP^+ by MAO-B

2.4.4 Known inhibitors of MAO

One of the first MAO inhibitors to be discovered was iproniazid (**25**) and during the 1950-1960's, it and other MAO inhibitors were introduced into the clinic as antidepressants. Although these drugs were shown to possess antidepressant activity, their clinical usefulness was limited due to

liver toxicity and what became known as the so called "cheese reaction" (Figure 2.7) (Youdim & Weinstock, 2004).



25 Iproniazid

MAO-A is the major form of MAO in the intestine and stomach. MAO-A and MAO-B to a lesser extent, metabolise tyramine and other indirectly acting sympathomimetic amines which are present in food, like certain cheeses and wine to inactive substances. Since these amines cause a significant release of noradrenaline when taken up into the circulatory system, inhibition of MAO can lead to a severe hypertensive crisis due to the excessive amounts of noradrenaline being released. It is because of these side effects that the development and evaluation of other MAO inhibitors were brought to an end (Youdim & Weinstock, 2004).

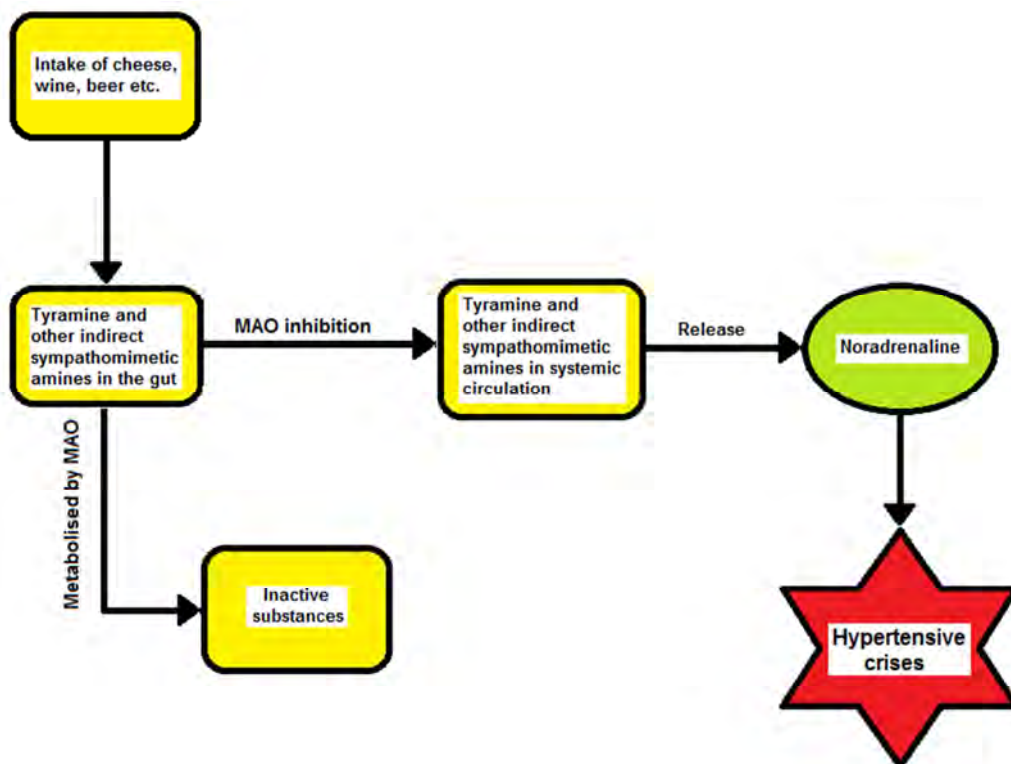
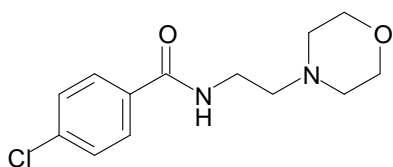


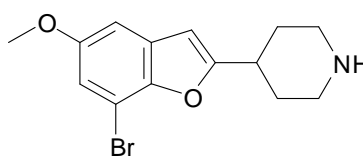
Figure 2.7: Mechanism of the so called "cheese reaction"

Reversible inhibitors of MAO-A can, however, reduce the risk of developing a hypertensive crisis. The rationale behind this is that tyramine can displace the inhibitor (if acting reversibly)

from the MAO enzyme and as a result allow some metabolism to occur (Tipton, 1997). As a consequence new reversible MAO-A inhibitors like moclobemide (**26**) and brofaromine (**27**) were developed for the treatment of depression (Nagatsu, 2004) and today, MAO inhibitors can be classified as either irreversible or reversible inhibitors.

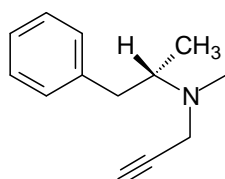


26 Moclobemide

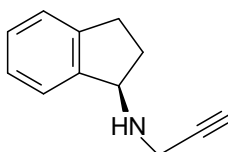


27 Brofaromine

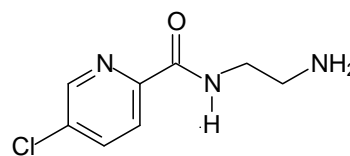
In contrast to MAO-A, MAO-B inhibitors lack an antidepressant effect and are thought to be effective in Parkinson's disease. Selegiline (**13**), rasagiline (**14**) and lazabemide (**15**) are selective MAO-B inhibitors currently available for the treatment of Parkinson's disease (Fernandez & Chen, 2007).



13 Selegiline



14 Rasagiline



28 Lazabemide

Selegiline and rasagiline are irreversible selective MAO-B inhibitors that have shown potential disease modifying effects in experimental models and clinical studies (Fernandez & Chen, 2007). Both increase dopamine levels, reduce the dose of levodopa needed for treatment of Parkinson's disease and improve the profile of dopamine needed for continuous stimulation of dopamine receptors (Riederer *et al.*, 2004). Currently, selegiline can be used as adjunctive therapy in Parkinson's disease patients which are experiencing motor complications while receiving optimised dopaminergic treatment (Pahwa *et al.*, 2006). There have also been reports that selegiline exert neuroprotective effects. Studies have shown that selegiline can prevent neurodegeneration with at least seven accepted mechanisms (Gerlach *et al.*, 1996). For example, selegiline may decrease the formation of free radicals (hydrogen peroxide) when DA is metabolised (Cohen & Spina, 1989). Regardless of the above mentioned benefits, selegiline unfortunately does not possess good bioavailability and has amphetamine-like metabolites that may produce adverse effects.

The second-generation MAO-B inhibitor rasagiline is a safe effective option for management of Parkinson's disease throughout the disease spectrum due to the fact that it is up to 10 times more potent and has less potential for toxicity than selegeline (Fernandez & Chen, 2007; Finberg *et al.*, 1999). This is because rasagiline is not a propargyl amphetamine derivative like selegeline and therefore no amphetamine-like adverse effects are associated with it (Rascol *et al.*, 2005). The neuroprotective activity of rasagiline has also been examined in several *in vitro* and *in vivo* studies (Finberg *et al.*, 1999). In these studies rasagiline had similar neuroprotective and apoptotic activity and was 15-20% more effective as a neuroprotector than selegeline (Goggi *et al.*, 2000).

Like rasagiline, lazabemide also is not metabolized to active metabolites and was found to be safe and well tolerated in patients with Parkinson's disease (Parkinson's disease study group, 1993, 1994). Lazabemide is a short acting reversible and highly selective inhibitor of MAO-B and is significantly more effective ability to inhibit oxidative damage than selegeline (Mason *et al.*, 2000).

2.4.5 The three-dimensional structure of MAO-B

The data obtained from the evaluation of the three-dimensional structure of MAO-B provides important insight into the relationship between structure and function so that more effective and specific inhibitors of MAO-B can be developed (Son *et al.*, 2008). Together with the binding model it also provides insight into the mechanism of enzyme catalysis.

The crystal structure of human MAO-B were only recently determined, which provided much needed information about the enzyme. The high resolution crystal structure showed that MAO-B crystallises as a dimer and that the active site contains a substrate as well as a smaller entrance cavity (Figure 2.8) (Hubalek *et al.*, 2005).

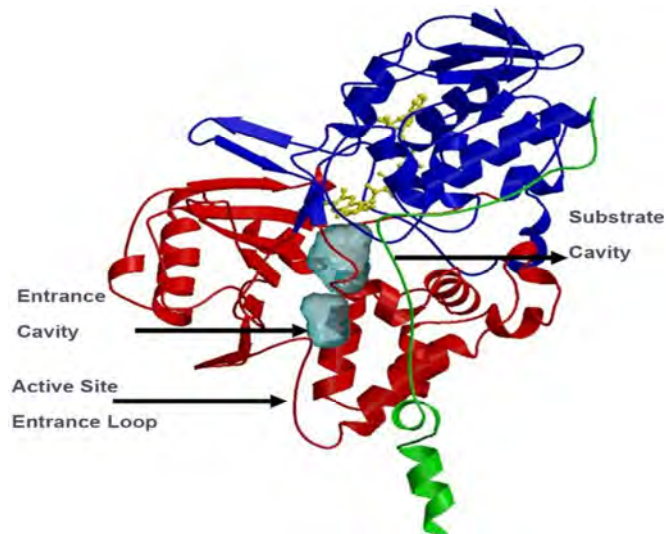


Figure 2.8: The active site of a monomeric unit of human MAO-B containing both a substrate and entrance cavity (Edmondson *et al.*, 2007).

The substrate cavity is flat and exhibits a volume of 390 Å³. A number of aliphatic and aromatic amino acids outline this cavity making it a highly hydrophobic active site. The entrance cavity is similarly hydrophobic and is situated between the protein surface and the active site. It exhibits a volume of 290 Å³ which is lined with the following residues: Phe 103, Pro 104, Trp 119, Leu 164, Leu 167, Phe 168, Leu 171, Ile 199, Ile 316 and Tyr 326 (Binda *et al.*, 2001).

An Ile 199 sidechain (loop 99-112) proximal to the membrane binding region serves as a "gate" which separates the entrance cavity from the substrate cavity. It has been suggested that diffusion of a substrate into the active site can only occur if loop 99-112 move momentarily after the substrate reaches the entrance cavity (Figure 2.8). A substrate must therefore access the catalytic site from the protein surface which is oriented towards the membrane. The so called "gate", residue Ile 199, has shown to be important in defining the inhibitor specificity of MAO-B seeing that it can either exist in an open or closed form, depending on the substrate or bound inhibitor (Binda *et al.*, 2001, Edmondson *et al.*, 2007, Hubalek *et al.*, 2005).

When looking at the membrane binding region it can be observed that MAO-B is tightly bound to the outer mitochondrial membrane and that it forms dimers on the mitochondrial surface through monomer-monomer interactions (Figure 2.8). This membrane attachment is formed by the C-terminal amino acids 461-520 (Mitoma & Ito, 1992). It has been suggested that MAO-B contains additional membrane interactions as various deletions of the C-terminal did not eliminate the ability of the enzyme to bind to the membrane (Binda *et al.*, 2001).

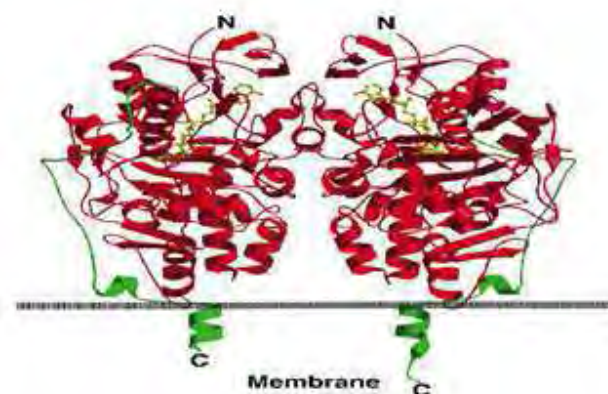
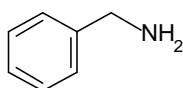


Figure 2.9: MAO-B forming dimers on the mitochondrial surface through monomer-monomer interactions

Since there are substantial data on the interaction of benzylamine and its analogs with the enzyme, attempts have been made to model the binding of this substrate in the active site (Walker & Edmondson, 1994) (Figure 2.10). According to this model the benzylic carbon of benzylamine, which undergoes flavin dependant oxidation, binds in a highly conserved position in front of the flavin N5-C4a locus (3.6 Å away from N5) (Fraaije & Mattevi, 2000). The flat shape of the binding cavity further restricts the orientation of the aromatic ring which forces the amine to bind between the phenolic side chains of Tyr 398 and Tyr 435. An aromatic caged environment is thus formed by these residues and the flavin (Binda *et al.*, 2001). The deprotonated substrate therefore binds to MAO-B since no interaction between the substrate nitrogen atom and any anionic residues can be detected (Figure 2.10) (Miller & Edmondson, 1999).

A



B

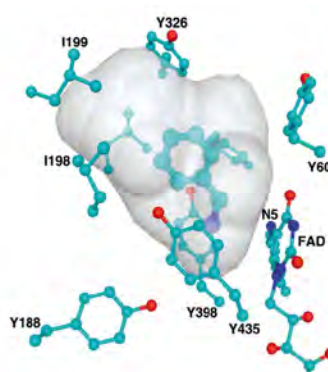


Figure 2.10: (A) The structure of benzylamine (B) Model for the binding of benzylamine to human MAO-B

The structure of MAO-B provides insight into mechanistic aspects of catalysis, which may lead to the improved design of new MAO-B inhibitors.

2.4.6 Catalytic cycle of MAO-B

MAO-B catalysis can be described as an oxidative deamination reaction of amines to imines (Figure 2.11). There are two absolute important factors necessary for the above mentioned reaction namely the covalently bound FAD co-factor present in MAO-B and oxygen (Edmondson *et al.*, 2004).

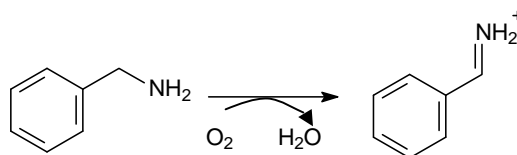


Figure 2.11: Oxidative deamination reaction of amines to imines (Edmondson *et al.*, 2004)

During the catalytic reaction the FAD co-factor is reduced while the amines are oxidized (hence the importance of oxygen) to form the imine product. Structural data show that before a general catalytic reaction can take place the substrate must travel a distance of ~ 20 Å from the entry point of the enzyme to the necessary flavin responsible for the catalysis (Edmondson *et al.*, 2004).

The exact mechanism for the electron transfer from the amine to the flavin in MAO catalysis is still not clear, although two theoretical mechanisms have been suggested (Edmondson *et al.*, 2004, Binda *et al.*, 2001). One of the first mechanisms that were proposed was the single

electron transfer (SET) mechanism by Silverman and co-workers (1995) (Figure 2.12)

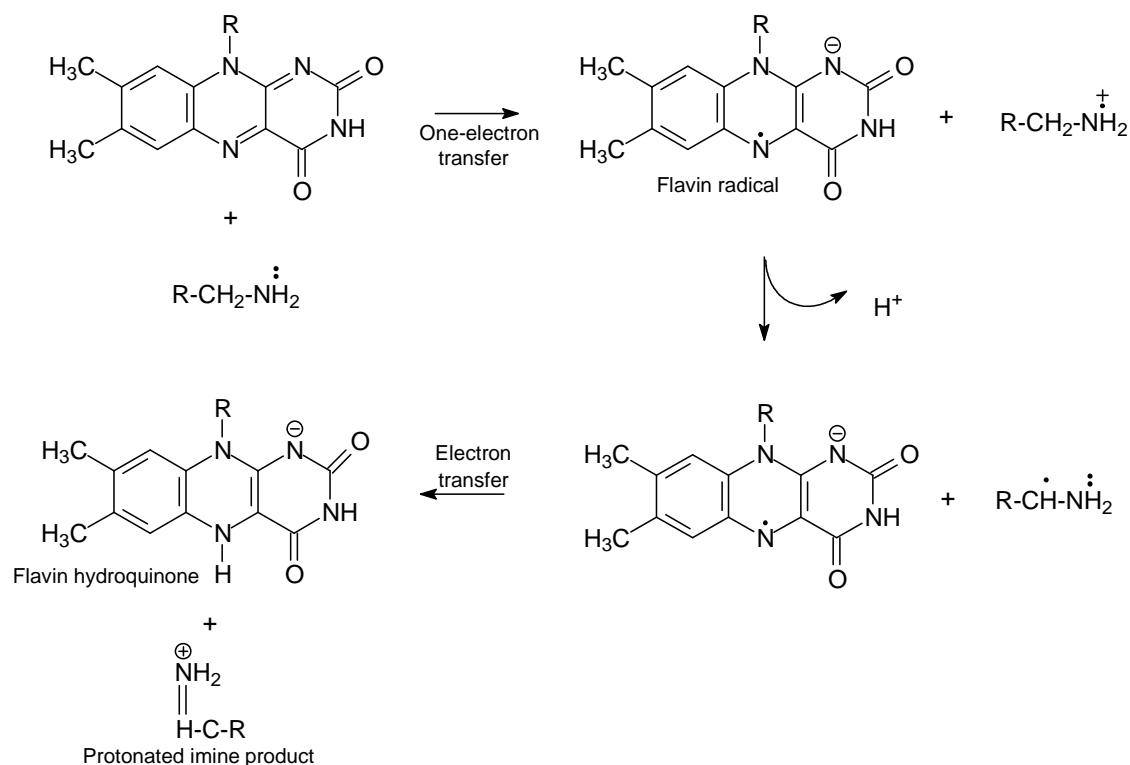


Figure 2.12: Single electron transfer (SET) mechanism by Silverman and co-workers (1995)

In the first step of the SET mechanism an aminium cation radical as well as a flavin radical is formed when the lone electron pair on the amine nitrogen undergo a one electron oxidation. It has been proposed that the aminium cation radical contains an acidic $\alpha\text{-C-H}$ that allows a basic amino acid residue (in the active site) to abstract a H^+ and form the reduced flavin and imine product. However, this was contradicted when structural data on MAO-B illustrated that in the no amino acid residues could perform such a role in the active site. Furthermore, several other studies showed that the SET mechanism for amine oxidation is improbable (Edmondson *et al.*, 2004).

In 2004, Edmondson and co-workers provided a second mechanism called the polar nucleophilic mechanism (figure 2.13). This mechanism is consistent with the current structural data available for MAO-B. In this mechanism the N-5-position of the flavin is transformed into a strong base when the deprotonated amine attacks the flavin at the 4a-position in a nucleophilic manner. This then leads to the abstraction of the $\alpha\text{-pro-R-H}$ (H^+) by N-5 of the FAD (Edmondson *et al.*, 2004).

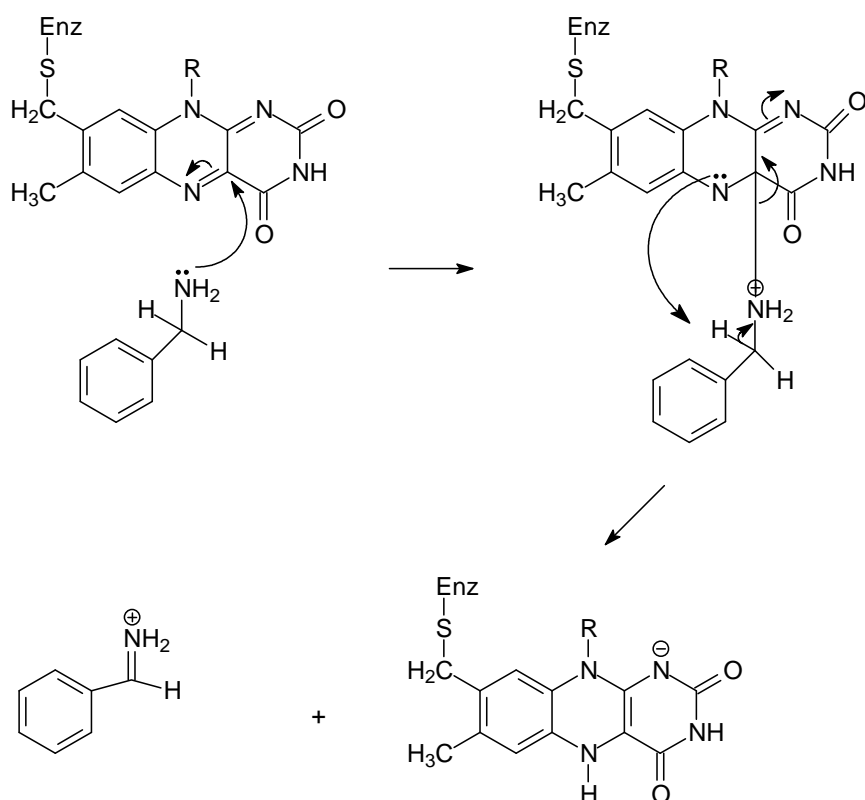


Figure 2.13: The polar nucleophilic mechanism (Edmondson *et al.*, 2004).

Edmondson *et al.* (2004), postulated that MAO-B catalysis rather proceeds via a polar nucleophilic mechanism than a SET mechanism. However, as with most postulated enzyme mechanisms, it is not unanimously accepted by all workers in the field, and is a subject of considerable debate.

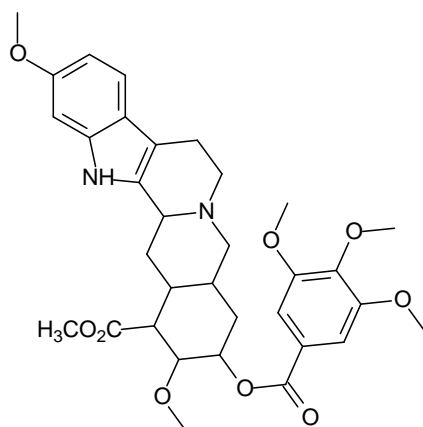
2.5 Adenosine A_{2A} antagonists

The use of adenosine A_{2A} antagonists for controlling the motor symptoms of Parkinson's disease has recently attracted attention as an alternative to traditional treatments. As previously mentioned, adenosine A_{2A} receptors are selectively localised in the cell bodies and terminals of the GABAergic neurons of the indirect pathway (Schiffmann *et al.*, 2007; Schiffmann *et al.*, 1991). The excessive excitability of the indirect output pathway, present in Parkinson's disease, should be decreased by the antagonism of the A_{2A} receptor with a selective antagonist and alleviate the motor symptoms of the disease (Mori & Shindou, 2003).

Proof of concept has been illustrated in animal models as well as in the clinical setting. For example, administration of a selective adenosine A_{2A} antagonist, istradefylline (KW 6002) (1) to

rodents (Shiozaki *et al.*, 1999) and non-human primates (Kanda *et al.*, 1998a) improved motor disability without inducing dyskinesia in these animals (Kanda *et al.*, 1998b). The observed improvement could definitely be ascribed to the blockade of the A_{2A} receptor as the increase in locomotor activity caused by the administration of KW 6002 was reversed by a selective A_{2A} receptor agonist (Kanda *et al.*, 1998b). These results suggest that therapy with KW 6002 (**1**) could be beneficial in the treatment of bradykinesia (Fernandez *et al.*, 2010).

Like bradykinesia, two other primary features, parkinsonian rest tumor and muscle rigidity, might also improve with A_{2A} blockade (Schwarzschild *et al.*, 2006). Muscle rigidity induced in rodents by the dopamine depleting agent reserpine (**29**) can be reduced by an A_{2A} antagonist or eliminated by a synergistic combination of a A_{2A} antagonist plus levodopa (Wardas *et al.*, 2001). Also, parkinsonian rest tumor, which is relatively resistant to dopamine replacement therapy, was counteracted in rodent models of parkinsonian tremor. This model was validated in a clinical setting as a combination of the subthreshold dose of levodopa and the A_{2A} antagonist KW 6002 counteracted resting tremor more effectively than they did other clinical symptoms of PD (Bara-Jimenez, *et al.*, 2003).



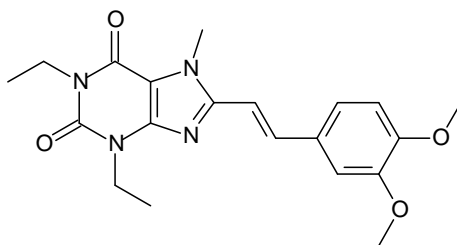
29 Reserpine

During clinical trials, in a proof-of-concept study conducted in 15 Parkinson's disease patients, the acute effects of KW 6002 were evaluated in combination with levodopa infusions (Bara-Jimenez *et al.*, 2003). Although KW 6002 (**1**) provided no antiparkinsonian benefit when given as monotherapy, or in combination with an optimal dose levodopa infusion, it improved the motor response when administered with a low dose levodopa infusion. Moreover, this response was achieved with 45% less dyskinesia and all cardinal parkinsonian signs significantly improved (Bara-Jimenez *et al.*, 2003). These studies demonstrated symptomatic improvement

in patients with relatively advanced Parkinson's disease, who already developed dyskinetic motor complications (Schwarzschild *et al.*, 2006).

A second exploratory clinical trial of moderately advanced Parkinson's disease patients on levodopa also demonstrated a prolonged levodopa efficacy half-time with addition of KW 6002 (1). Phase II and III clinical trials, indicated that KW 6002 reduces "off" time and increases "on" time with dyskinesia, but this was principally non-troublesome dyskinesia (Bara-Jimenez *et al.*, 2003).

In addition to these clinical trials, one of the most exciting prospective roles for A_{2A} receptor antagonists as a novel therapy for Parkinson's disease, is their potential to attenuate dopaminergic neurodegeneration and act as neuroprotective agents (Jenner *et al.*, 2009).



1 Istradefylline, KW 6002

2.5.1 A_{2A} antagonists in neuroprotective therapy

John Phillis was the first to propose the impact of adenosine A_{2A} receptors in the control of neural damage in a model of cerebral ischemic injury (Gao & Phillis, 1994). It was confirmed that, either the genetic elimination, or the pharmacological blockade of A_{2A} receptors, conferred a robust neuroprotection in animal models of brain ischemia (Chen *et al.*, 1999, Monopoli *et al.*, 1998). This was later extended to a variety of situations that had in common the deleterious impact of chronic noxious insults to adult brain tissue, such as glutamate excitotoxicity (Domenici *et al.*, 2007; Popoli *et al.*, 2003; Stone & Behan, 2007), epilepsy (Jones *et al.*, 1998, Lee *et al.*, 2004, Zeraati *et al.*, 2006), free radical toxicity (Behan & Stone, 2002), MPTP toxicity (Chen *et al.*, 2001; Ikeda *et al.*, 2002; Xu *et al.*, 2002) or 6-hydroxydopamine toxicity (Chen, *et al.* 2001; Ikeda *et al.*, 2002). The increased release of the stimulating transmitters might play a crucial role in the death of neurons resulting from excitotoxicity (Popoli *et al.*, 2003; Chen *et al.*, 2001). Adenosine A_{2A} antagonists could therefore provide a neuroprotective effect in slow, progressive degenerative disorders, such as Parkinson's disease, where excitotoxicity could play some pathogenic role (Müller & Ferré, 2007).

There is also some epidemiological proof of the neuroprotective properties of A_{2A} antagonists. A study of 8004 Japanese-American men reported an inverse relationship between the risk of developing Parkinson's disease and the consumption of the non-selective adenosine antagonist caffeine. Men who reported a daily consumption of 793.7 g or more of coffee, compared to men with no coffee consumption, had a fivefold reduction in age- and smoking-adjusted risk of contracting Parkinson's disease. A similar inverse relationship between the consumption of caffeinated (but not decaffeinated) coffee and the risk of developing Parkinson's disease supported this finding within several other large, prospective, more ethnically diverse cohorts (Ascherio *et al.*, 2001). These epidemiologic studies have clearly established a relationship between increased caffeine consumption and decreased risk of developing Parkinson's disease in males. The ability of caffeine to confer neuroprotection was linked to its ability to inhibit A_{2A} receptors and further supports the use of A_{2A} receptor antagonists as neuroprotective agents in Parkinson's disease (Jenner *et al.*, 2009).

The mechanism of this underlying ability of A_{2A} receptors to impact on brain tissue damage is still a matter of debate (Cunha *et al.*, 2008). One hypothesis suggest that during antagonism of A_{2A} receptors, blood vessels dilate, blood platelets aggregate, and the amount of neutrophils is decreased, resulting in neuroprotection (Morelli & Wardas, 2001). Several other mechanisms were suggested for the neuroprotective action of adenosine receptor antagonists, such as a decrease in microglia activation and/or decreased release of cytokines (Morelli & Wardas, 2001).

Unfortunately, there are also contradictory results regarding the neuroprotective effects of A_{2A} antagonists (Fredduzzi *et al.*, 2002; Agnati *et al.*, 2004). Furthermore, the neuroprotective effects of A_{2A} receptor antagonists such as 8-(3-chlorostyryl)caffeine may be ascribed to their ability to inhibit MAO-B, rather than their ability to antagonise the A_{2A} receptor (Müller & Ferré, 2007). Whether adenosine A_{2A} receptor antagonists will exert a neuroprotective effect in slow, progressive neurodegenerative diseases is therefore still an open question. Consequently, experimental and clinical data are sufficient to support the role of adenosine A_{2A} antagonists in the symptomatic, but not the pathogenic treatment of Parkinson's disease (Müller & Ferré, 2007). The introduction of A_{2A} antagonists may be a promising new alternative in the treatment of Parkinson's disease; however, there are some important factors which should be considered when designing these drugs.

2.5.2 Factors to consider in the design of A_{2A} antagonists

2.5.2.1 Adenosine receptors: function, distribution and the consequences of antagonism.

Extracellular adenosine plays an important physiological role and it initiates most of its effects through the activation of four guanine nucleotide-binding protein (G-protein)-coupled receptor (GPCR) subtypes, namely A₁, A_{2A}, A_{2B} and A₃. Each subtype has its own unique pharmacological profile and is primarily coupled to the cyclic adenosine monophosphate (cAMP) second messenger system (Hernan *et al.*, 2002; Fredholm *et al.*, 2001; Fredholm *et al.*, 2005). All four adenosine receptor subtypes have been considered as potential therapies for neurodegenerative (Morelli *et al.*, 2009; Sebastiao & Ribeiro, 2009), cardiac (Mustafa *et al.*, 2009; Headrick & Lasley, 2009), inflammatory and immune disorders (Wilson *et al.*, 2009; Blackburn *et al.*, 2009), as well as cancer (Fishman *et al.*, 2009; Katritch *et al.*, 2010). These four receptors play an essential role in responding to adenosine in the central nervous system (Dunwiddie & Masino, 2001; Jacobson & Gao, 2006), and by administration of drugs to mice with targeted deletions, it has been possible to delineate a number of physiological and pathophysiological processes where one or more adenosine receptors are involved. There's already a long list of such processes and since it is increasing each year, it is likely that this list will further lengthen. In the table below a partial list of some of these processes can be seen (Fredholm, 2010).

Table 1: Partial list containing physiological and pathophysiological processes of adenosine receptors.

Adenosine receptor subtype:	Processes
A ₁ receptors	Decreased renal blood flow; tubuloglomerular feedback; reduced heart rate; sleep; analgesia, preconditioning; inhibition of neurotransmitter release, lipolysis and insulin/glucagon release.
A _{2A} receptors	Neurodegeneration; locomotion; wakefulness; immunosuppression; angiogenesis; inhibition of platelet aggregation; vasodilatation.
A _{2B} receptors	Pain; preconditioning; vascular integrity.

A ₃ receptors	Increased mast cell activation; white cell chemotaxis; inflammatory pain; airway contraction
--------------------------	--

The therapeutic relevance of adenosine A_{2A} antagonists in Parkinson's disease has already been discussed. However, the effects of the concurrent blockade of other adenosine receptor subtypes (which may have an influence on the symptoms of Parkinson's disease) should always be considered, since selectivity of synthesised compounds cannot always be guaranteed. The A₃ receptor subtype is distributed widely in the brain, but when compared to the other subtypes, the concentration of A₃ receptors is significantly lower than the other adenosine receptors. The adenosine A_{2B} receptor subtype is also widely distributed in the central nervous system but differs from the A_{2A} receptor in terms of location and pharmacologic properties (Romanowska & Komoszyński, 2002; Zalewska-Kaszubska, 2002). Thus, antagonism of both the A₃ and A_{2B} adenosine receptor subtypes should in theory have minimal or no influence on the symptoms of Parkinson's disease. On the other hand, binding data revealed that, besides the A_{2A} receptor, the A₁ receptor subtype is also prevalent in the basal ganglia. A₁ receptors have a relatively high expression throughout the brain with the highest densities found in the striatum (similar to A_{2A} receptor subtype, which is expressed almost exclusively in this region), thalamus, cerebral cortex, hippocampus and stratum (Svenningsson *et al.*, 1997). Furthermore, A₁ receptors antagonistically and specifically modulate the binding and functional characteristics of dopamine D₁ receptors, much like the A_{2A}-D₂ interaction (Ferré *et al.*, 1997). Antagonism of the adenosine A₁ receptor would facilitate DA release in the striatum and potentiate DA-mediated responses the same as antagonism of the adenosine A_{2A} receptors. As seen in Table 1, the A₁ receptor also plays a role in neurotransmitter release as A₁ activation depresses cholinergic, noradrenergic and GABAergic transmission. Therefore, blocking both adenosine A₁ and A_{2A} receptors would be synergistic as the antagonism of adenosine A₁ receptors would facilitate dopamine release and antagonism of the adenosine A_{2A} receptor would enhance postsynaptic responses to DA (Shook *et al.*, 2012).

A₁ receptors also play important roles in cognitive function, memory formation and have been implicated in antidepressant action. The antagonism of A₁ receptors facilitate dopamine release and potentiate dopamine mediated responses within the striatum. In particular, blockade of both adenosine A_{2A} and A₁ receptors has potential in the treatment of Parkinson's disease, which presents with both motor disability and cognitive impairment (Normile & Barraco, 1991; Costenla

et al., 1999; Shook *et al.*, 2012). In fact both mental and motor impairment scores improved with the administration of theophylline (a nonselective adenosine receptor antagonist), in a short, open-labeled study in Parkinson's disease patients (Mally & Stone, 1994). The selective adenosine A_{2A} antagonist KW 6002, showed little or no cognitive improvement in animal models of Parkinson's disease, suggesting that the A_1 receptor could provide added benefit to Parkinson's disease patients. Thus, compounds that present with dual activity (both A_1 and A_{2A}) may be preferred since adenosine A_1 activity may contribute to Parkinson's disease therapy (Shook *et al.*, 2012).

As previously mentioned, adenosine A_{2A} receptors are abundant in the striatum and other nuclei of the basal ganglia, as well as in the nucleus accumbens and olfactory bulb (Figure 2.14), where they are always co-localized with dopaminergic D_2 receptors (Rosin *et al.*, 2003)

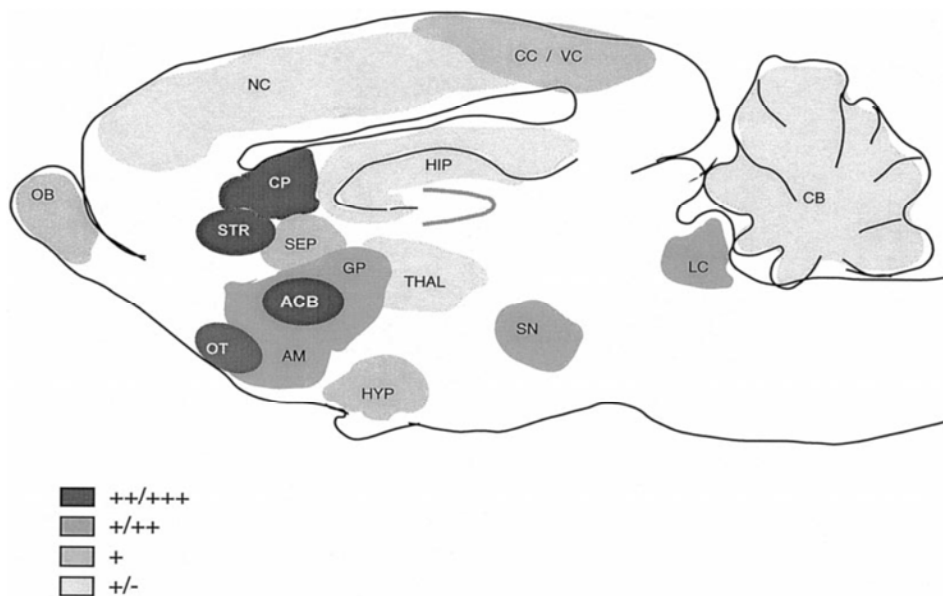


Figure 2.14: Expression and distribution of adenosine A_{2A} receptors in the brain. The highest concentration of A_{2A} receptors are found in the striatum, nucleus accumbens and olfactory tubercles, but also in other brain regions such as hippocampus, cortex and extended amygdala, as illustrated by differentially shaded areas. Shading density correlates with receptor density. ACB: nucleus accumbens; AM: extended amygdala; CC/VC: visual and cingulate cortex; CB: cerebellum; CP: caudate putamen; GP: globus pallidus; HIP: hippocampus; HYP: hypothalamus; LC: locus coeruleus; NC: neocortex; OB: olfactory bulbs; OT: olfactory tubercle; SEP: septum; SN: substantia nigra.; STR: striatum; THAL: thalamus; ++/+++ : high density; +/++ : moderate density; +/- : low density; -/no labeling: no expression detected (Moreau & Huber, 1999).

Other than the central nervous system (CNS), A_{2A} receptors are expressed in a wide variety of organs, including major peripheral tissues e.g. liver, heart, lungs and the immune system (Ledent *et al.*, 1997; Lee *et al.*, 2003). The possibility therefore exists that there could be pathophysiological consequences to the use of A_{2A} antagonists, stemming from the peripheral inhibition of A_{2A} receptors.

The A_{2A} receptor is important in several processes which includes: mediating vasodilatation in various vascular bed, including coronary blood flow, contributing to increased respiratory drive in systemic hypoxia (Fredholm, 2007) and support synthesis of new blood vessels via the generation of vascular endothelial growth factor and/or by other mechanisms (Adair, 2005). Adenosine is known to regulate many immune functions, which is perhaps the most important factor to consider with regard to potential adverse events in the use of A_{2A} antagonists (Bours *et al.*, 2006; Akkari *et al.*, 2006). For example, highly altered T-lymphocyte-mediated inflammatory responses were found in mice with targeted deletions of A_{2A} receptors (Ohta & Sitkovsky, 2001) and the T-cell-mediated inflammatory response, which is present in a variety of tissues and cells, is strongly reduced with the administration of drugs that activate adenosine A_{2A} receptors (Akkari *et al.*, 2006; Lappas *et al.*, 2005). Nevertheless, from a clinical perspective it is important to know that this appears to remain a theoretical possibility rather than a clinical concern and that there have been no reports of impairments of the immune system or inflammatory change in either the toxicological studies or in the clinical development of the most advanced A_{2A} antagonist candidate, istradefylline (Jenner *et al.*, 2009).

2.5.2.2 Second messenger system and its implication for assays

A_{2A} receptors are coupled to G_s proteins and activation stimulates adenylate cyclases which results in increased levels of cAMP. Adenylate cyclases are enzymes that are stimulated or inhibited as a result of direct interaction with G-protein alpha subunits. The role of the adenylate cyclases is to convert adenosine triphosphate (ATP) to cAMP and inorganic pyrophosphate. It's difficult to conduct receptor signaling studies for the A_{2A} receptor in non-recombinant cells, since, when compared with other G_s -coupled receptors, its ability to stimulate adenylate cyclase is relatively small (Moreau & Huber, 1999). This is an important factor to consider when conducting cAMP measurement assays, since results would be harder to obtain and interpret because of the lack of cAMP that is formed.

After stimulation of Gs coupled receptors, intracellular cAMP can then modulate different signaling cascades, for example activate the protein kinase A pathway or inhibit the MAP kinase (mitogen-activated protein kinase, MAPK) (Moreau & Huber, 1999).

The second messenger system plays a role during haloperidol induced catalepsy which in this study, is the *in vivo* assay used to determine if synthesised compounds are antagonists of the adenosine A_{2A} receptor. During the administration of haloperidol, the inhibitory effect of the dopamine D₂ receptors on cAMP is reduced. Furthermore, the binding of physiological adenosine to the stimulatory A_{2A} receptors causes an increase in cAMP levels which in turn results in catalepsy (Figure 2.15 B). Administration of an A_{2A} antagonist will then decrease the stimulatory effect of the A_{2A} receptors and cAMP levels will be reduced, causing catalepsy to be reversed (Figure 2.15 C).

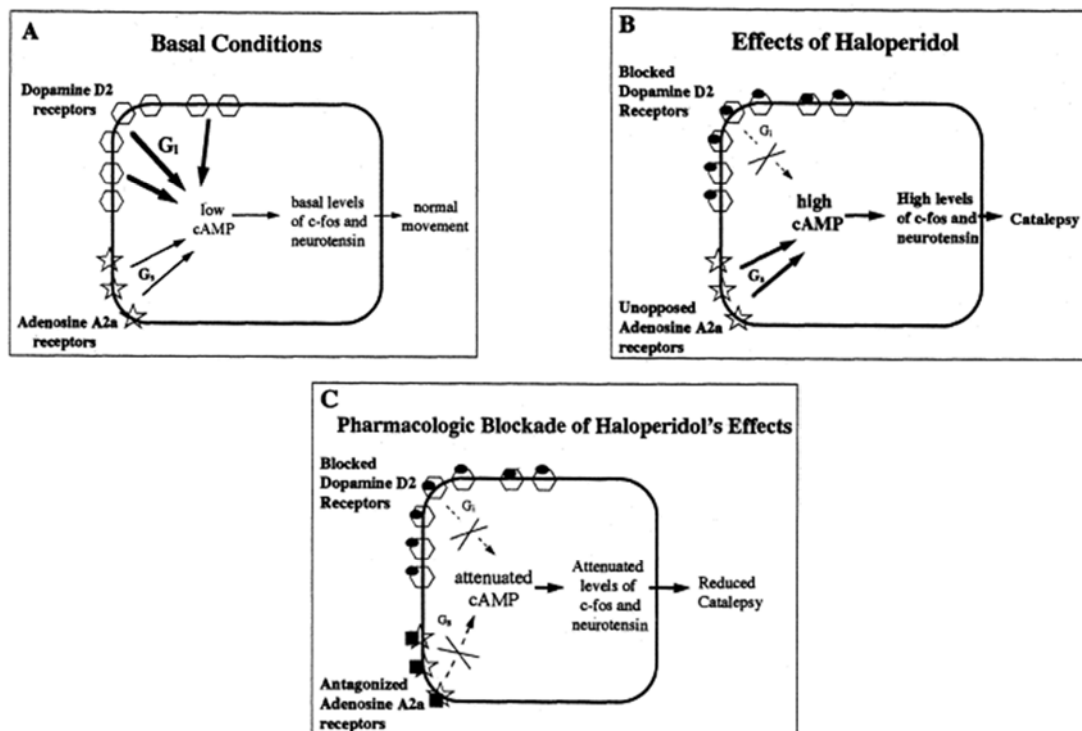


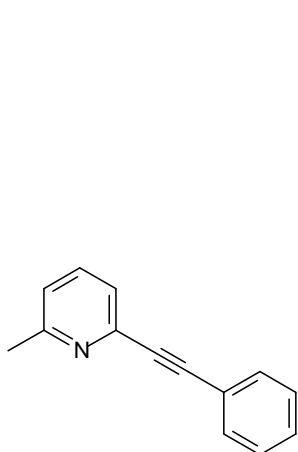
Figure 2.15: Figure illustrating the effects of haloperidol and an adenosine A_{2A} antagonist on cyclic AMP levels (Ward & Dorsa, 1999).

2.5.2.3 The relationship of A_{2A} receptors and other neurotransmitters

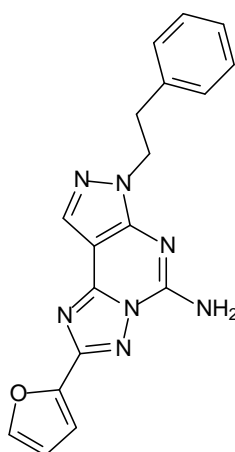
As previously stated, adenosine A_{2A} receptors have strong interactions with dopamine D₂ and adenosine A₁ receptors. The activation of adenosine A_{2A} receptors enhances cAMP production while the stimulation of dopamine D₂ receptors results in inhibition of cAMP formation. Thus,

antagonism of adenosine A_{2A} receptors can both mimic and potentiate the effects of dopamine D_2 receptor agonists (Lee *et al.*, 2002).

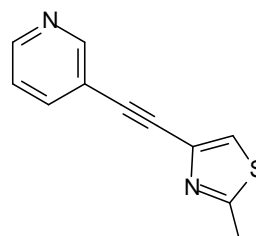
In addition, there is also an interaction with the metabotropic glutamate receptor (mGlu5), which itself is a candidate target as a new symptomatic and neuroprotective anti-parkinsonian therapy (Schwarzschild *et al.*, 2006). The adenosine A_{2A} receptor has a strong synergistic relationship with the mGlu5 receptor. A recent study has shown that low doses of a selective mGlu5 antagonist MPEP (**30**) and the selective A_{2A} antagonist KW 6002 (**1**) stimulate locomotor activity to a greater degree than the sum of the effects of each drug on its own. The expression of this synergy between adenosine A_{2A} and glutamate at the behavioral level was observed in both normal and parkinsonian mice (Kachroo *et al.*, 2005). Evidence of these positive interactions on specific motor deficits such as akinesia has recently been shown by Coccorello *et al.* (2004), who found that chronic administration of subthreshold doses of A_{2A} and mGlu receptor antagonists alleviates motor executive deficits in rats with bilateral 6-OHDA (6-hydroxydopamine) lesions of the striatum and that co-stimulation of A_{2A} and mGlu receptors synergistically modulate the quinpirole-induced turning behavior in 6-OHDA-lesioned rats (Popoli *et al.*, 2001). Moreover, parkinsonian muscle rigidity in rats was synergistically diminished by the acute joint administration of the A_{2A} and mGlu5 receptor antagonists SCH58261 (**31**) and MTEP (**32**) respectively (Wardas *et al.*, 2001; Ossowska *et al.*, 2005). In conclusion, these results show that combined chronic or acute blockade of A_{2A} and mGlu receptors potentiates their beneficial effect in PD models.



30 MPEP



31 SCH58261

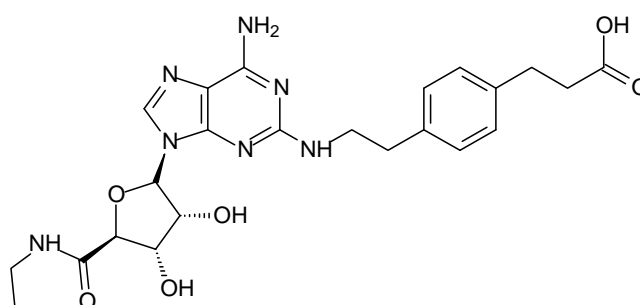


32 MTEP

It follows from the above-mentioned functional role of striatal A_{2A} receptors that A_{2A} receptor antagonists can provide a new therapeutic approach for Parkinson's disease. Firstly, by

blocking the adenosine A_{2A} receptor, the effects of drugs like levodopa can be potentiated and secondly, A_{2A} antagonists can potentially decrease glutamate-dependant excitation of GABAergic enkephalinergic neurons, which is highly increased in patients with Parkinson's disease because of the dopamine depletion (Müller & Ferré, 2007). Therefore, the future development of bivalent ligands, able to activate D₂ and block adenosine A_{2A} receptors or antagonize both A_{2A} and mGlu5 subtypes, would be a promising strategy for the treatment of Parkinson's disease.

The A_{2A} receptor-regulated cAMP second messenger system also plays a crucial role in cyclooxygenase-2 (COX-2) gene regulation in microglial cells. The A_{2A} receptor agonist CGS21680 (**33**) induces increased levels of COX-2 mRNA and the synthesis of prostaglandin E₂ (PGE₂) in these cells (Fiebich *et al.*, 1996). This may link the A_{2A} receptor to the inflammatory response thought to play a role in neurodegenerative events involved in Alzheimer's disease (Moreau & Huber, 1999).



33 CGS21680

2.5.2.4 Crystal structure of the A_{2A} receptor

GPCRs have an inherent structural flexibility because of their numerous thermodynamic conformations (Cohen *et al.*, 2002; Kobilka & Deupi, 2007). When extracted by detergent from lipid membranes this flexibility manifests itself as thermal instability and this is one of the primary challenges in generating crystal structures of GPCRs (Magnani *et al.*, 2008; Serrano-Vega *et al.*, 2008). Despite the crystallographic challenges, the crystal structure of the human A_{2A} adenosine receptor in complex with the subtype-selective high-affinity antagonist ZM 241385 (**2**) (Ongini *et al.*, 1999; Poucher *et al.*, 1995) has recently been determined (Jaakola *et al.*, 2008). ZM 241385 occupies a pocket in the receptor which is almost perpendicular to the membrane plane. This is a significantly different position in the transmembrane network when

compared to the β -adrenergic ligands and retinal, previously used for the design of A_{2A} antagonists.

Some of the important interactions between the human A_{2A} receptor and ZM 241385 are illustrated in Figure 2.16. These interactions include for example: anchoring of the bicyclic triazolotriazine core of ZM 241385 by an aromatic stacking interaction with Phe 168, an aliphatic hydrophobic interaction with Ile 274 and hydrogen bonding interactions of the exocyclic amine group with Asn 253 and Glu 169.

The furan ring occurs in many adenosine A_{2A} receptor antagonists. This moiety is located deep in the ligand binding cavity where it hydrogen bonds to Asn 253 and forms a water-mediated interaction with His 250. Hydrophobic interactions of the furan ring system include those with Leu 249 and His 250. The furan ring is ~ 3 Å away from the highly conserved Trp 246 which is an important residue in receptor activation (Audet & Bouvier, 2008). It is speculated that the hydrophobic interactions between this residue and the furan ring of ZM 241385 will hinder the structural rearrangement necessary for activation and so constrain the receptor in an inactive state (Jaakola *et al.*, 2008). Mutations of Glu 169, Asn 253, Ile 274 and His 250 result in either a decrease or loss of agonist and/or antagonist binding, indicating the importance of these residues for receptor functionality (Kim *et al.*, 1996; Kim *et al.*, 1995).

A hydrogen bond with an ordered water molecule is formed by the hydroxyl group of the phenolic substituent that extends from the ethylamine chain of ZM 241385 while the phenyl ring has hydrophobic interactions with Leu 267 and Met 270. Tremendous substituent flexibility exists in this area of the pharmacophore as demonstrated in a recent study on new antagonists for the A_{2A} adenosine receptor (Mantri *et al.*, 2008).

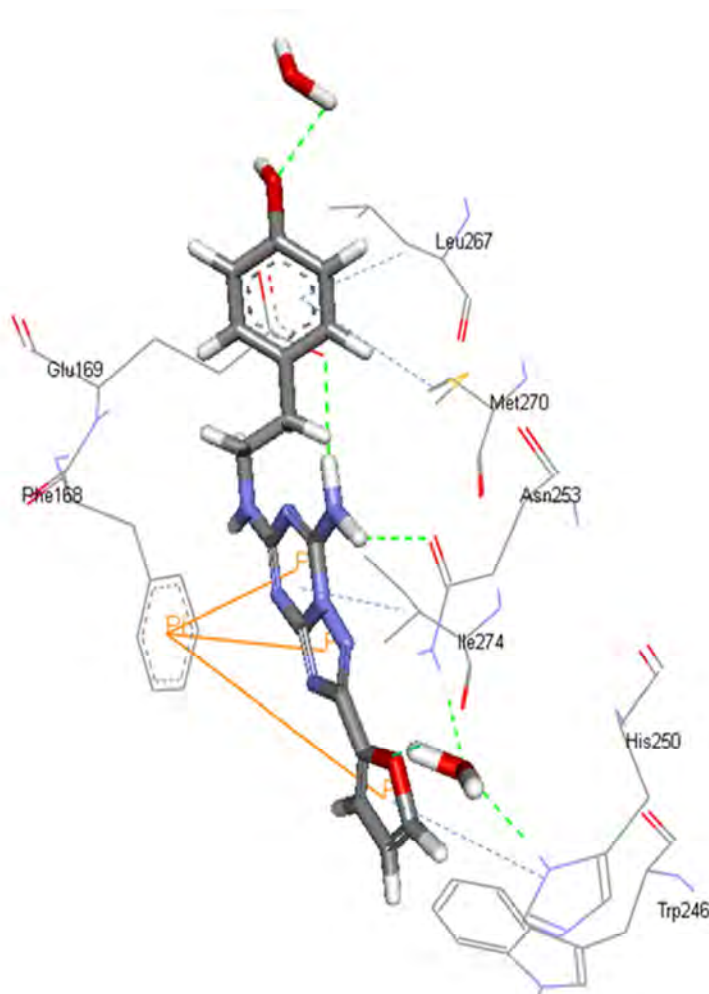
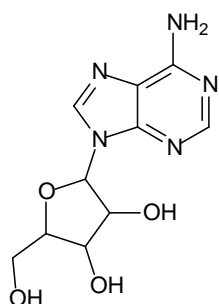
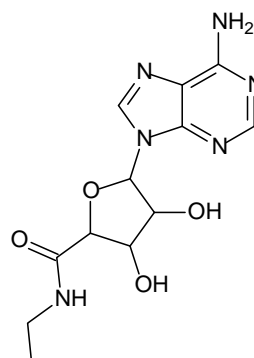


Figure 2.16: Schematic representation of some interactions between the human A_{2A} receptor and ZM 241385 at the ligand-binding cavity. Green lines: hydrogen bonding interactions; Blue lines: hydrophobic interactions; Orange lines: π stacking

In a more recent study by Lebon *et al.* (2011) two crystal structures of the thermostabilised human adenosine A_{2A} receptor bound to its endogenous agonist adenosine (**34**) and the synthetic agonist NECA (**35**) were presented. The two agonists bind in a virtually identical manner and there are also similarities with the binding observed for the antagonist, ZM 241385. The binding of the adenine core of the agonists for example, are similar to the binding observed for the triazolotriazine ring of ZM 241385. Glu 169 and Asn 253 further form hydrogen bonds with the exocyclic amine of adenosine in a similar way as observed for the exocyclic amine group of ZM241385, while Phe 168 has the same aromatic stacking interaction (Lebon *et al.*, 2011) with the heterocyclic ring system.



34 Adenosine



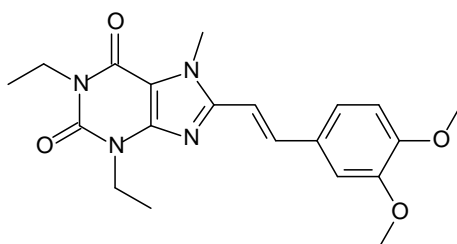
35 NECA

Given the similarities, there is also one major structural difference between the agonists and ZM 241385. The presence of a furan substituent on the triazolotriazine in the antagonist differs from the agonists which contains a ribose substituent linked with adenine. In contrast to ZM 241385 this ribose moiety forms hydrogen bonds with Ser 277 and Histidine 278. Furthermore the ribose moiety has Van der Waals interactions with other residues than ZM 241385. In particular, upon agonist binding, valine 84 has to shift its position owing to a steric clash with the ribose ring. The differences in binding between ZM 241385 and the agonists indicate that residues such as Ser 277 and His 278 have a further key role in the activation of the receptor (Lebon *et al.*, 2011).

The A_{2A} adenosine ligand-bound crystal structures suggest that there is not a general, family-conserved receptor binding pocket for GPCR's and thus the availability of the specific human A_{2A} receptor crystal structure offers the unique opportunity of designing A_{2A} antagonists with increased selectivity for this important drug target (Jaakola *et al.*, 2008).

2.5.2.5 Scaffolds and pharmacophores

Since 1996, after the utility of the A_{2A} antagonists as antiparkinsonian agents have been proposed, a number of A_{2A} antagonists have been developed and several classes of compounds have been patented. Most of the A_{2A} receptor antagonists belong to two different chemical classes, namely the xanthine derivatives (and analogs) and amino-substituted heterocyclic compounds (Müller & Ferré, 2007). KW 6002 (istradefylline) (1) is a xanthine derivative already in phase III clinical trials for the treatment of Parkinson's disease and has a styryl moiety on position 8.



1 Istradefylline, KW 6002

Unfortunately, the 8-styryl xanthine moiety is associated with photochemical instability. Since xanthine-based structures have already been optimized and explored to a large extent, research on A_{2A} antagonists with non-xanthine like structures was stimulated (Mantri *et al.*, 2008). These include compounds containing the aminopyrimidine scaffold that will form the focus of this study.

There exist several pharmacophore models that could be used during the design of A_{2A} antagonists. Mantri and co-workers (2008), for example, took previously published antagonists with high selectivity and affinity for the A_{2A} adenosine receptor, superimposed these and constructed the pharmacophore model indicated in Figure 2.17 (Mantri *et al.*, 2008).

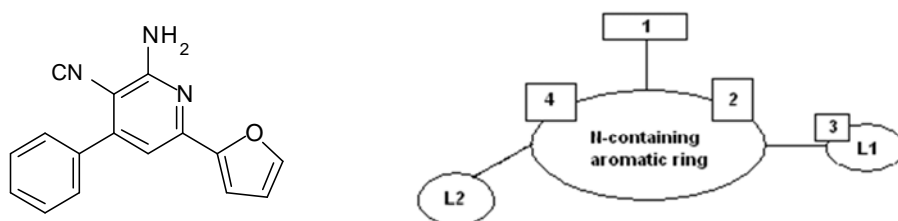


Figure 2.17: a) Compound synthesised by Mantri and co-workers (2008) b) Simplified superimposed pharmacophore model. 1: represents a H-bond donating region; 2, 3 and 4: H-bond accepting and electron-rich regions, respectively. L1 and L2: lipophilic regions (Mantri *et al.*, 2008).

According to the pharmacophore illustrated in Figure 2.17, the central monocyclic ring should have an aromatic character, as this is essential for A_{2A} receptor affinity. A nitrogen atom, which acts as a hydrogen bond acceptor, at position 2, is also required for optimal activity (Matasi *et al.*, 2005; Mantri *et al.*, 2008). The model suggests a nitrile function or group at position 4, and it is stated that the L2 pocket cannot accommodate extended bulky lipophilic groups, as this would result in a loss of activity for both adenosine A_1 and A_2 receptors (Mantri *et al.*, 2008).

Hoffmann-La Roche also proposed a pharmacophore for an adenosine A_{2A} receptor antagonist structure related to adenine (Figure 2.18). As for the pharmacophore in Figure 2.17, the

lipophilic pocket proved to be important for affinity and selectivity, while heteroaromatic and aromatic residues were tolerated in the 6-position, although a 2-furyl residue was optimal for A_{2A} affinity (Müller & Ferré, 2007).

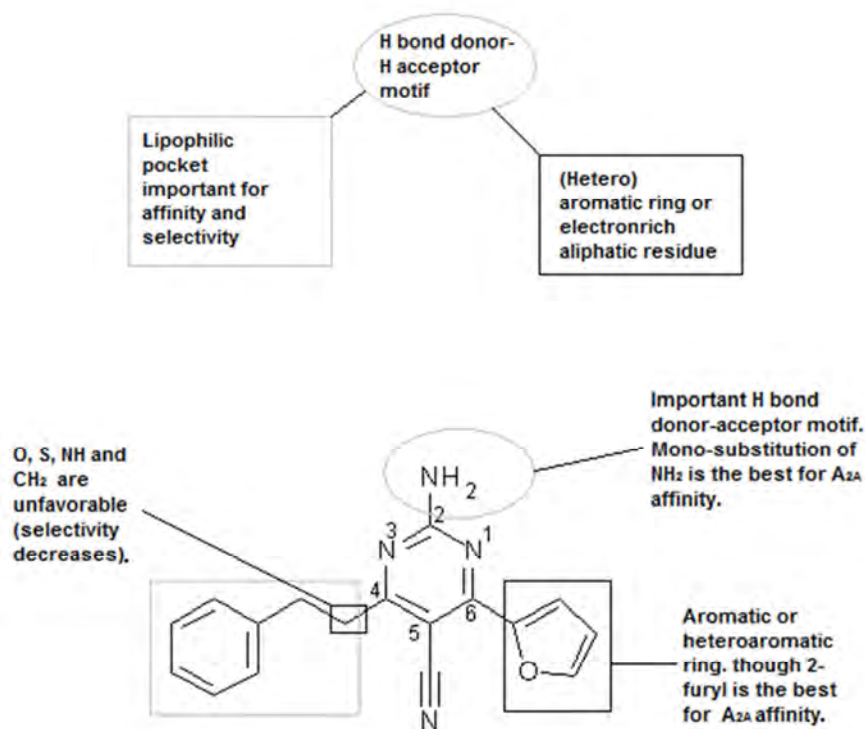


Figure 2.18: Pharmacophore model proposed by Hoffmann-La Roche for adenosine A_{2A} receptor antagonists structurally related to adenine (Müller & Ferré, 2007)

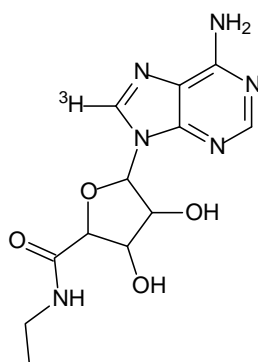
However, Richardson and co-workers (2006) reported that a furan group is prone to oxidative metabolism and thus may carry a safety liability. Reactive intermediates can form during the metabolism of unsubstituted furans which can react and form protein adducts and result in liver toxicity and other adverse effects (Dalvie *et al.*, 2002; Slee *et al.*, 2008a,b). The potential risk of oxidative metabolism and covalent adduct forming can be decreased by using a 5-methylfuran moiety to replace the furan without loss of selectivity or potency. Another promising alternative to the furyl group is thiazole derivatives (Gillespie *et al.*, 2009; Slee *et al.*, 2008a,b), as well as a propyn-1-yl or 2-pyridyl residues which may also be effective in certain instances (Müller & Ferré, 2007).

2.6 *In vivo* and *in vitro* studies

Given the potential for A_{2A} receptor antagonists as a therapeutic approach in the treatment of disorders such as Parkinson's disease, an investigation of the effects of A_{2A}-selective antagonist in suitable *in vitro* and *in vivo* models are required.

In vitro assays

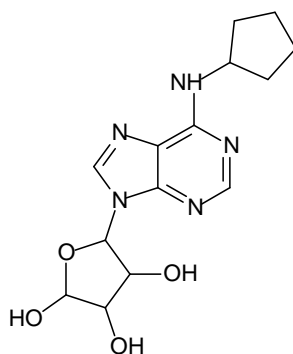
During *in vitro* assays the affinity of new molecules for adenosine A_{2A} receptors is studied by using radioligand binding technology. The radioligand binds to its receptors, and the radioactivity is used as a measure of binding. Thus, the higher the affinity of the test molecule, the more radioligand is displaced from the binding site (in this case adenosine A_{2A} receptors) which will result in a low radiation measurement. When the molecule has a low affinity for the receptor it will result in a high radioactive measurement (Baraldi *et al.*, 1995). Homogenates of rat or mouse brain striatal membranes (where A_{2A} receptors are abundant) are used as a source of A_{2A} receptors, or alternatively, membranes prepared from cells with recombinant expression of adenosine A_{2A} receptors. To have the most effective and rapid methodology for *in vitro* assays, it is necessary that the ligand used during the radioligand binding study should be selective and have a high-affinity for the target receptor. Several radioligands are employed in *in vitro* assays. These include [³H]NECA ([³H]5'-N-ethylcarboxamide-adenosine) (**36**), (a non-specific adenosine antagonist which will be used during this study), CGS 21680, [³H]XAC, [³H]PD, and [³H]KF 17837 which have all been described to label adenosine A_{2A} receptors in rat/mouse striatum (Baraldi *et al.*, 1995).



36 [³H]NECA

[³H]NECA (**36**) is known to bind to both the A₁ and A₂ subtypes of adenosine receptors in rat striatal membranes (Bruns *et al.*, 1986), which is in contrast with the above mentioned statement regarding the high selectivity ligand necessary for the most effective results.

However, although [^3H]NECA is a non-selective adenosine A_1/A_2 receptor ligand, CPA (N^6 -cyclopentyladenosine) (**37**), a adenosine A_1 receptor agonist with a higher affinity for A_1 receptors than [^3H]NECA, is used to selectively eliminate the A_1 component of binding by displacing [^3H]NECA from the A_1 receptors and leaving only adenosine A_{2A} receptors available for radioligand and test compound binding.



37 CPA

During a radioligand assay, synthesised compounds are screened for A_{2A} affinity by adding it to a suspension containing striata and CPA (**37**). The striatal membranes abstracted from laboratory animals such as rats, are treated with adenosine deaminase to completely remove all endogenous adenosine that might still be present. [^3H]NECA has also been reported to interact with non-selective receptors. Thus, the amount of radioactivity measured in a [^3H]NECA binding assay can be seen as the total binding to adenosine A_{2A} and other non-specific receptors. A second experiment (without test compound) is therefore done to determine the quantity of [^3H]NECA binding to non-selective receptors. The amount of radioactivity measured for non-specific binding in the second experiment is subtracted from the radioactivity of total binding in the first experiment to determine the specific binding of the test compound to adenosine A_{2A} receptors (Baraldi *et al.*, 1995).

***In vivo* assays**

Functional assays (*in vivo*) will be necessary to understand the characteristics of the compounds under examination, since binding studies (*in vitro*) do not discriminate between antagonists and agonists or illustrate whether any activity at all is present (Baraldi *et al.*, 1995). The *in vivo* assays are performed by systemic administration of the D_2 antagonist haloperidol to rats to induce catalepsy or inhibition of locomotor activity, which serves as a model of parkinsonian symptoms. A low dose of haloperidol is used to suppress locomotor activity while a

higher dose is used to induce catalepsy. Adenosine A_{2A} antagonism results in restoring normal behavior to baseline measures by reversing catalepsy. The use of the haloperidol-induced catalepsy assay as indicator of adenosine A_{2A} antagonism has been utilised in several studies (Antoniou *et al.*, 2005; Kafka & Corbett, 1996; Malec, 1997; Moo-Puc *et al.*, 2003). Locomotor activity should also be effectively restored as indicated by literature which states that A_{2A} antagonists effectively restored locomotor activity (Correa *et al.*, 2004; Ishiwari *et al.*, 2007; Salamone *et al.*, 2008).

There are several other animal models of Parkinson's disease which can also be used to determine the *in vivo* activity of a test compound. This includes the model of reserpine-induced akinesia, the DA depleted 6-hydroxydopamine (6-OHDA) lesion model of drug-induced rotation as well as the MPTP-treated non-human primate model (Shook *et al.*, 2012).

2.7 Summary

Although Parkinson's disease was first described almost two centuries ago, it is only recently that we have begun to understand its neurobiological causes and the complex nature of the functional deficits that it entails (Zigmond & Burke, 2005). Today a number of new strategies aimed at improving the quality of life of patients with Parkinson's disease are being researched. One such a strategy involves the antagonism of A_{2A} receptors as a novel therapy for Parkinson's disease. Therapy with A_{2A} antagonists seems warranted when the postmortem evidence of A_{2A} receptor alteration, the dyskenesiogenic effect when combined with levodopa treatment and encouraging initial clinical trial results are considered. Furthermore, the possibility that A_{2A} receptor antagonists have additive or synergistic activity when combined with dopaminomimetic drugs or drugs with mGlu5 receptor antagonistic activity is especially appealing (Morelli *et al.*, 2007). Thus A_{2A} antagonism clearly offers a uniquely hopeful and realistic opportunity for improving Parkinson's disease treatment in the future (Schwarzchild *et al.*, 2006).