Structure-Affinity Relationship Study of Novel Imidazoline Ligands at Imidazoline Binding Sites and α-Adrenoceptors

by

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Abstract

Many drugs containing an imidazoline moiety are thought to bind to both imidazoline binding sites (IBS) and α-adrenoceptors (α-AR) to mediate their therapeutic effects. To aid in better characterisation of these binding sites, the structure-affinity relationships of several new series of imidazoline containing ligands with regard to activity at α-AR and IBS were explored in this project. Radioligand binding was used to investigate the affinity and selectivity of these ligands for similar receptor types, α_1- and α_2-AR and I_1- and I_2BS, in rat whole brain and kidney membranes. In an MP series of compounds, the minor structural modifications investigated in this study appeared to favour I_2BS selectivity in general. Additionally substituents with low steric bulk (like chloro and methyl) at ortho position of the aromatic ring in MP compounds maintained affinity and selectivity at corresponding receptive sites. Similar observation was made with compounds (derivatives of marsanidine, a selective partial α_2-AR agonist) in TCS/TCA series where halogen and methyl substitutions were well tolerated with respect to α_2-AR affinity, although these ligands were nonselective in nature. Among the compounds of AW series (fluorinated derivatives of marsanidine and its heteroarylmethyl analogue), AW-21 with fluorine substituted at C-7 on the heteroaromatic ring displayed high nanomolar affinity and selectivity for α_2-AR versus other receptor types. Further *in vivo* assessment using brain microdialysis showed that AW-21, when administered intraperitoneally, reduced extracellular noradrenaline levels in rat frontal cortex in a dose related manner. Moreover, AW-21 rapidly induced sedation in rats following systemic administration indicating that it can cross the blood-brain barrier. Taken together, preliminary data suggests that AW-21 possesses favourable binding and pharmacological profiles, indicating its potential to be a suitable candidate for selective α_2-AR positron emission tomography (PET) ligand.

Preface

Some of the research for this thesis forms part of international collaborations with Professor Maria Pigini (University of Camerino, Italy) and Professor Franciszek Saczewski (Medical University of Gdansk, Poland). Their laboratories were responsible for the design and synthesis of the ligands used for my biological and neurochemical experiments. All radioligand binding studies and *in vivo* experiments and subsequent results presented in this thesis are my original work. Part of my results from Chapter IV is presented in a collaborative paper entitled "Fluorinated analogues of marsanidine, a highly alpha2-AR/imidazoline I1 binding site-selective hypotensive agent. Synthesis and biological activities". The paper has been submitted to European Journal of Medicinal Chemistry and is currently under review pending acceptance and publication. The paper is co-authored with Professor Saczewski's co-workers and my supervisor.

To my parents for their immense love and trust and endless support

“Research is what I'm doing when I don't know what I'm doing.”

Wernher von Braun (1912-1977)

Acknowledgements

Firstly, I would like to thank my supervisor Dr Alan Hudson for his guidance, insight and continuous support and for giving me ample opportunities to present my work at conferences. I would also like to thank my supervisory committee members, Dr Bradley Kerr and Dr Ian Winship, for their support.

I would also like to thank Professors Maria Pigini and Franciszek Saczewski for kindly synthesising the compounds studied in this project. A special thanks to Dr Maggie Lalies for guiding me patiently throughout and helping me with *in vivo* work and HPLC analyses.

Much appreciation goes to Judy, Joy, Sharon, Jen and specially to Ray for their help and support throughout my time at the Department of Pharmacology.

Most importantly I would like to thank my family who, despite being many hundreds of miles away, has always been supportive and on my side whenever I needed them. Also, thanks to my friends in Edmonton for making my stay over here full of fond memories that I will cherish all through my life.

Last but not the least, I would like to thank the Discovery Grant of NSERC for providing financial support to my research work.

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List of abbreviations

2-BFI = 2-(2-Benzofuranyl)-2-imidazoline

α-AR = α-adrenoceptors

α_1-AR = α_1-adrenoceptors

α_2-AR = α_2-adrenoceptors

aCSF = Artificial cerebrospinal fluid

ADC = Arginine decarboxylase

ANOVA = Analysis of variance

BBB = Blood-brain barrier

BU224 = 2-(4,5-dihydroimidaz-2-yl)-quinoline

Ca^{2+} = Calcium

CDS = Clonidine displacing substance

CH_3 = Methyl

CHO = Chinese hamster ovary

CNS = Central nervous system

DA = Dopamine

DAG = Diacylglycerol

DMI = Desipramine

ECD = Electrochemical detectionf

ERK = Extracellular signal-regulated kinase

^{18}F = Fluorine-18

FST = Forced swim test

HPLC = High performance liquid chromatography

IAA-RP = Imidazoleacetic acid-ribotide

IBS = Imidazoline binding sites

I_1BS = Imidazoline-1 binding sites

I_2BS = Imidazoline-2 binding sites

I_3BS = Imidazoline-3 binding sites

IC = Inhibitory concentration

ID = Inner diameter

i.p. = Intraperitoneal

IRAS = Imidazoline receptor antisera selected

K^+ = Potassium

K_D = Equilibrium dissociation constant

kDa = kilo Dalton

K_i = Inhibition constant

[L] = Concentration of radioligand

LC = Locus coeruleus

MAO = Monoamine oxidase

MAPK = Mitogen-activated protein kinase

MW = Molecular weight

NA = Noradrenaline

NRL = Nucleus reticularis lateralis

o- = Ortho

OD = Outer diameter

PC-PLC = Phosphatidylcholine-specific phospholipase C

PET = Positron emission tomography

RVLM = Rostral ventrolateral medulla

SAR = Structure-affinity relationship

S.E.M. = Standard error mean

VTA = Ventral tegmental area

Chapter I: General Introduction

1. Imidazoline Binding Sites

1.1. *Discovery of imidazoline binding sites*

The existence of imidazoline binding sites (IBS) had been hypothesised, some 30 years ago, from structure-activity relationship studies exploring the mechanism of action of clonidine, a centrally acting antihypertensive agent that contains an imidazoline moiety in its structure. It was thought for a long time that the hypotensive and bradycardiac effects of clonidine were mediated through partial agonism at α_2-adrenoceptors (α_2-AR) which inhibited central sympathetic outflow (see Szabo, 2002). The primary site of action of clonidine was found to be the rostral ventrolateral medulla (RVLM)* in the brainstem (Punnen *et al*., 1987; Schmitt and Schmitt, 1969). However, discrepancies remained in explaining clonidine's hypotensive mode of action via interactions with α_2-AR alone.

Bousquet *et al*. (1984) carried out a series of experiments where several α_2-AR drugs belonging to two main chemical classes (catecholamines and imidazolines) were microinjected directly into the nucleus reticularis lateralis (NRL) in the brainstem of anaesthetised cats. Interestingly, irrespective of their affinities for α_2-AR, α-methylnorepinephrine (a highly selective α_2-AR agonist) failed to produce any hypotensive effects whereas imidazoline containing drugs such as cirazoline and ST 587, like clonidine, reduced blood pressure in dose dependent manner. This implicated the presence of a new class of receptive sites other than α_2-AR in NRL that were involved in central control of blood pressure of clonidine-like agents. These binding sites were later termed as imidazoline binding sites due to their "imidazoline preferring" nature. Subsequent radioligand binding studies showed that only 70% of the total sites labelled by [^{3}H]*p*-

*also called nucleus reticularis lateralis (NRL)

aminoclonidine in bovine ventrolateral medulla membrane was sensitive to catecholamines, representing the α_2-AR population in the brain stem. The rest 30%, being insensitive to catecholamines, was only displaceable by imidazol(in)e containing compounds (Ernsberger *et al*., 1987). Also, a strong positive correlation was found between hypotensive actions of imidazoline containing agents and their affinity for imidazoline preferring sites, but not for α_2-AR (Ernsberger *et al*., 1990b), providing further support to the concept of discrete imidazoline selective binding sites.

It was soon realised that these imidazoline binding sites are heterogeneous in nature. Further studies with [^{3}H]idazoxan, an imidazoline containing α_2-AR antagonist, repeatedly labelled another group of nonadrenergic binding sites (named "idazoxan preferring sites" or "non-adrenoceptor idazoxan binding sites") that were insensitive to catecholamines and possessed high affinity for other imidazoline and related compounds but low affinity for clonidine (Michel and Insel, 1989). Given the vast amount of investigations going on in the field of "imidazoline receptor" research, a uniform nomenclature for these imidazoline recognition sites became imperative which was addressed in the first International Symposium on Imidazoline Receptors in 1992. Thus imidazoline binding sites were defined as nonadrenergic binding sites that recognise ligands containing an imidazoline nucleus or structurally related moiety (e.g. imidazole, imidazolidine, guanidine, oxazole, etc.; Figure 1.1) and classified into at least two subtypes based on their affinities for different radioligands (Michel and Ernsberger, 1992).

Those sites identified by [^{3}H]clonidine and/or [^{3}H]*p*-aminoclonidine with high affinity and involved in the regulation of blood pressure were called imidazoline-1 binding sites (I_1BS) while those preferentially labelled by [^{3}H]idazoxan were termed imidazoline-2 binding sites (I_2BS). In 1995, Ernsberger and colleagues suggested to designate I_1BS as receptors after reviewing all the

available pharmacological data on these sites thus far (Ernsberger *et al*., 1995). Although the term imidazoline-1 receptor is found in many publications, we would refer to them as I_1BS throughout this writing for the sake of simplicity. No such general consensus has yet been reached with regard to I_2BS. A third subtype of IBS has also been recognised and termed atypical or imidazoline-3 binding sites (I_3BS) which are found in the pancreatic β-cells and induce insulin secretion in a manner that is different from both I_1- and I_2BS-mediated physiology (Chan *et al*., 1994).

Since the concept of IBS was proposed by Bousquet and coworkers in 1984, much work has been carried out to characterise these sites to identify imidazoline binding proteins, endogenous and selective synthetic ligands, signal transduction pathways and their functional roles in normal physiology and in disease states. In addition a growing body of evidence suggests that these putative IBS may represent potential novel drug targets in diverse disease areas. The following sections in this review highlight some of these key findings in the field of I_1- and I_2BS, the two subtypes of IBS studied in this project.

Imidazole Imidazoline Imidazolidine Guanidine

Figure 1.1: Common pharmacophores interacting with imidazoline binding site system. R denotes different substituents (adapted from Dardonville and Rozas, 2004).

1.2. *The imidazoline-1 binding sites*

1.2.1. *Distribution*

Generally it is difficult to differentiate between α_2-AR and I_1BS since they appear to share certain degree of pharmacological and functional similarity in that most of the ligands display similar affinity toward both receptor systems and elicit similar physiological responses. Nonetheless using radioligand binding and autoradiography the presence of I_1BS has been reported in a number of mammalian tissues. Initially identified in the bovine brainstem (Ernsberger *et al*., 1987), I_1BS is present in the RVLM predominantly (Ernsberger *et al*., 1995) and in ventral tegmental areas (Ernsberger and Haxhiu, 1997) with low density in the cerebral cortex (Ernsberger *et al*., 1987). Interestingly, no I_1BS were found on astrocytes (Ernsberger *et al*., 1995). Peripherally, they are expressed in the kidneys (Ernsberger et al., 1990a; Ernsberger et al., 1995), platelets (Piletz *et al*., 1991), heart (El-Ayoubi *et al*., 2002), and placenta (Bagamery *et al*., 1999). I_1BS are also expressed in adrenal chromaffin and PC12 cells both of which lack α_2-AR (Ernsberger *et al*., 1995). Subcellularly I_1BS are localised in the plasma membrane fraction of RVLM tissue (Ernsberger and Shen, 1997), human platelets (Piletz and Sletten, 1993) and PC12 cells (Ernsberger *et al*., 1995). Presynaptic location of I_1BS in bovine brainstem neurons was reported for the first time by Heemskerk *et al*. (1998).

1.2.2. *Candidate imidazoline-1 binding site proteins*

The molecular structure of I_1BS protein has not yet been fully elucidated. A number of proteins of different sizes have been detected so far in different tissues across various species (see Table 1 in Escriba *et al*., 1999) using either of the two antibodies: one that was raised against idazoxan/clonidine binding proteins from bovine adrenal chromaffin cells (Wang *et al*., 1993)

and the other was a secondary antibody of anti-idiotypic type (anti-anti-idazoxan) (Bennai *et al*., 1996). Both the antibodies detected an 85 kDa protein in human and rat brain and PC12 cells which appeared to be a likely candidate for the full-length imidazoline receptor (Ivanov *et al*., 1998b).

Further studies using both antibodies isolated initially a partial form (Ivanov *et al*., 1998a) and later the full length (Piletz *et al*., 2000) cDNA clone from human hippocampal cDNA library. The full length cDNA was named imidazoline receptor antisera selected (IRAS) and the resulting 1504-amino acid protein, IRAS-1, was characterised as a 167 kDa protein band on Western blots (Piletz *et al*., 2000). In addition, no similarity was found between primary sequence of IRAS-1 and other proteins (like α_2-AR or MAO) known to bind imidazoline compounds (Piletz *et al*., 2000). Distribution of IRAS mRNA correlated well with the density of I_1BS across rat tissues (Piletz *et al*., 1999). Transfection of IRAS cDNA in Chinese hamster ovary (CHO) cells resulted in expression of high affinity I_1BS which exhibited high nanomolar affinity for moxonidine and rilmenidine (Piletz *et al*., 2000). A recent study reported that both the 33 and 85 kDa proteins found consistently in human platelets were likely to be the fragments of the full length 167 kDa IRAS protein (Zhu *et al*., 2003). In addition nischarin, an integrin binding protein, has been suggested to be the murine homologue of human IRAS protein and both of them were found to be transmembrane proteins (Alahari *et a*l., 2000; Zhang and Abdel-Rahman, 2006) as proposed previously for I_1BS (Ernsberger *et al*., 1995). Recent studies have shown that IRAS have antiapoptotic effects possibly by activating the PI3 kinase pathway (Dontenwill *et al*., 2003). Furthermore, knocking down nischarin expression in rat RVLM has been reported to reduce I_1BS mediated phosphorylated extracellular signal-regulated kinase (pERK)1/2 production in RVLM and rilmenidine's hypotensive response (Zhang and Abdel-Rahman, 2008). Therefore IRAS-1

represents a viable candidate of I_1BS although further molecular and pharmacological characterisation of this protein is required for complete identification.

1.2.3. *Proposed signal transduction pathway(s)*

Considerable progress has been made in identifying the signal transduction pathway and associated second messengers in producing I_1BS mediated responses. It was speculated that I_1BS were plasma membrane bound G-protein-coupled receptors (Ernsberger *et al*., 1995). This was supported by the observation that binding of [^{125}I]*p*-iodoclonidine to I_1BS in bovine RVLM membranes was sensitive to inhibition by nonhydrolyasable guanine nucleotide, Gpp(NH)p (Ernsberger and Shen, 1997). The prototypical I_1BS ligand clonidine failed to modify basal or forskolin stimulated cAMP levels and to activate phosphatidylinositol-specific phospholipase C and required very high concentrations to increase Ca^{2+} influx, suggesting that I_1BS did not act through the most common G-protein coupled systems (Regunathan *et al*., 1991).

Despite the negative results, Separovic and coworkers demonstrated that activation of I_1BS in PC12 cells by moxonidine led to increased levels of diacylglycerol (DAG) and phosphocholine, implicating activation of phosphatidylcholine-specific phospholipase C (PC-PLC) (Separovic *et al*., 1996; 1997). This effect was antagonised by both efaroxan, an I_1BS antagonist, and D609, a specific PC-PLC inhibitor (Separovic *et al*., 1996; 1997). In addition, hypotensive effect of intravenously administered moxonidine was abolished when D609 was microinjected into the rat RVLM (Separovic *et al*., 1997). The same group also reported that moxonidine stimulated release of prostaglandin E2 (Ernsberger *et al*., 1995) and its precursor, arachidonic acid, in PC12 cells via a phospholipase A2 independent pathway (Ernsberger, 1998). This led the author to speculate that arachidonic acid was released by an indirect mechanism possibly from PC-PLC

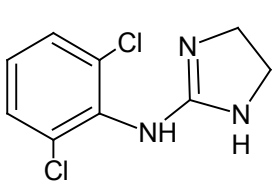
produced DAG (Ernsberger, 1998). Moreover, Zhang *et al*. (2001) reported on the downstream activation of mitogen-activated protein (MAP) kinase following stimulation of I_1BS coupled to PC-PLC. Interestingly, a study by Greney *et al*. in 2000 showed that benazoline, a selective I_1BS ligand (Bruban *et al*., 1999), was able to decrease forskolin induced cAMP levels in cell lines (PC12 and NG10815 cells) expressing I_1BS in a dose dependent manner which contradicted earlier reports (Regunathan *et al*., 1990; Regunathan *et al*., 1991). This indicates that I_1BS may be coupled to multiple signaling pathways and additional work is required to understand these mechanisms.

1.2.4.*Selective ligands of imidazoline-1 binding sites*

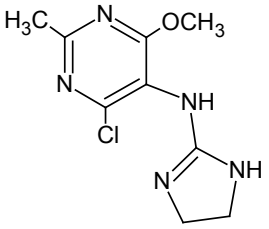
Owing to the lack of established molecular structure of an I_1BS protein, research in this field has heavily relied on synthetic ligands that interact with I_1BS, most of which also exhibit affinity towards α_2-AR with varying degree of selectivity. The prototype clonidine is nonselective in nature and shows high affinity for both α_2-AR (particularly α_{2A}-AR) and I_1BS (Ernsberger *et al*., 1993; Piletz *et al*., 1996). Clinically used new centrally acting antihypertensive agents, rilmenidine and moxonidine (Figure 1.2), exhibit high I_1BS affinity with certain degree of selectivity over α_2-AR (Ernsberger *et al*., 1993; Piletz *et al*., 1996) which is attributed to their improved side effect profiles in comparison to clonidine (Eglen *et al*., 1998).

A number of imidazoline containing compounds have been synthesised so far in order to obtain selective ligands for I_1BS that will aid in characterising functional aspects of this putative receptive site. A series of pyrroline analogues were synthesised by Bousquet laboratory which exhibited high selectivity for I_1BS with almost no affinity for α_2-AR. Of these, LNP 509 and S23515 induced hypotension when given intracisternally in the brain stem of anaesthetised

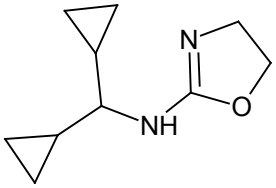
rabbits (Bruban *et al*., 2001; Schann *et al*., 2001). Interestingly, intravenous administration of these compounds did not produce any response possibly due to lack of entry into the brain. Recently reported LNP 630, with high nanomolar affinity for I_1BS and virtually no activity at any of the α_2-AR subtypes, is one of the first in its class to show potent antihypertensive response after intravenous administration into the rats (Schann *et al*., 2012).



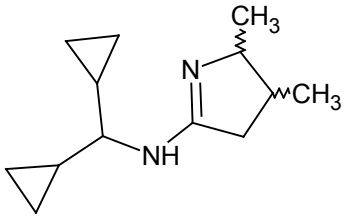
Clonidine



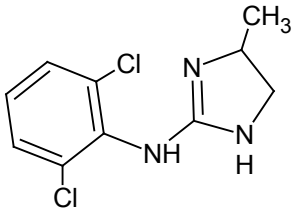
Moxonidine



Rilmenidine



LNP 509



LNP 630

Figure 1.2: Chemical structures of synthetic ligands interacting with I_1BS.

Gentili *et al*. (2003) reported about minor structural modifications in the imidazoline bearing ligands and their effect on receptor selectivity. They identified a high affinity I_1BS antagonist which was also selective over I_2BS along with α_2-AR. Phenyl substituted derivative of this compound was found to maintain selectivity versus I_2BS and α_2-AR but showed agonism at I_1BS (Gentili *et al*., 2005). Such novel ligands will facilitate molecular characterisation of I_1BS and

may also be useful leads in designing newer centrally acting antihypertensive agents with low side effects.

1.2.5. *Functional roles of imidazoline-1 binding sites*

Since the proposition of I_1BS (Bousquet *et al*., 1984), the primary physiological function associated with these putative sites was central regulation of blood pressure. Ample evidence indicates their involvement in eliciting hypotension centrally although controversy remains with regard to their exact mechanism of action. Clonidine-like drugs, given systemically or injected directly into the RVLM, induced hypotension that could be attenuated by microinjection into the RVLM of imidazoline preferring antagonists (idazoxan, efaroxan) whereas non-imidazoline antagonists (SKF 86466, yohimbine) did not have much effect (Ernsberger *et al*., 1990b; Haxhiu *et al*., 1994; Mayorov *et al*., 1993). Moreover, moxonidine and rilmendine, displaying some degree of selectivity for I_1BS over α_2-AR, are associated with lower incidence of adverse effects, namely sedation and dry mouth (Eglen *et al*., 1998). However, Szabo (2002) critically reviewed the mechanism of action of clonidine-like drugs and concluded that their action is best explained *via* interaction with α_2-AR rather than I_1BS. In addition, clonidine-like agents failed to reduce blood pressure in D79N mice, which lack functional α_{2A}-AR (MacMillan *et al*., 1996), indicating the importance of α_{2A}-AR subtype in mediating hypotension (Zhu *et al*., 1999).

Nevertheless, Tolentino-Silva *et al*. (2000) showed that moxonidine microinjected into the RVLM of D79N mice could induce hypotensive effects implicating an I_1BS mechanism. Furthermore, LNP 509 (a highly selective I_1BS ligand) reduced blood pressure in the same α_{2A}-AR deficient D79N mouse model (Bruban *et al*., 2002). A synergistic interaction between α_2-AR and I_1BS in decreasing blood pressure was also observed in the same study. In addition, the

hypotensive response induced by systemic administration of highly selective I_1BS ligand devoid of α_2-AR affinity, LNP 630, implicates strongly a physiological role of I_1BS in regulating blood pressure (Schann *et al*., 2012).

Apart from central regulation of blood pressure, pharmacological studies show that I_1BS are also involved in promotion of renal sodium excretion (Smyth and Penner, 1998) and regulation of intraocular pressure (Ogidigben and Potter, 2002). In addition, I_1BS stimulation in the heart induces release of atrial natriuretic peptide, a potent vasodilator, indicating possible peripheral mechanisms in blood pressure regulation along with central I_1BS (Mukaddam-Daher *et al*., 2006).

1.3. *The imidazoline-2 binding sites*

1.3.1. *Distribution*

Radioligand binding and receptor autoradiography using [^{3}H]idazoxan, [^{3}H]2-BFI or [^{3}H]BU224 demonstrated that I_2BS were found ubiquitously in the central nervous system (CNS) of various species (Anderson *et al*., 2005; MacInnes and Handley, 2005; Mallard *et al*., 1992; Robinson *et al*., 2002; Tesson and Parini, 1991) including glial cells (Martin-Gomez *et al*., 1996). They are located subcellularly in the outer membrane of mitochondria (Tesson and Parini, 1991). I_2BS are also abundant in the peripheral tissues particularly in the kidneys (Tesson *et al*., 1992), liver (Alemany *et al*., 1997; Tesson *et al*., 1992), adipocytes (Langin *et al*., 1990), and platelets (Piletz and Sletten, 1993).

1.3.2. *Proteins containing imidazoline-2 binding sites*

I_2BS are heterogeneous in nature and have been located on a number of proteins. Historically, they are associated with monoamine oxidase (MAO) (Tesson *et al*., 1995), a mitochondrial enzyme responsible for metabolism of monoamines, although there is much debate regarding the functional roles of I_2BS on MAO. High degree of similarity in amino acid sequence was found between I_2BS and MAO subtype A and B (MAO_A and MAO_B) derived from bovine, rat and human (Tesson *et al*., 1995). In addition, the same study demonstrated that transfecting MAO_A and MAO_B in yeast led to coexpression of I_2BS. Imidazoline containing ligands are able to inhibit MAO activity with varying potency (Carpene *et al*., 1995; Lalies *et al*., 1999). However, the binding site of these ligands is perhaps an allosteric site different from the catalytic site of the enzyme interacting with its substrate (Raddatz *et al*., 1997; Tesson *et al*., 1995).

Furthermore I_2BS seems to be predominantly located on MAO_B (Raddatz *et al*., 1997) though autoradiographical studies demonstrated that distribution of specific [^{3}H]2-BFI binding overlapped with those of MAO_A and MAO_B in rat brain (Eglen *et al*., 1998). In a MAO knockout mouse model, [^{3}H]idazoxan failed to label I_2BS when MAO_B expression, but not MAO_A, was blocked supporting a location of I_2BS solely on MAO_B (Remaury *et al*., 2000). In contrast, Anderson *et al*. (2006) showed a loss of I_2BS labelling by [^{3}H]idazoxan and [^{3}H]2-BFI in brain and kidney sections of MAO_A knockout mice. Interestingly it was also observed that the putative IBS endogenous ligand harmane (see section 1.5.3) labelled a subpopulation of non-MAO_A binding sites, suggesting presence of I_2BS in proteins other than MAO.

In fact, work from our group isolated an approximately 45 kDa protein, which was identified later as brain creatine kinase (an enzyme regulating energy homeostatis in cells), from rabbit

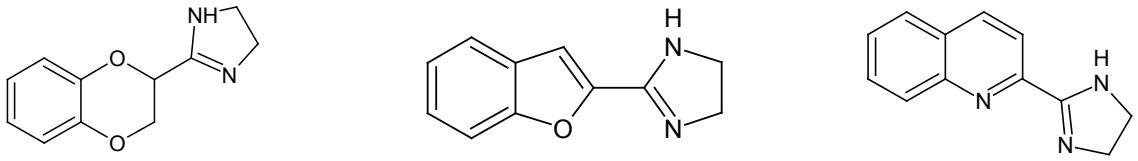
brain using affinity column with 2-BFI, a highly selective I_2BS ligand (Kimura *et al*., 2003). This was reproduced in 2009 - now isolating the same 45 kDa protein from both rabbit and rat brains and identifying it as brain creatine kinase using protein sequencing. Brain creatine kinase was found to exhibit high affinity for [^{3}H]2-BFI and its activity was strongly inhibited by the irreversible I_2BS ligand BU99006, confirming it as a novel protein that contained I_2BS (Kimura *et al*., 2009).

1.3.3. *Selective ligands of imidazoline-2 binding sites*

Like I_1BS, I_2BS research depends mostly on synthetic ligands interacting with these sites for molecular and pharmacological characterisation. However, the downside was that most of these ligands including idazoxan also displayed affinity for α_2-AR. Our group has successfully synthesised two imidazoline bearing ligands: 2-BFI (Hudson *et al*., 1997) and BU224 (Hudson *et al*., 1999a), exhibiting high affinity and selectivity for I_2BS with respect to both α_2-AR and I_1BS which greatly facilitated *in vitro* and *in vivo* studies aimed at elucidating functional roles of I_2BS (Figure 1.3). However, the nature (agonist/antagonist) of these ligands remains to be determined as the molecular structure of I_2BS protein has not yet been cloned. Interestingly a highly selective I_2BS ligand, BU99006 (Figure 1.3), binds irreversibly to I_2BS exhibiting antagonist-like properties (Tyacke *et al*., 2002) which would be useful in further characterising these sites.

Several studies mentioned that the density of I_2BS changes in several disease states which can be either a direct or indirect consequence of a particular disease and can act as a marker for disease state progression (see Table 2 in Garcia-Sevilla *et al*., 1999). Thus there has been considerable effort in developing positron emission tomography (PET) radioligand selective for I_2BS to aid in diagnosis of these disorders. Recent studies from the Nutt laboratory have reported on one such

promising candidate, ^{11}C-BU99008, which has high affinity for I_2BS with good selectivity versus α_2-AR, penetrated into the brain and distributed in I_2BS enriched regions in rhesus monkeys, indicating its potential for imaging I_2BS *in vivo* (Parker *et al*., 2014; Tyacke *et al*., 2012).



Idazoxan 2-BFI BU224

BU99006 BU99008

Figure 1.3: Chemical structures of synthetic ligands interacting with I_2BS.

1.3.4. *Functional roles of imidazoline-2 binding sites*

The functional roles of I_2BS depicted so far in the literature are mostly derived from the responses elicited by using selective I_2BS ligands. Nonetheless I_2BS have been implicated in a number of physiological and pathological conditions and they represent an attractive novel drug target in diverse disease areas.

Our group is particularly interested in the role of I_2BS in modulating monoamine release and turnover in the CNS in relation to psychiatric disorders like depression. The density of I_2BS was found to be altered in postmortem tissue studies from suicide/depressive victims (Garcia-Sevilla *et al*., 1999). In addition, *in vivo* neurochemical studies showed that compounds selective for I_2BS elevated extracellular monoamine levels in different regions of the brain. Using the

technique of *in vivo* microdialysis, our group had previously reported that systemic administration of I_2BS selective ligands such as 2-BFI, BU224, and BU98008 in rats increased extracellular noradrenaline in prefrontal cortex (Abu Ghazaleh *et al*., 2007; Hudson *et al*., 1999a; Nutt *et al*., 1997) and hippocampus (Lalies and Nutt, 1995), dopamine in striatum (Hudson *et al*., 1999a) and serotonin in dorsal raphe nucleus (Ugedo *et al*., 1999). However, the mechanism through which I_2BS selective ligands elevate extracellular monoamines in the brain is not yet clear. A probable explanation may be inhibition of the monoamine metabolising enzyme, MAO, though controversy remains. As mentioned earlier, imidazoline containing ligands like 2-BFI and BU224 inhibit MAO activity albeit with low potency in micromolar range (Carpene *et al*., 1995; Lalies *et al*., 1999). However, the highly selective irreversible I_2BS ligand, BU99006, failed to inhibit the activity of MAO (Paterson *et al*., 2003). Therefore, further studies must be carried out to find out the underlying mechanism behind such modulation.

Nevertheless, these *in vivo* findings mentioned above are further complemented by the results obtained in different behavioural paradigms. For instance, Finn *et al*. (2003) reported that BU224 significantly reduced immobility time of rats in the Porsolt forced swim test (FST) which was indicative of antidepressant-like behaviour. Similar decrease in immobility time of mice in the same behavioural model caused by pretreatment with 2-BFI was also demonstrated recently (Tonello *et al*., 2012). Although a previous study reported to find no antidepressant-like activity of BU224 in the FST in mice (O'Neill *et al*., 2001), combining overall *in vivo* and behavioural results suggest I_2BS as a potential novel drug target for the treatment of depression.

Besides elevating central monoamine levels, I_2BS is associated with modulation of pain and opioid addiction (see Li and Zhang, 2011). Both 2-BFI and BU224 exhibited antinociceptive effects in rat model of acute pain which was inhibited by nonselective imidazoline I_2/α_2

antagonist, idazoxan, but not by nonimidazoline α_2-AR antagonist, yohimbine (Li *et al*., 2011). Similar response was obtained with 2-BFI, but not BU224, in another rat model for acute phasic pain (Sampson *et al*., 2012). Another selective I_2BS ligand CR4056 demonstrated effective analgesic activity in both inflammatory and neuropathic pain models of rats (Ferrari *et al*., 2011). CR4056, one of the first in its class as a potential I_2BS analgesic agent, is currently under Phase I clinical trial to assess its safety in humans (Ferrari *et al*., 2011). In addition 2-BFI, BU224 and phenyzoline (another selective I_2BS ligand) were reported to potentiate morphine-induced analgesia when used in combination (Gentili *et al*., 2006; Li *et al*., 2011; Thorn *et al*., 2011) and the interaction between morphine and I_2BS selective ligands was synergistic (Li *et al*., 2011; Thorn *et al*., 2011). Moreover, chronic administration of 2-BFI with morphine inhibited the development of tolerance to morphine analgesia (Boronat *et al*., 1998). Furthermore, BU224 was capable of alleviating some of the physical symptoms associated with naltrexone induced morphine withdrawal syndrome (Hudson *et al*., 1999a). Therefore selective I_2BS ligands, alone or as adjunct to opioids, have potential in pain treatment and opioid addiction management.

In addition, I_2BS have been implicated in neurodegenerative diseases like Alzheimer's (Ruiz *et al*., 1993) and Huntington's diseases (Reynolds *et al*., 1996) and recently reported to provide neuroprotection in a rat stroke model (Han *et al*., 2010).

1.4. *Proposed endogenous ligands of imidazoline binding sites*

Given the discrete pharmacological existence of IBS, the search for endogenous ligands for these sites followed naturally since identification of endogenous ligands might produce leads with improved selectivity profiles that could be developed further as novel therapeutic agents. Several

compounds have been identified so far as endogenous IBS ligands including clonidine displacing substance (CDS), agmatine, harmane (a β-carboline), and imidazoleacetic acid-ribotide.

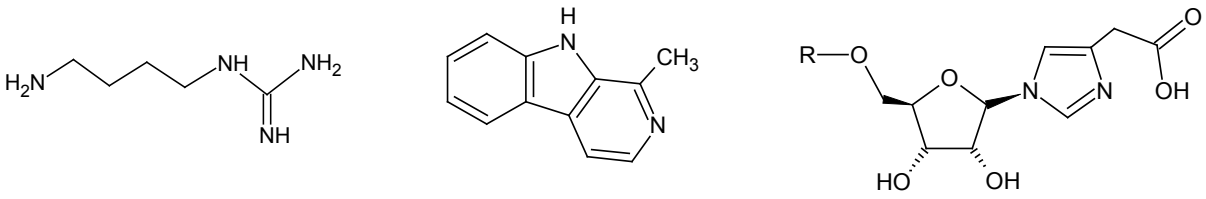
1.4.1. *Clonidine displacing substance – Endogenous ligand for IBS*

The first compound(s) suggested to be a likely candidate for endogenous ligand of IBS was clonidine displacing substance (CDS), an extract isolated and partially purified from rat and calf brains, which competitively displaced specific [^{3}H]clonidine binding in rat brain membranes, and hence the naming (Atlas and Burstein, 1984a, 1984b). In addition, CDS was found to be inactive at α_1- and β-adrenergic receptors (Atlas and Burstein, 1984a, 1984b). In 1986, Meeley and coworkers confirmed the presence of CDS-like substance in bovine brain (Meeley *et al*., 1986). Initially it was thought that CDS contained an endogenous clonidine-like substance that acted at α_2-AR. Subsequent experiments showed that similar to clonidine partially purified CDS extracts caused contraction of rat aorta (Synetos *et al*., 1991) and inhibition of rat *vas deferens* contraction (Diamant and Atlas, 1986) and of human platelet aggregation (Diamant *et al*., 1987) – all the effects attributed to activation of α_2-AR.

Further studies showed CDS could displace catecholamine insensitive [^{3}H]*p*-aminoclonidine binding in bovine ventrolateral medulla membranes, demonstrating a probable link to I_1BS (Meeley *et al*., 1986). However, when it was microinjected into the RVLM of rats, different groups reported contradictory results observing both increase (Atlas *et al*., 1987) and decrease (Meeley *et al*., 1986) in arterial blood pressure. In addition, CDS was found to inhibit binding of [^{3}H]idazoxan and [^{3}H]2-BFI to rabbit renal basolateral and rat brain membranes, respectively, indicating possible interaction with I_2BS (Coupry *et al*., 1990; Parker *et al*., 1999). Moreover, Chan *et al*. (1997) demonstrated enhancing effect on insulin secretion by CDS in rat islets of

Langerhans similar to efaroxan (I_3BS agonist) which was blocked by KU14R (I_3BS antagonist), further linking CDS to I_3BS activity.

Despite many efforts in characterising CDS as an endogenous ligand of IBS, its identity still remains elusive. As yet, it is known that CDS has low molecular mass that chemically is neither a primary amine (e.g. a catecholamine) nor a peptide (Atlas and Burstein, 1984a, 1984b), and has been detected in the brain (Atlas and Burstein, 1984a, 1984b; Meeley *et al*., 1986) and various peripheral tissues (Hensley *et al*., 1989; Meeley *et al*., 1992). Differences in biological activity exhibited by CDS may be accounted for usage of different tissue sources and slight variations in extraction processes that result in variations in CDS extract (crude or partially purified) composition of its active principle(s) and impurities. This is illustrated in two recent studies by Pinthong and coworkers who demonstrated bovine lung and brain derived CDS may response differently in the same functional model and that methanolic extracts of CDS, devoid of monovalent cations and histamine (impurities), failed to elicit any α_2-AR responses (Pinthong *et al*., 2003a; Pinthong *et al*., 2003b). Thus a more uniform approach in characterising CDS and isolating its active principles is required to confirm its identity. Nevertheless, to date three separate compounds (Figure 1.4) have been proposed to be the endogenous ligand of IBS, two of which isolated from CDS extracts, that will be detailed in the following sections.



Agmatine Harmane Imidazoleacetic acid-ribotide (R = PO_3H)

Figure 1.4: Chemical structures of proposed endogenous ligands for IBS.

1.4.2. *Agmatine*

Attempts to isolate and characterise the active component of CDS led to the detection of agmatine as a bioactive compound in mammalian brain (Li *et al*., 1994). Agmatine, which is synthesised from decarboxylation of L-arginine by arginine decarboxylase (ADC) and metabolised in several pathways, fulfilled not all but some of the criteria for a CDS. It was found in the same study that agmatine was capable of binding to both α_2-AR and I_1- and I_2BS though with low micromolar affinities and could stimulate catecholamine release from adrenal chromaffin cells which was attributed to interaction with IBS (Regunathan *et al*., 1993). Moreover, Li *et al*. (1994) demonstrated for the first time that ADC, the biosynthetic enzyme of agmatine, was present in rat brain. However, subsequent studies with agmatine did not produce much convincing evidence to confirm it as an endogenous ligand of IBS. For instance, when administered centrally agmatine failed to elicit characteristic fall in blood pressure as seen with clonidine or moxonidine (Head *et al*., 1997; Sun *et al*., 1995).

Although agmatine and CDS appears to behave as different entities with regard to current lines of evidence (Raasch et al., 2001), agmatine has its own biological actions at several molecular targets (including IBS) that may be useful therapeutically in the treatment of multiple diseases as recently reviewed by Piletz *et al*. (2013). Among many of its modulatory functions, agmatine is found to increase insulin secretion from pancreatic β-cells (Sener *et al*., 1989), enhance morphine induced analgesia (Thorn *et al*., 2011) and may regulate dependence and tolerance to chronic morphine use (Aricioglu-Kartal and Regunathan, 2002), precipitate antidepressant and anxiolytic effects (Aricioglu and Altunbas, 2003a) and has also been proposed as a novel neurotransmitter (Piletz *et al*., 2013).

1.4.3. *Harmane*

The β-carboline harmane[#] was proposed to be a putative endogenous ligand for IBS by Hudson *et al*. (1999b) where it was shown to bind with good affinity to both I_1- and I_2BS in low nanomolar range in rat kidney and brain membranes, respectively. In addition harmane displayed low affinity for α_2-AR in rat whole brain membranes (Husbands *et al*., 2001). The same group successively isolated harmane as an active component of purified CDS derived from bovine lung (Parker *et al*., 2004). Presence of β-carbolines like harmane has been shown in mammalian tissues including the brain (Robinson *et al*., 2003) although the exact biosynthetic pathway is not yet fully elucidated.

Unlike agmatine, harmane produced dose dependent hypotensive effect, similar to clonidine, when microinjected into the rat RVLM. This effect was blocked by efaroxan (I_1/α_2 antagonist) indicating possible I_1BS interaction (Musgrave and Badoer, 2000). Hamane is a potent inhibitor of monoamine oxidase subtype A (MAO_A) enzyme (Rommelspacher *et al*., 1994) and, similar to I_2BS selective ligands 2-BFI and BU224 (Nutt *et al*., 1997), could modulate monoamine levels in specific brain regions (Adell *et al*., 1996). Moreover, harmane demonstrated both antidepressant and anxiolytic effects and critically modulated learning and memory functions in rats in different behavioural models, though further studies are needed to confirm any I_2BS interaction in mediating these responses (Aricioglu and Altunbas, 2003b; Celikyurt *et al*., 2013). Aricioglu-Kartal *et al*. (2003) also reported that harmane attenuated the severity of the signs of naloxone precipitated morphine withdrawal syndrome in rats. In addition, harmane induced insulin release from human pancreatic islet cells (Cooper *et al*., 2003; Morgan *et al*., 2003). Although the response differed from that produced by efaroxan, it was inhibited by KU14R suggesting I_3BS action along with other possible mechanisms (Squires *et al*., 2004). Hence, harmane appears to

[#]referred sometimes as harman in the literature

be a strong candidate and requires further characterisation to establish it as an endogenous ligand for IBS.

1.4.4. *Imidazoleacetic acid-ribotide*

The phosphoribosyl-pyrophosphate conjugate of imidazole acetic acid called imidazoleacetic acid-ribotide (IAA-RP) has recently been proposed as an endogenous mediator of IBS owing to some degrees of similarity in physiocochemical properties between IAA-RP and yet-unidentified substance(s) in CDS (Prell *et al*., 2004). IAA-RP is found in mammalian brain including the brainstem and, in particular, in the RVLM (Friedrich Jr. *et al*., 2007; Prell *et al*., 2004). Like a neurotransmitter, it exhibits Ca^{2+}-dependent release from synaptosomes (Prell *et al*., 2004). A recent study demonstrated its modulatory role in hippocampal synaptic transmission which is thought to be mediated via activation of IBS (Bozdagi *et al*., 2011). Prell and coworkers (2004) further showed that IAA-RP displaced [^{3}H]clonidine binding from adrenal medulla I_1BS albeit with low micromolar affinity and released arachidonic acid from PC12 cells – an I_1BS mediated response (Ernsberger, 1998). Moreover, it induced insulin secretion from rat and human pancreatic islet cells which was blocked by KU14R, corresponding to I_3BS function. However, microinjection of IAA-RP into rat RVLM caused hypertension dissimilar to the well characterised action of clonidine at RVLM (Bousquet *et al*., 1984). Besides no report has yet linked I_2BS functions with those of IAA-RP. Thus further studies are required to establish IAA-RP was a candidate for endogenous ligand of IBS.

2. α_2-Adrenoceptors

Adrenoceptors are plasma membrane receptors that belong to the seven transmembrane spanning family of G-protein coupled receptors and mediate physiological actions of the endogenous catecholamines, adrenaline and noradrenaline. Pharmacologically they are categorised into distinct α_1-, α_2- and β-adrenoceptors (Docherty, 1998). Of these, α_2-adrenoceptors (α_2-AR) constitute one of the most important receptor families mediating multiple biological responses of catecholamines and have been implicated in a number of pathophysiological conditions. Additionally the concept of IBS stemmed from work on α_2-AR pharmacology. Consequently, besides IBS our group is interested in imidazoline and related compounds that have the potential to interact with α_2-AR since both the receptor systems pose to be attractive drug targets in diverse therapeutic areas, particularly in the treatment of psychiatric disorders.

α_2-AR are widely distributed in almost all tissues centrally and peripherally and located on both pre- and postsynaptic membranes (Docherty, 1998; Robinson and Hudson, 2006). These cell surface receptors have well characterised signal transduction mechanism and second messenger system, (see Hein, 2006). α_2-AR are coupled to G-proteins of the $G_{i/o}$ type. Binding of endogenous agonist, adrenaline or noradrenaline, results in activation of this inhibitory G-protein which causes inhibition of adenylyl cyclase, decreasing cAMP production in the cell. Other effects include inhibition of voltage gated Ca^{2+} channels and activation of inwardly rectifying K^+ channels and MAP kinases, ERK1/2. Activation of α_2-AR results in various physiological functions including regulation of blood pressure, modulation of insulin release, sedation, analgesia, and thermogenesis (Robinson and Hudson, 2006). In addition, presynaptic α_2-AR acting as auto- and/or heteroreceptors inhibit the release of monoamine neurotransmitters

(noradrenaline, dopamine, and serotonin) from nerve terminals by a negative feedback loop (Robinson and Hudson, 2006).

2.1. *Subtypes of α_2-adrenoceptors*

α_2-AR are heterogeneous in nature. Using molecular biological and cloning techniques, three subtypes have been identified so far along with the genes encoding for the proteins – α_{2A} (Kobilka *et al*., 1987), α_{2B} (Lomasney *et al*., 1990) and α_{2C} (Regan *et al*., 1988). α_{2D}-AR represent the species ortholog of human α_{2A}-AR (Bylund *et al*., 1994). While all three subtypes are present in the peripheral tissues, brain expresses mainly α_{2A}- and α_{2C}-AR (Robinson and Hudson, 2006). Between the two, α_{2A}-AR is the predominant subtype widely distributed throughout the brain, primarily in the frontal cortex, brainstem, hippocampus, locus coeruleus and dorsal horn of the spinal cord (Talley *et al*., 1996; Uhlén *et al*., 1997). In addition, genetic study with α_2-AR subtype selective knockout mice revealed that the main presynaptic α_2-AR are of α_{2A} subtype, although some α_{2C}-AR are also present (Trendelenburg *et al*., 2001).

2.2. *Functional roles of α_2-adrenoceptors in the CNS*

As mentioned earlier, α_2-AR are involved in a multitude of biological activities although it is difficult to render functions specific to a particular subtype owing to the lack of subtype selective ligands. However, genetic approach with α_2-AR subtype(s) specific knockout mice models has greatly aided in identifying the functional roles of each subtype (Link *et al*., 1996; MacMillan *et al*., 1996). These models also revealed that most of the physiological and pharmacological functions of α_2-AR are mediated principally through α_{2A}-AR subtype (MacMillan *et al*., 1996; Lakhlani *et al*., 1997).

One of the crucial functions mediated by α_2-AR (as auto- or heteroreceptors) in the central nervous system is the modulation of neurotransmitter release. α_2-AR agonists such as clonidine activate the autoreceptors on noradrenergic cell bodies and presynaptic membrane resulting in reduced cell firing rates (Cedarbaum and Aghajanian, 1977) and inhibition of neurotransmitter release from nerve terminals (L'Heureux *et al*., 1986; Maura *et al*., 1992), respectively. In contrast, α_2-AR antagonists like idazoxan and yohimbine block this inhibitory effect and elevate extrasynaptic noradrenaline levels in the brain (Dennis *et al*., 1987; Thomas and Holman, 1991). In addition, presynaptic α_2-AR acting as heteroreceptors exert inhibitory effects on the release of other monoamines (serotonin and dopamine) from nerve terminals (Tao and Hjorth, 1992; Gresch *et al*., 1995). Studies with functionally deficient α_{2A}-AR mice showed that α_{2A}-AR mainly mediate these regulatory effects (Altman *et al*., 1999; Trendelenburg *et al*., 2001). However, presynaptic α_{2C}-AR may also participate in the inhibition of neurotransmitter release (Hein *et al*., 1999; Trendelenburg *et al*., 2001) and have been identified as the major regulatory receptor in the release of adrenaline from chromaffin cells in adrenal medulla (Brede *et al*., 2003).

The regulatory role of α_2-AR in releasing neurotransmitters is particularly interesting to our group owing to its pharmacological implication in the field of psychiatric disorders like depression. Most antidepressant drugs function by modulating brain monoamine levels particularly serotonin and noradrenaline (Elhwuegi, 2004). Mirtazapine, an α_2-AR antagonist, is used clinically in the treatment of depression (Elhwuegi, 2004). Therefore, selective imidazoline bearing ligands interacting with α_2-AR may be useful as antidepressants.

Apart from controlling neurotransmitter release, α_2-AR are classically implicated in the central regulation of blood pressure by activating sympathoinhibitory pathway at the level of brainstem

although there is much debate regarding the role of I_1BS in producing hypotension (Szabo, 2002). α_{2A}-AR are the primary mediators of central hypotensive effects while peripherally α_{2B}-AR cause vasoconstriction, producing the characteristic of antihypertensive agents acting *via* α_2-AR : initial hypertensive phase (by α_{2B}-AR) followed by long lasting hypotension (by α_{2A}-AR) (Link *et al*., 1996; MacMillan *et al*., 1996; Altman *et al*., 1999). Clonidine is the prototypical centrally acting antihypertensive drug with both α_2-AR and I_1BS affinity (Ernsberger *et al*., 1987). Though selective to certain degree for I_1BS, both moxonidine and rilmenidine also exhibit high nanomolar affinities for α_2-AR (Ernsberger *et al*., 1993; Piletz *et al*., 1996). Recent work from our laboratory identified a highly selective imidazoline ligand, marsanidine, and its 7-methyl analogue which are potent hypotensive agents (Sączewski *et al*., 2008) with diuretic and natriuretic properties (Wróblewska *et al*., 2013) and can serve as useful leads in the development of centrally acting antihypertensive agents.

In addition α_2-AR, particularly α_{2A}-AR, are attributed to the sedative effects produced by α_2-AR agonists (Lakhlani *et al*., 1997) which may be useful in intensive care as sedative, hypnotic and analgesic (Sanders and Maze, 2007). However, sedation may also pose a disturbing side effect of the α_2-cardiovascular agents (Timmermans and van Zwieten, 1982).

3. Aims of the project

Characterisation of IBS with selective ligands is crucial for understanding their possible roles as novel drug targets. Therefore, the primary aim of this project is to explore the structure-affinity relationships (SAR) of several series of novel imidazoline compounds with regard to activity at both α-adrenoceptors (α-AR) and IBS. One of these new series investigated herein was synthesised by Professor Maria Pigini's group in Italy (designated as MP series). In these

compounds the effect of minor structural modifications on preferential recognition of a particular receptor system was investigated.

The other series of compounds studied in this project were synthesised by Dr Franciszek Sączewski's group in Poland. Of these, compounds in the TCS/TCA series were derivatives of a previously characterised highly selective α_2-AR ligand, marsanidine (Sączewski *et al*., 2008). In these TCS/TCA marsanidine derivatives, the effects of incorporating various substituents into the heteroaromatic ring with regard to α_1-/α_2-AR and IBS affinity and selectivity were explored.

During the course of the project, an interesting observation made in TCS/TCA series was that ligands containing halogen substituents retained good affinity for α_2-AR. Inspired by this observation, a further "AW series" of compounds was synthesised by Sączewski's group in an attempt to identify a ligand with high affinity and selectivity for α_2-AR which can be further developed as a potential α_2-AR selective PET radiotracer. Thus in this AW series the influence of fluorination at different positions of the heteroaromatic rings for α_2-AR affinity and selectivity was investigated.

One final aspect of this project was to further evaluate the most α_2-AR selective ligand in AW series *in vivo* where its effects on extracellular monoamine levels in specific brain regions were studied in conscious freely moving rats using brain microdialysis. This would also help determine whether this compound will have the potential to be developed as a ligand for future PET studies to image central α_2-AR *in vivo*.

Chapter II: Structure-Affinity Relationship Study of MP Series of Compounds

Introduction

The concept of imidazoline binding sites (IBS) had been derived in the early 1980s from studies involving the mechanism of actions at α_2-AR of clonidine-like hypotensive drugs (Head and Mayorov, 2006). Subsequently, it was demonstrated that the antihypertensive effects mediated by imidazoli(di)ne containing agents like clonidine and its analogues were not only due to activation of α_2-AR in the brainstem but also due to interactions with a distinct group of imidazoline-preferring nonadrenergic binding sites that were not activated by catecholamines (Bousquet *et al*., 1984). Since then many developments were made in the field of imidazoline receptor research in terms of identifying proteins containing IBS, endogenous and selective synthetic ligands for IBS and their diverse roles in normal physiology and during disease states (reviewed in Chapter I). To date, pharmacological studies have characterised at least three distinct subtypes of IBS – I_1, I_2 and I_3 – based on physiological functions and binding affinities to different radioligands. Several compounds have been proposed as the endogenous ligand of IBS including agmatine (Li *et al*., 1994), harmane (Parker *et al*., 1999), and imidazoleacetic acid-ribotide (Prell *et al*., 2004).

Among the subtypes of IBS I_1BS, which are labelled preferentially by [^{3}H]clonidine and [^{3}H]-*p*-aminoclonidine (Dardonville and Rozas, 2004), are localised in synaptic plasma membranes (Heemskerk *et al*., 1998) and involved in the regulation of blood pressure (Head and Mayorov, 2006). The prototypical clonidine, used clinically as an antihypertensive agent, displays good affinity towards both I_1BS and α_2-AR (particularly α_{2A}-AR subtype) (Piletz *et al*., 1996) and lowers blood pressure by acting centrally at the rostral ventrolateral medulla (RVLM) in the

brainstem (Bousquet *et al*., 1984). However clonidine exhibits side effects like sedation and dry mouth attributed to its interaction with α_2-AR (Eglen *et al*., 1998; Timmermans and van Zwieten, 1982). Incidence of such side effects is reduced with the clinical use of antihypertensives like rilmenidine and moxonidine (Head and Mayorov, 2006) which, although bind with good affinity to both α_2-AR and I_1BS, are relatively more selective for I_1BS (Ernsberger *et al*., 1993; Piletz *et al*., 1996). Recent studies have identified two highly selective I_1BS ligands (LNP 509 and LNP 630) which exert potent hypotensive effects *in vivo* despite lacking affinity for α_2-AR, thus highlighting important role played by I_1BS in regulating blood pressure (Schann *et al*., 2001; Schann *et al*., 2012). Therefore development of highly selective ligands for I_1BS over α_2-AR may prove to be beneficial in better managing cardiovascular disorders.

In contrast to I_1BS, I_2BS are recognised preferentially by [^{3}H]idazoxan (Dardonville and Rozas, 2004) and located mainly on the outer mitochondrial membrane in central and peripheral tissues (Tesson and Parini, 1991; Tesson *et al*., 1992). I_2BS have been identified thus far on several crucial proteins including both isoforms of monoamine oxidase (MAO_A and MAO_B) as allosteric sites (Tesson *et al*., 1995) and brain creatine kinase (Kimura *et al*., 2009). Previous *in vivo* microdialysis studies have shown I_2BS selective ligands like 2-BFI (Hudson *et al*., 1997) and BU224 (Hudson *et al*., 1999a) to elevate extracellular levels of noradrenaline in rat frontal cortex (Nutt *et al*., 1997) and reduce immobility times in Porsolt forced swimming paradigm (Finn *et al*., 2003; Tonello *et al*., 2012). In addition, several lines of studies have provided evidence about the involvement of I_2BS in chronic and neuropathic pain and modulation of morphine analgesia as well as tolerance and opioid addiction (Li and Zhang, 2011). Hence ligands acting via I_2BS appear to have therapeutic potential in the treatment of diverse diseases like depression, pain, opioid addiction etc to name a few.

Although IBS appear to be attractive drug targets, their molecular and pharmacological characterisation are hindered owing to the fact that most of the ligands used lack in selectivity with respect to α_2-AR and also to the two main IBS subtypes, I_1 and I_2. Selective ligands for I_2BS investigation like 2-BFI and BU224 were reported previously (Hudson *et al*., 1997; Hudson *et al*., 1999a). Much effort has been devoted by Pigini and coworkers to obtain IBS selective ligands and elucidate structure-activity relationships (SAR) influencing affinities with respect to α- and imidazoline receptor systems. Some of the early studies reported that removal of the cyclopropyl ring of cirazoline, which is an imidazoline containing α_1-AR agonist/α_2-AR antagonist (Ruffolo and Waddell, 1982) with high I_2BS affinity (Pigini *et al*., 1997), did not affect the affinity for I_2BS but reduced that for α_1-AR showing that simple structural modifications might lead to separation of α-AR and IBS activity (Brasili *et al*., 1995). This is followed by a series of intense SAR studies exploring biological characteristics of the molecular structure illustrated in Figure 2.1 which contains an imidazoline ring substituted at position 2 by the bridge and an aromatic ring. Indeed, several studies have reported that minor chemical modifications in the bridge affected preferential recognition of a particular receptor system while substitution of the aromatic ring affected ligand affinity and functional activity.

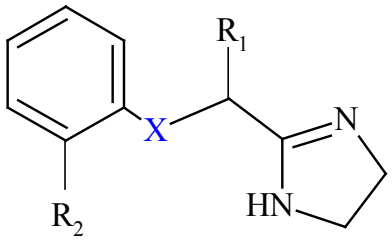


Figure 2.1: Basic structure of the compounds in MP series consisting of an imidazoline nucleus connected to the aromatic ring by a two-atom bridge. R_1 and R_2 are substituents on bridge and aromatic ring respectively. X can be O, N, S, C etc.

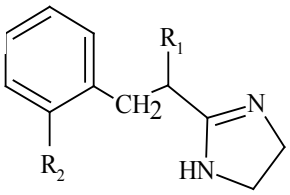
Previous studies have shown that in the cirazoline derivative described above which is also an α_2-AR antagonist (Gentili *et al*., 2002), replacing O with –NH in the bridge resulted in compounds with similar affinities at both α_2-AR (K_i = 72.4 nM) and I_2BS (K_i = 33.1 nM) (Pigini *et al*., 1997). Drastic reduction in I_2BS affinity (K_i = 2692 nM) was observed with –OCH(CH_3) bridge in the cirazoline derivative (Gentili *et al*., 2002). Interestingly, in this derivative of cirazoline (with or without a CH_3 substitution in the bridge) adding a phenyl ring in the ortho position of the aromatic ring changed the functional profile at α_2-AR, yielding an agonist (Gentili *et al*., 2002). On the other hand, replacing O in the bridge with isosteric –CH_2 abolished α_1-AR activity and reduced affinity for α_2-AR. The resulting compound phenyzoline displays increased selectivity for I_2BS over α_2-AR and also with respect to I_1BS (I_2BS K_i = 2.5 nM; α_2/I_2 = 794; I_1/I_2 = 1479) (Gentili *et al*., 2003; Pigini *et al*., 1997).

Gentili *et al*. (2003) carried out further modification with a CH_3 substitution on C-1 of phenyzoline's ethylene bridge which increased affinity for I_1BS (K_i = 5 nM) while decreasing that for I_2BS (K_i = 933 nM), thus producing a highly selective I_1BS ligand over I_2BS (I_2/I_1 = 186) and also α_2-AR (α_2/I_1 = 708). In addition when this compound was resolved into its two optical isomers, the (*S*)-(–) enantiomer exhibited high affinity and selectivity for I_1BS versus I_2BS whereas the (*R*)-(+) isomer only showed poor binding affinity towards both I_1BS and I_2BS. These results of reversed enantioselectivity highlighted stereospecific requirements with respect to I_1BS and I_2BS binding. In contrast to the changes made above, inserting a double bond in the bridge and thereby limiting conformational freedom leads to a compound named tracizoline that has high affinity for I_2BS (K_i = 1.82 nM) with unprecedented high selectivity over α_2-AR (α_2/I_2 = 7762) (Pigini *et al*., 1997). Furthermore Pigini's group has recently demonstrated that ligands bearing the common pharmacophores in Figure 2.1, which were rationally designed to interact

with both α_2-AR and I_2BS, represent novel multifunctional tools that may be useful therapeutically in the management of opioid withdrawal and associated depressive disorder, possibly with lower side effects (Del Bello *et al*., 2013).

Given the therapeutic potential of ligands which are either selective for α_2-AR or a particular subtype of IBS or exhibiting multitarget interaction, in the current study we aim to investigate a diverse series of imidazoline bearing compounds of the type shown in Figure 2.1. On the basis of previous observations in SAR studies of this particular basic molecular structure, Dr Maria Pigini's group (University of Camerino, Italy) rationally designed these new ligands (MP series illustrated in Figure 2.2) containing minor modifications in the bridge and/or different substituents on the aromatic ring with the aim to obtain novel compounds highly selective for I_1BS or ones that display multitarget activity. Therefore in this chapter we sought to explore potential biological activities of compounds in MP series by assessing their affinities and hence selectivity for both receptor systems: α-adrenoceptors (α_1-AR and α_2-AR) and IBS (I_1BS and I_2BS) using *in vitro* radioligand binding assays in rat whole brain and kidney membranes.

A)



MP 108: X = CH_2, R_1 = CH_3, R_2 = H

MP 512: X = CH_2, R_1 = CH_3, R_2 = CH_3

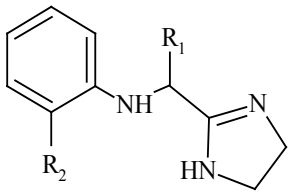
MP 513: X = CH_2, R_1 = CH_3, R_2 = 

MP 514: X = CH_2, R_1 = CH_3, R_2 = 

MP 515: X = CH_2, R_1 = CH_3, R_2 = 

MP 516: X = CH_2, R_1 = CH_3, R_2 = Cl

B)



MP 51: X = NH, R_1 = H, R_2 = H

MP 96: X = NH, R_1 = H, R_2 = 

MP 133: X = NH, R_1 = CH_3, R_2 = H

MP 960: X = NH, R_1 = H, R_2 = CH_3

MP 961: X = NH, R_1 = H, R_2 = Cl

MP 970: X = NH, R_1 = H, R_2 =

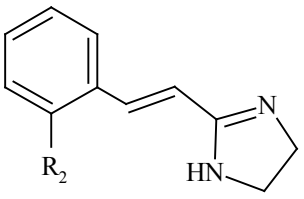
$CH_2CH{=}CH_2$

MP 971: X = NH, R_1 = H, R_2 =

$CH_2CH_2CH_3$

Figure 2.2: Chemical structures of imidazoline bearing compounds in MP series. A) Compounds containing $CH_2CH(CH_3)$ bridge; B) Compounds containing $NHCH_2$ bridge (except MP 133)

C)



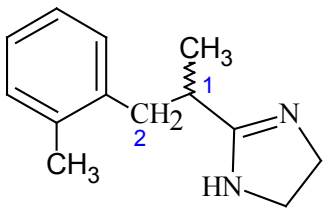
MP 56: R_2 = Cl

MP 957: R_2 = $CH_2CH{=}CH_2$

MP 958: R_2 = 

MP 972: R_2 = CH_3

D)



MP (+/–) 512

Figure 2.2 (contd.): Chemical structures of imidazoline bearing compounds in MP series. C) Compounds containing carbon-carbon double bond in the bridge; D) MP (+/–) 512: stereoisomers showing the chiral centre at C-1 on bridge.

Materials and Methods

Preparation of rat whole brain and kidney crude P2 membranes

Rat whole brain and kidney crude P2 membranes were prepared according to the procedure by Lione *et al*. (1998). All procedures were carried out at 4°C unless otherwise stated. Whole brain and/or kidneys were obtained postmortem from Sprague-Dawley rats (male, 250-300 g) and homogenised in 10 volumes (w/v) of ice cold sucrose buffer (50 mM Tris-HCl, 320 mM sucrose and 1 mM $MgCl_2$, pH 7.4) using a Polytron homogeniser (Polytron PT 1020350D, Brinkmann Instruments). The homogenates were centrifuged (Sorvall RC-5B Plus) at 1000 *x* g for 10 minutes and the precipitate discarded. The supernatant was recentrifuged at 32000 *x* g for 20 minutes to yield supernatant, which was discarded, and crude membrane pellet (P2). The resulting pellets were resuspended in 30 mL of ice cold assay buffer (50 mM Tris-HCl and 1mM MgCl2, pH 7.4) and spun at 32000 *x* g for 20 minutes. In this way the P2 membrane pellets (brain or kidney) were washed twice and finally they were stored at -80°C until use. Prior to radioligand binding studies, the membrane pellets were thawed, resuspended in assay buffer at room temperature and washed two more times by centrifuging at 32000 *x* g for 20 minutes at 4°C to remove any possible endogenous inhibitors of binding.

In vitro radioligand competition binding assays

Competition radioligand binding assays were employed to assess the affinities of the newly synthesised MP compounds (Figure 2.2) for four different receptors: α_1- and α_2-AR, I_1BS and I_2BS. Rat whole brain membranes were used to carry out α_1- and α_2-AR and I_2BS binding assays whereas rat kidney membranes were used for I_1BS assays. The membrane pellet (brain or kidney) was finally resuspended in assay buffer (50 mM Tris-HCl and 1mM $MgCl_2$, pH 7.4) at

room temperature. Rat brain membrane aliquots (400 µL) were incubated across a range of concentrations (0.1 nM to 100 µM) of the displacing ligand (test compound) in presence of receptor specific radioligand (1nM), [^{3}H]prazosin (α_1-AR), [^{3}H]RX821002 (α_2-AR) and [^{3}H]2-BFI (I_2BS) in a final volume of 500 µL. Nonspecific binding was determined by 10 µM of phenylephrine (α_1-AR), rauwolsine (α_2-AR) and BU224 (I_2BS). In case of I_1BS binding assays, [^{3}H]clonidine (3 nM) was bound to rat kidney membrane aliquots in presence of rauwolsine (10 µM) to mask any α_2-AR binding; nonspecific binding was determined by rilmenidine (10 µM). Under these conditions, the sites labeled in the kidney membrane represent a model of central I_1BS binding sites (Parker *et al*., 1998).

Incubation for each concentration of the test compound was carried out in triplicates at room temperature and allowed to reach equilibrium for 45 minutes. Bound and free ligands were separated by rapid filtration through presoaked (0.5% polyethyleneimine in distilled water) glass fiber filters (Whatman GF/B, Brandel Inc) using a cell harvester (Brandel M-24). Filters were washed twice with 5 mL of ice cold assay buffer. Membrane bound radioactivity remaining in the filters was then measured in a liquid scintillation counter (Beckmann LS 6500) by adding 3 mL of scintillation cocktail (Ecolite, MP Biomedicals).

Statistical Analysis

Binding data for individual experiments were analysed by iterative nonlinear regression curve fitting procedures using GraphPad Prism for Windows version 5.01 (GraphPad Software, San Diego, CA) to yield IC_{50} values (concentration of test compound needed to displace 50% specific binding of the radioligand). Where appropriate K_D (equilibrium dissociation constant) values of

the specific radioligands were available, IC_{50} values of displacing ligands were converted to K_i (inhibition constant) using the Cheng-Prussof equation given below (Cheng and Prusoff, 1973).

$$K_i = IC_{50} / \{1+ ([L]/K_D)\}$$

where [L] and K_D refer to the radioligand

Affinity values of the test compound for each receptor were finally expressed as mean ± S.E.M of three to four separate experiments.

Materials

The following chemicals were purchased: Tris-HCl base, polyethyleneimine, (R)-(-)-phenylephrine HCl, and rauwolsine HCL (Sigma-Aldrich); sucrose (Fisher Scientific, New Jersey, USA); $MgCl_2$ hexahydrate (EMD Chemicals, Darmstadt, Germany). BU224 (2-(4,5-dihydroimidaz-2-yl)-quinoline) was synthesised by Dr Stephen Husbands (University of Bath, UK). Rilmenidine was a kind gift from Servier Laboratories, France.

[^{3}H]Prazosin (specific activity 83.6 Ci/mmol), [^{3}H]RX821002 (specific activity 51.2 Ci/mmol) and [^{3}H]clonidine (specific activity 57.8 Ci/mmol) were bought from Perkin Elmer, USA. [^{3}H]2-BFI, 2-(2-benzofuranyl)-2-imidazoline, (specific activity 42.3 Ci/mmol) was purchased from Moravek Biochemicals, USA. The imidazoline bearing compounds under investigation in MP series were designed and synthesised by Dr Maria Pigini's group (University of Camerino, Italy).

Results

Binding affinities and selectivity of the newly synthesised compounds in MP series for α_1- and α_2-AR, as well as I_1BS and I_2BS, were investigated in rat whole brain and kidney membrane preparations. Affinity data of all the compounds for four different receptors are summarised in Table 2.1.

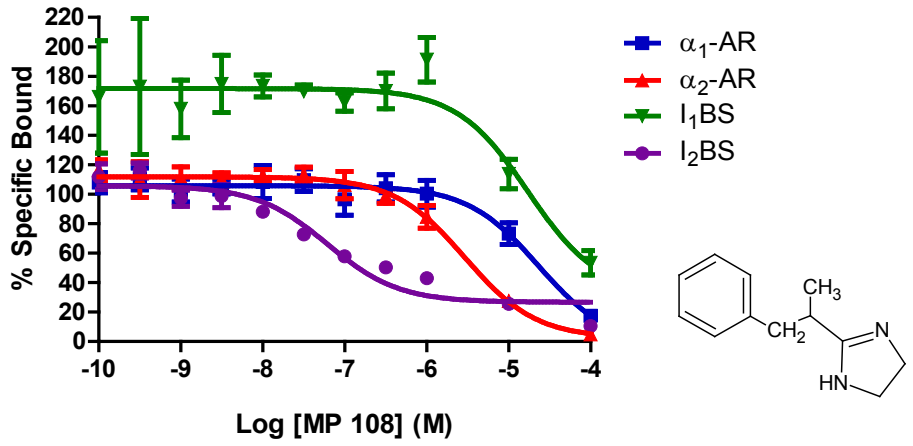
MP 108, MP 512 to MP 516

As shown in Figure 2.2, these compounds contain $–CH_2CH(CH_3)$ bridge linking the imidazoline moiety and the ortho-substituted aromatic ring. In general, they displayed good nanomolar affinity for I_2BS (K_i values ranging from 14.6 to 43.9 nM) and moderate to low affinity for other receptor types, except for MP 513, MP 514 and MP 515 which exhibited low affinity at all four receptors. MP 108 with unsubstituted aromatic ring showed good affinity towards I_2BS (K_i = 43.9±10.11 nM) with the highest selectivity for I_2BS with respect to I_1BS (selectivity ratio: I_1/I_2 = 663) in this subgroup of compounds within the series (Figure 2.3A). Substituting chlorine at ortho position of the aromatic ring resulted in MP 516 which exhibited the highest I_2BS affinity in this subgroup (K_i = 14.64±6.231 nM, α_2/I_2 = 57, I_1/I_2 = 217). Similarly MP 512 with an ortho methyl ($–CH_3$) substituent into its aromatic ring retained high affinity at I_2BS (K_i = 24.17±0.3283 nM) with good selectivity over the other IBS subtype, I_1BS (I_1/I_2 = 392) (Figure 2.3B).

MP 512 was resolved into its optical isomers to investigate the effect of chiral centre with respect to affinities at IBS subtypes. Interestingly, both the enantiomers MP(+)-512 and MP(–)-512 exhibited good affinity for I_2BS (K_i = 24.02±15.24 and 30.15±15.65 nM respectively). However, MP(+)-512 showed greater than 300-fold selectivity for I_2BS versus I_1BS compared to MP(–)-

512 (~ 100-fold). Therefore it appears that the selectivity exhibited by the racemate MP 512 for I_2BS over I_1BS is mostly due to the enantiomer MP(+)-512.

A)



B)

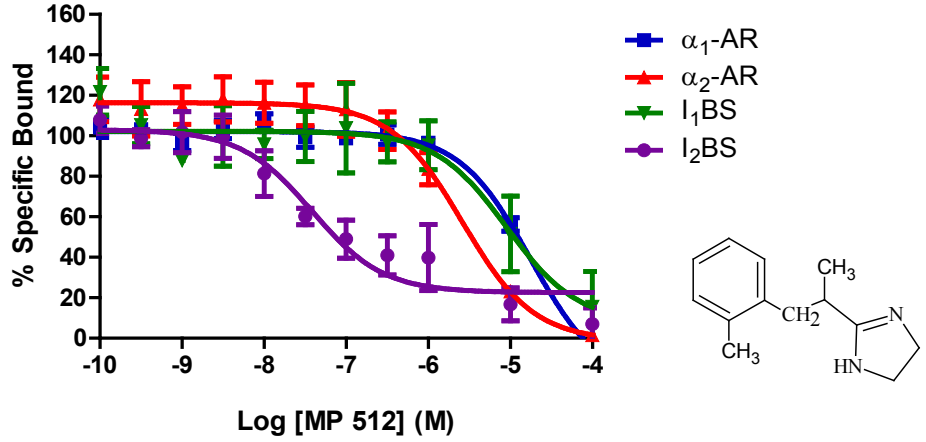


Figure 2.3: Binding curves of (A) MP 108 and (B) MP 512 at α_1-AR (■), α_2-AR (▲), I_1BS (▼) and I_2BS (●). Incubation was performed in triplicates across a range of concentration (0.1 nM to 100 μM) of the displacing ligands. Data represent the mean ± S.E.M. of 3 to 4 separate experiments for each receptor type. Inset: chemical structures of respective ligands.

Other MP compounds in the series

As seen previously by Pigini *et al*. (1997), similar effects on α_2-AR and I_2BS affinity were observed when $–CH_2$ was replaced by –NH in the bridge linking imidazoline moiety and aromatic ring. MP 51 which did not have any substitution at the aromatic region exhibited good affinity at both α_2-AR and I_2BS with K_i values of 69.03±7.026 and 51.67±5.629 nM, respectively (Figure 2.4A). Conversely, introduction of a methyl substituent on C-1 in the bridge as in MP 133 lowered affinity for all four receptors tested. Substituting $–CH_3$ at ortho position of the aromatic ring as in MP 960 increased affinity for both α_2-AR and I_2BS (K_i = 25.33±2.805 and 16.42±5.167 nM respectively) as compared to the unsubstituted analogue, MP 51. Binding affinities of MP 961 for α_2-AR and I_2BS were further increased (K_i = 19.73±1.157 and 8.773±1.714 nM respectively) with ortho-chloro (*o*-Cl) substitution on the aromatic ring (Figure 2.4B). On the other hand, allyl ($–CH_2CH=CH_2$) or propyl ($–CH_2CH_2CH_3$) substitution in MP 970 and MP 971 respectively had little effect on I_2BS affinity but slightly reduced α_2-AR affinity making these compounds scarcely selective for I_2BS over α_2-AR. In contrast to the linear substitutions made on the aromatic ring mentioned so far, introducing an ortho phenyl substituent as in MP 96 resulted in moderate to low binding affinities for α_2-AR and I_2BS in submicromolar range (Figure 2.4C). Interestingly, MP 96 exhibited high affinity for I_1BS (IC_{50} = 28.56±18.94 nM; I_2/I_1 = 83) although it only shows partial displacement. In addition, some of the compounds in this subgroup recognised α_1-AR with good affinity in nanomolar range with MP 96 again showing the highest α_1-AR affinity (K_i = 35.67±10.61 nM) within the entire series.

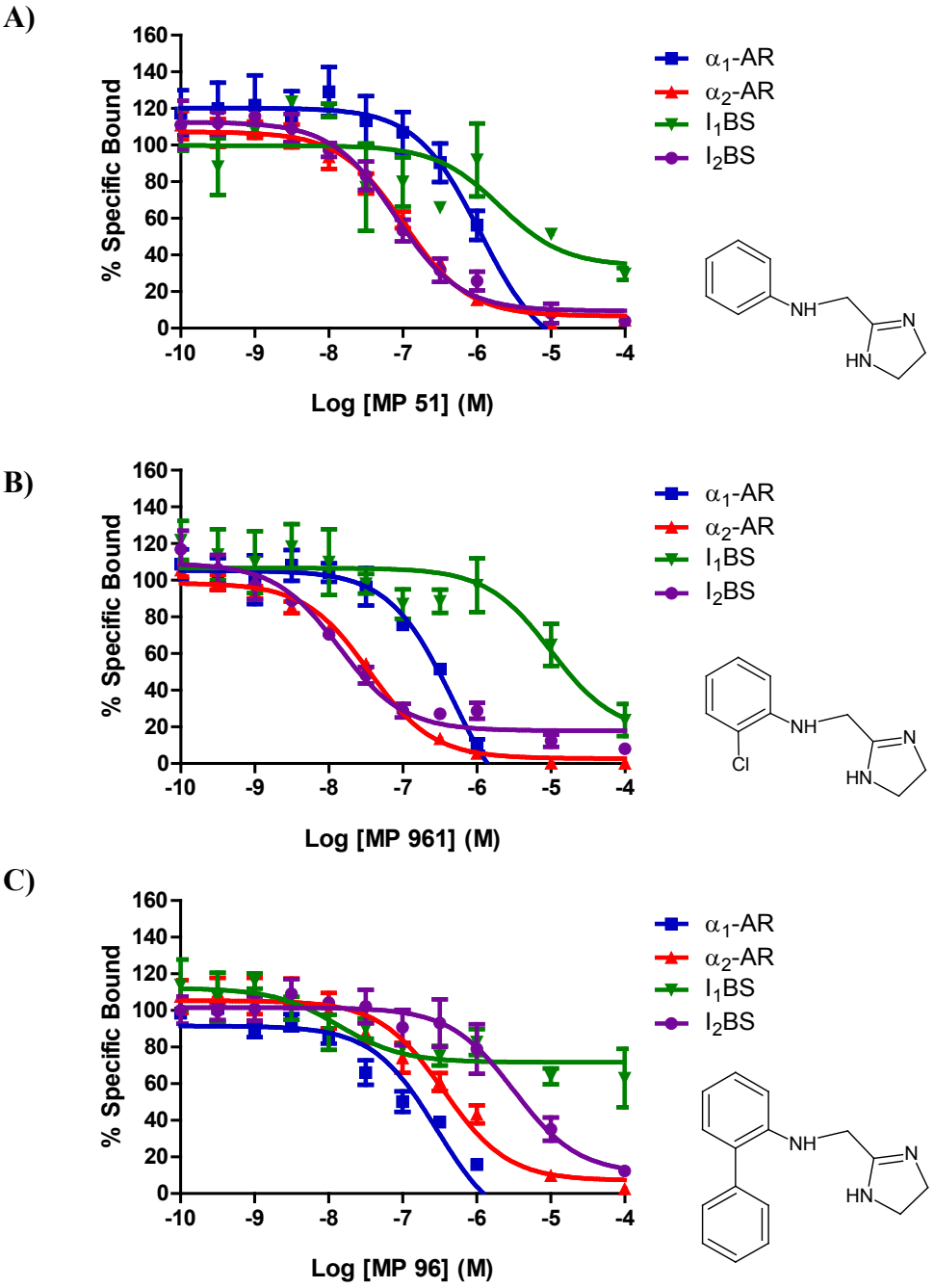
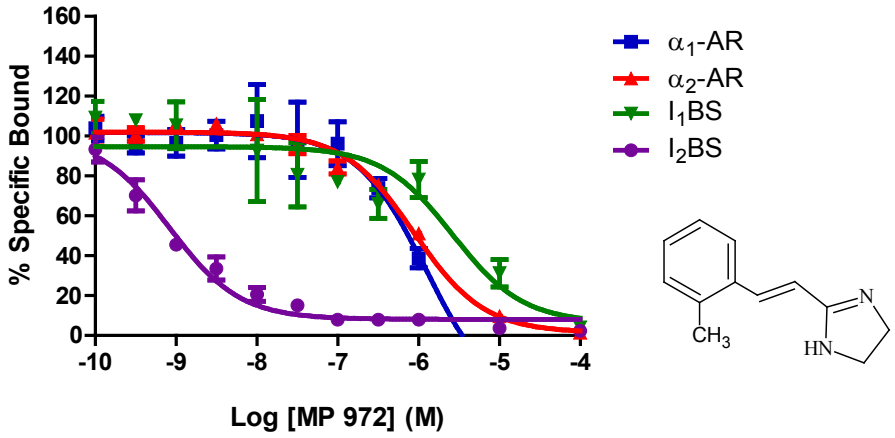


Figure 2.4: Binding curves of (A) MP 51, (B) MP 961 and (C) MP 96 at α_1-AR (■), α_2-AR (▲), I_1BS (▼) and I_2BS (●). Incubation was performed in triplicates across a range of concentration (0.1 nM to 100 µM) of the displacing ligands. Data represent the mean ± S.E.M. of 3 to 4 separate experiments for each receptor type. Inset: chemical structures of respective ligands.

The remainder of the compounds in MP series all contained double bond in the bridge and ortho substituents in the aromatic ring. Like the parent compound tracizoline, all the substituted compounds in this subgroup exhibited very high affinity for I_2BS (K_i values ranging from 0.769 to 11.2 nM) and mostly low affinities for other receptor types. Of these, MP 972 containing an ortho $–CH_3$ substitution into the aromatic ring displayed the highest I_2BS affinity (K_i = 0.7693±0.3854 nM) along with the highest selectivity with respect to α_2-AR and I_1BS (α_2/I_2 = 619; I_1/I_2 = 6892) within the whole series (Figure 2.5A). MP 957 and MP 56 with allyl and chloro substitution respectively also showed high affinity in nanomolar range for I_2BS (K_i = 5.047±1.144 and 1.595±1.103 nM respectively) although their selectivity versus I_1BS was lower than that of MP 972. Interestingly, insertion of a phenyl ring at the ortho position of the aromatic ring in MP 958 still retained some affinity towards I_2BS but moderate affinity for α_1-, α_2-AR and I_1BS, resulting in much lower I_2BS selectivity versus other receptor types within this subgroup of compounds (Figure 2.5B).

A)



B)

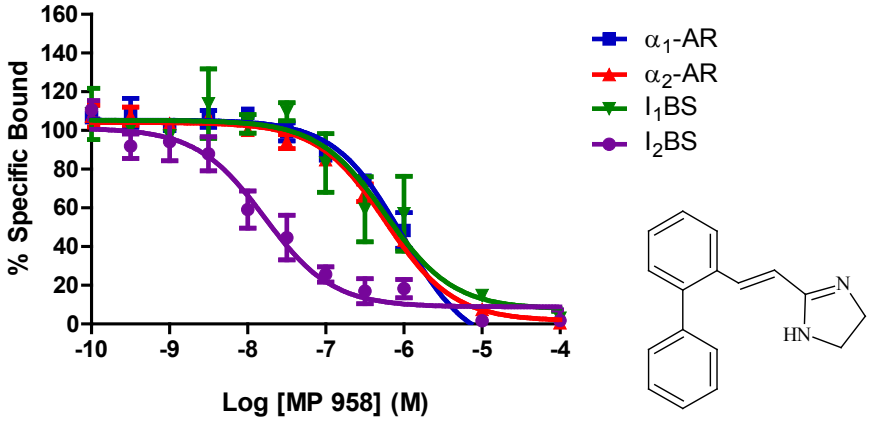


Figure 2.5: Binding curves of (A) MP 972 and (B) MP 958 at α_1-AR (■), α_2-AR (▲), I_1BS (▼) and I_2BS (●). Incubation was performed in triplicates across a range of concentration (0.1 nM to 100 µM) of the displacing ligands. Data represent the mean ± S.E.M. of 3 to 4 separate experiments for each receptor type. Inset: chemical structures of respective ligands.

Table 2.1: Binding affinity data for the compounds in MP series. Data represent mean ± S.E.M. of 3 to 4 separate experiments performed in triplicate.

Compound	α_1 K_i (nM)	α_2 K_i (nM)	I_2 K_i (nM)	I_1 IC_{50} (nM)
Bridge: $-CH_2CH(CH_3)$				
MP 108	2653±246.9	1590±151.8	43.9±10.11	29107±18883
MP 512	2012±16.01	1420±151.8	24.17±0.3283	9473±724.8
MP (+) 512	4563±1327	1667±133.8	24.02±15.24	7787±2262
MP (-) 512	1166±93.01	848.7±60.62	30.15±15.65	2957±1811
MP 513	506±78.53	2870±72.11	313.0±114.8	6426±3350
MP 514	2030±291.6	7665±147.7	2210±1220	46300±12446
MP 515	534.0±89.00	2900±498.0	4435±1999	528.7±206.3
MP 516	1603±173.2	838.7±61.36	14.64±6.231	3173±1277
Bridge: $-NHCH_2$				
MP 51	146±7.638	69.03±7.026	51.67±5.629	5334±2691
MP 96	35.67±10.61	194.3±21.40	2363±1535	28.56±18.94*
MP 133	1773±396.1	3500±629.4	2247±327.3	4660±916.6
MP 960	77.93±13.67	25.33±2.805	16.42±5.167	23700±4130
MP 961	58.63±11.55	19.73±1.157	8.773±1.714	20050±19080
MP 970	113.0±8.083	162.3±3.528	14.01±6.715	314.7±137.1
MP 971	59.53±18.80	172.3±37.55	51.17±8.219	1944±594.6
Bridge: carbon-carbon double bond				
MP 56	239.3±52.84	562.7±38.25	1.595±1.103	4165±2246
MP 957	1146±301.4	3363±627.6	5.047±1.144	8306±5812
MP 958	152.3±25.25	328±48.95	11.20±2.875	974.3±563.6
MP 972	146.7±10.84	476.3±44.73	0.7693±0.3854	5302±2947

*partial displacement

Discussion

Imidazoline containing molecules depicted by the basic structure in Figure 2.2 were able to display different activity at α_2-AR and IBS. This is demonstrated by Pigini and coworkers in their numerous exhaustive SAR studies which are primarily focused at characterising IBS and identify novel compounds that may represent improvements over current options in various therapeutic areas. In fact, it was shown that minor chemical modifications made in the bridge connecting imidazoline moiety and aromatic ring may act as determinants in preferential recognition of a particular receptor system (Gentili *et al*., 2003) while substitutions introduced into the aromatic ring at ortho position affect ligand affinity and functional activity (agonism versus antagonism) (Gentili *et al*., 2002; Gentili *et al*., 2008).

Previous studies identified phenyzoline containing an ethylene ($–CH_2CH_2$) bridge as a selective I_2BS ligand with respect to I_1BS and α_2-AR (Gentili *et al*., 2003; Pigini *et al*., 1997). Although phenyzoline did not exert any analgesic effect when administered alone, it enhanced morphine induced analgesia in mice which could be prevented by pretreatment with idazoxan, a mixed I_2/α_2 antagonist (Gentili *et al*., 2006). Interestingly slight modification by substituting a $–CH_3$ on C-1 in the ethylene bridge [$–CH_2CH(CH_3)$] of phenyzoline gave rise to a high affinity and selective I_1BS ligand over I_2BS and α_2-AR (Gentili *et al*., 2003). Following resolution of CH_3-phenyzoline into its enantiomers demonstrated higher activity of (*S*)-(–) isomer at I_1BS than that of (*R*)-(+) isomer indicating that the chiral carbon might play a role with regard to I_1BS affinity and selectivity. Further *in vivo* study in anaesthetised rabbits showed that the racemate did not exert any hypotensive effect when administered alone but could antagonise blood-pressure lowering effects of clonidine indicating that it probably acts as an antagonist at I_1BS. Insertion of an ortho phenyl substituent into the aromatic ring of CH_3-phenyzoline maintained moderate

affinity at I_1BS (K_i = 155 nM) and high selectivity over α_2-AR (α_2/I_1 = 513) although selectivity against I_2BS reduced as compared to the unsubstituted analogue (I_2/ I_1 = 43) (Gentili *et al*., 2005). However, the interesting modulation to note here was reversal of functional activity, from antagonist to agonist, of the *o*-phenyl-substituted ligand at I_1BS. In fact the new ligand was found to reduce blood pressure in anaesthetised rats. In addition it was demonstrated that only the (*S*)-(+) isomer of this newly found I_1BS agonist possessed hypotensive activity whereas the (*R*)-(–) isomer appeared to be inactive showing the importance of enantioselectivity in I_1BS activation.

In the current study a series of novel imidazoline bearing compounds related to the type shown in Figure 2.2 have recently been synthesised and assessed *in vitro* for receptor affinity and selectivity using rat whole brain and kidney membranes. These compounds differ in terms of minor modifications in the bridge between imidazoline moiety and aromatic ring which contained substituents of various nature at the ortho position. The unsubstituted derivative MP 108 containing $–CH_2CH(CH_3)$ bridge displayed good affinity for I_2BS as well as high selectivity (> 600-fold) for I_2BS versus I_1BS. Its selectivity for I_2BS over I_1BS is also the highest among the analogues (MP 512 to MP 516) containing similar modification in the bridge. However, this is in contrast to previously obtained data by Gentili *et al*. (2003) who showed that $–CH_3$ substitution on C-1 in the bridge led to high affinity I_1BS ligand with reduced I_2BS affinity as mentioned earlier. Differences in selectivity for the two IBS subtypes observed in the current and previous studies may be due to methodological or species variation used for determining receptor affinity. Conversely, low affinity for I_1BS shown in the present study may explain the lack of hypotensive effects observed earlier (Gentili *et al*., 2003) when CH_3-phenyzoline (i.e. MP 108 in the current study) was administered alone in anaesthetised rabbits. However, the possibility of MP 108 to

interact with both I_1BS and I_2BS perhaps with different functional activity at the two IBS sites cannot be discarded. Therefore, further experiments with similar methodology should be carried out to account for any possible species/tissue variability along with *in vivo* studies for pharmacological characterisation of the ligand at both IBS subtypes.

As mentioned earlier for this subgroup of MP compounds [bridge: $–CH_2CH(CH_3)$], ortho substitution of the aromatic ring resulted in compounds which mostly exhibited higher affinity for I_2BS but lower I_1/I_2 selectivity as compared to the unsubstituted analogue, MP 108. Introduction of aromatic substituents which are considerably large and bulky as in MP 513, MP 514 and MP 515 appear to be detrimental to affinity at both I_1BS and I_2BS possibly due to unfavourable interaction with receptive sites owing to steric hindrance. The effect of steric hindrance becomes more evident when small substituent-containing MP compounds, MP 512 (*o*-CH_3) and MP 516 (*o*-Cl), in this subgroup exhibit high I_2BS affinity and also maintain good selectivity versus I_1BS (~ 400- and 200-fold). In contrast to the results obtained previously with optical resolution of racemates with one isomer being the active form (eutomer) and the other inactive (distomer) at a particular IBS subtype (Gentili *et al*., 2003), both the enantiomers of MP 512 in the present study were high affinity ligands at I_2BS. However, MP(+)-512 exhibited considerably higher selectivity for I_2BS over other receptor types than MP(–)-512 hence demonstrating enantioselectivity at I_2BS with respect to I_1BS. Further study is required to confirm any reverse enantioselective activity of the isomers at IBS subtypes.

A subgroup of compounds in the present study contained –NH linkage in the bridge giving rise to less selective ligands which have comparable affinities at both α_2-AR and I_2BS. This lower I_2/α_2 selectivity or vice-versa as seen beginning with MP 51 (containing unsubstituted aromatic ring) is in line with previously obtained affinity data with similar modification in the bridge by

Pigini *et al*. (1997). Introduction of a CH_3 substituent in the bridge of MP 133 proved to be detrimental for both α-adrenoceptors and IBS activities. As seen earlier in the present study, ortho substitution of the aromatic ring with small groups like methyl and chloro with low steric bulk (MP 960 and MP 961 respectively) again proved to be favourable for α_2-AR and I_2BS affinities. On the other hand, enhanced steric bulk with *o*-phenyl substitution appear to have unfavourable interaction at α_2-AR and particularly I_2BS demonstrated by moderate to low affinity values at these receptors obtained for MP 96. This modulation in affinity by phenyl group substitution is in accord to previously obtained results (Gentili *et al*., 2002).

Another minor structural modification carried out was insertion of a double bond in the bridge which restricts flexibility of the carbon chain. Such modification that limits conformational freedom of the compound produced tracizoline with very high I_2BS affinity along with high selectivity over both α_1- and α_2-AR (Pigini *et al*., 1997). Tracozoline also shows some selectivity for I_2BS with respect to I_1BS although to a much lesser extent (Quaglia *et al*., 1999). In the present study a few tracizoline analogues were studied which contained different ortho substituents on the aromatic ring. MP 972 with an *o*-CH_3 substitution exhibitied the highest affinity for I_2BS in the entire series which was roughly two times higher than the parent tracizoline. It retains good selectivity over α_1- and α_2-AR though less than that of the unsubstituted parent molecule. However, MP 972 displays unprecedented selectivity for I_2BS versus I_1BS (~ 7000-fold). Substituents like chlorine with low steric bulk seem favourable in this subgroup also for maintaining high I_2BS affinity and selectivity as observed earlier in the present study. Introduction of an *o*-phenyl substituent with increased steric bulk lowered selectivity for I_2BS versus other receptor types. Thus conformational restriction in the ligand plays a crucial

role in pertaining selectivity for I_2BS sites that is favoured by small ortho substituents made on the aromatic ring.

Conclusion

The clinical significance of identifying ligands showing high affinity and selectivity for one particular receptor system over the other of similar type is usually related with lower incidence of side effects (e.g. I_1BS or I_2BS selectivity over α_2-AR). On the other hand, potential multifunctional tools interacting with similar receptor systems (e.g. I_2BS and α_2-AR) represent novel approach in managing conditions like opioid withdrawal symptoms associated with other comorbid disorders such as depression. Compounds explored in the present study were rationally designed taking into account SAR knowledge obtained previously. In general, ligands containing wholly carbon bridge with methyl substitutions show high affinity for I_2BS which contradicts previous studies where they were found to be selective for I_1BS. Presence of –NH in the bridge produced ligands with comparable affinities at I_2BS and α_2-AR. On the other hand, limiting conformational freedom at the bridge led to very high I_2BS affinity with marked selectivity over other receptor types. Overall small substituents endowed with low steric bulk at the ortho position of the aromatic ring appear to be favourable in maintaining affinity and selectivity at target receptor sites. In this chapter, it was further confirmed that slight structural modifications (such as flexibility and steric hindrance) may affect affinity and selectivity of the ligand towards α-adrenoceptor and IBS. In addition, several highly selective I_2BS ligands were identified along with a few compounds displaying multitarget interaction. These ligands should be explored further in *in vivo* settings to evaluate their potential in different therapeutic areas like chronic pain and opioid addiction management with associated depressive disorders in order to find out novel tools which provide improvements over current therapies.

Chapter III: Structure-Affinity Relationship Study of a novel series of Marsanidine Derivatives

Introduction

Imidazole and imidazoli(di)ne containing agents constitute a major class of therapeutics acting via α_2-AR that are present in the central nervous system and in peripheral tissues (Gentili *et al*., 2007). These biologically active compounds are widely used as antihypertensive, antidepressant, sedative, anxiolytic, and analgesic agents (Gentili *et al*., 2007). In addition to α_2-AR interaction, these versatile imidazoline bearing ligands have been proposed to exert their pharmacological actions via imidazoline binding sites (IBS) (Eglen *et al*., 1998). First proposed as nonadrenergic binding sites that are insensitive to catecholamines and recognise drugs with imidazoline moiety such as clonidine (compound A, Figure 3.1) and its analogues (Bousquet *et al*., 1984), IBS have been shown to be present in both central and peripheral tissues in several species including human (Regunathan and Reis, 1996). At least three subtypes of IBS have been identified – I_1BS which are found in the brainstem and associated with the regulation of blood pressure (Bousquet *et al*., 1984); I_2BS that are present predominantly in the brain and liver and modulates monoamine turnover (Alemany *et al*., 1997); and I_3BS that are located in pancreatic β-cells and regulates secretion of insulin (Chan *et al*., 1994).

Although there is much debate with regard to the role of I_1BS in producing hypotensive effects of clonidine-like drugs as reviewed by Szabo (2002), several lines of evidence indicate otherwise supporting the involvement of I_1BS in producing antihypertensive effects and there might be potentiating interaction between the two receptor systems in inducing such effects (Head and Mayorov, 2006). On the other hand, I_2BS have been implicated in various disease states such as depression, opioid withdrawal, pain, and Alzheimer's, Huntington's and Parkinson's diseases

(Garcia-Sevilla *et al*., 1999; Li and Zhang, 2011; Reynolds *et al*., 1996; Ruiz *et al*., 1993). Several ligands like 2-BFI and BU224, which are highly selective for I_2BS over I_1BS and α_2-AR (Hudson *et al*. 1997, 1999a), have been reported to exhibit antidepressant activity (Finn *et al*., 2003; Tonello *et al*., 2012), antinociceptive effects (Li *et al*., 2011; Sampson *et al*., 2012) and enhance morphine induced analgesia *in vivo* in different animal models (Li *et al*., 2011; Thorn *et al*., 2011). It is hypothesised that compounds acting selectively at I_1- or I_2BS over α_2-AR and *vice versa* are expected to have better efficacy with lower side effect profile than nonselective agents and may be useful research tools in characterising particular receptor systems (Eglen *et al*., 1998).

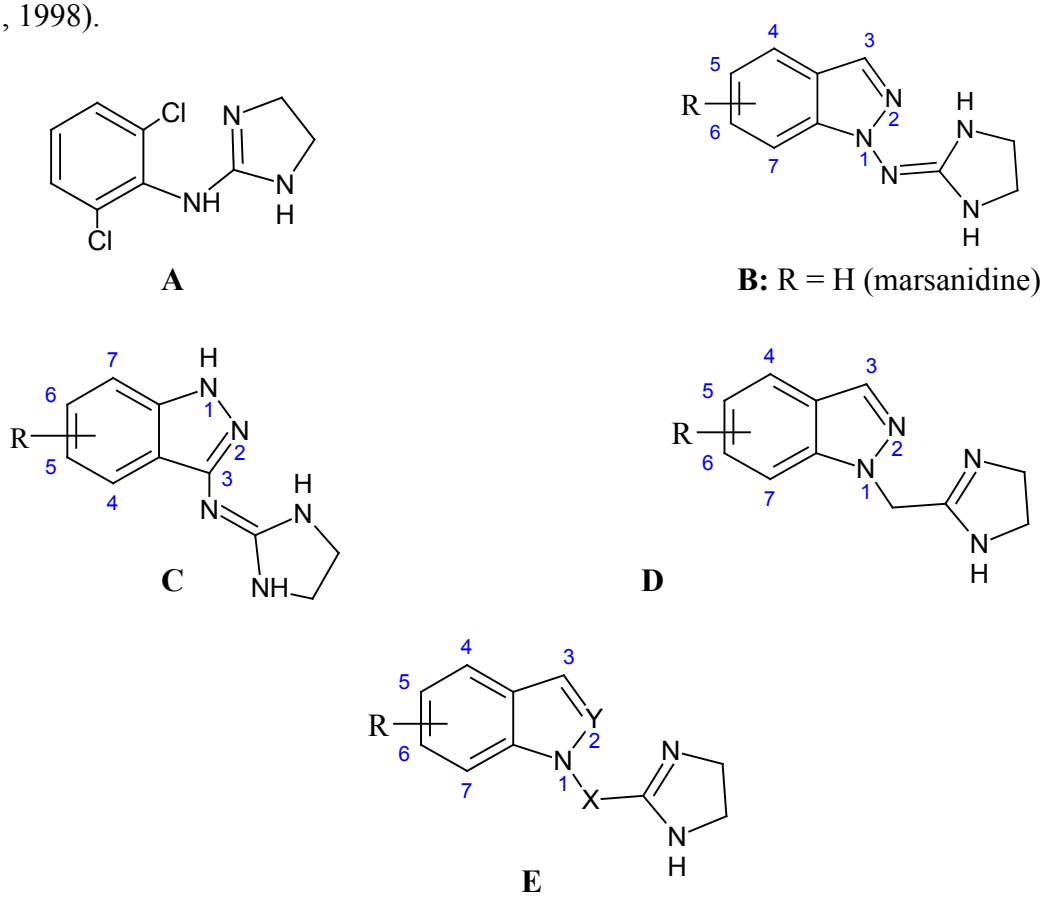


Figure 3.1: Chemical structures of some selective α_2-AR ligands. **A)** Clonidine; **B)** marsanidine; **C)** positional isomer of marsanidine; **D)** heteroarylmethyl derivative of marsanidine. **E** represents general structure of marsanidine derivatives in TCS/TCA series under investigation.

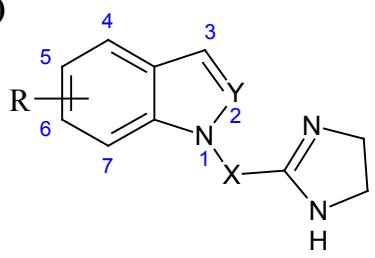
Given the therapeutic potential of agents acting at α- and/or imidazoline receptor systems, our group in collaboration with Saczewski and coworkers has made significant efforts in synthesising, identifying and characterising novel imidazoline containing ligands that would be selective for α_2-AR or I_1- or I_2BS. Past investigations on ligands containing an imidazoline ring that is substituted at position 2 by an aryl or heteroaryl moiety with or without a spacer/bridge have yielded interesting results. Compounds in which the imidazoline ring was directly substituted at position 2 by a phenyl/aryl/heteroaryl ring generally showed reduced α-adrenergic affinity and increased affinity for imidazoline binding sites (Anastassiadou *et al*. 2001; Saczewski *et al*. 2003, 2006).

Previous studies reported about a potent imidazoline based partial α_2-AR agonist, marsanidine (compound B, Figure 3.1; K_i = 14 nM) which also exhibited high selectivity for α_2-AR versus both I_1- and I_2BS (I_1/α_2 =3879; I_2/α_2 = 1203) (Sączewski *et al*., 2008). Its 7-methyl analogue was also found to display nanomolar affinity for α_2-AR (K_i = 53.5 nM) with moderate to low affinity for I_1- and I_2BS, respectively (Sączewski *et al*., 2008). The same study also demonstrated that 7-methyl-marsanidine was more effective in producing hypotensive effects *in vivo* in rats as compared to the parent marsanidine. In addition, positional analogues of marsanidine of type C (Figure 3.1) retained high α_2-AR affinity with relatively high I_1/α_2 selectivity ratios and the 4-methyl congener elicited potent hypotensive effects when administered systemically in anaesthetised rats (Sączewski *et al*., 2011). Recent studies investigating biological activities of marsanidine derivatives of type D with partially restricted conformational freedom also exhibited moderate to high affinity for α_1- and α_2-AR with respect to I_1- and I_2BS (Saczewski *et al*., 2012).

In this chapter we sought to explore biological activities of another series of marsanidine derivatives of type E (Figure 3.1) where the imidazoline ring is substituted at position 1 of the

heteroaromatic ring via a $–CH_2$ or –N= bridge. These novel compounds in TCS/TCA series (Figure 3.2) were kindly designed and synthesised by Dr Franciszek Saczewski's group (Medical University of Gdańsk, Poland). In the present study the importance of the nature and position of substitution introduced into the heteroaromatic nucleus was also investigated by testing *in vitro* binding affinities of the ligands at α_1- and α_2-AR and I_1- and I_2BS in rat whole brain and kidney membranes.

A)



TCS-207: X = CH_2, Y = CH, R = 7-Br

TCS-209: X = CH_2, Y = CH, R = 6-Cl

TCS-210: X = CH_2, Y = CH, R = 5-Br

TCS-213: X = CH_2, Y = CH, R = 7-Cl

TCS-214: X = CH_2, Y = CH, R = 7-F

TCS-215: X = CH_2, Y = CH, R = 4-Br

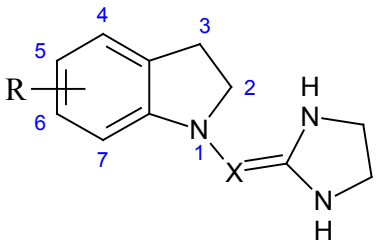
TCS-216: X = CH_2, Y = N, R = 7-F

TCS-219: X = CH_2, Y = CH, R = $7\text{-}CH_3$

AK-93: X = N, Y = CH, R = $7\text{-}CH_3$

TCA-831: X = N, Y = CH, R = 7-Cl

B)

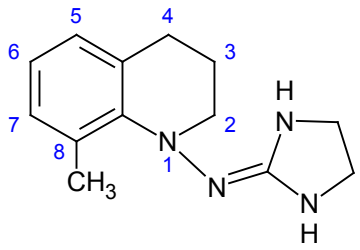


TCA-795: X = N, R = $2\text{-}CH_3$

TCA-809: X = N, R = 4-Cl

TCA-912: X = N, R = $7\text{-}CH_3$

C)



TCS-936

Figure 3.2: Chemical structures of novel marsanidine derivatives in TCS/TCA series under investigation.

Materials and methods

Preparation of rat whole brain and kidney crude P2 membranes

Whole brain and kidney crude P2 membranes were prepared from male Sprague-Dawley rats as outlined in Chapter II.

In vitro radioligand competition binding assays

Competition radioligand binding assays were performed similarly as described in Chapter II to assess the affinities of the newly synthesised compounds (Figure 3.2) in TCS/TCA series using rat brain (for α_1, α_2-AR, and I_2BS) and kidney membranes (for I_1BS). Briefly, brain or kidney membrane aliquots were incubated with a range of concentrations (0.1 nM to 100 μM) of the displacing ligand in presence of receptor specific radioligand for 45 minutes. Incubations were terminated by rapid filtration and membrane bound radioactivity remaining in the filters was then determined by liquid scintillation counting.

Statistical Analysis

Binding data for each experiment were analysed using iterative nonlinear regression curve in GraphPad Prism for Windows version 5.01 (GraphPad Software, San Diego, CA) to yield IC_{50} values and subsequently K_i values as described before. Affinity values (K_i or IC_{50} where appropriate) of the test compound for each receptor were finally expressed as mean ± S.E.M of three to four separate experiments.

Materials

Chemicals required were similar to the ones listed in Chapter II unless mentioned here. [^{3}H]RX821002 (specific activity 60.0 Ci/mmol) and [^{3}H]2-BFI (specific activity 67.0 Ci/mmol)

were purchased from Amersham Int, UK. All the marsanidine derivatives in TCS/TCA series were synthesised by Dr Franciszek Saczewski's group (Medical University of Gdańsk, Poland) in the form of water soluble HCl salts except for AK 93 and TCS 219 which were synthesised as HBr salts.

Results

Binding affinity and selectivity of the novel marsanidine derivatives in TCS/TCA series for α_1- and α_2-AR and closely related receptor types, I_1- and I_2BS, were investigated using rat whole brain and kidney membranes. A summary of the results of receptor binding assays is given in Table 3.1.

The newly prepared compounds in TCS/TCA series contained substituents of mainly two types, halogen and methyl, at different positions on the heteroaromatic ring. In general all the compounds in this current series exhibited good to high nanomolar affinity for α_2-AR (K_i values ranging from 0.7533 to 94.50 nM) except for TCS-210 displaying moderate affinity (α_2K_i = 596.7±270.4 nM). Introduction of halogen substituents (chlorine, bromine or fluorine) at various positions on the heteroaromatic ring (C4 to C7) appears to maintain good α_2-AR affinity. Of these, substitution at position 7 yielded ligands with high affinity for α_2-AR as seen in TCS-214 (7-F; K_i = 26.80±11.13 nM), TCS-213 (7-Cl; K_i = 6.343±2.471 nM) and TCS-207 (7-Br; K_i = 18.30±5.543 nM). However, most of these halogen substituted ligands failed to exhibit selectivity for α_2-AR with respect to other three receptor types. Interestingly TCS-210, which contained a 5-bromine substitution on the heteroaromatic ring, showed the highest affinity for I_2BS (K_i = 30.33±4.775 nM) within the entire series and moderate to good selectivity ratios over α_2-AR and I_1BS, respectively (α_2/I_2 = 20; I_1/ I_2 = 347) (Figure 3.3A). TCS-216 with a 7-fluoro substitution displayed good affinity for α_2-AR (K_i = 40.47±19.25 nM) with good selectivity over I_1BS only (I_1/α_2 = 303) (Figure 3.3B). Similar to the compounds mentioned so far with $–CH_2$ bridge, –N= bridge containing TCA-809 and TCA-831, with chlorine substituted at position 4 and 7 respectively on the heteroaromatic ring, showed high affinity for α_2-AR. TCA-809 exhibited the second highest α_2-AR affinity and selectivity over I_1BS (K_i = 3.1±0.3651 nM; I_1/α_2

= 1864) among the halogen substituted ligands in the series; however, it was only scarcely selective for α_2-AR versus I_2BS (I_2K_i = 49.77±4.649 nM; I_2/α_2 = 16) (Figure 3.3C). On the other hand, TCS-831 showed high affinities for both α_1- and α_2-AR (K_i = 31.87±5.874 and 9.950±2.519 nM, respectively) with moderate to good selectivity over IBS (I_1/α_2 = 95; I_2/α_2 = 272).

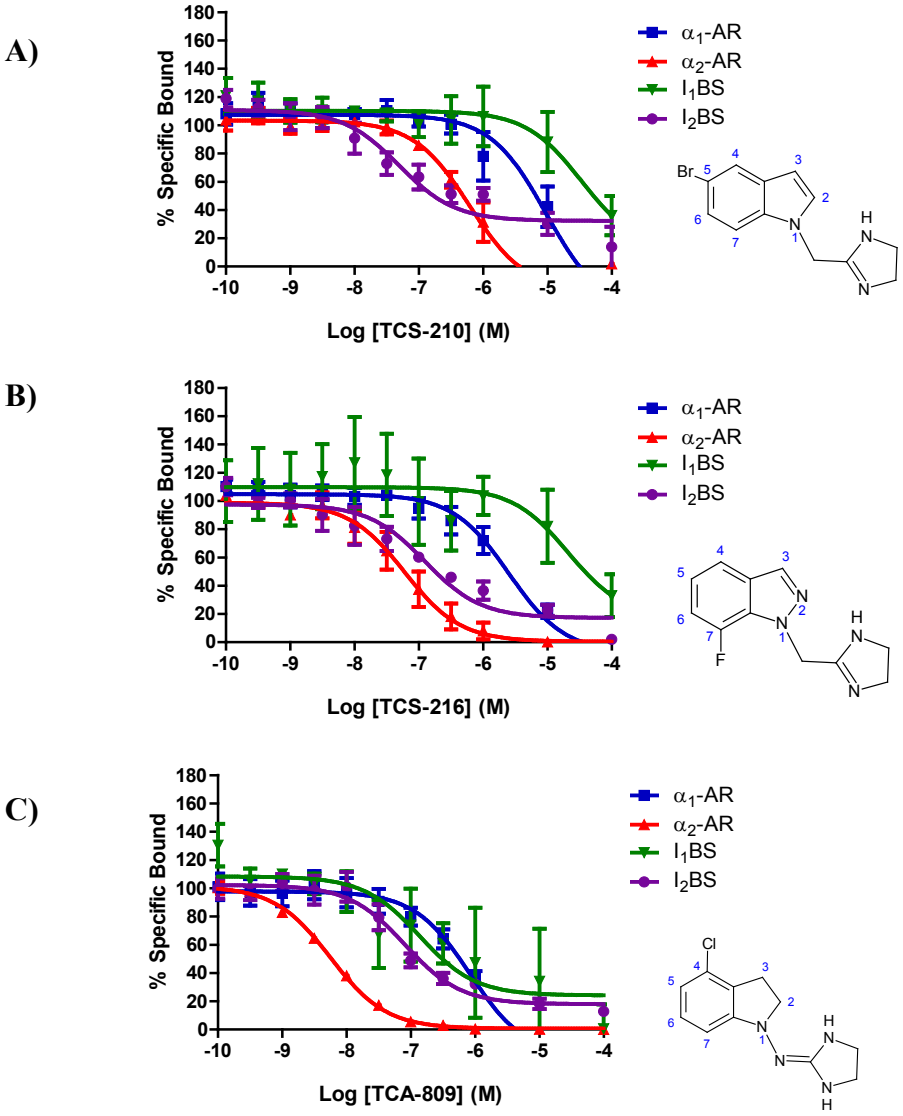


Figure 3.3: Binding curves of (A) TCS-210, (B) TCS-216 and (C) TCA-809 at α_1-AR (■), α_2-AR (▲), I_1BS (▼) and I_2BS (●). Incubation was performed in triplicates across a range of concentration (0.1 nM to 100 µM) of the displacing ligands. Data represent the mean ± S.E.M. of 3 to 4 separate experiments for each receptor type. Inset: chemical structures of respective ligands.

Substituting a methyl group (CH_3) at different positions on the heteroaromatic ring did not appear to affect affinity of the ligands for α_2-AR. TCA-795 which contained partially saturated heteroaromatic moiety with 2-CH_3 substitution exhibited high affinity at α_2-AR in nanomolar range (K_i = 6.087±2.489 nM) but lacked in selectivity. Interestingly CH_3 substitution at position 7 again produced ligands with high affinity for α_2-AR. Among all the ligands investigated in the present study, TCA-912 displayed the highest affinity for α_2-AR (K_i = 0.7533±0.1099 nM) and the highest α_2-AR selectivity with respect to I_1BS affinity (I_1/ α_2 = 2057); however, it also exhibited high affinities at α_1-AR and I_2BS (Ki = 27.07±4.611 and 48.33±5.932 nM, respectively) (Figure 3.4B). Its structural congener TCA-936 displayed comparable affinities at both α_2-AR and I_2BS (K_i = 52.40±9.193 and 62.67±5.053 nM, respectively). On the other hand, AK-93 containing a 7-CH_3 substitution and –N= bridge showed high α_2-AR affinity (K_i = 12.09±2.373 nM) along with good selectivity versus both I_1- and I_2BS (I_1/ α_2 = 591; I_2/α_2 = 210) (Figure 3.4C). In contrast to AK-93, TCS-219 with a 7-CH_3 substitution and –CH_2 bridge displayed high affinity at both α_1- and α_2-AR ((K_i = 24.97±4.267 and 16.63±2.782 nM, respectively) and moderate affinities at I_1- and I_2BS (Figure 3.4A).

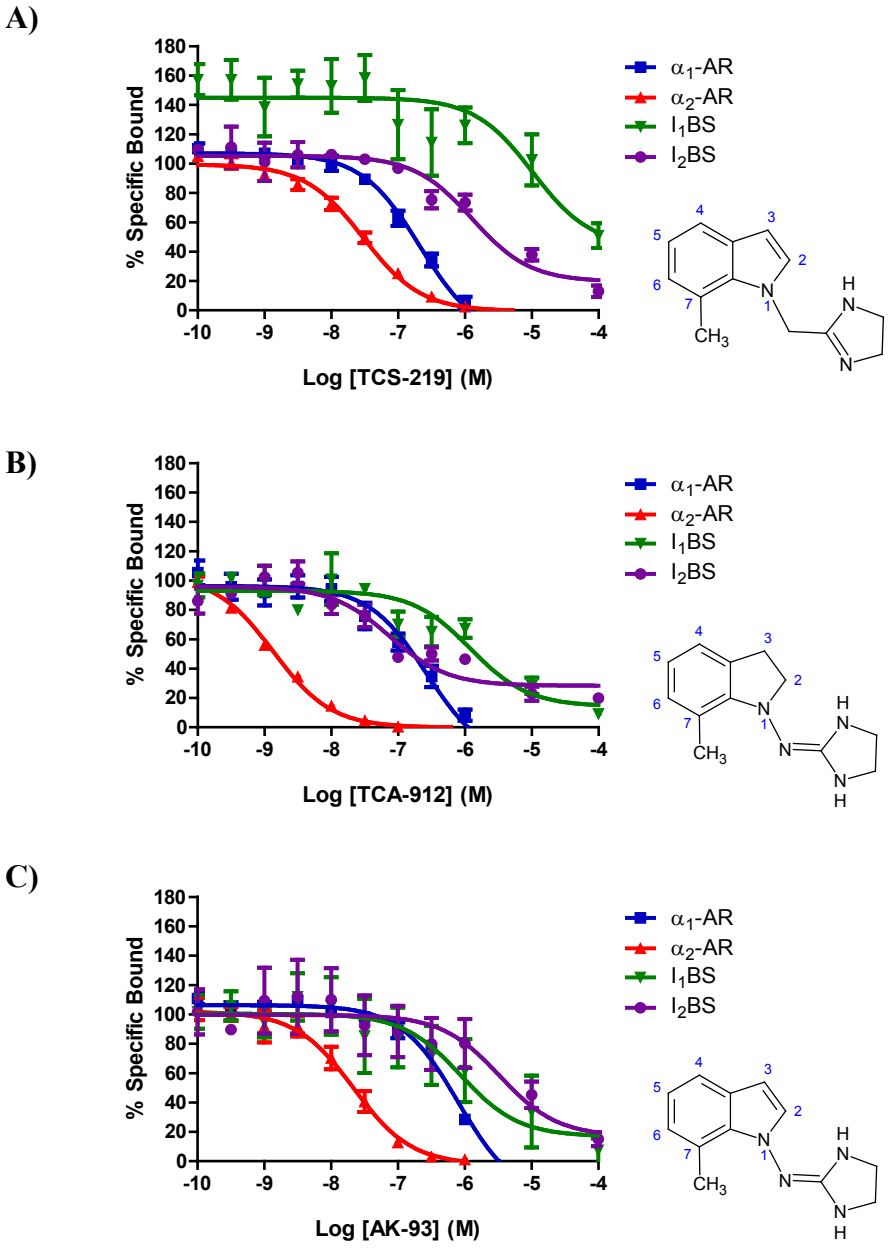


Figure 3.4: Binding curves of (A) TCS-219, (B) TCA-912 and (C) AK-93 at α_1-AR (■), α_2-AR (▲), I_1BS (▼) and I_2BS (●). Incubation was performed in triplicates across a range of concentration (0.1 nM to 100 μM) of the displacing ligands. Data represent the mean ± S.E.M. of 3 to 4 separate experiments for each receptor type. Inset: chemical structures of respective ligands.

Table 3.1: Binding affinity data for the compounds in TCS/TCA series. Data represent mean ± S.E.M. of 3 to 4 separate experiments performed in triplicate.

Compound	α_1 K_i (nM)	α_2 K_i (nM)	I_2 K_i (nM)	I_1 IC_{50} (nM)
TCS-207	7.907±0.7928	18.30±5.543	254.3±133.2	79.20±8.240
TCS-209	228.7±94.81	94.50±33.68	48.50±12.06	5582±4560
TCS-210	1348±884.3	596.7±270.4	30.33±4.775	10514±8102
TCS-213	6.823±0.5752	6.343±2.471	71.07±2.038	10290±2390
TCS-214	35.93±8.577	26.80±11.13	63.07±17.75	8837±4215
TCS-215	257.7±77.37	84.40±11.47	178.7±20.99	468.0±121.7
TCS-216	405.7±243.0	40.47±19.25	119.5±18.30	12247±5558
TCS-219	24.97±4.267	16.63±2.782	1159±713.9	1004±694.6
AK-93	166.0±47.71	12.09±2.373	2536±950.5	7152±6775
TCA-795	30.20±2.401	6.087±2.489	431.7±276.1	39.70±6.062
TCA-809	106.0±14.01	3.100±0.3651	49.77±4.649	5777±5662
TCA-831	31.87±5.874	9.950±2.519	2708±1022	949.7±532.8
TCA-912	27.07±4.611	0.7533±0.1099	48.33±5.932	1543±1233
TCA-936	528.0±238.8	52.40±9.193	62.67±5.053	3076±2647

Discussion

Efforts to synthesise imidazoline containing compounds selective for α-adrenoceptors (α_1- or α_2-AR) or IBS (I_1- or I_2BS) have been the focus of our research for many years given the immense potential of these receptors/binding sites as therapeutic targets. It is postulated that highly selective ligands for either of the receptor systems (including their subtypes) would be beneficial as novel therapeutic agents with minimum side effects and as research tools in elucidating possible functional roles of these putative drug targets (particularly IBS) in normal physiology and in disease states. For instance, lack of highly selective ligands for I_1BS over α_2-AR has been a limitation in resolving the debate concerning mechanism of action of centrally acting antihypertensive drugs like clonidine and its analogues since these compounds are regarded as mixed α_2/I_1 agonists (Dardonville and Rozas, 2004; Eglen *et al*., 1998). In contrast, several imidazoline bearing compounds such as tracizoline, benazoline (Pigini *et al*., 1997), 2-BFI (Hudson *et al*., 1997), BU224 (Hudson *et al*., 1999a), etc have been synthesised in recent years that display high affinity as well as high selectivity for I_2BS over α_2-AR, facilitating pharmacological characterisation of I_2BS as a novel drug target.

Owing to similar functional profiles and the fact that structurally diverse groups of ligands exhibit affinity towards both α-adrenoceptors and IBS, it is hypothesised that some degree of similarity exists between the binding pockets of the two receptive sites (Hieble and Ruffolo, 1995). Minor chemical modifications incorporated in the structures of imidazoline containing ligands are able to alter preferential recognition of a particular receptor system as seen in the previous chapter. Earlier studies suggested that ligands preferring a more rigid and planar conformation tend to be selective for I_2BS with respect to α_2-AR (Dardonville and Rozas, 2004). This is supported by SAR studies of compounds containing phenyl or various heterocyclic rings

directly substituted at position 2 of the imidazoline moiety that retained high I_2BS affinity and showed low or no affinity for α_2-AR (Anastassiadou *et al*. 2001; Saczewski *et al*. 2003, 2006). On the contrary, recent studies from our group revealed marsanidine, consisting of a heteroaromatic nucleus connected to position 2 of the imidazoline ring by –N= bridge, to be a potent and highly selective partial agonist of α_2-AR (Sączewski *et al*., 2008). Its 7-methyl analogue, which is a mixed α_2/I_1 agonist, proved to be a highly potent hypotensive agent that can be used as a lead structure for developing new centrally acting antihypertensives (Sączewski *et al*., 2008). Further SAR studies with positional analogues (Sączewski *et al*., 2011) and $–CH_2$ bridge containing heteroarylmethyl derivatives (Saczewski *et al*., 2012) of marsanidine confirmed that the =C–H group at position 3 in the heteroaromatic ring of marsanidine was important in hydrogen bonding or stacking interactions with an aromatic ring side chain in the binding pocket of receptor protein.

A novel group of imidazoline containing compounds (TCS/TCA series) related to marsanidine and its heteroarylmethyl derivatives have been recently synthesised and assessed *in vitro* for receptor affinity and selectivity in rat brain and kidney membranes. Ligands in this series consisted of a heteroaromatic ring connected to an imidazoline moiety via a $–CH_2$ or –N= bridge and monosubstituted at different positions on the heteroaromatic ring with a halogen or methyl group. In general the derivatives in TCS/TCA series were endowed with good to high α_2-AR affinity depending on the substitution pattern.

All the halogen (F, Cl, and Br) substituted compounds (except for TCS-210) retained good affinity in nanomolar range for α_2-AR like the parent molecules. However, most of them failed to be selective for α_2-AR over α_1-AR and I_2BS. Introducing a halogen substituent at position 7 on the heteroaromatic ring seems to favour high binding affinity, especially at α_2-AR. This is in

agreement with previously evaluated marsanidine derivatives including the positional isomers where it was shown that halogen substitution at position 6 or 7 on the heteroaromatic ring usually maintained high α_2-AR affinity (Sączewski *et al*., 2011; Saczewski *et al*., 2012). Among the halogens introduced into position 7 of the heterocyclic ring (from TCS-207 to TCS-215), the 7-Cl substituted derivative TCS-213 (with $–CH_2$ bridge) exhibited the highest affinities for both α_1- and α_2-AR. Its 7-Cl congener TCA-831 (with –N= bridge) also showed similar high affinities at α-adrenoceptors but tend to be more moderately selective versus I_2BS than TCS-213. TCS-216 with a 7-F substitution on the slightly modified heteroaromatic ring showed high α_2-AR affinity and seemed to possess good selectivity over I_1BS (~300-fold) but scarce selectivity versus α_1-AR and I_2BS. Interestingly TCS-210, which contained a 5-Br substitution, displayed the highest I_2BS affinity in nanomolar range among all the compounds in the series and was also selective (~300-fold) versus I_1BS. Moreover, partial hydrogenation of the heteroaromatic ring did not affect affinity of the ligand for α_2-AR as seen in TCA-809. In fact TCA-809 with 4-Cl substitution displayed the highest affinity for α_2-AR (~4.5 times higher than marsanidine) among all the halogen substituted derivatives. Thus electron withdrawing halide substituents on the heteroaromatic moiety appear to be well tolerated with regard to α_2-AR affinity.

Similar to halogen substitution, introduction of a methyl (CH_3) substituent with low steric bulk into the heteroaromatic ring proved to be favourable towards α_2-AR interaction with affinities comparable or higher than the parent molecules (except for TCA-936). However, the methyl substituted ligands also turned out to be nonselective in nature with respect to affinity for α_1-AR and I_2BS. This is in accord with previous studies assessing marsanidine and its analogues where methyl substituted derivatives tend to possess low selectivity ratio for α_2-AR over α_1-AR and I_2BS (Sączewski *et al*., 2008, 2011). Unlike 7-methyl marsanidine which was a mixed α_2/I_1

ligand (Sączewski *et al*., 2008), AK-93 with slight modification in the heteroaromatic ring displayed good selectivity for α_2-AR over both I_1- and I_2BS. TCS-219, structurally differing from AK-93 only in the bridge atom connecting imidazoline ring and heteroaromatic moiety, showed higher affinities at both α_1- and α_2-AR as compared to its 7-methyl congener studied previously (Saczewski *et al*., 2012). Interestingly, the partially saturated heteroaromatic moiety containing derivatives displayed high affinities at α_2-AR though nonselective in nature. Of these, TCA-912 also with 7-methyl substitution showed the highest affinity towards α_2-AR and the highest I_1/α_2 selectivity ratio (greater than 2000-fold) within the whole series of compounds. Likewise TCA-795 with 2-methyl substitution exhibited high α_2-AR affinity though it was a mixed α_2/I_1 ligand. This again indicates that leaving the =C–H group at position 3 unaltered in the heteroaromatic ring may be important for interaction with the binding pocket of the receptor proteins. In addition, such ligands may be useful as a lead for the development of novel centrally acting antihypertensive agents provided they can cross the blood-brain barrier. Therefore these ligands require further *in vivo* characterisation.

Conclusion

The compounds described so far in the present study extend the structure-affinity relationship (SAR) knowledge of marsanidine-like derivatives. In this chapter it was shown again that minor modifications in the structure of the imidazoline bearing ligands affect their preferential recognition for a particular receptor system whereas nature and position of substituents on the heteroaromatic nucleus may alter affinity for receptor proteins. Both halide and methyl substituents were well tolerated with regard to α_2-AR affinity as seen previously with marsanidine derivatives, although most of these ligands lack selectivity for α_2-AR over other three receptor types. Substitution at position 7 appears to be favourable for α_2-AR activity. The

7-methyl substituted marsanidine derivative with partially hydrogenated heteroaromatic ring, TCA-912, displayed the highest affinity for α_2-AR along with the highest I_1/α_2 selectivity ratio. Since it also showed high affinity for α_1-AR and I_2BS, it would be interesting to further study this compound *in vivo* to find out about its pharmacological effects. On the other hand, introducing electronegative halogen substituents at different positions on the heteroaromatic ring produces ligands with good to high α_2-AR affinity and, therefore, is not detrimental towards α_2-AR activity. This inspired us to consider marsanidine-like compounds as lead structures for the development of a prospective imidazoline based ligand with high affinity and selectivity for α_2-AR versus other receptor types that can be radiolabeled and used routinely as an α_2-AR selective positron emission tomography (PET) radiotracer (discussed in Chapter IV).

Chapter IV: Evaluation of AW Series of Compounds – Fluorinated Marsanidine Derivatives

Introduction

Alpha-2 adrenoceptors (α_2-AR), located on both pre- and postsynaptic neurons in the central and peripheral nervous systems, belong to the superfamily of G-protein coupled receptors (G_i/G_o type) and mediate actions of endogenous catecholamines, adrenaline and noradrenaline. They are involved in a broad array of physiological functions including regulation of blood pressure, regulation of insulin release, sedation, analgesia, and thermogenesis. Ligands acting as agonists and antagonists at these receptors have a wide variety of therapeutic application in human and animals. To date three subtypes of α_2-AR have been identified, α_{2A}, α_{2B}, and α_{2C}, among which α_{2A}-AR is the predominant subtype present in the CNS and is mostly attributed to the well-recognised physiological roles of α_2-AR (for detailed review see Chapter I).

Centrally located presynaptic α_2-AR, as auto- and/or heteroreceptors, mainly regulate the release of monoamine neurotransmitters (norepinephrine, serotonin, dopamine) from nerve terminals by negative feedback mechanism and are implicated in several central nervous system disorders including major depression (Elhweugi, 2004; Meana *et al*., 2012; Robinson and Hudson, 2006). Dysfunction of central α_2-AR has long been postulated as one of the key components in pathophysiology of depressive disorders (Cottingham and Wang, 2012). Indeed, studies of α_2-AR utilising radiolabeled agonists, but not antagonists, on platelets from depressed patients and human postmortem brain tissue obtained from depressed/suicide victims, repeatedly showed selective upregulation of high affinity conformational state α_2-AR (Callado *et al*., 1998; García-Sevilla *et al*., 1981, 1986, 1987; Gurguis *et al*., 1999; Meana *et al*., 1992; Ordway *et al*., 1994), along with enhanced G-protein coupling and receptor activity (Gonzalez-Maeso *et al*., 2002;

Valdizán *et al*., 2010). On the other hand, chronic treatment with antidepressants affecting noradrenergic system results in downregulation of α_2-AR density as investigated upon platelets, human postmortem and rodent brain tissues (De Paermentier *et al*., 1997; García-Sevilla *et al*., 1981, 1986, 1987, 2004; Subhash *et al*., 2003). Therefore, quantification of central α_2-AR *in vivo* may prove to be beneficial in terms of investigating the pathological condition, diagnosing the disease and assessing antidepressant treatment efficacy.

Positron Emission Tomography (PET) is a versatile non-invasive imaging technique that can quantitatively measure biodistribution, concentrations and functions of target proteins (receptor, transporter, enzyme or plaque) in living organisms including humans by using radioligands usually labeled with one of the two short-lived positron emitters, carbon-11 or fluorine-18 ($t_{1/2}$ = 20 and 110 minutes, respectively). In order to be a suitable candidate for imaging and measuring any central protein target *in vivo*, a prospective PET radiotracer must fulfill a wide range of challenging criteria among which demonstrating high affinity and selectivity for the target and adequate ability to cross the blood-brain barrier (BBB) are fundamental (Pike, 2009). A steady stream of radiolabeled α_2-AR specific ligands, which are mostly antagonists, has been continually designed and studied in animals and humans for the past 20 years albeit limited in success (Table 4.1; references therein). Of these, a recent study exploring cerebral binding pattern of ^{11}C-yohimbine in living pigs showed consistency with the expected pig brain distribution of α_2-AR with the binding rapidly reaching equilibrium in the brain (Jakobsen *et al*., 2006). Another study focused on developing potential α_{2A}-AR subtype specific PET radiotracer, [^{11}C]MPTQ, which was able to cross the BBB and accumulate in brain regions enriched with α_{2A}-AR as seen in PET scans in anaesthetised baboons (Prabhakaran *et al*., 2010). Although the

results are promising, there is yet no effective radiotracer to be used routinely for imaging central α_2-AR in humans with PET.

Table 4.1: Summary of candidate radiotracers to date studied *in vivo* to image central α_2-AR with PET (listed chronologically as they appeared in literatures).

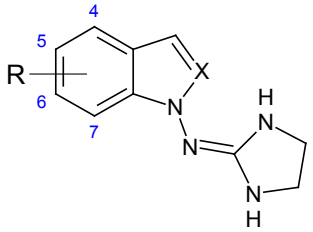
Ligand	Nature	*In vivo* model	Highlights/Limitations	Reference(s)
[^{11}C]WY-26703	Antagonist	Rodents and monkey	High nonspecific binding; fast washout with lack of retention in α_2-AR enriched brain regions	Pleus *et al*. (1992)
[^{3}H]RX821002	Antagonist	Rats	Differential localisation of specific binding observed	Hume *et al*. (1992)
[^{18}F]Fluoroatipamezole	Antagonist	Rats	Low specific signal	Ref 17 in Prabhakaran *et al*. (2010)
[^{3}H]RS-79948-197	Antagonist	Rats	Signal localisation similar to but more specific than *in vitro* [^{3}H]RX821002 distribution	Hume *et al*. (1996)
[^{11}C]MK-912	Antagonist	Rodents and rhesus monkey	Slow washout with lack of retention in α_2-AR enriched brain regions	Shuie *et al*. (1998)
[O-methyl-^{11}C]RS-15385-197	Antagonist	Rat and human	Inadequate brain extraction of the radioligand	Hume *et al*. (2000)
[*N*-methyl-^{11}C]mianserine	Antagonist	Swine	Low regional specificity	Marthi *et al*. (2002a)
[*N*-methyl-^{11}C]mirtazapine	Antagonist	Porcine and human	Rapidly enters into the brain; slow plasma metabolism; some degree of region specific binding present	Marthi *et al*. (2002b,2004)
[^{11}C]R107474	Antagonist	Rats	Poor *in vivo* binding specificity	Van der May *et al*. (2006)
[^{11}C]Yohimbine	Antagonist	Pigs	Binding pattern similar to expected α_2-AR distribution in pigs	Jakobsen *et al*.(2006)
[^{11}C]MPTQ	Antagonist	Baboon	Able to enter into the brain and accumulate in α_2-AR enriched regions	Prabhakaran *et al*. (2010)

Our group has made considerable effort in identifying and characterising structure-activity relationship of novel imidazoline based compounds that are selective for α_2-AR and/or IBS given the growing interest in potential therapeutic applications of ligands interacting with these two receptor systems. Previous studies of our group exploring biological characteristics of imidazoline containing ligands identified 1-[(imidazolidin-2-yl)imino]indazole (marsanidine, **1**) and its heteroarylmethyl analogue, 1-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-1H-indazole (**2**), (Figure 4.1) as potent α_2-AR ligands (α_2 K_i = 14 and 18.5 nM, respectively), which also displayed high selectivity for α_2-AR over I_1BS and I_2BS (Table 4.2) (Sączewski *et al*., 2008; Saczewski *et al*., 2012). Moreover, recent studies have confirmed marsanidine to be a partial α_2-AR agonist (Sączewski *et al*., 2011) with potent hypotensive and diuretic activity (Sączewski *et al*., 2008; Wróblewska *et al*., 2013). In addition, most of the halogen (F, Cl, or Br) substituted derivatives of marsanidine discussed in the previous chapter (TCS/TCA series) exhibited high affinity for α_2-AR with varying degree of selectivity over α_1-AR, I_1BS and I_2BS. Hence, given the huge interest in developing an α_2-AR selective PET radiotracer that would facilitate the possibility of directly studying central α_2-AR in living patients suffering from depressive disorders, we decided to explore the potential of marsanidine and its heteroarylmethyl analogue as lead structures for developing a PET ligand selective for α_2-AR.

1 **2**

Figure 4.1: Structure of previously reported marsanidine (**1**) and its heteroarylmethyl analogue (**2**): lead compounds for the present study (Sączewski *et al*., 2008; Saczewski *et al*., 2012).

In this chapter we sought to study the biological activity of a diverse series of fluorinated derivatives (AW-20 to AW-41, Figure 4.2) of the above mentioned lead compounds which were kindly designed and synthesised by Dr Franciszek Saczewski's group (Medical University of Gdańsk, Poland). We explored the influence of fluorination on different positions of the heteroaromatic nucleus by assessing their affinities for α_2-AR using *in vitro* competitive radioligand binding assays. The affinities of these newly synthesised compounds for α_1-AR as well as I_1BS and I_2BS were also investigated in order to identify compounds exhibiting similar or may be higher selectivity for α_2-AR than that of **1** and **2**. Compounds displaying both high affinity and selectivity for α_2-AR versus α_1-AR, I_1BS and I_2BS were further evaluated in *in vivo* settings to characterise their pharmacological actions at central α_2-AR in specific brain regions (discussed in the next chapter).



AW-20, X= N, R= 4-F

AW-21, X= N, R= 7-F

AW-25, X= N, R= 6-F

AW-26, X= N, R= 5-F

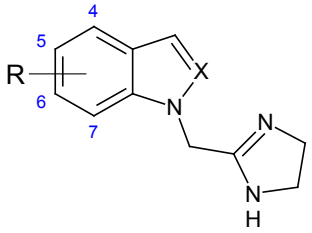
AW-35, X= N, R= 4,5,6,7-tetraF

AW-28, X= C, R= 4-F

AW-29, X= C, R= 5-F

AW-30, X= C, R= 6-F

AW-31, X= C, R= 7-F



AW-22, X= N, R= 4-F

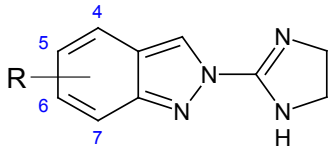
AW-23, X= N, R= 6-F

AW-27, X= N, R= 5-F

AW-32, X= C, R= 4-F

AW-33, X= C, R= 5-F

AW-34, X= C, R= 6-F



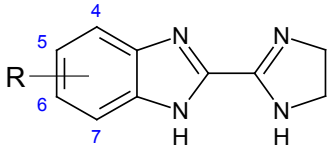
AW-24, R= 4-F

AW-36, R= 5-F

AW-37, R= 6-F

AW-38, R= 7-F

AW-39, R= 4-Cl,5-F



AW-40, R= 5-F

AW-41, R= 4,5,6-triF

Figure 4.2: Chemical structures of fluorinated derivatives in AW series. Fluorine is substituted at different positions (C4 to C7) on the heteroaromatic ring of the lead compounds.

Materials and methods

Preparation of rat whole brain and kidney crude P2 membranes

Whole brain and kidney crude P2 membranes were prepared from male Sprague-Dawley rats as outlined in Chapter II.

In vitro radioligand competition binding assays

Competition radioligand binding assays were performed similarly as described in Chapter II to assess the affinities of the newly synthesised fluorinated derivatives (Figure 4.2) in AW series for four different receptors: α_1- and α_2-AR, I_1BS and I_2BS. Rat whole brain membranes were used to carry out α_1-, α_2-AR and I_2BS binding assays whereas rat kidney membranes were used for I_1BS assays. Briefly, brain or kidney membrane aliquots were incubated with increasing concentration (0.1 nM to 100 μM) of the displacing ligand in presence of receptor specific radioligand for 45 minutes. Following rapid filtration which terminated incubation, membrane bound radioactivity remaining in the filters was then determined by liquid scintillation counting.

Statistical Analysis

Binding data for individual experiment were analysed by iterative nonlinear regression curve fitting procedures using GraphPad Prism for Windows version 5.01 (GraphPad Software, San Diego, CA) to yield IC_{50} values and subsequently K_i values as described before. Affinity values of the test compound for each receptor were finally expressed as mean ± S.E.M of three to four separate experiments.

Materials

Chemicals required were similar to the ones listed in Chapter II unless mentioned here. The fluorinated derivatives of marsanidine and its analogue (AW-20 to AW-41) were synthesised by Dr Franciszek Saczewski's group (Medical University of Gdańsk, Poland) in the form of water soluble hydrochloride salts.

Results

Binding affinities and selectivity of the newly synthesised fluorinated derivatives (AW series) of **1** and **2** for α_1- and α_2-AR, as well as I_1BS and I_2BS, were investigated in rat whole brain and kidney membrane preparations. Affinity data of all the compounds for four different receptors are summarised in Table 4.2. Binding data of the lead compounds (**1** and **2**) from previous studies are also included for useful comparison (Sączewski *et al*., 2008; Saczewski *et al*., 2012).

As shown in Figure 4.2, fluorine was substituted in different positions (C4 to C7) on the heteroaromatic ring of **1** and **2** which resulted in varying degree of affinity for different receptor types. In general, derivatives of both the lead compounds containing monofluorosubstituted heteroaromatic nuclei (and –N= or $–CH_2$ connecting bridge) showed high nanomolar affinity for α_2-AR (K_i values ranging from 7.1 to 68.4 nM), except AW-20 and AW-22 which displayed moderate α_2-AR affinity (K_i = 416±16.17 and 187.3±40.26 nM, respectively). Of all the compounds assayed AW-30, AW-31 and AW-34 exhibited the highest affinities for α_2-AR over other receptor types with K_i values of 7.073±0.3886, 12±0.7371 and 15.9±1.002 nM, respectively (Figure 4.3). However, AW-30 and AW-34 were not selective in nature since both of them also displayed high affinity for α_1-AR (K_i = 70.77±9.51 and 87.83±6.016 nM, respectively), although their binding affinities for both I_1BS and I_2BS were low in submicromolar range.

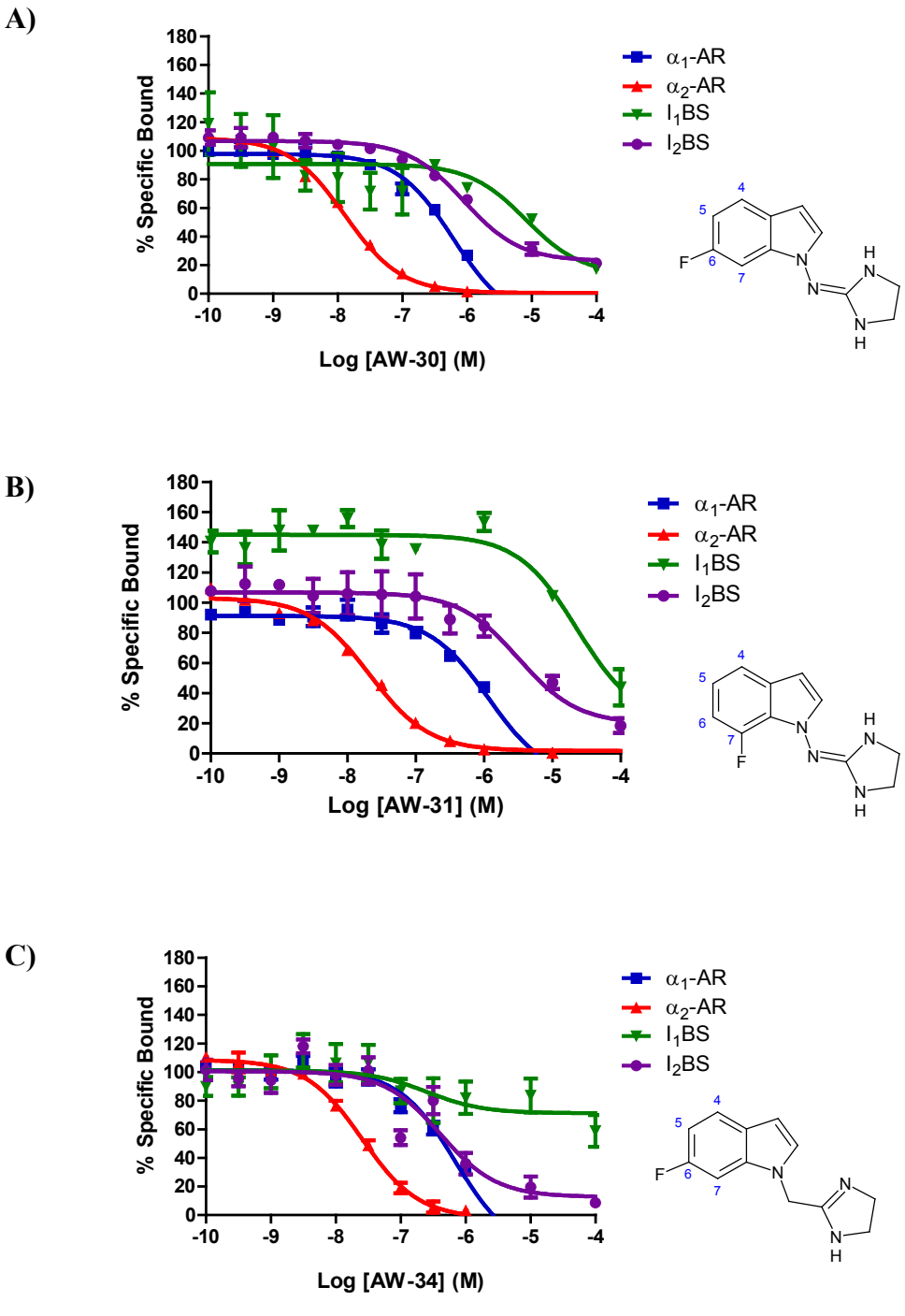


Figure 4.3: Binding curves of (A) AW-30, (B) AW-31 and (C) AW-34 at α_1-AR (■), α_2-AR (▲), I_1BS (▼) and I_2BS (●). Incubation was performed in triplicates across increasing concentration (0.1 nM – 100 µM) of the displacing ligands. Data represent the mean ± S.E.M. of 3 to 4 separate experiments for each receptor type. Inset: chemical structures of respective ligands.

All the monofluorosubstituted derivatives of the lead compound marsanidine (**1**) displayed moderate to low binding affinities for I_2BS; these compounds tend to be more selective for α_2-AR over I_2BS similar to the parent molecule (selectivity ratio for α_2-AR calculated as I_2/α_2 ranging from 14 to 11263). In particular, AW-21 and AW-25 showed high affinity for α_2-AR with K_i values of 30.97±1.927 and 26.20±6.304 nM, respectively (Figure 4.4). These two fluorinated ligands also displayed the highest selectivity for α_2-AR over I_2BS within the entire AW series with selectivity ratios (I_2/α_2) of 11263 and 838, respectively. Furthermore, they showed moderate to good selectivity for α_2-AR over α_1-AR and I_1BS. It is interesting to note that AW-35, with four fluorine atoms substituted on the heteroaromatic ring of marsanidine, failed to show good affinity at any of the receptors.

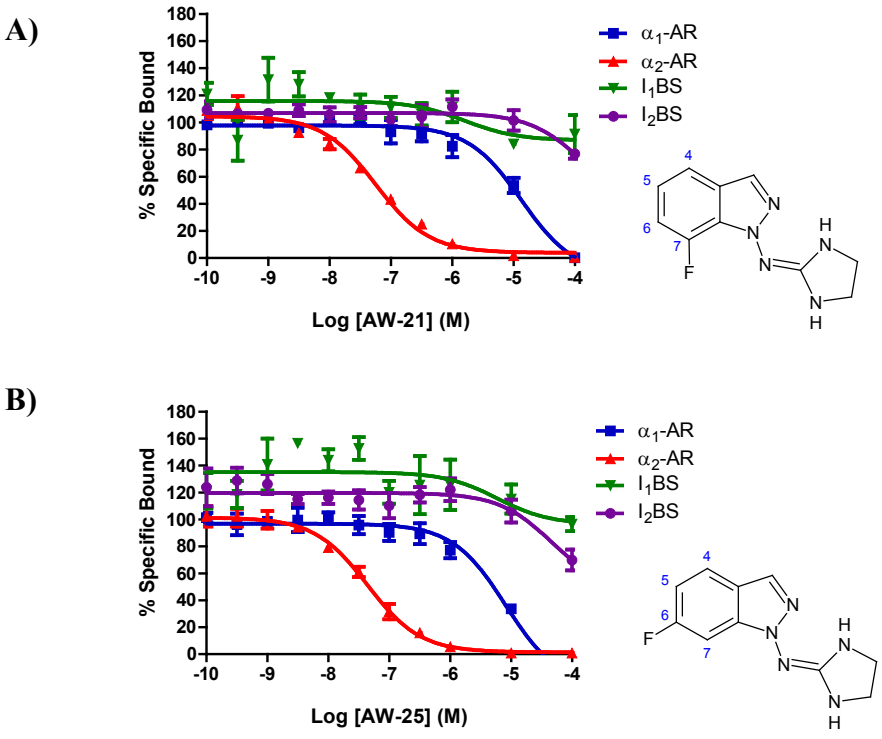


Figure 4.4: Binding curves of (A) AW-21 and (B) AW-25 at α_1-AR (■), α_2-AR (▲), I_1BS (▼) and I_2BS (●). Incubation was performed in triplicates across increasing concentration (0.1 nM – 100 μM) of the displacing ligands. Data represent the mean ± S.E.M. of 3 to 4 separate experiments for each receptor type. Inset: chemical structures of respective ligands.

In contrast, the monofluorosubstituted derivatives of the lead compound **2** (an analogue of marsanidine) not only showed high α_2-AR affinity but also exhibited high affinity towards I_2BS at nanomolar concentrations, except AW-32 and AW-34 which had moderate affinity for I_2BS. In particular, AW-23 and AW-27 displayed the highest affinities for I_2BS (K_i = 8.193±3.439 and 10.38±5.448 nM, respectively) within the entire series and AW-33 showed comparable affinity at both α_2-AR (K_i = 68.43±8.476 nM) and I_2BS (K_i = 66.27±14.73 nM) (Figure 4.5). However, these compounds proved to be relatively nonselective for the target protein α_2-AR.

In comparison to the above mentioned ligands, the fluorinated derivatives of the lead compound marsanidine lacking a –N= or $–CH_2$ bridge between the heteroaromatic ring and imidazoline moiety (AW-24 and AW-36 to AW-41) were found to bind with low micromolar affinity to α_2-AR but with high nanomolar affinity to I_2BS (except AW-36). The monofluorosubstituted ligands in this subgroup of compounds within the series, AW-37 and AW-40, showed the highest selectivity for I_2BS versus α_2-AR (α_2/I_2 = 669 and 329, respectively) (Figure 4.6 A and C). Unlike AW-35 which was inactive at all four receptors tested, disubstituted AW-39 (Cl at C4 and F at C5) and trisubstituted AW-41 (F at C4, C5 and C6) retained good affinity and overall selectivity for I_2BS (K_i = 48.17±14.42 and 70.33±9.848 nM, respectively) (Figure 4.6 B and D).

Throughout the series, affinity of the fluorinated compounds for α_1-AR was moderate to low except for AW-30, AW-32 and AW-34 displaying high α_1-AR binding in nanomolar range. Similarly, binding affinities for I_1BS were also low among all the compounds excepting AW-26 and AW-33 which showed high affinity for I_1BS (IC_{50} = 91.65±16.54 and 32.14±24.84 nM, respectively) apart from good α_2-AR activity.

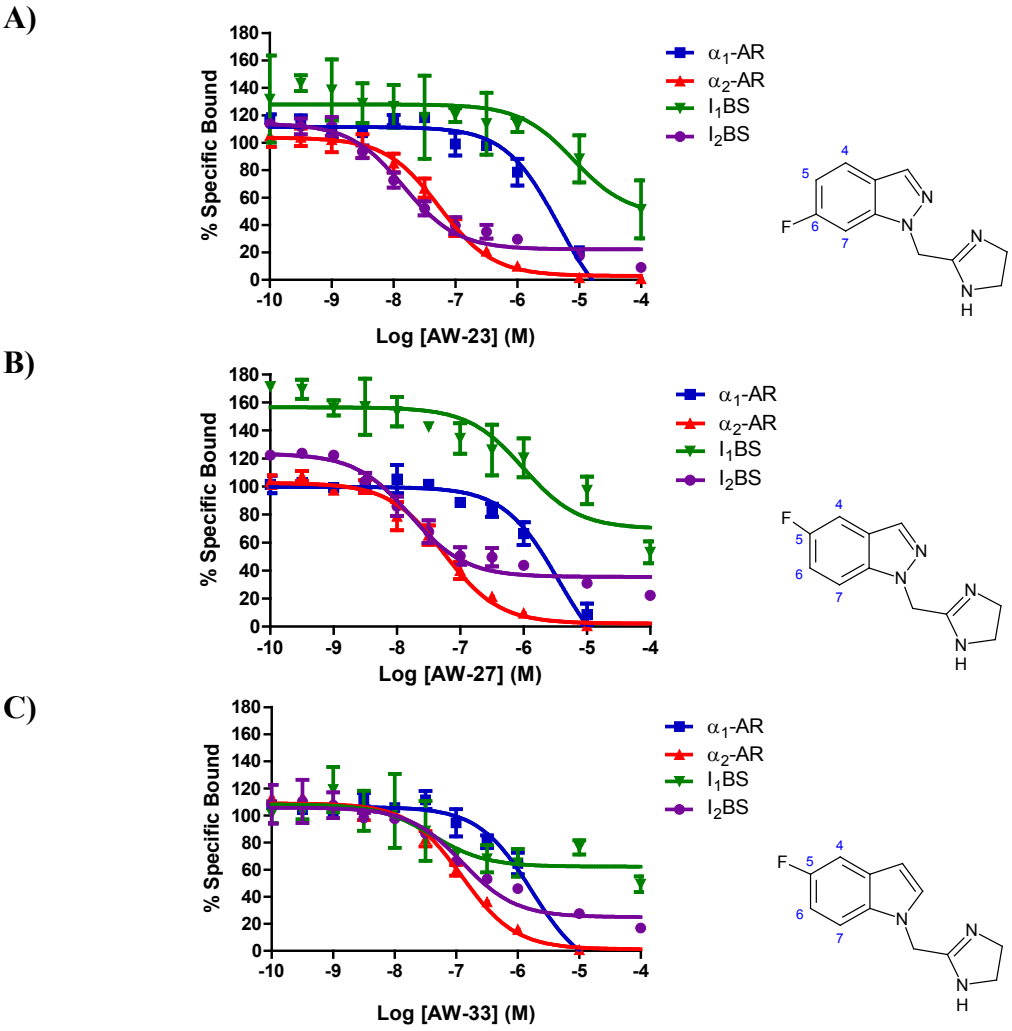


Figure 4.5: Binding curves of (A) AW-23, (B) AW-27 and (C) AW-33 at α_1-AR (■), α_2-AR (▲), I_1BS (▼) and I_2BS (●). Incubation was performed in triplicates across increasing concentration (0.1 nM – 100 µM) of the displacing ligands. Data represent the mean ± S.E.M. of 3 to 4 separate experiments for each receptor type. Inset: chemical structures of respective ligands.

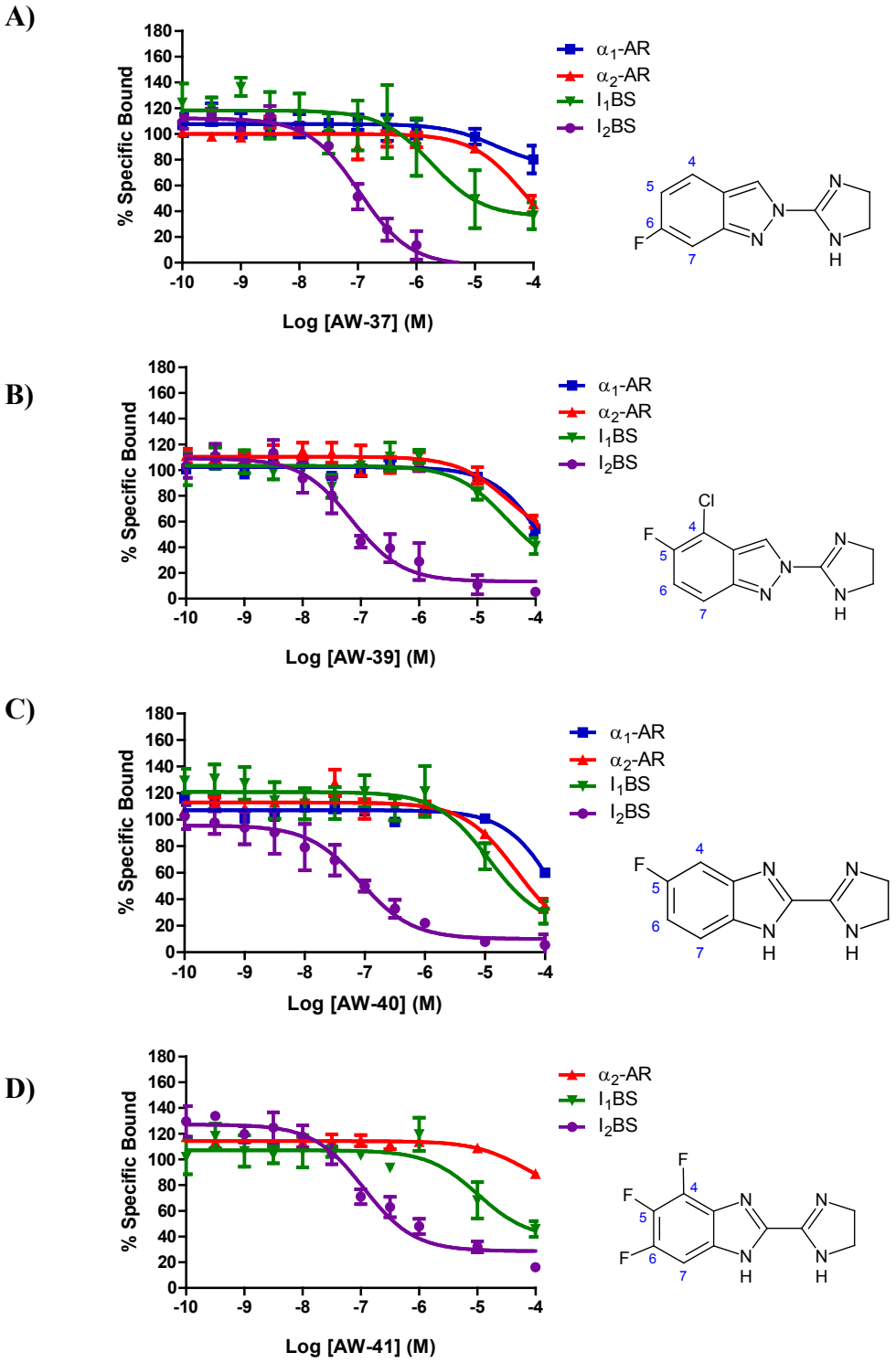


Figure 4.6: Binding curves of (A) AW-37, (B) AW-39, (C) AW-40 and (D) AW-41 at α_1-AR (■), α_2-AR (▲), I_1BS (▼) and I_2BS (●). Incubation was performed in triplicates across increasing concentration (0.1 nM – 100 µM) of the displacing ligands. Data represent the mean ± S.E.M. of 3 to 4 separate experiments for each receptor type. Inset: chemical structures of respective ligands.

Table 4.2: Binding affinity data for the fluorinated derivatives in AW series. Data represent mean ± S.E.M. of 3 to 4 separate experiments performed in triplicate. [a]Affinity values of marsanidine (**1**) from Saczewski *et al*. (2008). [b]Affinity values of heteroarylmethyl analogue of marsanidine (**2**) from Saczewski *et al*. (2012). [c]ND: not determined. [d]na: not active at 100 μM concentration of test compound.

Compound	α_1 K_i (nM)	α_2 K_i (nM)	I_2 K_i (nM)	I_1 IC_{50} (nM)
1[a]	ND[c]	14.05±2.7	16900±5900	54550±16730
2[b]	272±42	18.5±5.9	19400 (n=1)	42200 (n=1)
AW-20	3807±1324	416.0±16.17	6177±4962	14776±7893
AW-21	1625±475.2	30.97±1.927	348833±306639	7740±5522
AW-22	534.7±118.4	187.3±40.26	21.87±4.703	1765±1200
AW-23	518.3±159.9	29.73±3.036	8.193±3.439	33456±24023
AW-24	2367±505.5	17533±2858	55.00±16.00	3941±2156
AW-25	918.3±52.61	26.20±6.304	21967±3735	20675±20513
AW-26	2320±293.1	64.33±4.397	5703±2656	91.65±16.54
AW-27	476.3±267.5	32.17±9.603	10.38±5.448	2419±2057
AW-28	438±146.5	63.6±8.561	2127±351.8	241±7.024
AW-29	165±25.48	23.4±1.986	335.7±103.5	22557±15338
AW-30	70.77±9.51	7.073±0.3886	422.7±43.21	5270±2659
AW-31	167±26.1	12±0.7371	1087±432.9	15230±6203
AW-32	27.56±18.52	46.97±5.16	249±94.11	14601±8150
AW-33	306.7±121.2	68.43±8.476	66.27±14.73	32.14±24.84
AW-34	87.83±6.016	15.9±1.002	239±70.31	788.5±381.1
AW-35	19793±18003	3717±220.2	22733±817.2	5827±3402
AW-36	3430±1230	3007±308.7	180±40.07	4457±1402
AW-37	3023±1031	43433±16146	64.88±6.405	5219±4645
AW-38	3440±1200	1553±327.5	51.83±17.65	5456±2284
AW-39	5313±2037	20733±7665	48.17±14.42	38033±12693
AW-40	41233±23557	18900±3643	57.47±20.25	11475±1851
AW-41	na[d]	20677±13362	70.33±9.848	7731±6595

Discussion

Given the rapidly growing interest in the development of a PET radiotracer to selectively label central α_2-AR, we decided to explore the potential of the fluorinated derivatives of marsanidine and its heteroarylmethyl analogue (lead compounds **1** and **2**, respectively) which were potent and highly selective α_2-AR agonists (Sączewski *et al*., 2008; Saczewski *et al*., 2012). As mentioned earlier, most of the previously studied potential PET radioligands for α_2-AR were antagonists, which is in contrast to our current choice of lead compounds that are agonists at the receptor. Literatures show contradictory results obtained when α_2-AR density was determined in diseased states in platelets or human postmortem brain tissue employing radioligand agonists and antagonists. Studies which used radiolabeled agonists for α_2-AR reported increased α_2-AR density, enhanced G-protein coupling and receptor activity, whereas those employing antagonists found no alteration in α_2-AR density in the diseased state as compared to controls (Meana *et al*., 2012; Cottingham and Wang, 2012). By carefully accounting for methodological differences (agonist versus antagonist binding), it was concluded that depressive disorder is probably associated with a selective increase in the high affinity conformational state of α_2-AR being labeled preferentially by agonists (also indicative of enhanced G-protein coupling) instead of an overall increase in density of the receptor in any conformation (active or inactive) that is labelled with equal affinity by antagonists (Cottingham and Wang, 2012). Thus, a PET radioligand acting as an agonist at α_2-AR may be beneficial in assessing the actual functional change in receptor density in normal versus disease states.

Site of fluorination on the heteroaromatic ring is crucial in determining the affinity and selectivity of the compound for the target receptive site as reported by Kirk (1995). It was shown that affinity and selectivity of fluorinated adrenergic agonists were dependent on the position of

fluorine substituent on the aromatic ring of catecholic or phenolic adrenergic agonists (Kirk, 1995). In all cases fluorine at position 2 markedly reduced α-adrenoceptor affinity whereas substitution at position 6 were detrimental for β-adrenoceptor affinity; however, 5-fluoro substituents of norepinephrine retained affinity for both α- and β-adrenoceptors. Hence a new series of fluorinated derivatives of marsanidine and its analogue were synthesised to find out the optimum site of fluorination on the heteroaromatic ring without any loss of affinity and with similar or may be higher selectivity profile than the parent compounds for α_2-AR. Radioligand binding was used to assess for their affinity and selectivity for the target protein, α_2-AR, versus other similar receptor types, α_1-AR, I_1BS and I_2BS, in rat brain and kidney membrane preparations.

In general, we observed that substitution of one fluorine atom on the heteroaromatic ring of both **1** and **2** resulted in compounds with good to moderate affinity for α_2-AR. Fluorination at position 4 seems detrimental for α_2-AR affinity since both AW-20 and AW-22 displayed moderate α_2-AR affinity with K_i values 10- to 30-fold higher than that of the lead compounds (Sączewski *et al*., 2008; Saczewski *et al*., 2012). On the other hand, fluorine substitution on C6 or C7 of the heteroaromatic ring favours α_2-AR binding as seen in AW-30, AW-31 and AW-34. It is interesting to note that substituting more fluorine on the heteroaromatic ring of marsanidine at adjacent positions (AW-35) resulted in low affinity at all four receptors possibly due to formation of a large and bulky molecule that is unable to interact favourably with the receptor.

In addition to exhibiting high α_2-AR affinity, the fluorinated derivatives of **2** were also highly potent at I_2BS unlike the parent molecule which showed good selectivity for α_2-AR over I_2BS (selectivity ratio I_2/α_2 >2000 fold) (Saczewski *et al*., 2012). Therefore, the nonselective nature of these derivatives makes them unsuitable as candidates for α_2-AR PET ligand. In contrast, the

fluorine substituted marsanidine (**1**) derivatives displayed high binding affinity for α_2-AR but only moderate to low affinity for I_2BS. Hence, their relative selectivity for α_2-AR versus I_2BS makes them promising as prospective candidates for PET radiotracer. Of particular interest are the two compounds, AW-21 and AW-25. Although the affinities of AW-21 and AW-25 for the target protein α_2-AR is roughly two times less than that of marsanidine, they exhibited the highest selectivity for α_2-AR over I_2BS among all the compounds in the series. In fact, AW-21 is almost 10-fold more selective towards α_2-AR than marsanidine with respect to I_2BS affinity (Sączewski *et al*., 2008). In addition, its moderate selectivity for α_2-AR versus α_1-AR and I_1BS makes AW-21 a suitable candidate for a potential PET radiotracer.

In contrast to the above mentioned ligands, fluorinated derivatives of marsanidine which did not contain a –N= or $–CH_2$ bridge between the heteroaromatic ring and imidazoline moiety showed low affinity for α_2-AR irrespective of site of fluorination and this disqualifies them as potential candidates for α_2-AR PET radiotracer. With the exception of AW-36, this subgroup of marsanidine derivatives exhibited high affinity along with high selectivity for I_2BS relative to α_2-AR. These findings seem to agree with previously evaluated compounds where linking various aromatic rings directly to position 2 of the imidazoline moiety resulted in increased affinity for imidazoline binding sites while generally reducing α-adrenergic affinity (Anastassiadou *et al*., 2001).

Although they are termed as marsanidine derivatives here, binding profile of similar compounds had also been reported earlier by Saczewski and coworkers. Previous studies showed that the parent compound with unsubstituted ring had moderate I_2BS affinity whereas its 4-Cl substituted analogue displayed high affinity and selectivity for I_2BS over α_2-AR which was reasoned in part due to preference for a more planar conformation favouring I_2BS binding (Saczewski *et al*.,

2003). This can be compared to the 4-F congener in the current series AW-24 which also retained high affinity and selectivity for I_2BS versus α_2-AR, although to a lower extent than that of the 4-Cl derivative reported earlier. Interestingly, the disubstituted ring of the 4-Cl analogue studied currently AW- 39 (4-Cl and 5-F) retained good affinity for I_2BS. Another study by the same group in 2006 reported on a group of imidazoline compounds with high I_2/α_2 selectivity ratio that are structurally analogous to AW-40 and AW-41 investigated in the current study. Although the 4-Cl derivative in this case showed high I_2/α_2 selectivity, substitution of more chlorine on the aromatic ring at adjacent positions reduced the compound's affinity for I_2BS (Saczewski *et al*., 2006). In contrast, the mono- and trifluorosubstituted AW-40 (F on C5) and AW-41 (F on C4, C5 and C6) retained good affinity towards I_2BS over α_2-AR. These highly selective I_2BS compounds may prove to be useful tools in further characterising I_2BS functions, although they lack good affinity for α_2-AR making them unsuitable as α_2-AR PET radiotracer.

Conclusion

Development of a PET radiotracer selective for α_2-AR has been studied extensively in recent years. PET ligands must have affinity and be highly selective for the target protein. We chose two previously reported highly selective α_2-AR agonists as lead compounds and prepared several fluorinated derivatives which were studied *in vitro* for their affinity and selectivity for α_2-AR over α_1-AR and I_1BS and I_2BS. Fluorination at position 6 and 7 on the heteroaromatic ring of marsanidine seemed to favour α_2-AR binding. We identified AW-21 and AW-25 exhibiting high α_2-AR affinity at nanomolar concentrations of which AW-21 displayed the highest I_2/α_2 selectivity ratio (>10000) along with moderate selectivity versus α_1-AR and I_1BS. Therefore the 7-F derivative of marsanidine AW-21 shows favourable binding profile at and overall selectivity for α_2-AR, making it suitable for further *in vivo* studies (see Chapter V).

Chapter V: *In vivo* Study on the Effects of AW-21 and Marsanidine on Monoamine Release at Frontal Cortex

Introduction

Perhaps the most studied physiological function of central α_2-AR is their inhibitory role in the regulation of monoamine neurotransmitter release. When stimulated by endogenous adrenaline and noradrenaline (NA), presynaptic α_2-AR present on noradrenergic neurons (autoreceptors) couple to G-proteins of the G_i/G_o type (Robinson and Hudson, 2006). This leads to inhibition of adenylyl cyclase (reducing cAMP) and voltage-gated Ca^{2+} channels and activation of inwardly rectifying K^+ channels and MAPK signaling cascade, resulting in inhibition of neurotransmitter release from nerve terminals (Hein, 2006). In addition, α_2-autoreceptors which are present on neuronal cell bodies (somatodendritic autoreceptors) may reduce NA release by decreasing cell firing rates (Cedarbaum and Aghajanian, 1977). Apart from regulation of NA release by α_2-autoreceptors, another group of presynaptic inhibitory α_2-AR (heteroreceptors) can also be found on nerve terminals of neurons that release neurotransmitters other than NA (Gilsbach and Hein, 2012; Robinson and Hudson, 2006).

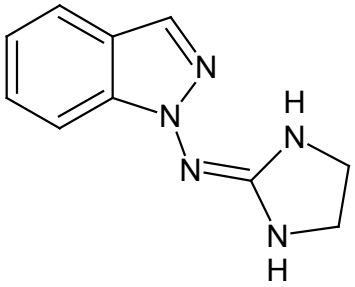
Exogenous α_2-AR agonists mimic this inhibitory effect while antagonists cause a blockade of such inhibition (Nutt *et al*., 1997). It has been confirmed in both *in vitro* and *in vivo* studies that α_2-AR agonists such as clonidine reduce extracellular NA levels in different brain regions (Dalley and Stanford, 1995; L'Heureux *et al*., 1986; Maura *et al*., 1992; van Veldhuizen *et al*., 1993). On the other hand, numerous studies have also reported elevation of extracellular NA levels following administration of α_2-AR antagonists such as idazoxan and yohimbine (Abercrombie *et al*., 1988; Dennis *et al*., 1987; L'Heureux *et al*., 1986; Thomas and Holman, 1991). Furthermore, *in vivo* neurochemical studies have shown α_2-AR agonists and antagonists

to modulate concomitant changes in extracellular dopamine (DA) levels along with altering NA release in different brain regions in rats and mice (Gobert *et al*., 1997; Gresch *et al*., 1995; Kawahara *et al*., 2001; Yavich *et al*., 1997).

The technique of brain microdialysis has been one of the most commonly employed methods to monitor changes in neurotransmitter release in *in vivo* studies. Microdialysis is essentially a sampling technique that can be used to measure dynamic changes of endogenous (neurotransmitters, hormones, metabolites, etc.) and exogenous substances (e.g., drugs) in the extracellular fluid of central and peripheral tissues in both animals and humans (Anderzhanova and Wotjak, 2013; Li *et al*., 2006). The basic principle of microdialysis is to mimic the function of a capillary blood vessel where a thin dialysis probe with semipermeable membrane at the tip is implanted at the tissue of interest and perfused with physiological fluid at a low flow rate. As a result, exchange of soluble substances between the extracellular fluid compartment and the fluid within the probe takes place by simple diffusion along a concentration gradient through the semipermeable membrane. The concentric semipermeable dialysis membrane of known pore size usually allows small molecules to diffuse freely, restricting large molecules (e.g., tissue enzymes) and thus providing relatively clean samples containing analytes of interest. Samples are subsequently collected and concentration of analytes determined using appropriate analytical techniques in order to draw conclusions about biochemical changes in respect of any behavioural, pharmacological or genetic interventions. This widely used technique is applied not only to monitor *in vivo* neurochemical changes but also to associate changes in behaviour with neurotransmission, assess biochemical changes following local administration of drugs through the probe and study pharmacodynamics and pharmacokinetics of drugs.

In the previous chapter, a series of fluorinated derivatives of marsanidine (Figure 5.1A) and its heteroarylmethyl analogue was evaluated in order to identify a ligand selective for α_2-AR which may be further developed as a prospective α_2-AR PET radiotracer. Preliminary binding assays identified the 7-fluoro derivative of marsanidine AW-21 (Figure 5.1B) to show favourable binding profile at α_2-AR. AW-21 displayed high affinity in nanomolar range for α_2-AR along with high selectivity versus I_2BS and moderate selectivity over α_1-AR and I_1R (α_2 K_i = 31 nM, selectivity ratio α_2/I_2 > 10,000), thus fulfilling the primary criterion for a candidate PET ligand.

A)



B)

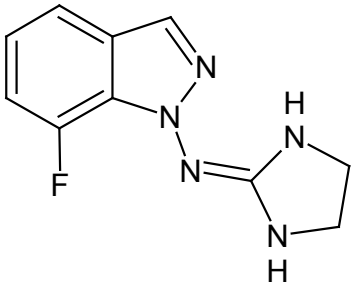


Figure 5.1: Chemical structures of (A) marsanidine and (B) its 7-fluoro derivative, AW-21.

Apart from showing high affinity and selectivity for the target protein, a potential PET ligand must also be able to reach its central target protein *in vivo*, that is, be able to cross the blood-brain barrier (BBB) in adequate concentration (Pike, 2009). Endothelial cells making up the BBB are connected by complex tight junctions strictly limiting movement of substances into the brain except for small (<400 Da) lipophilic molecules. In addition, highly efficient efflux pumps are present in the BBB that remove xenobiotics from the CNS adding another layer of restriction to entry of molecules into the brain (Shannon *et al*., 2013).

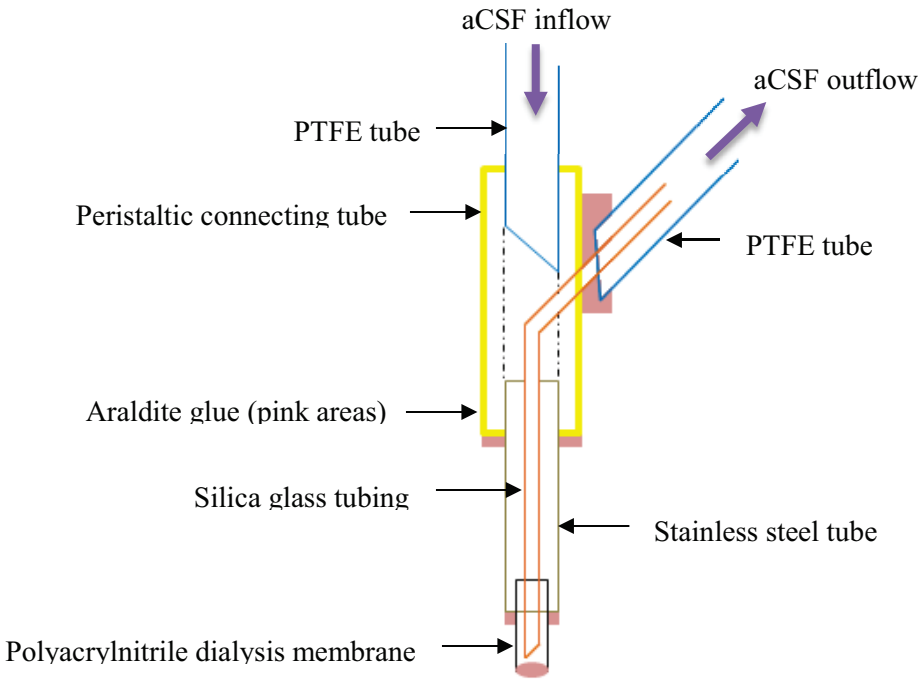
Therefore, in this chapter we sought to further evaluate the potential of AW-21 as a candidate PET ligand for α_2-AR by investigating its ability to cross the BBB as well as its pharmacological effects on extracellular monoamines (NA and DA) in the frontal cortex of conscious freely moving rats using the technique of *in vivo* brain microdialysis. To date *in vivo* pharmacological properties of the parent compound marsanidine have not been assessed with microdialysis. Therefore, marsanidine was included in the present study for useful comparison. In addition, any overt behavioural changes in the animals following drug administration were investigated.

Materials and method

Construction of microdialysis probe

Concentric dialysis probes were constructed in-house using thin-walled stainless steel tube (23 gauge) with silica tubing (OD: 0.17 mm) and polyacrylnitrile membrane (exposed length: 4 mm, ID: 0.2 mm, OD: 0.3 mm, cut off MW 40,000) (Figure 5.2). The *in vitro* recovery rate of the probes ranged between 10 - 15%.

A)



B)

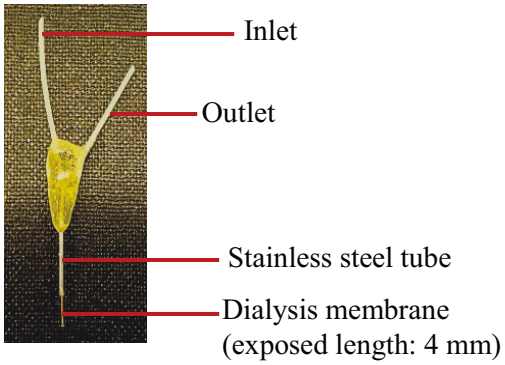


Figure 5.2: Microdialysis probe constructed in-house and used in the present study. (A) Diagrammatic representation. Arrows (→) represent the direction of aCSF (+ DMI) flow through the probe. (B) Photograph of a probe.

Animals

Dialysis experiments were carried out using male Sprague-Dawley rats (final weight 280-300 g) obtained from Bioscience, University of Alberta, Canada. Animals were held in a temperature controlled environment (23°C) on a 12-hour light/dark cycle with free access to food and water. They were acclimatised to laboratory environment and to regular handling for 7 days prior to surgery. Animal care and all experimental procedures described below were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee: Health Sciences for the University of Alberta.

Surgical implantation of microdialysis probe

Before beginning surgery, dialysis probes were placed carefully to be held by a probe clip and perfused with deionised water pumped at a flow rate of 2 μL/min. Rats were anaesthetised with a mixture of ketamine HCl (75 mg/kg) and xylazine (10 mg/kg) given intraperitoneally and placed in a stereotaxic frame with the incisor bar set at -3.5 mm. Throughout the duration of surgery, animals remained under anaesthesia with additional doses of ketamine/xylazine mixture if required and their body temperature was maintained at 37°C using a homeothermic heating blanket.

Microdialysis probes were implanted stereotaxically into the rat frontal cortex as previously described by Abu Ghazaleh *et al*. (2007). An incision was made on the scalp running anterior to posterior and pericranium removed to expose the bony surface of the skull which is then slightly roughened making bregma (reference point) visible. Two anchor screws were bored adjacent to probe placement for better contact with cement mixture. Probe implantation location at the frontal cortex was calculated relative to bregma (+1.2 mm lateral, +3.0 mm anterior) using the

Rat Brain Atlas of Paxinos and Watson (1986). Once the probe was aligned directly above the site of implantation, a small 1 mm hole was drilled through the skull at that point. The probe was then lowered to gently touch the dura and vertical measurements were made to calculate the depth (-5.0 mm relative to dura) of probe placement. Piercing the dura with a fine pointed needle, the probe was finally lowered to a position according to the depth measurements made earlier. Dental acrylic cement was applied onto the exposed skull and around the probe to secure the position of the implanted probe. Rats were then removed from the frame and left to recover overnight in home-box with food and water *ad libitum*.

Microdialysis

Following 24 hours recovery, the implanted probes were perfused with artificial cerebrospinal fluid (aCSF) composed of 147 mM NaCl, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$ and 3 mM KCl using a syringe infusion pump 22 (Harvard Apparatus) at a flow rate of 2 µL/min. The NA reuptake blocker desipramine (DMI) at a concentration of 1 µM was included in the perfusion medium from the beginning of experiments to enhance detectability of NA and DA (Meana *et al*., 1997). Dialysate samples were collected every 20 minutes in conscious freely moving rats. Extracellular monoamine levels were taken to be stable when contents in three consecutive samples did not differ by more than 5% of one another. Once basal NA and DA levels were established, rats were administered with saline (0.9% NaCl), marsanidine (1 mg/kg), or AW-21 (0.1 or 1 mg/kg) *via* intraperitoneal (i.p) route at time zero and sampling continued for a further 3 hours.

Analysis of dialysate samples

Dialysate samples collected were immediately subjected to analysis for monoamine contents using two independent reverse phase high performance liquid chromatography (HPLC) systems

(one for NA and the other for DA) coupled with electrochemical detection (ECD). The general principle of reverse phase HPLC includes separation of components in a mixture based upon their relative chemical interactions with nonpolar stationary phase (chromatography column). The isocratic mobile phase was delivered through the system under high pressure by a dual piston pump. Dialysate samples which were injected into the system *via* a manual injection valve flowed through the column along with the mobile phase. Each component in the sample travels through the column at different speed, being retained depending upon several factors which include structural interaction of each component, composition of the mobile phase and composition of the packing material of the column. As a result, separation of components in the sample occurs, each one eluting from the column at different times. The output from the column is then directly analysed using an ECD which either oxidises or reduces the separated compounds from the column depending upon the potential applied across the electrodes of the cell, thus producing a change in current measured by the ECD. This change in signal is recorded as a measurable peak on the chart recorder and its size is dependent upon the amount of compound producing the signal. Thus the concentration of a substance in a sample is determined by interpolating the peak height obtained from a standard curve.

For NA assay, mobile phase A consisted of 2 g/L sodium acetate, 3.1 g/L citric acid and 500 mg/L octanesulphonic acid in deionised water. Methanol (13% v/v) was added and pH adjusted to 5.0 using 10 M NaOH. Mobile phase A was delivered onto a reverse phase analytical column (Hichrom, ODS 3 μm of 12.5 cm length, ID: 4.6 mm) at a flow rate of 1.0 mL/min. A dual 5014A electrode cell paired to a Coulochem II (ESA) electrochemical detector was used to detect NA in the sample. The first electrode of the cell was set at a potential of -100 mV while maintaining the potential of second electrode at +250 mV. The signal from this second electrode

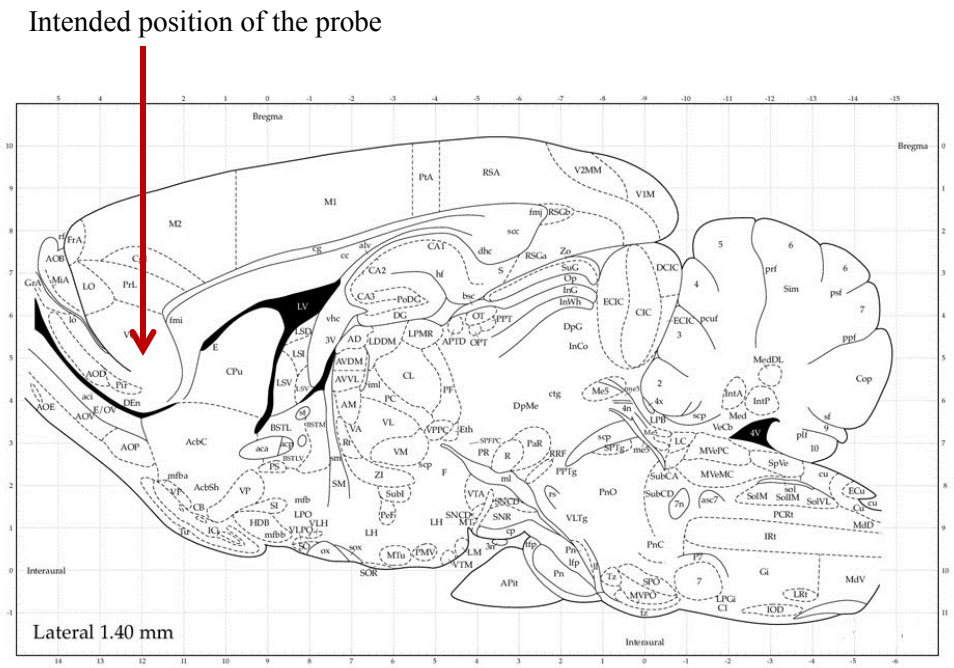
was output to the chart recorder. Under these conditions the retention time of NA was 5.5 minutes.

For DA assay, mobile phase B was prepared by dissolving 10.92 g/L sodium dihydrogen orthophosphate and 270 mg/L octanesulphonic acid in deionised water to which methanol (11% v/v) was added (pH 4.6). Mobile phase B was delivered onto another reverse phase analytical column (Beckman, ODS 3 μm of 7.5 cm length, ID: 4.6 mm) at a flow rate of 1.0 mL/min and extracellular DA present in the sample was detected in a similar way as described above using electrode cell coupled to a detector. However, in this case the second electrode potential was set at +300 mV. The retention time of DA was 5 minutes under these conditions.

Verification of probe placement

After microdialysis experiment was over, rats were euthanized by Euthanyl given i.p. Following euthanasia, the probes were carefully pulled out of the brains which were then rapidly removed and frozen at -70°C. On a later date, each brain was sectioned using a sharp blade to visualise a thin tract left by the probe and confirm its placement at the frontal cortex using the rat brain atlas (Figure 5.3).

A)



B)

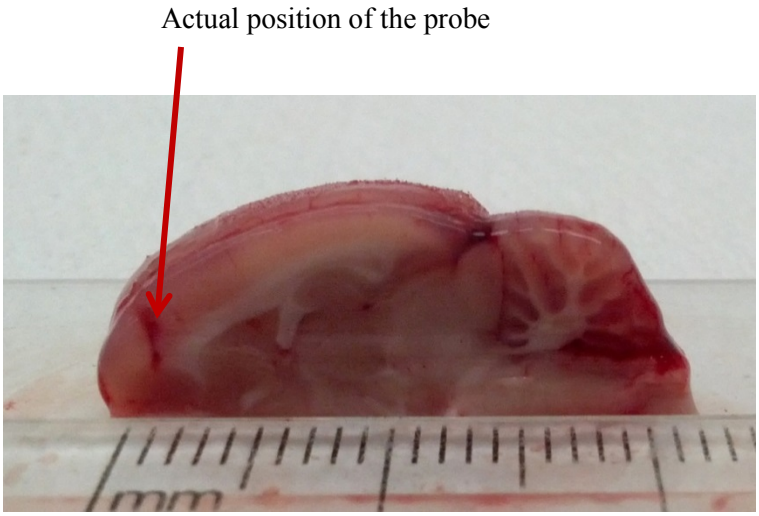


Figure 5.3: Verification of microdialysis probe placement in rat frontal cortex. (A) Schematic illustration of intended position of the probe in frontal cortex according to Rat Brain Atlas of Paxinos and Watson (1986). (B) Photograph of saggital section of rat brain showing the actual position seen as a thin tract in frontal cortex.

Statistical analysis

All statistical analyses were performed using GraphPad Prism for Windows version 5.01 (GraphPad Software, San Diego, CA). NA and DA contents in the dialysate samples at each time point were expressed as mean ± S.E.M. (n = 6 per group) and presented as percentage of basal. Absolute data obtained were statistically analysed using one-way repeated measures analysis of variance (ANOVA) followed by *post hoc* Dunnett's multiple comparison test to determine any significant differences within a group from basal values (t = 0 minutes) following drug treatment. The level of significance was set at $p < 0.05$.

Materials

Ketamine HCl (100 mg/mL) and Euthanyl (pentobarbital sodium 240 mg/mL) were purchased from Bimeda-MTC Animal Health Inc. (Cambridge, ON). Xylazine (100 mg/mL) was obtained from Bayer Inc. (Toronto, ON). Desipramine hydrpcloride, dopamine hydrochloride and L-(-) noradrenaline bitartrate salt monohydrate were purchased from Sigma Aldrich. Marsanidine and AW-21 were kindly synthesised by Dr Franciszek Saczewski's group (Medical University of Gdańsk, Poland).

The following components were required for in-house probe construction: solva trans tubing (Elkay Laboratory Products Ltd, Basingstoke, UK), glass silica tube (SGE Analytical Science Pty Ltd, Victoria, Australia), hypodermic stainless steel tube (A-M Systems Inc, Sequim, WA) and hospalpolyacrylnitrile dialysis membrane (Hospal Medical, New Jersey, USA).

Results

The functional activities of AW-21 and its parent compound marsanidine on NA and DA release in frontal cortex were assessed in conscious freely moving rats using *in vivo* brain microdialysis. Tables 5.1 and 5.2 summarise the levels of extracellular NA and DA respectively in frontal cortex following i.p. drug administration at time zero after establishing the basal neurotransmitter levels.

As illustrated in Figure 5.4A, no significant change was observed in extracellular NA level from basal in saline treated control animals throughout the time course of the experiment. The parent compound marsanidine at a dose of 1 mg/kg body weight produced only a slight decrease in extracellular NA levels (maximum by about 15% at 40 minutes) in rat frontal cortex compared to basal values. Systemic administration of AW-21 at a low dose of 0.1 mg/kg caused a slight decrease in extracellular NA levels (maximum by about 17% at 20 minutes) when compared to basal. At a higher dose of 1 mg/kg, AW-21 significantly reduced cortical NA levels as compared to basal values between 20 and 80 minutes post drug administration. The maximal effect was observed at 40 minutes where extracellular NA levels were reduced by about 73%. Extracellular NA level returned to baseline at 120 minutes following treatment. However, significant overshoot of NA from basal level in rat frontal cortex was observed at 160 and 180 minutes.

Further evaluation was carried out to examine the effect of AW-21 and marsanidine on DA release in rat frontal cortex as shown in Figure 5.4B. In saline treated animals, DA level was slightly lower than baseline at the latter part of the experimental time course. Intraperitoneal administration of 1 mg/kg marsanidine produced very little changes in extracellular DA levels compared to basal. Only a small transient fall of about 11.5% from basal, though not significant, in DA level was observed at 80 minutes post drug administration. Systemic administration of

AW-21 at 1 mg/kg caused slight reduction in extracellular DA levels (maximum by 20% at 100 minutes) when compared to basal.

Apart from changes in extracellular monoamine levels in the frontal cortex, it was observed that peripheral administration of AW-21 at both the doses (0.1 and 1 mg/kg) induced rapid onset of sedation in rats. Similarly 1 mg/kg marsanidine treated rats showed a lack of coordinated movement that was soon followed by sedation.

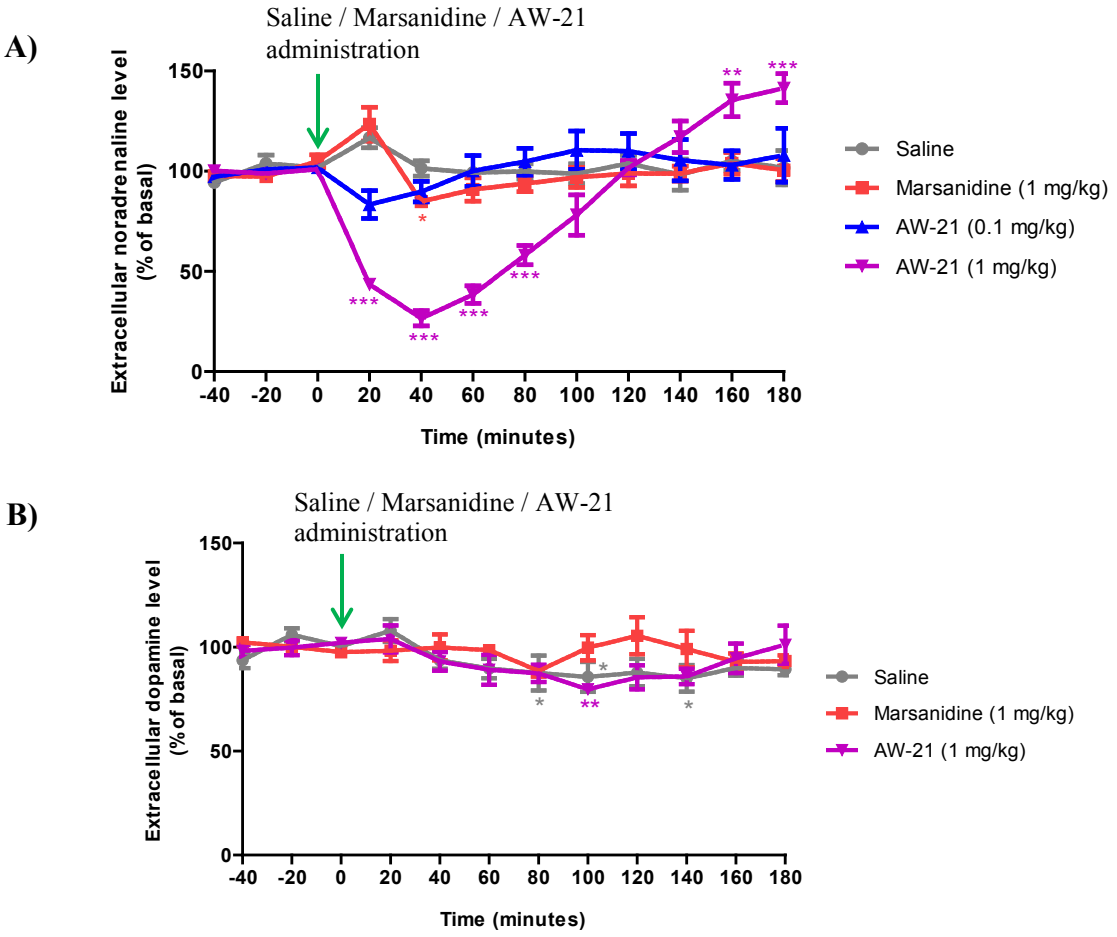


Figure 5.4: Changes in extracellular (A) NA and (B) DA levels in rat frontal cortex following systemic (i.p.) administration of saline, marsanidine and AW-21. Results are expressed as mean ± S.E.M (n = 6 per group) and presented as percentage of basal. Statistical analyses were performed using one-way repeated measures ANOVA followed by Dunnett's *post hoc* test. *p < 0.05, **p < 0.01 and ***p <0.001 denotes significant changes in monoamine levels (NA/DA) within a treated group compared to basal at time t = 0 minutes.

Table 5.1: Changes in extracellular NA levels in rat frontal cortex following systemic administration (i.p.) of saline, marsanidine and AW-21. Data is represented as mean ± S.E.M. (n = 6 per group). Statistical analyses were performed on absolute values using one-way repeated measures ANOVA followed by Dunnett's *post hoc* test. **p* < 0.05, ***p* < 0.01 and ****p* <0.001 denote significant changes in NA levels within a treated group compared to basal at time t = 0 minutes.

Time (min)	**Extracellular NA level (fmol / 40 µL sample)**			
	Saline	***Marsanidine (1 mg/kg)***	***AW-21 (0.1 mg/kg)***	***AW-21 (1 mg/kg)***
0	53.63±9.568	51.86±3.215	56.05±10.42	42.43±4.114
20	60.13±8.950	60.45±3.866	44.18±8.388	18.52±2.290***
40	54.46±10.21	41.87±2.566*	50.09±10.72	11.55±2.668***
60	52.67±9.446	44.86±3.590	57.88±13.67	16.88±3.353***
80	52.27±8.575	45.96±1.687	59.64±13.09	25.19±4.342***
100	52.16±8.810	47.69±2.998	60.01±11.82	34.1±7.242
120	54.01±8.629	48.64±3.488	59.91±11.65	43.07±5.245
140	53.96±11.44	48.88±3.555	57.92±11.95	49.22±6.121
160	57.05±11.77	51.49±4.367	58.15±12.97	58.62±9.115**
180	54.32±9.908	49.37±3.013	61.4±15.73	60.67±8.530***
One way repeated measures ANOVA	$F(9,54) = 1.841$ $p = 0.0689$	$F(9,54) = 4.136$ $p = 0.0002$	$F(9,54) = 1.762$ $p = 0.0838$	$F(9,54) = 34.54$ $p = <0.0001$

Table 5.2: Changes in extracellular DA levels in rat frontal cortex following systemic administration (i.p.) of saline, marsanidine and AW-21. Data is represented as mean ± S.E.M. (n = 6 per group). Statistical analyses were performed on absolute values using one-way repeated measures ANOVA followed by Dunnett's *post hoc* test. *$p < 0.05$ and **$p < 0.01$ denote significant changes in DA levels within a treated group compared to basal at time t = 0 minutes.

Time	**Extracellular DA level (fmol / 40 µL sample)**		
	Saline	***Marsanidine (1 mg/kg)***	***AW-21 (1 mg/kg)***
0	35±8.759	22.77±2.127	29.03±2.761
20	36.25±7.744	23.15±3.048	29.96±3.524
40	32.26±7.527	23.3±2.738	26.68±2.908
60	30.97±7.765	22.89±1.843	25.61±3.137
80	30.39±7.805*	20.52±1.600	25.41±3.507
100	30.08±8.097*	22.69±0.8839	22.89±2.564**
120	30.72±8.016	23.86±1.409	24.58±2.883
140	30.31±8.670*	22.51±1.524	24.7±2.754
160	31.48±8.330	21.38±1.338	26.57±2.496
180	30.96±7.783	21.73±2.023	28.46±2.953
One way repeated measures ANOVA	$F(9,54) = 4.109$ $p = 0.0002$	$F(9,54) = 0.6330$ $p = 0.7928$	$F(9,54) = 3.078$ $p = 0.0028$

Discussion

In the previous chapter several fluorinated derivatives of marsanidine were investigated for their affinity at α_2-AR in an attempt to identify a potential α_2-AR PET ligand. Preliminary radioligand binding assays using rat whole brain and kidney membranes identified a 7-fluoro substituted derivative of marsanidine, AW-21, to exhibit high affinity and selectivity for α_2-AR over other similar receptor types (α_1-AR, I_1R and I_2BS). In the present study AW-21 was further evaluated *in vivo* for its pharmacological effects on monoamines using *in vivo* brain microdialysis to determine whether it can cross the BBB and produce similar neurochemical effects to that of known α_2-AR agonists. The parent compound marsanidine, a known imidazoline based highly selective partial α_2-AR agonist (Sączewski *et al*., 2008; Sączewski *et al*., 2011), has not been previously studied *in vivo* for its pharmacological effects on monoamine release and/or turnover. Therefore, marsanidine was included in the current study for useful comparison.

Several regulatory mechanisms control the synaptic NA levels in the brain including presynaptic α_2-AR autoregulation. Previous *in vitro* and *in vivo* studies have invariably demonstrated α_2-AR agonists like clonidine or more selective dexmedetomidine when administered locally or systemically to exert an inhibitory effect on NA release *via* presynaptic α_2-AR located on noradrenergic axon terminals (autoreceptors) (Dalley and Stanford, 1995; Gobert *et al*., 1998; L'Heureux *et al*., 1986; van Veldhuizen *et al*., 1993) which can be reversed by administration of α_2-AR antagonists like RX 821002 (Meana *et al*., 1997). It was further revealed using knockout mice that the NA release in the prefrontal cortex is predominantly regulated by presynaptic α_{2A}-AR subtype (Ihalainen and Tanila, 2002; Trendelenburg *et al*., 2001). In the present study systemic administration of both marsanidine and its fluorinated derivative AW-21 reduced extracellular NA levels in rat frontal cortex. In addition, AW-21 caused a dose-related reduction

in extracellular NA levels in rats. Therefore this is in accord to well established effects of α_2-AR agonists on cortical NA release from nerve terminals. However, marsanidine being a partial agonist (Sączewski *et al*., 2011) exerted its inhibitory effect to a lesser extent on NA release when compared to AW-21 at the same dose of 1 mg/kg. Interestingly, slight elevation in extracellular NA levels following i.p. injection were observed in saline and marsanidine treated rats. Such a transient NA rise may be the result of mild stress induced by animal handling (Ma and Morilak, 2005).

The frontal cortex is densely innervated by noradrenergic projections arising mainly from locus coeruleus (LC), a small nucleus located in the pons which is the major noradrenergic nucleus of the brain and also the exclusive source of cortical NA. LC plays a central role in the maintenance of wakefulness and regulation of autonomic functions (Samuels and Szabadi, 2008a). In addition to presynaptic terminal location, inhibitory α_2-AR are present on both cell bodies and dendrites of LC noradrenergic neurons (Cedarbaum and Aghajanian, 1977). Activation of these somatodendritic α_2-AR which are mainly of α_{2A}-subtype (Mateo and Meana, 1999) reduces LC activity by hyperpolarising neuronal membrane *via* increased K^+ efflux leading to decreased spontaneous neuronal firing rates and attenuation of NA release in projecting regions (Samuels and Szabadi, 2008b; Svensson *et al*., 1975; Williams *et al*., 1985). Local infusion of α_2-AR agonist clonidine directly into the LC through microdialysis probe causes a reduction in extracellular NA levels in the LC as well as in the prefrontal cortex of conscious rats (Kawahara *et al*., 2001; Pudovkina *et al*., 2001). Systemic administration of drugs does not restrict the location of pharmacological action of the ligand as opposed to local administration. Hence the reduction in NA levels in frontal cortex observed in the present study may be alternatively due to

interaction of marsanidine or AW-21 with somatodendritic α_2-autoreceptors at the level of LC along with presynaptic terminal α_2-AR in frontal cortex.

Moreover, it was observed that marsanidine or AW-21 treated rats were rapidly sedated following systemic administration. Sedation is one of the classic functions mediated by α_2-AR predominantly attributed to the action of α_{2A}-AR subtype (Hunter *et al*., 1997; Lakhlani *et al*., 1997). Clinically used α_2-AR agonists, clonidine and dexmedetomidine, are well known for their potent sedative effects in human and animals, the mechanism of which was shown due to inhibition of LC neurons *via* activation of somatodendritic α_{2A}-AR in LC (Gilsbach and Hein, 2012; Samuels and Szabadi, 2008b). This further suggests marsanidine and AW-21 were able to exert their sedative effects probably at the level of LC. Given that the drugs were administered in the rats peripherally *via* i.p. route, they appear to exert a centrally mediated sedative effect indicating that both compounds were able to cross the BBB. This further highlights the potential of AW-21 as a candidate PET ligand for α_2-AR since ability to cross the BBB is one of the fundamental properties a prospective PET ligand must exhibit as mentioned earlier. However, additional experiments are necessary to confirm their subtype selective interaction in mediating the observed central neurochemical alterations and behavioural changes in the animals.

Interestingly, near the end of experimental time frame there is a significant overshoot of extracellular NA levels over baseline indicating a rebound effect. Carefully observing the changes in the levels of extracellular NA (Figure 5.4A) over time following drug administration, it can be speculated that as the inhibitory effect of AW-21 through presynaptic α_2-AR on NA release from nerve terminals wears off, perhaps there is an increase in synaptic release of NA. This may also be accompanied by an increase in neuronal firing rates at the level of LC since the sedative effect appears to cease around the same time frame. An additional augmenting effect

behind such elevation of extracellular NA levels may be presence of the NA reuptake inhibitor DMI in the perfusion medium. However, further studies need to the carried out to fully understand the mechanistic basis of these changes.

As mentioned earlier, another group of inhibitory α_2-AR called heteroreceptors are located presynaptically on nerve terminals of non-adrenergic neurons which when activated can modulate the release of many neurotransmitters including monoamines like serotonin and dopamine (Gilsbach and Hein, 2012; Robinson and Hudson, 2006). In the present study effects on DA release in rat frontal cortex of one dose (1 mg/kg) of marsanidine and AW-21 were investigated. Following systemic administration, both the compounds reduced DA levels in frontal cortex. However, the extent of extracellular DA modulation appears to be much less than that of NA. Of the two, marsanidine exerted an overall weaker effect on lowering extracellular DA levels than its fluorinated derivative AW-21. It should also be noted that the timecourse of DA response was slower than that of NA in terms of achieving maximal reduction in extracellular monoamine levels post treatment. A minor effect of time on extracellular DA levels was also observed in saline treated rats.

Dopaminergic projections in the frontal cortex originate from ventral tegmental area (VTA) (Lindvall *et al*., 1974). Reports from various lines have shown a dopamine-noradrenaline interaction in the cortex. Previous *in vitro* and *in vivo* studies employing α_2-AR agonists administered either locally or systemically resulted in a decrease in cortical DA levels whereas α_2-AR antagonists increased extracellular levels of DA (Gobert *et al*., 1997; Gobert *et al*., 1998; Gresch *et al*., 1995; Kawahara *et al*., 2001; Trendelenburg *et al*., 1994; Yavich *et al*., 1997). Thus the current study supports the hypothesis that DA release in the frontal cortex is under (tonic) inhibitory control of presynaptic α_2-heteroreceptors present on dopaminergic nerve

terminals though it seems that the compounds under investigation here exert a weak effect on DA modulation. It has been suggested that the heteroreceptors on non-adrenergic terminals are mainly of α_{2A}-subtype (Trendelenburg *et al*., 1994). Gobert *et al*. (1998) demonstrated that non-subtype selective α_2-AR agonist, dexmedetomidine, and antagonist, RX 821002, decreased and increased respectively DA in frontal cortex measured by *in vivo* microdialysis. These effects were mimicked when preferential α_{2A}-AR agonist and antagonist, guanabenz and BRL 44408 respectively, were employed indicating that α_{2A}-heteroreceptors regulate cortical DA release. In addition, knockout studies in mice suggest a role of α_{2A}-adrenergic heteroreceptors in modulating extracellular DA levels in frontal cortex although involvement of α_{2C}-AR as heteroreceptors cannot be excluded (Bücheler *et al*., 2002; Ihalainen and Tanila, 2002). Despite several lines of evidence pointing towards presynaptic α_2-heteroreceptor regulation of DA release, location of these receptors on dopaminergic neurons and their regulatory mechanism require further verification.

Subsequently, alternative mechanisms in DA modulation have also been proposed. Recent studies have shown that extracellular DA may be removed by NA transporters located in noradrenergic nerve terminals reducing synaptic DA levels (heterotransporter regulation) which can be blocked by NA reuptake inhibitors (Gresch *et al*., 1995; Pan *et al*., 2004). However, this is unlikely since DMI, a NA reuptake blocker, was already present in the perfusion medium. Others have suggested co-transmission of both NA and DA from cortical noradrenergic terminals (Devoto *et al*., 2001; Devoto *et al*., 2004). A complex auto- and heteroreceptor control on monoamine release exists in the frontal cortex. Therefore, further studies must be carried out in frontal cortex and other brain areas to fully elucidate dopamine-noradrenaline modulatory

properties of AW-21 and its parent compound marsanidine and also to explore any direct/indirect influence of other monoaminergic/neurotransmitter system on NA and/or DA release.

Conclusion

Our preliminary *in vivo* microdialysis data shows AW-21, acting as an agonist, reduces extracellular NA level in rat frontal cortex in a dose-related manner and shares similar functional properties with the known α_2-AR agonists. Although both marsanidine and AW-21 at the same dose of 1 mg/kg reduced cortical NA, the effect of AW-21 was much greater compared to that of marsanidine which is a partial agonist. In addition, both marsanidine and AW-21 had little effect on extracellular DA levels in the rat frontal cortex. Moreover, sedation induced by marsanidine and AW-21 indicates that they are able to cross the blood-brain barrier following peripheral administration. Taken together, the present study suggests that AW-21 has favourable pharmacological properties at α_2-AR and can penetrate the blood-brain barrier, further highlighting its potential as a prospective α_2-AR selective ligand for *in vivo* PET imaging.

Chapter VI: General Discussion

The field of IBS has grown since Bousquet and coworkers in 1984 first suggested the existence of binding sites preferably interacting with ligands of one particular chemical class (imidazoline and related structures). However, to date IBS containing proteins have not been cloned. Thus characterisation of these putative sites depend on the use of a combination of agents with differing affinities for each receptive site which sometimes may give rise to confounding results. Subsequently it is crucial to synthesise highly selective ligands that would be useful research tools for investigating functional roles of these sites. Therefore, the primary aim for this thesis was set to explore the structure-affinity relationships of several series of newly synthesised imidazoline bearing ligands with regard to their *in vitro* binding activity at α-adrenoceptors (α_1- and α_2-AR) and IBS (I_1- and I_2BS) in rat whole brain or kidney membranes.

The first series of compounds investigated in this project was MP series synthesised by Pigini's group (Chapter II) whose work focuses on the synthesis and identification of novel selective ligands for α_2-AR or subtypes of IBS containing the basic structure where an imidazoline nucleus is linked to an aromatic ring via a two-atom bridge. Previous SAR studies have shown that minor chemical modifications in the bridge determine preferential recognition of a particular receptor system whilst the nature of substituents introduced into the aromatic ring affect affinity and subsequent functional activity of the ligands (Gentili *et al*., 2002; Gentili *et al*., 2003). In the present study we explored the binding profile of compounds in MP series, which were rationally designed (based on previous SAR studies) to interact selectively with subtypes of IBS or show multitarget interactions.

Overall, compounds in MP series showed higher affinity for I_2BS than I_1BS and α_2-AR. This includes the ligands containing a methyl substitution on C-1 in the bridge. However, the unsubstituted derivative MP 108, which exhibited high selectivity for I_2BS over I_1BS, contradicted previous results where the same ligand (CH_3-phenyzoline) showed high affinity and selectivity for I_1BS versus I_2BS (Gentili *et al*., 2003). Thus the present study suggests that the lack of hypotensive response observed earlier with CH_3-phenyzoline (Gentili *et al*., 2003) may not be due to its antagonistic effects but rather due to its low affinity at I_1BS. Ligands with limited conformational freedom at the bridge owing to presence of a double bond exhibited very high affinity and marked selectivity at I_2BS. This is in agreement with previous studies by other groups suggesting a more planar structure is favourable for I_2BS interaction (Saczewski *et al*., 2003). On the other hand, compounds containing –NH in the bridge were nonselective in nature showing high and comparable affinities at both α_2-AR and I_2BS. Additionally, small substituents (like methyl and chloro) with low steric bulk at the ortho position of the aromatic ring were well tolerated in terms of affinity and selectivity at respective receptors while those with enhanced steric bulk exhibited unfavourable interactions and low affinity at the target receptors.

Subsequent radioligand binding studies were carried out on compounds in TCS/TCA series which was synthesised by Saczewski's group (Chapter III). These compounds were derivatives of previously established highly selective partial α_2-AR agonist, marsanidine (Sączewski *et al*., 2008). Here, the importance of the nature and pattern of substitution on the heteroaromatic ring was explored with regard to α_2-AR affinity and selectivity in particular. The compounds in TCS/TCA contained either a halide (fluorine, chlorine or bromine) or a methyl group substituted at different positions on the heteroaromatic ring. As seen with MP compounds, ligands in the TCS/TCA series with these substituent groups, which are endowed with low steric bulk,

generally display good to high affinity for α_2-AR. Substitution of a halide or methyl group at position 7 of the heteroaromatic ring appears to be particularly favourable for interaction with α_2-AR. However, most of the ligands in this series were nonselective in nature and displayed good affinity for other receptor types as well.

Many groups, including our own, have attempted to develop prospective candidates for selective α_2-AR PET radiotracer. Although the results obtained thus far are promising, there is yet no α_2-AR PET radiotracer for routine use. Halogen substituted compounds in the TCS/TCA series maintained high affinity for α_2-AR. This observation inspired us to select two lead structures: marsanidine and its heteroarylmethyl analogue, which are highly selective α_2-AR ligands characterised previously by our group (Sączewski *et al*., 2008; Saczewski *et al*., 2012). Since fluorine-18 (^{18}F) is one of the positron emitters that label a PET radiotracer (Pike, 2009), a new series of fluorinated derivatives of the leads (AW series) was synthesised (Chapter IV). Here fluorine was substituted on the heteroaromatic ring at different positions (C-4 to C-7) to find out the optimum site of fluorination that would produce ligand(s) with high affinity and possibly selectivity for the target protein, α_2-AR – the primary criterion for a candidate PET ligand.

In AW series of compounds, introducing fluorine substituent at position 6 or 7 on the heteroaromatic ring favoured α_2-AR binding similar to what was in the previous study with TCS/TCA series. However, fluorinated derivatives of the heteroarylmethyl analogue of marsanidine were nonselective and exhibited high I_2BS affinity in general, making them unsuitable as candidates for an α_2-AR PET ligand. Also, the subgroup of compounds lacking the one-atom bridge between imidazoline moiety and heteroaromatic ring showed low affinity for α_2-AR, disqualifying them as candidates for α_2-AR PET ligand. Instead, these compounds displayed high affinity and selectivity for I_2BS over α_2-AR, possibly attributed to their more

planar structure as seen previously with compounds with similar structure (Saczewski *et al*., 2003; Sączewski *et al*., 2006). On the contrary, fluorinated derivatives of the lead, marsanidine, generally showed high nanomolar affinity and selectivity for α_2-AR. In particular AW-21 and AW-25 exhibited the highest selectivity for α_2-AR over I_2BS. Of the two, AW-21 was roughly 10 times more selective for α_2-AR than its parent molecule. Hence the preliminary *in vitro* binding profile of AW-21 suggests that it may be suitable as a prospective candidate for an α_2-AR selective PET ligand.

AW-21 was further characterised *in vivo* (Chapter V) to determine its pharmacological action on brain monoamine levels and, more importantly, whether it can cross the BBB – another fundamental criterion to be met by any candidate PET ligand (Pike, 2009). The effects of systemic (i.p.) administration of AW-21 on monoamine levels (NA and DA) in frontal cortex were investigated in conscious freely moving rats using brain microdialysis. The parent compound marsanidine was included in the study for useful comparisons.

AW-21 reduced extracellular NA levels in rat frontal cortex in a dose related manner. Similar decrease in NA levels was observed following i.p. administration of marsanidine though to a much lesser extent than AW-21 perhaps due to partial agonism of marsanidine (Sączewski *et al*., 2011). The results are in agreement with previous *in vivo* studies which showed other α_2-AR agonists to lower extracellular NA levels in different brain regions in rats possibly by acting at presynaptic α_2-AR and exerting an inhibitory effect on NA release from nerve terminals (Dalley and Stanford, 1995; Gobert *et al*., 1998; L'Heureux *et al*., 1986; van Veldhuizen *et al*., 1993). Alternatively AW-21 and marsanidine may also act at the somatodendritic α_2-AR at the level of LC and reduce neuronal firing rates, thereby decreasing cortical NA levels (Kawahara *et al*., 2001; Pudovkina *et al*., 2001). In addition, LC inhibition appears to be likely due to rapid

induction of sedation observed in the rats following AW-21 or marsanidine administration. This also indicates that AW-21 is able to cross the BBB when given peripherally, highlighting its potential as a candidate α_2-AR PET ligand. Both AW-21 and marsanidine, at the higher dose, reduced extracellular DA levels slightly in the rat frontal cortex. Although previous studies suggested an α_2-heteroreceptor mediated inhibitory effect on cortical DA levels (Gobert *et al*., 1998; Kawahara *et al*., 2001; Trendelenburg *et al*., 2001), the ligands under investigation in the current study exerted weak effect on DA modulation. Overall, the present study indicates AW-21 can cross the blood brain barrier and exhibit favourable pharmacological properties at α_2-AR, suggesting that it is suitable for further investigation as a candidate for selective α_2-AR PET radiotracer.

Overall the findings in this project extend the SAR knowledge of imidazoline containing ligands that will aid in better characterising the elusive family of IBS and understand their therapeutic potential as novel drug targets. We have provided further support that minor chemical modifications in the structure of these ligands may act as determinant in preferential recognition of a particular receptive site. For instance, as seen in the binding studies of the current project, structural modifications in ligands that give them a more rigid and planar conformation favours I_2BS interaction. Moreover, small substituents with low steric bulk generally appear to favour binding interaction in terms of affinity and selectivity for a particular receptor system, α-adrenoceptors (α_1- and α_2-AR) and IBS (I_1- and I_2BS). In the current project, some highly selective ligands like TCA-912 (at α_2-AR) and MP 972 (at I_2BS) were identified along with a few ligands exhibiting comparable affinities at both α_2-AR and I_2BS (MP 960 and MP 961). It would be interesting to study these ligands further in appropriate *in vivo* models to account for any potential pharmacological effects. In addition to SAR evaluation, a fluorinated marsanidine

derivative AW-21 was identified having favourable *in vitro* binding and *in vivo* pharmacological properties at α_2-AR. In addition, studies showed that AW-21 could cross the BBB. Therefore, from preliminary data AW-21 appears to be a suitable candidate for the development of a potential PET radiotracer selective for α_2-AR. Further evaluation of AW-21 will be carried out by radiolabelling it with ^{18}F and studying its pharmacokinetics in animal models by Professor Mika Scheinin's group at Turku University, Finland.

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