1.0 Introduction

According to the World Health Organization (WHO), about 450 million people worldwide suffer from mental, behavioural and neurological and substance use disorders (WHO, 2010). The dopamine (D) and serotonin (5-hydroxytryptamine; 5-HT) pathways in the central nervous system (CNS) are putative drug targets for treating neuropsychiatric disorders, including depression, anxiety, bipolar disorder, schizophrenia and drug abuse.

Antipsychotic drugs (APDs) are the main therapeutic intervention in psychotic illness. Notably, the atypical antipsychotic drugs, such as clozapine, risperidone, olanzapine and ziprasidone are commonly used treatments for schizophrenia (Newman-Tancredi et al., 2007). Meltzer et al., (2003) have suggested that antagonism of both D$_2$ and 5-HT$_{2A/2C}$ receptors is associated with lowered extrapyramidal effects (EPS) such as neuromuscular disturbance liability and improved capacity to alleviate negative symptoms (alogia, flattening affect, social withdrawal) and cognitive impairments (sensory-gating, working, verbal memory). Although the introduction of APDs was a major advance in the treatment of schizophrenia, there is still a need for improvement, as they have a tendency to cause weight gain and a significant number of patients remain resistant to treatment.

Naturally occurring aporphine alkaloids and their synthetic derivatives have served as leads for the development of new neurotropic drugs. Of these, (R)-apomorphine (APO) and its derivatives have high affinity and specificity for dopaminergic receptors and may be a valuable template for the search of subtype selective ligands for diseases like Parkinson’s disease, schizophrenia, attention-deficit
hyperactivity disorder and erectile dysfunctions (Sipos et al., 2008). However, despite its high intrinsic agonism activity and rapid onset of anti-parkinsonian effects, APO suffers from poor bioavailability, metabolic instability and potential central emetic side-effects (Liu et al., 2008).

In contrast, compounds such as bulbocapnine, corytuberine, boldine and glaucine that share the (S)-configuration exhibit neuroleptic-like actions in mice upon subcutaneous injection, suggesting that they may act as dopamine antagonists (Asencio et al., 1999). However, orally administered boldine has very short plasma half life and is rapidly glucuronidated in the liver, a behavior that may explain its relatively weak and short-lived systemic actions and certainly limits its potential clinical usefulness (Sobarzo-Sanchez et al., 2000).

In the present study, a combination of both computational and biomolecular screening approaches have been used to evaluate the affinity of aporphines at dopamine (D₁ and D₂) and serotonin (5-HT₁A and 5-HT₂A) receptors to identify selective monopotent as well as multipotent ligands for dopaminergic and serotonergic receptors. To accomplish this, a homology model of the rat 5-HT₂A receptor was built based on the crystal structure of the human β₂-adrenergic receptor followed by in silico screening of potential compounds using aporphines as the reference point. Subsequently, filtration based dopamine (D₁ and D₂) and serotonin (5-HT₁A and 5-HT₂A) radioligand receptor binding assays were optimised to test the compounds selected from the docking studies across the dopamine (D₁ and D₂) and serotonin (5-HT₁A and 5-HT₂A) receptors. Thus, findings in this study could have important implications for the treatment of dopamine and serotonin circuitry dysfunction.
1.1 Aim and Objectives of the Research

The aim of the research is to identify active aporphines with significant activity at the receptors that are involved in central nervous system (CNS) disorders such as schizophrenia and depression. The specific objectives are:

1. To construct a 3D molecular model of the rat 5-HT\textsubscript{2A} receptor against the known crystal structure of the human \(\beta_2\)-adrenergic receptor (PDB ID: 2RH1) and to investigate the ligand-receptor interactions of standard 5-HT\textsubscript{2A} ligands.
2. To search for potential 5-HT\textsubscript{2A} receptor active ligands based on aporphines.
3. To optimise assays for the characterization of dopamine (D\textsubscript{1} and D\textsubscript{2}) and serotonin (5-HT\textsubscript{1A} and 5-HT\textsubscript{2A}) receptor binding activity.
4. To evaluate the affinity of aporphines at dopamine (D\textsubscript{1} and D\textsubscript{2}) and serotonin (5-HT\textsubscript{1A} and 5-HT\textsubscript{2A}) receptors to identify selective monopotent as well as multipotent ligands.
2.0 Literature Review

2.1 G-Protein Coupled Receptors (GPCRs)

2.1.1 G-Protein Coupled Receptor (GPCR) Overview

Currently, almost 30% of the marketed prescription drugs act on GPCRs as agonists or antagonists by activating or inhibiting the receptors, respectively (Overington et al., 2006) (Table 2.1). They still remain among the most profitable therapeutic targets (Hopkins and Groom, 2002). GPCRs have been categorized into six classes based on the nature of their ligands and sequence similarity. They are Class A (Rhodopsin-like), Class B (Secretin receptor family), Class C (Metabotropic glutamate/pheromone), Class D (Fungal mating pheromone receptor), Class E (Cyclic AMP receptor) and Class F (Frizzled/Smoothened) (George et al., 2002; Kolakowski, 1994; Lagerstrom and Schioth, 2008; Pierce et al., 2002). Of these, class A (Rhodopsin-like) receptors containing biogenic amines, nucleotides, odorants, lipids, proteases and neuropeptides hormones have been studied extensively.

GPCRs are integral membrane proteins composed of seven membrane-spanning segments helices connected by intracellular and extracellular loop segments, as well as an extracellular N-terminus and an intracellular C-terminus (Figure 2.1). There are several highly conserved residues or sequence motifs across the GPCRs including the LXXXD motif in the transmembrane 2, the DRY motif at the end of the transmembrane 3 and the NPXXY motif on the transmembrane 7 (De La Nuez Veulens and Rodríguez, 2009). Upon binding to a GPCR, a ligand induced conformational change promotes its coupling to a heterotrimeric G-protein (alpha (Gα), beta (Gβ), and gamma (Gγ) subunits), leading to the activation of various effector proteins that allow the release of second messenger molecule.
Table 2.1: Examples of some prescription drugs which target GPCRs for the indicated condition or disease state (adapted from Leifert et al., 2010).

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Generic name</th>
<th>G-protein coupled receptors</th>
<th>Disease/Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zyprexa</td>
<td>Olanzapine</td>
<td>Serotonin 5-HT₂ and Dopamine</td>
<td>Schizophrenia, Antipsychotic</td>
</tr>
<tr>
<td>Risperdal</td>
<td>Risperidone</td>
<td>Serotonin 5-HT₂</td>
<td>Schizophrenia</td>
</tr>
<tr>
<td>Claritin</td>
<td>Loratidine</td>
<td>Histamine H₁</td>
<td>Rhinitis, Allergies</td>
</tr>
<tr>
<td>Imigran</td>
<td>Sumatriptan</td>
<td>Serotonin 5-HT₁B/₁D</td>
<td>Migraine</td>
</tr>
<tr>
<td>Cardura</td>
<td>Doxazosin</td>
<td>β₁-adrenoceptor</td>
<td>Prostate hypertrophy</td>
</tr>
<tr>
<td>Tenormin</td>
<td>Atenolol</td>
<td>β₁-adrenoceptor</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>Serevent</td>
<td>Salmeterol</td>
<td>β₂-adrenoceptor</td>
<td>Asthma</td>
</tr>
<tr>
<td>Duragesic</td>
<td>Fentanyl</td>
<td>Opioid</td>
<td>Pain</td>
</tr>
<tr>
<td>Imodium</td>
<td>Loperamide</td>
<td>Opioid</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Cozaar</td>
<td>Losartan</td>
<td>Angiotensin II</td>
<td>Hypertension</td>
</tr>
<tr>
<td>Zantac</td>
<td>Ranitidine</td>
<td>Histamine H₂</td>
<td>Peptic ulcer</td>
</tr>
<tr>
<td>Cytotec</td>
<td>Misoprostol</td>
<td>Prostaglandin PGE₁</td>
<td>Ulcer</td>
</tr>
<tr>
<td>Zoladex</td>
<td>Goserelin</td>
<td>Gonadotrophin releasing factor</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>Requip</td>
<td>Ropinirole</td>
<td>Dopamine</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>Atrovent</td>
<td>Ipratropium</td>
<td>Muscarinic</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
</tbody>
</table>
Figure 2.1: Schematic diagram of GPCRs with seven transmembrane regions (TM 1–TM 7) connected by intracellular and extracellular loop segments, as well as an extracellular N-terminus and an intracellular C-terminus (adapted from Bockaert and Pin et al., 2010).

2.1.2 G-Protein Coupled Receptor (GPCR) Crystal Structures

Structural designation of GPCRs, particularly the heptahelical transmembrane region, is very challenging as their greasy hydrophobic surfaces do not readily make the regular intermolecular contacts required for high quality crystallization (Kanagarajadurai et al., 2009). To date, relatively a few three dimensional (3D) crystal structures of GPCRs have been obtained (Table 2.2). The structural data from these studies provide basis information on the drug interactions at molecular level and crystallization of other GPCRs which allow rational approach for drug design.
Table 2.2: List of the three dimensional crystal structures of GPCRs.

<table>
<thead>
<tr>
<th>Receptor structure</th>
<th>PDB ID</th>
<th>Resolution (Å)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine rhodopsin</td>
<td>IF88</td>
<td>2.8</td>
<td>Palczewski et al., 2000</td>
</tr>
<tr>
<td>Bovine rhodopsin</td>
<td>1HZX</td>
<td>2.8</td>
<td>Teller et al., 2001</td>
</tr>
<tr>
<td>Bovine rhodopsin</td>
<td>1L9H</td>
<td>2.6</td>
<td>Okada et al., 2002</td>
</tr>
<tr>
<td>Bovine rhodopsin</td>
<td>1U19</td>
<td>2.2</td>
<td>Okada et al., 2004</td>
</tr>
<tr>
<td>Bovine rhodopsin</td>
<td>1GZM</td>
<td>2.65</td>
<td>Li et al., 2004</td>
</tr>
<tr>
<td>Bovine rhodopsin photoproduct</td>
<td>2I35-37</td>
<td>4.15</td>
<td>Salom et al., 2006</td>
</tr>
<tr>
<td>Bovine rhodopsin</td>
<td>2G87</td>
<td>2.6</td>
<td>Nakamichi and Okada, 2006a</td>
</tr>
<tr>
<td>Bovine rhodopsin</td>
<td>2HPY</td>
<td>2.8</td>
<td>Nakamichi and Okada, 2006b</td>
</tr>
<tr>
<td>Bovine lumirhodopsin</td>
<td>2PED</td>
<td>2.95</td>
<td>Nakamichi et al., 2007</td>
</tr>
<tr>
<td>Bovine isorhodopsin</td>
<td>2J4Y</td>
<td>3.4</td>
<td>Standfuss et al., 2007</td>
</tr>
<tr>
<td>Human β2 adrenoceptor</td>
<td>2R4R/S</td>
<td>3.4/3.7</td>
<td>Rasmussen et al., 2007</td>
</tr>
<tr>
<td>Squid rhodopsin</td>
<td>2Z73</td>
<td>2.5</td>
<td>Murakami and Koyama, 2008</td>
</tr>
<tr>
<td>Squid rhodopsin</td>
<td>2ZIY</td>
<td>3.7</td>
<td>Shimamura et al., 2008</td>
</tr>
<tr>
<td>Human β2 adrenoceptor</td>
<td>3D4S</td>
<td>2.8</td>
<td>Hanson et al., 2008</td>
</tr>
<tr>
<td>Turkey β1 adrenoceptor</td>
<td>2VT4</td>
<td>2.7</td>
<td>Warne et al., 2008</td>
</tr>
<tr>
<td>Bovine rhodopsin</td>
<td>3CAP</td>
<td>2.9</td>
<td>Park et al., 2008</td>
</tr>
<tr>
<td>Bovine rhodopsin</td>
<td>3DQB</td>
<td>3.2</td>
<td>Scheerer et al., 2008</td>
</tr>
<tr>
<td>Human adenosine2A</td>
<td>3EML</td>
<td>2.6</td>
<td>Jaakola et al., 200</td>
</tr>
<tr>
<td>Human dopamine D3</td>
<td>3PBL</td>
<td>2.89</td>
<td>Chien et al., 2010</td>
</tr>
<tr>
<td>Human CXCR4</td>
<td>3ODU/EO/E6/E8/E9</td>
<td>2.5/2.9/3.2/3.1/3.1</td>
<td>Wu et al., 2010</td>
</tr>
<tr>
<td>Human histamine H1</td>
<td>3RZE</td>
<td>3.1</td>
<td>Shimamura et al., 2011</td>
</tr>
<tr>
<td>M3 muscarinic acetylcholine</td>
<td>4DAJ</td>
<td>3.4</td>
<td>Kruse et al., 2012</td>
</tr>
<tr>
<td>Human M2 muscarinic acetylcholine</td>
<td>3UON</td>
<td>3.0</td>
<td>Haga et al., 2012</td>
</tr>
</tbody>
</table>
2.2 Dopamine (D) and Serotonin (5-hydroxytryptamine; 5-HT) Receptors

2.2.1 Dopamine (D) Receptors

Dopamine functions as a catecholamine neurotransmitter and neurohormone, are found extensively in central nervous system, and constitute about 80% of the total brain content (Vallone et al., 2000). Dopamine receptors are G-protein-coupled receptors that can be further classified into D1-like (D1, D5) and the D2-like (D2, D3, D4) subfamilies, based on their pharmacological, structural and biochemical properties (Andersen et al., 1990; Niznik and Van Tol, 1992; Vallone et al., 2000). The D1-like receptor subtypes exert its signal by activating Gs proteins, which stimulate adenylyl cyclases and increases cyclic adenosine monophosphate (cAMP) accumulation (Monsma et al., 1990; Sunahara et al., 1991), whereas the D2-like receptor subtypes mediate its actions by activating Gi0-proteins, which inhibits adenylyl cyclases and decreases cyclic adenosine monophosphate (cAMP) accumulation (Bunzow et al., 1988; Sokoloff et al., 1990; Van Tol et al., 1991). Among the many types of dopamine receptors, D1 and D2 are widely expressed in the brain (Dziedzicka-Wasylewska, 2004).

2.2.2 Serotonin (5-hydroxytryptamine; 5-HT) Receptors

Serotonin (5-hydroxytryptamine; 5-HT) is predominantly located in the periphery as a neuromodulator of blood vessel tone and gastrointestinal tract, and only about 1–2% of the body’s serotonin is found in the brain as the tryptaminergic neurotransmitter (Skop et al., 1994). On the basis of their genetic, pharmacological and functional properties, serotonin receptors are clustered into seven main families, comprising a total of 14 distinct subtypes (Barnes and Sharp, 1999; Hoyer et al., 2002; Peroutka, 1993). With the exception of the ionotropic 5-HT3 receptor (Derkach et al.,
of the other serotonin receptors are G protein-coupled metabotropic receptors and act through intracellular signaling pathways to hyperpolarize (5-HT_1) or depolarize (5-HT_2A/5/6/7) their host cells (Barnes and Sharp, 1999). Of the different subclasses of serotonin receptors, 5-HT_1A and 5-HT_2A are distributed extensively in many regions of the brain (Hoyer et al., 1994).

### 2.2.3 Clinical Significance of Dopamine and Serotonin Receptors

The biogenic amine neurotransmitters, dopamine and serotonin are responsible for regulating diverse array of psychological and physiological functions including control of appetite, behaviour, cardiovascular system, cognition, emotion, endocrine secretion, motor, memory, mood, perception and reward (Emilien et al., 1999; Jones and Blackburn, 2002; Missale et al., 1998; Nichols and Nichols, 2008). Alteration in dopaminergic and serotonergic neurotransmission in central nervous system have been proposed to play a fundamental role in treating neuropsychiatric disorders including schizophrenia, depression, anxiety, Parkinson’s Disease and related disorders (De Almeida et al., 2008; Giovanni et al., 2010). Numerous psychotropic drugs including antidepressants, antipsychotics, anxiolytics and hallucinogens have been implicated as the main therapeutic intervention for psychotic illness which exert their actions via interactions with dopamine (D_1 and D_2) and serotonin (5-HT_1A and 5-HT_2A) receptors (Alex and Pehek, 2007).

Several D_2 agonists including apomorphine, pramipexole, ropinirole, piribedil, rotigotine and the ergot alkaloids pergolide, bromocriptine and cabergoline have been used in the treatment of Parkinson’s diseases (Emilien et al., 1999; Güldenpfennig et al., 2005; Morgan and Sethi, 2006; Rascol et al., 2006). D_1 receptors agonist also has
been targeted in the treatment of Parkinson’s diseases although its use is limited by the occurrence of dyskinesias (Mailman et al., 2001). In both preclinical models and clinical trials, the role of 5-HT\textsubscript{1A} agonists and 5-HT\textsubscript{2A} antagonists has been implicated as potential antiparkinsonian and antidyskinetic agents (Huot et al., 2011).

First generation antipsychotics like chlorpromazine and haloperidol act primarily as D\textsubscript{2} antagonists are ineffective against negative and cognitive symptoms and inducing extrapyramidal motor side effects (EPS). Second generation antipsychotics including clozapine, risperidone, olanzapine, sertindole, quetiapine and ziprasidone are antagonists at both 5-HT\textsubscript{2A} and D\textsubscript{2} receptors with minimal EPS burden and effective in alleviation of negative symptoms and cognitive functions. Third generation antipsychotics such as perospirone, aripiprazole, lurasidone, bifeprunox and cariprazine possessing combined D\textsubscript{2} partial agonism or antagonism and 5-HT\textsubscript{1A} agonism is also associated with a lower EPS liability and better efficacy in treating negative and cognitive symptoms (Abi-Dargham and Laruelle, 2005; Akhondzadeh, 2006; Gardner et al., 2005; Mailman and Murthy, 2010; Meltzer et al., 2003; Miyamoto et al., 2005; Newman-Tancredi and Kleven, 2010).

Blockade of 5-HT\textsubscript{2A} receptors by antidepressant drugs such as trazodone, nefazodone and mirtazapine are widely used treatment for depressive illness (Celada et al., 2004; Richelson, 2001). Activation of 5-HT\textsubscript{1A} receptors by 8-OH-DPAT, buspirone, gepirone and tandospirone revealed a significant antidepressant effects in various animal models (Koek et al., 2001; Matsuda et al., 1995; Schreiber et al., 1994; Wieland and Lucki, 1990). Additionally, azapirone class of drugs including buspirone and its analogs ipsapirone, gepirone and tandaspirone also exert anxiolytic actions via activation of 5-HT\textsubscript{1A} receptors (Barradell and Fitton, 1996; Boyer and Feighner, 1993;
Csanalosi et al., 1987; Goa and Ward, 1986). Also, amoxapine is an antidepressant drug which act as weak competitive antagonist of D₂ receptors (Richelson, 2001).

2.3 Computer Aided Drug Discovery

2.3.1 Structure-based Drug Design (SBDD)

Structure-based drug design (SBDD) has emerged as a valuable pharmaceutical tool in medicinal chemistry. Remarkable contributions have been made by SBDD in the field of cancer chemotherapy, drug resistant infections and neurological diseases. The general process of SBDD involves both the synthesis of new derivatives and the evaluation of their binding to the target structure either through computational docking or elucidation of the target structure as a complex with the lead compound (Grey and Thompson, 2010). A prerequisite for this approach is a high-resolution (< 2.5 Å) three-dimensional structure of the target, obtained through X-ray crystallographic analysis, in the presence or absence of the drug candidate (Grey and Thompson, 2010).

2.3.2 Homology Modelling

The process of homology or comparative modelling involves identification of the known 3D structure of a protein template, sequence alignment of the protein to be modelled (target) and the template protein, model building for the target based on the 3D structure of the template and the alignment and refining, validation or evaluation of the models (Marti-Renom et al., 2000). The rate of success, quality and the reliability of the homology models depend on the level of sequence identity between the template and the target (Chothia and Lesk, 1986; Moult et al., 1999; Venclovas et al., 2001). As
a rule of thumb, the target protein should share at least 30% of sequence identity with the template structure (Figure 2.2) (Baker and Sali, 2001).

For the majority of the last decade, rhodopsin served as the template for homology modelling of other GPCRs. Rhodopsin is structurally stabilized by being covalently bound to a prosthetic group, 11-cis-retinal that sustains the receptor in the inactive conformation (Okada and Palczewski, 2001). In contrast, all other GPCRs are activated by diffusible ligands, expressed at relatively low levels in native tissues and having greater functional and structural plasticity (Cherezov et al., 2007). Thus, functionally and structurally more reliable templates are desired to obtain GPCR models which can give further insight into ligand binding and activation mechanisms. Hence, with the acquisition of the structure of the first human β2-adrenoceptor, the world of GPCR modelling has undergone tremendous changes especially with respect to more authentic approximations of the ligand binding sites of biogenic amine receptors.
Figure 2.2: Accuracy and application of protein structure models. Shown are the different ranges of applicability of comparative protein structure modelling, threading and de novo prediction; the corresponding accuracy of protein models; and their sample applications (adapted from Baker and Sali, 2001).
2.3.3 Molecular Docking

Molecular docking is a computational process of searching for a ligand that is able to fit both geometrically and energetically the binding site of a protein (Teodoro et al., 2001). In general, the best fit for the ligand-protein complex relies on several parameters obtained from the protein and ligand atomic coordinates, such as geometrical complementarities, atomic van der Waals radius, charge, torsion angles, intermolecular hydrogen bonds and hydrophobic contacts, which results in numerous possible conformations or poses of a ligand in the target’s binding site. As such, the best docking position is chosen considering the scoring functions, which are able to evaluate intermolecular binding affinities or binding free energies and the population of the cluster (number of conformations belonging to the same cluster, as such repetition is considered a signal of a local minimum) of the ligand-protein complex (De Azevedo Jr and Dias, 2008).

2.4 Receptor Binding Assays

2.4.1 Fundamental of Receptor–ligand Interactions

According to the law of mass action, the binding of a radioligand to a receptor can be described as a biomolecular association reaction, in which a radoligand [L] binds reversibly to a single binding site on the receptor [R]. In the absence of analyte [A], the radioligand [L] binds to the receptor [R] to form a radioligand receptor complex [RL] (Equation 1). At equilibrium, the ratio of \( k_{-1}/k_{+1} \) is denoted as the dissociation constant \( (K_d) \), which is inversely proportional to the ligand affinity towards the receptor. Binding occurs when the radioligand and receptor collide due to diffusion with the proper collision energy and orientation. The radioligand receptor complex remains bound
together over a certain time period depending on the affinity of the receptor and radioligand for one another.

**Equation 1:**

\[
\frac{k_{eq}}{k_{de}} [R] + [L] \leftrightarrow [RL]
\]

The origin of radio-receptor binding assays depends on the competition between an analyte \([A]\) and the radioligand for binding to a receptor (De Jong et al., 2005). As shown in **Equation 2**, the presence of the analyte \([A]\) leads to the formation of two receptor complexes, the radioligand receptor complex \([RL]\) and the analyte receptor complex \([RA]\). The analyte competes with the radioligand for the same binding site on the receptor. The concentration at 50% inhibition \((IC_{50})\) value, which indicates the analyte \([A]\) concentration that displaces 50% of the bound radioligand can be determined, if the analyte concentration is varied and both the receptor and radioligand concentrations are kept constant. The inhibition constant \((K_i)\) of the analyte can be calculated using Cheng-Prusoff equation (**Equation 3**) (Cheng and Prusoff, 1973).

**Equation 2:**

\[
[R] + [L] + [A] \leftrightarrow [RL] + [RA]
\]

**Equation 3:**

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

### 2.4.2 Radioligand Receptor Binding Assays

Radioligand receptor binding assays have been utilized in drug discovery and development to identify potential lead compounds. The first example of a quantitative radio-receptor binding assay was published by Lefkowitz et al., (1970) based on the
same principle as the radioimmunoassay described by Yalow and Berson (1959). Traditionally used in vivo or whole animal assays as high volume screening tools have been replaced by receptor binding assays due to their reliability, cost-effectiveness and rapid nature (Sweetnam et al., 1993). The basic principle of receptor binding studies is to assess the ability of potential drugs to interfere with the specific binding of a radioligand to its receptor. After attaining equilibrium with receptors, excess free radioligand must be separated rapidly from the bound radio-receptor complex. In contrast to centrifugation and other techniques, filtration is the most efficient and convenient method of separating free from bound radioligand, because it requires less handling and manipulation of samples (Mckinney, 1998). The bound complex is retained on glass fiber filters and the free radioligand passes through. The filter-bound radioactivity was counted using microplate scintillation.

2.5 Aporphine Alkaloids

2.5.1 An Overview of Aporphines

The naturally occurring aporphine alkaloids constitute a broad subgroup of benzylisoquinoline compounds, with more than 500 structures reported to date (Zhang et al., 2007). Examples of different classes are aporphines, proaporphines, secoaporphines, oxoaporphines, dehydroaporphines, 7-hydroxyaporphines, aporphine dimers and aristolactams. They are widely distributed in many plant species including Annonaceae, Lauraceae, Monimiaceae, Menispermaceae, Hernandiaceae, Ranunculaceae and others.
2.5.2 Structural Diversity of Aporphines

![Figure 2.3: General structure of aporphines](image)

Aporphines are a diverse group of tetracyclic bases derived by direct bonding between the A and D aromatic rings of the benzylisoquinoline nucleus (Figure 2.3). Many natural aporphines are exist as R-(−) or S-(−) configuration, depending on the stereochemistry of C-6a and usually substituted at position 1 and 2 by hydroxyl, methoxy or methlenedioxy groups. The tetracyclic skeleton can also be substituted at position 9, 10 and 11, and less frequently at positions 3 and 8 and in a few cases, the position 7 or 4 is oxygenated. The nitrogen atom at position 6 is usually tertiary in the base form but may also be quaternary, less frequently acetylated or formylated (Stevigny et al., 2005).

2.5.3 Pharmacological Properties of Aporphines

Aporphines exhibit a wide range of interesting pharmacological properties including antioxidant, antiplatelet, antitumor, anticonvulsant, antiplasmodial, antineoplastic, antimalarial, antiprotozoal, antipolio virus, cytotoxic and antiparkinsonian effects (Guinaudeau et al., 1994; Rios et al., 2000). Of these, R-(−)-Apomorphine (APO; Figure 2.4), the semisynthetic or total synthetic prototypical
aporphine (Neumeyer et al., 1970), is a well established dopamine agonist and has been marketed for the treatment of Parkinson’s disease and erectile dysfunction (Picada et al., 2002). Despite its high intrinsic agonist activity and fast onset of anti-parkinsonian effects, APO suffers from poor bioavailability, short duration of action and potential central emetic side-effects due to its oxidation potential (Subramony, 2006). In addition, although the catechol moiety contributes to the high dopaminergic activity by H-bonding with dopamine receptors it is not physico-chemically stable (Liu et al., 2008). Thus, to improve APO’s pharmacological profile, there is a need for further development of novel compounds by bioisostERICALLY replacing the catecholic component with a more stable functional group.

Likewise, S-(+)-boldine (Figure 2.4), the major active alkaloidal constituent in the leaves and bark of the Chilean boldo tree (Peumus boldus Molina, Monimiaceae) (O'brien et al., 2006) has been commercially used for hepatic dysfunction and as an ingredient in choleretics and laxatives. In spite of the greater in vitro binding affinity of boldine, its in vivo activities are weaker than those of glaucine, which is interpreted as a result of its poor access to the central nervous system. Therefore, it has been suggested that halogenation of boldine derivative would be expected to give a better absorption and distribution properties, being more lipophilic, as well as being more stable metabolically (Asencio et al., 2005). O-methylation of one or both of the hydroxyl groups of boldine as exhibited by predicentrine and glaucine also enhances the pharmacokinetic profile (Figure 2.4). Chung et al., (2009b) also reported that boldine shows competitive binding to 5-HT$_{2A}$ receptors.
Figure 2.4: Structures of aporphines
3.0 Computational Investigation of the 5-HT2A Receptor Binding Interactions of Standard Ligands and Aporphines

3.1 Introduction

3.1.1 5-HT2A Receptor (5-HT2AR) Structure

The 5-HT2 receptor family consists of three subtypes, 5-HT2A, 5-HT2B and 5-HT2C, which are similar in terms of molecular structure, pharmacology and signal transduction pathways (Barnes and Sharp, 1999). All three subtypes are single protein molecules with a high degree of homology within the seven transmembrane domains and coupled via heterotrimeric GTP binding protein of the Gq/11 type to increase the hydrolysis of inositol phosphates and elevate intracellular calcium (Hoyer et al., 2002). However, due to high sequence conservation within the transmembrane region, it has been historically difficult to develop subtype selective ligands. This task has been made even more challenging in the absence of X-ray crystal structures for any of serotonin receptors. 5-HT receptors share a common molecular architecture with other members of the GPCR. The 5-HT2A receptor structure consists of an extracellular domain, a membrane domain and an intracellular domain. The extracellular domain comprises the extracellular loops (ECL) ECL1, ECL2 and ECL3 between transmembrane (TM) helices TM 2 and 3, TM 4 and 5, and TM 6 and 7, respectively. The membrane domains possess seven transmembrane helices, TM1–TM7. The intracellular domain comprises the C-terminus and the intracellular loops (ICL) ICL1, ICL2 and ICL3 between TM 1 and 2, TM 3 and 4, and TM 5 and 6, respectively, and a highly conserved disulfide bond linking TM3 and ECL2 in nearly all GPCRs.

Site-directed mutagenesis studies associated with molecular modelling studies have led to the identification of a number of residues that are probably involved in
ligand binding and effector coupling features of the 5-HT$_{2A}$ receptor (Baxter et al., 1995; Boess and Martin, 1994; Saudou and Hen, 1994). A number of 5-HT$_{2A}$ receptor models have been constructed based on analogy with rhodopsin and bacteriorhodopsin and subjected to site directed mutagenesis (Almaula et al., 1996a; Almaula et al., 1996b; Choudhary et al., 1993; Holtje and Jendretzki, 1995; Kristiansen and Dahl, 1996; Roth et al., 1997a; Westkaemper and Glennon, 1993). Another X-ray crystal structure of GPCR, human $\beta_2$-adrenergic receptor that belongs to amine subfamily has also been used as a template to build 5-HT$_{2A}$ homology model (Kanagarajadurai et al., 2009; Pecic et al., 2010).

Most distinct models of the 5-HT$_{2A}$ receptor structure share the following properties: an electrostatic interaction between the positively charged amine moiety of the agonists and antagonists with a negatively charged aspartic acid residue Asp155(3.32) (Wang et al., 1993), interactions with polar/charged side chain groups including Ser159(3.36) (Almaula et al., 1996b) and Ser239(5.43) (Braden and Nichols, 2007) and van der Waals interactions with aromatic residues such as Trp151(3.28), Phe243(5.47), Phe244(5.48), Trp336(6.48), Phe339(6.51), Phe340(6.52), Trp367(7.40) and Tyr370(7.43) which form a hydrophobic pocket surrounding the agonist or antagonist molecule (Choudhary et al., 1995; Kroeze et al., 2002; Roth et al., 1997b; Shapiro et al., 2000). Structural information from these studies has shown the importance of the conserved residues in the binding mode of both agonists and antagonists and also provides detailed knowledge about the ligand-receptor interactions.
3.2 Materials

Silicon graphics workstations, Sybyl modelling software, Autodock4.0 docking, Discovery Studio 3.1 and LigPlot 4.4.2 softwares.

3.3 Methodology

3.3.1 Sequence Alignments

FASTA sequences used in the analysis were retrieved from the European Bioinformatics Institute website (http://www.ebi.ac.uk) database. Sequence alignments were generated with CLUSTALW2 software (http://www.ebi.ac.uk/Tools/clustalw2/) (Thompson et al., 1994). Conserved residues among the 5-HT family with respect to 5-HT2A receptor were resolved through sequence comparison.

3.3.2 Model Construction

A homology model for the rat 5-HT2A receptor was constructed using Sybyl 7.1 (Tripos, St. Louis, MO, USA) on a Silicon Graphics O2 station. The FASTA sequence rat 5-HT2A receptor (ID: P14842) was submitted for BLASTp analysis to search for a suitable template. The template and target sequences were aligned using CLUSTALW2. The alignment was then manually refined using JALVIEW 2.4 to ensure alignment of the known conserved motifs and minimise gaps in the transmembrane regions. The final alignment was used as the input to the Sybyl 7.1 software package to build the model. Molecular mechanics minimisations were performed using CHARMM (Brooks et al., 1983) with a nonbonded interaction cutoff = 8 Å and a distance dependent dielectric constant $\varepsilon = 4$. The output structure was validated using PROCHECK
(http://nihserver.mbi.ucla.edu/SAVES/) (Laskowski et al., 1993) to assess the stereochemical quality by Ramachandran plot analysis.

### 3.3.3 Docking Procedure

Molecular docking was conducted using the Lamarckian genetic algorithm (Morris et al., 1998) implemented in Autodock4.0. 3D structures of the 5-HT$_2$A standard ligands were taken from the ZINC database (http://zinc.docking.org) (Irwin and Shoichet, 2005). Prior to docking, the structures of the rat 5-HT$_2$A receptor and the compounds were prepared using AutoDock Tools interface. The polar hydrogen atoms were added to the receptor structure, non-polar hydrogen atoms were merged, missing atoms were repaired, Kollman charges were added and the resulting structure was saved as a pdbqt file. On the other hand, Gasteiger charges were added to each of the compound structure and these were then saved as separate pdbqt file. Using the Autogrid utility, the binding cavity was defined to cover the active site with a size of 128 x 128 x 128 points and a grid spacing of 0.2 Å. A genetic algorithm was calculated for 100 runs with a population size of 150 and a total number of 2,500,000 evaluations. Based on the generated clusters using the MGL Autodock tools 1.5.2, the docked conformations were visualized using the 3D window of Discovery Studio 3.1 (Accelrys, Inc., San Diego, CA, USA). Hydrogen bonds, hydrophobic contacts and distances were plotted by LigPlot 4.4.2 (Wallace et al., 1995).

### 3.3.4 Compound Selection for Docking

The ZINC database with approximately ~3.7 million compounds was used as a source of compounds (http://zinc.docking.org) (Irwin and Shoichet, 2005). A set of 69
compounds were selected based on boldine as the backbone structure with at least 90% similarity, drug-like properties (molecular weight \( \leq 500 \), number of rotatable bonds \( \leq 8 \), number of hydrogen bond donors or acceptors \( \leq 10 \), log P value from -4 to 5, net charges from -5 to 5, polar desolvation value from -400 to 1 kcal/mol, apolar desolvation value from -100 to 40 kcal/mol, polar surface area of 150\( \AA^2 \)) and purchasable. The selected compounds were further filtered by discarding compounds with unusual substitutions not found in naturally-occurring aporphines including compounds with \( R_3 \)-methylamine, \( R_3 \)-hydroxymethyl, \( R_6 \)-dimethyl and \( R_6 \)-oxoethyl substituents (Figure 3.1). The remaining 52 compounds were then subjected for docking using the generated rat 5-HT\(_{2A}\) receptor model according to protocol described previously (see Section 3.3.3).

Figure 3.1: General structure of naturally-occurring aporphines with possible substitution sites indicated as \( R_1 \)–\( R_{11} \).
3.4 Results and Discussion

3.4.1 Selection of Templates

Following BLASTp query, the human \(\beta_2\)-adrenergic receptor structure (PDB ID: 2RH1) was selected as the template sequence. This receptor belongs to the group of GPCRs with almost 30% sequence similarity with the 5-HT\(_2A\) receptor, thus it is functionally connected to the family of serotonin receptors. In contrast, bovine rhodopsin shares a low sequence similarity to 5-HT\(_2A\) receptor with less than 20% and it is a retinal-binding protein, functionally totally different from the 5-HT\(_2A\) receptor (Palczewski et al., 2000).

3.4.2 Sequence Analysis

Alignment of the rat 5-HT family receptor sequences revealed that they possess 19–68% identity (Figure 8.1 in Appendix A). The analysis revealed the presence of motif [T/S]XSI at transmembrane 3, F[Y/F]XP at transmembrane 5, WXPFF at transmembrane 6, WXGY and SX\(_2\)NPX\(_2\)Y at transmembrane 7 and DRY at intracellular loop 2. Alignment of the multiple species of 5-HT\(_2A\) receptor including human, orang utan, Rhesus macaque, bovin, pig, dog, rat, mouse and Chinese hamster showed that they share 88–97% identity (Figure 8.2 in Appendix A). The major discrepancies occur at the N-terminus and C-terminus where there is very little conservation of sequence. However, these regions are less likely to affect the putative ligand binding site. The rat and human 5-HT\(_2A\) receptors displayed 91% sequence identity (Figure 8.3 in Appendix A). A detailed comparison of their sequences indicated that all the species specific residues are found in the loop regions apart from Thr82, Ile150 and Ala242 which are located in transmembrane regions 1, 3 and 5,
respectively. The rat 5-HT$_{2A}$ receptor is closer to rat 5-HT$_{2C}$ than rat 5-HT$_{2B}$ with 48% and 38 % identity respectively (Figure 8.4 in Appendix A).

3.4.3 Model Construction

Chain A of the human $\beta_2$-adrenergic receptor crystal structure (PDB: 2RH1) was used as a basis structure after excision of the lysozyme adduct. A rat 5-HT$_{2A}$ receptor homology model was constructed from the substitution of amino acids in the 3D structure of the human $\beta_2$-adrenergic receptor and the insertion and/or deletion of amino acids according to the final sequence alignment using Sybyl 7.1. Internal water molecules were ignored during model building. The conserved disulfide bond between Cys148 in TM3 and Cys227 in extracellular loop 2 (ECL2) was maintained during the building of rat 5-HT$_{2A}$ homology model. A protein loop search was used to fill the gaps in the intracellular and extracellular loops, the C-terminus and the N-terminus by the Biopolymer loop search facility in Sybyl 7.1 with suitable fragments from its protein database based on PDB structures.

3.4.4 Model Evaluation

The output structure was energy minimised using the CHARMM force field in order to remove as many as possible of any of unfavourable bond lengths, bond angles, torsion angles or non-bonded interactions that may have been produced during the modelling process. After the refinement process, the full-length structure was submitted to PROCHECK to verify the integrity of the structure. All together 89.4% of the residues were in the most favourable region, 9.9% in the additionally allowed region and 0.5% in the generously allowed region (Figure 8.5 in Appendix B).
3.4.5 Docking

In order to validate the model and computational methods, docking was performed with standard 5-HT\textsubscript{2A} ligands which are classified as endogenous ligands (serotonin), agonists (tryptamine, 5-methoxytryptamine (5-MeOT), \((R/S)\)-isomers of 2, 5-dimethoxy-4-iodoamphetamine (DOI)) and antagonists (spiperone, risperidone, haloperidol, ritanserin and ketanserin).

All the standard ligands were observed to bind within the active site. Three sets of residues were involved: Asp155 forming an electrostatic interaction with the protonated amine group of the agonists and antagonists, an aromatic network consisting of the conserved residues (Trp336, Phe339, Phe340 and Tyr370) and non-aromatic residues including Val156, Ser131, Ile152, Ser159, Thr160, Leu229, Val235, Gly238, Ser239, Ala242, Asn343 and Val366. In the case of serotonin, residues from transmembrane regions 3, 5 and 6 (Figure 8.6(a) in Appendix C) were seen to be involved in binding interactions. The protonated amine moiety was able to form an electrostatic interaction with Asp155, whereas the hydroxyl group was hydrogen interacted with Thr160. Johnson et al., (1994) reported an interaction of Ser239 with the hydroxyl group. The van der Waals interactions were also observed between the endogenous ligand and the residues Trp336, Phe339 and Phe 340.

The agonists interacted with similar residues from transmembrane regions 3, 5, 6 and 7 as for serotonin (Figure 8.6(b)–(e) in Appendix C). However, the antagonists were found to bind with a wider range of residues from transmembrane regions 2,3,5,6,7 and extracellular loop 2 (ECL2) (Figure 8.7(a)–(e) in Appendix C) with a
number of residues in common such as Trp151, Asp155, Ser159, Thr160, Leu229, Val235, Gly238, Ala242, Trp336, Phe339, Phe340 and Tyr370.

These observations were consistent with experimental data and also indicated that the conformation of the ligand and a preference of residues in certain helices are influenced by the type of the ligand. The electrostatic interaction with the Asp155 residue has been found to be essential for agonist and antagonist binding (Wang et al., 1993). Almuala et al., (1996b) have suggested that Ser159 in transmembrane 3 helps in anchoring the charged amino group of serotonin. Mutation of Ser239A causes a significant reduction in affinity of serotonin, but has less effect on 5-MeOT and (R/S)-DOI affinity and no effect on ketanserin, ritanserin and spiperone affinity (Johnson et al., 1997; Shapiro et al., 2000; Shapiro et al., 2002). Other molecular modelling studies on serotonin receptors have suggested that aromatic residues in the transmembrane region, Trp151, Phe243, Phe244, Trp336, Phe339, Phe340, Trp367 and Tyr370 involved in van der Waals interactions and formed a hydrophobic pocket surrounding the agonist or antagonist ligands (Choudhary et al., 1995; Kroeze et al., 2002; Roth et al., 1997b; Shapiro et al., 2000).

3.4.6 Compound Selection from Docking Studies

It is well known that aporphinods can bind to at least three types of neurotransmitters including adrenergic, dopaminergic and serotonergic receptors. Therefore, identification of compounds that have favourable affinity and selectivity for 5-HT$_{2A}$ receptor as therapeutic drugs and chemical leads is pivotal. Hence, in this study, boldine based on aporphine skeleton with S-configuration was selected as the reference
point because it was thought to bind with high affinity to 5-HT$_{2A}$ receptor (Chung et al., 2009b).

An investigation into the ligand-receptor interactions for the 5-HT$_{2A}$ receptor revealed the formation of an electrostatic interaction and hydrogen bonding between the protonated amine group of (S)-boldine and the carboxylate group of the conserved Asp155 (Figure 3.2). This observation was in agreement with the previous work reported by Indra et al., (2002) who had also demonstrated that the conserved Asp155 in transmembrane region 3 forms a strong reinforced electrostatic interaction with the protonated amine group of aporphines. On the other hand, the hydrophobic part of the (S)-boldine that is defined by tetracyclic core (ring A, B, C, D) was surrounded by residues from transmembrane domains 3, 5, 6 and 7 and extracellular loop 2 including the non-aromatic residues (Val156, Ser159, Thr160, Leu229, Val235, Ser239, Ala242 and Val366) and aromatic residues (Phe234, Trp336, Phe339, Phe340 and Tyr370). Of these, Val156, Ser159, Thr160 and Phe234 involved in polar interactions or hydrogen bonding and Leu229, Ser239, Ala242, Trp336, Phe339, Phe340 and Tyr370 involved in van der Waals interactions. This is consistent with the work by (Braden et al., 2006; Choudhary et al., 1993; Roth et al., 1997b) who reported the importance of the conserved aromatic residues (Trp336, Phe339, Phe340 and Tyr370) in the binding of 5-HT$_{2A}$ receptor.
Through virtual screening against the 5-HT$_{2A}$ receptor model, 13 purchasable compounds with higher or comparable activity to (S)-boldine were identified (Table 3.1). Of these, four of them ((R) and (S)-laureline, (S)-nuciferine and (S)-isothebaïne) were only available in racemic form. The selected compounds were assayed to determine their binding affinities towards dopamine (D$_1$ and D$_2$) and serotonin (5-HT$_{1A}$ and 5-HT$_{2A}$) receptors.

**Figure 3.2:** Proposed binding mode of (S)-boldine in the 5-HT$_{2A}$ receptor. The residues involved in hydrogen bonding, electrostatic or polar interactions were represented in pink circles with their hydrogen bonds with their main chains (blue dashed arrow), side chains (green dashed arrow), charge interactions (pink dashed double-ended arrow) and residues involved in van der Waals interactions (green circles).
Table 3.1: Computed $K_i$ values of the selected compounds.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>6a</th>
<th>R₁</th>
<th>R₂</th>
<th>R₆</th>
<th>R₉</th>
<th>R₁₀</th>
<th>R₁₁</th>
<th>$K_i$ (µM)</th>
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<tr>
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<td>OMe</td>
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<tr>
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<td>Pr</td>
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<td></td>
<td></td>
<td></td>
<td>5.82</td>
</tr>
<tr>
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<td>OH</td>
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<td>6.82</td>
</tr>
<tr>
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<tr>
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<td>OMe</td>
<td></td>
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NPA=$N$-propylapomorphine; 2-OH-NPA=2-hydroxy-$N$-propylapomorphine

Conclusion

A 3D molecular model of the rat 5-HT$_{2A}$ receptor was constructed by homology modelling against the human $\beta_2$-adrenergic receptor. All standard 5-HT$_{2A}$ receptor ligands were accommodated well at the putative ligand binding cleft, however there were slight variations in their interactions due to the different functional groups present. The constructed model is competent to be used as the starting structure to predict ligand-receptor interactions at the molecular level and future drug design. A set of 13 compounds were selected to determine their activities across the dopamine and serotonin receptors using (S)-boldine as the backbone structure.
4.0 Optimisation and Evaluation of Filtration Based Receptor Binding Assays for Dopamine (D₁ and D₂) and 5-Hydroxytryptamine (5-HT₁A and 5-HT₂A)

4.1 Introduction

4.1.1 Properties of Receptor Binding Assays

Among the heterogeneous assays, filtration based receptor binding assays have been used widely for the biochemical identification and pharmacological characterization of the receptors. This assay is a traditional and trusted method which offer the advantages of less colour quenching, larger sample volumes, higher efficiency than the scintillation proximity assays and with a fewest development steps (De Jong et al., 2005). There are two components of the radioligand binding including the specific binding to a targeted receptor sites and nonspecific binding to other receptor sites. The nonspecific binding is determined in the presence of excess unlabelled ligand to block the receptor binding sites of interest. Specific binding is defined as the difference between total binding observed in the absence of the unlabelled ligand and nonspecific binding.

It is important to meet the desired binding criteria for validating the radioligand binding assays (Mckinney, 1998; Scheinin et al., 1994). These are the following criteria:

- the binding should be reversible and saturable with low ligand concentrations.
- nonspecific binding should be lower (less than 20% of the total binding).
- total binding should be equal or less than 10% of the radioligand added.
- more than 80% of the specific binding observed at $K_d$ concentration of the radioligand.
• the binding should reach equilibrium and stable at the each concentration of the radioligand.

• the tissue and subcellular distribution of the specific binding should be consistent.

• correlation between the drug affinity \textit{in vitro} and pharmacological potency \textit{in vivo}.

• characteristic drug displacement with both agonists and antagonists.

The binding parameters of a ligand to its receptor also might be altered by other assay conditions including incubation time and temperature, buffer pH, filter plate type, washing times and concentration of radioligand, unlabelled ligand and membrane.

4.1.2 Selection of Receptor Source

In the brain, the D\textsubscript{1} and D\textsubscript{2} receptors are highly concentrated in the striatum and are also present at lower levels, in several other brain regions including the amygdala, cortex, globus pallidus, (Jackson and Westlind-Danielsson, 1994) hippocampus, hypothalamus, thalamus, substantia nigra and ventral tegmentum (Beaulieu and Gainetdinov, 2011; Missale \textit{et al.}, 1998; Weiner \textit{et al.}, 1991). The D\textsubscript{2} receptors are observed to be lower in cortical areas (De Keyser \textit{et al.}, 1988; Hall \textit{et al.}, 1991; Lidow \textit{et al.}, 1989). Conversely, D\textsubscript{2} receptors are expressed at higher level in thalamus, hippocampus and brainstem (Hurd \textit{et al.}, 2001; Jackson and Westlind-Danielsson, 1994; Weiner \textit{et al.}, 1991). There is only a minimal amount of D\textsubscript{1} and D\textsubscript{2} receptors in the cerebellum (Beaulieu and Gainetdinov, 2011; Camps \textit{et al.}, 1989). Radioligand-binding studies have shown that rat striatum is used widely as a D\textsubscript{1} and D\textsubscript{2} receptor source, where they are distributed abundantly (Billard \textit{et al.}, 1984 ; Lo, 2007). Thus, this study
was carried out using striatum from male Sprague Dawley (SD) rat brain as D₁ and D₂ receptor source.

Within the brain, 5-HT₁A receptors are mainly found both pre- and postsynaptically, particularly with highest expression in the cerebral cortex, hippocampus, septum, amygdala and raphe nucleus and with lower levels of expression in the basal ganglia and thalamus (Barnes and Sharp, 1999; El Mestikawy et al., 1991; Hoyer et al., 2002; Pazos and Palacios, 1985a). On the other hand, 5-HT₂A receptors are primarily located postsynaptically, where they are most densely distributed throughout the layers of cortex, notably in the neocortex, but they are found with much lower levels in brainstem, hippocampus and striatum (Barnes and Sharp, 1999; Cornea-Hebert et al., 2002; Hoyer et al., 2002; Pazos et al., 1985b; Xu and Pandey, 2000). Radioligand binding studies have shown that either rat cerebral cortex or hippocampus is used as a 5-HT₁A receptor source (Arro et al., 2001; Chung et al., 2009a; Hall et al., 1985; Nénonéné et al., 1994; Nénonéné et al., 1996) in contrast to either rat prefrontal cortex or cerebral cortex as receptor source for 5-HT₂A receptors (Chung et al., 2009b; Harms et al., 2000; Hoyer et al., 1985). This study was conducted using the cerebral cortex from male Sprague Dawley (SD) rat brain as the 5-HT₁A and 5-HT₂A receptor source due to the higher yield of cerebral cortex.

4.1.3 Selection of Radioligand

A number of in vitro binding assays with radiolabelled drugs containing tritium, [³H] or iodine, [¹²⁵I] have previously been developed to characterise the interaction of ligands with different types of receptors. Tritium is the radiolabel most commonly used because it has a long half life and is less hazardous, meaning that fewer handling precautions are required and larger quantities can be stored and disposed of. The choice
of radioligand used depends on if possessing high specific activity, affinity and selectivity toward the receptor being studied. The radioligand also should be pure and chemically stable in the assay medium used during incubation.

Quantitative autoradiographic as well as \textit{in vitro} and \textit{in vivo} binding studies have revealed the specificity and selectivity of $[^3\text{H}]$ SCH 23390 in labelling D$_1$ receptors (Andersen and Groenvald, 1986a; Billard \textit{et al.}, 1984; Dawson \textit{et al.}, 1986). Thus, $[^3\text{H}]$ SCH 23390 is designated as the most potent and selective antagonist radiotracer in ascribing the pharmacological and regional characteristics of D$_1$ receptors (Hyttel, 1983; Iorio \textit{et al.}, 1983).

On the other hand, in case of D$_2$ receptors, $[^3\text{H}]$ spiperone has been used in both \textit{in vitro} and \textit{in vivo} binding experiments as a potent antagonist radioligand with reasonable selectivity over D$_2$ receptors (Bischoff and Gunst, 1997; Chung \textit{et al.}, 2005; Kalkman \textit{et al.}, 2001; Lo, 2007; Madras \textit{et al.}, 1988). In addition, $[^3\text{H}]$ spiperone is also used for the visualization of D$_2$ receptor in rat brain using quantitative autoradiography (Boyson \textit{et al.}, 1986).

For 5-HT$_{1A}$ receptors, the prototypical radiolabelled agonist, $[^3\text{H}]$ 8-OH-DPAT has been used extensively to characterize their physiological, biochemical and behaviours properties (Gozlan \textit{et al.}, 1983; Hall \textit{et al.}, 1985). It has also been widely used as a specific radioligand for both \textit{in vitro} and \textit{in vivo} studies of the 5-HT$_{1A}$ receptor (Assie and Koek, 1996; Chung \textit{et al.}, 2005; Chung \textit{et al.}, 2009a; Hamon \textit{et al.}, 1984). and for quantitative autoradiographic analysis to determine the regional distribution of 5-HT$_{1A}$ receptors in rat brain (Pazos and Palacios, 1985a).
In the case of 5-HT$_{2A}$ receptors [³H] ketanserin has been found to be the radioligand of choice for \textit{in vitro}, \textit{in vivo} and autoradiographical studies to investigate the neuroanatomical localization, pharmacological characterization and functions of the receptor throughout the central nervous system (Assie \textit{et al.}, 1988; Chung \textit{et al.}, 2009b; Harms \textit{et al.}, 2000; Leysen \textit{et al.}, 1982; López-Giménez \textit{et al.}, 1997; Pazos \textit{et al.}, 1985b).
4.2 Materials

4.2.1 Chemicals and Instrumentation

$[^3]$H 8-OH-DPAT (150 Ci/mmol), $[^3]$H ketanserin HCl (67 Ci/mmol), $[^3]$H SCH 23390 (60 Ci/mmol) and $[^3]$H spiperone (15 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Bradford protein reagent, tris(hydroxymethyl)aminomethane, bovine serum albumin (BSA, fraction V), spiperone, serotonin hydrochloride, butaclamol hydrochloride, SCH 23390 hydrochloride, buspirone hydrochloride, (±)-propranolol hydrochloride, dicyclomine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvent and reagents were of analytical grade and purchased from Fisher Scientific (Loughborough, Leicestershire, UK).

96-well flat bottom Falcon plates were purchased from Becton Dickinson Labware (Franklin Lakes, NJ, USA). TopSeal-A, Bottom Seal, MicroScint-O scintillation cocktail, UniFilter GF/C plates and UniFilter GF/B plates were supplied by Perkin Elmer (Wellesley, MA, USA).

The instruments used included a Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany) and a glass-teflon motor-driven Glas-Col homogenizer (Terre Haute, Indiana, USA), an ultra high speed centrifuge (Beckman Coulter's Optima L-100 K Ultracentrifuge, Brea, CA, USA), a UV-vis spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan), an incubator (Friocell 55, MMM Medcenter Einrichtungen GmbH, Grafelfing, Germany), a FilterMate Cell Harvester, a TopCount NXT microplate scintillation and a luminescence counter (Perkin Elmer).
4.3 Methodology

4.3.1 Preparation of Dopamine (D₁ and D₂) and 5-Hydroxytryptamine (5-HT₁A and 5-HT₂A) Membrane Receptors

All animal use techniques were in accordance with the guiding principles for the care and use of research animals promulgated by the Ethics Committee on Animal Welfare and Experimentation, Faculty of Medicine, University of Malaya, Malaysia (Reference number: FAR/05/11/2007/CLY (b) (R)). Male Sprague Dawley rats weighing approximately 200–400 g were killed by cervical dislocation and their brains were removed rapidly and stored at -80°C prior to use. Either rat striatum (for D₁ and D₂ receptors) or rat cerebral cortex (for 5-HT₁A and 5-HT₂A receptors) was dissected on ice, weighed and placed in 20 volumes (weight/volume) of ice-cold 50 mM Tris-HCl buffer at pH 7.4 (Figure 4.1). The tissues were homogenized using an Ultra-Turrax homogenizer and a glass-teflon motor driven Glas-Col homogenizer at 2400 rpm on ice and centrifuged at 40,000g for 45 min at 4 °C. The supernatant was then discarded and the pellet was resuspended, homogenized and centrifuged for another 45 min. The resulting supernatant was discarded and the pellet was re-homogenized and incubated at 25 °C for 20 min to destroy endogenous dopamine or serotonin before being re-centrifuged as above. The final pellet was resuspended in 3 volumes of the same buffer.
Figure 4.1: Diagrammatic representations of dissection procedure for rat brain. Dotted lines indicate positions of initial sections (adapted from Glowinski and Iversen, 1966).
4.3.2 Protein Determination

A standard curve for Bovine Serum Albumin (BSA) was constructed for the range of 0–2.0 mg/ml. The assay contained of 0.1 ml of protein standard and 0.1 ml of tissue sample, respectively. Thereafter, 3.0 ml of Bradford reagent was added and gently vortexed to ensure even mixing. The reaction was allowed to continue for 45 min at room temperature before the absorbance was measured at the wavelength 595 nm. Protein concentration estimations were calculated from the BSA standard curve plotted using PRISM® software, version 3.0 (Graphpad software, Inc, San Diego, CA, USA).

4.3.3 Receptor Binding Assays

Filtration based radioligand binding assays of dopamine (D₁ and D₂) and 5-hydroxytryptamine (5-HT₁A and 5-HT₂A) receptors were carried out according to the methods published previously (Billard et al., 1984; Chung et al., 2009b; Chung et al., 2009a; Lo, 2007; Soo, 2006) with minor modifications.

4.3.3.1 Optimisation of Dopamine (D₁ and D₂) and 5-Hydroxytryptamine (5- HT₁A and 5-HT₂A) Receptor Binding Assays

All assays were performed in triplicate in a total volume of 250 µl according to the protocol summarized in Table 4.1. To optimise a variety of assay conditions, the reaction mixtures containing 200 µl of membrane suspension in 50 mM tris-HCl buffer at different pH values was incubated with 25 µl of radioligand for different periods of time at a variety of controlled temperatures. Nonspecific binding was determined by parallel incubations with 25 µl of reference ligand. Incubations of the assay mixtures
were terminated by rapid filtration through GF/C or GF/B filters using a FilterMate Cell Harvester and washed with 200 µl of ice-cold 50 mM Tris-HCl buffer at pH 7.4. The filters were then dried at room temperature overnight and sealed at the bottom. Subsequently, 25 µl of MicroScint-O scintillation cocktail was added into each well and the plates were sealed on top. The radioactivity retained on the filters was quantified using a micro plate scintillation counter for 1 min per well.

### 4.3.3.2 Saturation Experiments

For the determination of $K_d$ and $B_{\text{max}}$ values by saturation binding assays, the reaction mixtures containing 200 µl of either striatum (30 µg/well (D₁) and 50 µg/well (D₂)) or cerebral cortex (100 µg/well (5-HT₁A and 5-HT₂A)) were incubated with 25 µl of radioligand (0.003–1.0 nM [³H] SCH 23390 for D₁ receptor, 0.003–2.0 nM [³H] spiperone for D₂ receptor, 0.020–1.0 nM [³H] 8-OH-DPAT for 5-HT₁A receptor or 0.003–0.8 nM [³H] ketanserin for 5-HT₂A receptor). Nonspecific binding was determined by parallel incubations with 25 µl of reference ligand (1 µM SCH 23390 for D₁, 100 µM butaclamol hydrochloride for D₂, 100 µM serotonin hydrochloride for 5-HT₁A or 100 µM spiperone for 5-HT₂A). Incubations of assay mixtures (90 min at 22 °C (D₁ and 5-HT₂A), 80 min at 22 °C (D₂), 90 min at 25 °C (5-HT₁A)) were terminated by rapid filtration through a GF/C filter using a FilterMate Cell Harvester and washed with 200 µl of ice-cold 50 mM Tris-HCl buffer at pH 7.4 (4 times (D₁ and 5-HT₂A), 5 times (D₂), 3 times (5-HT₁A)). The filters were then dried at room temperature overnight and sealed at the bottom. Subsequently, 25 µl of MicroScint-O scintillation cocktail was added into each well and the plates were sealed on top. The radioactivity retained on filters was quantified using a micro plate scintillation counter for 1 min per well.
4.3.3.3 Competition Experiments

For the determination of $K_i$ values by competition binding assays, the reaction mixtures containing of 200 µl of either striatum (30 µg/well ($D_1$) and 50 µg/well ($D_2$)) or cerebral cortex (100 µg/well (5-HT$_{1A}$ and 5-HT$_{2A}$)) were incubated with 25 µl of 0.5 nM [$^3$H] SCH 23390 ($D_1$), 0.8 nM [$^3$H] spiperone ($D_2$), 0.4 nM [$^3$H] 8-OH-DPAT (5-HT$_{1A}$) or 0.5 nM [$^3$H] ketanserin HCl (5-HT$_{2A}$) and 25 µl of the standard unlabelled ligands or DMSO. Each of the standard unlabelled ligand or DMSO interference was tested at 9–12 varied concentrations. Nonspecific binding was determined by parallel incubations with 25 µl of reference ligand (1 µM SCH 23390 for $D_1$, 100 µM butaclamol hydrochloride for $D_2$, 100 µM serotonin hydrochloride for 5-HT$_{1A}$ or 100 µM spiperone for 5-HT$_{2A}$). The incubations were terminated, washed and dried and the radioactivity quantified as described earlier for the saturation experiments.

4.3.3.4 Z' factor

For the determination of Z' factor, the reaction mixtures containing 200 µl of either striatum (30 µg/well ($D_1$) and 50 µg/well ($D_2$)) or cerebral cortex (100 µg/well (5-HT$_{1A}$ and 5-HT$_{2A}$)) were incubated with 25 µl of 0.5 nM [$^3$H] SCH 23390 ($D_1$), 0.8 nM [$^3$H] spiperone ($D_2$), 0.4 nM [$^3$H] 8-OH-DPAT (5-HT$_{1A}$) or 0.5 nM [$^3$H] ketanserin HCl (5-HT$_{2A}$). Nonspecific binding was determined by parallel incubations with 25 µl of reference ligand (1 µM SCH 23390 for $D_1$, 100 µM butaclamol hydrochloride for $D_2$, 100 µM serotonin hydrochloride for 5-HT$_{1A}$ or 100 µM spiperone for 5-HT$_{2A}$). The incubations were terminated, washed and dried and the radioactivity quantified as described earlier for the saturation experiments.
Table 4.1: Summary of the values used for the different parameters involved in optimising the protocols for the radioligand binding assays.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>D₁</th>
<th>D₂</th>
<th>5HT₁ₐ</th>
<th>5HT₂ₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor source (SD rat)</td>
<td>striatum</td>
<td>striatum</td>
<td>cerebral cortex</td>
<td>cerebral cortex</td>
</tr>
<tr>
<td>Membrane (µg protein/well)</td>
<td>0–100</td>
<td>0–100</td>
<td>0–110</td>
<td>0–110</td>
</tr>
<tr>
<td>Radioligand</td>
<td>[³H] SCH 23390</td>
<td>[³H] spiperone</td>
<td>[³H] 8-OH-DPAT</td>
<td>[³H] ketanserin</td>
</tr>
<tr>
<td>Radioligand (nM)</td>
<td>0.003–1.0</td>
<td>0.003–2.0</td>
<td>0.020–1.0</td>
<td>0.003–0.8</td>
</tr>
<tr>
<td>Reference ligand</td>
<td>SCH 23390 HCl</td>
<td>butaclamol HCl</td>
<td>serotonin HCl</td>
<td>spiperone</td>
</tr>
<tr>
<td>Reference ligand (µM)</td>
<td>10⁻⁴–10⁻¹³</td>
<td>10⁻²–10⁻¹³</td>
<td>10⁻²–10⁻¹³</td>
<td>10⁻⁴–10⁻¹³</td>
</tr>
<tr>
<td>buffer pH (50 mM Tris-HCl)</td>
<td>7.4, 8.0, 9.0</td>
<td>7.4, 8.0, 9.0</td>
<td>7.4, 8.0, 9.0</td>
<td>7.4, 8.0, 9.0</td>
</tr>
<tr>
<td>Filter plate type</td>
<td>GF/C,GF/B</td>
<td>GF/C,GF/B</td>
<td>GF/C,GF/B</td>
<td>GF/C,GF/B</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>0–300</td>
<td>0–300</td>
<td>0–180</td>
<td>0–120</td>
</tr>
<tr>
<td>Incubation temperature (°C)</td>
<td>4, 22, 37</td>
<td>4, 22, 37</td>
<td>4, 25, 37</td>
<td>4, 22, 37</td>
</tr>
<tr>
<td>Washing times</td>
<td>1–12</td>
<td>1–10</td>
<td>1–9</td>
<td>1–10</td>
</tr>
<tr>
<td>References</td>
<td>Billiard et al., 1984</td>
<td>Lo, 2007</td>
<td>Chung et al., 2009a</td>
<td>Chung et al., 2009b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Soo, 2006</td>
<td>Lo, 2007</td>
</tr>
</tbody>
</table>
4.4 Data and Statistical Analysis

The percentage of specific binding of radioligand in the presence of standard competitors or solvents was calculated using a standard data reduction algorithm as follows: \( \frac{[B - NSB]}{[TB - NSB]} \times 100 \) where B is the binding in the presence of standard competitor or solvents, NSB is the non-specific binding in the presence of excess reference ligand and TB is the total binding.

The data from the radioligand binding assays was analyzed by non-linear regression and curve fitting programmes using prism Version 3.0 (GraphPad Inc., San Diego CA, USA). Equilibrium dissociation constant for the radioligand \((K_d)\) and maximal binding capacity \((B_{\text{max}})\) values were determined from saturation binding assays with one-site binding hyperbola analysis using the equation, Specific binding = \( B_{\text{max}} \times \frac{[L]}{K_d + [L]} \), where [L] was the concentration of radioligand used. The concentration causing 50% inhibition of specific binding \((IC_{50})\) values was determined from competition binding assays with one-site competition binding analysis using the equation, Specific binding = Bottom + (Top - Bottom) / \( 1+10^{(\log \text{IC}_{50} - \log [A])} \), where [A] was the log concentration of competitor. \( K_i \) values were calculated from the \( IC_{50} \) values using the Cheng and Prusoff (1973) equation, \( K_i = IC_{50} / (1 + [L] / K_d) \), where [L] was the concentration of radioligand used.

The quality of the assays was evaluated using Z’ factor according to the equation: \( 1-(3SD_{TB}+3SD_{NSB}) / (\text{Mean}_{TB} - \text{Mean}_{NSB}) \) as described by Zhang et al., (1999), where SD_{TB} is the standard deviation of total binding, SD_{NSB} is the standard deviation of non-specific binding, Mean_{TB} is the mean of total binding, and Mean_{NSB} is the mean of non-specific binding.
4.5 Results and Discussion

4.5.1 Protein determination

The protein concentration of dopamine (D₁ and D₂) and 5-hydroxytryptamine (5-HT₁A and 5-HT₂A) receptors was determined by extrapolation of the absorbance value using the standard curve (Figure 4.2) developed with the BSA standards. The estimated protein concentration of the receptors was used to prepare the membranes required for subsequent experiments.

![Standard curve of BSA concentration against absorbance for use in protein determination.](image)

Figure 4.2: Standard curve of BSA concentration against absorbance for use in protein determination.

4.5.2 Effects of Different Physico-Chemical Properties on Dopamine (D₁ and D₂) and 5-Hydroxytryptamine (5-HT₁A and 5-HT₂A) Receptor Binding Assays

Initial optimisation of various parameters was carried out, particularly radioligand, membrane and unlabelled ligand concentration, buffer pH, filter plate type, incubation time and temperature, washing times and solvent interference to maximize total binding and minimise non-specific binding of the receptors.
4.5.2.1 Selection of Membrane Concentration

The choice of membrane concentration in incubation media is critical for receptor binding assays. Generally, the total amount of radioligand bound should be less than 10% of the total amount of radioligand added to avoid ligand depletion. Bylund and Toews (1993) reported that as the concentration of the membrane increased, the ratio of specific to nonspecific binding increased because a larger portion of the nonspecific binding is to glass fiber filters. The membrane concentration should be high enough so that at least 1000 cpm are bound when all receptors are occupied with radioligand, as this allows reasonable accuracy in counting the radioactivity at the lowest concentration of radioligand. Conversely, the membrane concentration also should not be too high because elevated membrane concentration could cause filter clogging and prolonged aspiration time (Harms et al., 2000).

The effect of increasing the membrane concentration in the D1, D2, 5-HT1A and 5-HT2A binding assays is shown in Figure 4.3(a), 4.4(a), 4.5(a) and 4.6(a) respectively. The specific binding of D1, D2, 5-HT1A and 5-HT2A receptors observed at 30 µg of protein/well, 50 µg of protein/well, 100 µg of protein/well and 100 µg of protein/well each yielded 90%, 89%, 90% and 92% of the total binding respectively. Under saturating conditions, the observed nonspecific binding was about 8–11%, suggesting that there is no significant depletion of the radioligand during the course of the assay. Thus, the membrane concentration of 30 µg of protein/well for D1, 50 µg of protein/well for D2 and 100 µg of protein/well for 5-HT1A and 5-HT2A were selected for subsequent assays to achieve maximal signal to background ratio binding.
4.5.2.2 Selection of Unlabelled Ligand and its Concentration

The choice of the unlabelled ligand and its concentration to determine nonspecific binding is critical in receptor binding assays. Usually, the unlabelled ligand should be chemically different from the radioligand to avoid the labelling of specific nonreceptor sites (Bylund and Toews, 1993). The chosen unlabelled ligand should possess high specificity and selectivity towards the receptor being studied. As a general rule, the concentration of the unlabelled ligand should be 100 to 1000 fold in excess of its $K_d$ value (Hulme and Trevethick, 2010).

The amount of radioligand bound to the $D_1$ (Figure 4.3(b)), $D_2$ (Figure 4.4(b)), 5-HT$_{1A}$ (Figure 4.5(b)) and 5-HT$_{2A}$ (Figure 4.6(b)) receptors decreased with increasing concentration of unlabelled ligand until a point where no matter how much unlabelled ligand is added, the amount of radioligand bound remains constant. This remaining binding of radioligand represents the nonspecific binding. In the $D_1$, $D_2$, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor binding assays, this plateau was reached at 1 µM of $R$- (+)-SCH23390 hydrochloride, 100 µM of butaclamol hydrochloride, 100 µM of serotonin hydrochloride and 100 µM of spiperone respectively. These concentrations were used in subsequent assays to obtain minimal background binding.

4.5.2.3 Selection of pH

The pH is critical for receptor binding assays. Commonly, tris(hydroxymethyl) aminomethane (tris-HCl) is often used as the homogenization or assay buffer that yields maximum binding. The pH of the buffer should be in physiological range, between pH 7 and 8, so that the in vitro binding assays are conducted in an isotonic medium and to
be comparable with what is seen in vivo (Bylund and Toews, 1993). Furthermore, a wide range of buffer pH could affect the ligand receptor interaction due to changing of the degree of ionisation of groups within the receptor or ligand, which may influence both the equilibrium binding constant and the kinetic rate constant.

In general, pH variations from 7.4 to 9.0 caused a minimal variation in binding of the radioligand to the D₁ (Figure 4.3(c)), D₂ (Figure 4.4(c)), 5-HT₁A (Figure 4.5(c)) and 5-HT₂A (Figure 4.6(c)) receptors. Nevertheless, the highest level of total binding and lowest level of nonspecific binding were observed at pH 7.4 followed by pH 8.0 and pH 9.0. The signal to background ratio of pH 7.4 was slightly higher as compared to pH 8.0 and pH 9.0. Thus, subsequent assays for the D₁, D₂, 5-HT₁A and 5-HT₂A receptor were performed at physiological pH, 7.4 as it ensured the maximal signal binding and minimal background binding.

4.5.2.4 Selection of Filter Plate Type

Vacuum filtration is a widely used method in separating bound and free radioligand by rapid filtration through filter plates (Bylund and Toews, 1993). As a general rule, filtration is unsuitable for radioligands with $K_d >10^{-8} \text{ M}$ (Hulme and Trevethick, 2010). The most commonly used filter plates are GF/B (1.0 µm), GF/C (1.2 µm) and Durapore (0.22, 0.65, 1.0 µm). In some cases, pre-treatment of filter plates with polyethylenimine (PEI) gives them a positive surface charge that helps to capture negatively charged receptors and substantially improve the specific to nonspecific binding ratio (Hulme and Trevethick, 2010).
For the D₁ (Figure 4.3(d)), D₂ (Figure 4.4(d)), 5-HT₁A (Figure 4.5(d)) and 5-HT₂A (Figure 4.6(d)) receptors, total binding reached its highest value using the GF/C filters at all concentrations of radioligand. The nonspecific binding was also lower using the GF/C filters as compared to GF/B. Thus, subsequent assays were performed using GF/C filters for the D₁, D₂, 5-HT₁A and 5-HT₂A receptors.

4.5.2.5 Selection of Incubation Temperature

It is important to control the temperature to minimise the variability in receptor binding assays. Bylund and Toews (1993) suggested that room temperature (~ 22°C) is most suitable to perform the assays. However, some assays work better at physiological temperature (37°C), as this allow the results to be compared to in vivo ligand receptor behaviour. In some instances, receptor binding assays are carried out below room temperature, especially on ice (4°C) to prevent degradation of peptide ligands by proteolytic enzyme. Such temperature variation can produce conformational changes in receptors and other proteins in lipid bilayer and provide insight into the molecular nature of ligand receptor interaction.

Maximum total binding for the D₁ (Figure 4.3(e)), D₂ (Figure 4.4(e)), and 5-HT₂A (Figure 4.6(e)) receptors were achieved at 22°C and for 5-HT₁A (Figure 4.5(e)) achieved at 25°C with all concentrations of radioligand. In addition, the estimated level of nonspecific binding was also minimal for all the receptors. Decreases in total binding were measured at 4 and 37°C in all assays. Thus, the remaining assays were conducted at 22°C for the D₁, D₂, and 5-HT₂A receptors and 25°C for the 5-HT₁A receptor.
4.5.2.6 Selection of Incubation Time

In receptor binding assays, the assay medium must be incubated for sufficient time to ensure that equilibrium has been reached, where the rate of association of the ligand receptor complex is equal to the rate of dissociation. The time required for complexes to achieve equilibrium depends on the temperature of the radioligand (Bylund and Toews, 1993). Most commonly used radioligands reach steady state conditions within 20–60 minutes.

Equilibrium was obtained after approximately 90 minutes for the D₁ (Figure 4.3(f)), 5-HT₁A (Figure 4.5(f)), and 5-HT₂A (Figure 4.6(f)) receptors and 80 min for D₂ (Figure 4.4(f)) receptor. After these times the total and specific binding observed for each receptor was constant as a function of incubation time, suggesting that a steady state has been attained. Thus, all further assays were carried out with an incubation period of time of 90 minutes for the D₁, 5-HT₁A and 5-HT₂A receptors and 80 minutes for the D₂ receptor.

4.5.2.7 Selection of Washing Times

The method to separate bound and unbound radioligand after the incubation medium has attained equilibrium involves aspiration on to filters where the receptor and bound radioligand are trapped, while unbound radioligand passes through the filter plates. The filters are then washed repeatedly with ice cold buffer using a FilterMate Cell Harvester to remove unbound radioligand, as this reduces nonspecific binding (Bylund and Toews, 1993). As a rule of thumb, ice cold wash buffer is recommended as it can extend the usable range by up to 100 fold ( (Kₐ = 10⁻⁷–10⁻⁶ M) (Hulme and Trevethick, 2010).
For the D₁ (Figure 4.3(g)), D₂ (Figure 4.4(g)), 5-HT₁A (Figure 4.5(g)) and 5-HT₂A (Figure 4.6(g)) receptors, as the number of washing times increased, the counting for total binding and nonspecific binding decreased. At a certain point, further washings had only negligible effects on the nonspecific binding. Thus, 3 washings for the 5-HT₁A, 4 washings for the D₁ and 5-HT₂A and 5 washings for the D₂ receptor were adequate for efficient removal of any free radioligand without disruption of the bound complexes.

4.5.2.8 Solvent Interference

Due to its high solvent power, low chemical reactivity and toxicity and high flash point and boiling point, dimethyl sulfoxide (DMSO) has been used extensively to dissolve a great variety of chemical classes to evaluate their biological activities (Balakin, 2003). The range of concentrations in binding assays is prepared by serially diluting a compound from the initial DMSO stock solution, which is the highest concentration of compound. Incomplete solubility of the compounds at the highest concentration results in a shift of the concentration curve and the IC₅₀ will be estimated based on this ‘apparent’ concentration curve. The false, higher IC₅₀ will classify the compound as less active (Di and Kerns, 2006).

As the concentration of DMSO increased from 0.5–10% v/v, the percentage of inhibition increased for the D₁ receptor (Figure 4.3(h)). For the 5-HT₁A receptor (Figure 4.5(h)), the percentage of inhibition increased from 0.5–4% v/v and there were no significant trend from 4–10% v/v. Whereas, concentrations of DMSO as high as 10% had no effect for the D₂ (Figure 4.4(h)) and 5-HT₂A receptor (Figure 4.6(h)) binding assays. Thus, the concentration of DMSO chosen for the D₁, D₂, 5-HT₁A and 5-HT₂A receptor assays was up to 3% which gave less than 20% of inhibition.
Figure 4.3: Optimisation of D₁ receptor binding assay. Selection of (a) membrane concentration, (b) unlabelled ligand concentration, (c) pH, (d) filter plate type, (e) incubation temperature, (f) incubation time, (g) washing times and (h) DMSO effect. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=3) ± SEM.
Figure 4.4: Optimisation of D₂ receptor binding assay. Selection of (a) membrane concentration, (b) unlabelled ligand concentration, (c) pH, (d) filter plate type, (e) incubation temperature, (f) incubation time, (g) washing times and (h) DMSO effect. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=3) ± SEM. (c) – (h) adapted from Lo (2007).
Figure 4.5: Optimisation of 5-HT\textsubscript{1A} receptor binding assay. Selection of (a) membrane concentration, (b) unlabelled ligand concentration, (c) pH, (d) filter plate type, (e) incubation temperature, (f) incubation time, (g) washing times and (h) DMSO effect. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=3) ± SEM. (c), (e) – (h) adapted from Soo (2006).
Figure 4.6: Optimisation of 5-HT$_2A$ receptor binding assay. Selection of (a) membrane concentration, (b) unlabelled ligand concentration, (c) pH, (d) filter plate type, (e) incubation temperature, (f) incubation time, (g) washing times and (h) DMSO effect. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=3) ± SEM. (c) – (h) adapted from Lo (2007).
4.5.2.9 Saturation Experiments

Figure 4.7: Saturation binding curve of (a) [³H] SCH23390 for D₁ receptor, (b) [³H] Spiperone for D₂ receptor, (c) [³H] 8-OH-DPAT for 5-HT₁A receptor and (d) [³H] Ketanserin for 5-HT₂A receptor. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=3) ± SEM.

The respective binding of [³H] SCH23390 (Figure 4.7(a)), [³H] Spiperone (Figure 4.7(b)), [³H] 8-OH-DPAT (Figure 4.7(c)) and [³H] Ketanserin (Figure 4.7(d)) to D₁, D₂, 5-HT₁A and 5-HT₂A receptors was saturable, specific and of high affinity. The equilibrium dissociation constants (Kₐ) were estimated to be 0.40 nM ([³H] SCH23390; D₁), 0.86 nM ([³H] Spiperone; D₂), 0.17 nM ([³H] 8-OH-DPAT; 5-HT₁A), and 0.54 nM ([³H] Ketanserin; 5-HT₂A). The maximal binding capacities (B_max) were estimated to be 292.80 fmol/mg, 456.20 fmol/mg, 17.23 fmol/mg, and 74.98 fmol/mg for D₁, D₂, 5-HT₁A and 5-HT₂A receptors, respectively. These values were similar to those of (Billard et al., 1984; Chu et al., 2001) (D₁), (Chu et al., 2001; Cressee et al., 1977) (D₂), (Hoyer et al.,
1985; Peroutka et al., 1986) (5-HT_{1A}) and (Chung et al., 2009b; Leysen et al., 1982) (5-HT_{2A}).

4.5.2.10 Competition Experiments

Table 4.2 shows the $K_i$ values obtained from the competition experiments with standard unlabelled ligands on the D$_1$, D$_2$, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors. For comparison, Table 4.2 also includes previously reported $K_i$ values for the D$_2$, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors tested using a similar methodological approach.
For the D1 receptor binding assay, the relative potency for displacing $[^3\text{H}]$ SCH23390 was $R$-$(+)$-SCH23390 HCl > propranolol HCl > serotonin HCl > buspirone HCl > dicyclomine HCl (Table 4.2). Thus, $R$-$(+)$-SCH23390 HCl with a $K_i$ value of $1.03 \times 10^{-10}$ M is a suitable ligand for defining $[^3\text{H}]$ SCH23390 binding to the D1 receptor (Figure 4.8(a)). This value is very similar to $K_i$ value reported by Andersen and Braestrup (1986b).

For the D2 receptor binding assay, the relative potency for displacing $[^3\text{H}]$ Spiperone was butaclamol HCl > serotonin HCl > propranolol HCl > buspirone HCl > (±)-8-OH-DPAT > dicyclomine HCl (Table 4.2). Thus, butaclamol HCl with a $K_i$ value of $5.06 \times 10^{-8}$ M is a suitable ligand for defining $[^3\text{H}]$ spiperone binding to the D2 receptor (Figure 4.8(b)). This value is very similar to $K_i$ value reported by Lo (2007).

For the 5-HT$_{1\text{A}}$ receptor binding assay, the relative potency for displacing $[^3\text{H}]$ 8-OH-DPAT was (±)-8-OH-DPAT > serotonin HCl > buspirone HCl > propranolol HCl > scopolamine (Table 4.2). Thus, serotonin HCl with a $K_i$ value of $3.33 \times 10^{-9}$ M is a suitable ligand for defining $[^3\text{H}]$ 8-OH-DPAT binding to 5-HT$_{1\text{A}}$ receptor (Figure 4.8(c)) (Chung et al., 2009a; Hoyer et al., 1985; Peroutka et al., 1986; Schlegel and Peroutka, 1986).

For the 5-HT$_{2\text{A}}$ receptor binding assay, the relative potency for displacing $[^3\text{H}]$ ketanserin was spiperone > phentolamine HCl > buspirone HCl > serotonin HCl > (±)-8-OH-DPAT > (±)-propranolol HCl (Table 4.2). Thus, spiperone with a $K_i$ value of $5.51 \times 10^{-8}$ M is a suitable ligand for defining $[^3\text{H}]$ ketanserin binding to the 5-HT$_{2\text{A}}$ receptor (Figure 4.8(d)). This value is very similar to $K_i$ value reported by Chung et al., (2009b).
Table 4.2: \( K_i \) values obtained from competition experiments with standard unlabelled ligands on \( D_1 \), \( D_2 \), 5-HT\(_{1A} \) and 5-HT\(_{2A} \) receptors.

<table>
<thead>
<tr>
<th>Standard unlabelled ligands</th>
<th>Physiological mechanisms</th>
<th>( K_i ) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( D_1 )</td>
<td>( D_2 )</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>-</td>
<td>( 1.43 \times 10^{-6} )(^b)</td>
</tr>
<tr>
<td>spiperone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(+)-butaclamol HCl</td>
<td>( D_2 ) receptor antagonism</td>
<td>( 5.06 \times 10^{-8} )</td>
</tr>
<tr>
<td>( R )-(+)-SCH23390 HCl</td>
<td>( D_1 ) receptor antagonism</td>
<td>( 1.03 \times 10^{-10} )</td>
</tr>
<tr>
<td>propranolol HCl</td>
<td>( \beta )-adrenergic receptor antagonism and mixed 5-HT(_1)/5-HT(_2) antagonism</td>
<td>( 3.67 \times 10^{-7} )</td>
</tr>
<tr>
<td>serotonin HCl</td>
<td>5-HT receptor agonism</td>
<td>( 4.11 \times 10^{-7} )</td>
</tr>
<tr>
<td>buspirone HCl</td>
<td>5-HT(_{1A}) receptor agonism</td>
<td>( 5.34 \times 10^{-7} )</td>
</tr>
<tr>
<td>dicyclomine HCl</td>
<td>( M_1 ) muscarinic receptor antagonism</td>
<td>( 1.68 \times 10^{-6} )</td>
</tr>
<tr>
<td>phentolamine HCl</td>
<td>( \alpha )-adrenergic receptor antagonism and 5-HT(_{1A}) receptor antagonism</td>
<td>-</td>
</tr>
<tr>
<td>scopolamine</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data were taken from Soo, 2006\(^a\); Lo, 2007\(^b\); Peroutka, 1986\(^c\)
4.5.2.11 $Z'$ factor

To confirm the robustness of the assays, a $Z'$ analysis was performed for the D$_1$ (Figure 4.9(a)), D$_2$ (Figure 4.9(b)), 5-HT$_{1A}$ (Figure 4.9(c)) and 5-HT$_{2A}$ (Figure 4.9(d)) receptors each using 48 replicate values for both total binding and nonspecific binding. For each binding assay, the $Z'$ value yielded more than 0.5, which was within the acceptable range (0.5–1.0) and confirmed the robustness of the assay (Table 4.3). The percentage of coefficient of variability (CV) for total binding and nonspecific binding for each assay was less than 20, suggesting minimal variation and good reproducibility of the assay.

**Figure 4.9**: $Z'$ analysis for (a) D$_1$, (b) D$_2$, (c) 5-HT$_{1A}$ and (d) 5-HT$_{2A}$ receptors. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=48) ± SEM.
Table 4.3: Data from Z’ factor analysis for the D₁, D₂, 5-HT₁A and 5-HT₂A receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Z’ factor</th>
<th>S/B</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TB</td>
<td>NSB</td>
<td>TB</td>
</tr>
<tr>
<td>D₁</td>
<td>0.75</td>
<td>20.11</td>
<td>1028.08</td>
<td>51.13</td>
<td>72.86</td>
</tr>
<tr>
<td>D₂</td>
<td>0.74</td>
<td>6.44</td>
<td>1464.38</td>
<td>227.25</td>
<td>86.31</td>
</tr>
<tr>
<td>5-HT₁A</td>
<td>0.72</td>
<td>9.36</td>
<td>1021.92</td>
<td>109.17</td>
<td>71.08</td>
</tr>
<tr>
<td>5-HT₂A</td>
<td>0.75</td>
<td>7.56</td>
<td>1062.67</td>
<td>140.56</td>
<td>67.21</td>
</tr>
</tbody>
</table>

TB=Total binding, NSB=Nonspecific binding, S/B= Signal to background ratio; SD= Standard deviation; CV= Coefficient of variation

4.6 Conclusion

The optimised assay conditions for the dopamine (D₁ and D₂) and serotonin (5-HT₁A and 5-HT₂A) receptor binding assays were summarized in Table 4.4. Saturation binding assays of each receptor revealed a $K_d$ and $B_{\text{max}}$ of 0.40 nM and 292.80 fmol/mg (D₁), 0.86 nM and 456.20 fmol/mg (D₂), 0.17 nM and 17.23 fmol/mg (5-HT₁A), and 0.54 nM and 74.98 fmol/mg (5-HT₂A), respectively. The inhibition constant ($K_i$) for the respective competitive/reference ligands were 0.10 nM (SCH23390 HCl; D₁), 50.60 nM (butaclamol HCl; D₂, 3.33 nM (serotonin HCl; 5-HT₁A) or 5.10 nM (spiperone; 5-HT₂A). The respective Z’ values were 0.75, 0.74, 0.72 and 0.75 for the D₁, D₂, 5-HT₁A and 5-HT₂A receptors, indicated that the adopted assays were suitable, robust and reliable for medium throughput screening.
### Table 4.4: Optimised assay conditions for D₁, D₂, 5-HT₁A and 5-HT₂A receptors.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₁</td>
</tr>
<tr>
<td>Receptor source (SD rat)</td>
<td>striatum</td>
</tr>
<tr>
<td>Membrane (µg protein/well)</td>
<td>30</td>
</tr>
<tr>
<td>Radioligand (nM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reference ligand</td>
<td>R-(+)-SCH23390 HCl</td>
</tr>
<tr>
<td>Reference ligand (µM)</td>
<td>1</td>
</tr>
<tr>
<td>pH buffer (50 mM Tris-HCl)</td>
<td>7.4</td>
</tr>
<tr>
<td>Filter plate type</td>
<td>GF/C</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>90</td>
</tr>
<tr>
<td>Incubation temperature (°C)</td>
<td>22</td>
</tr>
<tr>
<td>Washing times</td>
<td>4</td>
</tr>
</tbody>
</table>
5.0 Virtual and Biomolecular Screening of Potential Ligands for 5-HT$_{2A}$ Receptor

5.1 Introduction

5.1.1 Structure Activity Relationship (SAR) of Aporphines

In the realm of central nervous system (CNS) activity, naturally occurring aporphines and their synthetic derivatives are known to have affinity and selectivity for adrenergic, dopaminergic and serotonergic receptors (Cabedo et al., 2009; Rios et al., 2000; Zhang et al., 2007). The structural modifications on the tetracyclic aporphine skeleton with varying substituents can lead to major changes in its pharmacological activity. Since there is no 3D X-ray structures for any of dopamine and serotonin receptors, the information gained from structure activity relationship (SAR) studies are important for the development of structure-based drug design.

For example, structure activity relationships of 2-substituted aporphines with aryl (Sondergaard et al., 2005), $O$-alkyl (Sipos et al., 2008), methylthio (Toth et al., 2006), or halogen (Neumeyer et al., 1990; Ramsby et al., 1989; Zhang et al., 2005) substituents suggest their potential to increase selectivity and affinity at D$_2$ receptors due to the existence of a lipophilic cleft or region on the surface of D$_2$ receptor proteins. 11-hydroxyaporphines, and especially their esters, have more prolonged behavioural arousal-inducing activity with far superior oral bioavailability (Csutoras et al., 2004). Aporphines containing a single hydroxyl group at C-11 have some affinity for the D$_1$ dopamine receptor and properties as D$_1$ antagonists (Schaus et al., 1990).

High affinities to 5-HT$_{1A}$ receptors are displayed by a number of aporphines carrying sterically and electronically different C-11 substituents (Hedberg et al., 1996).
The $N$-substituted 11-hydroxy-10-hydroxymethyl aporphine and 11-hydroxy-2-methoxy-10-methyl congeners displayed selective and potent affinity for the 5-HT$_{1A}$ receptor but low affinity at D$_1$ and D$_2$. These findings support that ortho-dihydroxy substitution in the aporphine D ring enhances affinity to dopamine receptors, whereas analogous methyl or hydroxymethyl substitution enhances interactions with serotonin 5-HT$_{1A}$ receptor (Si et al., 2007).

Removal of the 11-hydroxy group from the 2-methoxy-11-hydroxy-$N$-alkylaporphines decreased affinity at dopamine receptors, and afforded low affinity for the serotonin 5-HT$_{1A}$ receptor (Si et al., 2008). Alkylation of the 11-hydroxy group with an alkyl, but adding a hydroxy substituent on the 2-position also decreased the affinities at dopamine and serotonin receptors, supporting the assumption that 11-hydroxy substitution in aporphines is required for dopaminergic and serotonergic activity.
5.2 Materials

The test compounds were purchased from MolPort Ltd (Riga, Latvia). Details of all the other chemicals used are given in Section 4.2.1.

5.3 Methodology

5.3.1 Receptor Binding Assays

The compounds selected from the docking studies on the 5-HT$_{2A}$ receptor model were tested experimentally against the dopamine (D$_1$ and D$_2$) and serotonin (5-HT$_{1A}$ and 5-HT$_{2A}$) receptors according to protocol described previously (see Section 4.3.3.3).
5.4 Results and Discussion

5.4.1 Receptor Binding Assays

The compounds selected from the docking studies on the 5-HT$_{2A}$ receptor model were assayed in vitro for their ability to displace [$^3$H] SCH 23390, [$^3$H] spiperone, [$^3$H] 8-OH-DPAT, and [$^3$H] ketanserin binding to rat D$_1$, D$_2$, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors, respectively. The results are summarized in Table 5.1. The results indicate that (R)-roemerine has the most favourable affinity-selectivity profiles for the 5-HT$_{2A}$ receptor and has 20–400-fold higher affinity towards the 5-HT$_{2A}$ receptor than towards D$_1$, D$_2$ and 5-HT$_{1A}$ receptors (Figure 5.1(a)). This finding confirms previous work by Chen and co-workers which showed that (R)-roemerine has selectivity for 5-HT$_{2}$ receptors over D$_1$, D$_2$ and 5-HT$_{1}$ receptors (Chen et al., 1987). Compared to (R)-roemerine, all the compounds evaluated including the standard, (S)-boldine, have reduced affinity for the 5-HT$_{2A}$ receptor by 20- to 300-fold.

(S)-bulbocapnine is particularly selective for the D$_1$ receptor and has a 60–6000-fold greater affinity at the D$_1$ receptor than at the D$_2$, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors (Figure 5.1(b)). The observed results are in agreement with the work by Schaus and colleagues who reported only moderate to low affinity of (S)-bulbocapnine to D$_2$, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors due to the presence of the hydrophobic binding site at the D$_1$ receptor which accounted for the high antagonistic activity of (S)-bulbocapnine (Schaus et al., 1990). Likewise, (S)-boldine also showed higher affinity for the D$_1$ over D$_2$ receptor by 20-fold (Figure 5.1(c)), in agreement with the findings of a previous study (Asencio et al., 2005).
All compounds with the dihydroxy substitutions at position 10 and 11 including (R)-apomorphine (Figure 5.1(d)), (S)-apomorphine (Figure 5.1(e)) and (R)-N-propylapomorphine (Figure 5.1(f)) displayed potent affinity for the dopamine receptors (D1 and D2) than the serotonin (5-HT1A and 5-HT2A) receptors. (R)-apomorphine exhibited a 12-fold higher affinity at the D1 receptor and 11-fold higher affinity at the D2 receptor than its (S)-enantiomer. This observation is consistent with the previous report which demonstrated that the (R)-configuration was more favourable for high affinity at dopamine receptors, especially of the D2 type (Csutoras et al., 2004; Gao et al., 1990). Replacement of the N-propyl to N-methyl group as in (R)-N-propylapomorphine showed the higher selectivity by 19-fold for D2 over D1 receptor. This is in agreement with the previous study which proposed that the presence of the propyl cleft would accommodate a low energy conformation of the N-propyl substituent and enhanced the affinity at D2 receptor (Csutoras et al., 2004; Hedberg et al., 1996).

On the other hand, there is no significant difference in the binding of (±)-nuciferine, (±)-laureline and (R)-2-hydroxy-N-propylapomorphine for the D1, D2, 5-HT1A and 5-HT2A receptors. Lastly, (S)-isocorydine, (±)-isothebaine, (S)-corydine and (S)-corytuberine showed little or no affinity towards all four receptors.
Figure 5.1: Competition binding curves of (a) (R)-roemerine, (b) (S)-bulbocapnine (c) (S)-boldine, (d) (R)-apomorphine, (e) (S)-apomorphine and (f) N-propylapomorphine towards $[^3]$H SCH23390 for D$_1$ receptor (♦), $[^3]$H spiperone for D$_2$ receptor (■), $[^3]$H 8-OH-DPAT for 5-HT$_{1A}$ receptor (▲) and $[^3]$H ketanserin for 5-HT$_{2A}$ receptor (●). Results are represented as mean (n=3) ± SEM.
Table 5.1: Binding affinities of the selected ligand compounds towards rat D₁, D₂, 5-HT₁A and 5-HT₂A receptors.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>6a</th>
<th>R₁</th>
<th>R₂</th>
<th>R₆</th>
<th>R₁₀</th>
<th>R₁₁</th>
<th>Kᵢ ± SEM (nM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D₁</td>
<td>D₂</td>
<td>5-HT₁A</td>
</tr>
<tr>
<td>Boldine</td>
<td>S</td>
<td>OMe</td>
<td>OH</td>
<td>Me</td>
<td>OH</td>
<td>OMe</td>
<td>128 ± 10</td>
<td>2530 ± 350</td>
<td>2450 ± 390</td>
</tr>
<tr>
<td>Roemerine</td>
<td>R</td>
<td>O-CH₂-O</td>
<td>Me</td>
<td></td>
<td></td>
<td></td>
<td>1400 ± 100</td>
<td>22600 ± 2600</td>
<td>1170 ± 60</td>
</tr>
<tr>
<td>Bulbocapnine</td>
<td>S</td>
<td>O-CH₂-O</td>
<td>Me</td>
<td>OMe</td>
<td>OH</td>
<td></td>
<td>29.3 ± 5.0</td>
<td>185000 ± 2000</td>
<td>7260 ± 710</td>
</tr>
<tr>
<td>Laureline</td>
<td>±</td>
<td>O-CH₂-O</td>
<td>Me</td>
<td>OMe</td>
<td></td>
<td></td>
<td>1420 ± 360</td>
<td>8500 ± 320</td>
<td>520 ± 150</td>
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<tr>
<td>Nuciferine</td>
<td>±</td>
<td>OMe</td>
<td>OMe</td>
<td>Me</td>
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<td></td>
<td>820 ± 120</td>
<td>2050 ± 790</td>
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<tr>
<td>Apomorphine</td>
<td>R</td>
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<td>OH</td>
<td></td>
<td></td>
<td>17.5 ± 1.0</td>
<td>15.9 ± 2.0</td>
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<tr>
<td>Apomorphine</td>
<td>S</td>
<td>Me</td>
<td>OH</td>
<td>OH</td>
<td></td>
<td></td>
<td>221 ± 13</td>
<td>178 ± 7</td>
<td>2440 ± 620</td>
</tr>
<tr>
<td>NPA</td>
<td>R</td>
<td>Pr</td>
<td>OH</td>
<td>OH</td>
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<td></td>
<td>239 ± 11</td>
<td>12.4 ± 0.2</td>
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<td>2-OH-NPA</td>
<td>R</td>
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<td>Pr</td>
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<td>OH</td>
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<td>1320 ± 20</td>
<td>1220 ± 50</td>
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<td>Iso Corydine</td>
<td>S</td>
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<td>Me</td>
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<td>OH</td>
<td>1250 ± 200</td>
<td>140000 ± 7500</td>
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<tr>
<td>Isothebaine</td>
<td>±</td>
<td>OH</td>
<td>OMe</td>
<td>Me</td>
<td>OMe</td>
<td>OMe</td>
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<td>NE</td>
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<tr>
<td>Corydine</td>
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<td>OH</td>
<td>OMe</td>
<td>Me</td>
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<td>OMe</td>
<td>1800 ± 70</td>
<td>41300 ± 3500</td>
<td>27400 ± 400</td>
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<tr>
<td>Corytuberin</td>
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<td>OH</td>
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<td>Me</td>
<td>OMe</td>
<td>OMe</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
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</tbody>
</table>

The values shown for Kᵢ were the mean (n = 3) ± SEM.; NE = could not be estimated (10⁻⁴ or higher); NPA=N-propylapomorphine; 2-OH-NPA=2-hydroxy-N-propylapomorphine.
To gain a further insight into the binding characteristics of the highly selective 5-HT$_{2A}$ receptor compound ($R$)-roemerine, the ligand-receptor interactions were investigated using the generated 5-HT$_{2A}$ receptor model. The predicted binding pose for ($R$)-roemerine is shown in **Figure 5.2** with the ligand bound in the site bordered by transmembrane regions 3, 5, 6 and 7 (**Table 5.2**). In particular, ($R$)-roemerine was found to bind to the 5-HT$_{2A}$ receptor through a combination of a hydrogen bonding and electrostatic interaction formed between the protonated amino group at position $R_6$ of the ligand and the carboxylate group of the conserved residue Asp155 of the 5-HT$_{2A}$ receptor, a polar interaction involving side chain of amino acid residue Tyr370, a pi-cation interaction at Phe339 and additional van der Waals contacts between the aporphine rings and the aromatic residues (Phe234, Trp336, Phe339, Phe340 and Tyr370) and non-aromatic residues (Ser159, Thr160 and Val366). These observations were in agreement with site-directed mutagenesis experiments which have reported the importance of these amino acid residues for 5-HT$_{2A}$ receptor ligand binding (Choudhary *et al.*, 1993; Kristiansen *et al.*, 2000; Roth *et al.*, 1997; Shapiro *et al.*, 2000; Wang *et al.*, 1993).
Investigation into the ligand-receptor interactions involving the selected compounds revealed a significant difference in the binding affinity of (R)-roemerine towards 5-HT$_{2A}$ receptor versus D$_1$, D$_2$ and 5-HT$_{1A}$ receptors. Table 5.2 shows the complete list of residues and interactions involved in the binding of the selected aporphines with the 5-HT$_{2A}$ receptor. In case of (±)-laureline, (±)-nuciferine and (±)-isothebaine, the (S)-enantiomer was selected over the (R)-enantiomer because better binding affinities were observed towards 5-HT$_{2A}$ receptor in computational studies.

The results from this study showed that the (S)-boldine (Figure 3.2), (S)-bulbocapnine (Figure 8.8(a) in Appendix E), (S)-laureline (Figure 8.8(b) in Appendix E), (S)-apomorphine (Figure 8.8(e) in Appendix E), (S)-corydine (Figure 8.8(j) in Appendix E) and (S)-corytuberine (Figure 8.8(k) in Appendix E) also formed

**Figure 5.2:** Proposed binding mode of (R)-roemerine in the 5-HT$_{2A}$ receptor. The residues involved in hydrogen bonding, electrostatic or polar interactions were represented in pink circles with their hydrogen bonds with their main chains (blue dashed arrow), charge interactions (pink dashed double-ended arrow), pi interaction (orange line) and residues involved in van der Waals interactions (green circles).
hydrogen bonding and electrostatic interactions with the conserved Asp155 in 5-HT$_{2A}$ receptor. However, apart from (S)-laureline, they failed to interact via a pi-cation interaction as observed in (R)-roemerine. This may explain the unfavourable affinities of these compounds at the 5-HT$_{2A}$ receptor and the higher affinity-selectivity profile of (R)-roemerine at the 5-HT$_{2A}$ receptor. The 50-fold reduction in affinity at the 5-HT$_{2A}$ receptor observed for (S)-laureline seems to be due to the additional methoxy substitution at position 10.

On the other hand, (R)-apomorphine (Figure 8.8(d) in Appendix E), (R)-N-propylnorapomorphine (Figure 8.8(f) in Appendix E) and (R)-2-hydroxy-N-propylapomorphine (Figure 8.8(g) in Appendix E) were only able to form hydrogen bonds between the conserved residue Asp155 and substituents in the aporphine D-ring, particularly the hydroxyl group. Pi-cation interactions were also observed at Phe339 for (R)-apomorphine and at Phe234 for (R)-N-propylnorapomorphine and (R)-2-hydroxy-N-propylapomorphine. Similarly, the hydroxyl substituent in the aporphine D-ring of (S)-isocorydine (Figure 8.8(h) in Appendix E) was also hydrogen bonded with the Asp155, but without any pi-cation interaction. However, the lack of electrostatic interaction of the Asp155 with the protonated amino group of these ligands compared to (R)-roemerine led to poor binding and selectivity towards the 5-HT$_{2A}$ receptor.

(S)-nuciferine (Figure 8.8(c) in Appendix E) was only able to interact with Asp155 via an electrostatic interaction. Hydrogen bonding of the Asp155 with hydrogen from the protonated nitrogen and pi-cation interaction was not observed in (S)-nuciferine. Likewise, the conserved residue Asp155 failed to form electrostatic and hydrogen bond with the protonated amino group of the (S)-isothbeaine (Figure 8.8(i) in Appendix E). Instead of a pi-cation interaction, (S)-isothbeaine displayed a pi-pi
interaction at Phe234 together with hydrogen bonding between Gly238 and the hydroxyl group in the aporphine D-ring. The observed binding differs from (R)-roemerine which may suggest the lower affinity and selectivity for the 5-HT$_{2A}$ receptor.
Table 5.2: 5-HT$_{2A}$ residues found from the docking studies to interact with the selected aporphines

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<th>Compounds</th>
<th>Interactions</th>
<th>Electrostatic</th>
<th>H-bond</th>
<th>Polar</th>
<th>pi-cation</th>
<th>pi-pi</th>
<th>van der Waals Residues</th>
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<td>Asp155</td>
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<td>Ser159,</td>
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<td>Val156</td>
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<td>Asp155</td>
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<td>Phe339, Phe340,</td>
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<td>Asp155</td>
<td>Thr160, Gly238, Tyr370</td>
<td>Phe339</td>
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<td>Asp155, Ser239</td>
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<tr>
<th>van der Waals Residues</th>
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5.5 Conclusion

(R)-roemerine had been identified as a potential 5-HT$_{2A}$ receptor-selective compound. This suggest that preferential affinity for the 5-HT$_{2A}$ receptor results from that substitution at position 1 and 2, particularly with methylenedioxy group, non-substitution at position 10 and 11 and N-methyl substitution at position 6. Further investigation into the ligand-receptor interactions of (R)-roemerine also revealed that high 5-HT$_{2A}$ selectivity over D$_1$, D$_2$ and 5-HT$_{1A}$ receptors were probably due to the strong hydrogen bonding and electrostatic interactions involving the protonated amino group and a pi-cation interaction with Asp155 and Phe339, two of the conserved residues in the binding pocket of the 5-HT$_{2A}$ receptor.
6.0 General Conclusion, Limitations and Future Perspectives

A combination of structure based *in silico* and *in vitro* experiments were implemented in this study to identify potential ligands for dopaminergic and serotonergic receptors. A 3D homology model of the rat 5-HT$_{2A}$ receptor was first generated based on the crystal structure of the human β$_2$-adrenergic receptor as a structural template and further validated with standard 5-HT$_{2A}$ receptor ligands. Thereafter, a filtered set of aporphines using (S)-boldine as the backbone structure was docked into the generated 5-HT$_{2A}$ receptor model. A set of 13 aporphines with higher or comparable activity to (S)-boldine were selected for experimental testing.

A medium throughput screening assay for dopamine (D$_1$ and D$_2$) and serotonin (5-HT$_{1A}$ and 5-HT$_{2A}$) receptors based on radioligand receptor binding experiments was used to evaluate the affinity of the selected compounds towards these receptors. (R)-roemerine was found to have potent 5-HT$_{2A}$ affinity ($K_i = 62.1$ nM) and selectivity (20–400-fold higher) versus D$_1$, D$_2$ and 5-HT$_{1A}$ receptors. Investigation into the binding mode of (R)-roemerine revealed the importance of the hydrogen bonding and electrostatic interactions with the conserved residue Asp155 and a pi-cation interaction at Phe339. These observations suggest that (R)-roemerine could form the basis of the design of aporphinoid compounds with high affinity and selectivity towards the 5-HT$_{2A}$ receptor.

There were however, several limitations to this project. The application of structure based *in silico* methods using a homology model for the identification of new drugs is more challenging and inaccurate than docking to 3D X-ray structures. Potential reasons of error may be due to structural differences between the template and the
target, incorrect sequence alignment and difficulties in obtaining variable loop regions. Additionally, only a small number of compounds were screened due to limited availability.

On the other hand, although the *in vitro* radioligand receptor binding assays used were simple and rapid, errors may be introduced due to the limited time allowed to reach steady state and through the process of separating the bound and free ligand, drying the harvested filter plates and counting the filter plates.

In order to improve the *in silico* approach, the constructed homology model should be further refined using simulation techniques such as constrained molecular mechanics or molecular dynamics simulations and validated with known 5-HT$_{2A}$ receptor active ligands. Additionally, in an attempt to rationalize the observed affinity data and to predict ligand-receptor interactions at the molecular level, homology models of D$_1$, D$_2$ and 5-HT$_{1A}$ receptors also should be built. A larger set of screening compounds should be obtained from the database to dock into the newly refined model. A library could also be obtained through use of synthetic chemistry.

The receptor binding assays could be improved by using purified or cloned receptor in order to minimise the nonspecific binding and ultimately increase the sensitivity of the assay.
7.0 References


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of the 5-HT(2A) serotonin receptor but does not participate in activation via a "salt-bridge disruption" mechanism. *Journal of Pharmacology and Experimental Therapeutics*, **293**, 735–746.


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Schlegel, J. R. & Peroutka, S. J. (1986). Nucleotide interactions with 5-HT\textsubscript{1A} binding sites directly labeled by [\textsuperscript{3}H]-8-hydroxy-2-(DI-n-propylamino)tetralin ([\textsuperscript{3}H]-8-OH-DPAT). Biochemical Pharmacology, \textbf{35}, 1943.


Soo, W. K. (2006). Development and application of 96-well microplate lipoxygenase and 5-HT₁A receptor screening assays for bioactive extracts and compounds from Malaysian plants., Department of Pharmacy, Faculty of Medicine, MSc Thesis: 1–206, University of Malaya: Malaysia.


8.0 Appendices

A: Sequence Analysis

CLUSTAL 2.0.12 multiple sequence alignment

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| uniprot | P28565 | SHT1D_RAT | --------------------------- | SNRSNATAG-ANDPEVLQALRSILVLLISLITLAVT | 51 |
| uniprot | P30940 | SHT1F_RAT | --------------------------- | LNL | 39 |
| uniprot | P19327 | SHT1A_RAT | --------------------------- | QEPFGOGRNTSISDVTPFSYQVTLSTLGFLCAVE | 52 |
| uniprot | P35364 | SHT5A_RAT | --------------------------- | TLNPSELDEA- | 56 |
| uniprot | P35365 | SHT5B_RAT | --------------------------- | FSSGRSMSTFGPGLISREPPFS-ATLVTLLVLITAIAT | 68 |
| uniprot | P32305 | SHT7R_RAT | GFLVEQTAAPTFADPNDSGCGEQQNYKVEVWVIGSLITLT | 100 |
| uniprot | P14842 | SHT2A_RAT | EASNNITDANTNLSCGYLFPCLLSJLHDEQNSALTLYTVVINTA | 118 |
| uniprot | P08099 | SHT2C_RAT | --------------------------- | PVVAIVDTDN- | 131 |
| uniprot | P30994 | SHT2B_RAT | --------------------------- | AAEMGQTAEN- | 141 |
| uniprot | P62758 | SHT4R_RAT | --------------------------- | GFGYEHVVLITFAMVMAIL | 35 |
| uniprot | P31388 | SHT6R_RAT | --------------------------- | PGGPPAPGSGWVAACLTVIWLA | 42 |

**TI**

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| uniprot | P28565 | SHT1D_RAT | SNAFVITALTILLTTP-ANLYLASLTLTVDLSVLMVIPSTMYTT | 98 |
| uniprot | P30940 | SHT1F_RAT | INCLVITAITYRKLHHP-ANLYCISLVTDVFLVAVNLMSFISYTV | 86 |
| uniprot | P19327 | SHT1A_RAT | GNACVAAAILLESQNV-ANLYCISLVTDVFLVAVNLMSFISYTV | 101 |
| uniprot | P35364 | SHT5A_RAT | WNLVTVILDWVTVN- | 104 |
| uniprot | P35365 | SHT5B_RAT | WNLVTVILDWVTVN- | 104 |
| uniprot | P32305 | SHT7R_RAT | GNLCLVISVSFCVKKQRQ-YNVYSLVIALALSLVAVMVFYMSTVDGL | 143 |
| uniprot | P14842 | SHT2A_RAT | GNILVMAVSLEKLRQK-TNFSLFLILALMLGLFLNMLSTILTG-G | 138 |
| uniprot | P08099 | SHT2C_RAT | GNILVMAVSLEKLRQK-TNFSLFLILALMLGLFLNMLSTILTG-G | 138 |
| uniprot | Q62758 | SHT4R_RAT | GNILVMAVSLEKLRQA-TNFSLFLILALMLGLFLNMPAE | 117 |
| uniprot | P31388 | SHT6R_RAT | AnSSLILVICTOPABNT- | 83 |

**TI**

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| uniprot | P30940 | SHT1F_RAT | ESIMQGGLCDWLSVIICCTSIHLAIAIDRAT-DAVEYSAKR | 135 |
| uniprot | P19327 | SHT1A_RAT | NKWTQLVTCDFIALDVCLITSIHLAIAIDRAT-DIIVYNAK | 148 |
| uniprot | P35364 | SHT5A_RAT | RRQQLRQLCQIACWTDCCIASITNTALAILDRWET- | 153 |
| uniprot | P35365 | SHT5B_RAT | RQWFLFTHCCQIACWTDCCIASITNTALAILDRWET- | 153 |
| uniprot | P14842 | SHT2A_RAT | TWPLSLAINIYWDVLIFSASIMILCAIDRXVMVQAIP-1SISNFS | 187 |
| uniprot | P08099 | SHT2C_RAT | YWPLPLRYPCLVSIDLVIFSASIMILCAIDRXVMVQAIP-1SISNFS | 187 |
| uniprot | P30994 | SHT2B_RAT | ATWPLPLACPMWLVFSASIMILCAIDRHYAK-RPQANCNC | 166 |
| uniprot | Q62758 | SHT4R_RAT | DVWFGOECQFL-TSLVLDITSALFICLILIDRYAICQCLVLVRN | 133 |
| uniprot | P31388 | SHT6R_RAT | GVWVAGLCW- | 138 |

**TI**

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| uniprot | P28565 | SHT1D_RAT | TAGHAAMIAAWAISICBSPIILQ-QATAHEEMLCIVTNTS | 193 |
| uniprot | P30940 | SHT1F_RAT | TFFAINTYIVLDVPLFFMPA-QIQANSRND-QCICRCHRY | 180 |
| uniprot | P19327 | SHT1A_RAT | TPRHRTAVLISLGGFIPPLRQPM | 179 |
| uniprot | P35364 | SHT5A_RAT | RRRKVSNMILLWALSALIAPLFLGQETYESLSEEQQES | 199 |
| uniprot | P35365 | SHT5B_RAT | RRRSALMIAIWTALSALIAALIAPLFLGQEMEYRSLRQCVQF | 212 |
| uniprot | P32305 | SHT7R_RAT | NRGRCAILMSWLWLASITLFP-LFGAQWQVND-DRCVLSISQDO | 241 |
The lowest and highest sequence identity between any two sequences was between 19% and 68%, respectively.
Figure 8.2: Sequence alignment of 5-HT2A multiple species. A multiple sequence alignment indicates that the rat 5-HT2A receptor shares the lowest and highest sequence identity between 88% and 97% between any of two sequences. The major differences among the multiple species sequences were found at the N- and C-terminus ends.
**Figure 8.3:** Sequence comparison of rat 5-HT₂A against human 5-HT₂A. A close comparison of sequences between the rat and human 5-HT₂A indicated the species specific residues were found to be distributed in the loop regions (except three residues, Ala 82, Val 150 and Ser 242 (shaded in cyan), that were found in transmembrane 1, 4 and 5, respectively). The sequence identity between the rat and human 5-HT₂A receptor was 91%.
Figure 8.4: Sequence comparison of rat 5-HT2A against rat 5-HT2B and 5-HT2C sequences. The sequence identity of rat 5-HT2A with rat 5-HT2B and 5-HT2C is 38% and 48% respectively suggesting that the rat 5-HT2A is closer to 5-HT2C than 5-HT2B.
B: Ramachandran plot

Figure 8.5: Structure validation of the model using Ramachandran plot computed with the PROCHECK program. The result shows 89.4% in the most favourable region, 9.9% in the additionally allowed region, 0.5% in the generously allowed region and 0.2% in the disallowed region.
C: Binding Mode of Standard Ligands in the 5-HT$_{2A}$ Receptor Model

(a) The 5-HT$_{2A}$ receptor model in complex with endogenous ligand, serotonin

(b) The 5-HT$_{2A}$ receptor model in complex with tryptamine
(c) The 5-HT2A receptor model in complex with 5-MeOT

(d) The 5-HT2A receptor model in complex with (R)-(-)-DOI
(e) The 5-HT2A receptor model in complex with (S)-(−)-DOI

**Figure 8.6:** The 5-HT2A receptor model in complex with endogenous ligand and agonists (a) serotonin (b) tryptamine (c) 5-MeOT (d) (R)-(−)-DOI (e) (S)-(+) DOI. The residues involved in hydrogen bonding, electrostatic or polar interactions were depicted in pink circle with their hydrogen bonds (blue dashed arrow), charge interactions (pink dashed double-ended arrow) and residues involved in van der Waals interactions (green circle).
(a) The 5-HT$_{2A}$ receptor model in complex with spiperone

(b) The 5-HT$_{2A}$ receptor model in complex with risperidone
(c) The 5-HT2A receptor model in complex with haloperidol

(d) The 5-HT2A receptor model in complex with ritanserin
(e) The 5-HT2A receptor model in complex with ketanserin

**Figure 8.7:** The 5-HT2A receptor model in complex with antagonists (a) spiperone (b) risperidone (c) haloperidol (d) ritanserin (e) ketanserin. The residues involved in hydrogen bonding, electrostatic or polar interactions were depicted in pink circle with their hydrogen bonds (blue dashed arrow), charge interactions (pink dashed double-ended arrow) and residues involved in van der Waals interactions (green circle).
D: ZINC IDs and the Corresponding Compounds Names

Table 8.1: The ZINC IDs of the compounds selected from virtual screening.

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E: Binding Modes of the Selected Aporphines with the 5-HT$_{2A}$ Receptor Model

(a) Proposed binding mode of (S)-bulbocapnine in the 5-HT$_{2A}$ receptor

(b) Proposed binding mode of (S)-laureline in the 5-HT$_{2A}$ receptor
Proposed binding mode of (S)-nuciferine in the 5-HT$_2$A receptor

Proposed binding mode (R)-apomorphine in the 5-HT$_2$A receptor
(e) Proposed binding mode (S)-apomorphine in the 5-HT$_2A$ receptor

(f) Proposed binding mode of (R)-N-propylapomorphine in the 5-HT$_2A$ receptor
(g) Proposed binding mode of \((R)-2\)-hydroxy-\(N\)-propylapomorphine in the \(5\)-HT\(_{2A}\) receptor

(h) Proposed binding mode of \((S)\)-isocorydine in the \(5\)-HT\(_{2A}\) receptor
(i) Proposed binding mode of (S)-isothebaine in the 5-HT$_{2A}$ receptor

(j) Proposed binding mode of (S)-corydine in the 5-HT$_{2A}$ receptor
(k) Proposed binding mode of (S)-corytuberine in the 5-HT$_{2A}$ receptor

**Figure 8.8:** Proposed binding mode of (a) (S)-bulbocapnine (b) (S)-laureline (c) (S)-nuciferine (d) (R)-apomorphine (e) (S)-apomorphine (f) (R)-N-propylapomorphine (g) (R)-2-hydroxy-N-propylapomorphine (h) (S)-isocorydine (i) (S)-isothebaine (j) (S)-corydine (k) (S)-corytuberine in the 5-HT$_{2A}$ receptor. The residues involved in hydrogen bonding, electrostatic or polar interactions were represented in pink circles with their hydrogen bonds with their main chains (blue dashed arrow), side chains (green dashed arrow), charge interactions (pink dashed double-ended arrow), pi interaction (orange line) and residues involved in van der Waals interactions (green circles).
F: Abstracts of Research Presentations

Following is the abstract for oral presentation at the 1st Pharmaceutical Sciences Conference & Exhibition (PSCE), Penang, Malaysia, 27th–28th September 2010

COMPUTATIONAL INVESTIGATION OF THE 5-HT2A RECEPTOR BINDING INTERACTIONS OF STANDARD LIGANDS AND APORPHINES

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The 5-hydroxytryptamine 2A (5-HT2A) receptor has been implicated in numerous psychiatric disorders, including schizophrenia and depression. Several aporphines have been found to have 5-HT2A receptor binding activity. The aim of this study was to investigate the ligand-receptor interactions of standard 5-HT2A ligands and aporphines. A 3D molecular model of the rat 5-HT2A receptor was constructed by homology modelling against the known crystal structure of the human β2-adrenergic receptor (PDB ID: 2RH1). Docking studies were then conducted using Autodock4.0. The standard ligands and aporphines were found to interact with residues belonging to transmembrane domains 2, 3,5,6,7 and extracellular loop 2 (ECL2). The proposed binding interactions involve three sets of residues, (1) Asp155 (3.32) forming a hydrogen bond with the protonated amine of the ligands, (2) an aromatic network consisting of Trp151(3.28), Trp336(6.48), Phe339(6.51), Phe340(6.52), and Tyr370(7.43), and (3) Val156(3.33), Thr160(3.37), Leu229(ECL2), Gly238(5.42), Ser239 (5.43), Asn363(7.36) as possible hydrogen bond acceptors or donors. These findings are in agreement with currently available experimental data and suggest that the model has the potential to predict ligand-receptor interactions at the molecular level.
Following is the abstract for poster presentation at the 25th Scientific Meeting of The Malaysian Society of Pharmacology and Physiology (MSPP), University Putra Malaysia, Malaysia, 25th–26th of May 2011:

DEVELOPMENT OF FILTRATION BASED DOPAMINE₁ RECEPTOR BINDING ASSAY USING 96-WELL MICROPLATE

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The dopamine₁ receptor plays a pivotal role in the treatment of neuropsychiatric and neurodegenerative disorders such as schizophrenia and Parkinson’s disease. This study aims to develop a medium throughput screening assay for the search of potential dopaminergic ligands. A 96 well microplate was adopted in combination with Filterplate Cell Harvester and TopCount NXT. The optimised parameters consisted of 30 µg of protein/well of striatum in the presence of 0.5 nM of [³H] SCH23390 and 1 µM of SCH 23390 HCl. The equilibration time of ligand receptor interaction was reached after 90 min of incubation at 22°C. GF/C filter plates gave lower nonspecific binding compared to GF/B filter plates after 4 times washing with Tris-HCl buffer (50mM, pH 7.4). Saturation analysis of [³H] SCH23390 binding revealed mean $B_{max}$ and $K_d$ values of 293.7 fmol/mg protein and 0.41 nM respectively. Competition experiments with standard ligands include R-(+)-SCH23390 HCl ($K_i = 1.05 \times 10^{-10}$), propranolol HCl ($K_i = 3.66 \times 10^{-7}$), serotonin HCl ($K_i = 4.06 \times 10^{-7}$), buspirone HCl ($K_i = 5.38 \times 10^{-7}$) and dicyclomine HCl ($K_i = 1.69 \times 10^{-6}$). A $z'$ factor of 0.74 demonstrates the suitability, reliability and robustness of the assay for screening purposes.

**Keywords:** Central nervous system, dopamine₁ receptor, receptor binding assay, saturation experiment, competition experiment