Exploring the $\alpha_2$-adrenergic receptors with alkyl substituted bis-guanidinium diaryl derivatives

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Declaration

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December 2021
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Abstract

The burden of mental health conditions is on the rise globally. It is well documented that noradrenaline has a key role in memory, attention, stress, and regulation of emotions. The $\alpha_2$-adrenergic receptors ($\alpha_2$-ARs) are an attractive biological target for therapies for neurological conditions. Thus, $\alpha_2$-AR antagonists would block the activation of this autoreceptor potentially operating as therapeutic antidepressant agent. In a ligand-based drug design strategy we have investigated the effects on the binding affinity and ligand-receptor functional activity when releasing the rigid 2-aminoimidazolium moiety of lead compound 1, thus affecting steric and lipophilic properties. This aim will be achieved via computational studies, synthesis, and pharmacological evaluation.

Due to their relevance in depression, three inactive $\alpha_{2A}$-AR receptor templates were used in the docking study: a homology model developed by Prof. Olivella, and two crystal structures recently reported, one complexed with a partial agonist and another with an antagonist. Compounds 1 (lead, symmetric), 15 (asymmetric), 16 (asymmetric), 18 (symmetric) and 22c (symmetric) were used as ligands for standard rigid receptor and fit-induced docking studies. All docked compounds were orientated based on the ionic interaction with the aspartate residue D113\textsuperscript{3.32}, a known critical anchoring interaction with the $\alpha_2$-AR binding sites. Agonists 15 and 18 show interactions with S200\textsuperscript{5.42}, a known residue involved in agonist activity. Moreover, the interaction between the ligand and E94\textsuperscript{2.65} is common to the two known antagonists 16 and 22c and this could assist in directing the ligand away from TM5, avoiding receptor activation.

A new synthesis of di-Boc-protected mono-substituted thioureas was developed utilizing Mitsunobu reaction conditions. Additionally, five new compounds were prepared, characterised using $^1$H and $^{13}$C NMR, IR, and HRMS. The $\alpha_2$-AR binding affinities of these compounds were measured in human prefrontal cortex tissue using a competitive assay with the $\alpha_2$-AR selective radioligand [\textsuperscript{3}H]RX821002. When the ligands showed $\alpha_2$-AR affinity with $K_i < 100$ nM, functional [\textsuperscript{35}S]GTP\textsubscript{yS} assays were used to determine their activity (i.e. antagonist, agonist or inverse agonist). Encouragingly, one of the five newly synthesised compounds (22c) displayed a binding affinity of 95.50 nM (best of substituted bis-guanidinium ligands to date) and antagonist activity.

Considering past and present results with compounds 1, 16, 17 and 22c, a derivative containing both an imidazolium moiety and an ethyl-substituted guanidinium could be a very promising high affinity antagonist.
Abbreviations

ABC – ATP-binding cassette
AC – Adenylate cyclase
ACh – Acetylcholine
ADHD – Attention deficit/hyperactivity disorder
ADME – Absorption, distribution, metabolism, excretion
AR – Adrenoceptor / Adrenergic receptor
ATP – Adenosine triphosphate
BBB – Blood brain barrier
Ca\(^{2+}\) – Calcium ion
cAMP – Cyclic adenosine monophosphate
CNS – Central nervous system
CO – Carbon monoxide
CoMFA – Comparative molecular field analysis
COMT – Catechol-O-methyl transferase
CuCl\(_2\) – Copper (II) chloride
DBH – Dopamine β- hydroxylase
DCM – Dichloromethane
DEAD – Diethylazodicarboxylate
DNA – Deoxyribonucleic acid
DMF – Dimethylformamide
D\(_2\)O – Deuterium oxide
ESOL – Estimated solubility
GDP – Guanosine diphosphate
GI – Gastrointestinal
G-protein – Guanyl-nucleotide-binding protein
GPCR – G-protein-coupled receptor
G-score – Glide score
GTP – Guanosine triphosphate
HB – Hydrogen bond
HCl – hydrochloric acid
HgCl₂ – Mercury (II) chloride
HIA – Human intestinal absorption
HOMO – Highest unoccupied molecular orbital
HTS – High throughput screening
HPLC – High performance liquid chromatography
IC₅₀ – Half maximal inhibitory concentration
IMHB – Intramolecular hydrogen bonds
K⁺ – Potassium ion
Kᵦ – Affinity of radiolabelled ‘hot’ ligand
Kᵢ – Calculated dissociation constant of affinity of non-radiolabelled ligand
L-DOPA – L-3,4-dihydroxyphenylalanine
LUMO – Lowest unoccupied molecular orbital
MAO – Monoamine oxidase
MB-COMT – Membrane-bound catechol-O-methyl transferase
MeOD – Deuterated methanol
MDR – Multidrug resistance
MO – Molecular orbital
MR- Molecular refractivity
NA – Noradrenaline
NaH – Sodium hydride
NaHCO₃ – Sodium bicarbonate
NBO – Natural bonding orbital
N.D. – Not determined
NIS – N-iodosuccinimide
NMR – Nuclear magnetic resonance
NO – Nitric oxide
NT – Neurotransmitter
PAINS – Pan-assay interference compounds
PDB – Protein Data Bank
PFC – Prefrontal cortex
Pgp – permeability glycoprotein
PLC – Phospholipase C
PNMT – Phenylethanolamine N-methyltransferase
PNS – Peripheral nervous system
PPh$_3$ – triphenylphosphine
PS – Polymer supported
PSA – Polar surface area
QSAR – Quantitative structure-activity relationship
QTAIM – Quantum theory of atoms in molecules
Ro5 – Rule of five
SBDD – Structure based drug design
S-COMT – Soluble catechol-O-methyl transferase
SMNT – Small molecule neurotransmitter
TCT – Trichloro-1,3,5-triazine
THF – Tetrahydrofuran
TFA – Trifluoroacetic acid
TFAA – Trifluoroacetic anhydride
TPSA – Topological polar surface area
VMAT – Vesicular monoamine transporters
VS – Virtual screening
XL – Extracellular loop
W.H.O. – World Health Organisation
3D – 3-Dimensional
5-HT – Serotonin
5-HTP – 5-hydroxytryptophan
7-TM – Seven transmembrane
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1. Introduction

1.1 The Nervous System

In 1906, the Nobel Prize for Physiology or Medicine was awarded to Golgi and Ramon y Cajal for arguing that nerve cells are discrete entities that communicate by means of specialised contacts called synapses.\(^1\) However, it was not until the invention of the electron microscope in the 1950s that a visual identification of these synapses could be recorded. There are two types of nerve cells; neurons (specialised for electrical and chemical signalling over long distances) and neuroglia (supporting cells essential in developing the human brain).

Neurons are cells responsible for processing and transmitting signals within the human brain. They comprise of a cell body with dendrites, an axon usually covered by myelin and the terminals of the axon (Fig. 1.1).\(^2\) Neurons can be classified as:

1. Local circuit neurons or interneurons, which have typically relatively short axons
2. Afferent neurons, which carry information to the central nervous system.
3. Efferent neurons, which carry information away from the central nervous system.

Signals travel by electrical current starting on the dendrites and travelling along the axon to the terminals where, typically, they are transformed into a chemical signal (Fig. 1.1).\(^3\) The number of signals that a neuron receives depends on the complexity of its dendritic arbor,\(^1\) (i.e. the larger number of dendrites results in the cell body being innervated by a larger number of other neurons).

![Figure 1.1 Labelled diagram representing the structure of a neuron](image)

Typically, neurons lie adjacent to one another with no physical continuity; the extracellular space between each terminal is known as the synaptic cleft, thus the presynaptic and postsynaptic neurons communicate chemically via the secretion of molecules known as neurotransmitters.\(^1\) As each impulse reaches the terminal of the presynaptic neuron, one or
more neurotransmitters, are released transporting the corresponding message across the synapse to the postsynaptic neurons to influence biological activity around the body.

Chemical synapses are the most common, releasing chemical messengers by means of communication, however electrical synapses also exist.

1.1.1 The Central Nervous System (CNS)

The nervous system can be differentiated based on general function and anatomical distinction.

General function categorises the nervous system based on the sensory system (processes information from the environment, including visual and auditory senses), the motor system (responds to such information by generating movement or behaviour) and the associational systems (mediate the most complex brain functions).¹

This can then be further characterised into two main anatomical distinctions; the central nervous system (CNS) and the peripheral nervous system (PNS) (Figure 1.2). ⁴ The CNS includes the brain and the spinal cord, whereas the PNS includes sensory neurons linked to receptors at the body surface or deeper within the brain, motor axons connecting the brain and the spinal cord to skeletal muscles, and cells and axons that innervate smooth muscles, cardiac muscles and glands.¹

1.2 Synaptic Transmission

The most common type of synapse within the nervous system is the chemical synapse which functions through the release of neurotransmitters from synaptic vesicles. These neurotransmitters produce a secondary current flow by activating a specific receptor on the postsynaptic neuron, leading to a biological response.

As described by Dale Purves George et. al in the 2004 edition of Neuroscience, the stages in the signal transmission at the chemical synapses can be illustrated as follows:¹

“1. Firstly, an action potential arrives at the terminals of the presynaptic neuron.
2. This change in membrane potential results in the opening of voltage-gated calcium ion (Ca$^{2+}$) channels.

3. The abrupt concentration gradient of the Ca$^{2+}$ across the presynaptic membrane causes a rapid influx of the Ca$^{2+}$ into the terminal.

4. The sudden increase in Ca$^{2+}$ causes the synaptic vesicles to move to the axon terminals and fuse to the membrane releasing their content to the synaptic cleft. This process is known as exocytosis.

5. The freed neurotransmitters diffuse across the synaptic cleft, binding to specific receptors in the postsynaptic membrane, changing the ability of the ions to flow into (or out of) the cells.

6. The resulting neurotransmitter-induced current alters the conductance and the membrane potential of the postsynaptic neuron and increases or decreases the probability that the postsynaptic neuron will produce a new action potential."

Whether the postsynaptic action of a particular neurotransmitter is excitatory or inhibitory is determined by the nature of the neurotransmitter, the ionic permeability of the ion channel and the concentration of the permeant ions inside and outside of the cell.¹

Due to the rapid and dynamic nature of the events during synaptic transmission, the production and transport of the secretory vesicles from the cell body, to transport the necessary neurotransmitters, is far too slow. The time between the Ca$^{2+}$ influx and exocytosis in the nerve terminal is very short (from μs to ms).⁵ Thus, the synaptic vesicles are fused with the plasma membrane to be rapidly recycled via a clathrin-mediated process of endocytosis.⁵

1.2.1 Neurotransmitters

According to Kolb and Whishaw, the four experimental criteria used to characterise neurotransmitters are:

"1. The chemical must be synthesised in the neuron or otherwise be present in it.
2. When the neuron is active, the chemical must be released to produce a response.
3. The same response must be obtained when the chemical is experimentally placed on the target cell.
4. A mechanism must exist for deactivating the chemical when the reaction is complete."³

Using these criteria, researchers have been able to categorise the thousands of chemicals in the brain and isolate the neurotransmitters.
The neurotransmitters identified to date can be divided into three main categories: small-molecule neurotransmitters (SMNTs), neuropeptides and transmitter gases. SMNTs are mostly synthesised and packaged in the axon terminals and act relatively quickly at the synapses. This category mostly includes amines and amino acids. The classical SMNTs include acetylcholine (ACh) and monoaminergic NTs such as noradrenaline and dopamine.

Neuropeptides are multifunctional chains of amino acids that act as neurotransmitters. The process of transmitting information is relatively slow in comparison to that of the small-molecule neurotransmitters as they are mostly produced on the cell’s ribosomes, packaged by Golgi bodies and transported on the microtubule highway to the axon terminal. These include opioids and enkephalins.

Transmitter gases are the water-soluble gases such as nitric oxide (NO) and carbon monoxide (CO) and they are synthesised only when needed. Each gas diffuses from the site where it was made (i.e. the dendrites), easily crossing the cell membrane and immediately becoming active.

1.2.2 Catecholamines

Catecholamines are organic compounds consisting of a catechol moiety and an alkylamine side chain. They function in the human body as neurotransmitters and hormones as SMNTs. Examples of these catecholamines include dopamine, serotonin, adrenaline and noradrenaline. The pathway of the biosynthesis of these catecholamines was hypothesised by Hermann Blaschko in 1939 and confirmed in the 1950’s by Sydney Udenfriend using isotope experiments. Tyrosine hydroxylase was the last enzyme to be identified by Toshiharu Nagatsu, that participates in this biosynthesis (Fig. 1.3).

Tyrosine hydroxylase is the rate-limiting enzyme participating in the biosynthesis of the catecholamines found in neurons and endocrine cells. It uses molecular oxygen and
tyrosine as its substrate to synthesise L-3,4-dihydroxyphenylalanine (L-DOPA), in the cell’s cytoplasm. Whilst remaining in the cytoplasm, L-DOPA gets decarboxylated by the DOPA decarboxylase to form dopamine. This enzyme is also efficiently used to convert 5-hydroxytryptophan (5-HTP) to serotonin (5-HT).

From here, the dopamine neurotransmitter is taken up into the synaptic vesicle by proteins known as vesicular monoamine transporters (VMAT). VMAT’s ability to transport neurotransmitters is highly dependent on the energy available and the Na⁺ gradient of the neuronal membrane. Within the vesicles, dopamine β-hydroxylase (DBH) catalyses the synthesis of noradrenaline. As a result of an appropriate stimulus (Ca²⁺ influx), the vesicles transport to the end of the axon, fuse with the membrane and release noradrenaline into the synaptic cleft. This released noradrenaline activates the various adrenoceptors in the presynaptic (α₂-AR and β₂-AR) and postsynaptic (α₁-AR, β₁-AR and β₂-AR) membranes causing the appropriate post-synaptic reactions. Such reactions include protein kinase activation, protein phosphorylation or metabolism by catechol-O-methyl transferase (COMT).

Adrenaline is one of the major hormones of the sympathetic nervous system. It is classified as a hormone in comparison to noradrenaline, which is a neurotransmitter, because it is primarily produced in the adrenal glands and functions peripherally. When produced in the synaptic vesicles, adrenaline is transported back into the chromaffin granules for storage. Phenylethanolamine N-methyltransferase (PNMT) is the enzyme used by the body to catalyse the biosynthesis of adrenaline from noradrenaline. It is found primarily in the Figure 1.4 Diagram of a noradrenergic axonal terminal showing the release and reuptake of noradrenaline (NA).
adrenal medullary cells but also in neurons within the CNS that use adrenaline. Adrenaline is best known for the evolutionary survival instinct, the “fight-or-flight” response which is initiated in the brain when the eyes or ears register a threat. The sympathetic nervous system is then activated by the hypothalamus, resulting in a cascading effect through the body and finally the release of adrenaline into the bloodstream from the adrenal glands.\textsuperscript{12}

There are several possible fates of catecholamines in the CNS after they have been released back into the synapse from the postsynaptic cleft. The first possible fate is the catecholamine reuptake into the presynaptic neuron by means of the noradrenaline transporter and once in the neuron cytoplasm they can be stored in the vesicles again, where they are transported by the VMAT2.

The second is the metabolism of the neurotransmitters carried out by the monoamine oxidase (MAO) and COMT enzymes (Fig. 1.5). MAO is a flavin-containing enzyme located on the outer membrane of the mitochondria.\textsuperscript{5} It functions to oxidatively deaminate catecholamines to their corresponding aldehydes and removing neurotransmitters such as noradrenaline and serotonin from the brain.\textsuperscript{5} MAO exists as an A- and B-subtype, where the A-subtype preferentially metabolised noradrenaline and serotonin and the B-subtype metabolises tyramine and benzylamine. COMT functions similarly to MAO by producing the carboxylic acid through methylation and oxidation of the catecholamine, respectively.\textsuperscript{5} It is a ubiquitous enzyme observed in microorganisms, plants and animals.\textsuperscript{13} COMT exists as soluble (S-COMT) isoforms, which predominantly exists in the peripheral tissues, and membrane-bound (MB-COMT) isoforms, which is predominantly expressed in the mammalian CNS.\textsuperscript{14} The final product of both MOA and COMT metabolism of catecholamines, is a carboxylic acid which can be excreted in urine.

\begin{center}
\includegraphics[width=\textwidth]{figure1.5.png}
\end{center}

\textbf{Figure 1.5} (a) MAO metabolism and (b) COMT metabolism of noradrenaline

The third possible fate of noradrenaline, when released from the synapse, is to interact with the different post-synaptic and pre-synaptic noradrenergic receptors to transmit the corresponding signal.
1.3 Adrenergic Receptors

In general, neurotransmitters’ receptors can be divided into two main categories; ionotropic receptors for direct effect and metabotropic receptors for indirect effect.

Ionotrophic receptors allow the movement of charged atoms across the cell membrane when the membrane’s charge fluctuates. These receptors are ion channel receptors that contain a binding site for the neurotransmitter and a pore for the ions to travel through.³

The metabotropic receptors, such as the adrenergic receptors, consist of a single protein that spans the cell membrane with the neurotransmitter binding site.³ These receptors are all associated to a guanyl-nucleotide-binding protein (G-protein) hence being called G-protein coupled receptors (GPCRs). The comparative slowness of metabotropic receptor action reflects the fact that the signal is transmitted by multiple proteins sequentially to produce the final physiological response.¹

1.3.1 G-Proteins and G-Protein Coupled Receptors

GPCRs have a high pharmacological importance as 30% of all commercial drugs act by binding to these receptors.¹⁵ The receptor is embedded within the cell membrane in such a way that the peptide chain in the alpha-helix secondary structure winds back and forth seven times connected by loops. Thus, GPCRs are described as seven transmembrane (7-TM) domains, where each of these 7-TM helical sections are hydrophobic and are numbered I – VII starting from the N-terminal (Fig. 1.6).¹⁶,¹⁷ Additionally, the loops that connect the TM domains are labelled according to whether they are outside or inside the cell; thus, extracellular loops are called exo(1-3) and the intracellular loops are labelled endo(1-3).

![Figure 1.6 General structure of GPCRs including the 7-TM numbering I-VII, starting from the N-terminal. The extracellular loops are labelled exo(1-3) and the intracellular loops labelled endo(1-3)](16,17)
In general, the binding site for the neurotransmitter tends to be on the extracellular side of the receptor whereas the G-protein binding site is on the intracellular side and involves the C-terminal chain and part of the variable intracellular loop (labelled endo3 in Figure 1.6).15 The binding site for the neurotransmitters can vary for each type of GPCR. For example, the aminergic binding site associated with small molecule ligand binding tends to be deep within a binding pocket between the TM helices but the binding site for the larger peptide ligands would be closer to the surface as they require far more space and cannot enter deeply into the 7TM bundle.18

As mentioned, G-proteins are membrane-bound proteins constructed of three subunits (α, β, γ). The α-subunit has a binding pocket that binds to the guanyl-nucleotides and guanosine diphosphate (GDP) when in its resting state.15

The general activation of the GPCR and their signal transduction are as follows (Fig. 1.7):19

i) The neurotransmitter, or agonist, binds to the receptor. The induced fit causes the receptor to change shape revealing the binding site for the G-protein on the inner surface.

ii) The trimeric G-protein, containing GDP, binds to the receptor triggering a further shift in the proteins structure.

iii) This causes an exchange of GDP for guanosine triphosphate (GTP) at the α-subunit.

iv) The final conformational change of the G-protein weakens the links between the subunits and releasing an α-monomer containing GTP and a βγ-dimer.

![Figure 1.7 Visual representation of the signal transduction of a GPCR](image.png)
There are three main subtypes of the G-protein; G\textsubscript{i}/G\textsubscript{o}, G\textsubscript{s}, G\textsubscript{q}/G\textsubscript{11} with four possible \(\alpha\)-subunits. The \(\alpha\textsubscript{i}\)-subunit stimulates adenylate cyclase (AC) whereas the \(\alpha\textsubscript{o}\)-subunit inhibits AC and activates potassium (K\textsuperscript{+}) channels. The \(\alpha\textsubscript{o}\)-subunit activates the receptors that inhibit neural Ca\textsuperscript{2+} channels and the \(\alpha\textsubscript{q}\)-subunit activates phospholipase C (PLC).

The many different receptor-protein variations can lead to different biological responses. For example, adrenaline acts on the G\textsubscript{i/o} linked \(\alpha\textsubscript{2}\)-adrenoceptor (\(\alpha\textsubscript{2}\)-AR) leading to a contraction of smooth muscle.

### 1.3.2 \(\alpha\textsubscript{2}\)-Adrenoceptors

Adrenergic receptors, also known as adrenoceptors (AR), are described as GPCR 7-TM proteins, existing as \(\alpha\)- and \(\beta\)-subtypes. They function as biological targets for the endogenous catecholamine agonist, adrenaline and noradrenaline, resulting in a variety of controlled biological responses.

In 1905, the existence of such “receptive substances” that bind drugs or transmitters onto the cells initiating a chemical response was first published in the *Journal of Physiology* by John Newport Langley.\(^{20}\) Soon after, Paul Ehrlich proposed that these receptors were selective leading to the inspiration for his famous *side-chain theory*.\(^{21}\) It was John Jacob Abel, in 1897, who successfully isolated adrenaline, leading to W. B. Cannon’s *fight or flight theory* in the early 1900’s.\(^{22}\) Here he identified two chemical transmitters, sympathin E (which was excitatory in function) and sympathin I (which was inhibitory). However, it was not until 1948 that Raymond Ahlquist identified two distinct *adrenotropic* receptors (\(\alpha\) and \(\beta\)), now known as adrenergic receptors, describing the actions of adrenaline and the idea was established that a single sympathetic mediator produced excitatory and inhibitory responses in each receptor.\(^{23}\)

The \(\beta\)-ARs have now been categorised into three subtypes (\(\beta\textsubscript{1}\)-, \(\beta\textsubscript{2}\)- and \(\beta\textsubscript{3}\)-AR). With the \(\beta\textsubscript{2}\)-AR being one of the most extensively studied ARs, it was the first to have its X-ray crystal structure solved aiding with further research of the various subtypes of \(\alpha\)- and \(\beta\)-ARs such as by means of computational studies.\(^{23,24}\) However, the \(\beta\textsubscript{2}\)-AR shares only 50% homology with the \(\alpha\textsubscript{2}\)-AR. Thus, the more recent advancements in 2019 made by Lu Qu et al.\(^{25}\) and Chen et al.\(^{26}\) via the resolution of the crystal structure of the \(\alpha\textsubscript{2A}\)-AR and \(\alpha\textsubscript{2C}\)-AR subtype proved auspicious for this area of research.

The \(\beta\)-ARs are central to the overall regulation of cardiac function with \(\beta\)-AR stimulation being a primary control point for modulation of heart rate and myocardial contractility.\(^{23}\) However, the \(\beta\textsubscript{3}\)-AR subtype is unique as it is primarily associated with metabolic regulations. In a healthy heart, the \(\beta\)-AR downregulation appears to be specific to the \(\beta\textsubscript{1}\)-
subtype, the extent of which correlates with the severity of the heart condition. Furthermore, a correlation between aging and lower levels of the β1-AR subtype has been observed.23

In 1974, it was proposed that the α-AR should be subdivided into different subtypes: the α1-ARs, which tend to be postsynaptic receptors found on vascular smooth muscle; and α2-ARs which are predominantly associated with the presynaptic receptors and widely distributed across the CNS.27 Each of these receptors further differentiated in 3 subtypes; α1A, α1B, α1D, and α2A, α2B, α2C. The α1C-AR was removed as it demonstrated gross similarities to the α1A and thus, was recategorized as the α1A or α1A/C. The α1-AR couples with the Gq/11 proteins but the information on the exact role of each subtype is mostly limited to the receptors expressed in the vascular smooth muscles.28

The α2-AR is linked to the Gi-protein which αi-subunit inhibits the activation of adenylate cyclase; thus, preventing the formation of cAMP (which is required to open the ion channels through which Ca2+ can enter the neuron). As aforementioned, the influx of Ca2+ activates calmodulin inducing the exocytosis of the noradrenaline-containing synaptic vesicles thus releasing noradrenaline into the synaptic cleft.3 Therefore, the prevention of cAMP formation results in the activation of a negative feedback stopping the release of noradrenaline from the presynaptic neuron. For this reason, the overexpression of the α2-ARs and the selective increase in the high affinity conformation of the α2-ARs in the human brain has been linked as a causative factor of depression and other neurological conditions.29

To date, the individual pharmacological roles of these α2A-, α2B-, α2C-subtypes is still unknown due to the lack of highly subtype-selective ligands.29 As aforementioned, until December 2019, only the β-adrenergic receptor existed in a crystal structure, thus the development of subtype-selective compounds has proven challenging. With the localisation of the α2-AR being on the presynaptic neuron, it is the distribution of each of these subtypes within the body that has been used to further distinguish between them. The α2A-subtype is predominantly distributed across the locus coeruleus, but was also found in the brain stem, cerebral cortex, septum, hypothalamus, hippocampus and amygdala.30 The α2B-subtype has been identified solely in the thalamus as it is mostly localised in the smooth muscle, whereas the α2C-subtype, similar to the α2A, was distributed predominantly across the basal ganglia, olfactory tubercle, hippocampus, and cerebral cortex.30 This distribution profile indicates that when carrying out the pharmacological studies related to the treatment of brain disorders such as major depressive disorder and schizophrenia, the main focus should be on the α2A- and the α2C-subtypes.
1.4 Pharmacological Evaluation of Ligands of $\alpha_2$-AR

In vitro pharmacological studies are a vitally important phase of the drug discovery process and are used to experimentally assess the **affinity** and **activity** of a ligand to the $\alpha_2$-ARs.

The **affinity** of a drug for a receptor is a measure of how strongly that drug binds to the receptor. In the Rozas group, the compound’s binding affinity for the receptor ($K_i$) is measured using a radioligand competition binding assay in the human brain PFC tissue in collaboration with the group of Prof. Callado (Department of Pharmacology, University of the Basque Country UPV/EHU, Centro de Investigacion Biomedica en Red de Salud, Mental, CIBERSAM, Spain). This is done using the standard $\alpha_2$-AR radioligand, $[^3H]RX821002$, of known affinity and testing it against varying concentrations of the sample compound. The displacement of the standard ligand can be measured using a scintillation counter after the incubation period.

The ligand activity describes whether the compound is either an agonist or an antagonist. An agonist is a ligand that binds to a receptor and produces a physiological response. They exist as a full agonist (which reaches the maximal response capability of the system), partial agonist (does not reach the maximal response capability of the system and may act as an antagonist when competing for a receptor in the presence of a full agonist) or an inverse agonist. Some receptor systems display constitutive activity, thus are active in the absence of agonist. An inverse agonist would inhibit this constitutive activity and, as such, is said to display negative efficacy (Fig. 1.8). An antagonist stops/block the effects of an agonist and can be competitive or non-competitive. The competitive antagonists compete against the agonists for the receptor binding sites and their binding is mutually exclusive; however, the non-competitive antagonist can prevent the action of an agonist without influencing its binding.

![Figure 1.8 Dose-response curve illustrating the characteristics on an agonist, antagonist and an inverse agonist](image)

*Figure 1.8 Dose-response curve illustrating the characteristics on an agonist, antagonist and an inverse agonist.*

11
The compound’s activity as either an agonist or an antagonist in the particular case of the α₂-AR ligands can be determined using a functional binding assay, also referred to as a GTP exchange assay. Direct evaluation of the degree of G-protein activation upon ligand binding can be made by determining guanine nucleotide exchange using radiolabelled analogue of (GTP-γ-[³⁵S]) to observe agonist, antagonist or inverse agonist activity. Here, the phosphodiester bond that links the γ-phosphate to the rest of the nucleotide cannot be hydrolysed to reform GDP, and hence prevents the GTP binding protein from being inactivated, allowing for facile scintillation counting of the radiolabelled analogue. In the case of Callado’s group these assays are performed in human PFC tissue.

1.5 Previous Work within the Rozas Group

The Rozas group have been synthesising ligands to target the α₂-AR for over 20 years. These ligands contain the common feature of an aryl guanidinium or 2-aminoimidazolium with varying functionalisation of electron withdrawing or electron donating groups on the aromatic ring. Initially, the work focused on bis-2-aminoimidazolium and bis-guanidinium di-aryl derivatives and these compounds resulted in the discovery of lead compound 1 which shares the same di-aryl core as the previously existing anti-depressants, mirtazapine and mianserin (Figure 1.9). Through pharmacological studies carried out by the Callado group this compound was determined to be a poor α₁-AR antagonist but a potent α₂-AR agonist.

Based on these positive results, several mono- or bis-cationic molecules were synthesised in their hydrochloride salt form for ease of the pharmacological studies. Two main families of compounds were designed; Family A which contained the diaryl bis-cationic structure and Family B consisted of mono-cationic systems with mono-aromatic cores.

Figure 1.9 Structure, binding affinity and functional activity of Mianserin⁶⁶, Mirtazapine⁶⁶ and Lead Compound 1 containing the diaryl moiety
Research in Family A resulted in no increase in binding affinity of compound 1, however, compound 2 (Figure 1.10) was identified as the first di-aromatic molecule of the broad spectrum of molecules that resulted in antagonist activity in human PFC in vitro experiments as well as in vivo experiments in rat by micro-dialysis experiments.\(^{36}\)

![Family A](image1.png)  ![Family B](image2.png)

**Figure 1.10** General structures for Family A (diaryl bis-cationic) and Family B (mono-cationic with mono-aromatic core)

Family B research resulted in the preparation and identification of several antagonists with phenyl, pyridyl or thiophenyl cores (Figure 1.11).\(^{37}\)

![Compound 2](image3.png)

**Figure 1.11** Compound 2, the first twin molecule developed within the Rozas group acting as an antagonist

Through computational chemistry, a 3D pharmacophore was developed within the group, incorporating a wide range of antagonist ligands of the α₂-AR and used to design the next generation of compounds. This study demonstrated the importance of an R²-substitution at the cationic moiety in the form of N,N'-disubstituted arylguanidines and 4-substituted-2-
arylimino imidazolines. Thus, a new Family B’ focused on the hit-to-lead optimization of the previously synthesised compounds. This was done by monitoring the effects of varying the cationic moiety from a 2-aminoimidazoline to a mono- (8) or di-substituted (9) guanidinium moiety through which the pharmacological studies demonstrated α2-AR effects. Similarly, the effect of a biosteric change of the aryl core structure (10 and 11) was studied through functional assays and microdialysis (Figure 1.12). Thus, introduction of the 2-aminoimidazoline group in the 2-position of the pyridine ring (10) resulted in a dramatic drop in binding affinity and accordingly did not advance to the functional assay analysis. On the contrary, when the imidazoline substituent was introduced in the position 3 of the pyridine ring while keeping both substituents para to each other (11), an increment of binding affinity was observed.

![Chemical Structures](image)

**Figure 1.13** Hit-to-lead optimization through cationic moiety modification and biosteric changes to the aryl core structure of Family B’

Furthermore, they observed dramatic changes in ligand α2-AR activity caused by the minimal differences in structural isomers (see an example in Figure 1.13); thus, the minute change of the N-ethyl functional group to an N-dimethyl group resulted in a change from agonist to antagonist activity and a more enhanced binding affinity.39,40

![Chemical Structures](image)

**Figure 1.14** Structural isomers demonstrating the change in ligand activity based on minute changes to the ligand structure, a phenomenon commonly encountered by medicinal chemists
As previously mentioned, Family A consisted of the diaryl bis-cationic systems and the best example of this family is the lead compound 1, which is an agonist with a high Ki value (1.585 nM). Thus, following the proposed pharmacophore that involves introducing a second substituent in the guanidinium moiety, a new family A' was developed. Functionalisation assays and microdialysis studies drove to the identification of compound 16 as the antagonist with the most promising binding affinity to date.\(^{39}\)

![Molecular structures](image)

**Figure 1.15** Family A’ demonstrating the high affinity of di-aryl bis-cationic core structure. The functional activity for compound 17 was not determined (n.d.) because the binding affinity was too weak.
2. Objectives

The Rozas group have employed ligand-based drug design to synthesise ligands targeting the α₂-AR for over 20 years. The α₂-AR agonists and antagonists have been proven to be extremely promising in the pharmaceutical industry due to their cascading effect on noradrenergic neurotransmitters. The α₂-AR agonists have found use as anaesthetics (e.g. clonidine), treatments of attention-deficit/hyperactivity disorder (ADHD) and anti-hypertensive agents. Depression is one of the leading causes of illness worldwide and has been closely linked to low concentrations of monoaminergic neurotransmitters in the brain such as noradrenaline. Activation of presynaptic α₂-AR by the endogenous noradrenaline results in a decrease in the release of monoaminergic neurotransmitters. Therefore, the administration of α₂-AR antagonists leads to increased concentrations of brain monoamines and constitutes a viable strategy for the treatment for depression. As a result, the synthesis of an α₂-AR antagonists to block this overexpression of the receptor allowing for the gradual increase of noradrenaline in the synapse is very valuable.

The ligands synthesised within the Rozas group have contained a common feature of guanidinium or 2-aminoimidazolium which are attached to a mono- or di-aryl core structure with varying functionalization of electron withdrawing or electron donating groups. It is regularly observed within the development of GPCR-targeting ligands that a drastic change in functional activity (converting agonists to antagonists, or vice versa) stems from really minute changes to the ligands structure. Therefore, the main aim of this research was to investigate the effects on the α₂-AR binding affinity and functional activity when releasing the rigid 2-aminoimidazolium moiety of the lead compound (1) into N-alkyl substituted guanidinium groups, thus affecting steric and lipophilic properties (Figure 2.1). This aim will be achieved via the following objectives: synthesis, computational studies and pharmacological evaluation.

2.1 Synthetic Chemistry

As aforementioned the proposed compounds were chosen based on the current lead compound 1, using a ligand-based drug design strategy. Compound 1 is a diaryl symmetric bis-2-aminoimidazoline with a Ki of 1.585 nM, the highest binding affinity within the Rozas group to date, and it shows agonist functional activity (see Figure 2.2). The imidazoline moiety is a rigid cyclic structure which causes some steric clash when docked into the binding site of the α₂-AR. Through the course of this research, the effects of releasing this cyclic moiety into mono- and di-substituted guanidines probing the binding site of models of the α₂-ARs will be investigated (Figure 2.1).
The use of conveniently functionalized thioureas throughout the synthesis originally outlined by the Rozas group will be utilised to synthesise the symmetric bis-guanidine unsubstituted derivatives (R1 and R2 = H), mono-substituted and di-substituted derivatives (where, R1 and R2 = methyl or ethyl substituents).

Therefore, the following compounds have been proposed based on lead compound 1 (Figure 2.2).

![Schematic representation of the opening of the imidazoline moiety at location 1 and 2 forming A1 (N,N'-dimethyl-guanidine) and A2 (N-ethyl-guanidine), respectively](image)

**Figure 2.1** Schematic representation of the opening of the imidazoline moiety at location 1 and 2 forming A1 (N,N'-dimethyl-guanidine) and A2 (N-ethyl-guanidine), respectively

**Figure 2.2** The rationale behind the proposed compounds to be prepared and tested against the α2A-AR in vitro. Where, R1 – R4 = H / Me / Et

### 2.2 Computational Studies

The proposed compounds were theoretically studied to gain an understanding of their conformational and electronic features, and to predict how they would behave in the pharmacological tests with α2-AR. The introduction of this computational analysis of the desired compounds saves both time and money throughout the drug discovery process and has become known as computer-aided drug discovery.

Freeware tools such as Marvin (ChemAxon) and SwissADME were used to calculate the theoretical values of different physicochemical parameters of these compounds that can have an effect on their drug likeliness. Thus, parameters such as log-P, pK_a(H), aqueous solubility or hydrogen bonding descriptors (HB donors and HB acceptors), among others, were calculated.
Moreover, considering that the crystal structures of α₂A-AR and α₂C-AR were recently reported,[25,26] molecular docking was used to investigate the possible interactions between the proposed compounds and the α₂-AR binding sites. The molecules were prepared using the Maestro software and docked using Glide. The molecules were docked into three different α₂-AR models: α₂A-AR-MO (a homology model developed by Prof. Mireia Olivella from the Universitat de Vic in Spain, before any α₂-AR crystal structures were published), α₂A-AR-Y (crystal structure of the α₂A-AR in complex with a partial agonist), α₂A-AR-X (crystal structures of the α₂A-AR in complex with an antagonist). The chosen orientations of the docked compounds were based on the ionic interaction with the aspartate residue D113 as this has been reported to be a critical interaction with the α₂-AR binding sites.

2.3 Pharmacological Studies

In collaboration with Prof. Callado at the Department of Pharmacology at the School of Medicine in the University of Basque County UPV/EHU (Spain), in vitro pharmacological studies will be carried out of the successfully synthesised compounds to determine their affinity for the α₂-AR (Kᵢ values) and their functional activity on the receptor (agonist or antagonist) in human brain prefrontal cortex (PFC) tissue. These in vitro studies can only be carried out if the compounds synthesised are above 95% pure, thus a HPLC analysis will be carried out before sending the compounds to Prof. Callado.
3 Results and Discussion

3.1 Physicochemical properties

The comprehensive characterisation of physicochemical properties is a critical step in the development of drugs and the theoretical evaluation of these properties and their impact on absorption, distribution, metabolism and excretion (ADME) steps is a suitable approach to assess the drug likeliness of compounds before preparing them.

Physicochemical properties can be calculated to eliminate compounds that are likely to exhibit particular physical or toxicological hazards. These theoretical parameters, which are highly accessible and relatively simple, can allow for a thorough investigation of properties that can lead to a decrease in the failure rate of a compound in the drug discovery process. The pioneering research of using physicochemical properties to determine the high probability of a drug being orally available, is attributed to Christopher A. Lipinski and his development of the “Rule of five (Ro5)” general guidelines for oral drug-likeability. Further than those parameters used in the Ro5, there are different fundamental physicochemical properties that can be chosen based on the purpose and fate of the drug. The most common properties include logP, pKa(H), aqueous solubility, Polar Surface Area (PSA), number of rotatable bonds and hydrogen bonding descriptors (HB donors and HB acceptors).

In the present research, a number of physicochemical properties have been calculated for a series of derivatives of the lead compound 1 (Tables 3.1, 3.2, 3.3). These derivatives include previously synthesised compounds within the Rozas group (1, 15 – 18), newly synthesised compounds (19 – 22, 25, 28), and those for theoretically considered for future synthesis and evaluation (23, 24, 26, 27, 29 – 32). This was done using freeware tools such as SwissADME and ChemAxon’s Marvin, which rely on both physics-based methods and statistical empirical models such as quantitative structure-activity relationship (QSAR) analysis.

As aforementioned, the Lipinski Ro5 is a general guideline for the oral drug-likeness of a desired compound. These rules include: the molecular weight of the compound can be no more than 500 Da, no more than 5 hydrogen bond donors and no more than 10 hydrogen bond acceptors must be present in the molecule and the logP must be less than 5. Further expansions on this Ro5 include the Veber rule which states that the topological PSA must be below 140 Å² and the number of rotatable bonds must be less than or equal to 10. Each of these parameters will be discussed individually in this section; however, the explanation of the theory behind the computational methods used in the determination of the relevant parameters is beyond the scope of this study and thus will not be explained in detail, rather the appropriate reference will be provided.
Table 3.1 Calculated physicochemical parameters for all target compounds and mirtazapine (M) calculated using SwissADME

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<th>Code</th>
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<th>#HBD</th>
<th>Consensus LogP</th>
<th>#Rotatable bonds</th>
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</table>
3.1.1 HBD, HBA and Rotational Bonds

Each compound described in Table 3.1 demonstrated a molecular weight of less than 500 Da (calculated using OpenBabel®, version 2.3.0)\(^4\) which is the first of Lipinski’s rules that were obeyed. Additionally, the number of HBD and HBA for all compounds studied is also within the limits established in the Ro5 and the number of rotatable bonds is only larger than 10 for compounds 17, 20 and 32. To better visualised the fulfilment of the different drug-likeness rules, the hydrogen bond descriptors (HBA – all N and O atoms, HBD – all N-H and O-H groups), molecular weight and number of rotational bonds are displayed in Figure 3.1. From this graph is evident that most of the compounds studied have a good profile in terms of drug-like properties.

*Compounds previously synthesised within the Rozas Group*
3.1.2 Lipophilicity (LogP)

Lipophilicity is the affinity of a drug for a lipid medium and can be measured as the partition coefficient (P) that estimates the distribution of a drug between n-octanol (the organic phase) and an aqueous phase. This parameter is usually expressed as the LogP and SwissADME applied five different methods of calculating LogP:\(^{46}\)

1. XLOGP3, an atomistic method including corrective factors and knowledge-based library
2. WLOGP, SwissADME’s own implementation of a purely atomistic method based on the fragmental system of Wildman and Crippen
3. MLOGP, an archetype of topological method relying on a linear relationship with 13 molecular descriptors.\(^{49,50}\)
4. SILICOS-IT, a hybrid method relying on 27 fragments and 7 topological descriptors
5. iLOGP, SwissADME’s in-house physics-based method relying on free energies of solvation in n-octanol and water calculated by the Generalized-Born and solvent accessible surface area (GB/SA) model.

As seen in Table 3.1, the consensus LogP has been used for comparison of the compounds studied as it is representative of the mean LogP value of all five calculated results obtained by SwissADME. In accordance with the Ro5, each compound listed demonstrated a LogP value less than 5, thus indicating the possibility of the drug becoming a successful orally administered drug. Compounds 18, 15 and 23 had the lowest Log P values of 1.39, 1.67 and 1.68, respectively and compounds 20, 16 and 32 had the highest log P values of 4.00, 3.55 and 3.36, respectively. The addition of alkyl functional groups on the guanidinium derivatives increases the lipophilicity of the compound as seen by compound 18 (bis-unsubstituted guanidinium derivative, 1.39), compound 19 (bis-dimethyl guanidinium, 2.71) and compound 20 (bis-diethyl guanidinium, 4.00). Figure 3.2 displays the SwissADME consensus LogP values for all synthesised compounds and the commercially available mirtazapine (M). Due to the binding sites of receptors being hydrophobic in nature, the compounds typically succumb to the hydrophobic effect,\(^{51}\) i.e. when the hydrophobic molecules prefer to minimise the amount of exposure to the surface area of the surrounding water molecules and thus self-orientate to adopt the appropriate conformation within a hydrophobic environment.
3.1.3 PAINS and Molar Refractivity

In Table 3.2 other parameters of interest for the druggability of the compounds studied are gathered. For example, the number of pan-assay interference compounds (PAINS) is shown. PAINS are chemical compounds functionalities that are often associated to compounds that give false positive results in high-throughput screens. Figure 3.3 displays sample structures obtained from Capuzzi et al. containing multiple high PAINS alerting functional groups. None of the compounds studied contains any of these PAINS.

Another interesting parameter related to drug likeliness is the molar refractivity (MR) which relates the molecular weight, refraction index and density of a compound; the molar refractivity represents not only the real volume of the molecule, but also the dispersive forces that act in the drug-receptor interaction. The optimum MR values to increase the

![Consensus LogP](image)

**Figure 3.2** Comparison of all the SwissADME calculated LogP for all the synthesised compounds and mirtazapine (M)

**Figure 3.3** Sample compounds with multiple PAINS alerts taken from Capuzzi et al. Another interesting parameter related to drug likeliness is the molar refractivity (MR) which relates the molecular weight, refraction index and density of a compound; the molar refractivity represents not only the real volume of the molecule, but also the dispersive forces that act in the drug-receptor interaction. The optimum MR values to increase the
drug-likeness of the compound lies between 40-130. As seen from Table 3.2, each of the synthesised compound’s calculated MR values lie within the desired criteria.

Table 3.2. Calculated physicochemical parameters for all target compounds and mirtazapine (M) calculated using SwissADME (cont.)

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<th>CYP2C19 inh</th>
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</table>

3.1.4 Fraction Csp$^3$ and the Cytochromes P450 superfamily

The level of saturation could also have an impact in druggability; thus, the optimal ratio of sp$^3$ hybridized carbons over the total carbon count of the molecule (Fraction Csp$^3$) should be at least 0.25. As seen from the calculated values displayed in Table 3.2, compounds 18, 15, 23, 24, 25, 28 and 30 fall short of the recommended criteria outlined by SwissADME.
A different aspect on the drug-likeness of a potential drug relays in their metabolic stability. The cytochromes P450 superfamily is responsible to the metabolism of the drug compounds and their elimination from the body. Inhibition of these isoenzymes is certainly one major cause of pharmacokinetics-related drug-drug interactions leading to toxic or other unwanted adverse effects due to the lower clearance and accumulation of the drug or its metabolites.\textsuperscript{46,55} Thus, ideally, potential drugs should not be able to inhibit any CYP-type enzyme belonging to the cytochrome P450 superfamily. SwissADME gives an estimate of the potential of inhibition for a series of CYP enzymes and in most of the compounds studied the results show that they will not be able to inhibit these enzymes. Exceptions are compounds 1, 15, 17, 16, 20, 27, 29 and 32 which inhibit one or two of these enzymes.

### 3.1.5 PSA, BBB permeability and HIA

As mentioned before the Polar Surface Area (PSA) is a very useful parameter to assess drug-likeness of potential drugs since it is related to the ability of compounds to establish HBs which are one of the most usual interactions between drug and target. This parameter is calculated using the fragmental technique known as the topological polar surface area (TPSA),\textsuperscript{46,56} primarily considering oxygen and nitrogen as polar atoms and their corresponding hydrogens. TPSA provides results which are practically identical with the 3D PSA (the correlation coefficient between 3D PSA and fragment-based TPSA for 34 810 molecules from the World Drug Index is 0.99).\textsuperscript{56} It is a simple measure of the hydrogen-bonding capacity of a molecule through the sum of the fractional contributions to the surface area of all nitrogen and oxygen atoms.\textsuperscript{57} Molecules with a PSA less than 140 Å\textsuperscript{2} tend to have a good permeating ability through cell membranes.\textsuperscript{58} According to the results obtained from the SwissADME calculations all the compounds studied are within this limit.

However, for the particular case of molecules to penetrate the blood brain barrier (BBB), a PSA less than 90 Å\textsuperscript{2} is needed, preferentially between 60-70 Å\textsuperscript{2}.\textsuperscript{58} As seen in Table 3.3, mirtazapine has an extremely low TPSA (19.37 Å\textsuperscript{2}) which aids in the crossing of the drug through the cellular membrane of the glial cells in the BBB. Compounds 19, 20 and 27 demonstrated low enough TPSA values (72.84 Å\textsuperscript{2} each) to be considered probable candidates for crossing the BBB and target the receptors in the CNS. However, through previous experimental research within the Rozas group it is known that the related bis-guanidines or bis-2-aminoimidazolines can reach the brain.\textsuperscript{33}

SwissADME also evaluates the ability of compounds to undergo GI absorption by estimating their potential for human intestinal absorption (HIA). Calculated HIA probability for all compounds studied is shown in Table 3.3 indicating that all compounds have a high probability to be absorbed in the intestine.
Table 3.3. Calculated physicochemical parameters for all target compounds and mirtazapine (M) calculated using SwissADME and (*) Marvin. [S= soluble, MS= moderately soluble]

<table>
<thead>
<tr>
<th>Code</th>
<th>TPSA (Å²)</th>
<th>HIA</th>
<th>BBB perm.</th>
<th>Pgp substr.</th>
<th>pKah*</th>
<th>Ali Solubility Class**</th>
<th>#Heavy atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>19.37</td>
<td>High</td>
<td>✓</td>
<td>X</td>
<td>5.36/6.67</td>
<td>S</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>72.84</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>8.36/7.76</td>
<td>S</td>
<td>25</td>
</tr>
<tr>
<td>15</td>
<td>98.32</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>8.00/8.97</td>
<td>S</td>
<td>23</td>
</tr>
<tr>
<td>16</td>
<td>84.33</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>8.03/9.24</td>
<td>MS</td>
<td>26</td>
</tr>
<tr>
<td>17</td>
<td>95.82</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>9.52/8.91</td>
<td>MS</td>
<td>27</td>
</tr>
<tr>
<td>18</td>
<td>123.80</td>
<td>High</td>
<td>X</td>
<td>X</td>
<td>9.21/8.61</td>
<td>S</td>
<td>21</td>
</tr>
<tr>
<td>19</td>
<td>72.84</td>
<td>High</td>
<td>✓</td>
<td>✓</td>
<td>9.17/8.57</td>
<td>S</td>
<td>25</td>
</tr>
<tr>
<td>20</td>
<td>72.84</td>
<td>High</td>
<td>✓</td>
<td>✓</td>
<td>9.24/8.64</td>
<td>MS</td>
<td>29</td>
</tr>
<tr>
<td>21</td>
<td>95.82</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>9.42/8.82</td>
<td>S</td>
<td>23</td>
</tr>
<tr>
<td>22</td>
<td>95.82</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>9.46/8.85</td>
<td>MS</td>
<td>25</td>
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<tr>
<td>23</td>
<td>109.81</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>8.70/9.33</td>
<td>S</td>
<td>22</td>
</tr>
<tr>
<td>24</td>
<td>109.81</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>8.71/9.35</td>
<td>S</td>
<td>23</td>
</tr>
<tr>
<td>25</td>
<td>98.32</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>9.19/8.59</td>
<td>S</td>
<td>23</td>
</tr>
<tr>
<td>26</td>
<td>98.32</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>8.62/9.23</td>
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<td>25</td>
</tr>
<tr>
<td>27</td>
<td>72.84</td>
<td>High</td>
<td>✓</td>
<td>✓</td>
<td>9.21/8.60</td>
<td>MS</td>
<td>27</td>
</tr>
<tr>
<td>28</td>
<td>84.33</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>9.32/8.68</td>
<td>S</td>
<td>24</td>
</tr>
<tr>
<td>29</td>
<td>84.33</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>9.34/8.72</td>
<td>MS</td>
<td>26</td>
</tr>
<tr>
<td>30</td>
<td>95.82</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>8.84/9.44</td>
<td>MS</td>
<td>24</td>
</tr>
<tr>
<td>31</td>
<td>84.33</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>9.34/8.69</td>
<td>MS</td>
<td>25</td>
</tr>
<tr>
<td>32</td>
<td>84.33</td>
<td>High</td>
<td>X</td>
<td>✓</td>
<td>9.36/8.73</td>
<td>MS</td>
<td>27</td>
</tr>
</tbody>
</table>

**[S = soluble, MS = moderately soluble]

3.1.6 BOILED-Egg plot and the permeability glycoprotein (Pgp)

The BOILED-Egg plot (Figure 3.4) presents a correlation between calculated WLogP and calculated TPSA and is an intuitive simultaneous prediction of two key ADME parameters, brain access as BBB permeates or as passive gastrointestinal (GI) absorption. The compounds that are positioned within the white area are likely to undergo GI absorption and
those positioned within the yellow area are likely to be brain permeant. Both compartments are not mutually exclusive, and the outside grey region stands for molecules with properties implying predicted low absorption and limited brain penetration. In addition, SwissADME enables the estimation for a chemical to be a substrate of the permeability glycoprotein (Pgp), which is the most important ATP-binding cassette transporter responsible for an active efflux through biological membranes, e.g. from the GI wall to the lumen or exiting the brain (Table 3.3). ATP-binding cassette (ABC) transporters are a superfamily of membrane proteins that convert the energy gained from ATP hydrolysis into trans-bilayer movement of substrates either into the cytoplasm (import) or out of the cytoplasm (export) and are expressed ubiquitously in all kingdoms of life. Pgp was the first of the ABC transporter family to be discovered in the 1970s as a prototypic transporter involved in multidrug resistance (MDR) of cancer cells. Pgp remains a crucial factor in the drug development process as the expression of this efflux transporter in the GI tract and at the BBB limits oral absorption and CNS entry of many drugs. The graphical classification model provides also a visual representation of whether the compounds are a substrate of these Pgp (Pgp+, blue or Pgp-, red). According to the SwissADME calculations, all of the submitted compounds except compound 1 would efflux back into the bloodstream from the brain by the Pgp. However, from previous research carried out in the Rozas group, it is known that similar bis-guanidine and bis-2-aminoimidazoline derivatives can reach the site of action within the brain. This could be due to the protonation of both guanidinium moieties within the blood (pH 7.4) forming a di-cationic species rather than the neutral form required by the SwissADME programme for the calculations. Another causative factor relating to the transportation of the guanidine-containing derivatives through the BBB could be due to the presence of a carrier transporter as proposed in a webinar given in April 2020 by Douglas Kell. Kell et al., hypothesised that the typical passive diffusion of the lipid bilayer is, in fact, a myth and that the “uptake is mainly determined by biology, not physical chemistry”.

Figure 3.4 BOILED-Egg plot representing the correlation between WLogP and TPSA. The compounds in the white section are HIA active and those in the yellow area are passive BBB permeates. However, this plot also gives an indication as to whether the compounds are PGP+ (blue) or PGP- (red)
3.1.7 pK\textsubscript{Ah} and Water Solubility

To assess the basicity of the compounds studied, the ChemAxon’s programme, Marvin, was used to calculate the pK\textsubscript{ah} values of the guanidinium moieties of the compounds studied. According to the calculated results displayed in Table 3.3, there appears to be a trend in increasing basicity from compounds 21 (pK\textsubscript{ah} 9.42/8.82), 22 (pK\textsubscript{ah} 9.46/8.85) and 17 (pK\textsubscript{ah} 9.52/8.91) as the length of the alkyl chain in the guanidinium increases. This could be due to the increased inductive effect applied as the length of the alkyl chain increases and there is a greater electron donation present, however the differences are miniscule. A similar increase in pK\textsubscript{ah} is seen from compound 19 (pK\textsubscript{ah} 9.17/8.57) to compound 20 (pK\textsubscript{ah} 9.24/8.64). An outlier in this trend is the unsubstituted bis-guanidinium compound 15 (pK\textsubscript{ah} 9.21/8.61) which has a greater pK\textsubscript{ah} value than the dimethyl-guanidinium derivative 19 (maybe due to the increased steric hindrance for the protonation) and is equally as basic as compound 20.

The water solubility of a proposed compound is a fundamental factor in drug design and development. A poorly water-soluble drug tends to require a much higher dosing regimen than those which are water soluble when taken as orally administered drugs.\textsuperscript{62} The three methods to predict water solubility implemented in the SwissADME programme include: 1) Estimated Solubility (ESOL) model, which was derived from a set of 2874 measured solubilities using linear regression against nine molecular properties,\textsuperscript{63} 2) Ali Solubility which is adapted from Ali \textit{et al.},\textsuperscript{64} based on the general solubility equation, replacing melting point with TPSA, and 3) SILICOS-IT which is named after the company that developed it.\textsuperscript{46} As seen in Table 3.3, the method chosen for this set of compounds was the one developed by Ali \textit{et al.}, because had demonstrated a strong linear correlation between predicted and experimental values (R\textsuperscript{2} = 0.81);\textsuperscript{46} in our case, each of the proposed compounds displayed moderate to high solubility properties.

3.1.8 Conclusion

In summary, the physicochemical properties of the compounds proposed for this study were calculated with the help of computational tools. All compounds described met the necessary parameters of drug-likeness (i.e. MW, HBD, HBA, rotational bonds, logP), with the exception of compounds 17, 20 and 32 with the number of rotational bonds marginally above the desired 10 bonds. Thus, it is suggested that the rest of the compounds may become successful orally administered drugs.

Other parameters of interest for druggability such as PAINS, MR, and metabolic stability of the various compounds were also computed. Thus, none of the compounds studied contains any PAINS, and all compounds calculated MR values lie within the desired criteria.
Regarding cytochrome P450 metabolic related enzymes, the majority of the compounds studied do not inhibit these enzymes, with the exception of compounds 1, 15, 17, 16, 20, 27, 29 and 32 which inhibit one or two of these enzymes.

Moreover, according to the results obtained from the SwissADME calculations all the compounds studied are within the PSA limit for permeating ability to cross the cell membrane. However, for the particular case of molecules to penetrate the blood brain barrier (BBB), compounds 19, 20 and 27 demonstrated TPSA values too low to be considered probable candidates for crossing the BBB and target the receptors in the CNS (even though there is previous experimental evidence showing that related bis-guanidines or bis-2-aminoimidazolines can reach the brain). Regarding calculated water solubility, each of the proposed compounds displayed moderate to high solubility properties (Table 3.3).

Thus, from the results obtained it can be concluded that the compounds investigated fulfil most of the drug-like properties.

3.2 Synthesis

Over the last 20 years the Rozas group have developed a wide range of lead compounds targeting the α2-AR within the central nervous system to mediate excitatory functions of neurotransmitters. These compounds include mono- and bis-guanidinium or 2-aminoimidazolinium aromatic systems with different affinities towards the various α2-AR subtypes, primarily the α2A-AR and the α2C-AR which are the subtypes mostly expressed in human prefrontal cortex. Various pharmacological evaluations were carried out to measure the functional activity and binding affinity of the relevant hit compounds. Studies within Rozas group carried out by O’Donovan et al.,[29] and Rodriguez et al.,[36] showed that the size and lipophilicity of the cation had an impact on the binding affinity of the synthesised compound toward the α2-AR receptors (Table 3.4).
Table 3.4 Binding affinity (displayed in $K_i$) and functional activity of a range of both mono- and bis-cationic derivatives, previously synthesised and evaluated within the Rozas group

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>$R^1$</th>
<th>$R^2$</th>
<th>$K_i$ (nM)</th>
<th>Functional Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>33a</td>
<td>-NHEt</td>
<td></td>
<td>263.03</td>
<td>N.D.</td>
</tr>
<tr>
<td>33b</td>
<td>-NMe$_2$</td>
<td></td>
<td>87.096</td>
<td>Antagonist</td>
</tr>
<tr>
<td>33c</td>
<td>-NHEt</td>
<td></td>
<td>177.83</td>
<td>Agonist</td>
</tr>
<tr>
<td>33d</td>
<td>-NMe$_2$</td>
<td></td>
<td>38.019</td>
<td>Antagonist</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td>416.87</td>
<td>Agonist</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>13.183</td>
<td>Partial agonist</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>1.585</td>
<td>Agonist</td>
</tr>
</tbody>
</table>

As seen from the results previously obtained within the Rozas group for the mono-aryl guanidine and 2-aminoimidazoline derivatives (compounds 33a/b and 33c/d in Table 3.4, respectively), the change in regioisomers from the -NMe$_2$ to the -NHEt moiety had a large negative impact on the binding affinity toward $\alpha_2$-AR. This indicated that the increase in steric hindrance caused by the growth in chain length of the ethyl substituent and the decreased lipophilicity of the secondary amine moiety, are crucial factors to be considered when designing future compounds. Furthermore, the improved $K_i$ values observed in previous research when the guanidinium cation was replaced by the 2-aminoimidazolinium cation indicates that the ethylene bridge provides additional contacts for binding to the receptors active site, along with necessary increased lipophilicity while maintaining a
compact structure. Therefore, the objectives of this chapter are to discuss new di-aryl bis-cationic compounds with varying alkyl substitutions (i.e. mono- and di-ethyl or methyl) on the guanidinium moiety to probe the receptors’ binding site and investigate steric and lipophilicity effects on \( \alpha_{2A}-\text{AR} \) affinity and activity compared to the conformationally restricted 2-aminoimidazoline derivatives previously reported.

Guanidine derivatives have been extensively utilized in the field of medicinal chemistry due to their ability to form a wide variety of interactions within the body, ranging from ionic and H-bonding contacts to \( \pi \)-stacking. In addition, aromatic guanidines have been applied to a diverse range of therapeutic and biological applications. Examples include prevention of hyperglycaemia in diabetes type 2, inhibition of human platelet Na\(^+\)/H\(^+\) exchange, anti-obesity and eating disorder regulation, and radiotracers for imaging of Parkinson’s disease. The guanidine functional group’s high basicity, \( pK_{aH} = 13.6 \), means that protonation will occur at physiological pH and thus, this functional group has the unique ability to exist as neutral (guanidine), cationic (guanidinium), and anionic (guanidinate) entities, even though in the human body the cationic form will be the prevalent one.

Finally, the Rozas group has focused on the use of guanidine derivatives as \( \alpha_{2A}-\text{AR} \) ligands for the treatment of CNS disorders such as depression and schizophrenia through regulation of noradrenaline, and as DNA minor groove binders. Guanidines in their neutral form are commonly used as strong bases in organic synthesis and the specific H-bonding pattern of its conjugate acid (guanidinium cation) has led to the increased use of guanidine derivatives as catalysts. The increased stability of the guanidinium ion stems from the resonance through conjugation of the nitrogen lone pairs (Figure 3.5) where there is an abundance of H\(^+\) ions available for hydrogen bonding interactions and facilitating its ionic interactions with negatively charged biological systems.

**Figure 3.5 Resonance stabilisation of the guanidinium cation**

The crystal structure of guanidine was finally resolved 148 years after its first synthesis, showing two Y-shaped symmetry-independent molecules in a unit cell, interconnected by a H-bonding network. Furthermore, investigation into the conformational control induced by these intramolecular hydrogen bonds (IMHB) within 2-pyridoguanidine systems have been investigated within the Rozas group, along with the presence of crucial \( \pi \)-cationic interactions due to the aromaticity present within the structure.
3.2.1 Literature methods for the preparation of guanidine derivatives

The vast applicability of guanidines within biological and pharmaceutical sectors as well as building blocks for supramolecular chemistry, synthetic receptors, sensors and catalysis,\textsuperscript{77} has increased the demand for multiple synthetic routes towards guanidine derivatives. The most common approach includes the use of thioureas and isothioureas as guanidylating agents reacting with primary, and some secondary, amines. The majority of molecules described in this family of compounds were synthesised – or partially synthesised – via the Kim and Qian method.\textsuperscript{78} As described in Section 3.2.2.2, this method required the coordination of mercury (II) chloride (HgCl\textsubscript{2}) to the sulfur-containing thiourea, in the presence of triethylamine, to initiate the reaction with primary amines. This method was particularly useful for substrates with weak nucleophilic amino groups.

The use of toxic mercury chloride is undesirable within a chemical reaction, especially when the compound is destined for biological or pharmaceutical purposes. Copper (II) chloride (CuCl\textsubscript{2}) was also suggested as a possible thiophilic reagent by Kim \textit{et al}, and thus was later investigated by B. Kelly and I. Rozas.\textsuperscript{79} Kelly \textit{et al} determined that CuCl\textsubscript{2} was equally as satisfactory at producing high yields of the desired guanidine-containing compounds as its predecessor (Scheme 3.1). However, this thiophilic reagent was inconsistent in the activation of various substituted thioureas; this could be due to the fact that Hg\textsuperscript{2+} salts are more thiophilic than Cu\textsuperscript{2+} salts as they have lower LUMO, allowing for antibonding interactions toward sulfur's high energy HOMO.\textsuperscript{39} Furthermore, the removal of any excess HgCl\textsubscript{2} or the HgS biproduct has been very successful when filtered through a bed of celite, washed and purified via flash column chromatography. Therefore, HgCl\textsubscript{2} is still used within the Rozas’ group for research purposes, and commonly across other research laboratories.

\textbf{Scheme 3.1} Generic reaction scheme for the synthesis of guanidines using CuCl\textsubscript{2}

\[
\begin{align*}
\text{Boc}_2\text{S} & \quad \text{CuCl}_2, \text{NEt}_3 \\
\quad \text{DCM, 0 \textdegree C - r.t.} \\
\text{R'NH}_2 & \quad \text{i) 50\% TFA / DCM} \\
\text{Boc} & \quad \text{ii) H}_2\text{O, Amberlite} \\
\text{R'NH}_2 & \quad \text{H}_2\text{N}_2\text{NH}_2 \quad 2\text{HCl}
\end{align*}
\]

More recently (July 2019), the Kim and Qian method was again modified to replace the toxic HgCl\textsubscript{2} with the commercially available oxidant, iodine (Scheme 3.2). This I\textsubscript{2}-mediated approach produced the desired guanidine-containing derivatives with a 52-98\% yield.\textsuperscript{80} The lower yields were generally observed for sterically or electronically deactivated amines, due to the unstable nature of the reactive intermediates.\textsuperscript{80} This method was not suitable for any secondary amines that were explored.
Furthermore, Mukaiyama’s reagent has been frequently used as a promoter of the unreactive and sterically hindered aryl amines and the guanidylation of resin-bound amines (Scheme 3.3).\textsuperscript{81} Yong \textit{et al.} began their research into the development of a new thiophilic reagent due to the fact that the mercury sulphide biproduct made the Kim and Qian method inapplicable for solid-phase guanidylation. Unfortunately, Mukaiyama’s reagent tends to be restricted to mono-substituted guanidylation as bis-Boc-protected thiourea is needed to obtained high yields. Moreover, Mukaiyama’s reagent tends to be insoluble in many standard organic solvents resulting in the difficulty of removing any undesired side-products that may have generated.\textsuperscript{82}

Scheme 3.3 Reaction scheme illustrating the treatment of bis-Boc-thiourea with Mukaiyama’s reagent in the presence of benzylamine with a 91\% yield.

Ohara \textit{et. al.}, in 2009, published findings of the positive results obtained for the guanidylation of amines using N-iodosuccinimide (NIS) as a replacement promoter for the toxic mercury (II) chloride and Mukaiyama’s reagent.\textsuperscript{82} This form of reaction allowed for the guanidylation of primary and secondary amines via the use of various thioureas and di-Boc-S-methylisothiourea, respectively. According to Ohara \textit{et. al.}, NIS is a source of electrophilic iodine allowing for the stereoselective and regioselective reactions on various functional groups (Scheme 3.4).\textsuperscript{82} Furthermore, the proposed mechanism is similar to that of HgCl\textsubscript{2} as NIS should react as a Lewis acid and coordinate to the thiourea or S-methylisothiourea to initiate the reaction. The addition of the strong base, NEt\textsubscript{3}, would lead to the carbodiimide intermediate and thus upon interaction with the chosen amine, the desired guanidine would be obtained.
In 2002, Guisado et al. published a new polymer-supported method of guanidylation that avoided the use of the previously non-commercially available promoters that often required a multi-step synthesis. These guanidylating agents include pyrazole carboxamidine and its derivatives, S-alkyl thioureas, and N-triflyl guanidine. Furthermore, these previous methods often required a large excess of the starting amine to reach completion of the reaction if an efficient cleavage, in terms of purity, was required.

This new approach outlined by Guisado et al. combined the benefits of traditional solution phase chemistry with the application of polymeric reagents leading to the desired compounds in high throughput manner, without additional purification steps. Polymer-supported (PS) carbodiimide was chosen as its commercially available as well as the N,N'-bis-(tert-butoxycarbonyl)thiourea starting material which is readily synthesised in a one-pot procedure with multi-gram yields. For this reaction a base was not necessary, unlike the previous guanidylation methods described and could be carried out in DCM or DMF to achieve high yielding results. PS-trisamine was added to remove the small amounts of bis-(tert-butoxycarbonyl)carbodiimide side-product (Scheme 3.5).

**Scheme 3.4** Reaction scheme of the NIS-promoted guanidylation of A) primary and B) secondary amines

**Scheme 3.5** Reaction scheme for the guanidylation of a secondary amine from di-Boc-thiourea in the presence of i) PS-carbodiimide and ii) PS-trisamine for purification
Ultrasound energy has been used commonly throughout chemical synthesis as it assists in the reactions activation via a process known as acoustic cavitation. This process involves the expansion and contraction of small bubbles of gaseous substances which inevitably reach an unstable size and collapse. As these bubbles are small and rapidly collapse, they have been responsible for an enhancement in solubility, diffusivity, penetration and mass transportation of species in certain reactions. Pattarawarapan et al., investigated the use of ultrasound energy for the guanidylation of a variety of amines, using the inexpensive and easy-to-handle trichloro-1,3,5-triazine (TCT) as the dehydrosulfurization agent, in minimal amounts of solvent (Scheme 3.6). The yields obtained from the reactions were positively increased when the reactions were carried out under sonochemical conditions. Furthermore, it was observed that reducing the ratio of TCT to thiourea to 0.4:1 had little-to no effect on the yields obtained. Primary amines (e.g. benzyl- and alkylamines) and the sterically hindered disopropylamine reacted quickly and in high yield, suggesting that ultrasound energy could be used to overcome steric effects. The reactivity of the carbodiimide is the rate determining factor of these reactions.

Scheme 3.6 Reaction cycle illustrating the guanidylation process using TCT under sonochemical conditions. \( n = 1 \) is the first stage of the cycle. \( n = 2 \) is the second stage. \( n = 3 \) is the final cycle.

3.2.2 Preparation of the target bis-cationic diaryl derivatives

The Rozas group have designed and synthesised a large number of bis-guanidinium/2-aminoimidazolinium derivatives of varying linkers (NH, O, CH\(_2\), CO, SO\(_2\)); however, pharmacological testing indicated that none of the tested compounds displayed an \( \alpha_2 \)-AR
binding affinity stronger than that of the lead compound 1. Thus, the search for an α2-AR antagonist with a K smaller than 1.585 nM continues.

Aiming to identify key structural features involved in the design of the desired antagonist activity, computational studies were carried out in Rozas group. Comparative molecular field analysis (CoMFA) was the 3D QSAR method chosen as it is “based on the assumption that drug–receptor interactions are noncovalent and that changes in biological activity correlate with the changes in the steric and/or electrostatic fields of the drug molecules”\textsuperscript{15} Thus, a 3D pharmacophore was identified and hydrophobic extensions at the cationic moiety were determined to theoretically favour antagonist activity.\textsuperscript{39} Furthermore, the design of the compounds discussed in this section arose from the addition of the hydrophobic substituents on the guanidinium moiety paired with the di-aryl backbones as they generally afforded higher binding affinities.\textsuperscript{39}

The method most commonly utilised within the Rozas group for the guanidylation of alkyl or aryl amines is the Kim and Qian method,\textsuperscript{78} which, in our case, involves the reaction of the relevant mono- or di-substituted \textit{N,N'}-bis-(\textit{tert}-butoxycarbonyl)thiourea with 4,4'-methylenedianiline.

\subsection*{3.2.2.1 Preparation of Boc-protected thioureas}

Boc-protection of the thiourea is crucial for the mentioned Kim and Qian guanidylation method as it increases the electrophilicity of the carbon aiding the reaction with the poorly nucleophilic aryl-amines. According to these authors, the reaction goes via the initial formation of a highly electrophilic and short-lived bis-Boc carbodiimide intermediate.\textsuperscript{78} Furthermore, the addition of Boc groups make the resulting polar products easier to handle and purify via flash column chromatography.

In this project, Boc-protection of the chosen thiourea was a straight-forward, anhydrous reaction in which NaH (60% immersion in oil) activated the relevant thiourea, which was then reacted with di-\textit{tert}-butyl-dicarbonate (Scheme 3.7). The NaH needed to be quenched with NaHCO\textsubscript{3} saturated solution before the work-up could commence. However, the commercially available \textit{N,N'}-bis-(\textit{tert}-butoxycarbonyl)-S-methylisothiourea could be used instead of the unsubstituted \textit{N,N'}-bis-(\textit{tert}-butoxycarbonyl)thiourea, as a cost-effective and efficient alternative.
This Boc-protection method was also used to prepare the corresponding \(N,N'\)-substituted Boc-protected guanidines. However, when the Boc-protection was attempted using the \(N,N'\)-diethylthiourea there was a single water impurity signal at 1.55 ppm with an integration of 13 H with respect to the 18 H integration of the Boc-signal at 1.51 ppm. With extensive drying on the rotary evaporator and high vacuum over several hours, the water signal reduced but not completely removed. Thus, this thiourea starting material was assumed to be a highly hygroscopic compound. After exhausting time and resources attempting to remove the water impurity, it was decided to carry out the guanidylation reaction to see if there was a major impact on the guanidylation; however, only negative results were obtained (see Section 3.2.2.2).

Considering that this method of Boc-protecting the starting thiourea was unreliable for the \(N,N'\)-diethylthiourea and the \(N\)-alkylthioureas as it resulted in multiple side-products and impurities that were very difficult to separate via the usual column chromatography, alternative approaches were investigated.

A different approach was used to prepare Boc-protected \(N\)-methyl- and \(N\)-ethyl-thioureas (Scheme 3.8). Thus, Yin et al. had previously reported the preparation of \(N\)-Boc-\(N'\)-substituted thioureas by treatment of \(N,N'\)-di-Boc-substituted thiourea with NaH and trifluoroacetic anhydride (TFAA) in the presence of an amine.\(^{89}\) They proposed that the anion formed by deprotonation of the di-Boc protected thiourea is \(N\)-acylated to produce the \(N\)-Boc-\(N\)-trifluoroacetyl derivative, which undergoes nucleophilic attack by the amine during the second step (Scheme 3.8).\(^{39}\)
Even though this reaction had been previously used by Rozas group to yield the di-Boc protected N-substituted thioureas, in our case, only the mono-Boc protected N-methyl thiourea (24a) was obtained in moderate yields (~38%) (Scheme 3.9).

Although the introduction of the alkyl substituent was successful, the mono-Boc-protection was not satisfactory enough to fulfil the requirements of the guanidylation process and the following reaction was unsuccessful. From here, research was carried out to identify a method of synthesising a mono-substituted thiourea that maintained the electrophilicity of a di-Boc-thiourea.

Research uncovered that the Mitsunobu reaction was commonly used for synthesising substituted thioureas from primary and secondary alcohols. If successful, this method would provide a mono-substituted pseudothiourea that would also be bis-Boc-protected, thus providing the basis for an easier desulfurization during guanidylation. Moreover, the use of the pseudothiourea leaves only one amine available for alkylation, thus reducing the risk of unwanted side-products forming and purification would be simplified (Scheme 3.10).
The Mitsunobu reactions are the most widely used stoichiometric phosphorus mediated S_N2 reactions of alcohols with pro-nucleophiles such as carboxylic acids, sulphonamides, imides and thioureas. The reaction has been influential in medicinal chemistry and organic synthesis laboratories due to its broad scope, stereospecificity and mild reaction conditions. In 2015, Camp et al. published research on the solvent effects on the Mitsunobu reaction and it was determined that yields, particularly for sterically hindered alcohols, were often higher in non-polar solvents due to the slower rate of the side reactions making them less competitive. Typically, the rate constant for the formation of ethylbenzoate was 100 times greater when carried out in THF over acetonitrile. This reaction was used for the preparation of N-substituted Boc-protected pseudothioureas 21a and 22a. The reaction consisted of a one-pot procedure, reacting the appropriate alcohol with pseudothiourea in the presence of diethylazodicarboxylate (DEAD) and triphenylphosphine (PPh3).

The mechanistic details of the reaction, particularly the intermediate stages, are still subject of debate, but an estimation can be seen in Scheme 3.11. The activation of the alcohol is achieved by the reaction with the Morrison-Brunn-Huisgen zwitterion intermediate (A) which is formed in situ via the reaction of PPh3 and DEAD. A zwitterion is a chemical compound that results in both positive and negative charges.
Scheme 3.11 Suggested mechanism for the Mitsunobu reaction

However, the by-products formed in the reaction and the high energy nature of the azodicarboxylate reagent used in the reaction, limits its use and thus Mitsunobu couplings are often deserted when designing the final synthesis of an upscaled industrial-use synthesis.\textsuperscript{92} Since its discovery in 1967, the reaction has been used mostly in its original form; however, in more recent years attempts have been made to improve the catalysis of the reaction making it more suitable for industrial use. For example, in 2006 Toy et al. were the first to introduce the premise of azodicarboxylate recycling.\textsuperscript{92,95,96} The idea was not developed until 2013 when Taniguchi published an alternative oxidation system for an \textit{in situ} approach to azodicarboxylate recycling.\textsuperscript{97,98} However, neither approach addressed the phosphine oxide waste produced throughout the synthesis. Aldrich \textit{et al.} combined the O’Brien phosphine recycling method, developed from the catalytic Wittig reaction, with the Taniguchi azodicarboxylate recycling method creating a \textquotedblleft full catalytic system\textquotedblright.\textsuperscript{99,100} However, there were issues with the scope and reproducibility of Aldrich’s \textquotedblleft fully catalytic\textquotedblright approach.

In our case, the phosphine oxide by-product was only partially soluble in hexane; thus a recrystallisation from hexane was carried out before the column purification of the mixture...
since otherwise the by-product would precipitate disturbing the chromatographic silica due to the high ratio of hexane/ethyl acetate (9:1, respectively) needed to separate the alkyl-substituted pseudothiourea from excess DEAD. Alternatively, the use of diisopropylazodicarboxylate (DIAD) does not require such a polar system as it streaks from the baseline of the TLC; thus, the column can be carried out in a 1:1 ratio of hexane / ethyl acetate and the phosphine oxide by-product remains in solution.

An investigation into the use of this reaction to synthesise a disubstituted thiourea was carried out as an alternative to prepare the N,N'-diethyl pseudothiourea, however, after the first substitution there appears to be a change in electrophilicity of the thiourea and the second substitution never occurs, even with an increase in stoichiometric equivalents of base and alcohol. To conclude, the Mitsunobo reaction resulted in the successful synthesis of both the methyl- (21a) and ethyl- (22a) N-substituted Boc-protected pseudothioureas.

### 3.2.2.2 Guanidylation reactions

The Kim and Qian reaction is initiated via the activation of the corresponding thiourea by HgCl₂, followed by the presence of triethylamine to assist in the deprotonation of the amines on the 4,4'-methyleneedianiline backbone to generate the bis-Boc-protected product.

**Scheme 3.12 Kim and Qian method for guanidylation mechanism**

![Scheme 3.12 Kim and Qian method for guanidylation mechanism](image)

Although the use of toxic mercury chloride in the synthesis of compounds destined for medicinal purposes is proven undesirable, the efficacy of the reaction cannot be denied. In 2013, B. Kelly and I. Rozas published an alternative route for the guanylation of unreactive aryl-amine-containing compounds using CuCl₂ as the thiophilic salt with yields equally as
satisfactory as that of HgCl$_2$. However, CuCl$_2$ was not consistent in producing acceptable yields, unlike HgCl$_2$, therefore HgCl$_2$ is still used in the Rozas laboratory today. This could be due to the fact that Hg$^{2+}$ salts are more thiophilic than Cu$^{2+}$ salts as they have lower LUMO, allowing for antibonding interactions toward sulfur’s high energy HOMO.$^{101}$ Furthermore, the removal of any excess HgCl$_2$ or the HgS biproduct has been very successful when filtered through a bed of celite, washed and purified via flash column chromatography. Following this approach, the corresponding Boc-protected bis-guanidine diaryl derivatives have been synthesised as shown in Scheme 3.13.

**Scheme 3.13 Kim and Qian guanidylation reaction scheme and table of results for the synthesised compounds**

![Scheme 3.13 Image]

<table>
<thead>
<tr>
<th>Compound</th>
<th>R$^1$</th>
<th>R$^2$</th>
<th>R$^3$</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19b</td>
<td>CH$_3$</td>
<td>CH$_3$</td>
<td>-</td>
<td>56.65</td>
</tr>
<tr>
<td>20b</td>
<td>CH$_2$CH$_3$</td>
<td>CH$_2$CH$_3$</td>
<td>-</td>
<td>25.2</td>
</tr>
<tr>
<td>21b</td>
<td>CH$_3$</td>
<td>H</td>
<td>-</td>
<td>47.9</td>
</tr>
<tr>
<td>22b</td>
<td>CH$_2$CH$_3$</td>
<td>H</td>
<td>-</td>
<td>42.4</td>
</tr>
<tr>
<td>25a</td>
<td>H</td>
<td>H</td>
<td>CH$_3$</td>
<td>71.6</td>
</tr>
<tr>
<td>28a</td>
<td>CH$_3$</td>
<td>H</td>
<td>CH$_3$</td>
<td>67.4</td>
</tr>
</tbody>
</table>

The guanidylation forming 20b resulted in three spots being seen on the TLC (Rf: 0.51, 0.57, 0.64), likely due to a variation in the mono- and bis-Boc-protected thiourea and the mono- and bis-guanidylated products. Due to the Rf difference being less than 0.1, they were too difficult to separate by column chromatography. From here, the preparative TLC method was employed with the hopes of separating the three side products. The separation
remained extremely difficult and only minimal yields could be obtained from this technique. Due to the minimal amounts of product left after multiple attempts at purification, the product was next deprotected (see Section 3.2.2.3). If more time were available, more research would be invested into finding a means of isolating the product from the various by-products in the Boc-intermediate stage.

When researching methods for the synthesis of the symmetric mono-substituted bis-guanidine diaryl analogues, four possible methods were attempted. The first was based on the 2014 publication of Ríos Martinez et al. (Scheme 3.14), which investigated the synthesis of this type of structures in a three-step procedure: (i) preparation of the diaryl ethyloxycarbonyl protected bis-thioureas, (ii) formation of the ethyloxycarbonyl protected bis-guanidinium groups and (iii) elimination of the protecting groups. Following this synthetic approach, the 4,4'-methylenedianiline starting material that makes up the diaryl core of the final product was dissolved in dry DCM and cooled below 0 ºC before slowly adding an excess of ethoxycarbonyl isothiocyanate to yield compound 36a. In the original research, the NHCO and NHCONH linkers within the diaryl core were investigated instead of our methylene bridge. These linkers are stronger activating agents than the CH2 linker and therefore the reaction in our case never reached completion. A gravity column was carried out, but the product was only partially soluble in hexane and thus precipitated on the column in any ratio of hexane/ethyl acetate less than 1:1. Recrystallisation from hexane was attempted but the mono- and bis-substituted diamine systems had very similar solubilities and thus, separation was unsuccessful. Due to the lack of sufficient time to spend resolving this issue and investigating potential alternatives for the column chromatography solvent system, the next method for synthesising mono-substituted guanidines was explored.

Scheme 3.14 Reaction scheme for the first attempted synthesis of the mono-substituted bis-guanidine. Where R-NH2 is either methylamine (2 M solution in THF) or ethylamine hydrochloride.

The second approach involved the reaction of 4,4'-methylenedianiline with 1,1'-thiocarbonyl bis-imidazole at room temperature in DCM overnight to obtain the corresponding isothiocyanate (Scheme 3.15). Again, due to the poor activation of the weakly electron
donating CH$_2$ linker, the reaction did not go to completion. The product was purified via recrystallisation from hexane followed by gravity column chromatography (1:1 hexane/EtOAc), yielding compound 37a (97%). Compound 37a was reacted with tert-butyl-carbamate in the presence of NaH (60% immersion in oil) in dry THF. The subsequent reaction, however, was far less successful yielding only 16.2% of compound 37b as a pale-yellow solid which could be due to the combination of the poor reactivity of the CH$_2$ linker and the poor nucleophilicity of the carbamate caused by the surrounding bulky tert-butyl functional group. Due to the insufficient amount of product obtained, the first two steps needed to be repeated but this process was cut short during the purification of the first reaction due to the college closure because the Covid-19 outbreak. Upon returning to the laboratory the reaction was repeated; however, the purification used previously no longer yielded the desired product, thus the third approach was attempted.

**Scheme 3.15** The second approach towards the synthesis of mono-substituted bis-guanidines. Where R-NH$_2$ is either methylamine hydrochloride or ethylamine hydrochloride

![Scheme 3.15](image)

The third approach used the Kim and Qian guanidylation method, using N-(tert-butoxycarbonyl)-N'-methylthiourea as the guanidylating agent (Scheme 3.16). However, in the case of the preparation of compound 38b and despite the successful synthesis of the starting N-(tert-butoxycarbonyl)-N'-methylthiourea (38a), the lack of a second Boc-protecting group reduced the electrophilicity of the thiourea thus the reaction never progressed through the guanidylation stage.

**Scheme 3.16** Reaction Schemes for the third approach to the synthesis of mono-substituted bis-guanidines demonstrating the unsuccessful guanidylation using the Kim and Quan method.
Whilst analysing the synthesis of compound 38a, it was clear that the thiourea must be bis-Boc-protected. Furthermore, the use of the commercially available \(N,N\text{-bis-(tert-butoxycarbonyl)}\)-S-methylisothiourea (pseudothiourea) improved the efficacy of some reactions. This may be attributed to the methylation of the sulfur encouraging a more efficient desulfurization by mercury chloride (HgCl\(_2\)) forming a HgS by-product (Scheme 3.17). It was therefore concluded that the Mitsunobu reaction, described in Section 3.2.2.1 above, was the ideal approach to synthesise the mono-substituted thiourea prior to the Kim and Qian method for guanidylation.

**Scheme 3.17** Desulfurization of S-methylthiourea using HgCl\(_2\), followed by guanidylation of an amine

The asymmetric bis-guanidine compounds (25a and 28a) were synthesised using a two-part guanidylation with the Kim and Qian method (Scheme 3.18). First, the mono-guanidilation reaction conditions were set up using 1 equivalent of the \(N,N\text{-bis-Boc-dimethylthiourea}\) and 3 equivalents of the 4,4′-methylenedianiline, in the presence of HgCl\(_2\) (3 eq.) and NEt\(_3\) (6 eq.) in DCM. After purification by column chromatography yielding compound 39a (71.1%), the second guanidylation was carried out. Thus, 1 equivalent of compound 39a was reacted with 1.5 equivalents of the relevant thiourea in the presence of HgCl\(_2\) (3 eq.) and NEt\(_3\) (increase from 6 to 8 equivalents for the sterically hindered thiourea), yielding compounds 25a (71.6%) and 28a (64.7%). The final deprotection step shown in Scheme 3.18 is discussed in the next section.
Scheme 3.18 Asymmetrical synthesis of compounds 25b and 28b where \( R^1 - R^3 \) are Me or H. The diamine starting material was used in excess to assure mono-guanidylation.

\[
\begin{align*}
\text{R}^1 \text{N} & \text{N} \text{R}^1 \quad \text{(1 eq.)} \\
\text{Boc} & \\
\text{H}_2 \text{N} & \text{Boc} \\
\text{Am} & \text{H}_2 \text{N} & \text{Boc} \\
\text{DCM, r.t., o/h} & \\
\rightarrow & \\
\text{39a, } R^1 = \text{Me, } 71.1 \% \\
& \\
& \\
\end{align*}
\]

3.2.2.3 Preparation of the final salts

There are multiple recorded methods for Boc-deprotection to afford the desired salts. In this project, we initially used 1.25M solutions of HCl in methanol as it only required a 4 hour stirring period at 35 \(^\circ\)C to obtain the corresponding hydrochloride salt; however, the stronger acid caused the decomposition of the highly sensitive guanidine moiety. Other molarities could be attempted in the future along with the possibility of using HCl/dioxane as an alternative solvent.

The method that gave the most consistent results with the highest yields (>85%) was the use of trifluoroacetic acid (TFA). Thus, a 50% solution of TFA in DCM was used in excess and stirred for overnight, at room temperature to yield the guanidine trifluoroacetate salts. However, these salts are often insoluble in water. Thus, for the purpose of the pharmacological evaluations, an ion exchange was carried out by stirring the desired trifluoroacetate in excess deionised H\(_2\)O and activated Amberlite\textregistered IRA-400 resin – a polystyrene bead – in its chloride form. After 24-48 hrs of light stirring (not to damage the resin) and filtration, the guanidine hydrochloride salts were obtained. Complete ion interchange was checked using \(^{19}\)F NMR spectroscopy where the absence of any peaks in this spectrum confirms full conversion of TFA salt to the corresponding HCl salts.

In the particular case of compound 20b, when TFA was added to the product, the solution turned a shade of pink. This could have been an indication that some of the \( N,N^\prime\)-bis-(tert-
butoxycarbonyl)diethylthiourea starting material was still present, the colour being the result of a n-π* interaction between the TFA and the thiourea. Both ¹H and a ¹³C NMR spectra showed that a mixture was still present. After a final reverse phase column, the product was separated into the 4-bis-[(N,N'-diethyl)guanidino]diphenylmethane dihydrochloride (20c) and 4-amino-4'-[(N,N'-diethyl)guanidino]diphenylmethane hydrochloride (20d).

Scheme 3.19 Generic reaction scheme for the i) Boc-deprotection and ii) ion exchange to yield the desired hydrochloride salt, where R¹, R² = H, Me, Et, and table of results

<table>
<thead>
<tr>
<th>Compound</th>
<th>R¹</th>
<th>R²</th>
<th>R³</th>
<th>R⁴</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19c</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>98.2</td>
</tr>
<tr>
<td>20c</td>
<td>CH₂CH₃</td>
<td>CH₂CH₃</td>
<td>CH₂CH₃</td>
<td>CH₂CH₃</td>
<td>9.7</td>
</tr>
<tr>
<td>21c</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>67.5</td>
</tr>
<tr>
<td>22c</td>
<td>CH₂CH₃</td>
<td>H</td>
<td>H</td>
<td>CH₂CH₃</td>
<td>83.9</td>
</tr>
<tr>
<td>25b</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>CH₃</td>
<td>86.0</td>
</tr>
<tr>
<td>28b</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
<td>CH₃</td>
<td>94.3</td>
</tr>
</tbody>
</table>

Compounds 19b, 21b, 22b and 28b were all prepared in sufficient quantities and to the required purity specification to allow in vitro testing to be performed. These results are discussed in Section 3.4.
The α₂-ARs mediate a wide range of physiological functions making them highly attractive biological targets for drug discovery. Until December 2019, there was an absence of any α₂-AR subtype crystal structure, which had proven to be a major hindrance in the drug design and development process. To date, the Rozas group utilized homology models of the two most relevant α₂A-AR subtypes in their active and inactive forms developed by Rozas’ collaborator Prof. Mireia Olivella. Elucidation of the different physiological functions attributed to a given α₂-AR subtype remains challenging, largely due to the lack of subtype-selective ligands. However, due to the distribution of each of the subtypes across the CNS and PNS, the α₂A- and the α₂C-AR are most relevant due to their localisation within the human brain. Since the α₂A-AR is the most dominant, this has been the focus of the molecular docking studies here presented and discussed.

In December 2019, the following three crystal structures of the α₂-AR subtypes were resolved and have been recently deposited in the Protein Data Bank (PDB):

α₂A-AR- 6KUY- The crystal structure of the human inactive α₂A-AR in complex with a partial agonist, (S)-4-fluoro-2-(1H-imidazol-5-yl)-1-isopropylindoline (E39).25 The preparation and adrenergic activity of this ligand was described in a patent in 1996.103

α₂A-AR- 6KUX- Crystal structure of the inactive α₂A-AR – from the Spodoptera frugiperda in complex with an antagonist (E33). Research carried out by Uhlen et al. (1998) produced

![Figure 3.6](image-url)
the [\textsuperscript{3}H]RS79948-197 binding to rat \( \alpha_{2A} \), \( \alpha_{2B} \) and \( \alpha_{2C} \)-adrenoceptors with \( K_d \) values of 0.42, 0.18 and 0.19 nM, respectively.\textsuperscript{104}

\[ \alpha_{2C}-AR-6KUW \] The crystal structure of human \( \alpha_{2C} \)-AR G-protein coupled receptor in complex with antagonist (E33) in its inactive form.\textsuperscript{26,104}

The research-based pharmaceutical and medicinal chemistry industry have increasingly employed a wealth of molecular modelling methods within a variety of drug discovery programmes to study complex biological and chemical systems.\textsuperscript{105} Molecular modelling encompasses all theoretical and computational methods used to model or mimic the behaviour of molecules (Figure 3.8). Molecular docking is a specific computational technique used to explore ligand conformations within the binding sites of macromolecular targets and predicts the preferred binding orientation. This method plays an important role in structure-based drug design (SBDD) as it provides insights into the molecules binding behaviour as well as to elucidate fundamental biochemical processes. SBDD refers to the systematic use of structural data which are usually obtained using a biophysical technique (e.g. X-ray crystallography) or derived from computational homology modelling.\textsuperscript{105} Computational homology modelling refers to the technique used to prepare a model of a protein 3D structure from its amino acid sequence based on its similarity to a protein of known 3D structure.\textsuperscript{106} The \( \alpha_{2A} \)-AR-MO model is an example of a homology model, that was developed before the \( \alpha_{2A} \)-AR crystal structures were published in December 2019.
The availability of 3D macromolecular structures enables a diligent inspection of the binding site topology, including the presence of clefts, cavities and sub-pockets.\textsuperscript{105} Moreover, the electrostatic properties and potential binding interactions can also be carefully examined.

The process of identifying a target and synthesising an active compound with suitable characteristics (e.g. minimal toxicity, high bioavailability and a cost-effective synthesis) and developing it to be introduced to the market is a time-consuming, extremely complex and risky endeavour.\textsuperscript{107} Within academia, the high throughput screening (HTS) used to screen potential hit compounds and identify new lead compounds can be an extremely costly and time-consuming process. The introduction of molecular docking as a virtual screening (VS) system can be used instead of or as well as HTS to identify the new lead compounds in an efficient manner. To date, over 173,000 structures of potential targets have been registered on the RCSB Protein Data Base (PDB) and are available to academia and industry alike.\textsuperscript{108} Molecular docking was first introduced to the industry in the 1970s to assist with drug discovery tasks; however, more recent applications include the prediction of adverse side effects, pharmacology, drug repurposing and target fishing and profiling.\textsuperscript{109}

As previously mentioned, docking methods fit a ligand into a binding site by combining and optimizing variables such as steric, hydrophobic and electrostatic interactions.\textsuperscript{107} These ligands are then “scored” based on their potential as likely ligands for that receptor. Scoring functions are categorised in three main groups:

(i) \textit{Force-field-based scoring functions} estimate the binding energy by summing the contributions of bonded (stretching and bending) and non-bonded (electrostatic and van der
Waals) terms in a general master function.\textsuperscript{105} It applies molecular mechanics methods to calculate energy associated with each term of the force-field, sometimes using parameters/values calculated using quantum mechanics.\textsuperscript{110} Therefore, it is normal that inaccuracies appear when estimating entropic contributions or in the treatment of long-range effects involved in binding.\textsuperscript{111}

(ii) \textit{Empirical scoring function} refers to scoring functions where each of the terms describes one type of physical event involved in the formation of the ligand-receptor complex, \textit{i.e.} hydrogen bonding, ionic and apolar interactions, desolvation or entropic effects.\textsuperscript{105,112} Due to the simplicity of the employed energy terms, empirical functions are faster than the force-field-based methods and often preferred, even though they are less accurate. This research employed the empirical function GlideScore (G-Score), now referred to as binding affinity on autodock vina software, as the measure of ranking the docked compounds. However, the main disadvantage to empirical functions is that they depend heavily on the accuracy of the data used in the parameterization process.\textsuperscript{111}

(iii) \textit{Knowledge-based scoring function} uses pairwise energy potential values, extracted from known ligand-receptor complexes, to obtain a general scoring function. It is based on the inverse Boltzmann statistic principle where these potentials are constructed by observing the frequency with which two different atoms are found within a given distance in a structural data set.\textsuperscript{105,113} These different types of interactions observed in the dataset are classified and weighted according to their frequency of occurrence. The final score is given as a sum of these individual interactions.\textsuperscript{32,105}

In collaboration with Helene Mihigo, a PhD student within the Rozas group, the molecules seen in Section 3.3.1 were optimised using the Maestro software, the structures of the \(\alpha_{2A}\)-AR complexed with a partial agonist (6KUY) or with an antagonist (6KUX) were retrieved from the RCSB PDB to be used for docking studies, and docking of the ligands into the mentioned targets was performed using Glide. The chosen orientations of the docked compounds were based on the ionic interaction with the aspartate residue D113\textsuperscript{3,32} as this has been reported to be a critical interaction with the \(\alpha_2\)-AR binding sites.

Due to time constraints and their relevance in disease, only the three inactive \(\alpha_{2A}\)-AR receptor templates were chosen for the present docking study: the model developed by Prof. Olivella (\(\alpha_{2A}\)-AR-MO), and the two crystal structures recently reported \(\alpha_{2A}\)-AR-Y (complexed with a partial agonist) and \(\alpha_{2A}\)-AR-X (complexed with an antagonist). Thus, the appropriate standard docking experiments were performed using compounds 1 (\textit{lead}, symmetric), 15 (asymmetric), 16 (asymmetric), 18 (symmetric) and 22c (symmetric) as ligands. Considering that the standard docking studies assume a rigid receptor system, a fit-induced study was chosen for a selected set of compounds because this is not the case
for most receptors. It is common for a receptor to alter its orientation and binding site to better ‘fit’ the ligand. For this reason, false negatives can be achieved in the standard docking studies as the ligand may not bind well to the rigid system but is known to be active in vitro. Thus, based on the pharmacological results obtained (see Section 3.4), fit-induced docking studies were also performed with compounds 1, 22c and 16. These specific docking studies were carried out to investigate the possible differences and similarities of the interactions established between a known agonist (1) or antagonists (16 and 22c) with the receptor.

![Chemical structures of compounds 1, 15, 16, 18, and 22c](image)

**Figure 3.9 Structure of Compounds 1, 15, 16, 18 and 22c to be docked in the various α₂A-AR receptor templates**

### 3.3.1 Docking Results

As previously stated, compound 1 is the lead compound previously developed within the Rozas group and is used as a reference model for the remaining docking studies. As aforementioned, the binding affinity (kcal/mol), previously known as G-score, is an empirical scoring function that approximates the ligand binding free energy. The calculated binding affinity values for the previously synthesised compounds 1, 15 and 18 and the newly synthesised compound 22c are presented in Table 3.7, where a more negative value indicates a more favourable binding.
### Table 3.5 Autodock binding affinity values for compounds 1, 15, 18 and 22c when docked with the various receptor models

<table>
<thead>
<tr>
<th>Compound Number (Functional Activity)</th>
<th>Receptor Model</th>
<th>Binding Affinity (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Agonist)</td>
<td>α2A-AR-MO</td>
<td>-3.57</td>
</tr>
<tr>
<td></td>
<td>α2A-AR-Y</td>
<td>-5.93</td>
</tr>
<tr>
<td></td>
<td>α2A-AR-X</td>
<td>-3.97</td>
</tr>
<tr>
<td>18 (Agonist)</td>
<td>α2A-AR-MO</td>
<td>-5.26</td>
</tr>
<tr>
<td></td>
<td>α2A-AR-Y</td>
<td>-5.72</td>
</tr>
<tr>
<td></td>
<td>α2A-AR-X</td>
<td>-4.16</td>
</tr>
<tr>
<td>15 (Partial agonist)</td>
<td>α2A-AR-MO</td>
<td>-5.09</td>
</tr>
<tr>
<td></td>
<td>α2A-AR-Y</td>
<td>-4.87</td>
</tr>
<tr>
<td></td>
<td>α2A-AR-X</td>
<td>-4.61</td>
</tr>
<tr>
<td>22c (Antagonist)</td>
<td>α2A-AR-MO</td>
<td>-7.11</td>
</tr>
<tr>
<td></td>
<td>α2A-AR-Y</td>
<td>-3.74</td>
</tr>
<tr>
<td></td>
<td>α2A-AR-X</td>
<td>-2.58</td>
</tr>
</tbody>
</table>

For ease of purpose, the interacting residue’s name and the position that it occupies in the whole primary sequence of the protein receptor, alongside the Ballesteros–Weinstein nomenclature will be used as a special indexing system. The Ballesteros–Weinstein nomenclature is one of the most commonly adapted systems when discussing amino acids in GPCRs. The letter indicates which amino acid is being identified and the first digit refers to which of the seven transmembrane helices the amino acid belongs to. Finally, the number after the decimal point refers to the residue’s position with respect to the most conserved residue in that helix, which has been arbitrarily assigned the number 50.25

![Diagram](image)

**Figure 3.10 Example of Asp113 residue using the Ballesteros–Weinstein nomenclature**
Past research into these receptor models, by means of site directed mutagenesis and homology modelling studies, identified aspartate 113 (D3.32, from now on D113\textsubscript{3.32}) as one of the most pertinent residues in aminergic binding sites. This is due to the formation of a strong salt bridge between the anionic carboxylate of the aspartate and the cationic moieties of some known ligands (e.g. idazoxan or clonidine) at physiological pH, acting as an anchor within the binding site. Thus, D113\textsubscript{3.32} is the conserved residue involved in all aminergic and opioid receptors.\textsuperscript{25} Due to its vitality, any poses in the docking sequences that did not display a salt bridge formation with D113\textsubscript{3.32} were not taken into consideration.

Furthermore, it has been reported that the movement of the TM6 is the “hallmark” of GPCR activation and that partial agonists lack the necessary hydrophilic tails that can form bonds with the polar residues at the extracellular end of TM5 and TM6 to trigger this activation.\textsuperscript{115} It can therefore be suggested that when aiming for antagonist activity, the molecular docking would aim to avoid these key interactions with TM5 and TM6.

The previously synthesised compounds \textbf{15} and \textbf{18}, prepared and tested within the Rozas group, displayed similar interactions with the three targets used in the standard docking studies. Thus, hydrogen bonding with S200\textsubscript{5.42} was observed, which would correlate with their \[^{35}\text{S}]\text{GTPyS} binding functional assay results as α\textsubscript{2}-AR agonists. Moreover, these agonists displayed further similarities with an additional salt bridge and H-bonding interaction with the D192\textsuperscript{XL2} and E189\textsuperscript{XL2.51} residues, which lie on the extracellular loop 2 (XL2) directly linked to the TM5 (Figure 3.11).\textsuperscript{116}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.11.png}
\caption{Schematic of the basic GPCR structure; where TM 1-7 are the 7-transmembranes, ECL 1-3 and IL 1-3 are the extracellular and intracellular loops respectively.\textsuperscript{116}}
\end{figure}
The docking of lead compound 1 did not result in the expected interaction with S200 in any of the receptor models, but it did display the same interactions with the residues on XL2, thus potentially indicating a correlation between these interactions and the agonist behaviour of compounds 1, 15, 18, as they could be used to direct the ligands towards TM5.

Unique to the α2A-AR-Y receptor model of compound 18 is the introduction of H-bonding interactions at the isoleucine I190XL2.52 on the XL2 (Figure 3.14). The Schrödinger software utilised throughout this study automatically characterised the HB interactions displayed in Figure 3.14 etc. Figure 3.20 and 3.21 display the HB distance in angstrom (Å).

Thompson et al. and Laurila et al. reported that the residues at XL2.50, XL2.51 and XL2.52 may act as a lid covering the binding cavity and may interact with certain ligands to influence the binding mode of the receptor. The residues at position XL2.52, e.g. the I190XL2.52 residue of the α2A-AR, is directed downwards into the receptor and may result in subtype specific binding. Moreover, Ostopovici-Halip et al. stated that the negatively charged carboxyl side chain of D192XL2 could be used in designing ligands with substituents of opposite charge that can interact with these residue side chains. Furthermore, Jayaraman et al. reported the importance of E189XL2.51 in subtype selectivity as it influences...

Figure 3.12 Molecular docking pose for compound 1 and the α2A-AR-X target indicating the most important interactions
the space available for ligand binding. This data reports the possible importance of the XL2 in ligand selectivity in aminergic and other small molecule binding GPCRs.

Moreover, phenylalanine 412 (F7.39) has been identified as a potentially essential residue in α2A-AR agonist activity, acting as a “switching lid” of which the smaller ligands with less saturated ring systems induce closure of the “lid” to form an aromatic cage. Due to F412 7.39 being one of the three non-conserved residues within the α2A-AR, this phenomenon is considered unique to the α-ARs. Despite the computational docking of the antagonist 22c into the α2A-AR model showing a displacement of the π-π stacking interaction between F412 7.39 and the ligand, contradicting this reported data. Other aromatic residues F390 6.51, F391 6.52, F408 7.39, Y196 5.48, Y416 7.43 120 and W413 7.40 115, which vary in their position depending on the ligand and receptor model used, alongside F412 7.39 form the necessary π-π stacking and π-cation interactions with the di-aryl backbone of the ligand 22c. All this supports the good binding affinity shown by this compound into the α2A-AR (see Table 3.7).

Compound 15 had been previously synthesised in the Rozas group and determined to be a partial agonist via in vitro studies. Due to the asymmetry within the structure, two orientations were available in each of the receptor models. However, depending on the
presence of the necessary anchoring salt bridge with D113\(^{3.32}\), only one valid orientation was considered. In the particular case of the \(\alpha_{2A}\)-AR-Y target both up and down orientations demonstrated significant binding as seen in Figure 3.15 below. The up orientation refers to when the anchoring salt bridge is formed between D113\(^{3.32}\) and the imidazoline moiety, whereas the down orientation refers to when the salt bridge is formed between the guanidine moiety and the D113\(^{3.32}\) residue. This reinforces the theory that the conformational restraint provided by the imidazoline moiety results in a stronger binding interaction to drive the ligand within the receptors binding pocket for a better anchoring system than that of the free guanidine. In all three receptor models (\(\alpha_{2}\)-AR-MO, \(\alpha_{2}\)-AR-Y and \(\alpha_{2}\)-AR-X) the imidazoline forms the successful anchoring interaction; however the \(\alpha_{2}\)-AR-X orientation resulted in a worse binding affinity which may be due to the drastic increase in solvent exposure across the ligand (Appendix 21).
Figure 3.14 A) Computational representation of the interactions between the "up" conformation of compound 15 and the $\alpha_{2A}$-AR-Y receptor model. B) Visual representation of the interactions between the "down" conformation of compound 15 and the $\alpha_{2A}$-AR-Y receptor model.
As explained at the beginning of the section, induced-fit docking was carried out with the lead compound 1, previously synthesised compound 16 and the newly tested compound 22c. These particular ligands were chosen based on their structures, $K_i$ values obtained from the in vitro studies and their functional activity. Compound 1 was the lead compound to date with a good binding affinity ($K_i = 1.585 \text{ nM}$) but demonstrated agonist activity; compound 16 (asymmetric 2-aminoimidazoline/substituted guanidinium) was chosen due to its antagonist activity and excellent $K_i$ value ($0.794 \text{ nM}$); finally, bis-[(N-ethyl)guanidinium] 22c (antagonist with an average $K_i = 95.5 \text{ nM}$) was selected for comparison. Due to time constraints, only the $\alpha_{2A}$-AR-X receptor model was investigated as it is in complex with an antagonist and closely related to the desired activity of the receptor.

These studies were performed using the software Maestro (Schrödinger Inc.) and the $\alpha_{2A}$-AR-X receptor model retrieved from the RCSB PDB to be used as targets. Only poses where ligands which interact with D113$^{3,32}$ were considered. The binding affinity values calculated for each compound were larger (more negative) than those obtained in the standard docking as expected due to the induced-fit being more closely related to the actual binding to the receptor since the ligand can better orient itself within the adapting binding pocket over the rigid receptor models (Table 3.8).

**Table 3.6 Autodock binding affinity of compounds 1, 16, 22c, comparing the rigid receptor models with the induced-fit models of $\alpha_{2A}$-AR-X**

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Receptor model</th>
<th>Rigid Receptor Binding affinity (kcal/mol)</th>
<th>Fit-induced Receptor Binding affinity (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$\alpha_{2A}$-AR-X</td>
<td>-3.97</td>
<td>-7.44</td>
</tr>
<tr>
<td>16</td>
<td>$\alpha_{2A}$-AR-X</td>
<td>n.d.</td>
<td>-8.16</td>
</tr>
<tr>
<td>22c</td>
<td>$\alpha_{2A}$-AR-X</td>
<td>-2.58</td>
<td>-7.63</td>
</tr>
</tbody>
</table>

As previously discussed, lead compound 1 is a known agonist (Figure 3.17), and interestingly, when docked to $\alpha_2$-AR-X it did not display any binding interactions with the residues on TM5 or TM6 (e.g. S200$^{5,42}$) which are known to activate the receptor. Alongside the necessary anchoring salt bridge, a further salt bridge and H-bond with D192$^{XL2}$ and E189$^{XL2,51}$ were observed. As mentioned before, the XL2 is bound to TM5 and these interactions may assist in the resulting agonist activity when 1 is bound to the receptor. Further $\pi$-$\pi$ stacking and $\pi$-cation interactions between the diaryl backbone of the ligand and the F390$^{6,51}$ residue assist in increasing the binding affinity of the ligand.
Compound 16 is a known antagonist previously synthesised within the Rozas group, but no computational studies had been carried out on it to date. The autodock binding affinity obtained (-8.16 kcal/mol) correlates with the high binding affinity (Kᵢ = 0.794 nM) of the compound in vitro. As seen from Figure 3.17, this ligand-receptor complex also displays an additional salt bridge and H-bond interaction with E94.2,65

Surgand et al. (2006) suggested that E942,65, amongst other residues on TM1 and TM2 in the monoamine receptors, face antagonist ligands in the binding site, expanding this cavity and directing the ligand away from TM5 to avoid receptor activation.118,122 For this reason, it was expected that the new compound 22c would display this same salt bridge and H-bonding interaction with E942,65. This hypothesis held through when docked in the rigid receptor model α₂A-AR-X but failed when docked to the induced fit model, since the ligand formed a salt bridge with the E189XL2.51 residue instead. However, an induced-fit docking study was also carried out using the α₂A-AR-X structure (crystallized in complex with an

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**Figure 3.15 Induced-fit docking study of lead compound 1**

**Figure 3.16 Induced-fit docking study of compound 16**
 antagonist) as a template, looking only for poses in which the ligand interacts with E94\(^{2.65}\) and D113\(^{3.32}\). This particular pose was awarded an autodock binding affinity of -7.63 kcal/mol which was better than that of the previous orientation.

![Figure 3.17 Induced-fit docking of compound 22c in the α2A-AR-X in complex with an antagonist where only orientations with D113\(^{3.32}\) and E94\(^{2.65}\) were considered](image)

The characterisation of the different hydrogen bonds (HBs) established within the complex, distance and angles, have been carried out. By definition, the distance between the hydrogen and the acceptor atoms when a HB is formed has to be smaller than the sum of their corresponding van der Waals radii.\(^{123}\) The angles of the complex must be greater than 90° to be considered a HB, those nearing 180° are indicative of strong HBs.\(^{123}\) For compounds 1 (Figure 3.18) and 22c (Figure 3.20) the distance between the hydrogen and the acceptor atom has also been used as an indication of HB strength. Distances between 1.2 – 1.5 Å would correspond to very strong HB, 1.5–2.2 Å would be found in strong HB, and 2.0 – 3.0 Å would correspond to weak HB. As seen from Figures 3.18 and 3.20, most of the HB interaction are between 1.69 Å and 2.56 Å, indicating a range from weak-strong interactions.\(^ {123}\)

Two of the main methods used for characterising hydrogen bonds are the Quantum Theory of Atoms in Molecules (QTAIM) proposed by Prof. Richard Bader\(^{124}\) and the analysis of the natural bond orbitals (NBO) developed by Weinhold\(^{125}\). AIM theory defines chemical bonding and structure of a chemical system based on the properties of electron density in a particular point known as the bond critical point, corresponding to the saddle point in the electron density surface between the atoms.\(^ {123}\) This theory was applied to HBs in 1987 by
Bader et al.\textsuperscript{126} The NBO analysis is used to evaluate intermolecular interactions such as HBs.\textsuperscript{123} This analysis transfers the delocalized molecular orbitals (MO) to the localized MO and is usually used to characterise the donation from the lone pair to the antibonding orbital, obtaining the second order energy; thus, the strength of the donation represents the strength of the interaction. Had time allowed, a more in-depth computational study of the various hydrogen bonds would have been carried out.

\textbf{Figure 3.18} Induced-fit docking study of lead compound 1 displaying the HB bond length (Å). The shorter the bond length, the stronger the interaction.
When all three compounds (1, 16, 22c) were fit-induced docked into the α2AR-Y structure (crystallised in complex with a partial agonist, see Appendix 22 - 24) binding interactions with S200<sup>5.42</sup>, which is one of the most frequently reported residues on TM5 involved in receptor activation, were observed. Thus, it is queried that even though compounds 16 and 22c are known antagonists via in vitro studies, the receptor may be locked into a specific conformation when in complex with a partial agonist which results in these specific interactions. For this reason, a third induced-fit docking was carried out on the homology model of Prof. Mireia Olivella (α2AR-MO) as it was prepared without considering any ligand and may result in a clearer characterisation of the potential interaction between the synthesised ligands and the protein residues. Unfortunately, docking to this model did not show any obvious differences between the interactions of the agonist (1) and the antagonists (16 and 22c) and the active or inactive receptors. The main interactions present were H-bonding with S90<sup>2.45</sup> and N93<sup>3.33</sup>, along with several π-π or π-cation interactions with residues on the XL1 and XL2, e.g. W99<sub>XL1</sub>, W413<sub>7.40</sub>, K40<sub>9XL1</sub>. None of the orientations displayed interactions with the known agonist stimulation residues on TM5 (S200<sup>5.42</sup>).

In summary, these docking studies seem to indicate potential for the compounds proposed and synthesised and open the door to future improvements.

Figure 3.19 Induced-fit docking study of lead compound 22c displaying the HB bond length (Å). The shorter the bond length, the stronger the interaction.
3.4 Pharmacological Results and Structure-Activity Discussion

In vitro pharmacological assays were used firstly to evaluate whether the potential ligands of the α₂-AR synthesised during this study bind with an acceptable affinity ($K_i < 100 \text{ nM}$) to the receptor and, secondly to confirm the desired antagonist activity via functional activity assays. All these assays were performed at Prof. Callado’s laboratory (University of the Basque Country, School of Medicine, Leioa, Spain) and they were carried out using human brain tissue which gives a very realistic indication of the suitability of the compounds tested to act in such a complex environment.

3.4.1 Binding Affinity Evaluation

The binding affinities of the tested compounds were measured in human prefrontal cortex (PFC) tissue using a radioligand competitive binding assay with the α₂-AR selective radioligand $[^3\text{H}]RX821002$ (2-methoxyidazoxan; see Figure 3.5) at 2 nM concentrations.

This landmark method that ultimately enabled equivalent interpretations of the affinity for both antagonists and agonists was developed by Paton and Rang in 1965 and corrected by Cheng and Prusoff in 1973, in animal tissues. The Cheng-Prusoff correction allowed for the binding affinity of a non-radiolabelled ligand to be calculated. These studies utilize standard radiolabelled ‘hot’ ligands of known affinity ($K_D$) at a given concentration to calculate the affinity of a competing ligand over a range of concentrations. A graph of the percentage of the bound radioligand vs the negative logarithm of the unknown ligand concentration is plotted and used to determine the IC$_{50}$ (which is the concentration of the unknown ligand when half of the radioligand is displaced). The IC$_{50}$ itself is not a direct measure of the binding affinity as it is dependent on the concentration of the membrane used; however, it can be directly related to the dissociation constant of affinity ($K_i$) using the Cheng-Prusoff equation (Figure 3.5).

![Figure 3.20 A) The structure of the α₂-AR selective radioligand $[^3\text{H}]RX821002$ with a $K_i$ of 1.349 nM. B) Cheng-Prusoff equation where $K_i$ is the binding affinity of the non-radiolabelled ligand, $K_D$ is the binding affinity of the known radioligand, the IC$_{50}$ is determined from the previously obtained plot and represents the concentration of the unknown ligand when half of the radioligand is displaced, $[L_R]$ is the concentration of the radioligand](image)
In the present study these competitive α₂-AR binding assays were carried out in human brain prefrontal cortex tissue in in vitro assays developed by Rozas' collaborator Prof. Callado. The results obtained express as $K_i$ nM values can be seen in Table 3.5.

![Lead Compound 1](image1.png)  ![Family A](image2.png)  ![Family B](image3.png)

**Table 3.7** The α₂-AR binding affinities of both the previously synthesised compounds (1,15-18) and the newly synthesised (19c, 21c, 22c, 25b, 28b) measured as $K_i$ (nM)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Family</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RX821002</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.349</td>
</tr>
<tr>
<td>Idazoxan</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10.47</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>imidazoline</td>
<td>imidazoline</td>
<td>1.585</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>A</td>
<td>H</td>
<td>H</td>
<td>imidazoline</td>
<td>12.30</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>A</td>
<td>(CH₂)₂CH₃</td>
<td>H</td>
<td>imidazoline</td>
<td>0.794</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>B</td>
<td>(CH₂)₂CH₃</td>
<td>H</td>
<td>H</td>
<td>(CH₂)₂CH₃</td>
<td>355</td>
</tr>
<tr>
<td>18</td>
<td>B</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>147.9</td>
</tr>
<tr>
<td>19c</td>
<td>B</td>
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<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>17783</td>
</tr>
<tr>
<td>21c</td>
<td>B</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>263</td>
</tr>
<tr>
<td>22c</td>
<td>B</td>
<td>CH₃CH₃</td>
<td>H</td>
<td>CH₃CH₃</td>
<td>H</td>
<td>95.50</td>
</tr>
<tr>
<td>25b</td>
<td>B</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>CH₃</td>
<td>6456</td>
</tr>
<tr>
<td>28b</td>
<td>B</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
<td>CH₃</td>
<td>8511</td>
</tr>
</tbody>
</table>

The above data can be categorised into three groups to analyse the various effects on the α₂-AR binding affinity of the ligands caused by the variations in the guanidinium functional groups. In Group 1 the effect of the conformational restraint caused by the methylene bridge at the imidazoline moiety of compound 1 is investigated. This derivative is a lead compound within the Rozas group as it displays an excellent α₂-AR binding affinity ($K_i = 1.585$ nM) and
is an agonist as shown by the functional [³⁵S]GTPγS binding experiments, which will be discussed in the following section (Section 3.3.2). When compared to compound 16, which instead of an imidazoline on one end of the structure, has a propyl-substituted guanidine, the binding affinity was greatly improved (Kᵢ = 0.794 nM). This seems to indicate that increased conformational freedom at one of the cationic moieties is favoured within the binding site of the α₂-AR receptor, since the propyl group is free to orient itself inside the binding site in a preferred manner. Considering the computational studies to be discussed in Section 3.4, it can be seen that in the best-pose of docked compound 16 within the α₂A-AR (see Figure 3.17, Section 3.4.1) the propyl group loops back towards the guanidine due to the increased steric hindrance of the lengthy sidechain. However, when the imidazoline ring was substituted by propyl-substituted guanidines at both ends of the diaryl core the α₂-AR binding affinity decreased drastically (Kᵢ = 355 nM), indicating that the methylene bridge of at least one of the imidazoline moieties forces a desired orientation of the whole molecule to drive the initial binding interactions deep within the binding site.

In Group 2 the increased length of the substituents (i.e. aliphatic from compounds 18 (H atom), 21c (CH₃), 22c (CH₃CH₂), 17 (CH₃CH₂CH₂)) is analysed. Only the symmetrical compound 22c (Kᵢ = 95.50 nM) with two ethyl-substituted guanidines was shown to have an α₂-AR binding affinity good enough to be considered for the functional [³⁵S]GTPγS binding assays. This result appears to be an outlier in the trend and could be related to the similarities of the ethyl-substituted guanidine to the imidazoline’s five-membered ring (similar number of C atoms attached to the guanidine-like system). Compound 18 (unsubstituted bis-guanidinium derivative) showed the next best α₂-AR binding affinity (Kᵢ = 147.91 nM), followed by the methyl-substituted guanidine 21c (Kᵢ = 263.03 nM) and the propyl-substituted guanidine 17 (Kᵢ = 345.81 nM) symmetric derivatives. From this trend in α₂-AR binding affinity it could be predicted that an increase in the chain length of the alkyl substituents would result in more steric hindrance within the receptor’s binding site. Moreover, when considering the results concluded from the analysis of Group 1 and Group 2, modifications to compound 22c could result in a further increase to its α₂-AR binding affinity if an asymmetrical compound could be synthesised with an imidazoline moiety and the ethyl-substituted guanidine as seen in Figure 3.6.

![Figure 3.21 A future compound to be investigated based on the results from group 1 and group 2](image)

Finally, in Group 3 the comparison between the closed imidazoline ring and open alkyl substituted guanidines at breaking points (A) and (B) as shown in Figure 3.7 is discussed.
As expected from the outcomes of Group 1 analysis, there was a decrease in the α₂-AR binding affinity by opening the methylene bridge of the imidazoline ring at both ends of the molecule. There was a drastic increase in the binding for compound 19c ($K_i = 17782\ nM$) which correlates with the increased steric bulk caused by the dimethyl-substituted guanidine. Furthermore, in the computational studies presented in Section 3.4, it can be seen that both the ethyl- and propyl-substituted guanidines’ preferred orientation exhibits the alkyl chain curling back towards the guanidine; however, this conformation is not possible in the case of the dimethyl-substituted guanidine system. The remaining tested compounds, 25b ($K_i = 6456\ nM$) and 28b ($K_i = 8511\ nM$), were both asymmetrical molecules containing the dimethyl-guanidine in one side and both of them gave very low α₂-AR binding affinities further indicating the disfavouring of guanidine arrangement. Even the slight increase in the $K_i$ values of compound 1 to compound 22c additionally suggests the benefit of further investigating the asymmetric compound proposed in Figure 3.6 above.

![Figure 3.22 Schematic representation of the ring opening possibilities. Incision at the A) position resulted in the mono-ethyl substituted guanidinium and the B) position resulted in the dimethyl substituted guanidine](image)

### 3.4.2 Functional Activity Assays

Functional $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding experiments are used to determine whether the tested ligands are antagonists, agonists or inverse agonists toward the α₂-ARs. These assays directly measure the guanine nucleotide exchange of G proteins, an early event after GPCR activation, which is not subjected to amplification or regulation by other cellular processes. A non-hydrolysable radiolabelled analogue of GTP ($[^{35}\text{S}]\text{GTP}\gamma\text{S}$) is used to facilitate measurement of GPCR activation after the addition of a known agonist by measuring the amount of radiolabelled $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ is bound to the cell membrane after washing away the unbound $[^{35}\text{S}]\text{GTP}\gamma\text{S}$. This particular GTP is labelled on the γ-phosphate with $^{35}\text{S}$, thus hydrolysable GTP cannot be used as it would convert to GDP too quickly, removing this radiolabelled γ-phosphate and then escaping detection.

GPCRs exhibit constitutive activity which refers to the ability of a GPCR to undergo agonist-independent isomerization from an inactive/resting (R) state where the GPCR is uncoupled
from the G-proteins, to an active (R*) state. The first evidence for this constitutive activity of GPCRs was obtained for the δ opioid receptor (Koski et. al, 1982)and β2-adrenoceptor (Cerione et. al, 1984). The full agonist stabilizes the active R* state of GPCRs. The conformational change in GPCRs associated with the R to R* isomerization enables the dissociation of GDP from the G-proteins; thus, resulting in the GDP to [35S]GTPγS exchange which can be monitored during the functional assay. In the case of full inverse agonists, they maximally stabilize the R state and reduce basal GDP/[35S]GTPγS exchange. Finally, inverse agonists block and decrease the activity of an agonist which, prior to the development of highly sensitive model systems and GPCR mutants, were often mistaken for antagonists. Antagonists do not alter the activity of the GPCR, rather they block both the inhibitory effects of an inverse agonist and the excitatory effects of the agonist.

 Compounds which displayed an affinity less than 1 μM (Ki < 100 nM) were subjected to in vitro [35S]GTPγS binding experiments in human prefrontal cortex tissue to determine their nature as agonists or antagonists in the laboratory of Prof. Callado, and the results obtained are displayed in Table 3.6.

**Table 3.8 Results for the [35S]GTPγS exchange functional assay in PFC human tissue.** See Table 3.5 for the general structures of families A and B.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Family</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>[35S]GTPγS Binding Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RX821002</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td>Antagonist</td>
</tr>
<tr>
<td>Idazoxan</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td>Antagonist</td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>H</td>
<td>H</td>
<td>Imidazoline</td>
<td>Imidazoline</td>
<td>Agonist</td>
</tr>
<tr>
<td>15</td>
<td>A</td>
<td>H</td>
<td>H</td>
<td>Imidazoline</td>
<td>Imidazoline</td>
<td>Agonist</td>
</tr>
<tr>
<td>16</td>
<td>A</td>
<td>(CH₂)₂CH₃</td>
<td>H</td>
<td>Imidazoline</td>
<td>Imidazoline</td>
<td>Antagonist</td>
</tr>
<tr>
<td>18</td>
<td>B</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Agonist</td>
</tr>
<tr>
<td>22c</td>
<td>B</td>
<td>CH₂CH₃</td>
<td>H</td>
<td>CH₂CH₃</td>
<td>H</td>
<td>Antagonist</td>
</tr>
</tbody>
</table>

As seen in Table 3.5 (Section 3.3.1), most of the newly synthesised compounds did not show the level of α₂-AR binding necessary to carry out the in vitro [35S]GTPγS functional assay. Compounds 19c, 21c, 25b, 28b, had binding affinities (Ki) >100 nM largely due to the unfavoured methyl groups present in the guanidine systems. Only the symmetrical bis-guanidinium compound 22c demonstrated a strong enough binding to the α₂-AR to be tested in the functional assay. Satisfactorily, this compound showed to act as an α₂-AR antagonist, not modifying the basal [35S]GTPγS binding to the receptor, which was desired.
as it would result in blocking the activity of an agonist at the receptor and could be used as a potential antidepressant. In Section 3.4, the computational studies performed with compounds 16 and 22c suggest that the alkyl chain preferred to orient itself towards the guanidine in a pseudo-cyclic formation. These compounds were both α2-AR antagonists contrary to the lead compound 1 which was an agonist; this may suggest that the conformational freedom of the guanidinium substituents in these two compounds may be identified as preferred antagonist features in comparison to the rigid imidazoline moieties of the agonist. We have previously seen how such minute changes in ligand structure can cause a drastic change in the ligand’s functional activity at the α2-AR receptor. The new compound 22c had a high α2-AR binding compared to the other bis-guanidines studied; thus, it is expected that the addition of an imidazoline ring at one end could result in a new hit compound (Figure 3.6, Section 3.3.1) in further studies and following the trend of compounds 16 and 22c, it could also result in the target antagonist activity.
4. Conclusions and Future Work

The aim of this research was to investigate the effects on the binding affinity and ligand-receptor functional activity when opening the rigid 2-aminoimidazolium moiety of the lead compound 1 thus affecting steric and lipophilic properties. This was done by exploring the potential drug-likeness of the compounds prepared by calculating a number of pharmacokinetic and physicochemical parameters, synthesising five new compounds followed by an *in vitro* pharmacological evaluation of the binding affinity and functional activity of the compounds prepared and finally studying the binding interactions between various ligands and the $\alpha_{2A}$-AR via molecular docking.

4.1 Physicochemical parameters

Using different software such as SwissADME and Chemaxon, relevant parameters (e.g. Ro5 parameters, rotatable bonds, TPSA, metabolic stability towards cytochrome P415 enzymes, solubility, ability to cross GI or BBB membranes) were calculated for all the compounds proposed indicating their suitability as potential drugs.

4.2 Synthesis

Through the course of this study, five new compounds were synthesised (19c, 21c, 22c, 25b, 28b). It was determined that the Kim and Qian method for guanidylation was the most effective in the synthesis of these compounds. As described in Section 3.2.2.2, this method required the coordination of mercury (II) chloride ($\text{HgCl}_2$) to the sulfur of a suitable thiourea, in the presence of triethylamine, to initiate the reaction with primary amines. For the unsubstituted guanidinium derivatives (25b and 40a) there was no need to synthesise any starting materials as the commercially available $N,N'$-bis-($\text{tert}$-butoxycarbonyl)-$\text{S}$-methylisothiourea was a cost-effective and efficient alternative. For the rest of the compounds prepared, the di-Boc-protected $N,N'$-dimethylthiourea (19a, 54.69%) and $N,N'$-diethylthiourea (20a, 74.63%) had to be prepared. Thus, the Boc-protection is a straightforward, anhydrous reaction in which NaH (60% immersion in oil) activated the relevant thiourea, which was then reacted with di-$\text{tert}$-butyl-dicarbonate. However, this method of preparing the Boc-protected thiourea was unsuccessful for the methyl- and ethyl-substituted thioureas (21a and 22a), and thus, alternative approaches were attempted to prepare the mono-substituted bis-guanidylated products (Section 3.2.2.1). Finally, a new method for the preparation of mono-substituted di-Boc protected thioureas within the Rozas group was established using the Mitsunobu reaction conditions. This method provided a mono-
substituted pseudothiourea that was also bis-Boc-protected, thus providing the basis for an easier desulfurization during guanidylation. Moreover, the use of the pseudothiourea leaves only one amine available for alkylation, thus reducing the risk of unwanted side-products forming and purification is simplified. This method was used to synthesize 21a and 22a with good yields (80.8% and 94.8%, respectively).

Boc-deprotection was carried out using a 50% solution of TFA in DCM, in excess and stirred overnight, at room temperature to yield the guanidine trifluoroacetate salts. However, since these salts are insoluble in water what is a requisite for the pharmacological evaluations, an ion exchange was carried out using activated Amberlite® IRA-400 resin in its chloride form. Accordingly, the guanidine hydrochloride salts of compounds 19c, 21c, 22c, 25b, 28b were obtained in good yield (>85%).

**Scheme 4.1 General reaction scheme illustrating the successful synthesis of the five final compounds**

![Scheme 4.1 General reaction scheme illustrating the successful synthesis of the five final compounds](image)

4.3 Computational Studies

The docking studies were carried out using the software Maestro (Schrödinger Inc.). The molecules were docked into three different α2-AR models: α2A-AR-MO (an homology model developed by Prof. Mireia Olivella from the Universitat de Vic in Spain, before any α2-AR crystal structures were published), α2A-AR-Y (6KUY: crystal structure of the α2A-AR in complex with a partial agonist), α2A-AR-X (6KUX: crystal structures of the α2A-AR in complex with an antagonist). The crystal structures of the α2A-AR were retrieved from the RCSB PDB to be used for docking studies. Standard and induced-fit docking studies were carried out and the chosen orientations of the docked compounds were based on the ionic interaction with the aspartate residue D113 as this has been reported to be a critical anchoring interaction with the α2-AR binding sites. Both rigid receptor models and induced-fit docking were carried out on compounds 1, 16 and 22c. As expected, the autodock binding affinity

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related to those compounds were increased drastically based in the receptor’s ability to orient itself to better fit the ligand docked.

The previously synthesised agonists, 15 and 18, displayed H-bonding with S200\textsuperscript{5,42}, a known residue involved in agonist activity. Additional salt bridge and H-bonding interaction with the D192\textsubscript{XL2} and E189\textsubscript{XL2,51} residues were also common between compounds 1, 15, 18. The XL2, which is directly linked to TM5, could be participating in directing the ligand towards TM5 to initiate the receptor activation. Moreover, the salt bridge interaction between the ligand and the E94\textsuperscript{2,65} residue is a common denominator amongst the two known antagonists, 16 and 22c since these interactions could assist in directing the ligand away from TM5, thus avoiding receptor activation.

4.4 Pharmacological Studies

In collaboration with Prof. Callado at the Department of Pharmacology at the School of Medicine in the University of Basque County UPV/EHU (Spain), in vitro pharmacological studies were carried out of the successfully synthesised compounds to determine their affinity for the \(\alpha_2\)-AR (\(K_i\) values) and their functional activity on the receptor (agonist or antagonist) in human brain prefrontal cortex (PFC) tissue. These in vitro studies could only be carried out if the compounds to be tested are above 95% pure, thus the purity of all compounds prepared was proved to be within this threshold by HPLC analysis before sending the compounds to Prof. Callado’s laboratory.

The binding affinities of the tested compounds were measured in human PFC tissue using a radioligand competitive binding assay with the \(\alpha_2\)-AR selective radioligand \[^{3}H\]RX821002 (2-methoxyidazoxan) at 2 nM concentrations. Only one of the five newly synthesised compounds showed a strong enough binding affinity to be tested for its functional activity in the \[^{35}S\]GTP-\(\gamma\)-S exchange assay (22c, 95.50 nM). The corresponding SARs were deduced comparing different aspects of the compounds. First, comparison of the conformational freedom provided by the opening of the imidazolinium moiety to form the previously synthesised compound 16 was considered. The release of the conformational restraint on one end forming an asymmetrical product greatly improved the binding affinity from 1.585 nM (1) to 0.79 nM (16); however, by releasing both imidazolium moieties the binding affinity was negatively impacted resulting in a \(K_i\) of 355 nM (17). Therefore, it was concluded that maintaining one imidazolium moiety within the compound would assist in driving the initial binding, due to the conformational restraint potentially forming a strengthened anchoring system with the D113\textsuperscript{3,32} residue. Secondly, the increased carbon chain length appeared to be inversely proportional to the binding affinities for the symmetric compounds 18 (unsubstituted guanidines, 147.9 nM), 21c (methyl substituted, 263.03 nM), 17 (propyl substituted, 355 nM). From this trend in the data it would be predicted that the increase in
alkyl chain length would result in more steric hindrance within the receptor’s binding site. Compound 22c (ethyl substituted, 95.50 nM) was an outlier as it had a very promising binding affinity, which was unexpected for the symmetrical mono-substituted bis-guanidiniums. As seen in the computational studies, this substituent appeared to fold back towards the guanidinium, potentially occupying the desired space within the receptor and forming weak van der Waals contacts with the residues within the binding site. On the other hand, the binding affinities of the compounds decreased dramatically when the dimethyl substituted guanidinium derivatives were tested, with the symmetrical di-substituted molecule resulting in the highest Ki value (17782.79). This is indicative of the disfavoured conformation formed by the methyl substituents.

Only compound 22c had a binding affinity within the threshold necessary to test functional activity. As confirmed with the computational results, this compound was an antagonist which was the desired result for potential antidepressant activity. As aforementioned this compound achieved the lowest Ki value for binding affinity of all the alkyl substituted bis-guanidiniums (95.50 nM).

4.5 Future work

From the comparative study between the previously synthesised compounds 1, 16, 17 and the newly synthesised 22c it was determined that a further investigation into a compound containing both an imidazolium moiety and an ethyl-substituted guanidinium could potentially demonstrate a very promising binding affinity along with potential antagonist activity. Moreover, a more detailed computational study would be carried out on the characterization of the HBs of each of the compounds.

Figure 4.1 Compounds 41a and 42a theoretically fit the profile of some good binding compounds to the α2A-AR with antagonist activity
5. Experimental

5.1 General Materials and Methods

All solvents used in this project were analytical (HPLC) grade. All chemicals and reagents used were supplied by Merck (Sigma Aldrich), Fischer or VWR and were used as received. All glassware was pre-dried in an oven before use with anhydrous solvents. Anhydrous THF was obtained from the PureSolv MD-4EN Solvent Purification System (SPS) using molecular sieves. All glassware was washed with water and acetone before use. Silica gel 40 – 63 µm (Merck, 230-400 mesh) was used for gravity column chromatography. All compounds were subject to this purification unless stated otherwise. The analytical thin layer chromatography (TLC) was carried out with silica gel 60 (fluorescence indicator F254; Merck) or aluminium oxide 60 (F254, neutral; Merck) TLC plates and visualised under UV radiation (Spectroline, model ENF-204C/FE).

The proton, carbon and fluorine NMR spectra were carried out at room temperature using a Brucker 400 MHz UltraShield™. The spectra were recorded at 400 MHz and 600 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR. TMS was used as the internal standard reference ($^1$H, δ = 0.00 ppm) with the remaining chemical shifts appearing downfield from the reference. All Boc-protected samples were dissolved in deuterated chloroform (CDCl₃) and the final salts were performed in deuterated water (D₂O), unless stated otherwise. A rapid exchange occurs between the protons of the NH₂ substituents on the final hydrochloride salts and the deuterium within the D₂O NMR solvent, thus these proton signals do not appear on the attached spectra (See Appendices). The NMR data was processed using the Bruker TOPSPIN and MestReNova software.

FTIR spectra was obtained using a Perkin Elmer spectrum 100 FT-IR spectrometer and fitted with a Universal Attenuated Total Reflectance (ATR) sampling accessory and the ATR method was used throughout the project.

The melting point was determined using a Stuart Scientific Melting Point SMP1 apparatus.

Electrospray Ionisation (ESI) mass spectra were obtained in positive and negative modes, as required, using a Micromass time of flight (TOF) mass spectrometer with a WATERS 2690 HPLC autosampler using methanol or chloroform as the carrier solvent.

Reverse phase HPLC was used to determine that the purity of the final hydrochloride salts was above the minimum requirement of 95.0 %. This was done using a Varian ProStar system equipped with a Varian Prostar 335 diode array detector and a manual injector (20 µL), scanning wavelengths from 200 to 950 nm. For purity assessment, UV detection was performed at 245 nm and peak purity was confirmed using a purity channel. The stationary
phase consisted of an ACE 5 C18-AR column (150 mm × 4.6 mm), and the mobile phase used the following gradient system, eluting at 1 mL/min: aqueous formate buffer (30 mM, pH 3.0) for 10 min, linear ramp to 85% methanol buffered with the same system over 25 min, hold at 85% buffered methanol for 10 min.

5.2 Computational Details

The computational docking studies were carried out using the software maestro (Schrödinger Inc.). The ligands were generated via the LigPrep function with default ionizer and tautomerizer and the protein preparation was carried out using the Protein Preparation Wizard (PrepWizard) in Maestro.

Structures for the induced-fit docking were prepared using the Maestro software package and aligned using the Protein Structure Alignment module in Prime.

The structures of the α2A-AR complexed with a partial agonist (6KUY) or with an antagonist (6KUX) were retrieved from the RCSB PDB to be used for docking studies. Induced docking was carried out, and only ligands which interact with ASP113 were considered.

5.3 General Chemical Procedures

**Method A: Boc-protection of unsubstituted thiourea**

![Chemical structure](image)

To a solution of commercially available, unsubstituted thiourea (1.0 eq.) in dry THF, sodium hydride 60 % immersion in oil (4.5 eq.) was added carefully at 0 ºC with stirring. The reaction was slowly brought to room temperature and allowed to stir for 45 minutes. The reaction was then cooled back down to 0 ºC and di-tert-butyl-dicarbonate (2.2 eq., 57.794 µmol) was added. The reaction was brought back to room temperature and allowed to stir overnight.

Upon completion, the reaction was quenched slowly using NaHCO₃ saturated solution (10 cm³) and the solvent was reduced. The solid obtained was re-dissolved in DCM and washed with water (3x 20 cm³). The combined organic layers were dried over MgSO₄, filtered and concentrated in vacuum.

The product was then recrystallised from hot combination solvents, hexane and ethyl acetate, to obtain the purified product.
Method B: Boc-protection of N,N'-disubstituted thiourea

\[
\begin{array}{c}
\text{R} \overset{\text{S}}{\text{S}} \text{N} \overset{\text{N}}{\text{N}} \text{R} \\
\text{Boc}_2\text{O}, \text{NaH} \\
\text{anh. THF, r.t., 24 hr} \\
\end{array}
\rightarrow
\begin{array}{c}
\text{R} \overset{\text{S}}{\text{S}} \text{N} \overset{\text{N}}{\text{N}} \text{R} \\
\text{Boc} \\
\text{Boc} \\
\end{array}
\]

To a solution of commercially available N,N'-disubstituted-thiourea (1.0 eq.) in dry THF, sodium hydride 60% immersion in oil (4.5 eq.) was added carefully at 0 °C with stirring. The reaction was slowly brought to room temperature and allowed to stir for 45 minutes. The reaction was then cooled back down to 0 °C and di-tert-butyl-dicarbonate (2.2 eq.) was added. The reaction was brought back to room temperature and allowed to stir overnight.

Upon completion, the reaction was quenched slowly using NaHCO₃ saturated solution (25 cm³) and added to deionised water (150 cm³) and extracted with ethyl acetate. The combined organic phases were then washed with brine. The extracted organic phase was dried over MgSO₄, filtered and concentrated in vacuum.

The purified product was obtained by gravity column chromatography using the appropriate ratio of hexane and ethyl acetate.

Method C: Synthesis of mono Boc-protected N-substituted thiourea

Unsubstituted thiourea (1.0 eq.) was dissolved in dry THF (110 cm³) under argon gas and brought to 0 °C before adding the NaH 60% immersion in oil (4.5 eq.). The solution was stirred in the ice-bath for 5 minutes to allow the reagents to fully dissolve. Once complete the reaction was stirred at room temperature for 45 minutes before being cooled to 0 °C once more. Di-tert-butyl-dicarbonate (2.5 eq.) was added. After 30 minutes stirring at 0 °C, the ice bath was removed, and the reaction was allowed to stir overnight at room temperature.

After monitoring the formation of the N,N'-bis-Boc-thiourea by TLC, the reaction was cooled back to 0 °C and NaH 60% immersion in oil (1.7 eq.) was added carefully. After 1 hour, TFA anhydride (1.54 eq.) was added. After a further 1 hour of stirring at 0 °C, methylamine hydrochloride (1.54 eq.) was added and allowed to fully immerse in solution before the removal of the ice-bath. The reaction was gradually brought back to room temperature and allowed to stir overnight.
Upon completion, the reaction was cooled to 0 °C and quenched with deionised water (40 cm³), dropwise. The product was extracted using ethyl acetate (4 x 50 cm³) and the combined organic layers were washed with brine (4 x 50 cm³). The organic phase was dried over MgSO₄, filtered and concentrated under vacuum. Purification of the product by column chromatography using a hexane and ethyl acetate gradient mixture was carried out to afford the final product.

**Method D:** *General procedure for the bis-Boc-protected N-substituted pseudothiourea*³¹

\[
\begin{align*}
\text{DEAD} & \quad \text{PPh₃} \\
\text{anh. R-OH} & \quad \text{anh. THF} \\
\text{anhydrous} & \quad -5 \degree C - 0 \degree C, \text{o/n}
\end{align*}
\]

A solution of 1,3-Bis(tert-butoxycarbonyl)-2-methyl-2-pseudothiourea (1 eq.), anhydrous alcohol (1.5 eq.), and triphenylphosphine (1.5 eq.) in dry THF (15 cm³) under argon gas was cooled to -5 °C. Diethyl azodicarboxylate (3 eq.) was added dropwise at a rate such that the reaction mixture was completely colourless before the addition of the next drop. The reaction was then gradually brought to room temperature and stirred overnight. The reaction was monitored by TLC. Once complete, methanol (10 cm³) was added to the solution and the solvent was reduced. The product was purified by flash chromatography a hexane and ethyl acetate gradient mixture, yielding the final compound.

**Method E:** *General procedure for the synthesis of Boc-protected mono-guanidine derivative*⁴³

\[
\begin{align*}
\text{HgCl₂, NEt₃} & \quad \text{DCM} \\
\text{r.t., o/n}
\end{align*}
\]

4,4’-Methylenedianiline (3.0 eq.) was dissolved in DCM, cooling the mixture below 0 °C, and reacted with \(N,N’\)-di(tert-butoxycarbonyl)thiourea (1.0 eq.). The mixture was treated with HgCl₂ (1.5 eq.) and NEt₃ (6 eq.) and stirred for 15 minutes at 0 °C. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete.

The reaction was filtered through Celite and rinsed with DCM to remove any of the mercury by-product (mercury chloride and mercury sulfide mixture). The filtrate was extracted using
DCM (3 x 20 cm³) and then washed with deionised water (3 x 20 cm³). The combined organic layers were dried over magnesium sulphate (MgSO₄), filtered and concentrated using the rotary evaporator to obtain the crude product. The product was then purified by gravity column chromatography using the appropriate ratio of hexane and ethyl acetate.

**Method F: Synthesis of Boc-protected N,N'-disubstituted mono-guanidine derivative**

\[
\text{Boc}_2N\text{S}_2\text{Boc} + \text{H}_2\text{N}-\text{NH}_2 \xrightarrow{\text{HgCl}_2, \text{NET}_3, \text{DMF}} \text{R}_2\text{N}-\text{NH}_2
\]

4,4'-Methylenedianiline (3.0 eq.) was dissolved in DMF and cooled below 0 °C. The respective Boc-protected N,N'-disubstituted thiourea (1 eq.) was added to the mixture and then treated with HgCl₂ (1.5 eq.) and sodium bicarbonate (6 eq.). The reaction mixture was stirred for 15 minutes at 0 °C and then gradually brought back to room temperature and stirred overnight.

The reaction was monitored by TLC and once complete it was filtered through a bed of Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (2 x 20 cm³) and extracted using DCM (3 x 20 cm³) and deionised water (3 x 20 cm³). The combined organic layers were dried over magnesium sulphate (MgSO₄), filtered and concentrated using the rotary evaporator to obtain the crude product. The product was then purified by gravity column chromatography using the appropriate ratio of hexane and ethyl acetate.

**Method G: General procedure for the synthesis of Boc-protected disubstituted bis-guanidine derivatives**

\[
\text{Boc}_2N\text{S}_2\text{Boc} + \text{H}_2\text{N}-\text{NH}_2 \xrightarrow{\text{HgCl}_2, \text{NET}_3, \text{DMF}} \text{R}_2\text{N}-\text{NH}_2
\]

4,4'-Methylenedianiline (1.0 eq.) was dissolved in DMF at 0 °C and reacted with N,N'-di(tert-butoxycarbonyl)disubstituted thiourea (2.5 eq.). The mixture was treated with HgCl₂ (1.5 eq.) and an excess of sodium bicarbonate (6 eq.) and stirred for 15 minutes at 0 °C. The reaction was gradually brought back to room temperature and stirred overnight. The reaction was monitored by TLC analysis.

Upon completion the mixture was filtered through a bed of Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (2 x 20 cm³) and
extracted using DCM and deionised water (3 x 20 cm³). The combined organic layers were dried over magnesium sulphate (MgSO₄), filtered and concentrated using the rotary evaporator to obtain the crude product.

The product was then purified by gravity column chromatography using the appropriate ratio of hexane and ethyl acetate.

**Method H: General procedure for the synthesis of Boc-protected mono-substituted bis-guanidine derivatives**

![Chemical structure Diagram]

4,4'-Methylenedianiline (1.0 eq.) was dissolved in DCM below 0 °C and reacted with the respective N,N'-di(tert-butoxycarbonyl)monosubstituted thiourea (2.5 eq.). The mixture was treated with HgCl₂ (1.5 eq.) and an excess of triethylamine (6 eq.). The reaction was gradually brought back to room temperature and stirred overnight. The reaction was monitored by TLC analysis.

Upon completion the mixture was filtered through a bed of Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (2 x 20 cm³) and extracted using DCM and deionised water (3 x 20 cm³). The combined organic layers were dried over magnesium sulphate (MgSO₄), filtered and concentrated using the rotary evaporator to obtain the crude product.

The product was then purified by gravity column chromatography using the appropriate ratio of hexane and ethyl acetate.

**Method I: Synthesis of the hydrochloride salts – TFA method**

![Chemical structure Diagram]

The relevant Boc-protected sample (400 mg) was dissolved in an excess of 50% TFAA dissolved in DCM (20 cm³) and stirred at room temperature for 3-6 hours. The solvent was then reduced by rotary evaporation. The remaining TFA salt was re-dissolved in deionised water (20 cm³) and treated with Amberlite IRA 400 chloride form and allowed to stir gently for 48 hours at room temperature. When the reaction was judged complete using the TLC method, the product was extracted using DCM (3 x 20 cm³). The combined organic layers
were dried over MgSO₄, filtered and concentrated in vacuum to obtain the hydrochloride salt.

*Synthesis of 1,1'-Methylenebis(4-isothiocyanatobenzene)*

4,4'-Methylenedianiline (1.0 eq.) was dissolved in dry DCM and allowed to stir at 0 ºC for 10 minutes before the addition of 1,1'-thiocarbonyl bis-imidazole (2.2 eq.). The reaction was gradually brought to room temperature and stirred for 5 hours. The reaction was judged complete using the TLC method. The solvent was reduced by rotary evaporation to obtain a red-brown solid.

The product was purified using a 6:4 ratio of hexane and ethyl acetate, respectively, in a gravity column chromatography.

*Synthesis of 1,1'-(Methylene-di-4,1-phenylene)bis(thiourea)*

To a cooled solution of *tert*-butyl carbamate (2.5 eq.) in dry THF (8 cm³) at 0 ºC, NaH 60 % immersion in oil (9 eq.) was added. The reaction mixture was stirred at 0 ºC for 20 minutes before adding the previously synthesised 1,1'-Methylenebis(4-isothiocyanatobenzene). The solution was brought to room temperature and allowed to stir for 5 hours. The reaction was monitored by TLC until complete.

Once complete, the reaction was quenched with deionised water (5 cm³) and stirred for a further 15 minutes. The solvents were reduced by rotary evaporation and the solid obtained was re-dissolved in DCM and washed with deionised water (6 x 20 cm³). The crude product was purified using gravity column chromatography with an 8:2 ratio of hexane and ethyl acetate respectively.

*Synthesis of 1,1'-(Methylenedi-4,1-phenylene)-N-ethoxycarbonyl-bis(thiourea)*
To a solution of 4,4'-methyleneedianiline (1.0 eq.) dissolved in anhydrous DCM under argon, ethoxycarbonyl isothiocyanate (2.2 eq.) was added below 0 °C was added. After 15-20 minutes of stirring below 0 °C, the reaction was gradually brought back to room temperature and stirred overnight. The reaction was monitored by TLC and once deemed complete the solvent was removed. The product was purified by column chromatography using the appropriate hexane/ethyl acetate gradient.

5.3 Synthesis and Characterisation

\[ N,N'-(\text{Di-tert-butoxycarbonyl})-\text{thiourea (35a)} \]

Following Method A, at approximately 0 °C NaH 60 % immersion in oil (2,166 mg, 4.5 eq., 54.05 mmol) was added to a solution of thiourea (916 mg, 1 eq., 12.03 mmol) dissolved in dry THF (205 cm\(^3\)). The reaction mixture was brought to room temperature and stirred for 45 minutes. The reaction was cooled back to 0 °C and di-tert-butyl-dicarbonate (7,867 mg, 3 eq., 36.09 mmol) was added. The reaction was brought back to room temperature and stirred overnight.

Upon completion, the reaction was quenched slowly using NaHCO\(_3\) saturated solution (10 cm\(^3\)) and the solvent was reduced. The solid obtained was re-dissolved in DCM and washed with water (3x 20 cm\(^3\)). The combined organic layers were dried over MgSO\(_4\), filtered and concentrated in vacuum.

The product was then recrystallised from hot combination solvents, hexane and ethyl acetate, to obtain 35a (1.4156 g, 42.6%) a white crystalline solid.

Yield: 1.4156 g, 42.6%

Molar Mass: 276.35 g mol\(^{-1}\)

MP: 130 – 134 °C. (Lit. 131 – 135 °C)\(^{137}\)

\( \delta_H \) (400 MHz, CDCl\(_3\)): 1.54 (s, 18H, CH\(_3\)-Boc)

\( \delta_C \) (100 MHz, CDCl\(_3\)): 27.98 (CH\(_3\), Boc), 84.11 (q-C, Boc), 150.30 (C=O, Boc), 177.79 (C=S)
Using Method E, 4,4'-Methylenedianiline (3.0 eq., 1.024 mg, 5.166 mmol) was dissolved in DCM at 0 °C and reacted with \(N,N'\)-(tert-butoxycarbonyl)-thiourea (1.0 eq, 476 mg, 1.722 mmol). The mixture was treated with HgCl\(_2\) (1.5 eq., 701.28 mg 2.583 mmol) and NEt\(_3\) (6 eq., 1.44 cm\(^3\), 10.332 mmol, 0.726 g cm\(^{-3}\)) and stirred for 15 minutes at 0 °C. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete.

The reaction was filtered through Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (2 x 20 cm\(^3\)) and extracted using DCM and deionised water (3 x 20 cm\(^3\)). The combined organic layers were dried over magnesium sulphate (MgSO\(_4\)), filtered and concentrated using the rotary evaporator to obtain the crude product.

Gravity column chromatography was used to yield the purified product 40a (500.3 mg, 63.62%) using the appropriate ratio of hexane and ethyl acetate (8:2, respectively).

**Yield:** 500.3 mg, 63.62%

**Molar Mass:** 440.54 gmol\(^{-1}\)

\[\delta_H (400MHz, CDCl_3): \ 1.54 \ (d, \ 18H, \ CH_3-Boc), \ 3.86 \ (s, \ 2H, \ CH_2), \ 6.67 \ (d, \ 2H, \ J = 8Hz, \ H-2, \ H-6), \ 6.97 \ (d, \ 2H, \ J = 8Hz, \ H-2', \ H-6'), \ 7.15 \ (d, \ 2H, \ J = 8Hz, \ H-3, \ H-5), \ 7.52 \ (d, \ 2H, \ J = 8Hz, \ H-3', \ H-5').\]

\[\delta_C (100MHz, CDCl_3): \ 28.09 \ (CH_3), \ 40.50 \ (CH_2), \ 83.60 \ (q, \ Boc), \ 115.97 \ (Ar, \ H-2, \ H-6), \ 122.30 \ (Ar, \ H-2', \ H-6'), \ 129.23 \ (Ar, \ H-3, \ H-5), \ 129.84 \ (Ar, \ H-3', \ H-5'), \ 132.14 \ (q, \ Ar), \ 138.27 \ (q, \ C-N), \ 152.89 \ (C=N), \ 163.45 \ (C=O)\]

\(N,N'-(Di-tert-butoxycarbonyl)dimethylthiourea (19a)\)
Using Method B, sodium hydride 60 % immersion in oil (933 mg, 4.5 eq, 38.88 mmol) was added to a solution of \( N,N' \)-dimethylthiourea (1.0 eq, 900 mg, 8.64 mmol) in dry THF (200 cm\(^3\)) at 0 °C with stirring. The reaction was slowly brought to room temperature and allowed to stir for 45 minutes. The reaction was then cooled back down to 0 °C and di-\( \text{tert} \)-butyl-dicarbonate (4,148 mg, 2.2 eq, 19.01 mmol) was added carefully. The reaction was brought back to room temperature and allowed to stir overnight.

Upon completion, the reaction was quenched slowly using NaHCO\(_3\) saturated solution (25 cm\(^3\)) and added to deionised water (150 cm\(^3\)) and extracted with ethyl acetate. The combined organic phases were then washed with brine. The extracted organic phase was dried over MgSO\(_4\), filtered and concentrated in vacuum.

The product was purified by gravity column chromatography using the appropriate ratio of hexane and ethyl acetate (9:1, respectively) to yield 19a (1,436.7 mg, 54.69%) a yellow oil.

**Yield:** 1,436.7 mg, 54.69%

**Molar Mass:** 304.41 gmol\(^{-1}\)

\( \delta_H \) (400MHz, CDCl\(_3\)): 1.45 (s, 18H, CH\(_3\)-Boc), 3.46 (s, 6H, CH\(_3\))

\( \delta_C \) (100MHz, CDCl\(_3\)): 28.02 (CH\(_3\), Boc), 39.51 (C-N), 83.36(q-C, Boc), 151.13 (C=O, Boc), 191.21 (C=S)

\( \nu_{\text{max}} \) (ATR)/cm\(^{-1}\): 2978.59 (N-H), 2939.40, 1718.14 (C=O), 1428.16 (CH\(_3\)), 1394.08, 1368.72, 1317.15, 1275.81, 1250.26 (C-N), 1159.95 (C-O), 1095.15 (C=S), 1055.89, 980.87, 906.44, 850.78, 756.55, 661.57, 613.50

**HRMS** (m/z ESI\(^+\)): Calculated for C\(_{13}\)H\(_{24}\)N\(_2\)O\(_4\)S, [M] 304.15, Found: [M+Na\(^+\)] 327.1355

R\(_f\): 7:3 Hexane/Ethyl Acetate, 0.76

4-Amino-4'-[(2,3-di(\text{tert}-butoxycarbonyl)-\( N,N' \)-dimethyl)guanidino]diphenylmethane (39a)

\[ 39a \]
Using Method F, 4,4’-methyleneedianiline (977.42 mg, 3.0 eq., 4.93 mmol) was dissolved in DMF at 0 °C and reacted with 19a (500 mg, 1 eq., 1.64 mmol). The mixture was treated with HgCl₂ (667.89 mg, 1.5 eq., 2.46 mmol) and sodium bicarbonate (826.63 mg, 6 eq., 9.84 mmol) and stirred for 15 minutes at 0 °C. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete. The reaction was filtered through Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (2 x 20 cm³) and extracted using DCM and deionised water (3 x 20 cm³). The combined organic layers were dried over magnesium sulphate (MgSO₄), filtered and concentrated using the rotary evaporator to obtain the crude product.

Gravity column chromatography, using the appropriate ratio of hexane and ethyl acetate, was used to afford 39a (457.3 mg, 49.7%).

Yield: 457.3 mg, 46.7%

Molar Mass: 468.60 g mol⁻¹

δ_H (400MHz, CDCl₃): 1.50 (s, 9H, CH₃, Boc-9), 1.43 (s, 9H, CH₃, Boc-10), 2.64 (s, 3H, CH₃, H-7), 3.33 (s, 3H, CH₃, H-8), 3.86 (s, 2H, CH₂), 6.68 (d, 2H, J 8Hz, H-2, H-6), 6.81 (d, 2H, J 8Hz, H-2', H-6'), 6.99 (d, 2H, J 8Hz, H-3, H-5), 7.13 (d, 2H, J 8Hz, H-3', H-5').

δ_C (100MHz, CDCl₃): 28.22 (CH₃, Boc), 28.25 (CH₃), 36.16 (C-N), 40.48 (CH₂), 81.58 (q, Boc), 82.19 (q, Boc), 115.58 (Ar, H-2, H-6), 122.81 (Ar, H-2', H-6'), 129.61 (Ar, H-3, H-5), 129.74 (Ar, H-3', H-5'), 137.52 (q, Ar), 144.67 (q, C-N), 153.01 (C=N), 153.32 (C=O)

ν_max (ATR)/cm⁻¹: 3470.96 (NH), 3376.06 (NH), 2980.82 (CH₂), 1715.85 (C=O), 1693.68 (C=N), 1629.59, 1604.04, 1517.92, 1432.52 (CH₂), 1346.47, 1257.19 (C-N), 1135.83 (C-O), 1079.36, 948.57, 852.99, 832.53, 766.70, 725.66, 594.64

HRMS (m/z ESI⁺): Calculated for C₂₆H₃₆N₄O₄, [M] 468.27 Found: [M+Na]⁺ 491.2650

R_f: 7:3 Hexane/Ethyl Acetate, 0.25

4-Bis-[2,3-di(tert-butoxycarbonyl)-N,N’-dimethyl]guanidino]-diphenylmethane (19b)
Using Method G, 4,4'-methylenedianiline (374.2 mg, 1.0 eq., 1.89 mmol) was dissolved in DMF at 0 °C and reacted with 19a (2.5 eq., 1,435 mg, 4.72 mmol). The mixture was treated with HgCl₂ (1.5 eq., 1,537.8 mg, 5.664 mmol) and sodium bicarbonate (6 eq., 952.64 mg, 11.34 mmol) and stirred for 15 minutes at 0 °C. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete.

The reaction was filtered through Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (2 x 20 cm³) and extracted using DCM and deionised water (3 x 20 cm³). The combined organic layers were dried over magnesium sulphate (MgSO₄), filtered and concentrated using the rotary evaporator to obtain the crude product.

Gravity column chromatography was used to obtain the purified product 19b (788 mg, 56.65%) using the appropriate ratio of hexane and ethyl acetate (8:2, respectively).

**Yield:** 788 mg, 56.65%

**Molar Mass:** 738.93 g mol⁻¹

δ_H (400MHz, CDCl₃): 1.43 (s, 18H, CH₃, Boc-2,2'), 1.51 (s, 18H, CH₃, Boc-1,1'), 2.65 (s, 6H, CH₃, H-2, H-2'), 3.33 (s, 6H, CH₃, H-1, H-1'), 3.92 (s, 2H, CH₂), 6.80 (d, 4H, J 8Hz, H-2, H-2', H-6, H-6'), 7.11 (d, 4H, J 8Hz, H-3, H-3', H-5, H-5').

δ_C (100MHz, CDCl₃): 28.21 (CH₃, Boc), 28.24 (CH₃), 34.35 (C-N), 36.20 (C-N), 40.79 (CH₂), 81.67 (q, Boc), 82.30 (q, Boc), 81.67 (q, Boc), 120.92 (Ar, CH, H-2, H-2', H-6, H-6'), 129.70 (Ar, CH, H-3, H-3', H-5, H-5'), 136.98 (q, Ar), 144.74 (q, C-N), 148.62 (C=N), 152.99 (C=O)

ν_max (ATR)/cm⁻¹: 2979.16 (NH), 2934.94, 1705.80 (C=O), 1627.21 (C=N), 1604.77, 1504.39, 1433.88 (CH₃), 1336.77, 1323.74, 1248.30 (C-N), 1209.11, 1133.30 (C-O), 1078.96, 1014.19, 946.86, 854.48, 766.66, 733.96, 621.50, 591.71

HRMS (m/z ESI⁺): Calculated for C₃₉H₅₈N₆O₈, [M] 738.43 Found: [M+Na]⁺ 761.4212

R_f: 7:3 Hexane/Ethyl Acetate, 0.56

4-Bis[(2,3-dimethyl)guanidino]diphenylmethane dihydrochloride (19c)

Using Method L, the Boc-protected compound 19b (340 mg) was dissolved in an excess of 50 % TFAA in DCM (16 cm³) and stirred at room temperature for 3-6 hours. The solvent
was then reduced by rotary evaporation. The remaining TFA salt was re-dissolved in deionised water (16 cm³) and treated with activated Amberlite IRA 400 chloride form (1 g) and allowed to stir gently for 24 hours at room temperature.

When the reaction was judged complete using the TLC method, the product was extracted using DCM (3 x 20 cm³). The combined organic layers were dried over MgSO₄, filtered and concentrated in vacuum to obtain the hydrochloride salt, 19c (168 mg, 98.2%).

Yield: 168 mg, 98.2%

Molar Mass: 338.46 gmol⁻¹

δ_H (400MHz, D₂O): 2.88 (s, 12H, CH₃, H-1, H-2, H-1', H-2'), 4.06 (s, 2H, CH₂), 7.22 (d, 4H, J 8Hz, H-2, H-2', H-6, H-6'), 7.38 (d, 4H, J 8Hz, H-3, H-3', H-5, H-5').

δ_C (100MHz, D₂O): 28.70 (CH₃), 41.67 (CH₂), 127.28 (Ar-CH, H-2, H-6, H-2', H-6'), 131.54 (Ar-CH, H-3, H-5, H-3', H-5'), 134.43 (q, Ar), 141.93 (q, Ar, C-N), 157.17 (C=N)

ν_max (ATR)/cm⁻¹: 3446.47 (NH), 3220.32 (NH), 2969.47 (CH₂), 2342.93, 1806.43, 1617.70 (Ar, CH), 1597.63, 1511.95, 1455.77 (CH₃), 1414.97, 1369.83, 1251.56 (C-N), 1186.52, 1102.66, 1018.86, 802.08, 720.79, 655.69, 575.28, 568.66

HRMS (m/z ESI⁺): Calculated for C₁₉H₂₆N₆ [M] 338.22 Found: [M+H]⁺ 339.2292

HPLC Purity (> 95%): 99.05%

4-[2,3-(tert-Butoxycarbonyl)guanidino]-4'-[2,3-(tert-Butoxycarbonyl)-N,N'-dimethylguanidino]diphenylmethane (25a)

Using Method G, compound 39a (1.0 eq., 583 mg, 1.325 mmol) was dissolved in DMF at 0°C and reacted with N,N'-di(tert-butoxycarbonyl)-thiourea (1.2 eq, 461.7 mg, 1.59 mmol). The mixture was treated with HgCl₂ (1.3 eq., 467.7 mg 1.723 mmol) and NEt₃ (6 eq., 1.11 cm³, 7.95 mmol, 0.726 gcm⁻³) and stirred for 15 minutes at 0°C. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete.
The usual work-up was performed, followed by gravity column chromatography to yield the purified product 25a (674.1 mg, 71.56%) using the appropriate ratio of hexane and ethyl acetate (8:2, respectively).

**Yield:** 674.1 mg, 71.56%

**Molar Mass:** 710.87 g mol\(^{-1}\)

\(\delta_H\) (400MHz, CDCl\(_3\)): 1.51 (s, 18H, CH\(_3\), Boc-1), 1.44 (s, 18H, CH\(_3\), Boc-2), 2.64 (s, 3H, CH\(_3\), H-1), 3.33 (s, 3H, CH\(_3\), H-2), 3.92 (s, 2H, CH\(_2\)), 6.82 (d, 2H, J 8Hz, H-2, H-6), 7.13 (d, 2H, J 8Hz, H-2', H-6'), 7.15 (d, 2H, J 8Hz, H-3, H-5), 7.53 (d, 2H, J 8Hz, H-3', H-5'), 10.29 (br s, 1H, NH-1), 11.65 (br s, 1H, NH-2)

\(\delta_C\) (100MHz, CDCl\(_3\)): 28.09 (CH\(_3\)), 28.23 (CH\(_3\), Boc), 34.36 (q, C-N), 36.19 (q, C-N), 40.78 (CH\(_2\)), 79.57 (q, Boc), 83.66 (q, Boc), 120.89 (Ar, C-H, H-2, H-6), 122.30 (Ar, C-H, H-2', H-6'), 129.32 (Ar, C-H, H-3, H-5), 129.75 (Ar, C-H, H-3', H-5'), 134.84 (q, Ar), 137.72 (q, C-N), 153.01 (C=N), 153.50 (C=O)

\(\nu_{\text{max}}\) (ATR)/cm\(^{-1}\): 3262.16 (N-H), 3151.32 (N-H), 2978.78 (CH\(_2\)), 2933.76, 1711.33 (C=O), 1628.27 (C=N), 1604.51, 1561.13 (C-O), 1412.42 (CH\(_3\)), 1337.33, 1235.23, 1147.47, 1056.55, 947.03, 854.25, 807.38, 766.18

**HRMS** (m/z ESI\(^+\)): Calculated for C\(_{37}\)H\(_{54}\)N\(_6\)O\(_8\) [M] 710.40 Found: [M+Na]\(^+\) 733.3913

**R\(_f\):** 7:3 Hexane/Ethyl Acetate, 0.44

4-Guanidino-4'-[N,N'-dimethylguanidino]diphenylmethane dihydrochloride (25b)

Using Method L, the Boc-protected compound 25a (340 mg) was dissolved in an excess of 50 % TFAA in DCM (16 cm\(^3\)) and stirred at room temperature for 3-6 hours. The solvent was then reduced by rotary evaporation. The remaining TFA salt was re-dissolved in deionised water (16 cm\(^3\)) and treated with activated Amberlite IRA 400 chloride form (1 g) and allowed to stir gently for 24 hours at room temperature.

Following the usual work-up the hydrochloride salt, 25b (311 mg, 86.15%), was afforded.

**Yield:** 311 mg, 86.15%

**Molar Mass:** 310.41 g mol\(^{-1}\)
δ_H (400MHz, D_2O): 2.87 (s, 6H, CH_3, H-1, H-2), 4.05 (s, 2H, CH_2), 7.20 (d, 2H, J 8Hz, H-2, H-6), 7.25 (d, 2H, J 8Hz, H-2’, H-6’), 7.38 (d, 2H, J 8Hz, H-3, H-5), 7.40 (d, 2H, J 8Hz, H-3’, H-5’)

δ_C (100MHz, D_2O): 27.40 (CH_3), 40.12 (CH_2), 117.73 (Ar, q C), 120.89 (Ar, C-H, H-2, H-6), 126.34 (Ar, C-H, H-2’, H-6’), 126.73 (Ar, C-H, J-3, H-5), 130.22 (Ar, C-H, H-3’, H-5’), 132.40 (q, Ar), 141.22 (q, C-N), 141.46 (q, C-N), 155.69 (C=N)

ν_{max} (ATR)/cm^{-1}: 3124.05 (N-H), 2341.15 (CH_2), 1636.88 (C=N), 1618.26, 1598.55(Ar, CH), 1509.52, 1450.35 (Ar, CH), 1414.90 (CH_3), 1366.77, 1297.93 1249.76 (C-N), 1184.41 (C-O), 1159.41, 1111.28, 1051.78, 920.57, 868.36, 80.61, 771.85

HRMS (m/z APCI^+): Calculated C_{17}H_{22}N_6 [M] 310.19 Found: [M+H]^+ 311.1980

HPLC Purity (> 95%): 98.17%

_N,N'-(Di-tert-butoxycarbonyl)diethylthiourea (20a)_

Using Method B, sodium hydride 60 % immersion in oil (4.5 eq, 933 mg, 38.88 mmol) was added to a solution of _N,N'-diethylthiourea (1.0 eq, 900 mg, 8.64 mmol) in dry THF (200 cm^3) at 0 °C with stirring. The reaction was slowly brought to room temperature and allowed to stir for 45 minutes. The reaction was then cooled back down to 0 °C and di-tert-butyl-dicarbonate (2.2 eq, 4,148 mg, 19.01 mmol) was added carefully. The reaction was brought back to room temperature and allowed to stir overnight.

The usual work-up and purification yielded compound **20a** (942.9 mg, 74.63%) a yellow oil.

**Yield:** 942.9 mg, 74.63%

**Molar Mass:** 332.46 gmol^{-1}

δ_H (400MHz, CDCl_3): 1.34 (t, 6H, CH_3), 1.51 (s, 18H, CH_3-Boc), 4.13 (bd, 4H, CH_2)

δ_C (100MHz, CDCl_3): 13.75 (CH_3), 27.42 (CH_2), 28.07 (CH_3, Boc), 48.13 (C-N), 83.17 (q-C, Boc), 85.22 (q-C, Boc*), 146.67 (C=O, Boc*) 150.89 (C=O, Boc), 189.56 (C=S)

*Boc anhydride impurity signal*
\( v_{\text{max}} \) (ATR)/cm\(^{-1}\): 2978.08 (C-H), 2935.24, 1720.19 (C=O), 1458.49 (CH\(_3\)), 1363.11, 1313.66, 1251.30 (C-N), 1159.13 (C-O), 1101.26 (C=S), 1085.13, 1070.97, 993.61, 880.68, 846.14, 815.02, 774.94, 759.27, 738.99, 701.82, 658.09

**HRMS** (m/z ESI\(^+\)): Calculated for C\(_{15}\)H\(_{28}\)N\(_2\)O\(_4\)S [M] 332.18 Found: [M+Na\(^+\)] 355.1666

**R\(_f\)**: 7:3 Hexane/Ethyl Acetate, 0.77

4-Bis-[(2,3-di(tert-butoxycarbonyl)-N,N'-diethyl)guanidino]-diphenylmethane (20b)

Using Method G, 4,4'-methyleneedianiline (1.0 eq., 301.36 mg, 1.52 mmol) was dissolved in DCM at 0 °C and reacted with 20a (2.5 eq., 1,274 mg, 3.81 mmol). The mixture was treated with HgCl\(_2\) (3 eq., 1,240 mg, 4.57 mmol) and triethylamine (6 eq., 1.28 cm\(^3\), 9.15 mmol) and stirred for 15 minutes at 0 °C. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete.

The reaction was filtered through Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (2 x 20 cm\(^3\)) and extracted using DCM and deionised water (3 x 20 cm\(^3\)). The combined organic layers were dried over magnesium sulphate (MgSO\(_4\)), filtered and concentrated using the rotary evaporator to obtain the crude product. Gravity column chromatography and the preparative TLC method were used in an attempt to isolate the purified compound

**Yield:** 105.7, 8.76%

**Molar Mass:** 795.04 g/mol

\( \delta_H \) (400MHz, CDCl\(_3\)): 1.26 (t, 6H, CH\(_3\)**), 1.36 (s, 2H, Boc, thiourea*), 1.51 (s, 2H, CH\(_3\), Boc-bis*), 1.52 (s, 18H, CH\(_3\), Boc-mono), 3.88 (s, 2H, CH\(_2\)**), 3.89 (s, 2H, CH\(_2\)*), 4.13 (q, 3H, CH\(_2\)**), 6.51 (s, 2H, H-2, H-6), 7.10 (d, overlapping signal, 5H, J 8Hz, Ar-CH, mono and bis product), 7.26 (d, overlapping signal, 4H, J 8Hz, Ar-CH, mono and bis product).

\( \delta_C \) (100MHz, CDCl\(_3\)): 13.83 (CH\(_3\)), 28.15 (CH\(_3\), Boc, thiourea), 28.23 (CH\(_3\), Boc, bis*), 28.35 (CH\(_3\), Boc, mono**), 40.51 (CH\(_2\), mono**), 40.63 (CH\(_2\), bis*), 53.46 (CH\(_2\)), 80.39 (C-O), 118.75 (Ar, CH, H-2, H-6), 129.36 (Ar, CH, H-2', H-6', H-3', H-3, H-5, H-5'), 135.97 (q, C-Ar), 136.36 (q, C-Ar), 152.88 (C=N), 171.23 (C=S, thiourea)
*bis = bis-guanidinium product

**mono = mono-guanidinium side-product

\[ \nu_{\text{max}} \left( \text{ATR}/\text{cm}^{-1} \right) : 3344.45 \ (\text{CH}), \ 2977.33 \ (\text{CH}), \ 2932.58, \ 1744.87, \ 1719.75 \ (\text{C=O}), \ 1642.41 \ (\text{C=N}), \ 1604.26 \ (\text{Ar, C=N}), \ 1456.89 \ (\text{CH}_2), \ 1390.96 \ (\text{CH}_2), \ 1366.20, \ 1271.93, \ 1229.62 \ (\text{C-N}), \ 1136.91 \ (\text{C-O}), \ 1065.44, \ 1045.34, \ 1020.12, \ 917.29, \ 879.47, \ 852.1, \ 811.68, \ 764.02, \ 662.95 \]

HRMS (m/z APCI\(^+\)):
- Calculated for C\(_{43}\)H\(_{66}\)N\(_{6}\)O\(_{8}\) [M] 794.49 Found: [M+H\(^+\)] 795.5019
- Calculated for C\(_{28}\)H\(_{40}\)N\(_{4}\)O\(_{4}\) [M] 496.30 Found: [M+H\(^+\)] 497.3132

This synthesis resulted in a mixture of the thiourea starting material, bis-guanidinium symmetric compound and the mono-guanidinium compound to be separated via reverse phase column chromatography after the deprotection step (See compound 20c and 20d below).

4-Bis[(2,3-diethyl)guanidino]diphenylmethane dihydrochloride (20c)

Using Method J, the Boc-protected compound 20b (105.7 mg) was dissolved in an excess of 50 % TFAA in DCM (12 cm\(^3\)) and stirred at room temperature for 3-6 hours. The solvent was then reduced by rotary evaporation. The remaining TFA salt was re-dissolved in deionised water (20 cm\(^3\)) and treated with activated Amberlite IRA 400 chloride form (1 g) and allowed to stir gently for 24 hours at room temperature.

Following the usual work-up, with the addition of reverse phase column chromatography, the hydrochloride salt, 20c (5.7 mg, 9.66%), was afforded.

Yield: 5.7 mg, 9.66%

Molar Mass: 394.57 g mol\(^{-1}\)

\[ \delta_H \ (400\text{MHz, D}_2\text{O}) : \ 1.11 \ (t, \ 12H, \text{CH}_3, \ H-1, \ H-2, \ H-1', \ H-2'), \ 3.22 \ (q, \ 8H, \text{CH}_2, \ H-1, \ H-2, \ H-1', \ H-2'), \ 3.97 \ (s, \ 2H, \text{CH}_2), \ 7.16 \ (d, \ 4H, \text{J 8Hz}, \ H-2, \ H-6, \ H-2', \ H-6'), \ 7.32 \ (d, \ 4H, \text{J 8Hz}, \ H-3, \ H-5, \ H-3', \ H-5') \]

\[ \delta_C \ (100\text{MHz, D}_2\text{O}) : \ 13.36 \ (\text{CH}_3), \ 36.53 \ (\text{CH}_2), \ 40.16 \ (\text{CH}_2), \ 126.43 \ (\text{Ar, C-H}, \ H-2, \ H-6, \ H-2', \ H-6'), \ 130.20 \ (\text{Ar, C-H}, \ H-3, \ H-5, \ H-3', \ H-5'), \ 132.74 \ (q, \text{Ar}), \ 140.99 \ (q, \text{C-N}), \ 153.99 \ (\text{C=N}) \]
**HRMS** (m/z APCI⁺): Calculated for C_{23}H_{34}N_{6} [M] 394.28 Found: [M+H]⁺ 395.2920

4-Amino-4'-(N,N'-diethyl)guanidino)diphenylmethane hydrochloride (20d)

![Chemical Structure](image)

Using Method J, the Boc-protected compound 20b (105.7 mg) was dissolved in an excess of 50 % TFAA in DCM (12 cm³) and stirred at room temperature for 3-6 hours. The solvent was then reduced by rotary evaporation. The remaining TFA salt was re-dissolved in deionised water (20 cm³) and treated with activated Amberlite IRA 400 chloride form (1 g) and allowed to stir gently for 24 hours at room temperature.

Following the usual work-up and a reverse phase gravity column chromatography, the hydrochloride salt 20d (11.4 mg, 29.6%), was afforded.

**Yield:** 11.4 mg, 29.6%

**Molar Mass:** 296.42 g mol⁻¹

δ_{H} (400MHz, D_{2}O): 1.10 (t, 6H, CH₃, H-1, H-2), 3.31 (br q, 4H, CH₂, H-1, H-2), 3.83 (s, 2H, CH₂), 6.75 (d, 2H, J 8Hz, H-2, H-6), 7.08 (d, 2H, J 8Hz, H-2', H-6'), 7.12 (d, 2H, J 8Hz, H-3, H-5), 7.28 (d, 2H, J 8Hz, H-3', H-5')

δ_{C} (100MHz, D_{2}O): 13.32 (CH₃), 36.48 (CH₂), 39.85 (CH₂), 117.12 (q, C-N), 126.42 (Ar, C-H, H-2, H-6), 129.57 (Ar, C-H, H-2', H-6'), 130.03 (Ar, C-H, H-3, H-5), 132.40 (Ar, C-H, H-3', H-5'), 133.06 (q, Ar), 141.99 (q, C-N), 154.00 (C=N)

**HRMS** (m/z ESI⁺): Calculated for C_{18}H_{24}N_{6} [M] 296.20, Found: [M+H⁺]⁺ 297.2080

**HPLC Purity (≥95%):** 91.35%

1,1'-Methylenebis(4-isothiocyanatobenzene) (37a)

![Chemical Structure](image)

First, 4,4'-methylenedianiline (1.0 eq., 600 mg, 3.026 mmol) was dissolved in dry DCM and allowed to stir at 0 ºC for 10 minutes before the addition of 1,1'-thiocarbonyl bis-imidazole...
(2.2 eq., 1.186 mg, 6.66 mmol). The reaction was gradually brought to room temperature and stirred for 5 hours. The reaction was judged complete using the TLC method. The solvent was reduced by rotary evaporation to obtain a red-brown solid and purified using a 6:4 ratio of hexane and ethyl acetate, respectively, by gravity column chromatography yielding compound 37a (700 mg, 97%).

Yield: 700 mg, 97%

Molar Mass: 282.38 g mol⁻¹

δH (400MHz, CDCl₃): 7.17 (dd, 8H, J 8Hz, H-2, H-3, H-5, H-6, H-2', H-3' H-5', H-6'), 3.98 (s, 2H, CH₂)

δC (100MHz, CDCl₃): 40.99 (CH₂), 115.94 (C=N), 125.96 (Ar, C-H, J 8Hz H-2, H-6, H-2', H-6'), 129.99 (Ar, C-H, J 8Hz H-3, H-5, H-3', H-5'), 139.57 (q, C-N)

νmax (ATR)/cm⁻¹: 2925.84 (CH₂), 2853.72, 2069.65 (N=C=S), 1899.10, 1573.83, 1497.97, 1440.72, 1411.63, 1260.01, 1199.69 (C-N), 1171.67, 1105.83, 1016.32, 927.13, 864.50, 807.46, 783.16, 734.53

HRMS (m/z APCI⁺⁻): Calculated for C₁₅H₁₀N₂S₂ [M] 282.03 Found: [M+H] 281.0213

1,1'-{(Methylene-di-4,1-phenylene)bis(thiourea)} (37b)

NaH 60 % immersion in oil (9 eq., 544 mg, 22.68 mmol) was added to a cooled solution of tert-butyl carbamate (2.5 eq., 737 mg, 6.29 mmol) in dry THF (8 cm³) at 0 ºC. The reaction mixture was stirred at 0 ºC for 20 minutes before adding the previously synthesised 1,1'-methylenebis(4-isothiocyanatobenzene) (37a, 1 eq. 600 mg, 2.52 mmol). The solution was brought to room temperature and allowed to stir for 5 hours. The reaction was monitored by TLC until complete.

Once complete, the reaction was quenched with deionised water (5 cm³) and stirred for a further 15 minutes. The solvents were reduced by rotary evaporation and the solid obtained was re-dissolved in DCM and washed with deionised water (6 x 20 cm³). Purification by gravity column chromatography with an 8:2 ratio of hexane and ethyl acetate, respectively, afforded the pure product, 37b (211.0 mg, 16.2%) 

Yield: 211.0 mg, 16.2%
**Molar Mass:** 516.68 g mol⁻¹

δ_H (400MHz, CDCl₃): 1.55 (s, 18H, CH₃, Boc), 4.01 (s, 2H, CH₂), 7.23 (d, J 8Hz, 4H, Ar-CH, H-2, H-2', H-6, H-6'), 7.59 (d, J 8Hz, Ar-CH, H-3, H-3', H-5, H-5'), 7.97 (s, 2H, NH-1, NH-1'), 11.47 (s, 2H, NH-2, NH-2')

δ_C (100MHz, CDCl₃): 28.03 (CH₃, Boc), 40.99 (CH₂), 84.27 (C=O), 124.39 (Ar, C-2, C-2', C-6, C-6'), 129.33 (Ar, C-3, C-3', C-5, C-5'), 135.91 (Ar-qC), 139.23 (C-N), 151.86 (C=O), 178.21 (C=S)

ν_max (ATR)/cm⁻¹: 3170.00 (NH), 2977.5, 2928.3, 1710.97 (C=O), 1701.58, 1526.82 (C-O), 1508.81, 1365.89, 1352.64, 1251.12 (C-N), 1135.07 (C=S), 1015.82, 851.23, 764.01, 729.71, 695.72, 668.5, 595.1

HRMS (m/z ESI⁺⁻): Calculated for C₂₅H₃₂N₄O₄S₂ [M] 516.19, Found: [M+H]⁺ 515.1791

1,1'-((Methylenedi-4,1-phenylene)-N-ethoxycarbonyl-bis(thiourea) (36a)

![Chemical structure](image)

First, 4,4'-Methylenedianiline (1.0 eq., 550 mg, 2.75 mmol) was dissolved in anhydrous DCM under argon below 0 °C was added. Ethoxycarbonyl isothiocyanate (2.2 eq., 0.72 cm³, 6.10 mmol) was added and after approximately 15-20 minutes of stirring below 0 °C, the reaction was gradually brought back to room temperature and stirred overnight. The reaction was monitored by TLC and once deemed complete the solvent was removed. The product was purified by column chromatography using the appropriate hexane/ethyl acetate gradient, yielding compound 36a (136 mg, 10.7%).

**Yield:** 136 mg, 10.7%

**Molar Mass:** 329.42 g mol⁻¹

δ_H (400MHz, CDCl₃): 1.37 (t, 3H, CH₃), 3.99 (s, 2H, CH₂), 4.31 (q, 2H, CH₂), 7.18 (s, 4H, Ar-CH, H-3, H-3', H-5, H-5'), 7.20 (d, J 8Hz, 2H, Ar-CH, H-2, H-6), 7.59 (d, J 8Hz, H-2', H-6'), 8.05 (s, 1H, NH-1), 11.43 (s, 1H, NH-2)

δ_C (100MHz, CDCl₃): 14.20 (CH₃), 41.03 (CH₂), 63.10 (O-CH₂), 124.54 (Ar, C-2, C-6), 125.88 (Ar, C-2', C-6'), 129.29 (Ar, C-3, C-5), 130.08 (Ar, C-3', C-5'), 135.99 (Ar, q-C), 138.91 (Ar, q-C), 140.18 (C-N), 152.76 (C=O), 177.82 (C=S)
\( v_{\text{max}} \) (ATR)/\( \text{cm}^{-1} \): 3167.18 (NH), 2974.86, 2930.14, 1708.67 (C=O), 1700.97, 1515.89 (C-O), 1506.83, 1300.65, 1350.94, 1250.49 (C-N), 1126.03 (C=S), 1015.87, 871.46, 773.12, 758.31, 702.15, 684.20, 599.14

**HRMS** (m/z APCl\(^+\)): Calculated for C\(_{17}\)H\(_{19}\)N\(_3\)O\(_2\)S, Not Found: 329.12

**\( N\)-(tert-butoxycarbonyl)-\( N'\)-methyl thiourea (38a)**

![Chemical structure of 38a]

Using Method C, sodium hydride 60 % immersion in oil (1,183 mg, 49.26 mmol, 4.5 eq.) was added carefully to a solution of commercially available unsubstituted thiourea (500 mg, 6.57 mmol, 1.0 eq.) in dry THF (110 ml), at 0 °C with stirring. The reaction was slowly brought to room temperature and allowed to stir for 45 minutes. The reaction was then cooled back down to 0 °C and di-tert-butyl-dicarbonate (3,585 mg, 16.43 mmol, 2.5 eq.) was added. After 30 minutes stirring at 0 °C, the reaction was brought back to room temperature and allowed to stir overnight.

After monitoring the formation of the \( N,N'\)-bis-Boc-thiourea by TLC, the reaction was cooled back to 0 °C and NaH 60% immersion in oil (446.89 mg, 18.62 μmol, 1.7 eq.) was added carefully. After 1 hour, TFA anhydride (1.43 ml, 10.12 μmol, 1.54 eq.) was added. After a further 1 hour stirring at 0 °C, methylamine hydrochloride (683 mg, 10.12 μmol, 1.54 eq.) was added and allowed to fully dissolve in solution before the removal of the ice-bath. The reaction was gradually brought back to room temperature and allowed to stir overnight.

After approximately 16 hours the reaction was deemed complete and cooled to 0 °C, the reaction was quenched via the addition of adding deionised water (40 ml) dropwise to the mixture. Extraction of the product was carried out by using ethyl acetate (4 x 50 ml) and washed with brine (4 x 50 ml). The combined organic phases were dried over MgSO\(_4\), filter and concentrate under vacuum. Purification of the product was carried out by column chromatography using a hexane and ethyl acetate gradient mixture, yielding compound 38a (469 mg, 37.6%).

**Yield:** 469 mg, 37.6%

**Molar Mass:** 190.26 g\( \text{mol}^{-1} \)

**MP:** 101-104 °C
$\delta_H$ (400MHz, CDCl$_3$): 1.50 (s, 9H, Boc-CH$_3$), 3.19 (d, 3H, CH$_3$), 8.02 (bs, 1H, NH-2), 9.71 (bs, 1H, NH-1)

$\delta_c$ (100MHz, CDCl$_3$): 27.99 (CH$_3$, Boc), 32.07 (CH$_3$), 83.70 (q-C, Boc), 151.92 (C=O), 180.49 (C=S)

$\nu_{max}$ (ATR)/cm$^{-1}$: 3230.32, 3157.57, 2979.86, 2938.09, 1723.66 (C=O), 1512.33 (N-H), 1456.87 (CH$_3$), 1368.29, 1335.68, 1253.99 (C-N), 1143.59 (C-O), 1039.84, 1000.06 (C=S), 885.72, 765.54, 724.47

HRMS (m/z ESI$^{+/-}$): Calculated for C$_7$H$_{14}$N$_2$O$_2$S [M] 190.09, Found: [M+Na]$^+$ 213.0795
Methyl (Z)-N,N'-di(tert-butoxycarbonyl)-N-methylcarbamimidothioate (21a)

Using Method D, a solution of 1,3-bis-(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea (1 g, 1 eq., 3.44 mmol), anhydrous methanol (0.21 cm$^3$, 1.5 eq., 5.17 mmol), and triphenylphosphine (1.356 g, 1.5 eq., 5.17 mmol) in dry THF, under argon gas, was cooled to -5 °C. Diethyl azodicarboxylate (1.62 cm$^3$, 3 eq., 10.34 mmol) was added dropwise at a rate such that the reaction mixture was completely colourless before the addition of the next drop. The reaction was then stirred at room temperature overnight. The reaction was monitored by TLC. Once complete, methanol (10 cm$^3$) was added to the solution and then the solvent was reduced. The product was purified by flash chromatography a hexane and ethyl acetate gradient mixture, yielding compound 21a, 80.8%.

Yield: 846 mg, 80.8%

Molar Mass: 304.41 gmol$^{-1}$

Melting Point: oil

$\delta$$_H$ (400MHz, CDCl$_3$): 1.40 (d, 18 H, Boc-CH$_3$), 2.28 (s, 3 H, S-CH$_3$), 3.01 (s, 3H, N-CH$_3$)

$\delta$$_C$ (100MHz, CDCl$_3$): 15.26 (S-CH$_3$), 27.84 (CH$_3$, Boc$^1$), 27.90 (CH$_3$, Boc$^2$), 35.47 (N-CH$_3$),
81.56 (q-C, Boc$^2$), 82.14 (q-C, Boc$^1$), 151.78 (C=O, Boc$^1$), 157.76 (C=O, Boc$^2$), 180.49
(C=S)

$\nu$$_{max}$ (ATR)/cm$^{-1}$: 2978.43 (C=CH$_3$), 2933.27 (N=CH$_3$), 1922.31, 1838.67, 1714.01 (C=O),
1618.14 (N=C), 1550.34, 1459.11 (CH$_3$), 1425.24, 1393.67, 1365.94, 1332.26, 1241.2 (C-N),
1137.15 (C-O), 1059.7 (S-CH$_3$), 963.07, 854.7, 758.03, 711.09 (C=S), 664.03

HRMS (m/z ESI$^+$): Calculated for C$_{13}$H$_{24}$N$_2$O$_4$S [M] 304.15, Found: [M+Na]$^+$ 304.1352

Rf: 1.1 Hexane/Ethyl Acetate, 0.80

Methyl (Z)-N,N'-di(tert-butoxycarbonyl)-N-ethylicarbamimidothioate (22a)

A solution of 1,3-bis-(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea (1 g, 1 eq., 3.44 mmol), anhydrous ethanol (0.30 cm$^3$, 1.5 eq., 5.17 mmol), and triphenylphosphine (1.356
g, 1.5 eq., 5.17 mmol) in dry THF under argon gas was cooled to -5 °C. Diethyl azodicarboxylate (1.62 cm³, 3 eq., 10.34 mmol) was added dropwise at a rate such that the reaction mixture was completely colourless before the addition of the next drop. The reaction was then stirred at room temperature overnight. The reaction was monitored by TLC. Once complete, methanol (10 cm³) was added to the solution and then the solvent was reduced. The product was purified by flash chromatography a hexane and ethyl acetate gradient mixture, yielding compound 22a, 94.8%.

**Yield:** 1038 mg, 94.8%

**Molar Mass:** 468.60 gmol⁻¹

**Melting Point:** oil

δH (400MHz, CDCl3): 1.25 (t, 3H, CH₃), 1.51 (d, 18 H, Boc-CH₃), 2.39 (s, 3 H, S-CH₃), 3.59 (q, 2H, CH₂)

δC (100MHz, CDCl₃): 14.10 (CH₃), 15.54 (S-CH₃), 28.00 (CH₃, Boc¹), 28.08 (CH₃, Boc²), 43.98 (CH₂), 81.76 (q-C, Boc²), 82.11 (q-C, Boc¹), 151.70 (C=O, Boc¹), 157.94 (C=O, Boc²), 163.00 (C=S)

νmax (ATR)/cm⁻¹: 2978.43 (C-CH₃), 2933.27 (N-CH₃), 1922.31, 1838.67, 1714.01 (C=O), 1618.14 (N=C), 1550.34, 1459.11 (CH₃), 1425.24, 1393.67, 1365.94, 1332.26, 1241.2 (C-N), 1137.15 (C-O), 1059.7 (S-CH₃), 963.07, 854.7, 758.03, 711.09 (C-S), 664.03

**HRMS (m/z ESI⁺):** Calculated for C₁₄H₂₆N₂O₄S [M] 318.16, Found: [M+Na]⁺ 341.1507

Rf: 1:1 Hexane/Ethyl Acetate, 0.85

4-Guanidino-4'-'[(2,3-di(tert-butoxycarbonyl)-N-methyl)guanidino]diphenylmethane (43a)

Using Method E, 4,4'-methylenedianiline (3.0 eq., 390 mg, 1.97 mmol) was dissolved in DCM, and the mixture was cooled below 0 °C, and reacted with 21a (1.0 eq., 200 mg, 0.66 mmol). The mixture was treated with HgCl₂ (3 eq., 535 mg, 1.97 mmol) and NEt₃ (6 eq., 0.55 cm³, 3.94 mmol) and stirred for 15 minutes below 0 °C. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete.
The reaction was filtered through Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (2 x 20 cm³) and extracted using DCM (3 x 20 cm³) and deionised water (3 x 20 cm³). The combined organic layers were dried over magnesium sulphate (MgSO₄), filtered and concentrated using the rotary evaporator to obtain the crude product. The product was then purified by gravity column chromatography using the appropriate ratio of hexane and ethyl acetate.

**Yield:** 166 mg, 55.5%

**Molar Mass:** 454.57 gmol⁻¹

δ_H (400MHz, CDCl₃): 1.50 (s, 9H, CH₃, Boc), 1.43 (s, 9H, CH₃, Boc-10), 3.19 (s, 3H, CH₃), 3.82 (s, 2H, CH₂), 6.62 (d, 2H, J 8Hz, H-2, H-6), 6.94 (t, 4H, J 8Hz, H-2′, H-6′, H-3, H-5), 7.14 (d, 2H, J 8Hz, H-3′, H-5′).

δ_C (100MHz, CDCl₃): 27.64 (CH₃), 28.14 (CH₃, Boc), 40.47 (CH₂), 115.28 (Ar, H-3, H-5, H-3′, H-5′), 122.62 (Ar, H-2′, H-6′), 122.70 (Ar, H-2, H-6), 144.56 (q, C-N)

ν_max (ATR)/cm⁻¹: 3450.37, 3367.81 (NH), 3225.07 (NH), 2978.22 (CH₂), 2932.32, 1722.70 (C=O), 1641.91 (C=N), 1512.47, 1473.80, 1437.74 (CH₃), 1362.76, 1234.59 (C-N), 1139.85 (C-O), 1047.41, 924.06, 854.09, 813.78, 766.25, 699.71, 661.71, 630.05, 603.47, 571.83

**HRMS** (m/z APCI⁺): Calculated for C₂₆H₃₄N₄O₄ [M] 454.26, Found: [M+H]⁺ 455.2657

R_f: 1:1 Hexane/Ethyl Acetate, 0.37

4-4'-[(2,3-Di(tert-butoxycarbonyl)-N-methyl)guanidino]diphenylmethane (21b)

Using Method E, 4,4'-methylenedianiline (1.0 eq., 208 mg, 1.05 mmol) was dissolved in DMF below 0 ºC and reacted with compound 21a (2.5 eq., 800 mg, 2.63 mmol). The mixture was treated with HgCl₂ (3 eq., 855 mg, 3.15 mmol) and triethylamine (6 eq., 0.88 cm³, 6.30 mmol) and stirred in an ice-bath. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete.

The reaction was filtered through Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (4 x 50 cm³) and extracted using DCM and deionised water (5 x 50 cm³). The combined organic layers were dried over magnesium
sulphate (MgSO\textsubscript{4}), filtered and concentrated using the rotary evaporator to obtain the crude product.

Gravity column chromatography was used to obtain the purified product 21b (445 mg, 47.9%) using the appropriate ratio of hexane and ethyl acetate (7:3, respectively).

**Yield:** 445 mg, 47.9%

**Molar Mass:** 510.64 g mol\textsuperscript{-1}

\[ \delta_H \text{ (400MHz, CDCl}_3\text{):} \quad 1.41 \text{ (s, 18H, CH}_3\text{, Boc-1)}, \quad 3.16 \text{ (bs, 6H, CH}_3\text{)}, \quad 3.83 \text{ (s, 2H, CH}_2\text{)}, \]

6.89 (br s, 4H, H-2, H-6, H-2', H-6'), 7.15 (br d, 2H, H-3, H-5, H-3', H-5'), 11.08 (br s, 1H, NH)

**Amorphus intermediate compound resulting in broad signals**

\[ \delta_C \text{ (100MHz, CDCl}_3\text{):} \quad 27.67 \text{ (CH}_3\text{), 28.04 \text{ (CH}_3\text{, Boc), 34.76 \text{ (q, C-N), 40.62 \text{ (CH}_2\text{), 82.11 \text{ (q, Boc), 121.76 \text{ (Ar, C-H, H-2, H-6), 129.68 \text{ (Ar, C-H, H-3, H-5), 152.38 \text{ (C=N)}}}}\]

\[ \nu_{\text{max}} \text{ (ATR)/cm}^{-1}: \quad 2998.34, \quad 2933.14, \quad 2931.28, \quad 1751.44, \quad 1720.91 \text{ (C=O), 1655.15 \text{ (C=N), 1602.33 \text{ (Ar-CH), 1465.55 \text{ (-CH}_2\text{-)}}, \quad 1453.78 \text{ (Ar-CH), 1393.71 \text{ (CH}_3\text{), 1360.25, \quad 1277.39 \text{ 1229.96 \text{ (C-N), 1169.97 \text{ (C-O), 1110.31, \quad 1067.42, \quad 1043.34, \quad 1020.12, \quad 977.60, \quad 907.61, \quad 871.54, \quad 850.31, \quad 801.33, \quad 771.12, \quad 673.94}}\]

**HRMS (m/z ESI\textsuperscript{+}):** Calculated for C\textsubscript{37}H\textsubscript{54}N\textsubscript{6}O\textsubscript{8} [M] 510.40, Found: [M+H]\textsuperscript{+} 511.4082

**R\textsubscript{f}:** 1:1 Hexane/Ethyl Acetate, 0.56

**4-4'-[N-Methylguanidino]diphenylmethane dihydrochloride (21c)**

Using Method J, the Boc-protected compound 21b (444.6 mg) was dissolved in an excess of 50 % TFAA in DCM (20 cm\textsuperscript{3}) and stirred at room temperature for 3-6 hours. The solvent was then reduced by rotary evaporation. The remaining TFA salt was re-dissolved in deionised water (20 cm\textsuperscript{3}) and treated with activated Amberlite IRA 400 chloride form (1 g) and allowed to stir gently for 24 hours at room temperature.

Following the usual work-up the hydrochloride salt, 21c (182.3 mg, 67.5%), was afforded.

**Yield:** 182.3 mg, 67.5%
Molar Mass: 310.41 gmol⁻¹

δ_H (400MHz, D_2O): 2.80 (s, 6H, CH_3), 3.96 (s, 2H, CH_2), 7.16 (d, 2H, J 8Hz, H-2, H-6, H-2', H-6'), 7.31 (d, 2H, J 8Hz, H-3, H-5, H-3', H-5')

δ_C (100MHz, D_2O): 27.52 (CH_3), 40.14 (CH_2), 126.38 (Ar, C-H, H-2, H-6, H-2', H-6'), 130.21 (Ar, C-H, H-3, H-5, H-3', H-5'), 132.38 (q, Ar), 141.20 (q, C-N), 156.20 (C=N)

HRMS (m/z ESI⁺): Calculated for C_{17}H_{22}N_{6} [M] 310.19 Found: [M+H]^+ 311.1979

HPLC Purity (> 95%): 97.79%

4-Guanidino-4’-[(2,3-di(tert-butoxycarbonyl)-N-methyl)guanidino]diphenylmethane (44a)

Using Method E, 4,4’-methylenedianiline (3.0 eq., 250 mg, 1.26 mmol) was dissolved in DCM, and the mixture was cooled below 0 °C, and reacted with 22a (1.0 eq., 134 mg, 0.42 mmol). The mixture was treated with HgCl₂ (3 eq., 342 mg, 1.26 mmol) and NEt₃ (6 eq., 0.35 cm³, 3.94 mmol) and stirred for 15 minutes below 0 °C. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete.

The reaction was filtered through Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (2 x 20 cm³) and extracted using DCM (3 x 20 cm³) and deionised water (3 x 20 cm³). The combined organic layers were dried over magnesium sulphate (MgSO₄), filtered and concentrated using the rotary evaporator to obtain the crude product. The product was then purified by gravity column chromatography using the appropriate ratio of hexane and ethyl acetate, yielding compound 44a (227.6 mg, 24.5%)

Yield: 227.6 mg, 24.5%

Molar Mass: 368.48 gmol⁻¹

δ_H (400MHz, CDCl₃): 1.22 (br s, 3H, CH₃), 1.44 (s, 9H, CH₃, Boc), 3.68 (br s, 2H, CH₂), 3.79 (s, 2H, CH₂), 6.58 (d, 2H, J 8Hz, H-2, H-6), 6.89 (d, 2H, J 8Hz, H-2'), 6.96 (br d, 2H, H-6', H-3, H-5), 7.11 (d, 2H, J 8Hz, H-3', H-5').
δ_c (100MHz, CDCl₃): 27.65 (CH₃), 28.22 (CH₃, Boc), 34.90 (CH₂), 40.48 (CH₂), 82.23 (q, Boc), 115.58 (Ar, H-3, H-5 H3', H-5'), 121.80 (Ar, H-2, H-6), 129.70 (Ar, H-2', H-6'), 144.56 (q, C=N), 152.39 (C=N)

ν_max (ATR)/cm⁻¹: 3367.69 (NH), 2977.68 (CH₂), 2932.87, 1711.74 (C=O), 1606.57 (C=N), 1513.01, 1461.57 (CH₃), 1366.34, 1269.50 1231.58 (C-N), 1137.36 (C-O), 1065.51, 1021.03, 967.31, 913.78, 848.44, 812.54, 766.04, 72960, 645.21, 570.51

HRMS (m/z APCI⁺): Calculated for C₂₆H₃₆N₄O₄ [M] 368.27, Found: [M+H]⁺ 369.2810

R_f: 1:1 Hexane/Ethyl Acetate, 0.47

4-4'-[(2,3-Di(tert-butoxycarbonyl)-N-ethyl)guanidino]diphenylmethane (22b)

Using Method E, 4,4'-methylenedianiline (1.0 eq., 249 mg, 1.26 mmol) was dissolved in DMF below 0 °C and reacted with compound 22a (2.5 eq., 1000 mg, 3.14 mmol). The mixture was treated with HgCl₂ (3 eq., 1,026 mg, 3.78 mmol) and triethylamine (6 eq., 1.05 cm³, 7.56 mmol) and stirred in an ice-bath. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete.

The reaction was filtered through Celite and rinsed with DCM to remove any of the mercury by-product. The filtrate was washed with brine (4 x 50 cm³) and extracted using DCM and deionised water (5 x 50 cm³). The combined organic layers were dried over magnesium sulphate (MgSO₄), filtered and concentrated using the rotary evaporator to obtain the crude product.

Gravity column chromatography was used to obtain the purified product 22b (219 mg, 17.8%) using the appropriate ratio of hexane and ethyl acetate (7:3, respectively).

Yield: 129 mg, 17.8%

Molar Mass: 538.69 gmol⁻¹

δ_h (400MHz, CDCl₃): 1.22 (br s, 6H, CH₃), 1.44 (s, 18H, CH₃, Boc), 3.67 (br s, 4H, CH₂), 3.87 (s, 2H, CH₂), 6.96 (d, 2H, J 8Hz, H-2, H-6, H-2', H-6'), 7.10 (d, 2H, J 8Hz, H-3, H-5, H-3', H-5')
δc (100MHz, CDCl_3): 27.72 (CH₃), 28.10 (CH₃, Boc), 40.67 (CH₂), 43.09 (N-CH₂), 80.15 (q C, Boc), 82.14 (C-O), 121.92 (Ar, C-H, H-2, H-6, H-2’, H-6’), 129.70 (Ar, C-H, H-3, H-5, H-3’, H-5’), 152.34 (C=N), 171.30 (C=O)

νmax (ATR)/cm⁻¹: 2977.33 (CH₂), 2932.58, 1744.87, 1719.75 (C=O), 1642.41 (C=N), 1604.26 (Ar-CH), 1456.89 (Ar-CH), 1390.96 (CH₂), 1366.20, 1271.93, 1229.62 (C-N), 1136.91 (C-O), 1065.44, 1045.334, 1020.12, 967.20, 917.29, 879.47, 852.10, 811.68, 764.02, 662.95

HRMS (m/z ESI⁺): Calculated for C₂₉H₄₂N₆O₄ [M] 538.33 Found: [M+Na]⁺ 561.3197

Rₖ: 1:1 Hexane/Ethyl Acetate, 0.76

4-4’-[N-Ethylguanidino]diphenylmethane dihydrochloride (22c)

Using Method L, the Boc-protected compound 22b (219 mg) was dissolved in an excess of 50 % TFAA in DCM (20 cm³) and stirred at room temperature for 3-6 hours. The solvent was then reduced by rotary evaporation. The remaining TFA salt was re-dissolved in deionised water (20 cm³) and treated with activated Amberlite IRA 400 chloride form (1 g) and allowed to stir gently for 24 hours at room temperature.

Following the usual work-up the hydrochloride salt, 22c (115 mg, 83.9%), was afforded.

Yield: 115 mg, 83.9%

Molar Mass: 338.46 gmol⁻¹

δH (400MHz, D₂O): 1.13 (t, 6H, CH₃), 3.20 (q, 4H, CH₂), 3.97 (s, 2H, CH₂), 7.17 (d, 4H, J 8Hz, H-2, H-6, H-2’, H-6’), 7.32 (d, 4H, J 8Hz, H-3, H-5, H-3’, H-5’)

δc (100MHz, D₂O): 13.13 (CH₃), 36.47 (CH₂), 40.14 (CH₂), 126.37 (Ar, C-H, H-2, H-6, H-2’, H-6’), 130.20 (Ar, C-H, H-3, H-5, H-3’, H-5’), 132.47 (q, Ar), 141.161 (q, C-N), 155.16 (C=N)

HRMS (m/z APCI⁺): Calculated for C₁₉H₂₆N₆ [M] 338.22 Found: [M+H]⁺ 339.2293

HPLC Purity (> 95%): 96.37%
Using Method H, compound 39a (1.5 eq., 406 mg, 1.34 mmol) was dissolved in DCM below 0 °C and reacted with compound 21a (1.0 eq, 417 mg, 0.89 mmol). The mixture was treated with HgCl₂ (3 eq., 725 mg 2.67 mmol) and NEt₃ (8 eq., 0.99 cm³, 7.12 mmol, 0.726 g cm⁻³) and stirred for 15 minutes at 0 °C. The reaction was gradually brought back to room temperature while stirring and monitored by TLC analysis until complete.

The usual work-up was performed, followed by gravity column chromatography to yield the purified product 28a (434.5 mg, 67.4%) using the appropriate ratio of hexane and ethyl acetate (8:2, respectively).

**Yield:** 434.5 mg, 67.4%

**Molar Mass:** 624.78 g mol⁻¹

δ_H (400MHz, CDCl₃): 1.51 (s, 18H, CH₃, Boc-1), 1.44 (s, 9H, CH₃, Boc-2), 2.63 (s, 3H, CH₃, H-1), 3.25 (br s, 3H, CH₃, H-3), 3.32 (s, 3H, CH₃, H-2), 3.91 (s, 2H, CH₂), 6.79 (d, 2H, J 8Hz, H-2, H-6), 7.00 (br s, 2H, H-2', H-6'), 7.10 (d, 2H, J 8Hz, H-3, H-5), 7.16 (d, 2H, J 8Hz, H-3', H-5')

δ_C (100MHz, CDCl₃): 27.64 (CH₃), 28.13 (CH₃, Boc-2), 28.23 (CH₃, Boc-1), 36.09 (q, C-N), 40.74 (CH₂), 81.54 (q, Boc-2), 82.19 (q, Boc-1), 120.87 (Ar, C-H, H-2, H-6), 121.84 (Ar, C-H, H-2', H-6'), 129.65 (Ar, C-H, H-3, H-5), 129.76 (Ar, C-H, H-3', H-5'), 136.34 (q, Ar), 145.19 (q, C-N), 153.02 (C=N)

ν max (ATR)/cm⁻¹: 2978.11 (CH₂), 1710.15 (C=O), 1632.03 (C=N), 1470.60(C-O), 1430.44 (CH₃), 1338.47, 1237.23, 1135.50, 1079.61, 946.01 855.88, 766.21, 592.45

**HRMS (m/z ESI⁺):** Calculated for C₃₃H₄₈N₆O₆ [M] 624.36, Found: [M+H]⁺ 625.3711

Rf: 7:3 Hexane/Ethyl Acetate, 0.39
4-[[N-Methylguanidino]-4'-[N,N'-dimethylguanidino]diphenylmethane dihydrochloride (28b)

Using Method J, the Boc-protected compound 28a (434 mg) was dissolved in an excess of 50 % TFAA in DCM (25 cm³) and stirred at room temperature for 3-6 hours. The solvent was then reduced by rotary evaporation. The remaining TFA salt was re-dissolved in deionised water (25 cm³) and treated with activated Amberlite IRA 400 chloride form (1 g) and allowed to stir gently for 24 hours at room temperature.

Following the usual work-up the hydrochloride salt, 28b (183 mg, 94.3%), was afforded.

Yield: 183 mg, 94.3%

Molar Mass: 324.43 g mol⁻¹

δH (400MHz, D₂O): 2.80 (s, 9H, C-H₃), 3.97 (s, 2H, CH₂), 7.16 (t, 4H, J 8Hz, H-2, H-6, H-2', H-6'), 7.32 (d, 2H, J 8Hz, H-3, H-5, H-3', H-5')

δC (100MHz, D₂O): 13.13 (CH₃), 36.47 (CH₂), 40.14 (CH₂), 126.37 (Ar, C-H, H-2, H-6, H-2', H-6'), 130.20 (Ar, C-H, H-3, H-5, H-3', H-5'), 132.47 (q, Ar), 141.161 (q, C-N), 155.16 (C=N)

HRMS (m/z ESI⁺): Calculated for C₁₈H₂₄N₆ [M] 324.21  Found: [M+H]⁺ 325.2133

HPLC Purity (> 95%): 99.43%
6. References


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(120) Chapter V: A Comparison of the Antagonist Binding Sites of the Human Dopamine Receptors.


7. Appendix

Appendix 1 $^1$H NMR spectrum of final compound 19c in D$_2$O

Appendix 2 $^{13}$C NMR spectrum of final compound 19c in MeOD
Appendix 3 HPLC percentage purity check for compound 19c which displayed 99.05% purity
**Appendix 4** $^1$H NMR spectrum of final compound 20c in D$_2$O

**Appendix 5** $^{13}$C NMR spectrum of final compound 20c in D$_2$O
Appendix 6 $^1$H NMR spectrum of final compound 20d in D$_2$O

Appendix 7 $^{13}$C NMR spectrum of final compound 20d in D$_2$O
Appendix 8 HPLC percentage purity check for compound 20d which displayed 91.35% purity, less than the required 95% and therefore was not sent for pharmacological testing.
Appendix 9 $^1$H NMR spectrum of final compound 25b in D$_2$O

Appendix 10 $^{13}$C NMR spectrum of final compound 25b in D$_2$O
Appendix 11 HPLC percentage purity for compound 25b which displayed 98.17%
Appendix 12 $^1$H NMR spectrum for final compound 21c in D$_2$O

Appendix 13 $^{13}$C NMR spectrum for final compound 21c in D$_2$O
Appendix 14 HPLC percentage purity for compound 21c displaying 97.79%
Appendix 15 $^1$H NMR spectrum for the final compound 28b in D$_2$O

Appendix 16 $^{13}$C NMR spectrum for final compound 28b in D$_2$O
Appendix 17 HPLC percentage purity for compound 28b with 99.43%
Appendix 18 $^1$H NMR spectrum for final compound 22c in D$_2$O

Appendix 19 $^{13}$C NMR spectrum for final compound 22c in D$_2$O
Appendix 20 HPLC percentage purity for final compound 22c with 96.37%
Appendix 21 Molecular docking of compound 15 with $\alpha_{2A}$-AR-X with increased solvent exposure across the ligand (autodock binding affinity: $-3.15$ kcal/mol)

Appendix 22 Induced-fit docking of compound 22c with $\alpha_{2A}$-AR-Y receptor model in complex with a partial agonist (autodock binding affinity: $-9.54$ kcal/mol)
Appendix 23 Induced-fit docking of compound 16 with $\alpha_{2A}$-AR-Y receptor model in complex with a partial agonist (autodock binding affinity: -8.40 kcal/mol)

Appendix 24 Induced-fit docking of lead compound 1 with $\alpha_{2A}$-AR-Y receptor model in complex with a partial agonist (autodock binding affinity: -8.90 kcal/mol)