Modifying Pasireotide Analogues For Improved Oral Peptide Delivery

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For the little girl who wanted to be a scientist

(but more accurately wanted to wear a white lab coat)

Contents

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Abstract

Enhancing the oral bioavailability of peptide drug candidates is a major challenge in drug design. The gastrointestinal tract is a significant barrier to peptide absorption. This is attributed to rapid metabolic degradation and limited intestinal permeability. The aim of this thesis was to design, synthesise and assay a series of pasireotide cyclic peptide analogues to gain insight into the drivers of peptide absorption from the GI tract, ultimately to move towards improved oral bioavailability.

This thesis describes the systematic design of 27 pasireotide analogues that were synthesised using solid phase peptide synthesis. Pasireotide is an approved therapeutic drug on the market that has poor oral bioavailability. This peptide would be a direct comparison and a baseline for any improvements in absorption for the synthesised analogues. These new peptide analogues were designed to have varying degrees of lipophilicity, different stereochemistry, or a combination of both. Through systematic *in vitro* experimentation, including aqueous solubility, artificial membrane permeability, and metabolic stability in gastrointestinal and blood plasma, distinct correlations emerged. Predictably, a negative relationship between lipophilicity and aqueous solubility, and the influence of stereochemistry on permeability and metabolic resistance in gastrointestinal environments. The conformations assumed by each peptide analogue in solution dictate the exposure of labile moieties to the external environment, therefore highlighting the importance of conformational dynamics for physicochemical properties.

The extent of activity lost due to structural modifications of pasireotide analogues were measured using a novel BRET-based activity assay. From this study, it was found that the Lys residue in the peptide backbone is essential for the activity of pasireotide analogues. Removal of this residue renders the peptide inactive. Furthermore, the long-established pharmacophore of pasireotide, D-Trp-Lys, was challenged, since alternative stereochemical backbones could elicit stronger potency than that of analogues containing D-Trp-Lys. This again highlights the importance of judicious alteration of peptide conformation, this time with an impact on biological activity.

The *in vivo* uptake of select peptides B2, C2, D2 and D4 for oral delivery in male Sprague Dawley rats was studied. Each oral peptide was compared against an IV control to generate absolute bioavailability data. Peptide analogue B2 was also tested in an oral lipid-based formulation to explore the effects of an enabling formulation on peptide absorption. While conventional oral suspension formulations yielded low absorption rates akin to conventional therapeutic peptides such as octreotide, the incorporation of the most lipophilic peptide in a lipid-based formulation resulted in a two-fold increase in oral bioavailability, thereby showcasing tangible progress towards overcoming oral delivery hurdles with the aid of enabling formulations but further investigation is required given the low number of replicates for statical significance.

In summary, this thesis developed novel synthesised pasireotide analogues using a preclinical drug development pipeline, beginning with *in vitro* studies and through to *in vivo* studies in rats. We found that a balance must be struck between maintaining peptide activity and altering the structure to improve oral uptake. We found that peptide lipophilicity and changes in solubility were intrinsically linked to the likelihood of gut absorption, and this can be circumvented to some extent with lipid formulations. Stereochemistry plays a pivotal role in almost all aspects of successful drug delivery, including permeability, metabolic stability, and crucially bioactivity. Overall, this body of work advances our understanding of oral peptide therapeutic designs, offering valuable insights for future progress in the field.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Print Name: **Celine Yamfa Hung**

Date: **18th June 2024**

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Abbreviations

Bioluminescence Resonance Energy Transfer (BRET)

Calculated log P (cLogP)

Cell penetrating peptides (CPPs)

Cyclosporin A (CsA)

Cytochrome P450 (CYP)

Gastrointestinal (GI)

High-performance liquid chromatography (HPLC)

Hydrogen bond (H-bond)

Lipid-based Formulation (LBF)

Nuclear magnetic resonance spectroscopy (NMR)

Octreotide (OCT)

p-glycoproteins (P-gp)

Parallel artificial membrane permeability assay (PAMPA)

Pasireotide (PAS)

Permeation enhancers (PEs)

Self-emulsifying drug delivery system (SEDDS)

Salcaprozate Sodium (SNAC)

Solid phase peptide synthesis (SPPS)

Somatostatin (SST)

Total polar surface area (tPSA)

Ultra-performance liquid chromatography (UPLC)

Chapter 1 : Introduction

Drug discovery research is driven by chemistry, guided by pharmacology, and enabled by formulation. These three disciplines have significantly shaped the progress of medical research (1). Drug discovery can be loosely categorised into three key periods (2). The first commenced in the nineteenth century, where the basis of drug discovery relied on the serendipity of medicinal chemists. During this period, drugs now categorised as 'small drug molecules' were prevalent. The second period commenced in the early twentieth century, with the discovery of constructs like biologics and peptides. The third and current period is characterised by using computational modelling and high throughput screening for discovery and development of new therapeutic molecules (2). This thesis will focus on peptide therapeutics, a drug class that emerged in the twentieth century, yet remain a significant challenge to deliver through the oral route today.

This introductory chapter describes the background and context of this thesis within peptide therapeutic development. A brief overview of the historical significance of peptide therapeutics will be described followed by a discussion of the advantages of oral peptide delivery. A description of the current challenges to oral peptide delivery and the strategies used to combat these challenges will be described and an outline to the problem statement governing this study will be given. This chapter will conclude with this thesis' hypothesis and aims as well as an overview of the remaining five chapters in this thesis covering a detailed explanation of the methods used in this thesis, three experimental studies and an analysis of the findings and future directions of this thesis.

1.1. Peptide Therapeutics and their Utility

Drugs can be categorised into two major classes based on their size, small drug molecules and larger protein-based biologics. Small drug molecules tend to have molecular weights smaller than 500 Da and typically lack structural flexibility. These structural features lend themselves well towards facile synthesis and formulation of crystalline material but can suffer the consequence of limited specificity to their targets due to an inherent lack of flexibility in the binding pocket of their target. Biologics are drugs that have been derived from natural sources like microorganisms, plants, animals,

and humans. Examples of biologics include proteins, vitamins, vaccines, RNA, DNA and antibodies (3). Given their biological heritage, these drugs display high specificity to their targets but are typically administered via injectables due to their size and short half-lives (4).

Peptides can fall under 'biologics', however, they also sit in between small drug molecules and biologics, both size and pharmacological behaviour, and therefore can be considered as a separate category of drugs. Peptides are defined as amino acid sequences joined together by amide bonds that contain fewer than 50 amino acids (5). Like small drug molecules, peptides are small structures (on average ranging from 500 Da to 1300 Da) (6). Peptides share some key advantages with biologics, such as high activity, specificity, and low toxicity at their therapeutic targets. They also exhibit flexibility which allows for interaction with biological targets with shallow and or extended binding pockets that are not usually accessible to small molecule drugs (7). Like biologics, peptides are categorised as 'beyond rule of 5' and are usually delivered via parenteral routes of administration. This provides the most direct route to the site of action while also bypassing any first pass metabolism. Parenteral routes of administration include intravenous (directly into bloodstream), intramuscular (into the muscle), subcutaneous (into adipose tissue) and intradermal (into the dermis just below the epidermis) methods (8). Peptides are usually administered by subcutaneous and intramuscular routes, with some using the intravenous route (9).

Peptide therapeutics first came into prominence when it was found that exogenous peptide insulin could be used to treat diabetes with little to no toxicity to the patient (10). Prior to this discovery, diabetes was a condition with very poor life expectancy. The data on exactly how many people suffered from diabetes in the 1920s is difficult to determine, but it is estimated that between 0.5-2% of the population of industrialised countries were affected (11). At the time, there was no remedy for diabetes and often the supposed remedies did more harm than good to the patient. The medical standard for diabetes was so low that the life expectancy of juvenile diabetics was less than a year from diagnosis (11, 12). A revolutionary discovery by Banting et al. (10) in 1921 found that insulin extracted from a healthy dog and injected into the veins of a diabetic dog significantly improved its energy levels (11). This discovery opened the door for

peptide therapeutics and their utility in the pharmaceutical space. Just a year after its first isolation, insulin became the first commercial peptide drug and continues to treat patients to this day (13). Despite the remarkable success of insulin, it was not until the 1950s that the next peptide, corticotrophin, made it to the market (14). Discovery and the number of peptide drugs making it to market has accelerated since then with more than 80 peptide drugs having subsequently reached the market for a myriad of conditions and diseases (14). These include peptides like oxytocin for the inducement of labour, to vasopressin for the assistance and regulation of osmotic balance (15, 16). Peptides have many uses and can target a wide range of physiological disorders.

Although first discovered 100 years ago, peptide therapeutics is a growing market in 2024. Over the last 60 years, the approval rate of peptide therapeutics is trending upwards with a 7.7% increase of peptides on the global pharmaceutical market and climbing (14). In 2019, it was reported that peptide therapeutics occupy approximately 5% of the global pharmaceutical market with sales exceeding US$50 billion in that year (14). Peptide therapeutics were valued at US$601.9 million on the global market in 2023 and this is projected to increase to US$859.9 million by 2028 (17).

Peptides drugs can have a wide range of biological targets and as research in this field evolves their utility will grow. Peptide therapeutics are particularly beneficial for patients who suffer from chronic hormone deficiencies as hormone-based peptides are likely to naturally fit their therapeutics targets and can be used to supplement endogenous hormone levels in cases where levels are insufficient or compromised. G-protein coupled receptors (GPCR) currently represent 40% of total molecular targets of peptide entering clinical trials (18). Non-GPCR cell surface receptors such as cytokine receptors are also popular binding targets for peptides and make up approximately 20% of total targets that enter clinical trials (18). Antimicrobials, ion channels and other extracellular targets (e.g. adhesion molecules or secreted enzymes) make up most of the remaining peptide targets and a smaller portion of targets also include intracellular targets (18).

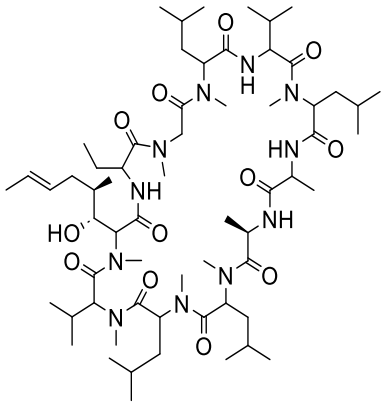
1.2. Oral Peptide Delivery

Oral delivery is often the first-choice administration route for formulators, particularly when multiple doses are required or the disease is chronic, requiring long term or even lifelong, treatment. The practical advantages to oral delivery over parenteral routes include better patient compliance, which can be attributed to a variety of negative factors associated with injections including, cost, perceived pain or discomfort, ease of administration and invasiveness. (19). Oral delivery also removes the risk of accidental injuries, improper use and biohazardous needle waste that can be consequential to using injection methods (14). There is a clear need for oral administration routes of peptides and this space remains an area of great interest in the drug delivery field.

As of 2017, there were at least 125 cyclic peptides that were reported to be orally absorbed by mammals to different extents (20). A 2020 report found that oral peptides occupied 12% of the approved/marketed peptide products and 9% of the development pipeline for peptides. Parenteral injection methods of peptides occupied 65% of the approved/marketed peptide products and 75% of the development pipeline (21). This report highlights the inherent challenge in oral peptide delivery with 88% of peptide therapeutics not developed (or undevelopable) as oral therapeutics. All of the top ten drugs defined by daily dose on the Pharmaceutical Benefits Scheme for the year 2022-23 were orally delivered small drug molecules (22). These therapeutics can use the Lipinski 'rule of 5' guidelines which predict the likelihood of a drug being successfully delivered via the oral route (23, 24). These five rules state that poor absorption or permeation is more likely when; there are more than 5 H-bond donors and 10 H-bond acceptors, the molecular weight is greater than 500 Da, and the calculated Log P (cLogP) is greater than 5 (23). In contrast, peptide therapeutics are typically larger in size and do not obey the rules established by Lipinski et al. and are therefore coined drugs 'beyond the rule of 5' (3).

However, some beyond rule of five peptide oral therapeutics have been developed. Key examples of peptides already on the market or in the late stages of clinical trials include oral formulations of cyclosporin A (CsA) and semaglutide (Figure 1.1).

(A) Cyclosporin A (CsA)



(B) Semaglutide

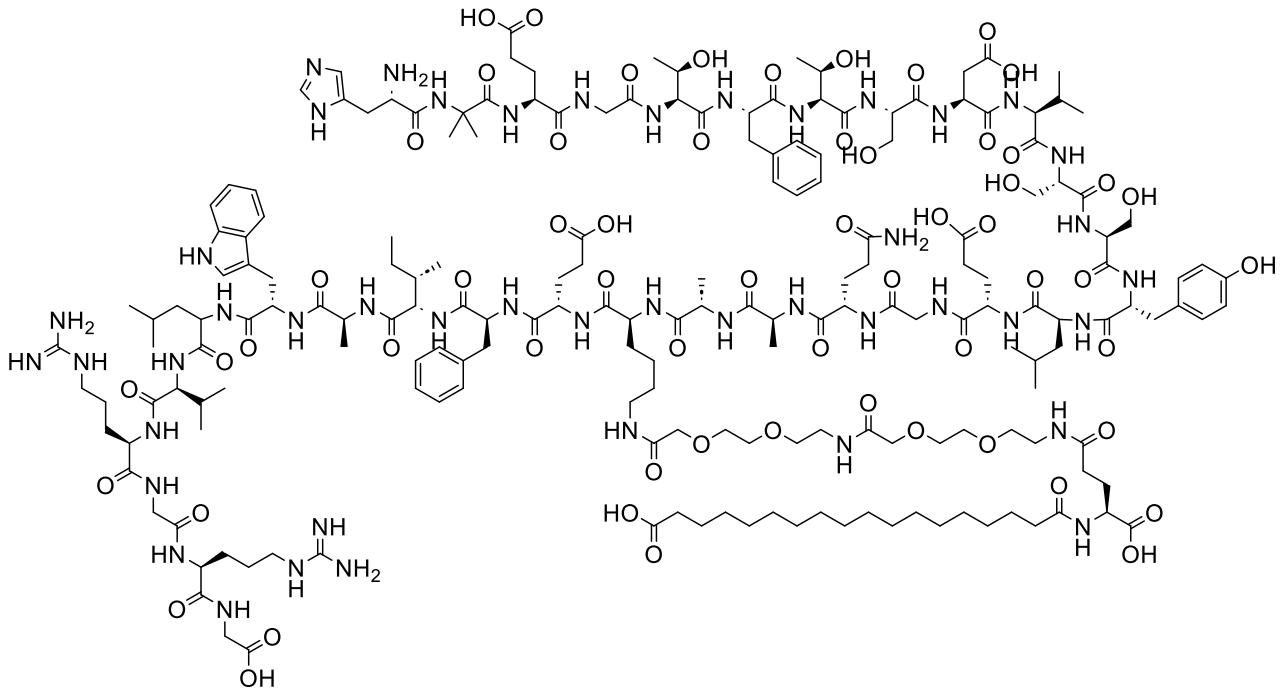


Figure 1.1 Chemical structures of cyclosporine A (A) and semaglutide (B), two orally deliverable peptides.

CsA was discovered in the 1970s from fungal metabolites and is a good immunosuppressant (25). The discovery of CsA revolutionised organ transplant therapy by preventing early graft loss due to rejection by the body (26). CsA is well known in the oral delivery space as the gold standard for oral peptide bioavailability. Good oral bioavailability is defined as more than 20% of drug present in the blood if the orally administered compound was to be measured in systemic circulation (14, 27). While a typical peptide, for example leuprolide acetate, has an oral bioavailability of less than 1% (28), when delivered in conjunction with lipid-based formulations, CsA has reported oral

bioavailability values of ~40% in the form of Sandimmune Neoral® (29). The role formulation plays in the successful delivery of CsA can be found in Section 1.4.4 (page 19) of this chapter. While CsA has good oral bioavailability, the same cannot be said about other types of cyclosporins. A review by Corbett et al. (29) reports that different subtypes of cyclosporin have different extents of absorption, and while cyclosporins are one of the best-studies class of membrane permeating peptides, the mechanism of absorption of these peptides remain not completely understood.

Semaglutide is a glucagon-like peptide 1 analogue that is used for the treatment of type II diabetes. Based on the previously successful peptide liraglutide, semaglutide was designed to be a once-a-week injection as opposed to the previously established once-a-day dose for liraglutide (30). This peptide has been on the market for some time in its injectable form (Ozempic®) but was recently approved by the FDA in an oral form (Rybelsus®) in 2019 (31) Semaglutide has an absolute bioavailability of 0.4-1% when co-formulated with salcaprozate sodium (SNAC) following oral administration (32). The permeability enhancer, SNAC, is reported to temporarily increase the epithelial flux without significant consequences to local or systemic toxicity by increasing lipophilicity from non-covalent macromolecule complexation (33, 34). The role of permeability enhancers can be found later in Section 1.4.4.3. This 2018 study was interesting for several reasons. Unlike most drugs, semaglutide in conjunction with SNAC was reported to be absorbed from the stomach using transcellular transport to cross the stomach lining (35). This is surprising as drug absorption from the stomach is known to be slower and less effective in comparison to absorption from the small intestine (36). It was also reported that the drug absorption of oral semaglutide in conjunction with SNAC was through the transcellular route, as opposed to paracellular routes commonly reported with alternative permeation enhancers like sodium caprate (34). What remains unclear is how the SNAC molecule is interacting with the stomach lining and whether the mechanism of action of this formulation will illicit permanent damage to the stomach lining.

Currently, the peptides that are successfully delivered orally require the assistance of enabling formulations to aid in gut absorption. The two examples given here, CsA and semaglutide, cover two very structurally distinct peptides. CsA is more

akin to a small drug molecule, highly lipophilic and relatively small compared to the majority of peptide therapeutics. Semaglutide looks more akin to most peptide therapeutics as it has a large molecular size and contains many ionisable amino acids. This highlights why it is so difficult to orally deliver peptides in their native forms. Despite the clear advantages of oral peptide delivery, there remain key challenges that candidate peptides must tackle prior to achieving oral bioavailability.

1.3. Challenges to Oral Peptide Delivery

Low peptide bioavailability through oral administration is attributed to several factors including degradation in the gastrointestinal tract and plasma, low gut absorption through permeability, and rapid elimination/clearance once in the bloodstream (Figure 1.2) (37). Each of these factors will be covered in more detail in the sections that follow.

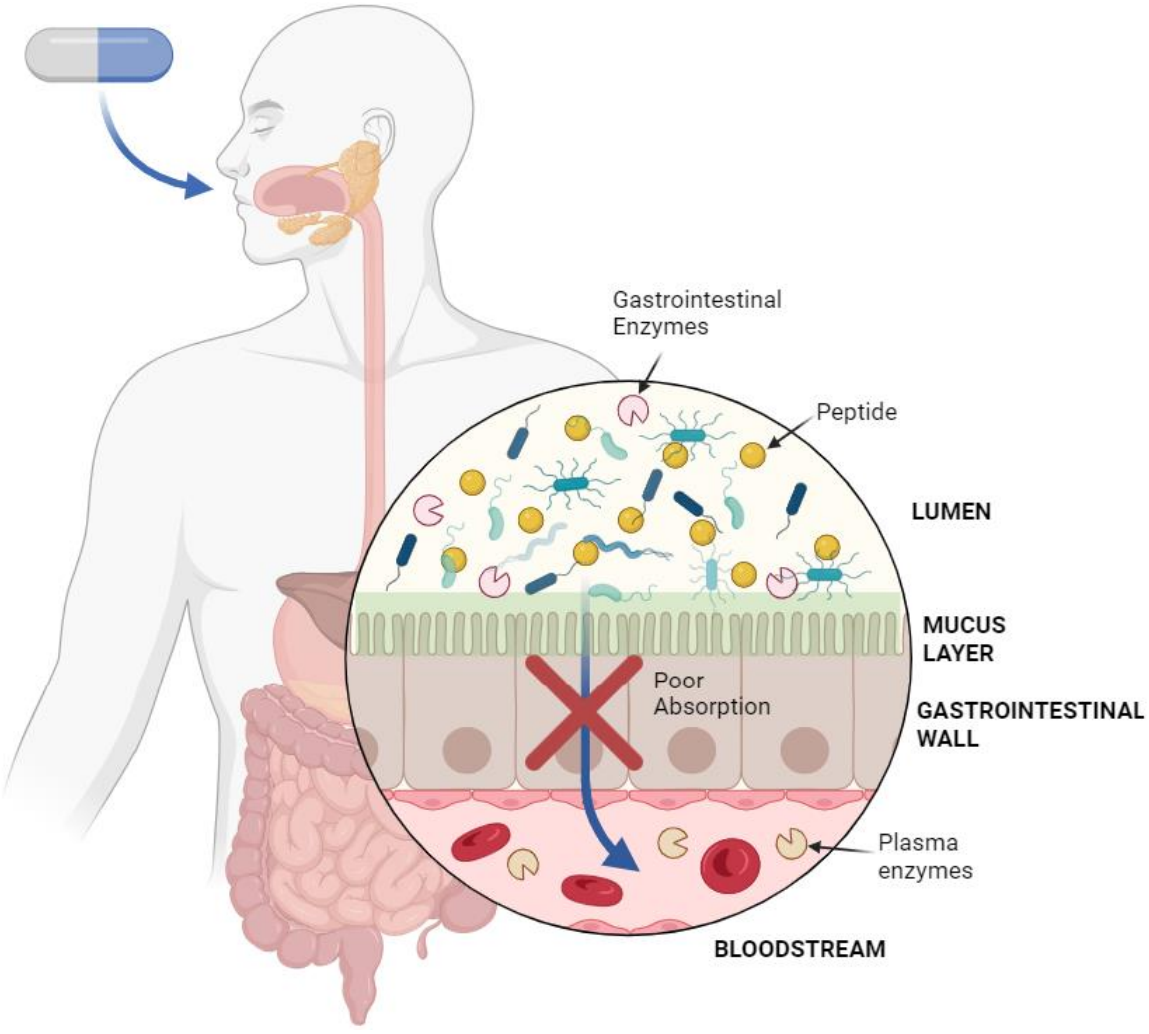


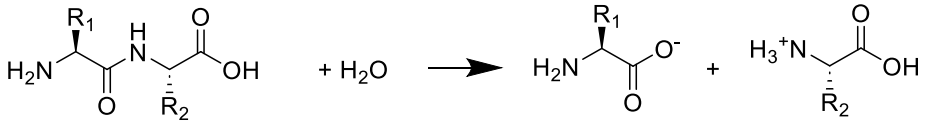
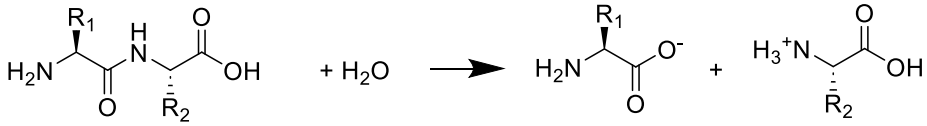
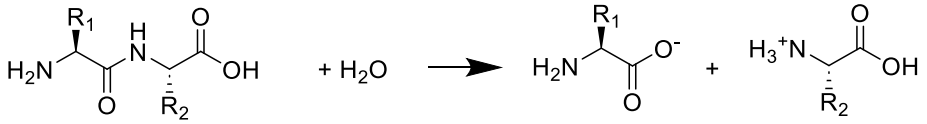
Figure 1.2 Challenges to oral peptide delivery from the gastrointestinal tract. Image was created with BioRender.com.

1.3.1. Metabolism of Peptides

Metabolism of peptides can occur at any stage after oral delivery; while traversing the gastrointestinal (GI) tract, in the bloodstream, in the liver, and other organs and tissues. Such susceptibility to metabolism means peptides often have short half-lives (between 15 seconds to 2 hours) in plasma (38).

The physiological function of the GI tract is to primarily digest food and other ingested materials which can then be subsequently absorbed as nutrients, and provide an efficient barrier to toxic materials, i.e. peptides, viruses and bacteria (5, 39, 40). To achieve these functions, the GI tract secretes many enzymes that cleave peptides into smaller fragments that can render the drug inactive. The GI tract also has a wide spectrum of pHs along its route, ranging from 2 in the stomach to 7 in the small intestine, requiring the peptide drug to withstand these pH extremes (41). These GI functions are clearly at odds with the oral delivery of larger molecules such as peptides and proteins as these structures are unlikely to remain intact throughout the digestion process Table 1.1 summarises the key GI enzymes and their localisation within the GI tract. Peptide drugs can be formulated to bypass GI degradation from enzymes like pepsin or the extreme acidic environment by utilising tablets or capsules that have enteric coatings which protect the drug from the external environment and release the drug in the small intestine (20, 42). The small intestine is the main site of absorption for drugs due to its large surface area, however, the intestines contain enzymes like trypsin and α-chymotrypsin which are secreted from the pancreas and can hydrolyse peptide bonds.

Table 1.1 Localisation of key enzymes found within the gastrointestinal tract (43, 44).

Enzyme	Location	Optimum pH	Cleaves
Pepsin	gastric mucosa	2	Carboxylic side of aromatic (e.g. Phe, Tyr, Trp) and hydrophobic (e.g. Leu) amino acids
	 R_1 group: Phe, Tyr, Trp, Leu		
Trypsin	Small intestinal lumen	8	Carboxylic side of Lys and Arg
	 R_1 group: Lys, Arg		
α-Chymotrypsin	Small intestinal lumen	9	Carboxylic side of Tyr, Phe, Trp and Leu
	 $R_{1/2}$ group: Tyr, Phe, Trp, Leu; $R_2 \neq$ Pro		

Should the peptide drug remain intact through the GI tract and cross through the gut wall into the bloodstream, the human body has over 550 proteolytic enzymes that can be found throughout the blood, liver, and kidneys (9, 38, 45). One of the advantages of peptide therapeutics are their ability to be flexible due to a high number of rotational bonds. This helps with their activity against a range of targets because they can assume a variety of conformations. However, through the lens of enzymatic stability, this flexibility can be a drawback, leaving peptides vulnerable to proteolytic degradation (37).

Orally administered drugs which are absorbed from the stomach or the intestine, are transported in the venous blood via the portal vein and pass through the liver before entering systemic circulation (20). Any metabolism performed by the liver prior to reaching systemic circulation is called first pass metabolism. Blood plasma itself also contains proteases like thrombin, plasmin and clotting factors that can hydrolyse peptides (20). Approximately 90% of the liver is comprised of hepatocyte cells which secrete a large variety of enzymes that drive hepatic first pass metabolism (46). An example of one of the classes of enzymes secreted by the liver are cytochrome P450 (CYP) enzymes which account for more than 75% of drug metabolism (46). These enzymes come in many isoforms and drugs like buspirone, cyclosporin, lovastatin, saquinavir, and verapamil exhibit poor oral bioavailability due to extensive CYP-mediated first-pass metabolism (46).

Once in systemic circulation, drug can be cleared via the kidneys and liver in the form of elimination and metabolism (47). The kidneys filter the blood and remove materials to the urine in a process called renal clearance (38). Renal clearance is a dynamic process involving glomerular filtration and reabsorption (48). For lipophilic drugs, passive reabsorption back into the systemic circulation will result in minimal renal clearance (48). Therefore, these lipophilic drugs are more likely to be cleared via the liver. The primary purpose of hepatic metabolism is to breakdown the peptide into more hydrophilic molecules that will not undergo tubular reabsorption and therefore can be eliminated from the body in the urine (47). For hydrophilic drugs, the filtered drug will be excreted into the urine unless the drug is actively reabsorbed back into the systemic circulation by a transporter-mediated mechanism (48). Drugs can also be secreted from the blood into the lumen of the proximal tubules, resulting in a renal clearance greater than the glomerular filtration rate (48). Therefore, even if a peptide drug has successfully crossed the GI tract intact, avoided first pass metabolism by the liver and manages to arrive in systemic circulation, there still runs the possibility of rapid clearance from plasma and from the body. Thus, repeat administration of peptide drugs is often needed to the target tissue to maintain the therapeutic effect, irrespective of delivery method.

The presence of proteolytic enzymes that can breakdown peptide drugs throughout the body necessitates innovative strategies such as structural modifications, enzyme inhibitors, and advanced delivery systems to enhance the stability and bioavailability of peptide drugs in the body (49).

1.3.2. Low Intestinal Absorption

GI absorption is the movement of compounds from the intestinal lumen into the bloodstream. Absorption of orally administered drugs primarily occur in the small intestine where its unique anatomical properties, namely microvilli and specific drug transporters, can facilitate drug transport into the bloodstream (50). The gut contains a layer of mucus that can prevent peptides from accessing the surface of the gut wall (51), however, should the peptide make it to the intestinal wall, there are several ways for drugs to be absorbed through the lipid membrane. These include transcellular passive diffusion, passive carrier-mediated uptake, paracellular diffusion between cell junctions and receptor mediated transport or endocytosis (Figure 1.3) (52).

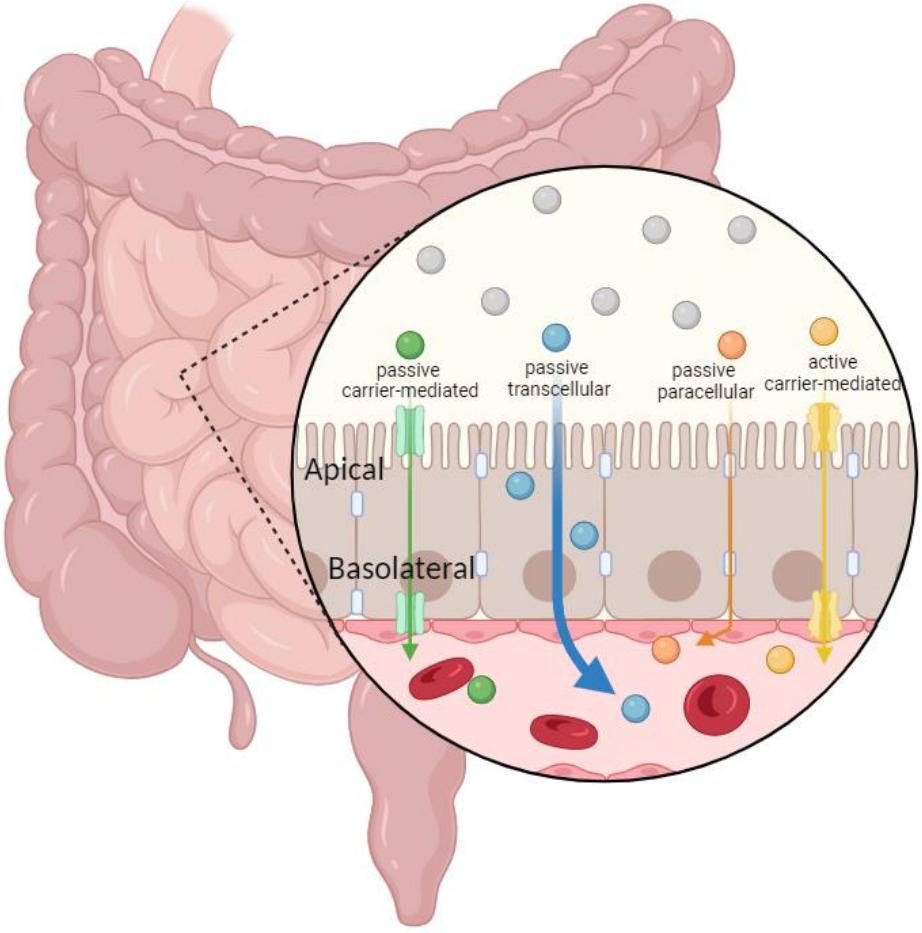


Figure 1.3 Intestinal absorption pathways: passive carrier mediated (green), passive transcellular (blue), passive paracellular (orange) and active carrier-mediated (yellow). Image was created with BioRender.com.

Passive transcellular diffusion is the movement of drug through the intestinal membrane without the use of any additional transporters or junctions. This process occurs down a concentration gradient from the apical to the basolateral side of the intestinal epithelium according to Fick's first law (50). This is the movement of solute from areas of high to low concentration in response to a chemical potential gradient and are energy independent (52). Passive transcellular diffusion is the predominant pathway for many small-molecule drugs that have good membrane permeability because of the extremely high surface area of the intestine. This route provides massive input of the drug into the system in all cases where the permeability is not dependent on transporters (25). This passive process requires drug molecules to partition across the lipid bilayer in the apical plasma membrane of intestinal epithelial cells and is strongly dependant on the physicochemical properties of the drug molecule. In general, the transcellular permeability of a solute is a complex function of such physicochemical properties as size, lipophilicity, hydrogen bond potential, charge, and conformation (52-54). A study by Camenisch et al. (53) described a sigmoidal relationship between lipophilicity (as a function of molecular size) and transcellular permeability, where they reported small peptides under 300 Da were too hydrophilic for any distribution but slightly higher molecular weight molecules were able to utilise transcellular transport before eventually plateauing in absorption due to solubility-limited absorption. Drugs that cross via the transcellular pathway normally have a good balance between optimal hydrophobicity and solubility (55).

While not technically an absorption pathway, cell penetrating peptides (CPPs) are a group of cationic or amphipathic peptides that can cross the cell membrane to deliver their payload into the cell. CPPs are often constructed with many 'Arg', 'His' or 'Lys' residues that range from 5-30 amino acids long (39). How these peptides cross membranes are not completely understood but it is suggested that their positively charged nature allows the peptide to disrupt the natural negatively charged lipid membrane to allow endocytosis into the cell (14, 20). A study trialling oral insulin in rats using conjugated- or electrostatically attracted CPP mixtures found that CPPs formulated with nanoparticles may be a method for epithelial internalisation but did not report any peptide presence in blood plasma (56, 57). In the scope of oral peptide

delivery, it is unlikely that these highly charged peptides will be absorbed from the gastrointestinal tract into the bloodstream.

The paracellular pathway is an aqueous route involving the passive movement of molecules between the narrow passages of adjacent intestinal epithelial cells restricted by the presence of tight junctions (52, 55). These tight junctions are channels that span about 80 nm long over the entire lateral side of the cell (55). Compounds can cross tight junctions via two pathways, the pore pathway and the leak pathway, and are differentiated by size and charge with regulation by immune cells (58). Pore pathway permeability is controlled by claudin proteins which form channels or barriers at the tight junction (58). These channels are charge-selective and size selective with a maximum diameter of solutes being 6 Å (58). The leak pathway allows molecules of up to 125 Å in diameter to cross the epithelial barrier but its mechanism is not well understood (58). These narrow spaces are designed to prevent the passage of large hydrophilic molecules, and therefore present a significant barrier to peptide absorption (50, 55). In addition, the total surface area of these paracellular spaces relative to the intestinal membrane is extremely low (~0.1%), so the overall contribution of unmodified paracellular diffusion in the drug absorption process is limited (50).

Carrier-mediated transport is the movement of compounds across a membrane with the assistance of channels or receptors. They can be controlled by two types; passive, which uses the same Fick's law principle as both transcellular and paracellular transport, and active carrier-mediated transporters which uses energy to move solute across the cell. Passive carrier-mediated transport (otherwise known as facilitated uptake) occurs according to a concentration gradient and is not energy-driven (50). Active carrier-mediated transport (otherwise known as receptor-mediated transport) utilises an array of peripherally and integrally associated proteins within the organized lipid matrix of a membrane. For some solutes, these proteins serve as specific transporters or receptors for uptake into the cell (50, 52). There are also transporters in the small intestine that prevent the uptake of compounds into the cell like p-glycoproteins (P-gp). P-gp are ATP-dependant transporters that are responsible for the efflux of compounds back into the gut lumen (59). It was found that P-gp prevented the absorption of cyclosporin when delivered orally by 10-fold in a study comparing the

absorption of cyclosporin in the presence of P-gp to that of inhibited P-gp (59). Carrier-mediated transport processes, either passive or active, may be subject to inhibition by both specific and non-specific interactions related to either drug-drug interactions, or the presence of food or food components (50).

1.4. Strategies for Improving Oral Peptide Delivery

Given the interest in oral peptide delivery, there are many strategies that have been explored to achieve this goal. These strategies are primarily focused on improving peptide permeability across lipid bilayers as well as stabilising the peptide against metabolic degradation. Two key strategies include reducing peptide flexibility and reducing the number of interactions between the peptide drug and its aqueous environment.

Drug flexibility can be measured by the number of rotatable bonds in the structure. Early research identified molecular weight as an important factor for predicting oral bioavailability (23, 60). However, Veber et al. (60) found that a complimentary factor to molecular size was the number of rotational bonds in the structure. As drug molecular weight increases, it is likely the number of rotational bonds also increases. A study by Veber et al. (60) found that among a group of drugs that all displayed oral bioavailability, there was an equal proportion of compounds both over and under 500 Da in molecular weight. What these compounds had in common was that despite molecular weight, they all contained less than 7 rotational bonds. In a similar fashion, this same study found that compounds of both size ranges (<500 Da and >500 Da) displayed low bioavailability when their rotational bonds were greater than 10. To reduce drug flexibility, two strategies have been explored in further detail, peptide cyclisation and intramolecular hydrogen bonding.

The passive permeability of peptides necessitates these drugs to transfer from highly polar aqueous environments to nonpolar interiors of the gut wall to again return to a polar environment. Peptides are intrinsically quite polar due to their amide bonds and therefore generally display good aqueous solubility by forming H-bonds with their aqueous environment. Peptide structures can be engineered to exhibit properties better

suited to oral delivery. Some of these properties include altering polar surface area (PSA) and lipophilicity.

Alternative strategies for improving oral peptide delivery include formulating the peptide within oral formulations like lipid-based formulations and utilising structural changes like D-amino acids to influence peptide conformation.

1.4.1. Cyclisation

There are many forms of cyclisation that peptides can undertake. These include cyclisation through sulphide containing amino acids like 'Cys' to form disulfide bridges, side chain interactions and 'head-to-tail' backbone cyclisation which binds both tail ends of a linear peptide to form an amide bond (Figure 1.4).

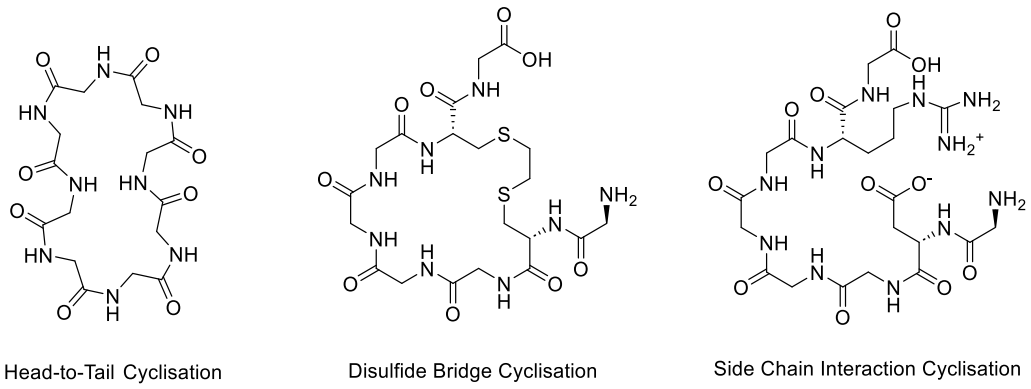


Figure 1.4 Types of structural peptide cyclisation.

Cyclisation adds conformational restraints to the peptide which can impact permeability, flexibility, and exposure to enzymatic degradation (both in the form of enzymes found in the external fluids but also from any exopeptidases present in the case of head-to-tail cyclisation) (38). Cyclic peptides also show improved chemical stability and longer biological half-life with little to no impact on potency (38, 60-62). Studies by Marastoni et al. (63) found that the cyclic analogue of Peptide T, a linear octapeptide, retained its bioactivity and proved to be highly resistant to degradation by plasma and brain enzymes. This trend can also be seen in the peptide somatostatin (SST) which in its native form has a half-life of less than 3 minutes (64). Pasireotide, a head-to-tail cyclic analogue of SST, has a half-life of 23 hours (65). A study by Okumu et al. (66) explored the permeability of three linear hexapeptides to their head to tail cyclic counterparts and found that all cyclic peptides performed better in Caco-2 permeability assays than the linear peptides. They predicted that this was due to the cyclic peptides being more

lipophilic to their linear counterparts, likely due to the absence of terminal amine and carboxylic acid groups. These cyclic peptides showed an increase in flux via the transcellular route of absorption. This same study also found that cyclisation by the formation of disulfide bridges also result in higher rigidity which in turn improved resistance to enzymatic cleavage of susceptible peptides bonds as seen between linear somatostatin and its cyclic counterpart analogue octreotide (67).

1.4.2. Intramolecular Hydrogen-Bonding

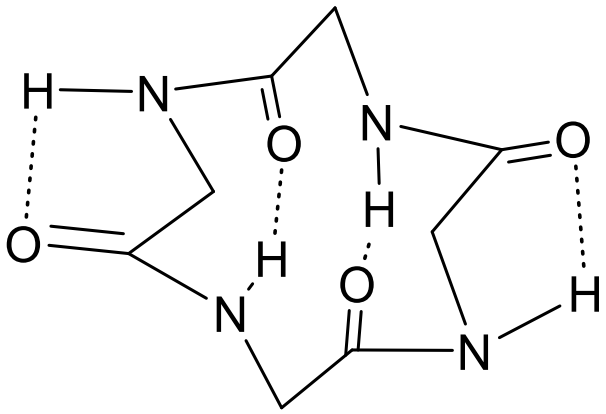


Figure 1.5 Example of intramolecular bonding within a peptide. Dashed lines present hydrogen-bonds.

Intramolecular hydrogen bonding (H-bonding) refers to the bonds that can form within the peptide molecule. This more frequently occurs for cyclic peptides as their structure better allows for this bonding to form by folding into itself. H-bonds can occur between the carbonyl and amine groups frequently found in amide bonds (Figure 1.5) (68). Intramolecular bonding reduces the flexibility of the peptide as they are held in place by the bonds forming within the cyclic structure. This reduces the number of interactions available for H-bond donors and acceptors to form with the solvent and lends itself better to lipid permeability.

1.4.3. Polar surface area (PSA) and Lipophilicity

Polar surface area and lipophilicity are molecular property descriptors reflecting the behaviour of the molecule in a solvent. Generally, peptides with low PSA show improved passive permeability. Initial research from Veber et al. (60) suggests that compounds with PSA equal to or less than 140 $Å^2$ will likely have good bioavailability. Computational models that try to predict peptide permeability often use PSA as a key property (41). Lipophilicity is the ability of a compound to dissolve in lipids or non-polar

solvents. It is widely established that lipophilicity is an important parameter for the permeability of drugs. The factors that contribute to peptide lipophilicity are varied and include amino acid content and hydrophobicity, which helps drive the peptide into the hydrophobic membrane core (27). Cyclic peptides already display an inherent increase in lipophilicity compared to its linear form from the absence of terminal amine and carboxylic acid groups, and there are further positive correlations between lipophilicity and cell permeability (27, 66). Computational studies by Wang et al. (69) sampled 62 cyclic hexapeptides and could draw correlations between peptide lipophilicity and their permeability from Caco-2 and PAMPA experiments. Hewitt et al. (70) observed that generally changes to peptide structures that led to a decrease in lipophilicity were detrimental to permeability, whereas those that retained or increased their lipophilicity showed better permeability performances in *in vitro* studies. Rand et al. (71) reports that the increased oral absorption they observed for their synthesised peptides was due to increased lipophilicity, measured by shake flask log D measurements as opposed to *in vitro* PAMPA or Caco-2 studies as reported by the previous two studies. This suggests that irrespective of how lipophilicity is measured, this property is highly correlated to peptide permeability.

However, peptides must strike a balance between being neither too hydrophilic nor too lipophilic. Peptides that are too lipophilic may readily move into the cell membrane but are unlikely to then leave the hydrophobic membrane core back into an aqueous environment which results in poor overall transport. Highly lipophilic peptides also run the risk of low aqueous solubility- a property which is critical for drug delivery. Hewitt et al. (70) noted in their study that highly lipophilic peptides were prone to low recovery due to poor solubility and aggregation issues during their *in vitro* permeability assays.

1.4.3.1. N-Methylation

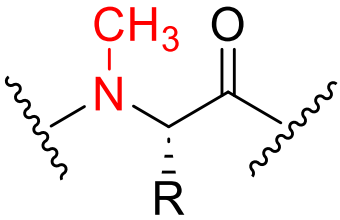


Figure 1.6 Example of N-methylation of an amino acid.

Researchers have explored the use of N-methylation to decrease PSA and increase lipophilicity. N-methylation is a chemical modification that involves the addition of a methyl group to nitrogen atoms within the amide bond of peptides (Figure 1.6). As a result of N-methylation, the peptide has an increased hydrophobic surface area and reduced PSA, as well as reduced H-bond interactions with solvent water molecules (27). N-methylation is common among peptide natural products, the best example is naturally derived cyclosporin A, the gold standard of oral peptide delivery. Cyclosporin A has extensive N-methylation along its backbone. Studies from Biron et al. (62) and Chatterjee et al. (72) both found that N-methylation can drastically improve the metabolic stability and intestinal permeability of peptides, with Chatterjee et al. reporting 10% oral bioavailability for a tri-N-methylated Veber-Hirschmann peptide analogue. There is a substantial impact on both the physical properties and conformation states of cyclic peptides when utilising N-methylation that can affect peptide activity (73). White et al. (73) synthesised a cyclohexapeptide containing three N-methyl groups which had an oral bioavailability of 28% in rats. However, White et al. also suggested that the addition of these N-methyl groups significantly impacted peptide conformation and that this can hinder the peptide activity, raising the question of whether peptides that are permeable using this strategy can retain their activity (73). In cases where lipophilic peptides that are highly N-methylated have been reported to have high membrane permeability, it is thought that those peptides are predominantly transported by mechanisms other than passive diffusion (27). A study by Marelli et al. (74) found that the absorption of polar N-methylated peptides is predominantly through carrier-mediated transport.

1.4.4. Formulation

Formulations can be used to enhance a peptide's likelihood of oral bioavailability. The formulation strategies explored in this section are ion-pairing salts, lipid-based formulations, and permeability enhancers.

1.4.4.1. Ion-pairing salts

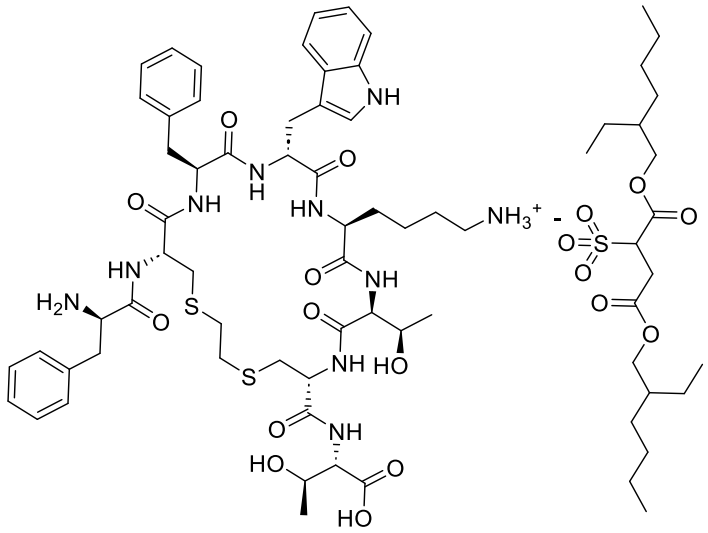


Figure 1.7 Lipophilic salt (docusate) ion paired octreotide.

Peptides are often ionisable and therefore are too polar to be permeable across a lipid membrane. To address this, the peptide's ionisable residue can be ion paired to a lipophilic salt to improve the drug's overall lipophilicity. Ion pairing a lipophilic salt with an ionisable drug has been seen to improve the drug's overall lipid solubility by more than 10-fold of the original salt form (75-78). Examples of lipophilic salts that have been previously used in conjunction with peptides include dodecyl sulphate, docusate, deoxycholate and decanoate salts (40, 79). Early studies found that ion pairing the peptide leuprolide acetate with decanesulfonate increased the 1-octanol-water partitioning of the peptide by 28-fold (80). More recent studies have also shown the improvement in permeability using ion-pairing in more modern *in vitro* permeability assays like PAMPA and Caco-2 (81). A study conducted by Bonengal et al. (40) found that octreotide-deoxycholate in SEDDS resulted in a 5% relative bioavailability to their IV formulations in pigs. However, ion-pairing lipophilic salts to ionisable peptides do not universally improve permeability measurements. Studies by Li et al. (78) found that the peptide octreotide when ion-paired with docusate (Figure 1.7) had no increase in measured permeability from both Caco-2 and *in situ* perfusion studies. However, ion-

paired peptides in conjunction with lipid-based formulations (LBF) demonstrated an enhancement in oral absorption, from 0.39 ng/mL for non-ion-paired octreotide in LBF to 4.64 ng/mL for ion-paired octreotide in LBF. Li suggests that this is likely due to higher partitioning and prolonged retention of drug within the lipid colloidal structures with the assistance of LBF (78).

1.4.4.2. Lipid-based Formulations (LBF)

Lipid based formulations for oral delivery are mixtures of oils, glycerides or surfactants, sometimes with the addition of co-solvents, which aid the aqueous solubility of poorly water-soluble drugs (82). These formulations allow their typically hydrophobic drug payloads to remain in a dissolved state throughout the transit through the gastrointestinal tract and have been found to improve the oral bioavailability of these poorly water-soluble drugs (82, 83).

Peptides do not fall under the poorly-water soluble category given their tendency for high hydrogen bonding potential with aqueous environments. However, as strategies to increase permeability also affect hydrophobicity, there comes a shift towards increasing lipophilicity and reducing water solubility. LBFs can aid in the solubility and oral delivery of these more lipophilic oral peptide candidates. The most well-known example of how formulation can aid oral delivery of peptides is Neoral® which contains the peptide cyclosporin A (CsA) (82, 84). Prior to its formulation in a lipid emulsion, CsA did not demonstrate promising oral bioavailability in humans. The first time a single dose of solid CsA was administered in humans, it was not absorbed, and no pharmacologically active levels of drug were found in the blood plasma (85). It was serendipity that the idea of dissolving the drug in olive oil to form an emulsion was had as a vehicle to transport dissolved CsA across the intestinal wall (85). From this idea, the first iteration of oral CsA on the market was developed in the form of Sandimmune®. Sandimmune® is a relatively crude LBF containing triglyceride oil, long-chain glycerides, and ethanol (86). This version of oral CsA has highly variable bioavailability and positive food effects which contributed to the risk of dosing patients outside the drug's therapeutic window (86). Its high variability is thought to be caused by this formulation's coarse emulsion in water and its reliance on digestion to promote absorption. In contrast, Neoral®, which is comprised of mono-, di-, triglycerides from refined corn oil, surfactant, propylene glycol and

ethanol, forms emulsions of droplets sizes less than 100nm. This nanoemulsion provides a more uniform droplet size for the peptide to diffuse and is less reliant on the digestion process for absorption (87). Neoral® is described as a self- micro emulsifying drug delivery system that has high bioavailability, less susceptibility to food effects and low patient effect variability (86). The formulation journey of orally deliverable CsA highlights the importance of formulation for the successful delivery of peptides. This one peptide went through a range of successes from initially not being orally absorbed without the aid of LBF, to achieving oral bioavailability but balancing this success with high variability between patients to finally settling into a robust formulation that has reduced variability between patients.

Since the successful formulation of CsA, the utility of lipid-based formulations for peptide drugs have been explored further and has even been found to protect peptides from enzymatic attack (28, 40, 49, 88, 89). Hintzen et al. (28) found that a self-emulsifying delivery system containing leuprorelin ion paired to oleate was able to protect the peptide from degradation from trypsin and α-chymotrypsin so that after 2 hours of exposure, 50-60% of the peptide remained intact compared to less than 20% of the ion pair alone. This same study reported a 17-fold improvement in oral bioavailability in rats compared to a leuprolide acetate solution. Similar observations were made by Ijaz et al. (88) who found that ion-paired peptide lanreotide with sodium deoxycholate in a self-nanoemulsifying delivery system could protect the drug payload against thiol-disulfide exchange reactions- a type of peptide metabolism (90).

1.4.4.3. Permeability enhancers (PEs)

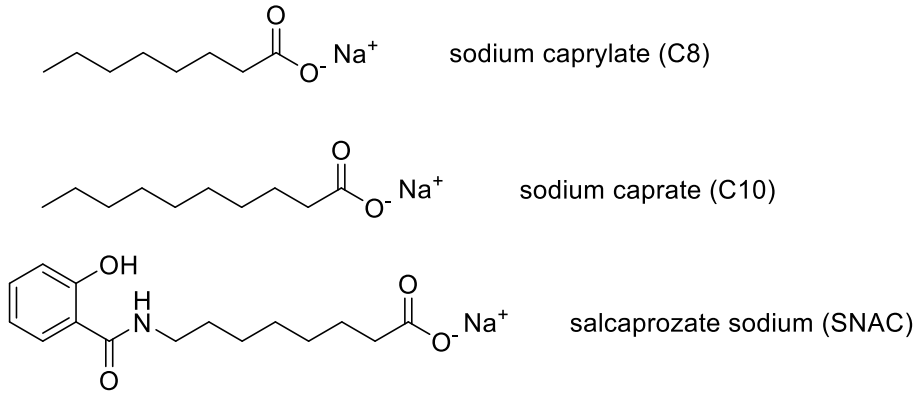


Figure 1.8 Examples of common permeation enhancers.

Permeability enhancers (PEs) are substances added to formulations to increase intestinal absorption by transiently altering the intestinal epithelial barrier to facilitate permeation of macromolecules with low oral bioavailability (79). Currently, the mechanism of action for PEs is not widely understood but it is thought to modulate tight junctions between the small intestine epithelial membrane (91). The concentration of PE as well the duration of exposure plays an important role in the extent of perturbation (91). Common PEs include medium chain fatty acid-based sodium caprate (C10), sodium caprylate (C8), and the C8 derivative salcaprozate sodium (SNAC) (Figure 1.8).

Currently, peptides like semaglutide and octreotide are available for oral delivery when co-formulated with permeation enhancers (35, 42). Semaglutide is currently sold under the name Rybelsus® and is co-formulated with SNAC. Studies found that the semaglutide-SNAC formulation has an oral bioavailability of 1.22% in dog models and a relative oral bioavailability of 0.4-1% via gastric absorption in humans (30, 35, 92). Studies have also explored the effect of SNAC in conjunction with octreotide. Fattah et al. (93) found that SNAC could improve the permeability of octreotide by up to 3.4-fold in the ileum using Ussing chamber assays. However, Fattah reports that these results were highly dependent on the composition of the buffer as SNAC can form interactions with buffer components. Oral octreotide is currently available with Transient Permeation Enhancer (TPE®) under the trade name Mycapssa™ (94). TPE® is an oily suspension of octreotide acetate which uses C8 as its primary PE in the formulation. Clinical trials studies found that this formulation has a relative oral bioavailability of ~0.7% for a 20 mg dose (94, 95). Despite such low oral bioavailability, both oral formulations for semaglutide and octreotide are effective due to their high potency and the structural modifications that increase their half-life (92).

There are however concerns about the prolonged use of PEs (91). It remains unknown as to whether PEs can cause irreversible epithelial damage or whether alternative species like pathogens can be co-absorbed along with the peptide drug. Given the GI tract is highly resistant to other irritants like spicy foods, drugs, alcohol etc., the PE effects appear to be reversible, but the long-term consequences have not yet been explored. For patients that require chronic and frequent treatment, it is unclear whether the intestinal epithelial damage-repair cycle is sustainable during repeat-dosing regimens or whether this could create conditions for allergies or autoimmune responses with prolonged use. While there are methods to model for safety and toxicity of PEs, these studies cannot thoroughly examine repeat-dosing consequences. PEs have shown promising results to improve intestinal absorption, further study is required to understand any side effects to this mechanism of absorption.

1.4.5. D-Amino Acids

D-amino acids are mirror images of the more common L-amino acids and have been widely reported in the literature to aid in stabilising peptides against enzymatic degradation, as well as in some cases, improve permeability (Figure 1.9) (38, 96-98). In nature, most amino acids found in peptides and proteins are L-amino acids. However, D-amino acids have unique properties that can be harnessed for specific purposes, including improving oral peptide delivery.

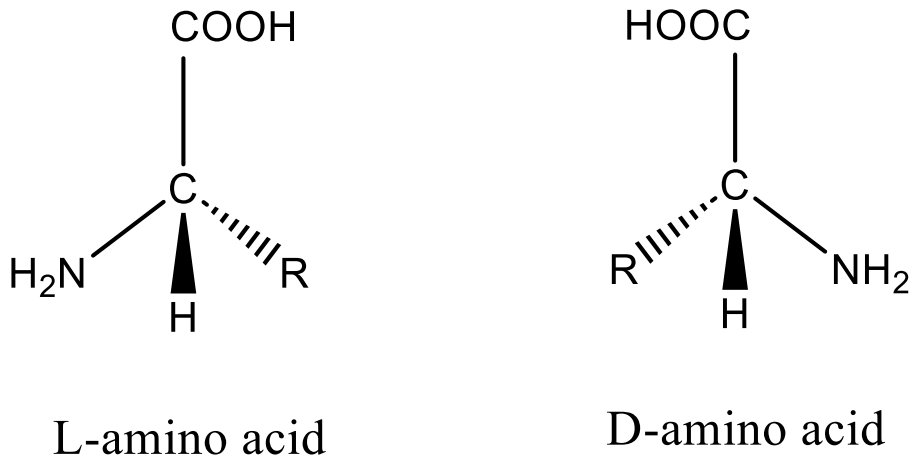


Figure 1.9 Example of L-amino acid vs D-amino acid.

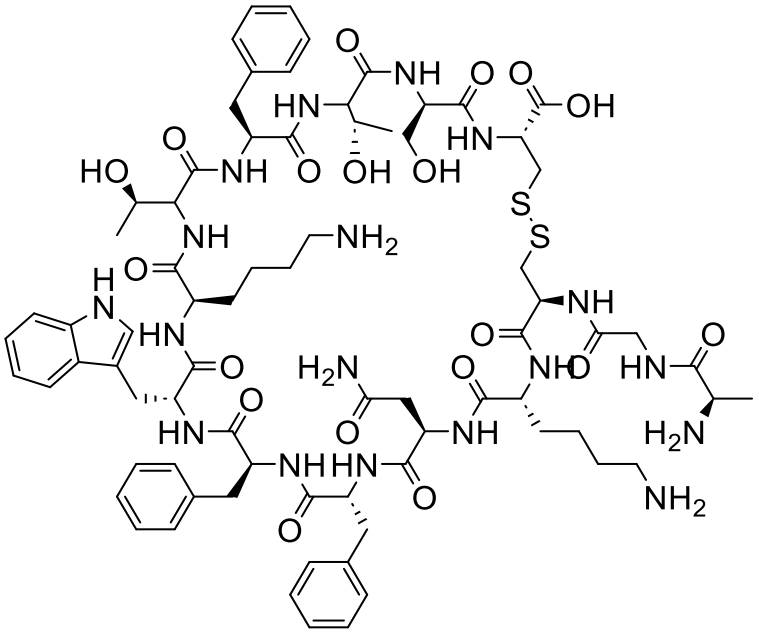
One significant difference between D- and L-amino acids is their susceptibility to enzymatic degradation. Naturally occurring proteolytic enzymes in the human body typically target L-amino acids for breakdown (5, 9). This prompted methods of peptide stabilisation which include the replacement of natural L-amino acids with D-amino acids, which are not susceptible to degradation by these naturally occurring proteolytic enzymes. This stability allows the peptide to remain intact in circulation for longer to improve pharmacokinetic properties. Studies by Agersø et al. (99) found that peptide vasopressin, which contains an L-Arg has a half-life of 10-35 mins in humans. When this amino acid is substituted with a D-Arg (desmopressin), the peptide has a half-life of 3.7 hours in humans. However, substitution of L-amino acids with D-amino acids is not a blanket method to withstanding degradation. Studies by Darlak et al. (100) and Rafferty et al. (101) both found that D-amino acids do not exclusively improve peptide stability (39). Darlak reported that D-amino acid substitutions of dermorphin were cleaved more rapidly than the parent peptides and Rafferty found no difference in in plasma half-life times after intravenous injection between L- and D- analogues of growth hormone-releasing factor (1–29)-amide.

The incorporation of D-amino acids can alter the conformation of peptides. In recent years it has been found that this structural modification may also facilitate interactions with biological membranes, making it easier for peptides to traverse cell membranes (102). Studies by Rezai et al. (68) found that two diastereoisomers, which were identical in both molecular weight and solubility, had membrane diffusion rates that differed by nearly two log units. This same observation was made by Ono et al. (103) where two identical peptides, except for one stereochemical change, differed by one order of magnitude in PAMPA assays. This vast difference in permeability from a single stereochemical change has piqued interest in how this structural modification can be used to further improve peptide permeability and therefore, oral delivery. However, studies have also reported that structural modifications like the incorporation of D-amino acids can affect the biological activity of the peptide therapeutic by impacting their binding to the target (13, 104, 105).

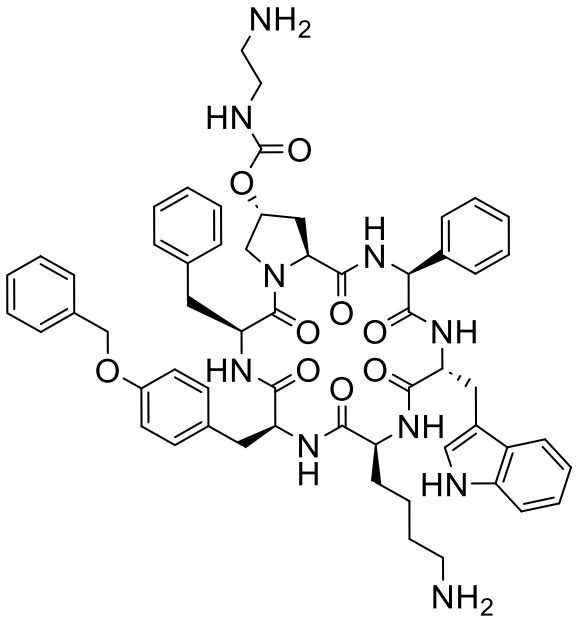
1.5. Problem Statement

Peptides are a rapidly growing therapeutic class that can target larger binding sites with higher sensitivity to conventional small drug molecules. The oral delivery of these drugs is desirable for patients from compliance, convenience, and cost perspectives. However, there are many challenges that peptide therapeutics face such as poor enzymatic stability, limited oral absorption and rapid clearance from the body. Making peptides orally bioavailable is a difficult task but it is far from impossible. This thesis will explore some key strategies known to improve drug permeability, namely lipophilicity and D-amino acids, to make specific structural changes to parent compound pasireotide, a cyclic somatostatin analogue. By altering a known pharmacologically active peptide, improvements to both permeability and enzymatic stability can be monitored, as well as any consequences to peptide bioactivity. Developing and testing the properties that are keys to unlocking oral delivery can be made using a mix of *in vitro* and *in vivo* methods. The challenge of this thesis will be to improve peptide permeability and oral bioavailability whilst still retaining the peptide bioactivity. There is a clear need for the oral delivery of peptides, but further research into the various factors currently inhibiting good oral bioavailability's is required before we can unlock a wider application of peptides to market using this route of administration.

1.6. Choice of Peptide Scaffold: Pasireotide



SRIF-14
Chemical Formula: $C_{76}H_{104}N_{18}O_{19}S_2$
Molecular Weight: 1637.90



Pasireotide
Chemical Formula: $C_{58}H_{66}N_{10}O_9$
Molecular Weight: 1047.23

Figure 1.10 Chemical structure of endogenous SRIF-14 and its analogue derivative pasireotide.

The work in this thesis is focussed on the model compound pasireotide. Pasireotide (PAS) was first reported in 2002 by Bruns et al. (65) as a novel cyclohexapeptide analogue of endogenous hormone somatostatin (SST) with unique antisecretory profiles (Figure 1.10). Somatostatin, previously known as somatotropin release inhibitory factor (SRIF), is an endogenous hormone discovered in 1973 by Brazeau et al. as having potent inhibitory effects on a variety of secretory hormones like growth hormone and insulin (106, 107). Occurring in two natural forms, SRIF-14 and SRIF-28, these compounds were seen to have an important role in the regulation of endocrine and exocrine secretion in many tissues (106, 107). The biological effect of SRIF-14 is mediated by five human G-protein coupled receptor subtypes numbered 1-5 (hSSTR1-5) (64, 65). All five SSTRs are prototypical class A GPCRs that belong to the rhodopsin-like family of receptors (64). SST receptors activate $G\alpha_{i/0}$ proteins which trigger the suppression of two critical secondary messengers: cAMP and cytosolic Ca^{2+} (64). Reduction of either cAMP or cytosolic Ca^{2+} leads to inhibition of secretion, and the simultaneous reduction of both secondary messengers by activation of SST receptors results in synergistic inhibitory effects on hormone release (64, 108).

While natural SRIF-14 and -28 have high binding affinities to all 5 subtypes, of all current commercially available analogues of SST, only pasireotide demonstrates high hSSTR5 affinity. Pasireotide can bind with high affinity (0.1–10 nM) to subtypes 1,2,3 and 5 of the human somatostatin receptors (109). Octreotide and lanreotide are two other examples of SST analogues also on the market used to treat disorders like acromegaly however these two analogues only bind to hSSTR2 with high affinity (109).

Pasireotide is currently sold under trade name Signifor® and was the first drug approved in the European Union for the treatment of Cushing's disease. Cushing's disease is caused by chronically high circulating levels of cortisol in the body from overactive corticotropin-secreting pituitary adenoma (109). This can result in obesity, hypertension, insulin resistance and an increased chance of mortality (109). Corticotropic tumour cells from patients with Cushing's disease express somatostatin receptors, particularly hSSTR5 (109). Studies exploring pasireotide show that patients who receive 0.9 mg subcutaneous doses twice daily demonstrate tumour shrinkages after 12 months of treatment (109, 110).

Although pasireotide is currently delivered subcutaneously, this therapeutic peptide shows the potential to be a strong candidate for oral peptide delivery. This peptide was selected as a good foundational scaffold for modification towards oral bioavailability due to its head to tail cyclic structure and prominently hydrophobic side chains (Figure 1.10).

Previous studies by Lokey et al. (98) found that peptides which demonstrate good permeability are possible to design, however, many of the peptides described in their work do not demonstrate bioactivity. Modifying pasireotide presents the opportunity to improve peptide oral bioavailability whilst also opening the door to explore the effect of any structural changes to peptide bioactivity. To simplify the design and synthesis of the peptide analogues, pasireotide was reduced to natural amino acids, namely the hydroxyproline was substituted with a proline, phenylglycine was substituted with phenylalanine and the benzyl group on the tyrosine residue was removed from the structure.

To understand how changes to L- or D- amino acids affect permeability, previous studies from our lab group performed a screening of the effect of single stereoisomer changes to peptide structure on permeability for each of the 6 amino acids in the sequence of pasireotide (*cyclo*[Pro-Phe-Trp-Lys-Tyr-Phe]). From that data, the five most permeable constructs were selected for further study in this thesis. Further details on this study can be found in Chapter 3 (Section 3.3.1, page 63).

1.6.1. Thesis Hypotheses and Aims

The hypothesis governing this study is that increasing lipophilicity and using unnatural D-amino acids will lead to improved metabolic stability and permeability of pasireotide analogues.

Hypothesis 1 (addressed in Chapter 3): That more lipophilic peptide analogues will improve permeability across a lipid bilayer.

Hypothesis 2 (addressed in Chapter 4): That increasing the lipophilicity of the analogues will reduce the potency and bioactivity of the peptide.

Hypothesis 3 (addressed in Chapters 5): That peptide analogues that demonstrate good *in vitro* permeability will demonstrate some absorption *in vivo*.

The aim of this thesis is to design, synthesise and assay a series of pasireotide analogues to gain insight into the drivers of peptide absorption from the GI tract and the consequent effect on stability and activity, ultimately to move towards improved oral bioavailability.

Aim 1: To design and synthesise a series of progressively more lipophilic analogues with varying stereochemical backbones. To measure synthesised peptides in *in vitro* solubility, permeability, and enzymatic stability assays.

Aim 2: To develop and validate a robust *in vitro* activity assay. To measure the IC_{50} values of synthesised peptides.

Aim 3: To dose select peptides *in vivo* to measure oral absorption and calculate pharmacokinetic properties. To understand the effect of formulation on oral absorption.

1.7. Thesis Outline

This thesis is broken down into six chapters. The general methods chapter details each of the experimental and analytical assays used in this study and methods will be briefly described in each experimental chapter for ease of review. Each experimental chapter address an integral aspect to oral delivery of peptides: solubility and permeability, activity, and *in vivo* performance. The final chapter will summarise findings and explore potential future work. Additional data for this thesis can be found in the Appendices (A-H, page 160).

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Chapter 2 : General Methods

This chapter describes the methods adopted by this research to achieve the aims and objectives stated in Chapter 1 (page 29) which is to investigate how lipophilic and D-amino acid structural changes to the peptide scaffold of pasireotide can be manipulated to achieve improved oral delivery. This chapter will describe the synthesis process for all peptide synthesised in this study. Peptide quantification and analytical validation methods will also be described in this chapter. All assays that pertain to certain studies will also be described in their relevant experimental chapters.

2.1. Peptide Synthesis

2.1.1. Materials

Acetonitrile (ACN), and methanol (MeOH) were purchased from Ajax Finechem (Tarren Point, NSW, AUS) and of analytical purity or high-performance liquid chromatography (HPLC) grade. Solvents were used without any pre-treatment. Water was obtained from a milli-Q purification system (Millipore, Bedford, MA, USA). Amino acids were purchased from ChemImpex (Wood Dale, IL, USA) unless otherwise stated. Amino acid (S)-2-(Fmoc-amino)heptanoic acid (Gly-2pent) was purchased from ACHEMBLOCK (Hayward, CA, USA)). Resin 2-chlorotrityl chloride (2-CTC), 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) and H-Benzotriazolium 1-[bis(dimethyl-amino)methylene]-5-chloro-hexafluorophosphate (1-),3-oxide (HCTU) were obtained from ChemImpex (Wood Dale, IL, USA). N,N-Diisopropylethylamine (DIPEA), triisopropylsilane (TIPS) and piperidine were purchased from Sigma–Aldrich (Castle Hill, NSW, AUS). N,N-Dimethylformamide (DMF), diethyl ether (Et2O) and dichloromethane (DCM) were purchased from Chemsupply (Port Adelaide, SA, AUS). Trifluoroacetic acid (TFA) was obtained from AusPep (Tullamarine, VIC, AUS).

2.1.2. Synthesis of Head to Tail Cyclic Peptides

Solid phase peptide synthesis (SPPS) is a technique developed in the 1960s to build long chains of amino acids using stepwise coupling and deprotection reactions (1). Contrary to solution phase synthesis, SPPS uses a polymer support resin to build the peptide chains. This allows unreacted reagents to be washed away between steps, limiting the need for purification steps. SPPS is reliant on protecting groups on one terminal end of the amino acids. These protecting groups allow only one end of the amino acid to be available for reaction to the linear peptide grounded to the support resin. Common protecting groups include Boc and Fmoc protecting groups. All the peptides synthesised in this study use Fmoc-based SPPS approach.

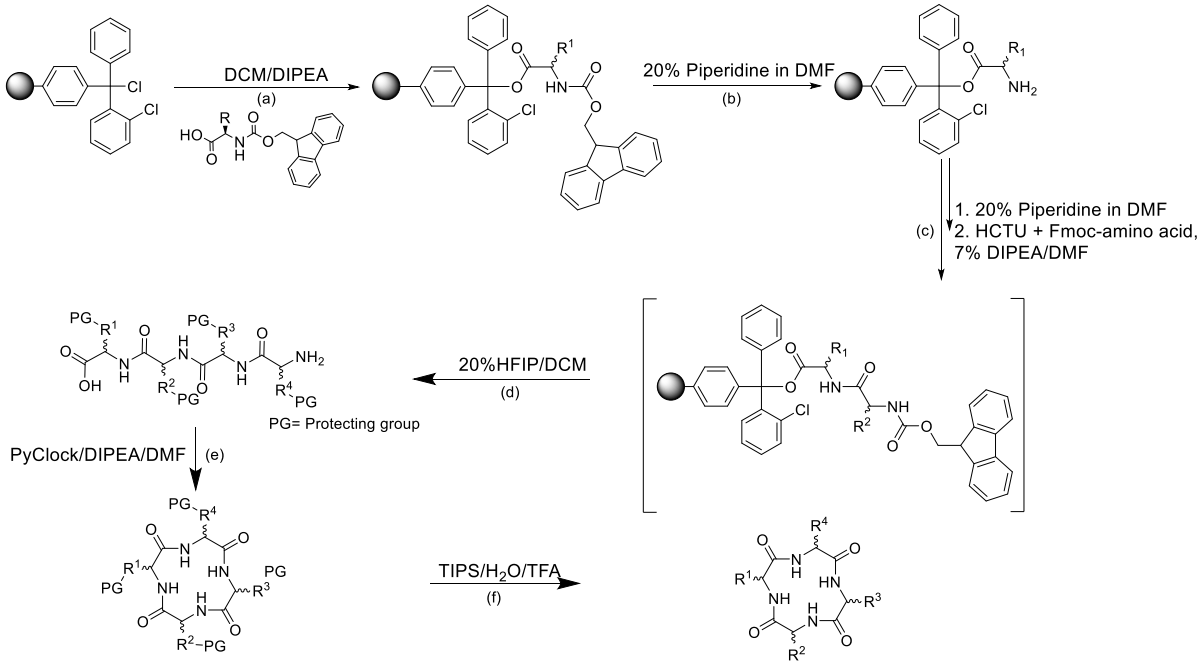
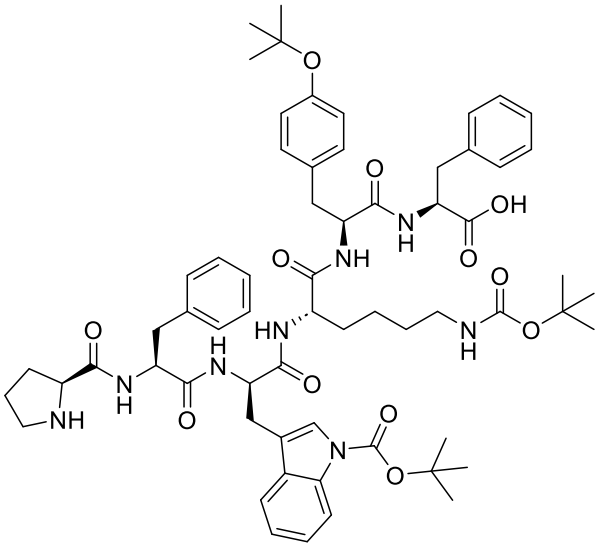


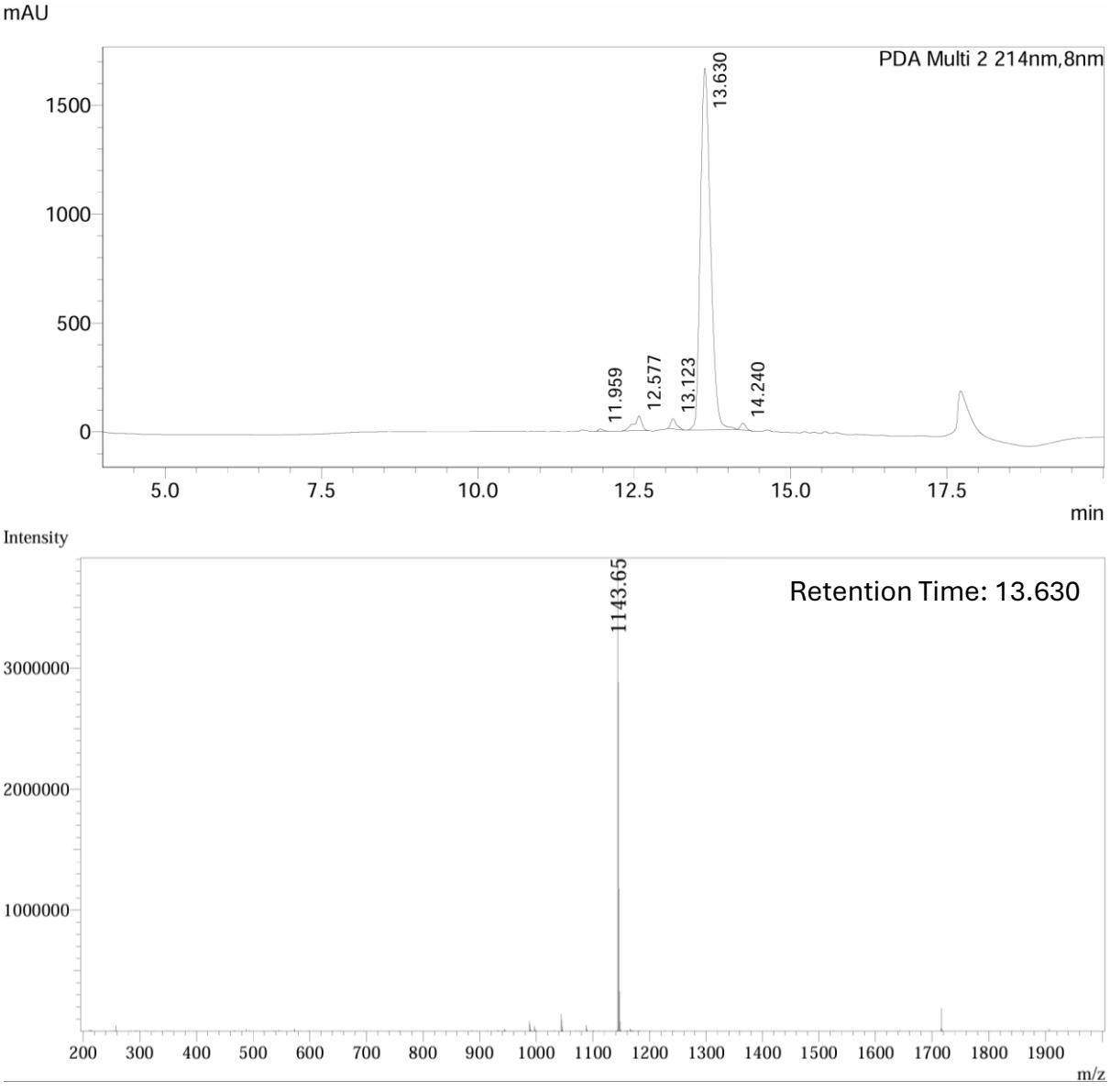
Figure 2.1 Peptide synthesis schematic using generic peptide scaffold. Synthesis steps include (a) Primary amino acid coupling (b) Fmoc-deprotection (c) Amino acid coupling (d) Resin cleavage (e) Head to Tail cyclisation (f) Protecting group deprotection.

Linear peptides were synthesised on 2-CTC resin by Fmoc (9-fluorenylmethoxycarbonyl)-based solid phase peptide synthesis. To couple the first amino acid, Fmoc-protected amino acid (1.5 eq) and DIPEA (3 eq) were dissolved in DCM, then added to the resin and shaken overnight at ambient temperature (250rpm, Innova® 2000 platform shaker, Eppendorf, Germany). The resin was washed with DCM and capped with methanol:DIPEA:DCM at 2:1:17. The Fmoc protecting group was removed with 20% v/v piperidine in DMF. For the remaining amino acid couplings, Fmoc-protected amino acid (3 eq) was activated with HCTU (3 eq) and DIPEA (6 eq) in DMF. The solution was added to the resin, and the reaction was maintained for 1hr at room temperature under shaking conditions (250rpm, Innova® 2000 platform shaker, Eppendorf, Germany). After completion of peptide assembly, peptides were washed with DMF, methanol and Et_2O, before being cleaved from the resin using 20% HFIP/DCM to yield linear peptide with sidechain protecting groups on select amino acids (tert-butyloxycarbonyl (Boc) groups on Lys and Trp; tert-butyl (tBu) groups on Thr and Tyr). Head-to-tail cyclization was performed overnight in DMF with HCTU (3 eq) and DIPEA (6 eq) and the reaction was monitored using LC-MS. Following removal of the solvent, side-chain protecting groups (Boc and tBu) were removed in TFA:triisopropylsilane (TIPS):water=95:2.5:2.5. Reaction progress was monitored using LC-MS, and example of which can be found in Figure 2.2.

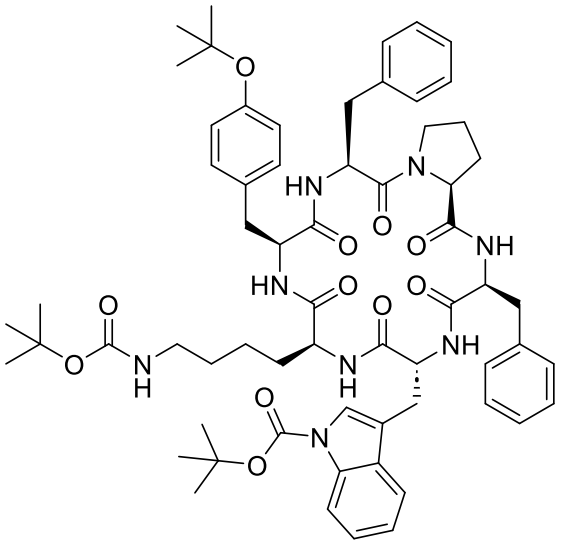
(A) Crude protected linear peptide A1



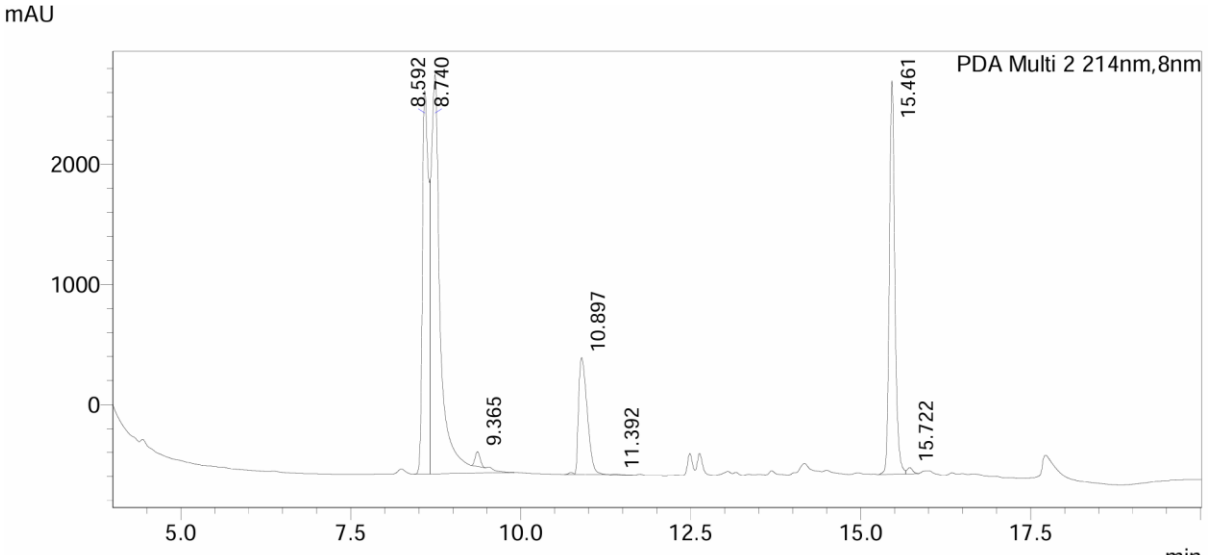
Chemical Formula: $C_{63}H_{82}N_8O_{12}$
Exact Mass: 1142.61

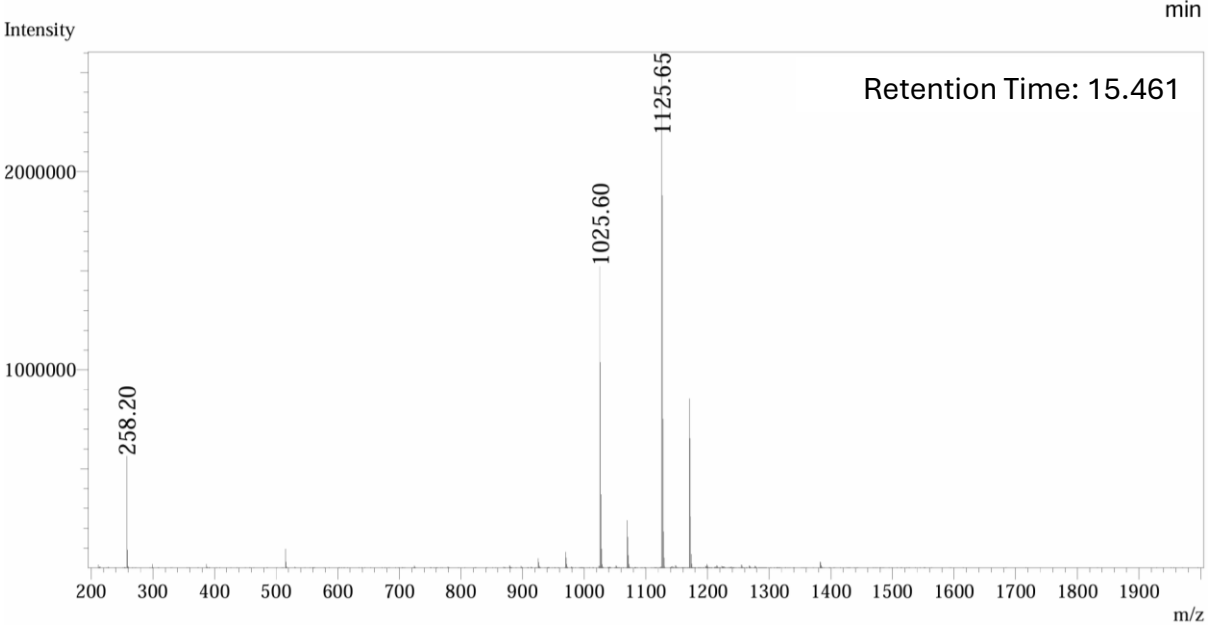


(B) Crude protected cyclic peptide A1

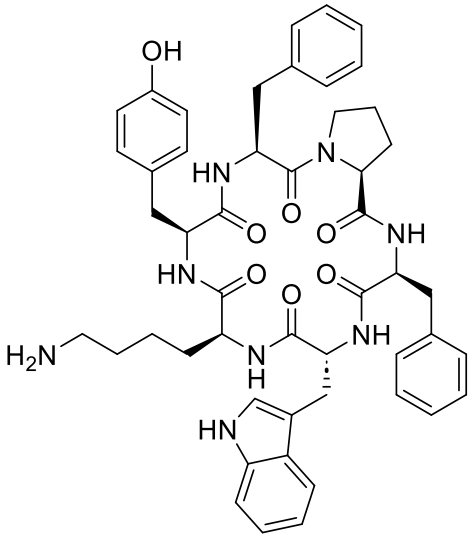


Chemical Formula: $C_{63}H_{80}N_8O_{11}$
Exact Mass: 1124.59

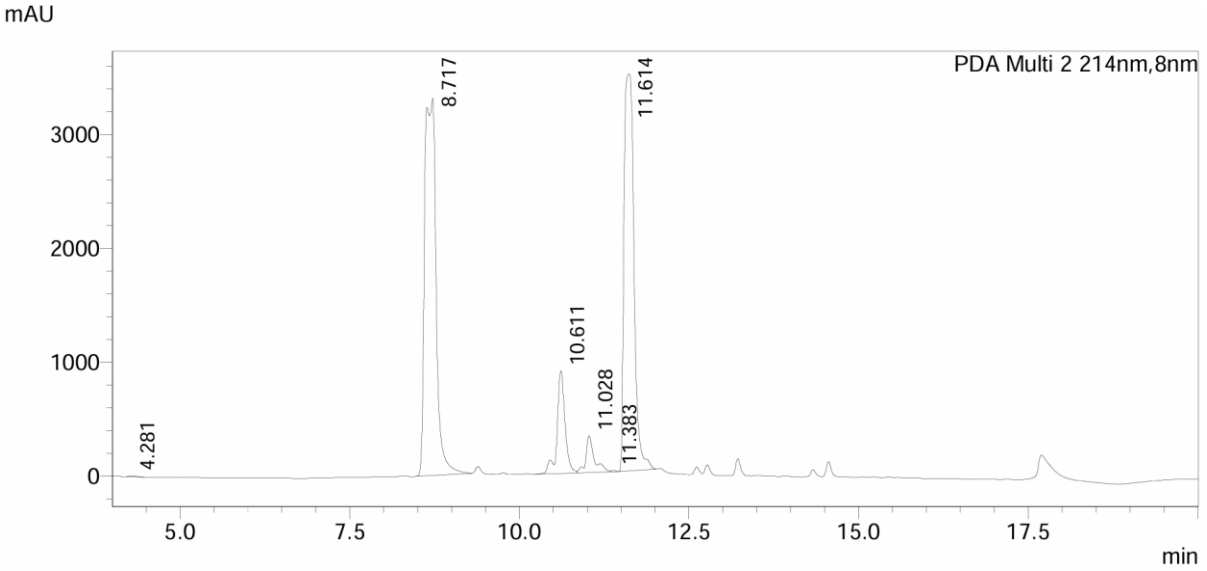


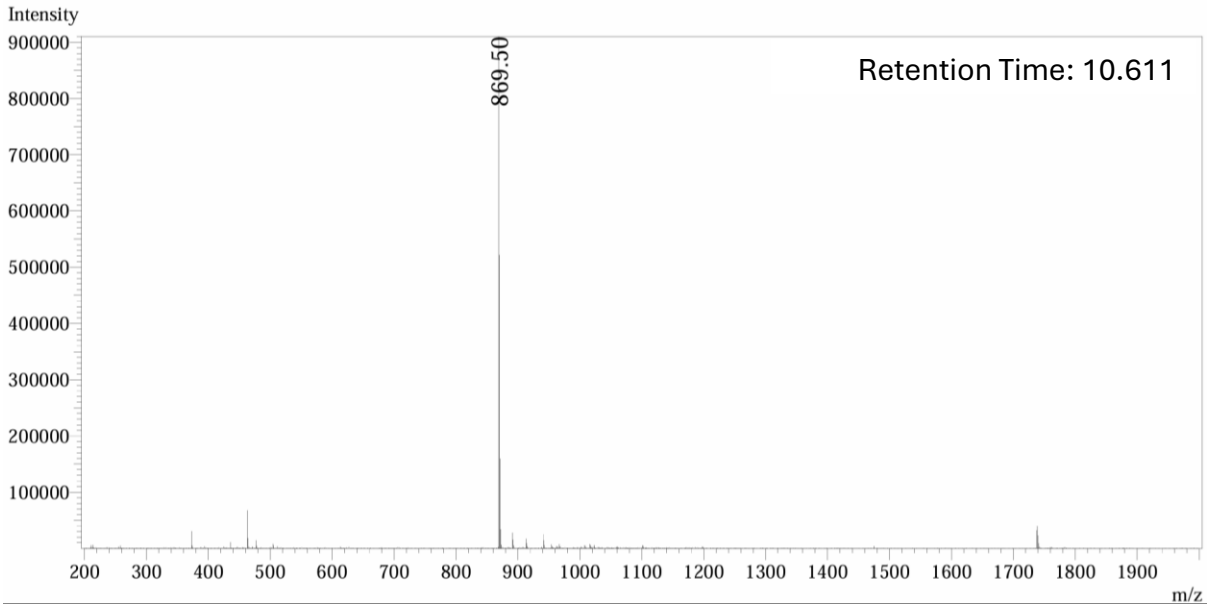


(C) Crude deprotected cyclic peptide A1



Chemical Formula: $C_{49}H_{56}N_8O_7$
Exact Mass: 868.43





(D) Purified deprotected cyclic peptide A1

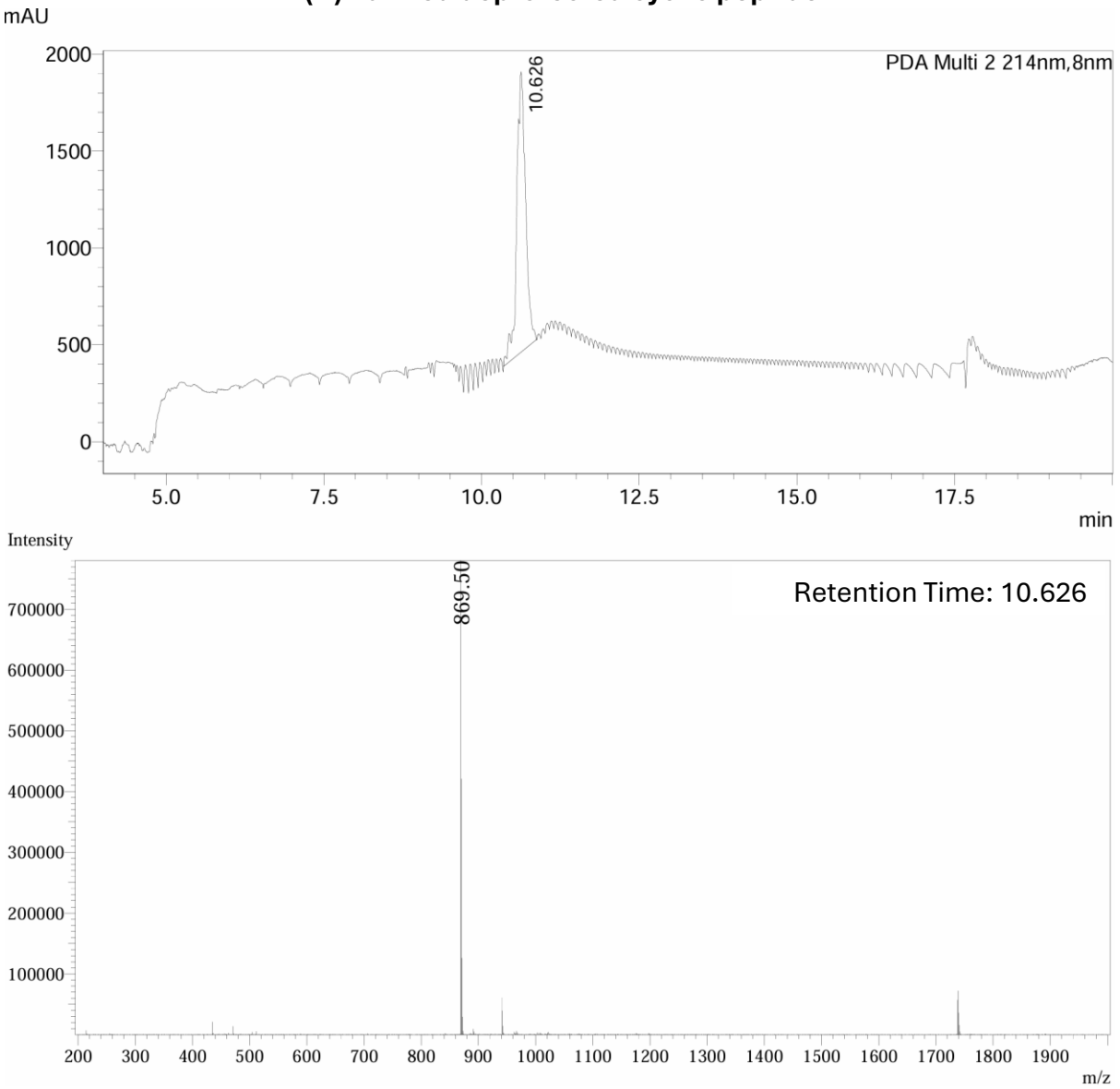


Figure 2.2 Peptide synthesis reaction monitoring of peptide A1 on LC-MS. Peptide structure and exact mass, chromatogram and mass spectra are given for each reaction stage. Stages are as follows A) Crude protected linear peptide, B) Crude protected cyclic peptide, C) Crude deprotected cyclic peptide and D) Purified cyclic peptide.

Cyclic peptides were purified using reverse-phase preparative HPLC (RP-HPLC) on an Agilent 1200 series HPLC system with an Agilent 1260 Infinity II Variable Wavelength Detector (VWD). The gradient used for separation was 30-80% solvent B over 30 minutes on a Luna® 10 µM C8(2) 100Å LC column 250 x 21.1mm (Phenomenex) at room temperature. The detector was set to 214 nm absorption wavelength and peptide fractions were collected manually. Purified peptides were recovered by lyophilization. In all cases, peptides were of a purity of greater than 95%, as determined by analytical LC-MS (Shimadzu LCMS-2020, Shimadzu Scientific, Japan). Fractions were analysed for peptide purity at an injection volume of 2 µL. The mobile phase was a gradient of solvent A and B (milli-Q water with 0.1% TFA, and acetonitrile with 0.1% TFA, respectively) with a flow rate of 1 mL/min at room temperature. The gradient used for separation was 20% solvent B from 0 to 4.00 min, B increasing linearly to 100% B from 4.0 to 14.00 min, B returning to 0% at 15.00 min from 15.00 to 20.00 minutes. The instrument was equipped with a Luna® 3 µM C8(2) 100Å LC column 100 x 2mm (Phenomenex) column. Peptide mass was confirmed by ESI-MS.

Trifluoroacetyl prodrugged peptides were synthesised using a method adapted from Barlow et al. (2) whereby base 1,3 lutidine was replaced with DIPEA. Briefly, the lysine-containing peptide was capped with a trifluoroacetate group by dissolving the peptide in DMF and treating with DIPEA and trifluoracetic anhydride. Reaction progress was monitored using ESI-MS and peptide was purified using HPLC.

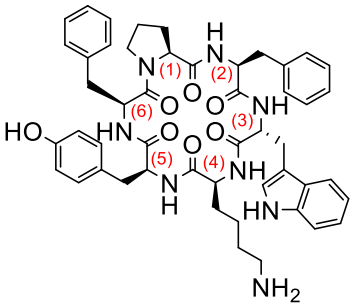
Analytical spectra of all synthesised peptides can be found in Appendix A (page 160).

2.1.3. List of Synthesised Peptides

A total of 27 peptides were designed and synthesised for this study. These analogues were categorised into series A-F based on peptide backbone structure. A summary of the structure of each synthesised peptide can be found in Table 2.1. Additional material where required was kindly synthesised by Travis Lay (Monash University).

*Table 2.1 Structure of each synthesised peptide. Each alphabetised set is organised by backbone structure. Amino acid positions are defined by numbers 1-6 starting at the 'Pro' residue and moving clockwise around the cyclic peptide. * Peptide A3 is adopted from Veber et al. (3).*

Pasireotide analogues



	cyclo[**1**	**2**	**3**	**4**	**5**	**6]**
A1	*cyclo[* Pro	Phe	D-Trp	Lys	Tyr	Phe]
A2	*cyclo[* Pro	Phe	D-Trp	Lys	Tyr(Bzl)	Phe]
A3*	*cyclo[* Pro	Phe	D-Trp	Lys	Thr	Phe]
A4	*cyclo[* Pro	Phe	D-Trp	Lys	Val	Phe]
A5	*cyclo[* Pro	Leu	D-Trp	Lys	Leu	D-Leu]
A6	*cyclo[* Pro	Phe	D-Trp	Gly(2-pent)	Tyr	Phe]
B1	*cyclo[* Pro	D-Phe	D-Trp	Lys	D-Tyr	Phe]
B2	*cyclo[* Pro	D-Phe	D-Trp	Ala	D-Tyr	Phe]
B3	*cyclo[* Pro	D-Phe	D-Trp	Nle	D-Tyr	Phe]
B4	*cyclo[* Pro	D-Phe	D-Trp	Gly(2-pent)	D-Tyr	Phe]
B5	*cyclo[* Pro	D-Phe	D-Trp	Lys($COCF_3$)	D-Tyr	Phe]
B6	*cyclo[* Pro	D-Phe	D-Trp	D-Ala	D-Tyr	Phe]
B7	*cyclo[* Pro	D-Phe	D-Trp	D-Nle	D-Tyr	Phe]
C1	*cyclo[* D-Pro	Phe	Trp	D-Lys	Tyr	D-Phe]
C2	*cyclo[* D-Pro	Phe	Trp	D-Ala	Tyr	D-Phe]
C3	*cyclo[* D-Pro	Phe	Trp	D-Nle	Tyr	D-Phe]
D1	*cyclo[* Pro	Phe	D-Trp	D-Lys	Tyr	D-Phe]
D2	*cyclo[* Pro	Phe	D-Trp	D-Ala	Tyr	D-Phe]
D3	*cyclo[* Pro	Phe	D-Trp	D-Nle	Tyr	D-Phe]
D4	*cyclo[* Pro	Phe	D-Trp	D-Ala	Tyr	Phe]
D5	*cyclo[* Pro	Phe	D-Trp	D-Nle	Tyr	Phe]
E1	*cyclo[* D-Pro	D-Phe	Trp	Lys	D-Tyr	Phe]
E2	*cyclo[* D-Pro	D-Phe	Trp	Ala	D-Tyr	Phe]
E3	*cyclo[* D-Pro	D-Phe	Trp	Nle	D-Tyr	Phe]
F1	*cyclo[* Pro	Phe	D-Trp	D-Lys	D-Tyr	D-Phe]
F2	*cyclo[* Pro	Phe	D-Trp	D-Ala	D-Tyr	D-Phe]
F3	*cyclo[* Pro	Phe	D-Trp	D-Nle	D-Tyr	D-Phe]

2.2. Drug Quantification with UPLC-MS/MS and Analytical Method Validation

2.2.1. Materials

Acetonitrile and formic acid were purchased from Merck (Bayswater, Victoria, Australia) at LC-MS grade quality. Internal standard, pasireotide, was purchased from Biocrick (Sichuan, China). Water was obtained from a milli-Q purification system (Millipore, Bedford, MA, USA).

2.2.2. Analytical Method

A Shimadzu LCMS 8050 system (Shimadzu Scientific Instruments, Kyoto, Japan) was used for quantitative analysis and consisted of a CBM-20A system controller, a DGU-20A5R solvent degasser, two LC-30AD pumps, a SIL-30AC autosampler, a CTO-20A column oven (held at 40 °C), and a triple quadrupole mass spectrometer with an electrospray ionization interface (ESI). The desolvation line (DL), the interface and the heat block were maintained at 250, 300, and 400 °C, respectively. The flow rate of the nebulizing gas, heating gas and drying gas were 3 L/min, 10 L/min, and 10 L/min, respectively.

For each sample, 2 µL was injected onto a Luna® Omega 3 µm PS C18 100 Å LC column 50 x 2.1mm (Phenomenex) coupled to a Phenomenex C18 4 x 2.0 mm guard cartridge. The mobile phase was a mixture of solvent A (milli Q water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) with a flow rate of 0.5 mL/min.

Each peptide analogue has unique instrument settings, a summary of which can be found in the Appendix B (page 164). For peptide B2, the chromatographic and mass spectroscopy conditions are as follows (Figure 2.3). Gradient elution: 10% B was held for 0.5 min before linearly increasing to 98% B in 2 min. Under these conditions, the retention times of peptide B2 and internal standard (IS) pasireotide were 2.183 and 1.567 min, respectively. Elution was immediately succeeded by a 0.5 min wash step at 98% B. Total run time was 3.5 min including the wash step. Data acquisition and peak integration were performed using Shimadzu software (Labsolutions, version 5.120).

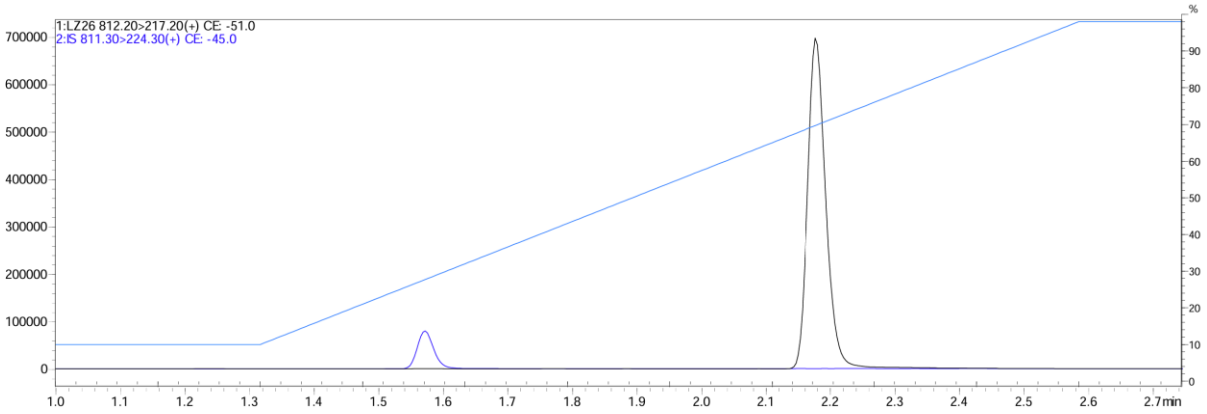


Figure 2.3 Chromatogram of peptide B2 and IS on LC-MS/MS. Peptide B2 is coloured black, IS (PAS) is coloured dark blue, gradient line is coloured light blue.

Unknown peptide concentrations were determined by interpolation from a weighted ($1/X^2$) calibration curve of peptide peak response plotted as a function of peptide concentration. Assay validation was conducted by multi-day matrix analysis of validation samples (n = 6 per group) of 10, 50 and 500 ng/mL peptide in blank plasma with IS. Accuracy, (n = 6) 99%; precision, (n = 6) <15%; linearity, R2 0.995 – 0.999; specificity, no interfering peaks in blank plasma extracts. The limit of quantitation was arbitrarily set at 4 ng/mL; the lowest concentration of the validated concentration range. Method validation values can be found in Appendix C (page 166).

2.3. Parallel Artificial Membrane Permeability Assay (PAMPA)

2.3.1. Materials

Phosphatidylcholine (L-α-Lecithin), dimethyl sulfoxide (DMSO) and dodecane were purchased from Sigma–Aldrich (Castle Hill, New South Wales, Australia). LC-MS grade acetonitrile (ACN) was obtained from Merck (Bayswater, Victoria, Australia) and used without any pre-treatment. Water was obtained from a milli-Q purification system (Millipore, Bedford, MA, USA). Filter plates were purchased from Millipore (Millipore, Bedford, MA, USA). Dulbecco's phosphate buffered saline 1x (DPBS) was purchased from Thermo Fisher (Gibco, Thermo Fisher Scientific, MA, USA).

2.3.2. Predictive permeability assay

Membrane permeability of the peptides was tested by *in vitro* parallel artificial membrane permeability assay (PAMPA) developed by Wohnsland et al. (4). This assay measures the passive diffusion of drugs across a semi-permeable membrane coated in lecithin and dodecane to mimic a biological membrane. In a method previously described by Li et al. (5), 5 µL of 1% lecithin in dodecane was used to coat a polyvinylidene difluoride membrane of a filter plate (Millipore) to form the artificial membrane. Each peptide for analysis was diluted prior to 4 mM stock solutions in DMSO. The donor phase contains 300 µL of solution containing 200 µM of peptide stock in DPBS buffer (final concentration of DMSO in donor phase is 5%). The receptor phase is overlayed on top of the donor phase and contains a 200 µL solution of 5% DMSO in DPBS. The plate was incubated for 16 hours at 37 °C.

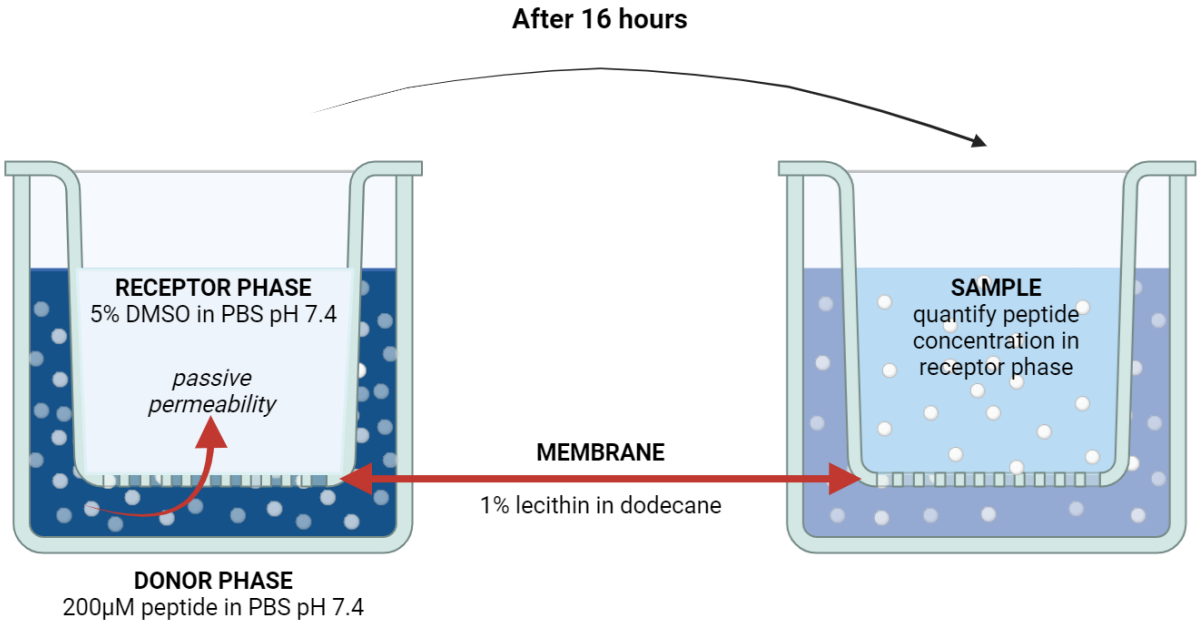


Figure 2.4 PAMPA assay. Image was made in BioRender.com.

Compound concentrations at equilibrium after 16 hours in both the donor and receptor wells were determined by UPLC analysis. The transmembrane permeability ($logP_e$) was calculated from the following equation as reported by Wohnsland et al. (4).

Equation 1 Permeability equation for PAMPA assay. Unit of $logP_e$ is cm/s.

$$logP_e = log\left\{C \times -ln\left(1 - \frac{[drug]_{acceptor}}{[drug]_{donor}}\right)\right\}$$

Equation 2 Definition of term 'C' from Equation 1 where VD represents donor volume, VA represents receptor volume.

$$C = \left(\frac{V_D \times V_A}{(V_D + V_A) \times area \times time}\right)$$

2.3.3. Peptide Quantification for PAMPA Assay using High Performance Liquid Chromatography (HPLC)

The UPLC system included a Shimadzu CBM-20A system controller, LC-30AD solvent delivery module, SIL-30AC auto sampler and a CTO-20AC column oven set at 40 °C, coupled to an PDA-M30A UV-detector and an RF-20AXS fluorescence detector (Shimadzu Corporation, Kyoto, Japan). A Luna® Omega 3 µm PS C18 100 Å LC column 50 x 4.6 mm (Phenomenex) was used. The mobile phase was a gradient of solvent A and B (milli-Q water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively) with a flow rate of 1 mL/min. The gradient used for separation was 10% B from 0 to 0.1 min, B increasing linearly to 98% B from 0.1 to 2.8 min, B returning to 10% at 3.6 min to re-equilibrate at 10% B from 3.6 to 4.0 minutes. Quantification was based on analyte florescence at excitation wavelength 300 nm and emission wavelength 338 nm.

2.4. *In vitro* stability assays

In vitro stability assays were performed to analyse the metabolic stability of the peptides in the presence of hydrolytic enzymes derived from the gastrointestinal tract and blood plasma.

2.4.1. Gastrointestinal Stability

2.4.1.1. Materials

Sodium taurodeoxycholate (>95%), porcine pancreatin extract (P7545, 8 × USP specifications activity), trizma® base, sodium chloride and calcium chloride dihydrate were purchased from Sigma–Aldrich (Castle Hill, New South Wales, Australia). Phosphatidylcholine (PC) (Lipoid E PC S, approximately 99.2% pure, from egg yolk) was obtained from Lipoid (Lipoid GmbH, Ludwigshafen, Germany). Octreotide acetate was purchased from Shanghai PI Chemicals Ltd. (Shanghai, China). Acetonitrile, ethanol, sodium hydroxide, hydrochloric acid, and formic acid (98-100%) were obtained from Merck (Bayswater, Victoria, Australia) and used without any pre-treatment. All chemicals and solvents were of analytical purity or high-performance liquid chromatography (HPLC) grade.

2.4.1.2. Enzymatic Degradation Assay using Pancreatin

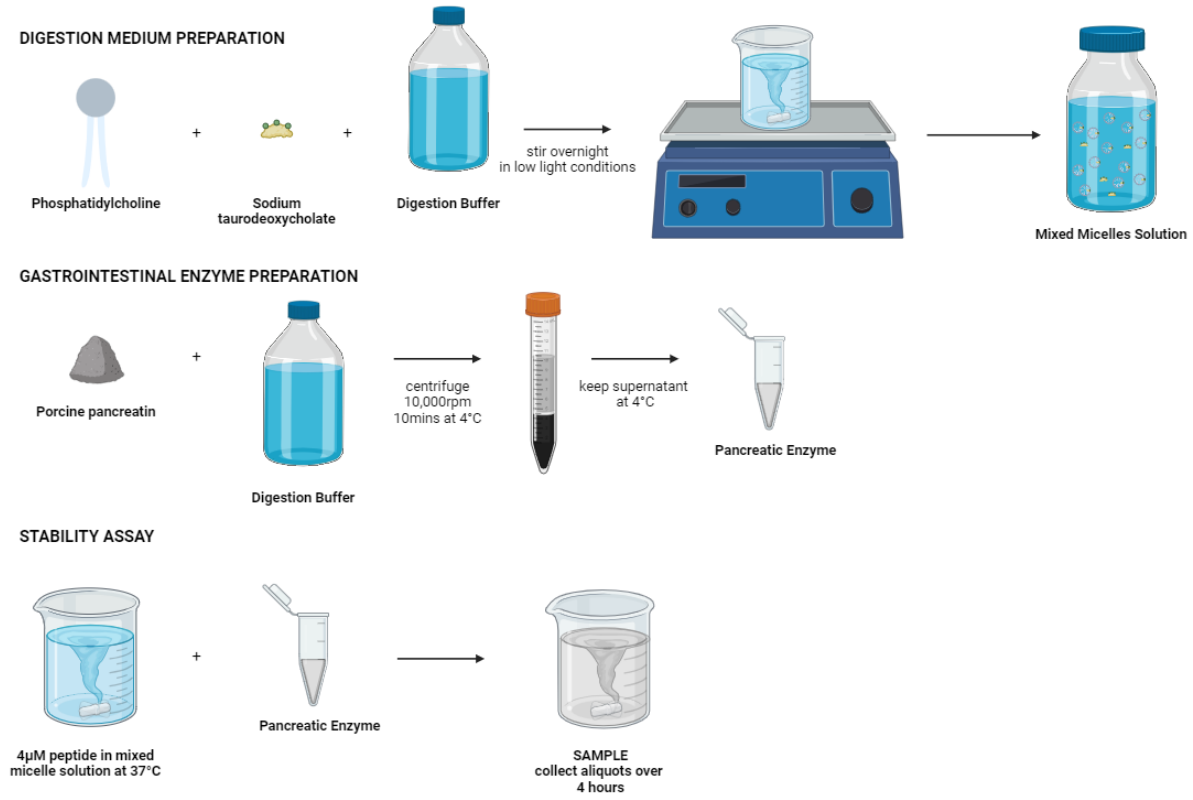


Figure 2.5 Gastrointestinal Stability Assay. Image was made in BioRender.com.

Simulated intestinal fluids previously described by Li et al. (6) was used to mimic digestion conditions with an excess of porcine pancreatin used to trigger enzymatic digestion. Octreotide was used as a positive control, as previous studies reported a consistent half-life of 1 hour under these conditions (5). Peptide at a concentration of 4 µM in a 1 mL system of digestion medium (Table 2.2) was equilibrated at 37 ºC for 10 minutes prior to enzyme addition. The sample aliquot removed prior to enzyme addition is T0 and the system is kept at 37 °C for the duration of the study. Further sample aliquots were taken at T5, 15, 30, 60, 120 and 240 mins post enzyme addition. Sample aliquots were quenched by 1:10 dilution in 0.1% formic acid in acetonitrile.

Table 2.2 Composition of Digestion Buffer.

Ingredient	**Concentration**	**Mass (g) for 1L**
Tris-maleate	2 mM	0.47
Sodium Chloride	150 mM	8.77
Calcium Chloride dihydrate	1.4 mM	0.21
Sodium hydroxide	Qs to pH 6.5 ± 0.1	
Milli Q water	Up to volume	

Table 2.3 Composition of Digestion Medium.

Ingredient	Concentration	Mass (g) for 250mL
Sodium taurodeoxycholate	3 mM	0.43
Phosphatidylcholine	0.75 mM	0.16
Digestion Buffer	Up to volume	

2.4.2. Plasma Stability

2.4.2.1. Materials

Fresh frozen rat plasma was obtained from the Monash Animal Research Platform (MARP, Melbourne, AUS). Somatostatin was purchased from Sigma–Aldrich (Castle Hill, New South Wales, Australia). Acetonitrile and formic acid (98-100%) were from Merck (Bayswater, Victoria, Australia) and used without any pre-treatment. All chemicals and solvents were of analytical purity or high-performance liquid chromatography (HPLC) grade.

2.4.2.2. Enzymatic Degradation Assay using Rat Plasma

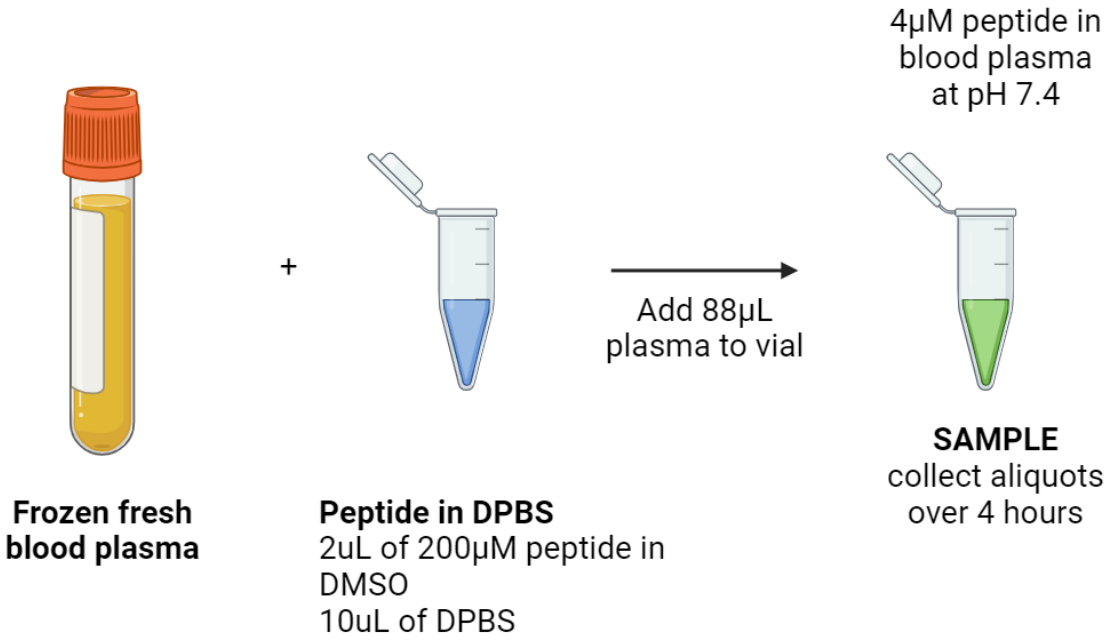


Figure 2.6 Plasma Stability Assay. Image was made in BioRender.com.

Metabolic stability was tested in rat plasma. Somatostatin was used as a positive control for the plasma stability assay as it has a well reported half-life of less than 3 minutes (7). Stability studies were conducted with peptide analogues at a final concentration of 4 µM and the estimated half-life was extrapolated from the results. In short, 10 µL of DPBS buffer was added to 88 µL rat plasma in an Eppendorf tube. 2 µL of

200 µM peptide stock solution dissolved in DMSO was added to the tube. Mixture was vortexed and incubated for 4 hours at 37°C with 10 µL aliquots collected at timepoints T0, 5, 15, 30, 60, 120 and 240 minutes. Proteins were precipitated from solution with a 1:10 dilution of 0.1% formic acid in acetonitrile. Samples were centrifuged at 4°C for 10 mins at 10,000 rpm and supernatant was analysed using LC-MS as previously described in Section 2.2 (page 43).

2.5. Structure – Activity Relationship

2.5.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) supplemented with sodium pyruvate and glutaMAX supplement, Dulbecco's phosphate buffered saline 1x (DPBS), Opti-MEM™, TrypLE™ Express Enzyme, Penicillin-streptomycin (P/S) (10,000 U/mL), Geneticin™ Selective Antibiotic (G418 Sulfate), Hank's balanced salt solution 1x (HBSS), HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid), lipofectamine LTX and lipofectamine messenger MAX were purchased from Thermo Fischer (Thermo Fisher Scientific, MA, USA). Heat inactivated Fetal Bovine Serum (FBS) was purchased from Bovogen Biologicals (Bovogen Biologicals, VIC, AUS). Hygromycin B extracted from streptomyces hygroscopicus, bovine serum albumin (BSA), forskolin (FSK) and tryphan blue were purchased from Sigma–Aldrich (Castle Hill, NSW, AUS). Rolipram was purchased from Stemcell Technologies (Vancouver, CA). T75 flasks were purchased from Corning® (Corning, NY, USA). White opaque 96-well and 384-well microplates were purchased from Perkin Elmer (Perkin Elmer, MA, USA). Absolute molecular grade ethanol was purchased from Ajax Finechem (Thermo Fisher Scientific, MA, USA). Human SSTR5 ACTOne™, which is a HEK-293 CNG cell line that expresses recombinant hSSTR5 receptor was purchased from Australian Biosearch (Wangara, WA, AUS).

2.5.2. Stable cell transfections

Flp-In human embryonic kidney (HEK 293) cells expressing hSSTR2 and hSSTR5 receptors were stably transfected by Dr Stewart Fabb (Monash University) from pEF5-SSTR2 and pEF5-SSTR5 vectors obtained by collaborators from the department of Drug Discovery Biology at the Monash Institute of Pharmaceutical Science.

Chinese hamster ovary (CHO-K1) Flp-In cells were obtained from the department of Drug Discovery Biology at the Monash Institute of Pharmaceutical Science. These cells were seeded onto a 6 well plate at a cell density of 3.85 x 105 cells/well in DMEM containing 10% FBS. Cells were left to grow overnight at 37 °C, 5% CO_2. In a sterile Eppendorf tube, 2.5 µg circular plasmid DNA (2.25 µg pOG44:250 ng expression plasmid) and 2 µl Plus reagent were added to Opti-MEM 1 medium to total 90 µl. Mixture was incubated at room temperature for 5 minutes. 5.8 µl of Lipofectamine LTX was added to each tube and incubated at room temperature for 15 minutes. Mixture is added dropwise into cells and incubated at 37 °C, 5% CO_2 for 48 hours. After 48 hours, the media was replaced. Based on the results of an antibiotic kill curve conducted, 800 µg/ml Hygromycin B was added to the media to kill non-transfected cells. Media was replaced every 3 days until colonies were observed. Once colonies were formed, cells were transferred to a T25 flask and cultured to 80% confluence for further cell culture.

2.5.3. Cell culture

Stably transfected cells were grown to 80% confluency in supplemented high glucose DMEM, containing 10% FBS and 1% glutamax within a T75 flask. Media was removed from the tissue culture flask and cells were rinsed with 10 ml DPBS. DPBS was removed and 2.5 ml of TrypLETM was added for 3-5 mins to assist in the detachment of cells from the flask. 7.5 ml of prewarmed media was added into the flask containing TrypLE™ and detached cells. The cells were transferred to a centrifuge tube and centrifuged for 5 minutes at 180 g at room temperature. The supernatant was removed from the tube and the pellet containing cells was resuspended in 10 ml of media. 20 µL of cells resuspended in media was transferred into an Eppendorf tube and mixed with 20 µL of trypan blue. 10 µL of this solution was transferred into a haemocytometer for cell counting. Based on the cell count, a stock solution was made for cell seeding and further assays. Every two weeks cells were placed under selection antibiotics to ensure correct expression. The selection for each cell line was as follows: HEK293 cells transfected by Dr Stewart Fabb (Monash University) used 200 µg/mL hygromycin B, CHO-k1 cells used 800 µg/mL hygromycin B and commercially bought HEK293 cells used 250 µg/ml G418 and 1 µg/ml Puromycin. Cells grow under incubation at 37 °C with 5% CO_2.

2.5.4. Development of *in vitro* activity assay

Two *in vitro* cell activity assays were developed during these studies.

2.5.4.1. Part A: LANCE Ultra cAMP Assay

Cell density of 25,000 cells/well were seeded into a 96-well flat bottom plate and placed into a 37 °C incubator at 5% CO_2 overnight. After 24 hours of incubation, growth medium was aspirated and replaced with stimulation buffer (80 µL) containing HBSS supplemented with 0.1% (w/v) BSA, 5 mM HEPES and 20 µM rolipram. The plates were placed back into a 37 °C incubator for 30 minutes. Each peptide for analysis was serially diluted in stimulation buffer at 10x the required concentration for the concentration curve (overall DMSO content was less than 1% in each concentration standard). After the 30-minute incubation, the 96 well plates were removed from the incubator and 10 µL of ligands at 10x the testing concentration in stimulation buffer were over a serial dilution was added into each well containing stimulation buffer and cells. The plates were placed back into a 37 °C incubator and left to incubate for a further 10 minutes after which 10 µL of 10x concentrated forskolin is added to each well at final concentration 10 µM for 20 minutes. The solution from the wells were aspirated and replaced with 50 µL of ice-cold absolute ethanol per well to terminate the reaction. Plates were left at room temperature overnight for the ethanol to evaporate. Upon ethanol evaporation, 200 µL of lysis buffer containing 0.1% BSA, 5 mM HEPES and 0.3% (v/v) Tween 20 was added into each well and placed on a shaker at 500 rpm for 15 minutes to lyse the cells and release accumulated cAMP. The amount of cAMP in the samples was quantified using Perkin-Elmer Life Sciences Lance ultra cAMP kit (Perkin Elmer, MA, USA). Briefly, lysate (5 µL) was transferred to a 384 well white opaque microplate (Perkin Elmer, MA, USA). The lysate was incubated at room temperature in low light conditions for 60 minutes with Europium cAMP Tracer (10 µL) and ULight Anti cAMP Solution (5 µL). Time resolved fluorescence for the samples was detected using Envision plate reader (Perkin Elmer, MA, USA) with excitation at 320 nm and emission at 615 nm.

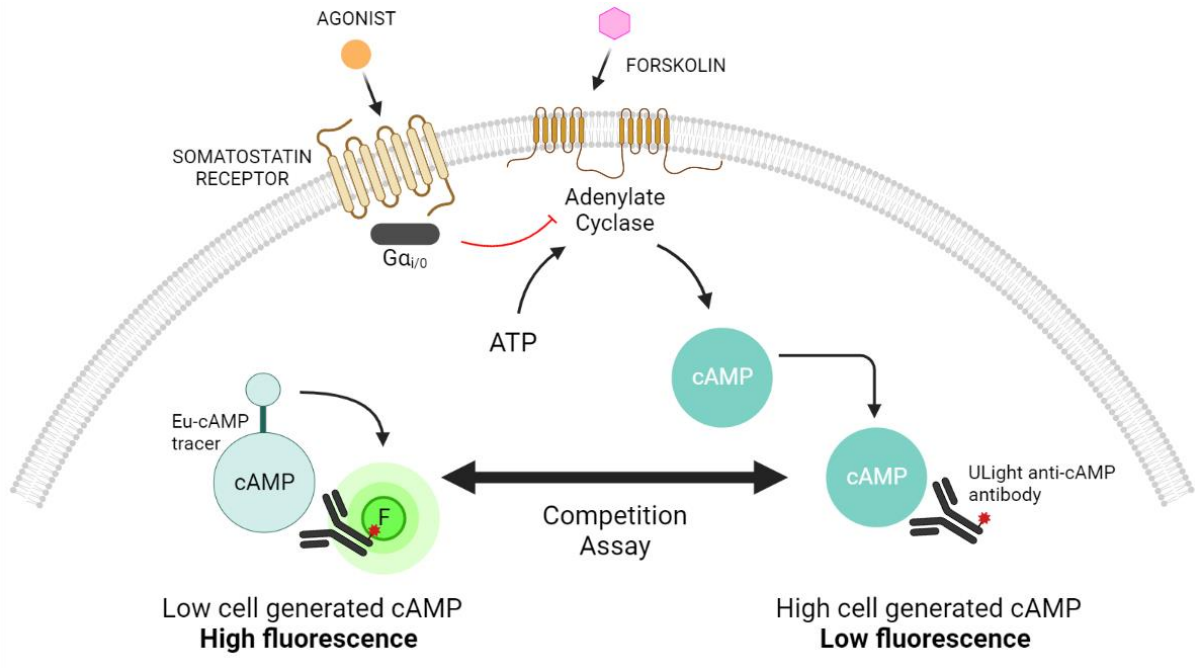


Figure 2.7 Schematic of the cAMP assay. Image was created in BioRender.com.

2.5.4.2. Part B: BRET-based Activity Assay

Stably transfected human SSTR5 ACTOne™ cells purchased from Australian Biosearch (Wangara, WA, AUS) were plated in a 6-well plate at 500,000 cells/well in DMEM supplemented with 10% FBS and 1% P/S. The following day, cells were transiently transfected with G-protein biosensor (pCMV-6xHis-Venus-GB1-nLuc (Tri-cistronic BRET sensor)). To perform the transfection, 4 µg/mL of G-protein biosensor in Opti-MEM and 6 µL/mL of lipofectamine messenger MAX in Opti-MEM were combined and left to incubate at room temperature for 20 mins. Then, mixture was added dropwise to each well at 500 µL/well. The cells were then placed back into a 37 °C at 5% CO_2 incubator for 24 hours. After 24 hours, media was aspirated from wells and cells are resuspended in phenol red-free DMEM (PRF-DMEM) containing 10% FBS and 25 mM HEPES and seeded into flat white bottom 96-well plates (approximately 55,000 cells/well) and placed back into a 37 °C at 5% CO_2 incubator for 24 hours.

Fluorescence was measured using PHERAstar FTX microplate reader (BMG LABTECH, VIC, AUS) set to 37 ºC, and luminescence was recorded at 528 nm and 460 nm simultaneously. Cell media was aspirated and replaced with 90 µL of PRF-DMEM containing 0.2% v/v Nano-Glo® Luciferase Assay Substrate and baseline readings were measured over 3 minutes. Ligands at 10x the testing concentration in PRF-DMEM were made over a serial dilution. After the 3-minute baseline readings, 10 µL of each dilution

is added and luminescence at 528 nm and 460 nm were recorded for 60 minutes. Activity is measured as a ratio between luminescence at 528 nm and 460 nm.

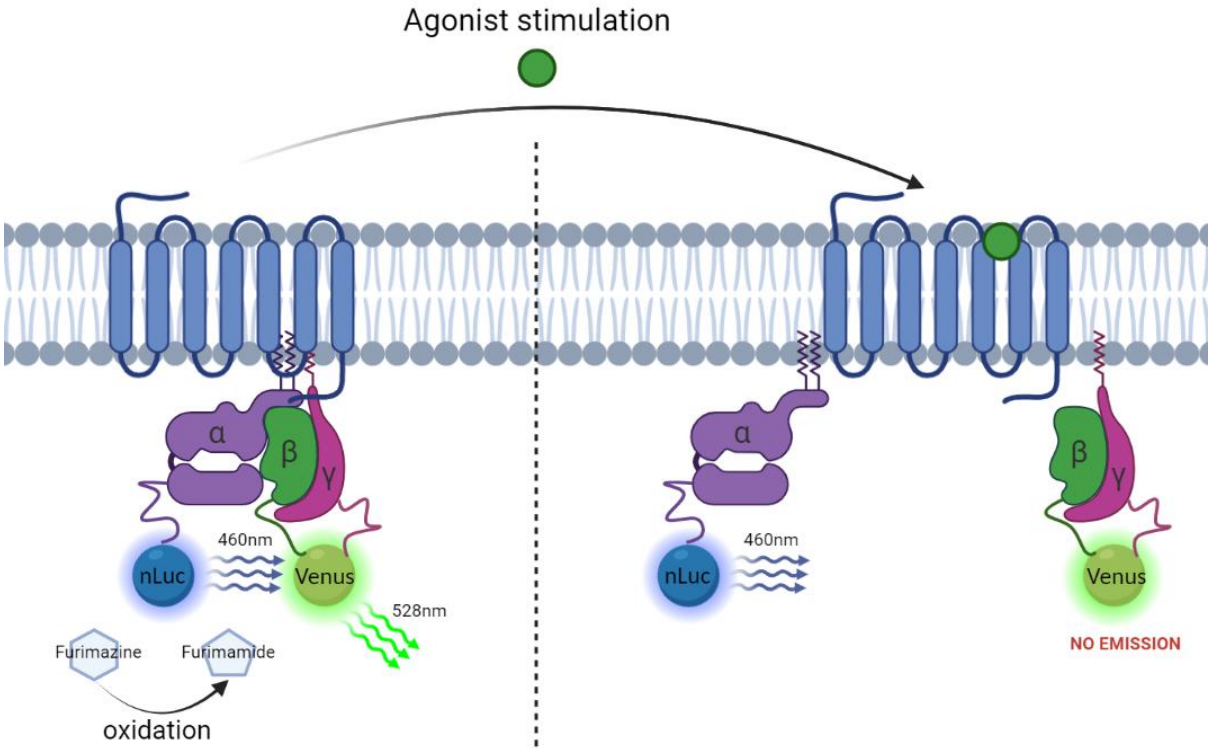


Figure 2.8 Schematic of the BRET-based Activity Assay. Image was created in BioRender.com.

2.6. Oral Bioavailability Studies

All *in vivo* studies were conducted at the Monash Institute of Pharmaceutical Sciences and used established procedures in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All surgical and experimental procedures were reviewed and approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee.

2.6.1. Formulation administration and sample collection

All animal studies were performed in accordance with the Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines. The ethic protocol number was AEC#26791 and the Agriculture Victoria Licence number was SPPL20181. The pharmacokinetics of each test compound were studied in male Sprague Dawley rats aged at 7-8 weeks. Animals were fasted overnight with access to food re-instated 4 hours post-dose. All animals had access to water ad libitum throughout both the pre- and post-dosing period. Samples of arterial blood, total faeces and urine were

collected up to 24 hr post-dose. Samples of carotid arterial blood at 200 µL per aliquot were collected using the Culex ABS system (BASi, West Lafayette, IN, USA) into borosilicate vials at 4 °C containing heparin as the anticoagulant. Blood samples were centrifuged, and supernatant plasma was removed. Plasma and urine samples were stored frozen at -80 °C until analysis by LC-MS. All rats were euthanized by administering a lethal IV dose of pentobarbitone (0.4 mL per rat) at the end of the sampling period.

For IV studies, the test compound was administered intravenously as a 10 min constant rate infusion via an indwelling jugular vein cannula (n = 3 rats per compound) at 1 mg/kg. Formulation concentration did not exceed 0.3 mg/mL and volume dosed was dependant on body weight of the rats. All IV formulations consisted of 1% ethanol and 5% Kolliphor EL in DPBS and were filter sterilised prior to administration. Aliquots were taken at 5, 10, 15, 30, 70, 160, 310, 430, 610, 970 and 1450 min timepoints.

Each test compound was administered orally by gavage at 3 mL/kg (n = 3 rats per compound). Each oral formulation was made to a peptide concentration of 3.3 mg/mL. Standard oral formulation was a uniform, fine, off-white suspension consisting of 0.5% (w/v) hydroxypropyl methylcellulose, 0.5% (v/v) benzyl alcohol and 0.4% (v/v) polysorbate 80 in Milli-Q water. Aliquots were taken at 15, 30, 60, 150, 240, 300, 420, 600, 960 and 1140 min timepoints. The self-emulsifying drug delivery system (SEDDS) formulation was a cloudy, faintly blue, solution consisting of 1.5% (v/v) absolute ethanol, 4% (w/v) Maisine cc, 5% (w/v) Kolliphor RH40 and 1% (w/v) propylene glycol in milli Q water. Each oral formulation was dosed at 10 mg/kg with an average of 0.8 mL of formulation given to each rat. Aliquots were taken at 15, 25, 30, 35, 40, 45, 50, 60, 90, 120 and 240 min timepoints.

Plasma proteins were precipitated from solution by ice cold 0.1% formic acid in ACN using a 1:10 dilution. The samples were when centrifuged at 10,000 rpm for 10 minutes and 45 µL of supernatant was transferred into Eppendorf tubes. The internal standard was then spiked into each plasma sample at 50 ng/mL and samples were run on the LC-MS using methods described in Section 2.2 (page 43).

2.7. References

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Chapter 3 : Effect of modulating lipophilicity and D-amino acids on *in vitro* permeability and enzymatic stability

3.1. Introduction

There is ongoing interest for biologics, like peptides, to be delivered orally rather than by injections (1). The primary advantage to oral delivery is improved patient compliance, which encompasses patients who find the cost of injectables too high and those with aversions to needles and pain. Some of the key challenges to oral peptide delivery include low intestinal absorption and high metabolism by enzymes (2). This study explores the influence of changing lipophilicity and peptide shape via the substitution of L-amino acids with D-amino acids on membrane permeability and metabolic stability.

In the gut, there are three key absorption mechanisms that compounds can use to cross the gastrointestinal membrane: transcellular, paracellular, and carrier-mediated transport (3). Each absorption pathway is affected by different factors and an in-depth exploration of these pathways can be found in Chapter 1 (page 11). In this thesis, we focus on targeting transcellular passive diffusion as the primary mechanism of absorption. In general, the transcellular permeability of a solute is a complex function dictated by physicochemical properties e.g. size, lipophilicity, hydrogen bond potential, charge, and conformation, and for any given drug, one of these factors may dominate over the others (3). Lipophilic small molecule drugs are predominantly absorbed through transcellular passive diffusion (2). This pathway allows larger payloads of drug to pass through the gut due to the small intestine's large surface area. Studies into the properties that exploit the transcellular absorption pathway have found that the permeability of drugs through the transcellular route are positively related to lipophilicity and negatively to both hydrogen bonding capacity and molecular size (4-8). This positive relationship between permeability and lipophilicity provides the foundational idea for this study.

Metabolism of peptides can occur at any point throughout the oral delivery route (9-11). Starting in the stomach, peptides are exposed to an extreme low pH environment

and enzymes like pepsin that hydrolyse peptides. Moving from the stomach and into the small intestines, there are also a wide variety of enzymes that breakdown peptides like trypsin, α-chymotrypsin, and exopeptidases (12). Should the peptide manage to remain intact through the GI tract and be absorbed into the bloodstream, the blood plasma also contains a wide range of enzymes that can metabolise peptides, for example thrombin, plasmin and clotting factors (13). Material absorbed from the gut travels via the bloodstream through the liver before reaching systemic circulation, with any clearance performed by the liver categorised as 'first pass metabolism'. Approximately 90% of the liver is comprised of hepatocyte cells which secrete a large variety of enzymes that drive hepatic first pass metabolism (14). An example of one of the classes of enzymes secreted by the liver are cytochrome P450 (CYP) enzymes (14). These enzymes come in many isoforms and drugs like buspirone, cyclosporine, lovastatin, saquinavir, and verapamil exhibit poor oral bioavailability due to extensive CYP-mediated first-pass metabolism (14). Of this list of drugs, cyclosporine and saquinavir are peptides or are peptide-like/peptide derivatives, suggesting that these drug types are susceptible to CYP degradation. Once in systemic circulation, the kidneys also filter the blood and remove materials in a process called renal clearance into the urine (15). Renal clearance is a dynamic process involving glomerular filtration and reabsorption (16). For lipophilic drugs, passive reabsorption back into the systemic circulation will result in minimal renal clearance (16). However, for hydrophilic drugs like peptides, the filtered drug will be excreted into the urine unless the drug is actively reabsorbed back into the systemic circulation by a transporter-mediated mechanisms (16). Drugs can also be secreted from the blood into the lumen of the proximal tubules, resulting in a renal clearance greater than the glomerular filtration rate (16).

This study will focus on the impact of lipophilicity and D-amino acids within cyclic peptides on *in vitro* peptide solubility, permeability, and stability. The cyclisation of peptides provides protection for labile amino acids through generation of a 3D structure. Cyclic peptides can be formed in a variety of ways including at the backbone, between sidechains, or through disulfide bridges from amino acids like Cys. Cyclic peptides show improved chemical stability and longer biologic half-life over straight chain peptides, with little to no negative impact on potency (15, 17-19). All the peptides in this study are

cyclised at the backbone. Backbone cyclisation reduces the risk of exopeptidase enzymatic degradation and increases the lipophilicity of the peptide by eliminating the exposed amine and carboxylic acid groups (4, 15). Studies from Okumu et al. found that cyclic hexapeptides were 2-3 times more likely to permeate a Caco-2 cell monolater than their linear analogue counterparts due to the higher lipophilicity from cyclisation (4).

Lipophilicity is a physicochemical property describing the affinity for a drug to be dissolved in a non-polar environment. The octanol/water partition coefficient (log $P_{o/w}$) can be used to describe a drugs lipophilicity by measuring the ratio of drug that has partitioned between a two-phase system of octanol and water (3). Today, this partition coefficient can be predicted using computational methods to calculate log P values called clogP. It is well established that peptides with high lipophilicities have better permeabilities across various *in vitro* assays, including PAMPA and Caco-2 studies (7, 8, 20-24). Apart from increased lipophilicity by peptide cyclisation, peptide lipophilicity can also be increased at the amino acid side chains by using hydrophobic side chains. Hydrophobic amino acids include Ala, Val, Ile, Leu, Met, Phe, Tyr and Trp residues. Stereochemical variations like utilising D-amino acids have also been found to improve permeability and peptide stability (25-31). D-amino acids are not commonly found in nature, allowing these structures to be less susceptible to proteolytic attack compared to their L-amino acid counterparts.

Previous unpublished work from our group explored the effect of D-amino acids in the pasireotide backbone on *in vitro* permeability. To do this the original pasireotide structure was simplified down to natural amino acids (Figure 3.1). The hydroxyproline residue was replaced with a Pro residue, the phenylglycine residue was replaced with a Phe residue and the benzyl protected tyrosine residue was replaced with a Tyr residue. Several amino acids have ionisable side chains at physiological pH. Examples of ionisable amino acids include Lys, Arg and His which have a positive charge, and Asp and Glu which have a negative charge at physiological pH. Charged groups within a peptide structure interact very strongly with water and are unlikely to possess any passive permeability. Therefore, the Lys residue was removed and replaced with Ala to better measure peptide permeability effects without the influence of ionisable Lys. Both L- and D- conformers for each of the six amino acids in the structure were synthesised to create

a database of 36 analogues. The *in vitro* permeabilities of these analogues were tested at n=1 and the results can be found in Appendix E (page 167). Based on the preliminary permeability data, five constructs were chosen for further exploration. The hypothesis governing this thesis is that lipophilicity can be further optimised to improve permeability across a lipid bilayer, and, that D-amino acids can be integrated into the peptide structure to move closer towards oral bioavailability.

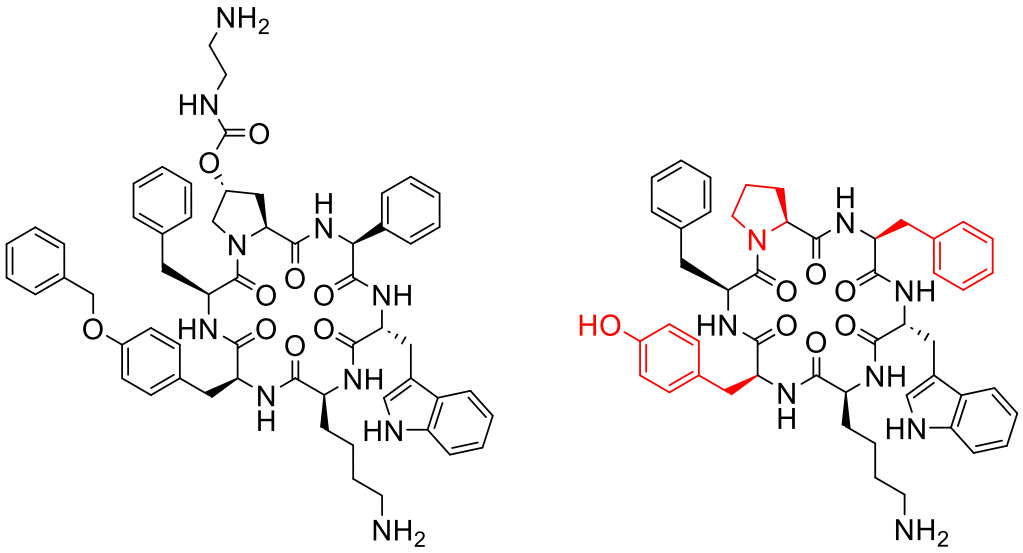


Figure 3.1 Structure of commercial pasireotide (left) and simplified pasireotide analogue A1 (right). Simplified pasireotide replaces Hyp with Pro, Phg with Phe and Tyr(Bzl) with Tyr. Changes to the structure are highlighted in red.

This study aims to achieve two objectives. The first objective is to design and synthesise a series of pasireotide analogues with increasing lipophilicity and differing stereochemical patterns. The second objective is to explore the physicochemical behaviours of these peptide analogues in *in vitro* permeability and solubility assays, as well as their enzymatic stability in simulated gastrointestinal fluids and rat plasma environments.

3.2. Methods

A detailed description of all the methods described in this chapter can be found in further detail in Chapter 2 (page 34). For clarity, a summarised version of each assay relevant to this chapter is provided in the following sections.

3.2.1. Solid Phase Peptide Synthesis

All peptide analogues synthesised for this study were made using manual Fmoc-based solid phase synthesis as previously described by Yue et al. (32). The process is summarised in Figure 3.2. Briefly, the linear peptide sequence was synthesised on a 2-CTC resin using a series of Fmoc protected couplings and piperidine deprotection cycles. Once the linear peptide was complete, the peptide was cleaved off the resin and backbone cyclised using pyclock in an overnight reaction. After reaction completion, any protecting groups on the amino acids were cleaved and the peptide was purified using RP-HPLC.

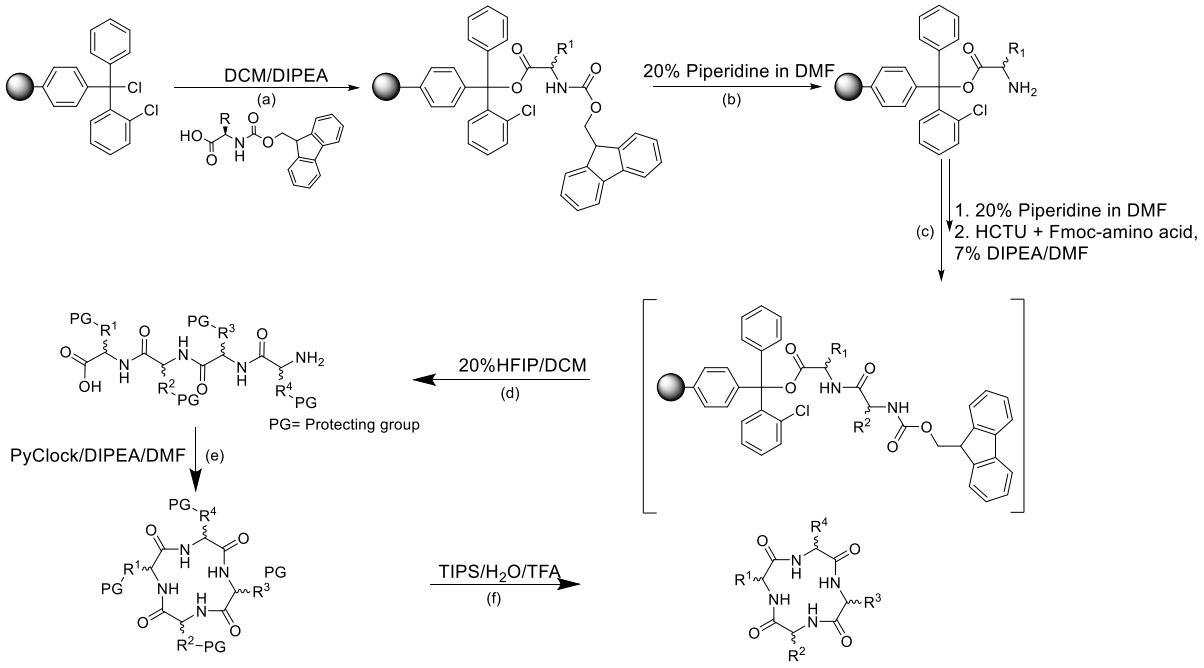


Figure 3.2 Generalised Synthesis Schematic. (a) Primary amino acid coupling (b) Fmoc-deprotection (c) Amino acid coupling (d) Resin cleavage (e) Head to Tail cyclisation (f) Protecting group deprotection.

3.2.2. Parallel artificial membrane permeability assay (PAMPA) and Solubility Assay

Membrane permeability of the peptides was tested by *in vitro* parallel artificial membrane permeability assay (PAMPA) developed by Wohnsland et al. (33). The method was previously described by Li et al. (34) but briefly, 5 µL of 1% lecithin in dodecane was used to coat a polyvinylidene difluoride (PVDF) membrane of a filter plate (Millipore) to form the artificial cell membrane (Figure 3.3). Stock peptide solutions (4 mM) were prepared in DMSO. The donor phase contained 300 µL of solution containing 200 µM of peptide stock in PBS buffer (final concentration of DMSO in donor phase is 5%). The

receptor phase is overlayed on top of the donor phase and contains a 200 µL solution of 5% DMSO in PBS. The plate was incubated for 16 hours at 37 °C.

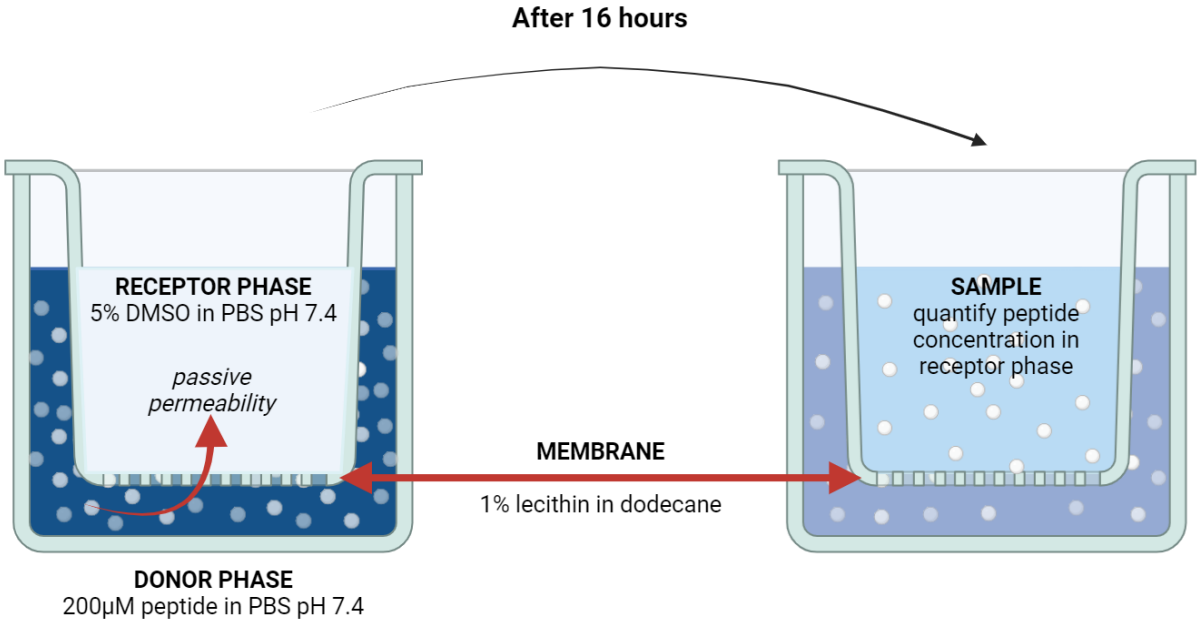


Figure 3.3 PAMPA assay. Image was made in BioRender.com.

Compound concentrations at equilibrium after 16 hours in both the donor and receptor wells were determined by UPLC analysis. Aqueous solubility values were measured in conjunction with the PAMPA assay. Due to limited material, the solubility values were measured at a maximum concentration of 200 µM in 5% DMSO in PBS. Peptides were incubated at 37 °C under shaking conditions for 16 hours and sample supernatant was measured using fluorescence UPLC at excitation wavelength 300 nm and emission wavelength 338 nm. Samples that measured at 200 µM can be assumed to be freely soluble at this concentration and therefore sufficiently soluble in aqueous environments.

3.2.3. Enzymatic Stability Assays

3.2.3.1. Gastrointestinal Stability

Simulated gastrointestinal fluid previously described by Li et al. (35) was used to mimic digestion conditions with an excess of porcine pancreatin used to trigger enzymatic digestion. Octreotide was chosen as a positive control, given its constant half-life of 1 hour under the experimental conditions (34). Peptide at a concentration of 4 µM in a 1mL system of digestion medium was equilibrated at 37ºC for 10 minutes prior to enzyme addition. The sample aliquot removed prior to enzyme addition is T0. Further samples were taken at T5, 15, 30, 60, 120 and 240 mins post enzyme addition. Sample aliquots were quenched by 1:10 dilution in 0.1% formic acid in acetonitrile. A graphical summary of the assay can be found in Figure 3.4.

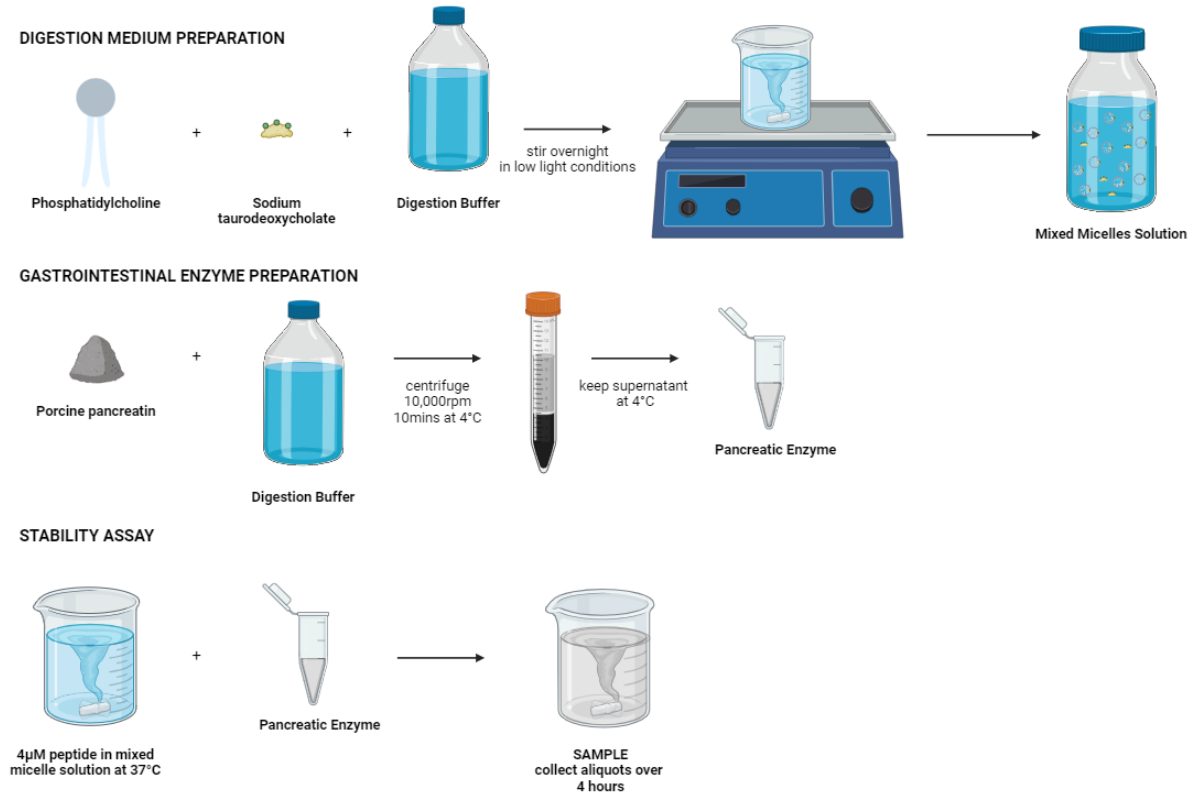


Figure 3.4 Gastrointestinal Stability Assay. Image was made in BioRender.com.

3.2.3.2. Plasma Stability

Fresh frozen rat plasma was obtained from the Monash Animal Research Platform (MARP, Melbourne, AUS). Somatostatin was used as a positive control for the plasma stability assay as it has a well reported half-life of less than 3 minutes in plasma (36). To prepare the plasma solution, 10 µL of PBS buffer was added to 88 µL of rat plasma in an Eppendorf tube. Following this, 2 µL of 200 µM peptide stock solution dissolved in DMSO and was added to the tube to obtain a final concentration of 4 µM in plasma (Figure 3.5). Mixture was vortexed and incubated for 4 hours at 37°C with 10 µL aliquots collected at timepoints T0, 5, 15, 30, 60, 120 and 240 minutes. Proteins were precipitated from solution with a 1:10 dilution of 0.1% formic acid in acetonitrile. Samples were centrifuged at 4°C for 10 mins at 10,000rpm and supernatant was analysed using LC-MS.

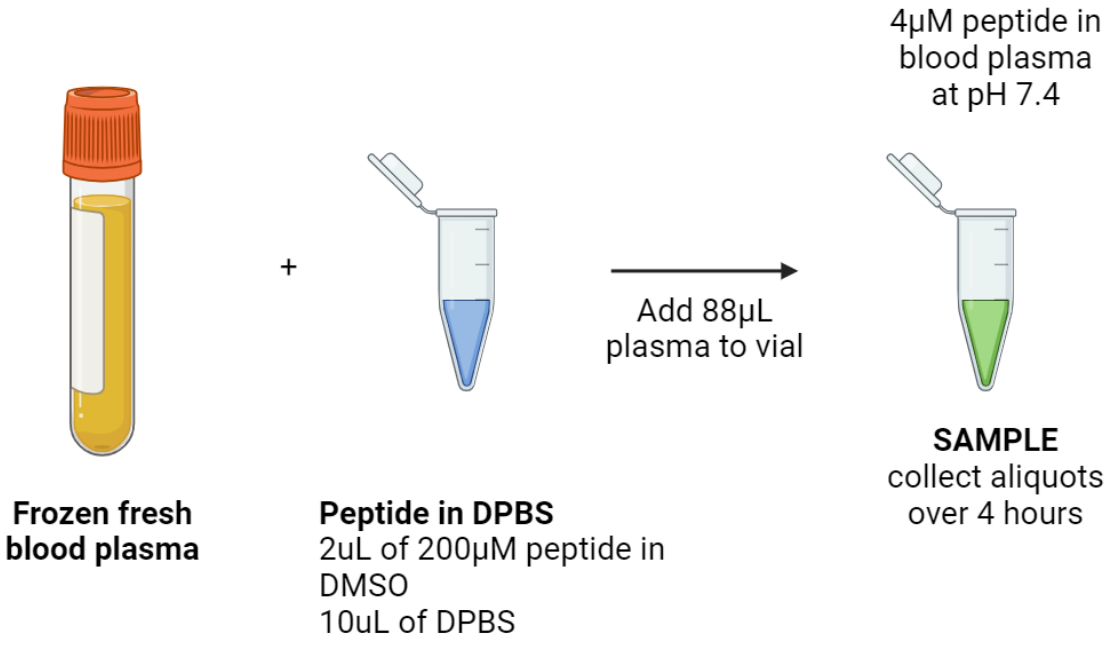


Figure 3.5 Plasma Stability Assay. Image was made in BioRender.com.

3.3. Results

3.3.1. Selection of peptide backbone scaffolds

The five most permeable backbones found from prior PAMPA studies performed by our group were chosen for further analysis in this current study. A summary of the backbones chosen can be found in Figure 3.6 (backbones A-F).

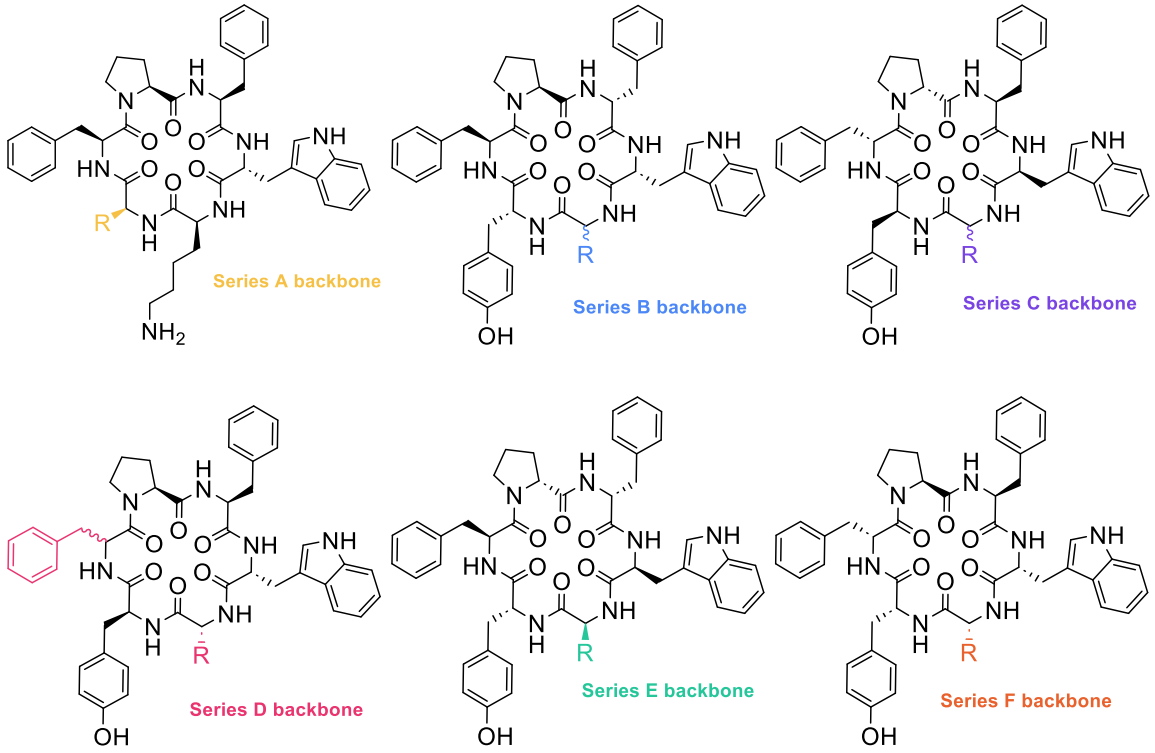


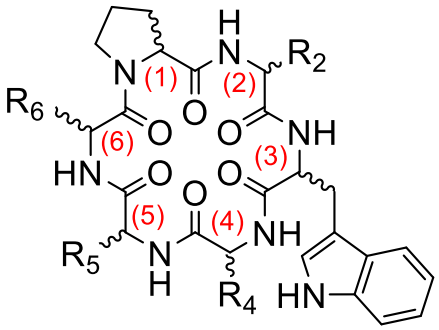
Figure 3.6 Chemical structures of the synthesised peptide backbones (series A-F). The change within each series is highlighted by their unique colour: yellow for series A, blue for series B, purple for series C, pink for series D, green for series E and orange for series F.

3.3.2. Synthesised peptides with systematic substitutions to alter lipophilicity and stereochemistry

A total of 27 peptides were designed and synthesised to fit the backbone series above. A summary of the structure of each synthesised peptide can be found in Table 3.1 and the rationale for each of the modification is discussed below. The LC-MS analysis of each synthesised product can be found in the Appendix A (page 160).

*Table 3.1 Synthesised peptides. Each peptide series (i.e. series A, B, C etc.) is organised by backbone structure. Amino acid positions are defined by numbers 1-6 starting at the Pro residue and moving in the N- to C-terminus direction around the cyclic peptide. *Peptide A3 is taken from Veber et al. (37). †Additional material synthesised by Travis Lay (Monash University).*

Pasireotide analogues



	cyclo[**1**	**2**	**3**	**4**	**5**	**6]**
A1	*cyclo[* Pro	Phe	D-Trp	Lys	Tyr	Phe]
A2†	*cyclo[* Pro	Phe	D-Trp	Lys	Tyr(Bzl)	Phe]
A3*†	*cyclo[* Pro	Phe	D-Trp	Lys	Thr	Phe]
A4	*cyclo[* Pro	Phe	D-Trp	Lys	Val	Phe]
A5	*cyclo[* Pro	Leu	D-Trp	Lys	Leu	D-Leu]
A6†	*cyclo[* Pro	Phe	D-Trp	Gly(2-pent)	Tyr	Phe]
B1	*cyclo[* Pro	D-Phe	D-Trp	Lys	D-Tyr	Phe]
B2†	*cyclo[* Pro	D-Phe	D-Trp	Ala	D-Tyr	Phe]
B3†	*cyclo[* Pro	D-Phe	D-Trp	Nle	D-Tyr	Phe]
B4†	*cyclo[* Pro	D-Phe	D-Trp	Gly(2-pent)	D-Tyr	Phe]
B5	*cyclo[* Pro	D-Phe	D-Trp	Lys($COCF_3$)	D-Tyr	Phe]
B6	*cyclo[* Pro	D-Phe	D-Trp	D-Ala	D-Tyr	Phe]
B7†	*cyclo[* Pro	D-Phe	D-Trp	D-Nle	D-Tyr	Phe]
C1	*cyclo[* D-Pro	Phe	Trp	D-Lys	Tyr	D-Phe]
C2†	*cyclo[* D-Pro	Phe	Trp	D-Ala	Tyr	D-Phe]
C3†	*cyclo[* D-Pro	Phe	Trp	D-Nle	Tyr	D-Phe]
D1	*cyclo[* Pro	Phe	D-Trp	D-Lys	Tyr	D-Phe]
D2†	*cyclo[* Pro	Phe	D-Trp	D-Ala	Tyr	D-Phe]
D3	*cyclo[* Pro	Phe	D-Trp	D-Nle	Tyr	D-Phe]
D4†	*cyclo[* Pro	Phe	D-Trp	D-Ala	Tyr	Phe]
D5	*cyclo[* Pro	Phe	D-Trp	D-Nle	Tyr	Phe]
E1	*cyclo[* D-Pro	D-Phe	Trp	Lys	D-Tyr	Phe]
E2	*cyclo[* D-Pro	D-Phe	Trp	Ala	D-Tyr	Phe]
E3†	*cyclo[* D-Pro	D-Phe	Trp	Nle	D-Tyr	Phe]
F1	*cyclo[* Pro	Phe	D-Trp	D-Lys	D-Tyr	D-Phe]
F2	*cyclo[* Pro	Phe	D-Trp	D-Ala	D-Tyr	D-Phe]
F3†	*cyclo[* Pro	Phe	D-Trp	D-Nle	D-Tyr	D-Phe]

Series A is comprised of six peptides beginning with peptide A1, the simplified structure of pasireotide. The focus of series A is to retain the pharmacophore motif of pasireotide, D-Trp-Lys (38), whilst increasing lipophilicity at alternative amino acids. Peptide A2 reintroduces the benzyl (Bzl) functional group, already found on pasireotide, back onto the Tyr5 residue as Bzl groups are intrinsically hydrophobic groups. A3 is a peptide previously published by Veber et al. (37) that has reported good potency at hSSTR2 somatostatin receptors. A4 acts as a comparative peptide to peptide A3 which interchanges the Thr5 residue with a more lipophilic Val5 residue. A5 is a peptide adapted from Schwochert et al. (39) where the pharmacophore of pasireotide was inserted into the backbone of naturally derived, orally bioavailable, phepropeptin A which contains primarily Leu residues (40). A6 is the only peptide in this series that alters the pharmacophore of pasireotide by replacing the Lys residue with a Gly(2-pent) residue. Gly(2-pent) contains a single alkyl chain at the side chain, the same alkyl length as Lys. This is based on a study by Nutt et al. (41) which explored the importance of Lys on the activity of somatostatin. This study found that there was significant loss of activity when Lys was replaced with alternative electrically charged side chains like Arg and His. This paper suggests that it is not merely the basic nature of Lys that contributes to the activity of somatostatin. This study alludes to the idea that steric interactions could be at play here and A6 addresses this hypothesis.

Series B is made of seven peptides with the backbone '*cyclo*(Pfw**R**yF)', where the R group at position 4 signifies the change in amino acid. Peptide B1 has a Lys residue in position 4 consistent with pasireotide. Peptide B2 changes this Lys residue at position 4 to an Ala residue. This is to compare the permeabilities of ionisable Lys and lipophilic Ala on permeability. Peptides B3 and B4 further increase the aliphatic chain at position 4 with Nle and Gly(2-pent) residues, respectively, again to explore the extent to which permeability can change with increasing lipophilicity at the side chain. Peptide B5 explores the effect of prodrugging on the permeability of ionisable peptides. This is adapted from work by Barlow et al. (42) who found the trifluoroacetyl group ($COCF_3$) was the most effective prodrug at improving peptide permeability. Here, the $COCF_3$ group was paired to the Lys residue at position 4. Peptide B6 compares the effect of a D-Ala stereochemical change at position 4 with that of the L-Ala4 of B2. Peptide B7 compares

the effect of increasing lipophilicity against peptide B6 whilst also comparing a D-Nle stereochemical change at position 4 with that of the L-Nle4 of B3.

Series C is comprised of three peptides with a backbone of *cyclo*(pFW**r**Yf). Peptides C1-C3 are enantiomers of B1-B3, respectively.

Series D is made of five peptides with the backbone 'cyclo(PFw**r**Yf)' where the R group at position 4 signifies the change in amino acid. Peptide D1 contains a D-Lys4 residue. Peptide D2 replaces this residue with a D-Ala4 residue to explore the effect of increasing lipophilicity on peptide permeability. Peptide D3 further increases this lipophilicity at position 4 with a D-Nle residue. Peptide D4 explores the effect of a L-Phe stereochemical change at position 6 with that of the D-Phe6 of peptide D2. Peptide D5 compares the effect of increasing lipophilicity against peptide D4 whilst also comparing a L-Phe stereochemical change at position 4 with that of D-Phe 4 in peptide D3 at position 6.

Series E and F are each comprised of three peptides. Series E is made of backbone '*cyclo*(pfW**R**yF)' where the R group at position 4 signifies the change in amino acid. Peptide E1 has a Lys residue at position 4. Peptide E2 changes this Lys residue to an Ala residue at position 4. Peptide E3 contains a further increase of lipophilicity by a Nle residue at position 4. Series F follows this same pattern with a different backbone '*cyclo*(PFw**r**yF)' to understand how backbone stereochemistry influences *in vitro* permeability and solubility.

3.3.3. *In vitro* peptide solubility and permeability of synthesised peptides

A summary of permeability and solubility for all constructs can be found in Table 3.2. This data set encompasses the measured PAMPA permeability values, the measured solubility values, and calculated Log P and tPSA values (calculated by ChemDraw v22.2.0). The PAMPA permeability value measures peptide permeability in cm/s. It is not straightforward to correlate a set logP value to determine whether a peptide is labelled as *in vitro* 'permeable' or 'not permeable', but Ahlbach et al. (43) have chosen -6.4 cm/s as the permeability threshold for 'good permeability' which is what this study will use for consistency. In a similar fashion, solubility is also difficult to define as 'good' or 'bad', but it is generally agreed that good solubility is when 1 g of material can be dissolved in 100 mL of solvent according to the United States Pharmacopeia (USP) (44). Here, 'good' solubility is defined as a measured concentration of 100 µM or higher. Both cLogP and tPSA are calculated values used to predict the likelihood of compound absorption.

*Table 3.2 Experimental PAMPA permeabilities and aqueous solubilities of the series of cyclic peptides synthesised. PAMPA values are expressed in cm/s and calculated using Equation 1. Solubility is expressed in μM concentration. *Calculated by Chemdraw V22.2.0.*

Pasireotide analogues	**PAMPA permeability, $LogP_e$, cm/s**	**Measured solubility, 5% DMSO in PBS, max conc 200 μM**	**cLogP***	**tPSA (Å^2)***
A1	-8.79 ± 0.28	187.3 ± 26.2	5.58	224
A2	-8.35 ± 0.45	89.8 ± 22.1	8.34	213
A3	-8.66 ± 0.32	≥200	4.11	224
A4	-8.33 ± 0.14	≥200	5.75	203
A5	-8.85 ± 0.04	184.4 ± 17.4	6.36	204
A6	-6.99 ± 0.42	10.7 ± 7.0	7.99	198
B1	-9.34 ± 0.16	180.8 ± 27.2	5.58	224
B2	-6.63 ± 0.10	103.6 ± 18.6	5.88	198
B3	-7.05 ± 0.42	11.8 ± 19.7	7.46	198
B4	-5.74 ± 0.18	3.4 ± 2.4	7.99	198
B5	-7.03 ± 0.07	25.0 ± 6.4	6.27	227
B6	-7.67 ± 0.24	15.3 ± 4.6	5.88	198
B7	-7.59 ± 0.43	7.9 ± 9.9	7.46	198
C1	-9.38 ± 0.43	≥200	5.58	224
C2	-6.75 ± 0.22	97.6 ± 15.0	5.88	198
C3	-6.23 ± 0.28	2.4 ± 1.9	7.46	198
D1	-8.53 ± 0.74	≥200	5.58	224
D2	-6.11 ± 0.07	29.9 ± 8.2	5.88	198
D3	-6.54 ± 0.36	5.2 ± 4.3	7.46	198
D4	-8.90 ± 0.20	66.5 ± 3.6	5.88	198
D5	-7.16 ± 0.24	8.4 ± 4.9	7.46	198
E1	-8.24 ± 0.87	185.2 ± 12.3	5.58	224
E2	-6.30 ± 0.28	35.3 ± 7.1	5.88	198
E3	-6.49 ± 0.29	8.6 ± 6.3	7.46	198
F1	-8.11 ± 0.20	182.4 ± 14.2	5.58	224
F2	-6.72 ± 0.13	53.7 ± 8.3	5.88	198
F3	-6.41 ± 0.29	8.4 ± 7.2	7.46	198

3.3.4. Aqueous solubility

Intestinal absorption is dependent on drug solubility in the gut lumen and permeability of the drug across the membrane (45, 46). A drug must first be in solution before it can permeate across the membrane, with any insoluble drug being cleared from the gut. Biologically active peptides are typically polar from their amide linking bonds and presence of ionisable side chains. These features contribute to good aqueous solubility. Both solubility and permeability are dependent on the physicochemical properties of the molecule, and general properties, such as the molecular size, lipophilicity and number of polar atoms present included in the molecule, are of importance. Unfortunately, it is often found that properties with a positive influence in one respect, have a negative impact elsewhere. For example, physicochemical properties positive for permeability typically have a negative influence on the solubility, and vice versa (47). Lipophilicity, which is the major driving force for the permeability, is one of the properties that most strongly restricts the aqueous solubility (48). Here, the effect of two properties on solubility and permeability were explored: total polar surface area (tPSA) and calculated logP (the calculated octanol/water partition coefficient).

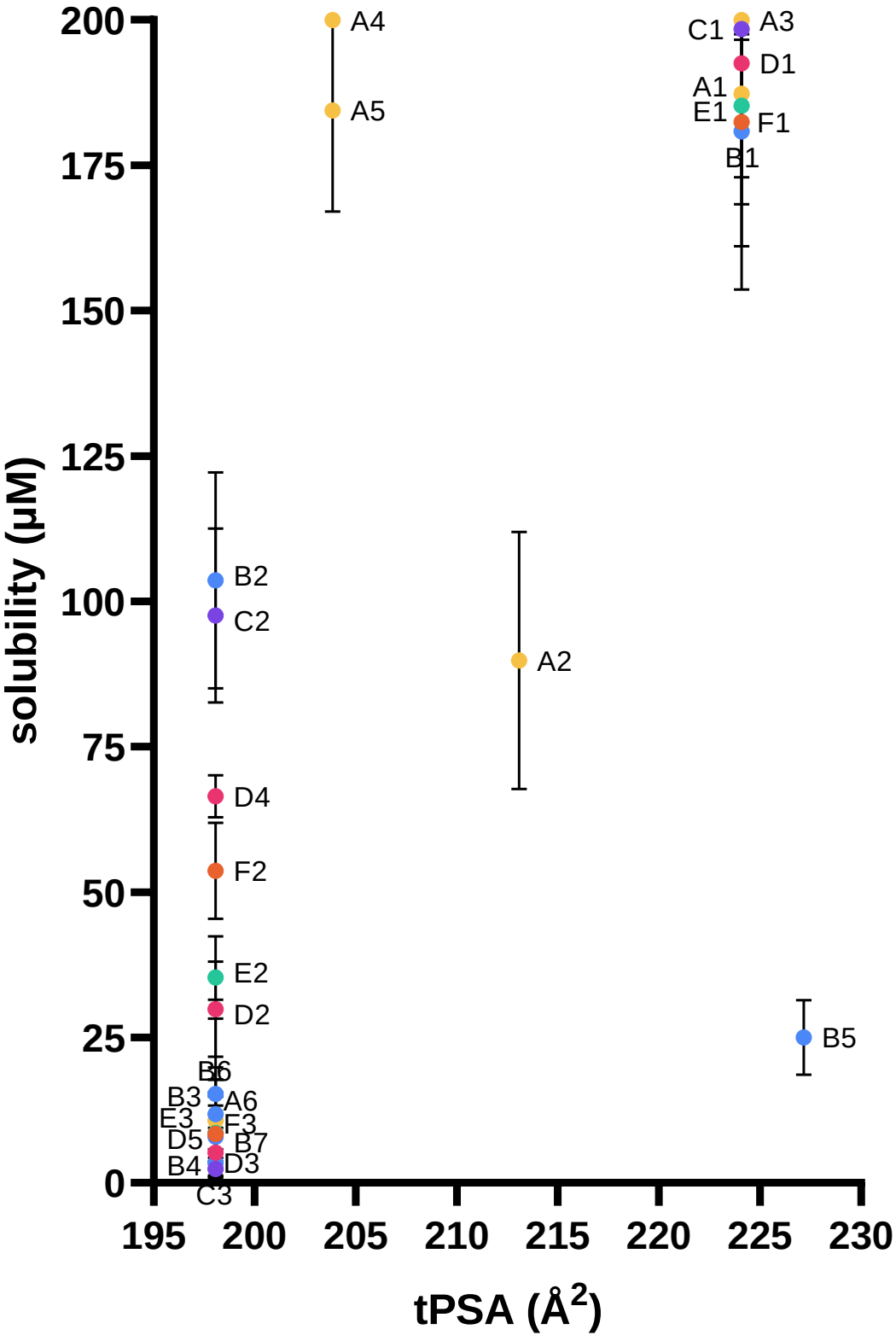


Figure 3.7 Calculated tPSA against measured aqueous solubility for all synthesised peptides. Each peptide series from A-F is coloured yellow, blue, purple, pink, and green, respectively. Data is expressed as mean ± SD, n=3.

Total polar surface area (tPSA) is the surface sum of all the polar moieties in a molecule and was calculated by ChemDraw (v22.2.0). Theoretically, as tPSA increases, aqueous solubility is predicted to increase in turn, creating a positive relationship between these two properties. From Figure 3.7, peptides that contain a Lys residue in their backbone (i.e. peptides in series A- yellow, and peptides that contain a 1 in their naming scheme) are highly soluble in the aqueous environment. This is reflected in their tPSA areas which trend higher than peptides without the Lys residue at position 4. The exceptions to this are peptides A4 and A5. Both peptides have more lipophilic side chains in their backbone, Val in position 5, and Leu in position 2, 5 and 6, respectively. This increase in lipophilicity reduces their calculated tPSA, yet the presence of Lys residue at position 4 keeps the peptide in solution with high aqueous solubility. The calculated tPSA was unable to distinguish the differences between peptides containing Ala, Nle or Gly(2-pent) residues at position 4. However, the effects of these side chains are seen in their measured solubilities with peptides containing smaller alkyl chains being more soluble than those with longer alkyl chains. In other words, the peptides with longer alkyl chains and therefore higher lipophilicity in the structure, had lower measured aqueous solubilities than peptides with shorter lipophilic chains and lipophilicity. Another outlier to this trend is peptide B5. This peptide has a Lys($COCF_3$) residue in position 4. This peptide has a high tPSA but low aqueous solubility. This suggests that tPSA on its own is not a good predictor of aqueous solubility.

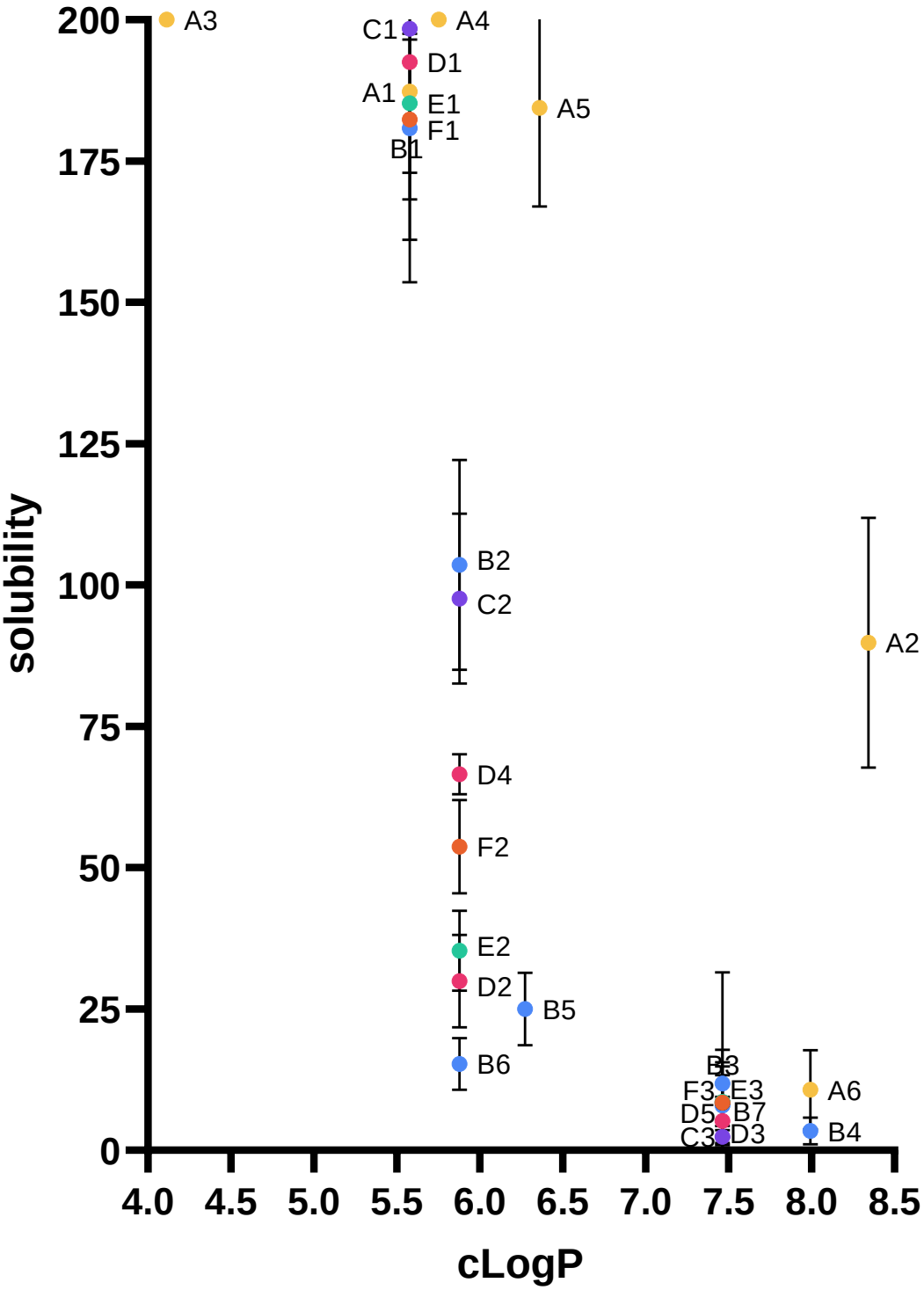


Figure 3.8 Calculated LogP against measured aqueous solubility. Each peptide series from A-F is coloured yellow, blue, purple, pink, and green, respectively. Data is expressed as mean ± SD, n=3.

Figure 3.8 represents the correlation between clogP (ChemDraw v22.2.0) and measured solubility. As cLogP increases, aqueous solubility should decrease, creating a negative relationship between these properties. The cLogP values can distinguish differences in peptide amino acid side chain composition with ionisable peptides having the lowest cLogP values, followed by peptides with Ala4, Nle4 and then Gly(2-pent)4, respectively. This differs from tPSA which groups all the peptides with aliphatic side chains together under one value. Generally, as clogP decreases, measured solubility increases linearly with solubility. There are several outliers including peptide A2 which has high cLogP due to the Bzl group but still has some solubility likely from Lys residue. There is also a large spread in solubility between peptides containing Ala sidechains. For example, peptides B2 and B6 differ by a single stereochemical change of L-Ala4 and D-Ala4, respectively. Their clogP values are identical but their solubilities differ at 104 μM and 15 μM, respectively. The same trend can also be seen between peptides D4 and D2 which differ by L-Phe6 and D-Phe6. Peptide D4 has a solubility of 66.5 μM and peptide D2 has a solubility of 29.9 μM. These examples highlight the importance of stereochemistry by demonstrating the differences in solubility between otherwise identical backbones by singular alterations of L- or D-amino acids.

3.3.5. Parallel artificial membrane permeability assay

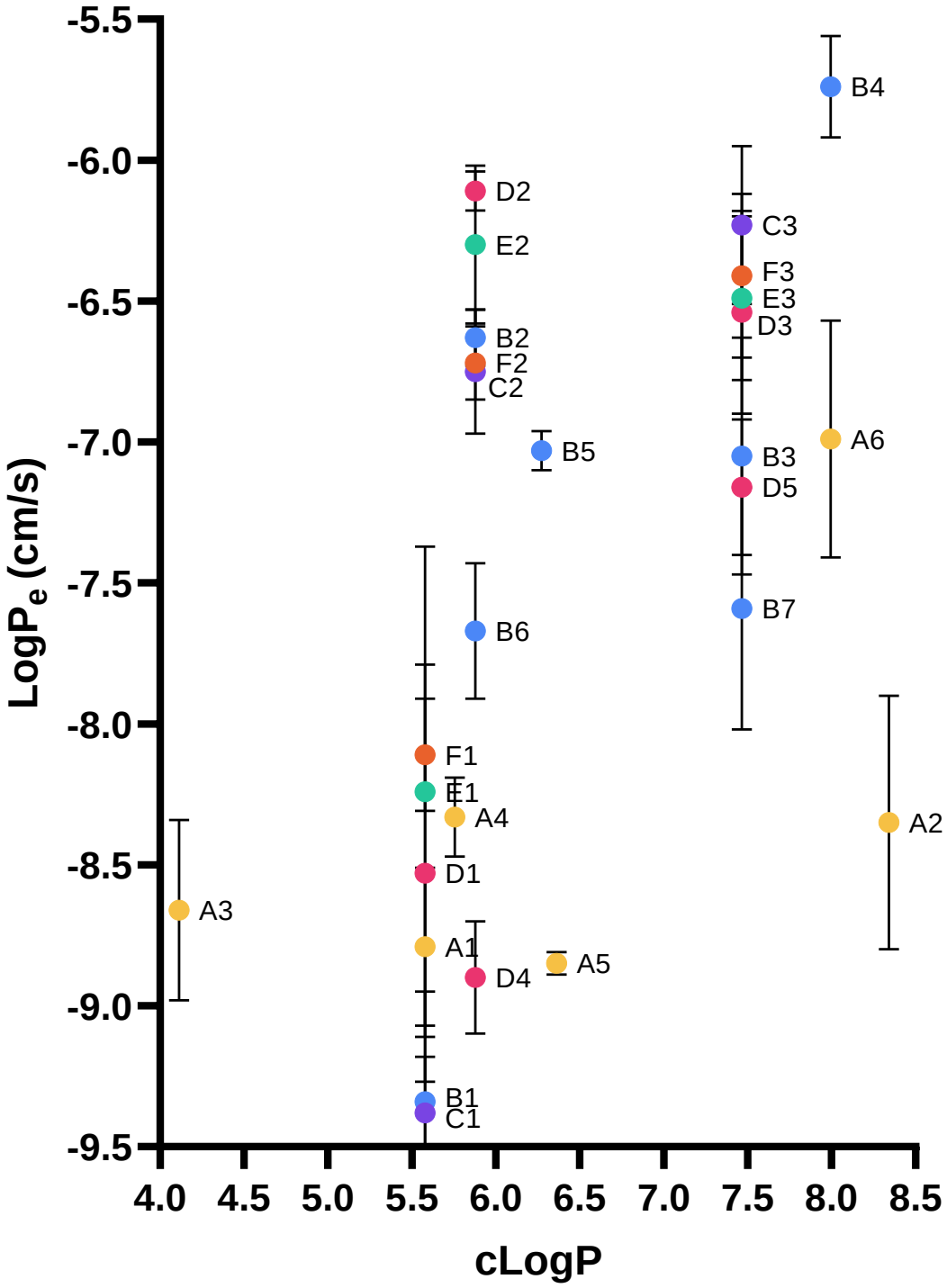


Figure 3.9 Calculated LogP vs Measured LogP$_e$. Each peptide series from A-F is coloured yellow, blue, purple, pink and green, respectively. Data is expressed as mean ± SD, n=3.

Figure 3.9 plots the cLogP of each analogue against its measured LogP. As clogP increases it is expected that the $logP_e$ also increases, creating a positive relationship. In a similar fashion to Figure 3.8, the cLogP values group the analogues into their respective sidechain groups (i.e. Lys, Ala, Nle, Gly(2-pent)). Generally, these values align with their measured permeability where low clogP values have low $logP_e$ values and vice versa. This is expected as both clogP and $logP_e$ values are measuring the same physicochemical property, permeability. However, outliers to this trend include some peptides within series A (e.g. peptide A2) and some measured permeability variability within peptides that have the same side chain functional groups (e.g. between peptides B2 (Ala4) and B6 (D-Ala4)). Once more, clogP appears to misjudge the impact of the Lys residue on the permeability of the peptide. This is seen in peptide A2 which contains a Tyr(Bzl) in position 5. This group contributes to a high clogP value but the $logP_e$ value demonstrates low permeability, likely due to the presence of the Lys residue. There is also variability between the measured permeability of structures with the same side chains but differing stereochemistry. An example of this is between peptides D2 and D4, which differs between D-Phe6 and L-Phe6. This single stereoisomer change results in a 100-fold increase in measured permeability.

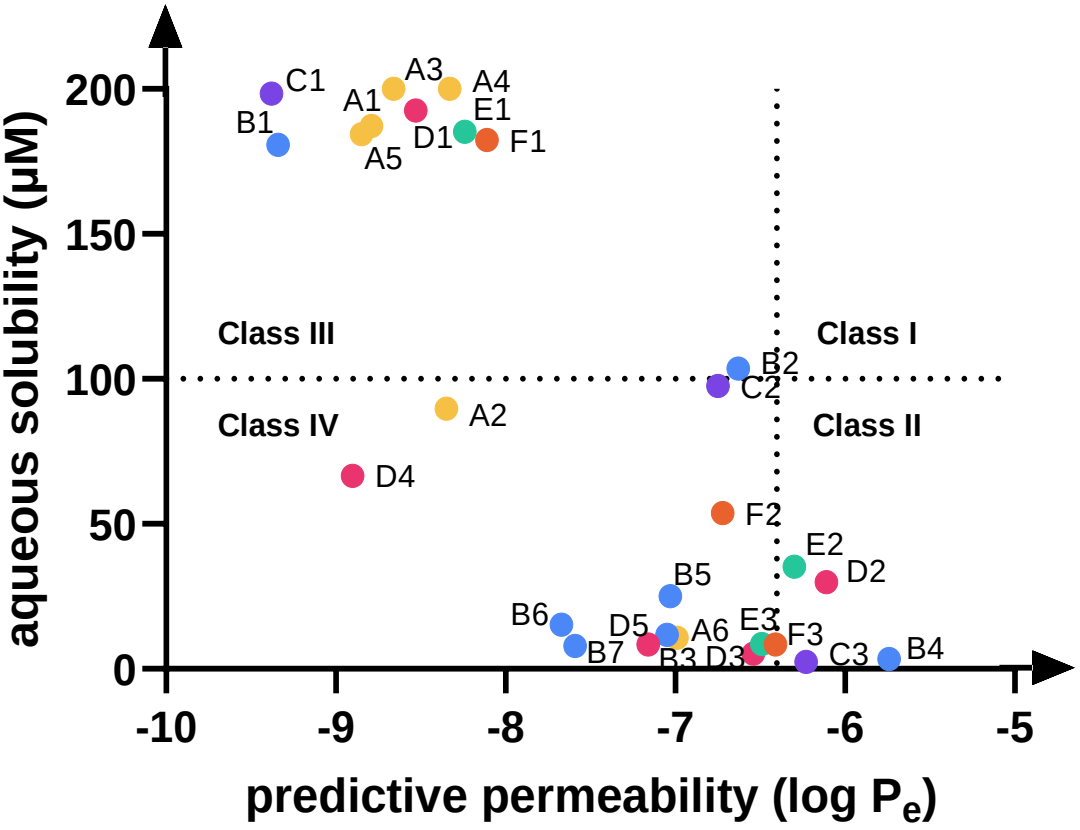


Figure 3.10 Scatter dot plot of experimental membrane PAMPA permeability against measured aqueous solubility. Each peptide series from A-F is coloured yellow, blue, purple, pink, and green, respectively. Vertical dotted line indicates threshold to 'good' in vitro permeability (log Pe -6.4) as reported by Ahlbach et al. (43). Horizontal dotted line indicates 50% of maximum solubility. Each quadrant is labelled Class I-IV following the biopharmaceutical classification system.

Figure 3.10 plots the measured PAMPA permeability against measured aqueous solubility. The dashed lines mimic a biopharmaceutical classification system which categorises drugs based on the physicochemical properties of permeability and solubility. Class I drugs are the most ideal for oral delivery and display high solubility and high permeability. Class II drugs are known as 'poorly water soluble' and have low aqueous solubility but high permeability. Class III drugs have high solubility and low permeability. Class IV drugs are colloquially known as 'brick dust' and are neither soluble nor permeable. Typically, peptides fall into either the class III or class IV groups with poor permeability being a common factor (49). Indeed here, the results show that peptides containing a Lys residue in the structural backbone, irrespective of L- or D-configuration of the peptide, tend to fall in the class III category with good solubility but poor permeability. Exceptions to this are peptides A2 and A6. A2 has a hydrophobic benzyl group on the Tyr5 residue, which reduces the peptide's solubility but is not sufficient to improve its permeability, potentially due to the bulky benzyl ring, placing it in the class IV category. The difference between peptides A1 and A2 is a nearly linear vertical drop in solubility with no change to permeability. Peptide A6 has a Gly(2-pent) residue at position 4 instead of the Lys residue. This removal of the ionisable side chain drastically reduces the solubility of the peptide with some increase in permeability. In comparison to peptide A1, A6 is a diagonal movement across the BCS system with a decrease in solubility and an increase in permeability. However, the improvement in permeability is not sufficient to push this peptide into the class II category. Seen within each series B-F, the peptides containing a Lys4 residues fall within the class III category. Replacement of the Lys residue in position 4 with either an Ala4 or the more lipophilic Nle4, irrespective of backbone chemistry, results in a positive and negative shift in both permeability and solubility, respectively. This moves these analogues from the Class III quadrant closer towards the Class II quadrant. However, there was varying success on whether these lipophilic analogues could cross over the good permeability threshold ($\log P_e$ of -6.4 or lower) or whether they remained in the Class IV category.

3.3.6. *In silico* modelling of Peptide B2

Computational modelling has evolved in recent years to be a useful tool in the discovery of new therapeutic agents (50). These methods are fast and cost-effective ways of screening for new drug candidates and predicting drug-target interactions. Here, molecular modelling was used to predict low energy conformations of peptide B2 using Maestro (Schrödnger LLC). Molecular modelling of stable peptide conformations was performed in both in aqueous and non-polar environments: water and chloroform (51). The conformational search finds as many energy minima conformations as possible within the specified parameters. The found conformations are listed in order of lowest energy minima to highest energy minima. The conformers shown in Figure 3.11 are the lowest energy minima conformations of peptide B2 found for each solvent (-593.920 kJ/mol and -570.293 kJ/mol in water and chloroform, respectively).

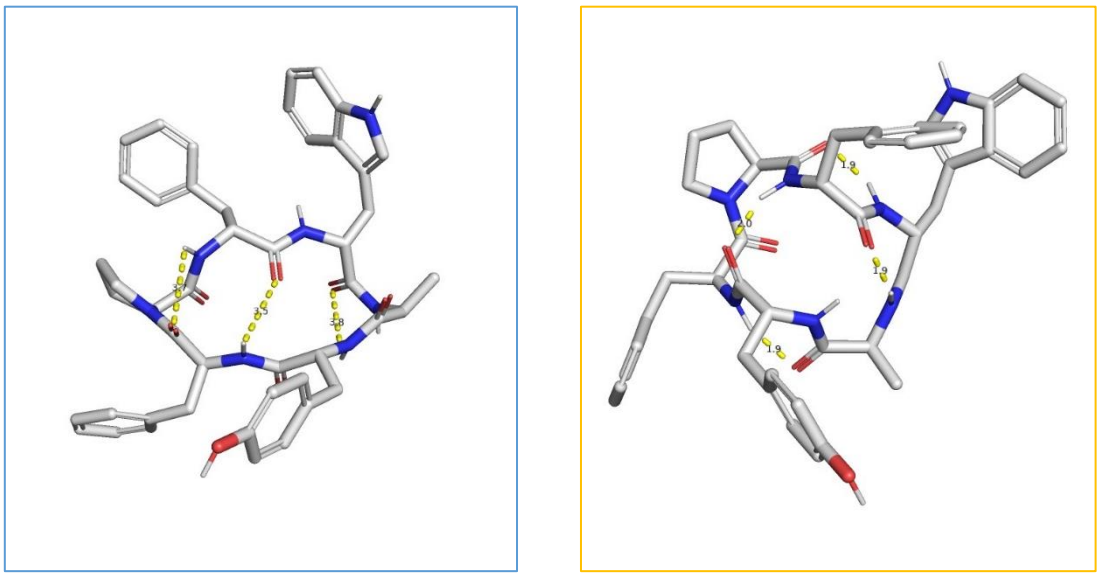


Figure 3.11 Lowest energy conformations of peptide B2 from in silico conformational search in water (left) and chloroform (right) using Maestro (Schrödnger LLC). Carbon, oxygen, and nitrogen atoms are coloured white, red, and blue, respectively. Hydrogen atoms, water molecules and chloroform molecules are not pictured.

3.3.7. *In vitro* enzymatic stability assay

It is essential that peptides remain stable to enzymatic metabolism before reaching their site of action. Based on the *in vitro* permeability and solubility results, peptides B2 and D2 within series B and D were determined as the most suitable candidates for oral delivery. Therefore, in this study we explored the stability of peptides in the presence of intestinal and plasma enzymes. Peptide series C was tested as the enantiomer pairs of peptide series B. The degradation profile of each peptide was assessed at a starting concentration 4 μM and tracked over 4 hours. Each sample was quantified using LC-MS.

Figure 3.12 shows the degradation profiles in simulated gastrointestinal fluid of select peptides within peptide series B (graph A), C (graph B) and D (graph C) against control peptide octreotide. Octreotide has a half-life of 60 mins in simulated gastrointestinal fluid (34). Porcine pancreatin was used to digest the peptides over a 4-hour study period. All tested peptides in graph A and C were relatively stable and did not degrade beyond 80% of starting material over the 4-hour window. Tested peptides within series C (graph B) were rapidly hydrolysed with half-lives of 145.6, 3.9 and 37.1 mins for C1, C2 and C3, respectively.

Figure 3.13 describes the stability of select peptides within series B, C and D in rat plasma against control peptide somatostatin. Somatostatin has a reported half-life of 2-3 minutes (52, 53). It was found that all peptides regardless of backbone, side chain composition or stereochemistry were stable and did not degrade beyond 90% of the initial concentration over the 4-hour study duration.

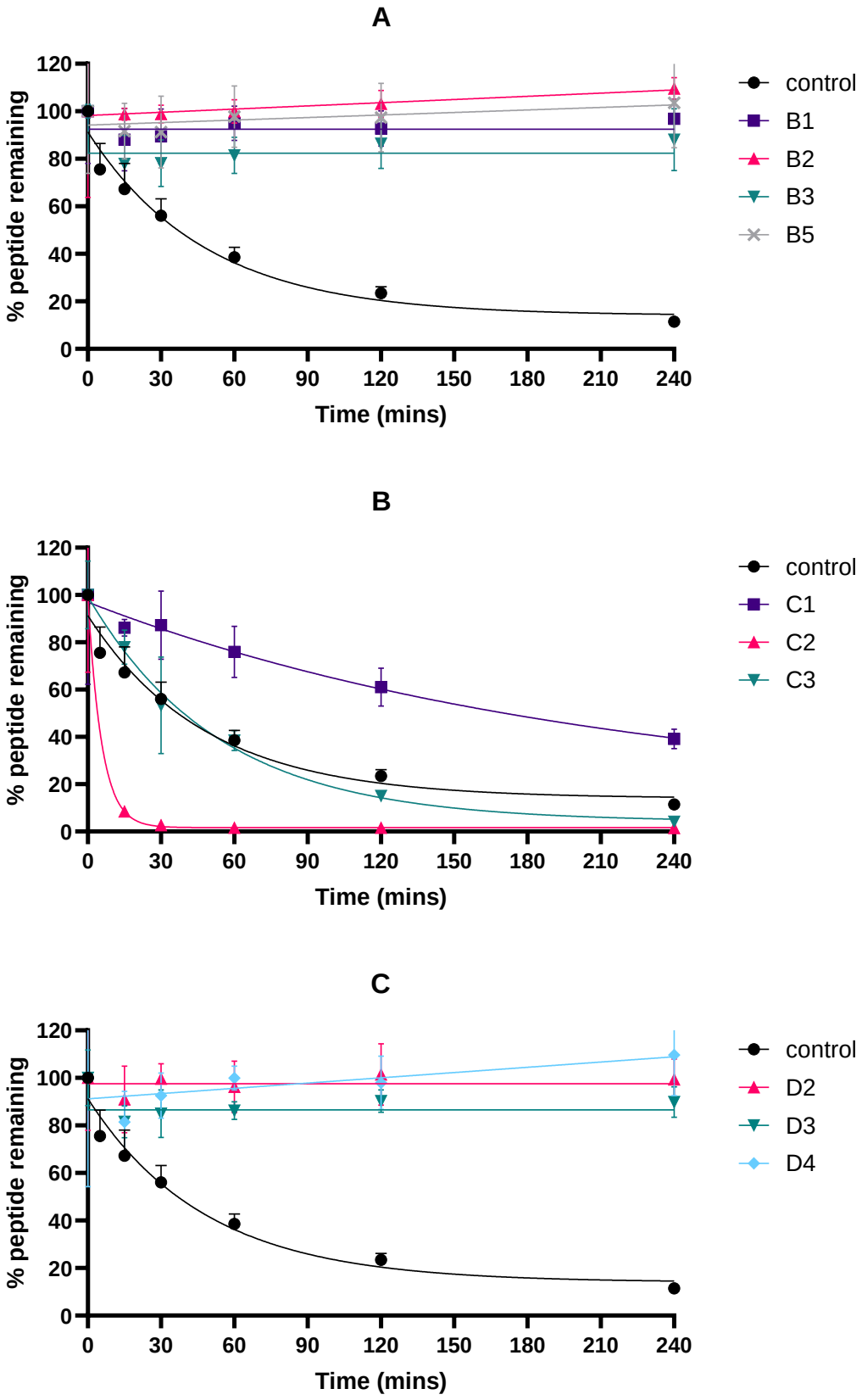


Figure 3.12 In vitro stability of peptides in simulated gastrointestinal fluid with pancreatin enzymes. Graph A depicts peptide series B, graph B depicts peptide series C and graph C depicts peptide series D. Control peptide was octreotide, and data is represented as mean ± SD, n=3.

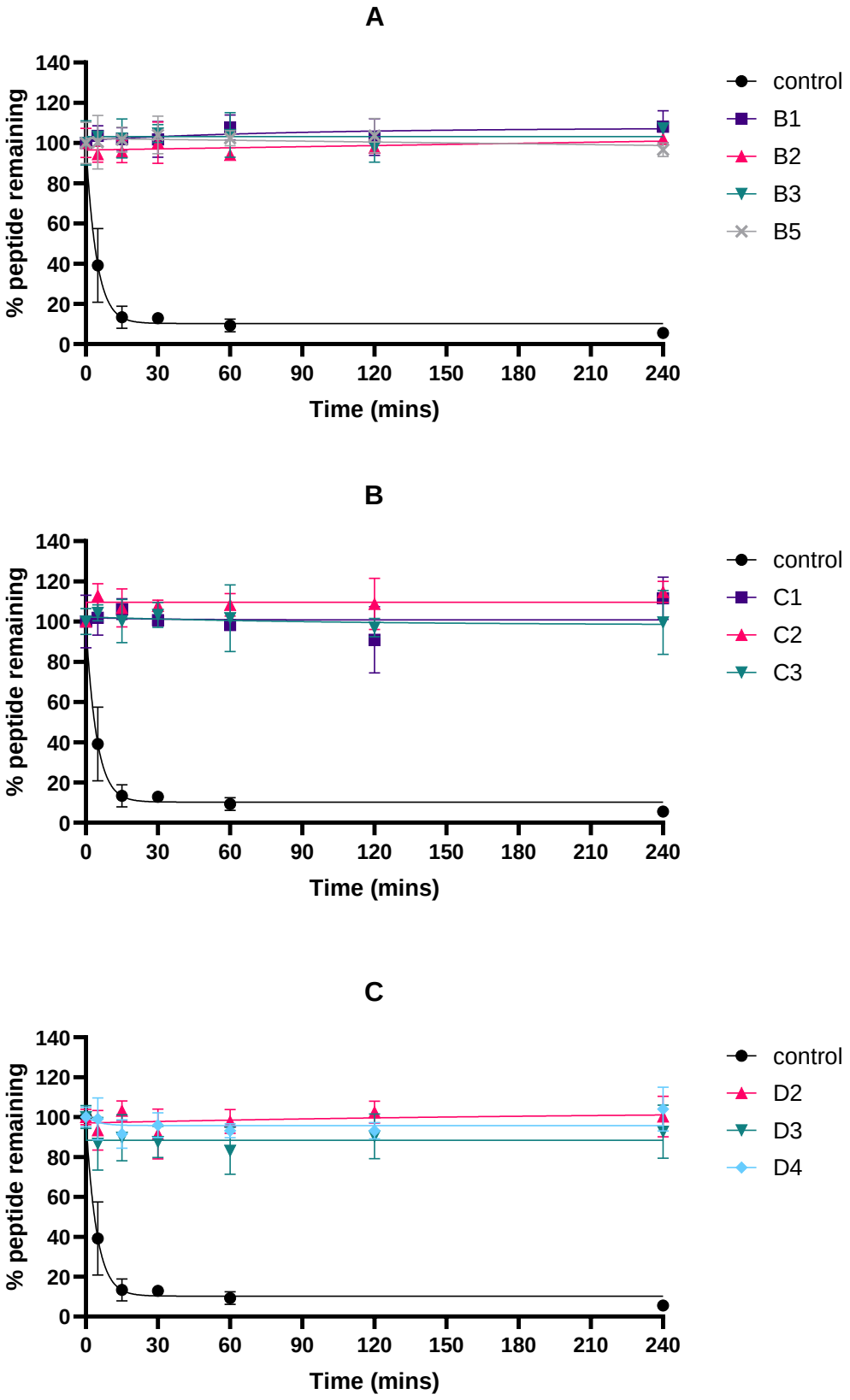


Figure 3.13 In vitro plasma stability profiles for peptides series B, C and D in rat plasma. Graph A depicts peptide series B, graph B depicts peptide series C and graph C depicts peptide series D. Control peptide was somatostatin. Data is represented as mean ± SD, n=3.

3.4. Discussion

Peptide permeability across the gut wall is a key challenge to the ongoing pursuit of oral delivery. The principle of 'like likes like' is a guiding phrase in many areas of pharmaceutics and was the basis for the structural changes made in this study. It is commonly reported in the literature that lipophilicity is positively related to permeability and negatively related to aqueous solubility (47, 54-56). The peptides synthesised in this study are categorised into series that each explore the effect of increasing lipophilicity on aqueous solubility, membrane permeability and enzymatic stability. Peptide solubility was measured in 5% DMSO in PBS buffer, permeability was measured in PAMPA assays and metabolic stability was measured in rat plasma and simulated gastrointestinal fluids with pancreatin.

3.4.1. Ionisable peptides aid in aqueous solubility but prevent passive permeability

Series A contains pasireotide analogues that retain the chiral backbone and pharmacophore of pasireotide. These peptides primarily contain a D-Trp residue in position 5 and a Lys residue at position 4 with the exception of peptide A6 which has a Gly(2-pent) amino acid at this position. The peptides within this series explore increasing lipophilicity at alternative amino acids that do not form part of the peptide's pharmacophore D-Trp-Lys. Peptides containing Lys do not tend to be permeable across a membrane due to its polarity, however, this study hypothesised that altering the amino acids outside of the pharmacophore towards increased lipophilicity would allow increased permeability potential despite the Lys residue.

In Figure 3.7 are plots of tPSA against measured aqueous solubility where tPSA calculates the total number of polar moieties in the structure. Theoretically, as tPSA increases, solubility should also increase. Many of the analogues in series A containing Lys have identical tPSAs except for A2, A4 and A5. These three peptides contain more lipophilic residues in their backbone, namely, Tyr(Bzl), Val and Leu, respectively. Although these peptides have lower calculated tPSA than others within series A, there was little impact on the peptide's aqueous solubility suggesting that lipophilicity at alternative side chains is not enough to counteract the ionisable Lys residue present and increase the total lipophilicity of the peptide. In a similar way, Figure 3.8 shows clogP

against measured solubility for all synthesised peptides. Unlike tPSA, the calculated clogP values were able to better distinguish between the amino acid side chains but even so, the peptides within series A that had high clogP values still had high solubility values except for peptides A2 and A6. In the case of peptide A2, the hydrophobic Bzl group on the Tyr residue is enough to increase cLogP by 3-log units and hinder the solubility of the peptide. Peptide A6 does not contain a Lys residue and shows that removing the ionisable Lys leads to lower solubility. This demonstrates that the polar Lys residue will likely keep the peptide in solution regardless of the lipophilicity of the surrounding amino acids.

Figure 3.9 and Figure 3.10 explore the impact of increasing lipophilicity on measured permeability and solubility. The performance of series A in Figure 3.9, which plots clogP against measured $logP_e$ shows that clogP does not linearly align with measured PAMPA permeability. Peptides with higher cLogP values did not necessarily have high measured permeabilities. As further seen in Figure 3.10, all the peptides containing a Lys residue in the backbone had high aqueous solubilities and did not achieve a permeability value deemed high enough for *in vivo* correlation (approx. log P_e of -6.4 cm/s) (43). Electrically charged side chains like Lys, Arg, His, Asp or Glu can form polar bonds in aqueous environments when in their ionised form and Lys is ionisable at physiological pH (41). The bonds that ionisable residues form with the aqueous environment are too strong to break, benefiting aqueous solubility but not allowing the peptides to cross into the lipid membrane layer. Reports in the literature show that peptides with low lipophilicity exhibit low permeability, and those with ionisable side chains like Lys residues have many solvent interactions (23).

This study has found that increasing lipophilicity at alternative amino acids while retaining the Lys in the peptide backbone will not significantly impact aqueous solubility. The bond that forms between ionisable Lys and its aqueous environment is too strong to break. It is important to note that a study by Bockus et al. (24) observed a similar trend where their attempts at sterically occluding polar groups via β-branching did not significantly improve permeability. They concluded that overall lipophilicity, solubility, and backbone flexibility had more impact on passive permeability. Therefore, the overall lipophilicities of structures containing Lys are still too low for permeability.

3.4.2. Lipophilicity has a negative relationship with solubility but correlates poorly with measured permeability

Although increasing lipophilicity is thought to improve membrane permeability, it can also incur serious oral delivery liabilities, such as poor water solubility. Many studies have tried to reconcile these effects (47) and while the goal of these synthesised pasireotide analogues was to increase lipophilicity for better permeability, the extent of any aqueous solubility lost was also observed. This study found that there is a negative relationship between lipophilicity of peptides in the form of both clog P and *in vitro* permeability, and their solubility.

All peptides containing a Lys residue and low lipophilicity were soluble in the aqueous environment. Concentrating on series B-F, all peptides containing Lys, identifiable by the '1' in their naming convention, had the lowest lipophilicities but the best aqueous solubilities. Replacing the Lys residue with an Ala ('1' to '2' in each series) resulted in an immediate reduction in tPSA and increase in clogP. This correlated to a decrease in solubility. Depending on the series there were differing extents to solubility loss ranging from 42% between peptides B1 and B2 to 84% loss between peptides D1 and D2. Further increases in lipophilicity from an Ala4 to a Nle4 saw further reduction in solubility again ranging from 48% to 97%. This confirms a strong correlation between lipophilicity and solubility suggesting an increase in lipophilicity has a direct negative impact on solubility.

The effect of increasing lipophilicity had a more varied response on peptide permeability. Replacement of the Lys residue with a more lipophilic Ala residue (B1 to B2) at position 4 resulted in a 1000-fold improvement in permeability. The same trends were observed with series C-F, where the substitution of Lys4 to Ala4 had significant improvements in permeability. The change between peptides C1 (Lys4) and C2 (Ala4) resulted in a 1000-fold increase in permeability, for peptides D1 (Lys4) and D2 (Ala4) it was a 100-fold increase in permeability, peptides E1 (Lys4) and E2 (Ala4) was a 100-fold increase and between peptides F1 (Lys4) and F2 (Ala4) it was a 100-fold increase in permeability. This suggests that the ionisable Lys residue strongly contributes to the peptide's permeability potential.

Furthermore, an increase in lipophilicity by addition of three methyl groups to position 4 (Ala4 to Nle4) had mixed effects on permeability. Permeability decreased between peptides B2 (Ala4) and B3 (Nle4) by 10-fold and yet permeability remains somewhat unchanged between peptides B6 (D-Ala4) and B7 (D-Nle4). Permeability increased between C2 (D-Ala4) and C3 (D-Nle4). Permeability between D2 (D-Ala4) and D3 (D-Nle4), each with a D-Phe6 residue, decreased yet increased between D4 (D-Ala4) and D5 (D-Nle4) which contains a L-Phe6 residue. Permeability decreased between peptides E2 (Ala4) and E3 (Nle4) and increased between peptides F2 (D-Ala4) and F3 (D-Nle). Therefore, a structural change of an Ala residue to a Nle resulted in varied permeability changes, demonstrating that simply increasing alkyl chain length and lipophilicity does not exclusively improve permeability.

Further increases to the alkyl chain at position 4, such as in the case between peptides B3 (Nle4) and B4 (Gly(2-pent)4) improved permeability by 100-fold. This is a 10-fold improvement between peptides B2 (Ala4) and B4 (Gly(2-pent)4), and a 10,000-fold improvement between B1 (Lys4) and B4 (Gly(2-pent)4). There are dangers to the peptides being too lipophilic as these peptides may get stuck in the lipid layer resulting in poor absorption. Peptides must be both soluble in aqueous and lipid environments to be orally deliverable and achieving this balance remains a significant challenge. Masking the ionisable group with a prodrug as was done with peptide B5 (Lys($COCF_3$)4) improved the permeability by 100-fold compared to B1 (Lys4).

Removing the ‘Lys’ residue results in an immediate improvement in passive permeability. This is on track to what we hypothesised where increasing lipophilicity will improve permeability. However, when comparing the change between peptides B2 (Ala4) and B3 (Nle4), and peptides B6 (D-Ala4) and B7(D-Nle4), which are further increases to the alkyl chain length, this has unique effects on permeability. This suggests that for passive permeability to be achieved it is not a simple principle of ‘like likes like’ that can be applied, but rather that the conformation a peptide takes in certain environments can affect its physicochemical properties. Similar observations were made by Saunders et al. (57) who explored the effect of heterocycles in place of the amide backbone which increases lipophilicity in the hopes of permeability. What they noticed was that the

permeability landscape of azole grafted macrocycles greatly depends on their 3D conformation in solution and the number of amide bonds which are exposed to solvent.

What does remain consistent however, is that increasing alkyl chain length at position 4 and the subsequent increase in hydrophobicity decreases the peptide's aqueous solubility linearly. What is also important to note is that while increasing lipophilicity in many cases can improve the permeability of the peptide, it does affect solubility. Solubility is incredibly important to drug delivery and a drug must first be soluble to be available for further action. This can also explain the high permeability values given to peptides with low solubility. For these peptides, it appears that majority of peptide is unable to dissolve in the aqueous medium and is precepting as a solid. It is the small amount of drug that can be dissolved that is then able to cross the membrane, skewing the permeability values slightly.

3.4.3. Peptide stereochemistry can influence physicochemical properties

Studies on peptides like CsA have contributed to the understanding that flexibility is an important factor for good oral delivery behaviour. Flexible structures allow the freedom for multiple conformers to be possible in both low and high polarity environments (21, 55, 58). This is advantageous as particular shapes can allow aqueous solubility and others more lipophilic solubility. Ability to be soluble in both environments is essential as drug must first be soluble in the gut lumen before adopting better lipophilic solubility to cross the gut membrane and readopt aqueous solubility in the bloodstream. In this study, D-amino acids were inserted at select stereoisomers in an otherwise identical backbone to understand the effect of peptide shape on solubility, permeability, and enzymatic stability. It was found that peptide shape can greatly impact physicochemical properties. While the methods used in this study cannot observe peptide shape or conformation in real time, these differences in physicochemical properties likely suggest that peptide analogues which demonstrate good membrane permeability are forming conformers that better suit lipid environments more easily than other analogues.

Figure 3.8 highlights the differences in measured solubility between peptides B2 and B6, two otherwise identical peptides with an L-Ala and D-Ala in position 4, respectively. Peptide B2 had a measured solubility of 103 μM in 5% DMSO in PBS and B6 had a solubility of 15 μM in the same solvent. This is an 85% reduction in solubility from this singular change. Increasing lipophilicity and the alkyl length from an Ala4 to a Nle4 residue in both cases demonstrate comparable trends to their Ala counterparts but to a lesser extent. Peptides B3 and B7 which have L-Nle and D-Nle at position 4, respectively, have a less overt reduction in solubility with a 33% reduction. This is likely due to the increase in lipophilicity intrinsically impacting solubility, limiting the solubilisation capacity of the peptides, and resulting in a smaller window to demonstrate this change in solubility. Interestingly, peptides B2 (Ala4) and B6 (D-Ala4) have similar measured permeabilities despite their solubility differences. This suggests that while peptide B2 can adopt conformations that allow better interactions with an aqueous environment, both peptide analogues can form the conformations required to permeate across the lipid membrane.

Figure 3.13 shows *in silico* modelling of peptide B2 in water and in chloroform. These images show that peptide B2, in chloroform, can form intramolecular bonds within the structure which limits the amount of hydrogen bonding interactions with the environment and may be allowing better permeability across the lipid membrane. On the other hand, peptide B2 in water does not form these intramolecular bonds, as the distance between hydrogen bond donors and acceptors are greater than 3 Å, the bond length limit of hydrogen bonds. This suggests that there are more bonds forming between the peptide and the aqueous environment as there are more available H-bonds within the structure itself. Similar observations were made by Bockus et al. (27) who found that permeability correlated well with the extent of intramolecular hydrogen bonding. This study reports that their synthesised peptide that exhibited high intramolecular bonding in low-dielectric solvent chloroform had oral bioavailability of 21% in rats. However, there are many limitations to *in silico* modelling and more specifically to conformational searching such as complexity around peptide flexibility and choosing the right parameters to run simulations. The limitations to *in silico* conformational searching play

a factor into the results shown in Figure 3.13, however in-depth discussion into the limitations to *in silico* modelling is outside the scope of this thesis.

In a similar fashion, Figure 3.8 highlights a similar difference in solubility between peptides D4 and D2. These peptides differ by a singular L-Phe to D-Phe at position 6 and have a 55% reduction in solubility, respectively. In contrast to peptides B2 and B6 however, these two peptides do not have similar measured permeabilities. In fact, these two peptides have a 100-fold difference in permeability with peptide D4 being impermeable at -8.90 cm/s and peptide D2 having a permeability value of -6.11 cm/s. According to these physicochemical properties, peptide D4 would be classified as category IV given its lack of solubility and permeability. In contrast, peptide D2 would be classified as category II as is it permeable but not well soluble. According to Albach et al. (43), peptide D2 has an *in vitro* permeability value high enough to allow for *in vivo* correlation. Once more, the same observations made between peptide D4 (D-Ala4, Phe6) and peptide D2 (D-Ala4, D-Phe6) can also be seen between the more lipophilic peptides D5 (D-Nle4, Phe6) and peptide D3 (D-Nle4, D-Phe6). Peptide D5 has a solubility value of 8.4 µM and a permeability value of -7.16 cm/s. Peptide D3 has a solubility value of 5.2 µM and a permeability value of -6.54 cm/s. Again, the solubility values between these two analogues are comparable yet their permeability values differ by approximately 5-fold. This difference is less than the observations made between peptides D4 and D2, yet the trend of a decrease in permeability between the single stereochemical remains consistent.

Both these examples demonstrate the importance of peptide shape on physicochemical properties like solubility and permeability. It is well established in the literature that membrane permeability relies on the ability to form intramolecular hydrogen bonds (24, 59). It is likely that the solubility and permeability differences between structures are due to certain shapes forming a different number of internal hydrogen bonds within the structure than the other stereoisomer. Similar observations by Bockus et al. (27) and Marelli et al. (28) also saw differences in permeability and solubility from singular changes to stereochemistry and in enantiomeric pairs. Bockus et al. (27) observed that it is the natural L-amino acid that demonstrates the higher solubilities than the D-amino acids. This observation was also seen in this study where

peptides that contain L-amino acids in the backbone rather than D-amino acids demonstrate greater solubilities. This supports the concept of conformational specific driven solubility and permeability.

Similar studies on stereochemistry have been found in the literature by Over et al. (60) who explored the *in vitro* solubility, permeability, and lipophilicity of eight stereoisomer analogues of T. cruzi growth inhibitors. In a similar fashion to the observations made here, there was a striking influence of stereochemistry on solubility and permeability. Again, studies by Schwochert et al. (39) explored naturally derived Phepropeptin C containing L-Pro with their analogue containing a D-Pro. This study found that the natural L-form showed higher cellular permeability than the D-form. However, the natural L-form of Pro exhibited similar aqueous solubility as the corresponding D-form, a phenomenon explained by solvent dependent conformational flexibility in the natural L-form.

Peptide flexibility allows a peptide to form a greater number of conformations in solution. This study shows that stereochemistry can influence peptide shape and allow the formation of internal hydrogen bonds. However, a thorough understanding of which conformation a peptide should adopt to achieve the perfect balance between solubility and permeability could allow for peptide rigidity to be advantageous. For example, naturally derived α-amanitin is a potent and bioavailable peptide that has a relatively rigid structure yet is able to be permeable using organic anion transport proteins (61). Although this absorption pathway is not passive diffusion across the lipid membrane, this lack of flexibility allows this peptide to be proteolytically stable. This shows that if a peptide can be “locked” in its bioactive, permeable, and soluble state, it has a high potential for oral delivery. This idea is supported by Neilson et al. (26) who state that conformation rigidity rather than flexibility results in higher membrane permeability, metabolic stability and oral bioavailability. This is consistent with less polar surface exposure to solvent and a reduced entropy penalty for transition between polar and nonpolar environments.

3.4.4. Peptide enantiomers can have different enzymatic resistance

The human gastrointestinal tract is designed to breakdown orally ingested food and clear waste from the body. This fundamental function hampers delivery of peptide drugs and enzymatic stability against these enzymes is important to allow the peptide's pharmacophore to remain intact for activity. In this study, the enzymes found in the small intestine and the blood plasma were tested against select peptide analogues for stability. Peptide series B, C and D were chosen for testing. Series B and C represent mirror enantiomers, and series D as the peptides in this series were highlighted as having good potential for further *in vivo* studies.

Of the three series tested for enzymatic stability, peptide series B and D were stable in simulated gastrointestinal fluid in the presence of pancreatic enzymes. The pancreatin used in this study is at 8 x the USP specifications for activity, meaning that enzyme was in excess, and the peptide was in a highly proteolytic environment. Despite this, all peptides tested within series B and D did not degrade further than 80% after 4 hours of digestion. In contrast, all the tested peptides within series C were susceptible to pancreatic degradation to varying degrees. Peptide C2 degraded the most rapidly with a half-life of 4 minutes, followed by peptides C3 and C1 with 37 mins and 145 mins, respectively. It appears that the increase in chain length at position 4 increases the time for degradation. This could be due to steric hindrance for enzyme degradation, hiding a vulnerable enzymatic hot spot of the peptide.

Peptides B2 (*cyclo*(PfwAyF)) and C2 (*cyclo*(pFWaYf)) are mirror enantiomers of each other yet peptide B2 was stable after 4 hours of digestion and peptide C2 was degraded within 4 minutes. This demonstrates how changes to peptide stereochemistry and shape can drastically affect the properties of the peptide. Bock et al. (61) discusses the importance of conformation on bioactivity and from this study it is clear that this is not exclusive to activity but also to solubility and permeability, as described earlier in this chapter but enzymatic stability as well. It is likely that the conformations formed by the peptides within series C allowed the labile features of the peptides to be more exposed to the environments and hence to the enzymes present compared to the peptides within series B. The degradation profile of peptide C2 was similar to that of the control peptide, octreotide, a peptide known to be unsuitable for oral delivery in an unaltered state (34). It appears that peptides B2 and C2 can form different conformations and peptide B2 is more likely to form a shape resistant to degradation whereas the shape peptide C2 is forming is more susceptible to degradation. Understanding how shape can influence properties is a key to unlocking oral peptide delivery.

All the peptides tested in rat plasma were stable with no degradation beyond 90% at 4 hours. This suggests that should these peptides remain intact within the GI tract and be permeable across the gut wall, these peptides would be stable in blood plasma.

Peptide B5 was a peptide analogue designed with a prodrug moiety to mask the ionisable 'Lys' residue which would cleave upon enzymatic activity. This did not prove to be the case as this peptide remains stable in both GI and plasma conditions. While a hydrophobic trifluoroacetyl group did increase the lipophilicity of the peptide, it did not significantly improve permeability and according to the paper by Goldberger et al. (62), the peptide prodrug will not readily cleave under physiological conditions.

3.5. Conclusion

Permeability and metabolic stability remain significant challenges to the oral delivery of peptides. To tackle these challenges a series of pasireotide analogues were successfully designed and synthesised using manual solid phase peptide synthesis to generate a series of lipophilic peptide analogues. These peptide analogues of pasireotide ranged in lipophilicity and stereochemistry. The peptides were tested in *in vitro* permeability and metabolic environments to observe their physicochemical properties in varying biological conditions. It was found that improvements towards permeability by way of increasing lipophilicity can have consequences on aqueous solubility. In addition, increasing lipophilicity does not guarantee improvement in membrane permeability. This study reveals that peptide conformation and shape plays an important role in both peptide permeability and metabolic stability. Single changes between stereoisomers can yield large differences in *in vitro* permeability and solubility studies. Unlocking the conformations required to achieve the properties needed for oral peptide delivery would greatly aid the understanding of what makes an oral peptide successful. Further exploration into how these shapes can have such marked differences is required, for example by studying the conformation of peptides in varying polar and non-polar environments by NMR spectroscopy.

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Chapter 4 : Effect of lipophilicity and stereochemistry on biological activity of pasireotide analogues

4.1. Introduction

Peptides are ideal drug candidates due to the specificity for their receptor targets with binding affinities that can far exceed small molecule alternatives. It is well established their flexibility can be an asset for peptide binding to their receptors by allowing interactions with biological targets with shallow or extended binding pockets (1). Peptide flexibility also allows peptides to adopt a variety of conformations in solution. However, not all these conformations will be biologically active, and the biologically active conformations may not be the major conformation adopted by the peptide (2). While small drug molecules can also bind to these complex binding pockets to elicit activity, often the potency is not comparable to using peptide drugs. For example, there are two drugs on the market that each treat hypoactive sexual desire disorder, bremelanotide (commercially sold as Vyleesi™) and flibanserin (brand name Addyi®). Bremelanotide is a peptide therapeutic with an EC_{50} value of 10 nM at MC4R receptors (3). The small drug molecular counterpart, flibanserin, has an EC_{50} value of 457 nM at 5-HT1A receptors (4). While the EC_{50} values of these two drugs cannot be directly compared due to differences in receptor type, there is still a large enough difference (50-fold) in activity between these two drugs to see that the peptide, bremelanotide, has a clear activity advantage over the small drug molecule flibanserin. Chapter 3 of this thesis has outlined the design rationale and synthesis of 27 pasireotide analogues. This chapter aims to determine the extent of bioactivity lost due to the modifications made in Chapter 3 using human somatostatin 5 receptors.

From as early as the 1970s, research into the biologically active conformation of somatostatin receptors was of interest to understand the structure-activity relationship between the peptide agonist (SRIF-14) and its receptor (2, 5). Knowledge of the biologically active conformations of somatostatin and SRIF-14 would allow the preorganisation of the peptide shape to promote better affinity in the active form. Early research into the biological motif necessary for SRIF-14 activity was performed by Arison et al. (2) who used NMR spectroscopy to determine whether the type II β-turn between Phe7-Trp8-Lys9-Thr10 residues in SRIF-14 would be beneficial to biological activity. This proved to be the case and as a result the natural Trp7 amino acid from somatostatin was replaced with its D-stereoisomer to stabilise this β-turn. The simple change in Trp amino acid stereoisomer from L- to D- energetically stabilises the 'U' shape adopted by the peptide with the β-turn. In addition to the change in the Trp residue, research has revealed the importance of the Lys8 residue for activity. Removal of the Lys8 or replacement with different amino acids rendered the peptide inactive (6, 7). As such, all current somatostatin analogues including octreotide, lanreotide and pasireotide, contain this D-Trp-Lys motif for activity. More recently it has been shown that the SSTR subfamily of receptors (and SSTR2 in particular) shows plasticity and kinetic effects in recognition of different ligands (8).

Studies into the utility of the amino acids outside of the pharmacophore have also been explored. The Phe6 and Phe11 residues, highlighted in blue (Figure 4.1), stabilise the biologically active conformer through hydrophobic bonding but do not interact themselves with the receptor (9, 10). Veber et al. (10) found that replacement of Phe6 and Phe 11 on SRIF-14 with Cys resides resulted in no loss of activity. Further studies by Vale et al. (11) found that replacement of these Phe residues with Ala resulted in a substantial loss of activity. This was attributed to the peptide being unable to remain in its active conformer in the absence of the Phe residues.

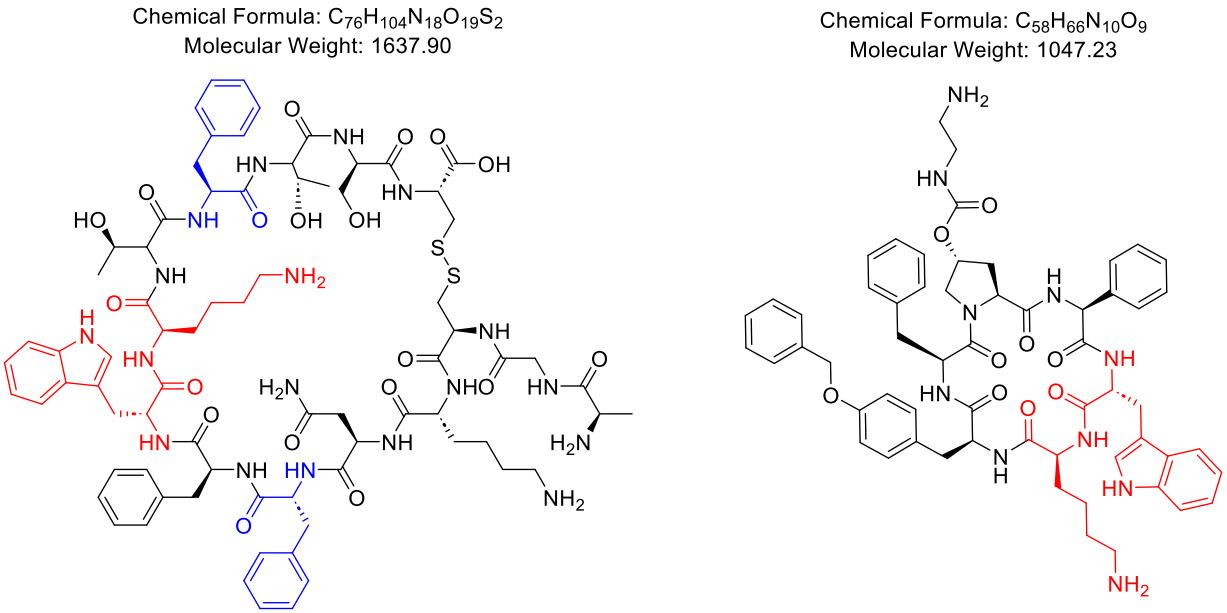


Figure 4.1 Chemical structures, formulas, and molecular weights of endogenous somatostatin (SRIF-14) (left) and pasireotide (right). The pharmacophore is highlighted in red (Trp-Lys). Stabilising Phe residues are highlighted in blue.

In 2022, cryo-EM structures of SRIF-14 and octreotide bound to hSSTR2 provided further insights into the biologically active conformations of ligand when bound to hSSTR2 (Figure 4.2) (8). This study found that for both SRIF-14 and octreotide agonists, the Trp residue buries into hydrophobic pockets of the receptor, breaking a hydrogen bond between glutamine (Q126) and tyrosine (Y273) residues within the binding pocket (denoted by pink residues in Figure 4.2). The Lys residues in both SRIF-14 and octreotide were confirmed to form a salt bridge with the Asp (D122) reside within the binding pocket. This is not unexpected as amine interactions with Asp residues within the binding pockets are essential in aminergic GPCRs, and this crystal structure further supports this observation (12). Given all somatostatin analogues contain the structural motif of Trp-Lys, these interactions may also occur in other peptide analogues.

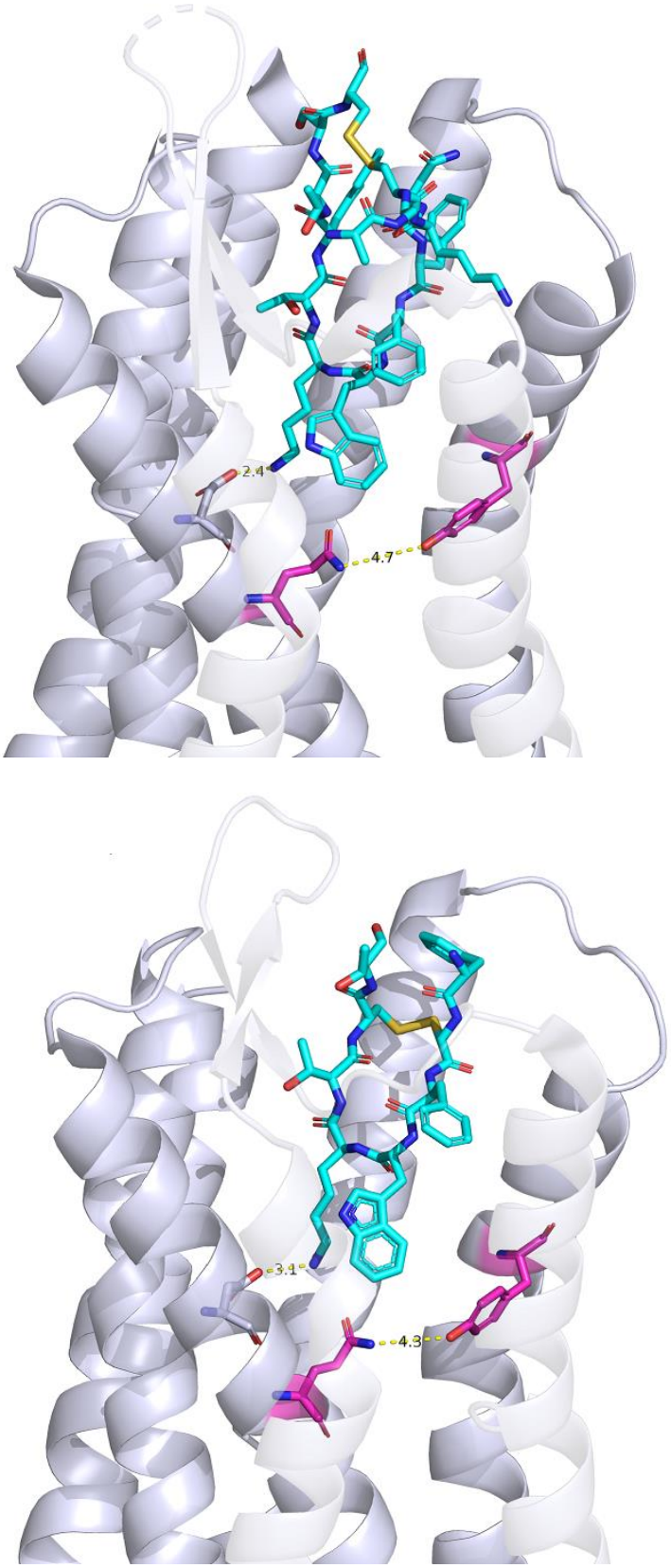


Figure 4.2 Cryo-EM structure of SRIF-14 (top) and octreotide (bottom) bound to hSSTR2 receptor. Adapted from Robertson et al. (8). The hSSTR2 receptor is shown in white represented by ribbon form with key interacting moieties represented in stick form. Peptides are in sticks representation. Carbon - cyan, Nitrogen - dark blue, oxygen - red, sulfur - yellow, hydrogens atoms are not shown. Dashed lines indicate interaction length in Å2.

Pasireotide (Figure 4.1) was designed in 2002 by Brun et al. (13) as a potent somatostatin analogue for hSSTR2 and hSSTR5 receptors. It differs from alternative somatostatin analogues by being particularly potent at hSSTR5 with 100-fold increased potency compared to lanreotide and a 40-fold increase in potency compared to octreotide (13). Pasireotide retains the established pharmacophore of 'D-Trp-Lys' and is only six amino acids long, rather than the 14 or 28 amino acid length of endogenous somatostatin peptides (14-16). This peptide contains a Pro1 residue that introduces conformational restraint by eliminating rotation about the carbon-nitrogen bond in the five membered ring (17). The structure also contains three benzene rings: phenylglycine in position 2, tyrosine-benzyl group in position 5 and phenylalanine in position 6. By targeting hSSTR receptors, a class of inhibitory receptors, pasireotide supresses cAMP cell production and downstream signalling to inhibit the secretion of hormones (18). Pasireotide is the first drug to be approved for the treatment of Cushing's disease, a rare disorder that impacts circulating levels of cortisol due to a corticotropin-secreting pituitary adenoma (19).

The overarching aim of this thesis is to move closer towards oral peptide delivery. Peptide analogues of pasireotide were designed and synthesised in Chapter 3 where we showed the effect of peptide conformation on *in vitro* permeability and its subsequent *in vitro* enzymatic stability. The literature reports of several lipophilic peptides that have been tested *in vivo* for oral bioavailability with some success (20-22). However, many of the peptide constructs in the literature that report good oral bioavailability are not bioactive peptides. While non bioactive orally bioavailable peptides are useful for furthering the understanding of factors that can influence the permeability of peptides, these peptides are not pharmacologically beneficial and therefore lack correlation to market and wider applications. The parent peptide of this thesis, pasireotide, is a bioactive peptide. Therefore, any activity lost due to modification of pasireotide can be measured. The pasireotide analogues chosen for this chapter represent two hypotheses, the first being that the increase in peptide lipophilicity at amino acids outside the pharmacophore can affect the bioactivity of the peptide. The second is that the loss of activity by modifying the pharmacophore in the pursuit of permeability can be measured. The specific aims of this chapter are to firstly develop and optimise a robust *in vitro*

activity assay, and secondly, to measure the IC_{50} values of select synthesised peptides for *in vitro* biological activity.

4.2. Methods

Two *in vitro* biological assays were developed to determine the IC_{50} values of synthesised pasireotide analogues; the cAMP assay (using Lance® Ultra cAMP kit) and a BRET assay. Two human embryonic kidney (HEK293) cell lines expressing hSSTR2 and hSSTR5 receptors, respectively, were transfected using DNA sequences obtained from collaborators at the Monash Institute of Pharmaceutical Sciences, both expression plasmids used a hygromycin resistance gene. These cell lines were chosen as the control peptides (pasireotide and octreotide) have different affinity for each receptor. Cells were grown under antibiotic selection pressure using 200 µg/mL hygromycin B. CHO-k1 cells expressing hSSTR2 and hSSTR5 receptors were transfected through the Flp-In system using lipofectamine LTX. Stably transfected populations were maintained with hygromycin B media supplementation at 800 µg/mL. Both cell types were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 1% glutamax and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humified environment at 37 °C/5% CO_2. Detailed methods used in this study can be found in Chapter 2 (page). The following sections briefly describe the methods that pertain to this experimental chapter.

4.2.1. Method A: cAMP-based Assay

Flp-In HEK293 cells or Flp-In CHO-k1 cells were seeded (25,000 cells/well) into a 96-well cell culture plate and incubated overnight in the DMEM medium at 37°C/5% CO_2. All incubation periods are performed at 37°C/5% CO_2. Growth media was replaced with stimulation buffer (80 µL) containing Hank's Balanced Salt Solution, 5 mM HEPES, 0.1% bovine serum albumin and 20 µM rolipram. Cells are incubated for 30 minutes before cells are treated with 10 µL of peptide ligand. Upon stimulation, cells were incubated for a further 10 minutes. Following this, 10 µL of 10 µM forskolin was added and further incubated for 20 minutes. After this incubation period, media was removed and 50 µL of ice-cold ethanol was added to each well to halt any further reactions. The ethanol was evaporated at room temperature overnight. The following day 200 µL of lysis buffer was added and shaken at 500 rpm for 10 mins.

The lysate (5 µL) was transferred to a 384-well optiplate. The amount of cAMP produced in each sample was determined using the Lance® Ultra cAMP Detection Kit. Briefly,10 µL of the Eu-cAMP tracer solution (1:200 dilution of the Eu-cAMP tracer stock in detection buffer) and 5 µL of U-light anti-cAMP solution (17:5000 dilution of U-light anti-cAMP stock in detection buffer)was added into each well containing the lysate in reduced light conditions, and the plate was left to incubate at room temperature for 1 hr. TRF (time-resolved fluorescence) for the samples was detected using an Envision Plate reader with excitation at 320 nm and emission at 615 nm. All data were analysed using GraphPad Prism 9. IC_{50} values represent the mean of the experiment performed in triplicate. IC_{50} estimates the half maximal inhibitory concentration, and its associated standard errors were determined by fitting the data using a non-linear regression analysis.

4.2.2. Method B: BRET-based Assay

Flp-In HEK293 cells were seeded (500,000 cells/well) into a clear 6-well cell culture plate and incubated overnight in the DMEM medium at 37°C/5% CO_2. All incubation periods are performed at 37°C/5% CO_2. The following day, cells are transiently transfected with 250 ng/well of the BRET biosensor DNA. For transfections, a ratio of 1 ng:2 µL DNA:Lipofectamine Messenger MAX was used. DNA and Lipofectamine were diluted in Opti-MEM (each in 250 µL/well of a 6 well plate) and incubated at room temperature for 5 minutes. DNA and Lipofectamine Messenger MAX dilutions were mixed and incubated for 20 minutes. Mixtures were then added to cells and left in an incubator (37°C/5% CO_2) overnight.

The following day, between 24-26 hours after transfection, cells were washed using DPBS and resuspended in phenol red-free DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, and 25 mM HEPES (complete PRF-DMEM). Cells were plated in white opaque 96-well microplates at 40,000 cells/well. PRF-DMEM was aspirated from each well and replaced with 90 µL of assay solution (ratio of 1 µL Nano-Glo® Luciferase Assay Substrate (Promega):450 µL PRF-DMEM). Plates were equilibrated at 37°C for 10-15 minutes in a PHERAstar® FSX microplate reader. After restarting the reader for ~3 minutes, the reader was paused, plate ejected, and 10 µL of 10x peptide dilutions were manually dispensed before reads were allowed to continue to completion.

4.3. Results

To measure the *in vitro* activity of the pasireotide analogues, two cell-based activity assays were developed and optimised. Each assay measures a different biological response to ligand binding. The cAMP activity assay measures cell generated cAMP upon ligand binding. The BRET activity assay measures G-protein dissociation upon ligand binding.

4.3.1. cAMP Activity Assay Optimisation

The cAMP assay was developed using the Lance Ultra cAMP kit as an *in vitro* activity assay for stably transfected cells expressing the receptor of interest. The cAMP assay uses a competition assay to measure the amount of cAMP generated by the cells. hSSTR receptors are G_i bound and therefore supress the production of cAMP when its agonist binds. This triggers a downstream reaction that blocks the adenylate cyclase in cells from producing cAMP. To measure the extent of cAMP that has been supressed, an adenylate cyclase stimulator, forskolin, is added to restimulate the production of cAMP. This process relies on first the knockdown of cAMP and then the re-stimulation of adenylate cyclase to calculate the potency of the peptide's inhibition.

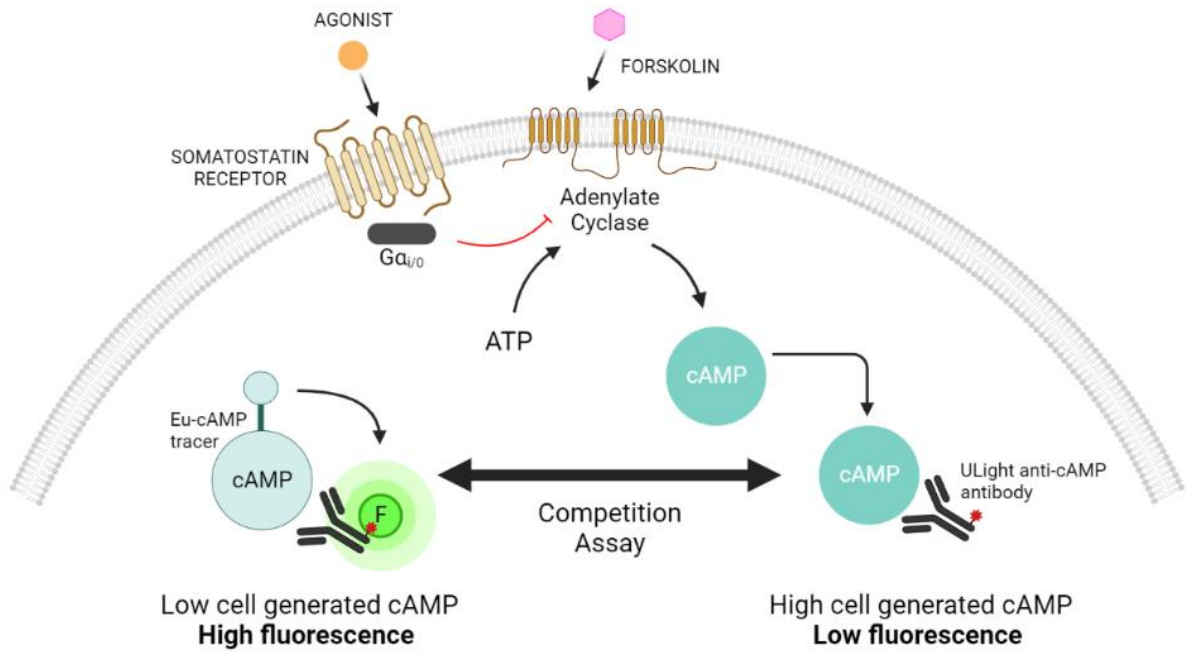


Figure 4.3 Lance Ultra cAMP assay. Image was created in BioRender.com.

To validate the functionality of the Lance Ultra cAMP kit, the adenylate cyclase stimulant forskolin was used to generate a dose-response curve. Forskolin generated consistent concentration curves for HEK293 cell lines each expressing hSSTR2 and hSSTR5 receptors, respectively, as shown in Figure 4.4. From these curves we found that a concentration of 10 μM of forskolin reduced the fluorescence by 90% and hence that concentration was used for subsequent experiments.

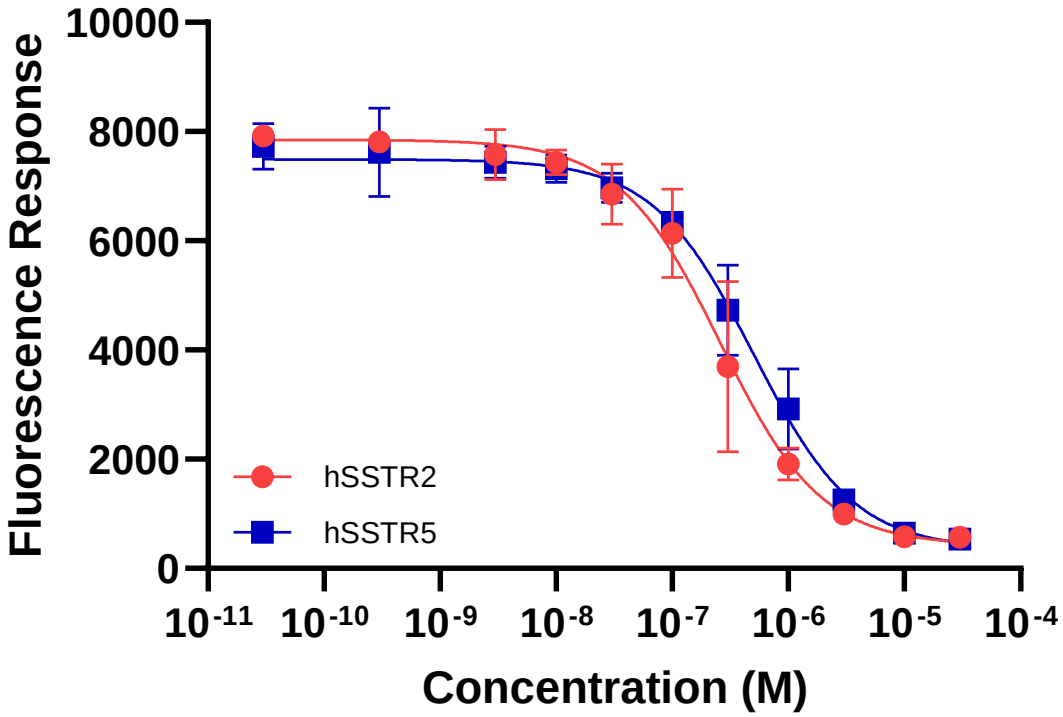
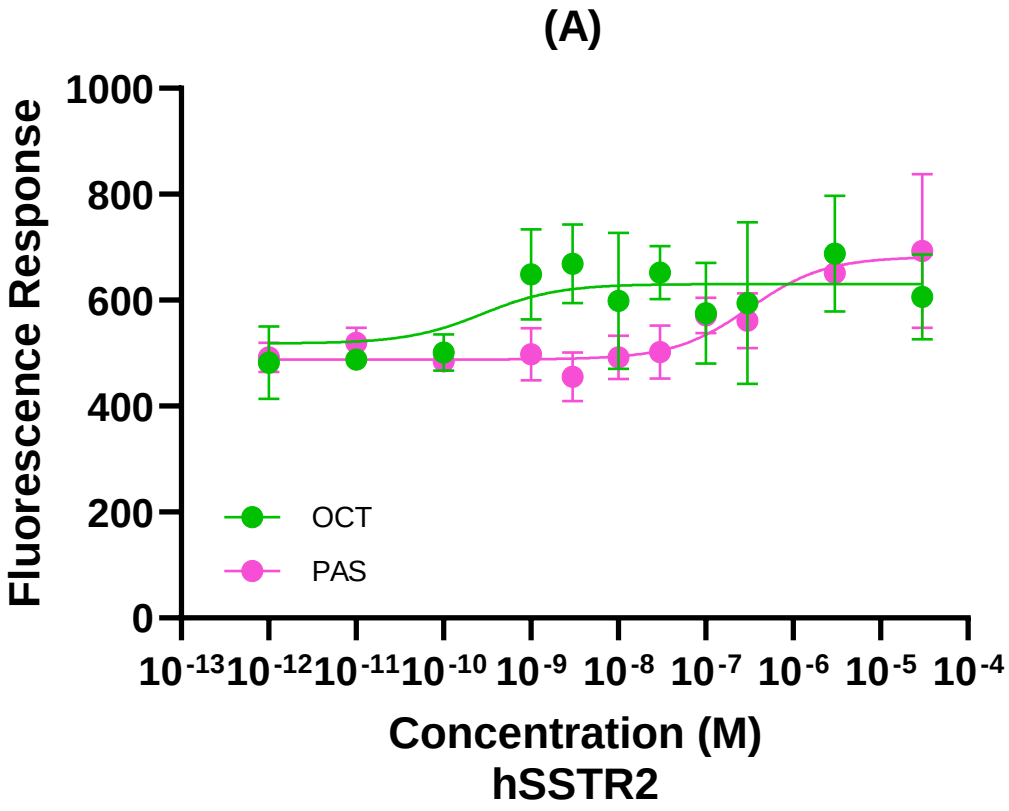


Figure 4.4 Dose-response curve of forskolin using stably transfected HEK293 cells expressing hSSTR2 (red) and hSSTR5 (blue). Data was obtained using LANCE® Ultra cAMP activity assay and expressed as mean ± SD, n=3.

Preliminary trials of commercial peptides pasireotide and octreotide under these initial conditions did not yield dose response curves with consistent dynamic ranges (Figure 4.5). This could be due to many factors including the range of the doses used, cell density, stimulation buffer type, phosphodiesterase inhibitor type and concentration, stimulation time, ligand binding time and forskolin concentration. A table summarising the different optimisation strategies can be found in the Appendix F (page 168).

Despite the significant time spent to develop a robust and consistent assay using the Lance® Ultra cAMP kit, no consistency could be obtained. In fact, when promising results were seen in one trial, the subsequent trials under the same conditions would not yield comparable results. An example of this is shown in Figure 4.6. The two trials depicted in this figure are both using HEK293 cells stably expressing hSSTR2 receptors with octreotide as the peptide agonist. The cell density was 35,000/well with stimulation buffer, HBSS, and phosphodiesterase inhibitor, rolipram. The stimulation times were identical at 30 mins, followed by 10 mins of peptide activation and 20 mins with forskolin. The only differing factor between these two trials was the cell passage number, the second assay being run two passages after the first.


(A)
Fluorescence Response
1000
800
600
400
200
0
OCT
PAS
10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5 10-4
Concentration (M)
hSSTR2

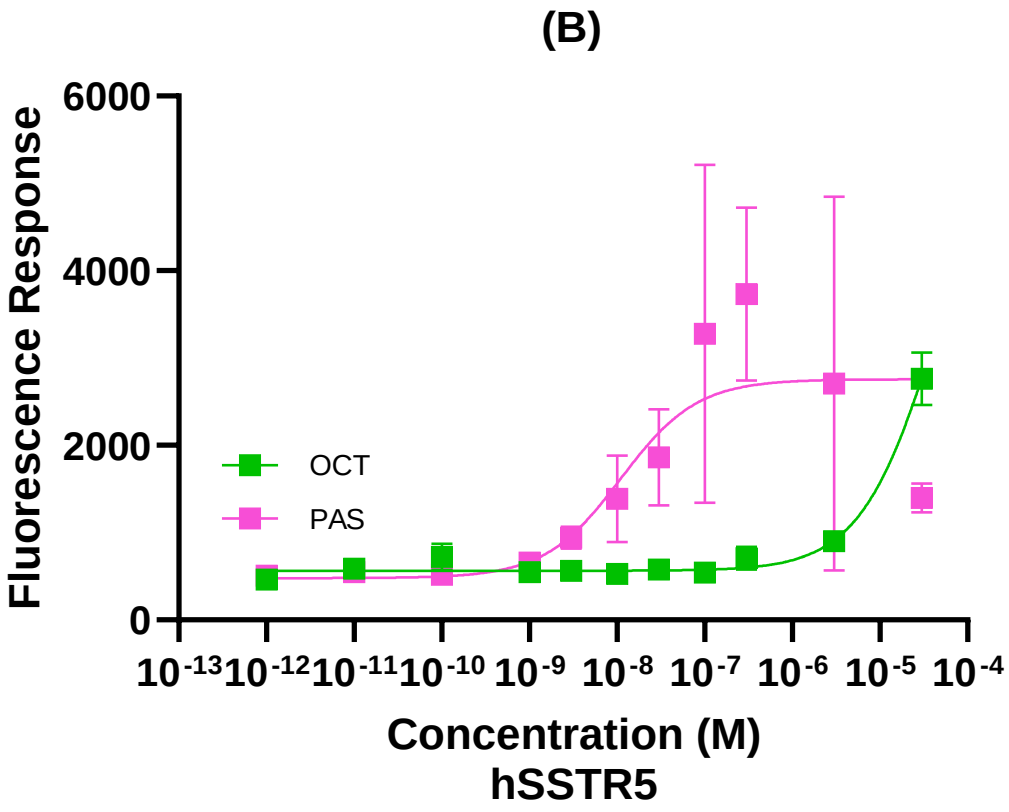

(B)
Fluorescence Response
6000
4000
2000
0
OCT
PAS
10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5 10-4
Concentration (M)
hSSTR5

Figure 4.5 Dose-response curves from LANCE® Ultra cAMP activity assay for OCT (green) and PAS (pink) using stably transfected HEK293 cells expressing hSSTR2 (A) and hSSTR5 (B). Data is expressed as mean ± SD, n=3.

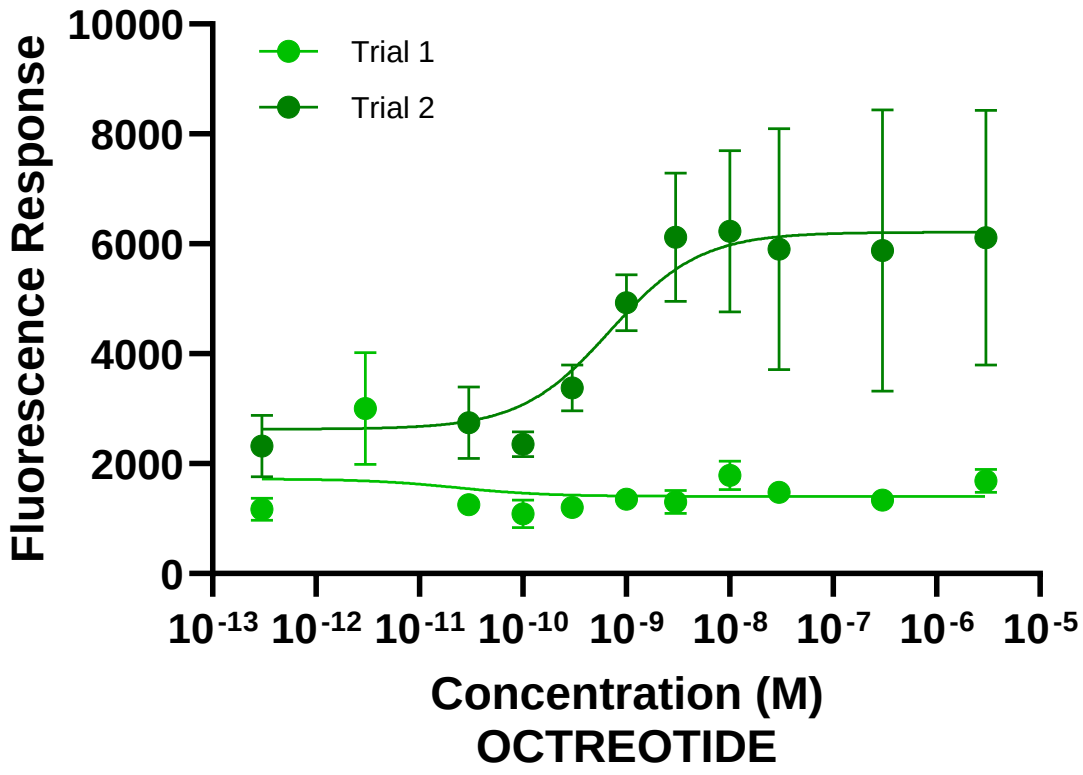


Figure 4.6 Dose-response curves of OCT (green) across two trials under identical conditions using the LANCE® Ultra cAMP activity assay. Both trials use stably transfected HEK293 cells expressing hSSTR2. Data is expressed as mean ± SD, n=3.

A possible explanation for this behaviour was that the receptor expression on the HEK293 cells were too low, providing variable signal strength and contributing to the high variability across replicates. Therefore, CHO-k1 cells were transfected with identical Flp-In vectors to yield higher receptor expression. Both receptor types and cell types were trialled using octreotide as the commercial agonist. Once more, the concentration responses demonstrated little dynamic range between peptide concentrations and high signal to noise ratios, irrespective of receptor type or cell type (Figure 4.7). The stimulation of adenylate cyclase using forskolin is an independent process from the binding of ligand to receptor. Given the forskolin concentration curves were consistent across both cell lines and all receptor types, this suggested that there was unlikely to be an issue with the cells themselves but rather the DNA sequences that were coding for the receptors. The DNA vectors were sent for sequencing, and it was found that both receptor types had key issues with their sequences (Figure 4.8).

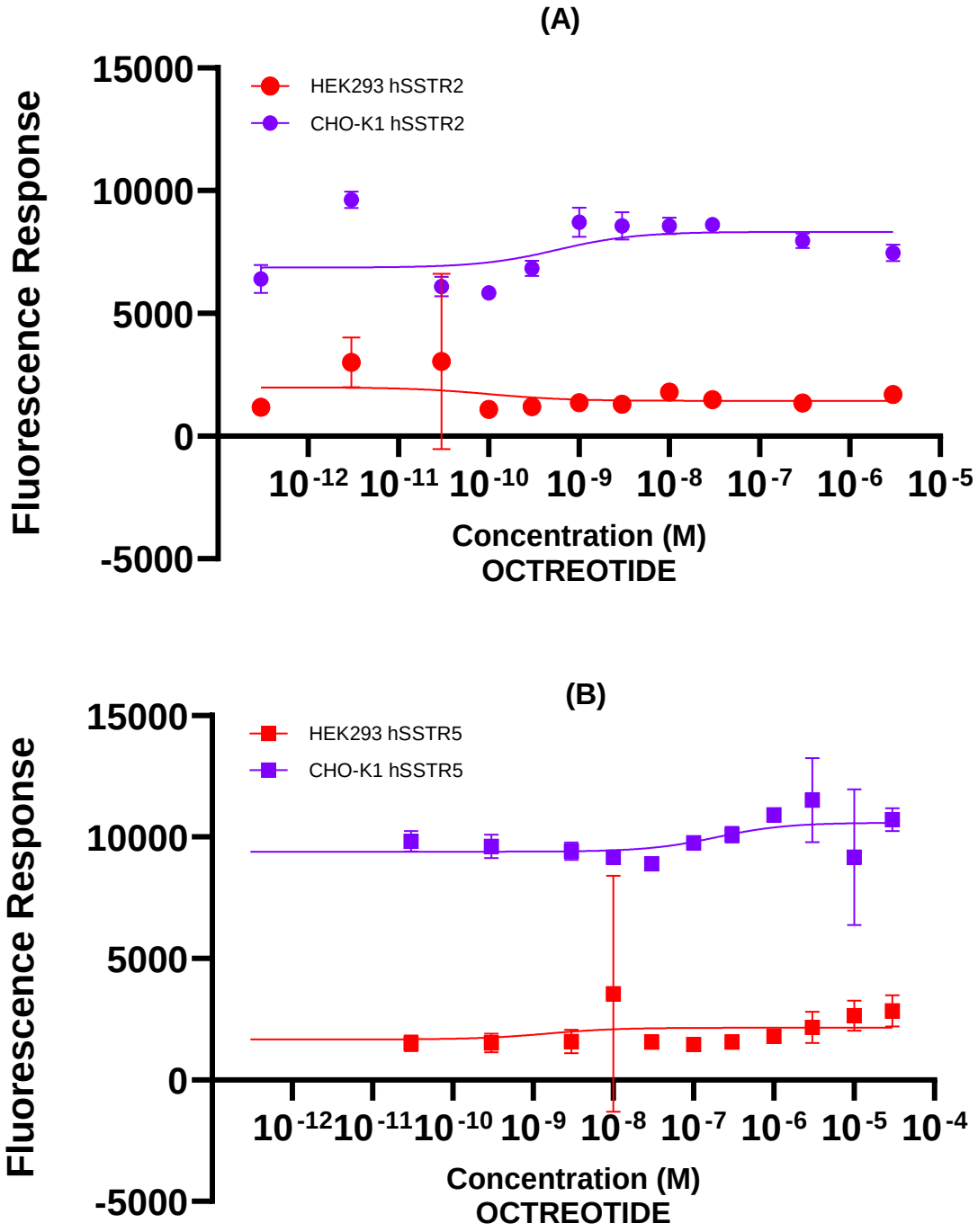


Figure 4.7 Comparison of dose-response curves obtained from LANCE® Ultra cAMP activity assay of OCT under two conditions. Graph A depicts the dose-response curves of OCT on stably transfected CHO-k1 cells (purple) and HEK293 cells (red) each expressing hSSTR2 receptors. Graph B depicts the dose-response curves of OCT on stably transfected CHO-k1 cells (purple) and HEK293 cells (red) each expressing hSSTR5 receptors. Data is expressed as mean ± SD, n=3.

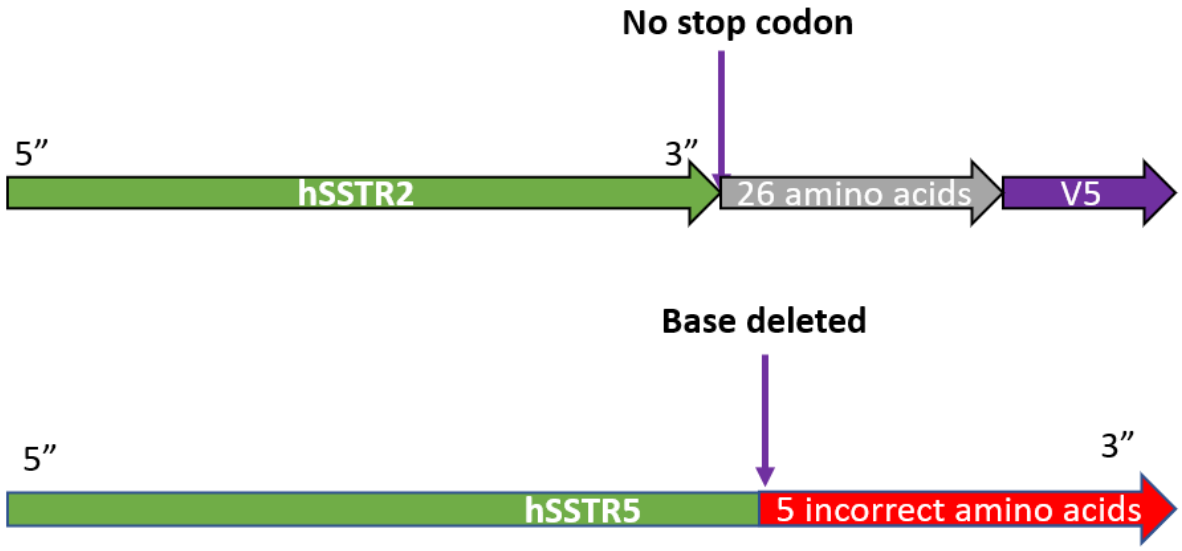


Figure 4.8 Gene sequence of hSSTR2 (top) and hSSTR5 (bottom). Correct sequence is coloured green, incorrect sequence is coloured red, additional amino acids are coloured grey and V5 tag is coloured purple.

The gene sequencing results of the hSSTR plasmids for both receptors showed that the hSSTR2 plasmids did not have a stop codon at the 3-prime end, but rather continued to code for 26 random amino acids before ending in a V5 tag. The hSSTR5 plasmids were more problematic as there was a base deletion resulting in the formation of five incorrect amino acids at the end of the sequence. One of these amino acids was a Cys residue which may form disulfide bonds with other areas of the receptors resulting in incorrect folding. These critical errors in the gene sequences meant that receptor function was dependent on tertiary structure and unreliable for ligand binding assays. In addition, as the cells had other means of cAMP production that were not mediated by hSSTR2/5 binding, the variable data may have been due to other pathways of cAMP production. Therefore, all cells were discarded and new validated HEK293 cells stably expressing hSSTR5 purchased from Australian Biosearch (Wangara, WA, AUS).

Following the results from the receptor gene sequencing, this assay was not continued due to the additional complexity. The cAMP assay remains a robust and reliable assay for EC_{50} measurements, however in the case of G_i receptors and IC_{50} measurements, the additional complexity for G_i receptors can lead to high variability between replicate trials and less reproducibility.

4.3.2. BRET Activity Assay Optimization

Following the purchase of new cells that have been validated for hSSTR activity, an alternative *in vitro* activity assay was developed that would be less susceptible to downstream cAMP production. The cAMP assay is additionally complex for G_i protein receptors as they require multiple inhibition and stimulations cycles to accurately measure cAMP suppression from peptide binding. This BRET-based activity assay is less variable compared to the cAMP assay as it is not affected by alternative productions of cAMP outside of ligand binding.

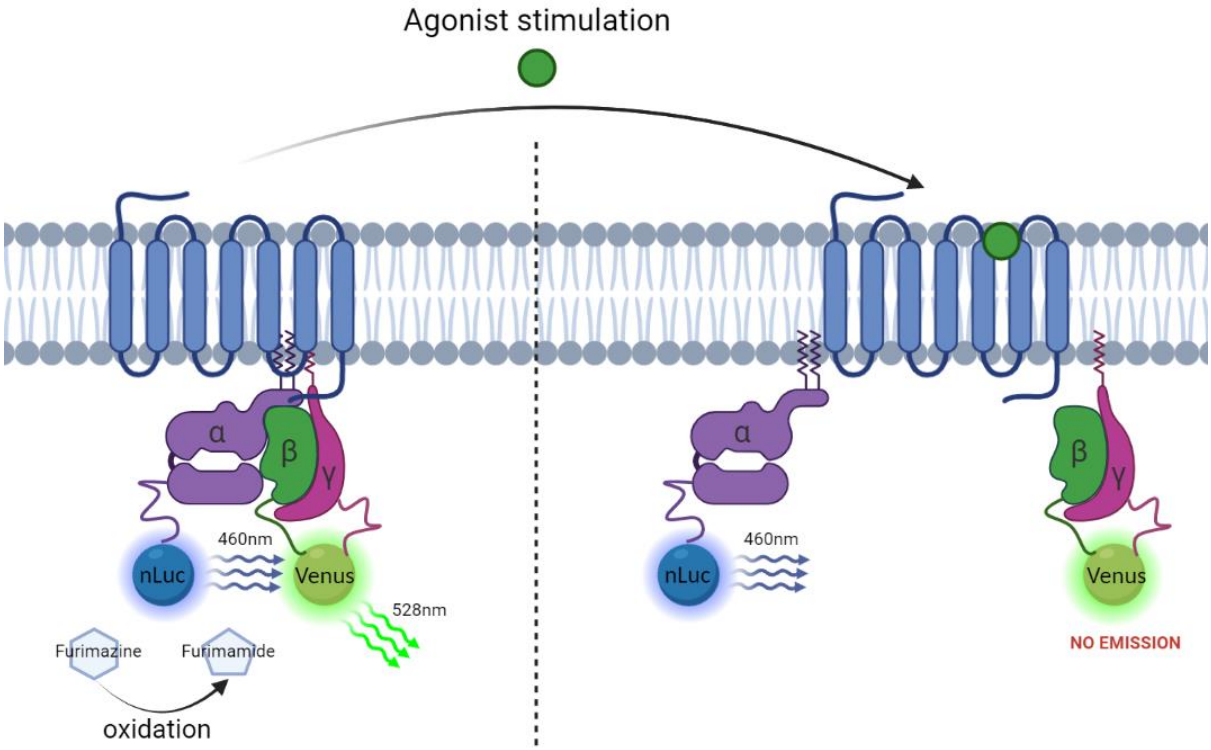


Figure 4.9 BRET activity assay. Image was made in BioRender.com.

The BRET-based assay measures the potency of the peptide based on binding to the hSSTR receptors (Figure 4.9). For this assay, cells are transiently transfected with DNA that codes for fluorescently tagged G-proteins (nLuc on the α-subunit and Venus on the β- and γ-subunits). In the presence of furimazine, nLuc fluoresces at 460nm. When the Gα- and Gβ/γ-subunits are in proximity (i.e. no ligand binding), Venus in turn fluoresces at 528nm. Upon ligand binding, the Gα and Gβγ subunits dissociate which causes the Venus to no longer fluoresce due to the loss of proximity. The fluorescence ratio between nLuc and Venus is measured and converted into a dose-response curve.

As with previous optimisation attempts, commercial analogues octreotide and pasireotide were used to establish robust and reproducible dose-response curves that would generate comparable IC_{50} values (Figure 4.10).

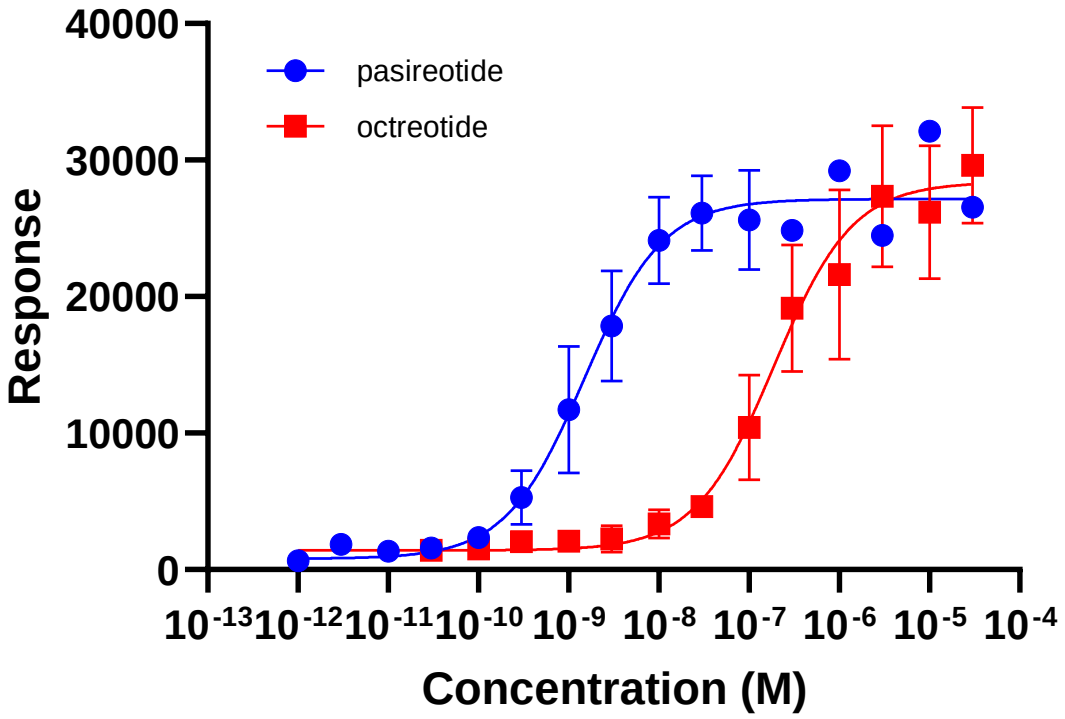


Figure 4.10 Dose-response curves of pasireotide (blue) and octreotide (red) produced using BRET activity assay on HEK293 cells expressing hSSTR5 receptors. Results are expressed as mean ± SD, n=3.

Table 4.1 shows a comparison between literature reported IC_{50} values and assay measured IC_{50} values for commercial pasireotide and octreotide. Literature values were obtained from a study by Bruns et al. (13) which used a competition binding experiment using CHO (COS) cell membranes expressing the hSSTR subtypes and Tyr_{11} [I^{125}] SRIF-14 as the SRIF receptor specific ligand. In contrast, this study's *in vitro* activity values were obtained using HEK293 cells expressing hSSTR5 receptors in a BRET-based binding assay. The values measured in the BRET binding assay were 10-fold higher than that reported by Bruns et al. for pasireotide and 30-fold higher for octreotide (Table 4.1). A difference in IC_{50} values is not unexpected as activity values can shift based on the conditions of the assay (e.g. cell type, measurement methodology etc.). Importantly, our data shows that octreotide has a lower affinity for hSSTR5 as expected.

Table 4.1 Comparison of measured IC_{50} values using BRET assay against literature reported IC_{50} values for commercial pasireotide and octreotide. Literature values were obtained using CHO (COS) cell membranes (13). Measured values were obtained using HEK293 cells and measured in n=3 and reported as mean ± SD.

Pasireotide analogues	**Literature IC_{50} (13) (nM)**	**Measured IC_{50} (nM)**
Pasireotide	0.16 ± 0.01	1.5 ± 0.7
Octreotide	6.3 ± 1.0	203.6 ± 76.6

4.3.3. BRET Activity Assay Results

A series of 27 peptides were designed and synthesised as described in Chapter 3. Among those peptides, a selection of analogues was chosen for further *in vitro* activity studies. A list of the selected peptides is shown in Table 4.2 along with the measured IC_{50} values using the developed BRET-based activity assay.

Table 4.2 Measured IC_{50} values of synthesised pasireotide analogues using BRET-based activity assay. IC_{50} values are reported in nM and performed in triplicate, n=3. Values are expressed as mean ± S.

Pasireotide analogues	**Structure**							**IC_{50} (nM)**
Pasireotide	***cyclo[***	**Hyp**	**Phg**	**D-Trp**	**Lys**	**Tyr(Bzl)**	**Phe]**	1.5 ± 0.7
A1	*cyclo[*	**Pro**	Phe	D-Trp	Lys	**Tyr**	Phe]	646.7 ± 280.0
A2	*cyclo[*	Pro	Phe	D-Trp	Lys	**Tyr(Bzl)**	Phe]	35.7 ± 9.6
A3	*cyclo[*	Pro	Phe	D-Trp	Lys	**Thr**	Phe]	1114 ± 149.9
A4	*cyclo[*	Pro	Phe	D-Trp	Lys	**Val**	Phe]	1127 ± 219.9
A5	*cyclo[*	Pro	**Leu**	D-Trp	Lys	**Leu**	**D-Leu]**	-
A6	*cyclo[*	Pro	Phe	D-Trp	**Gly(2-pent)**	Tyr	Phe]	-
B1	*cyclo[*	Pro	**D-Phe**	D-Trp	Lys	**D-Tyr**	Phe]	2269 ± 819.1
B2	*cyclo[*	Pro	D-Phe	D-Trp	**Ala**	D-Tyr	Phe]	-
B4	*cyclo[*	Pro	D-Phe	D-Trp	**Gly(2-pent)**	D-Tyr	Phe]	-
C1	*cyclo[*	**D-Pro**	Phe	**Trp**	**D-Lys**	Tyr	**D-Phe**]	1781 ± 168.3
C2	*cyclo[*	D-Pro	Phe	Trp	**D-Ala**	Tyr	D-Phe]	-
D1	*cyclo[*	Pro	Phe	D-Trp	**D-Lys**	Tyr	**D-Phe**]	4577 ± 553.5
D2	*cyclo[*	Pro	Phe	D-Trp	**D-Ala**	Tyr	D-Phe]	-
E1	*cyclo[*	**D-Pro**	**D-Phe**	**Trp**	Lys	**D-Tyr**	Phe]	3063 ± 1027
E2	*cyclo[*	D-Pro	D-Phe	Trp	**Ala**	D-Tyr	Phe]	-
F1	*cyclo[*	Pro	Phe	D-Trp	**D-Lys**	**D-Tyr**	**D-Phe]**	156.6 ± 56.5
F2	*cyclo[*	Pro	Phe	D-Trp	**D-Ala**	D-Tyr	D-Phe]	-

Pasireotide contains a hydroxyproline residue in position 1, a phenylglycine residue in position 2 and a benzyl group on the tyrosine at position 5. This peptide measured an IC_{50} value of 1.5 nM at the SSTR5 receptor. The reported IC_{50} of pasireotide is 0.16 nM (13) resulting in a 10-fold decrease in activity. This variation is not unexpected, and the reproducibility of the result suggests that the assay is robust and repeatable. As all subsequent analogues are compared to that of the commercial pasireotide using our BRET-assay, the difference between the literature value and the measured value is worth nothing but does not impact the stratification of peptide activity relative to the commercial peptide.

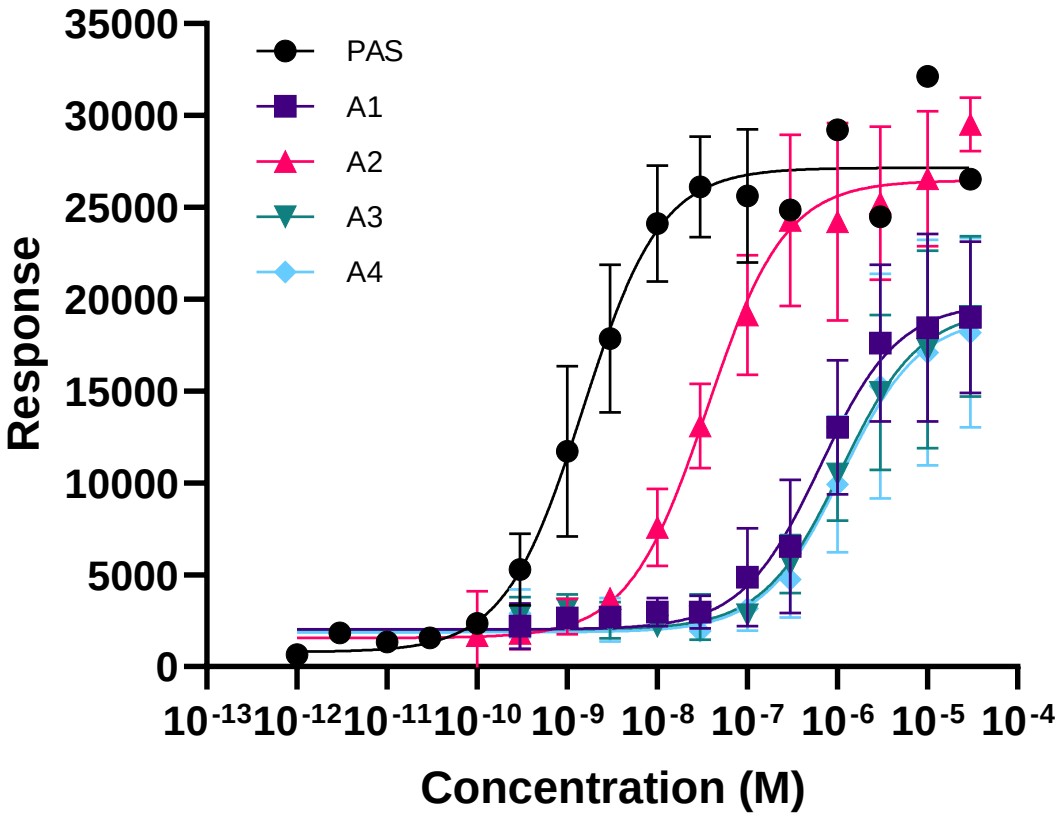


Figure 4.11 Dose-response curves obtained from BRET activity assay for PAS and each pasireotide analogue within Series A on stably transfected HEK293 cells expressing hSSTR5. Data is collected at n=3 and expressed as mean ± SD.

Series A contains the pasireotide pharmacophore 'D-Trp-Lys'. Peptide A1 is simplified by exchanging the unnatural amino acids with their canonical equivalents. This is done by replacing Hyp with Pro in position 1, Phg with Phe in position 2, and Tyr(Bzl) with Tyr in position 5. These changes caused a 425-fold decrease in activity (Figure 4.11). Peptide A2 reintroduces the Bzl group onto the Tyr residue and this change reintroduces activity from an IC_{50} of 690 nM to 34 nM. A2 is still 21-times less potent than commercial pasireotide. Analogue A3 is reported by Veber et al. (23) to be a potent cyclic hexapeptide analogue for somatostatin. Named compound 8 in the original paper, this peptide is structurally similar to simplified pasireotide (peptide A1) with the replacement of Tyr in position 5 with a Thr residue (23). Although reported in the literature as a potent analogue, this peptide is less active compared to peptide A1 at hSSTR5. In compound A4 the Thr5 residue is replaced with a more lipophilic Val5 residue. This peptide moves closer towards improving permeability by increasing lipophilicity. This change had little effect on activity with negligible changes in IC_{50} values, indicating this is a promising candidate for further study. Analogue A5 is derived from the structure of natural Phepropeptin A, a type of cyclic hexapeptide that were isolated from *Streptomyces* during a search for proteasome inhibitors (24). Peptide A5 uses the structure of Phepropeptin A (*cyclo*[Pro-Leu-D-Leu-Val-Leu-D-Phe]) and introduces the pharmacophore of pasireotide, D-Trp-Lys, into the backbone at positions 3 and 4, with an additional Leu in position 6 (*cyclo*[Pro-Leu-D-Trp-Lys-Leu-D-Leu]). Despite the presence of the pharmacophore of pasireotide, this peptide was not active *in vitro* at hSSTR5. Peptide A6 is the only peptide in this series that has an altered pharmacophore of pasireotide by replacing the Lys residue with a Gly(2-pent) residue. Gly(2-pent) contains a single alkyl chain at the side chain, the same alkyl length as Lys. This change was based on a study by Nutt et al. (6) which explored the importance of Lys on the activity of somatostatin and found that there was significant loss of activity when Lys was replaced with alternative positively charged side chains like ornithine, arginine, histidine, and ρ-amino phenylalanine. This paper suggests that it is not merely the basic nature of Lys that contributes to the activity of somatostatin and alludes to the idea that steric interactions could be at play here. We found that, peptide A6 was not biologically active at hSSTR5.

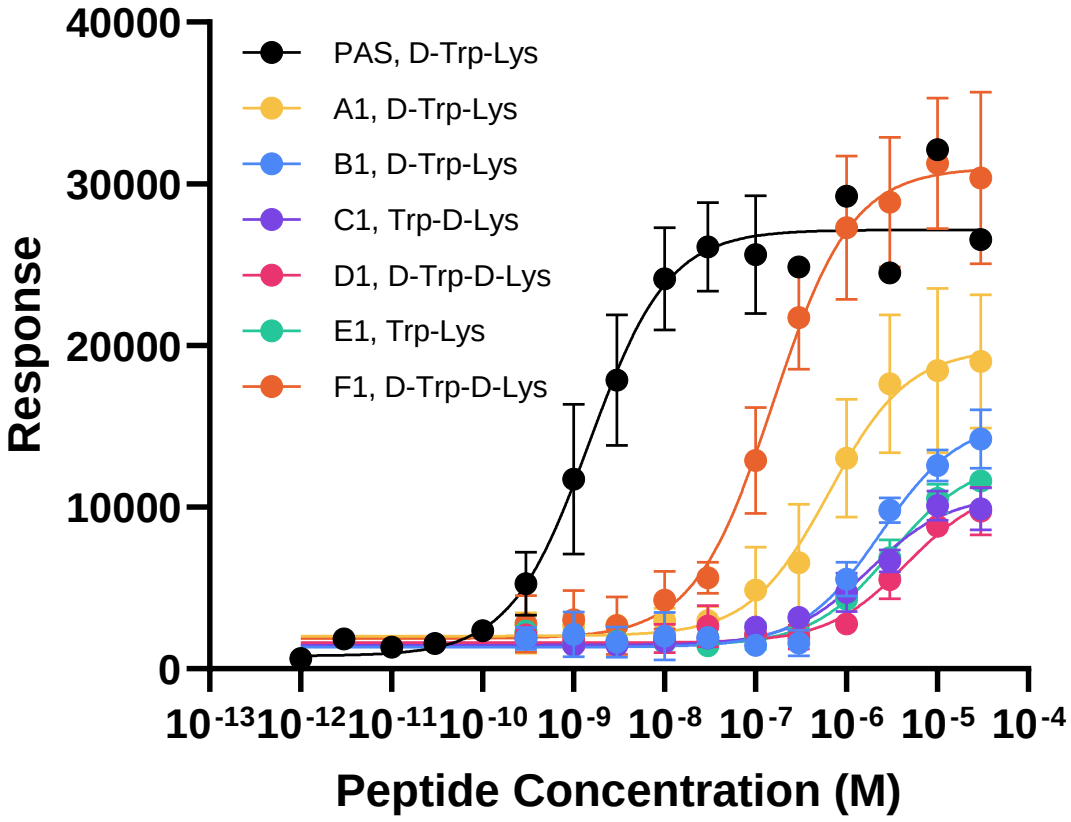


Figure 4.12 Dose-response curves obtained from BRET activity assay for PAS, A1, B1, C1, D1, E1 and F1 on stably transfected HEK293 cells expressing hSSTR5. Data is collected at n=3 and expressed as mean ± SD.

All peptide analogues containing an Ala in position 4 were not active. This further supports the idea that the presence of Lys is essential for somatostatin receptor activation. Figure 4.12 shows the *in vitro* activity of all permeable backbones with the Lys residue reintroduced in position 4. These peptides have differing stereochemical variations of Trp-Lys. Peptides B1, C2, D1 and E1 all had similar IC_{50} ranging from 1781 nM to 4577 nM. Comparatively, peptide A1 had slightly better potency with 646.7 nM. Interestingly, F1 had the most potent IC_{50} value with 156.6 nM.

4.4. Discussion

Balancing solubility and permeability are key determinants of oral absorption in the gut. However structural changes to a peptide to alter these physicochemical characteristics can have a detrimental effect on the biological activity (receptor binding and downstream signalling). This chapter aimed to examine in detail how structural changes to a pasireotide backbone could impact on receptor activation. The key amino acids for *in vitro* biological activity were explored to further the understanding that what makes pasireotide and its analogues active. To achieve this, two *in vitro* assays were developed, with a hSSTR5 specific BRET activity assay used to assess the potency of synthesised pasireotide analogues compared to that of the parent peptide.

4.4.1. An ionisable Lys residue is integral to activity

Since it was discovered, the active conformation of somatostatin has been of interest to researchers. It was from this early interest that the Lys residue was identified as an important moiety in the activity of the peptide. While little was understood about the mechanism of action that Lys had on peptide binding, many papers reported the lack of peptide activity observed when this residue was altered in the structure. As such, all current commercial analogues of somatostatin including pasireotide, octreotide and lanreotide, all retain the Lys residue in their structures. Here, we endeavoured to explore how increasing the lipophilicity of pasireotide analogues near or replacing the Lys residue impacts the *in vitro* activity of peptides.

Series A was designed and synthesised to be a selection of pasireotide analogues that retained the pharmacophore whilst moving towards increased lipophilicity. Within series A, the sole peptide without the Lys residue is peptide A6 which contains a Gly(2-pent) residue in its place. This change was inspired by Nutt et al. (6) who reported that there was significant loss of activity when Lys was replaced with alternative electrically charged side chains like Arg and His residues. This paper suggests that it is not merely the basic nature of Lys that contributes to the activity of somatostatin and alludes to the idea that steric interactions could be at play here. This was not the case here, as the Gly(2-pent) amino acid with an alkyl chain of the same length as Lys, did not elicit any biological activity suggesting that the amino group of the Lys is indeed having an ionisable function in the binding pocket. Recent cryo-EM images by Robertson et al. (8) show that

the Lys residue likely forms a salt bridge with an Asp residue in the receptor binding pocket and this interaction triggers further downstream signalling.

The importance of Lys is seen in the structure-activity relationship of series B-F, where all peptides that have Lys in position 4 are replaced by Ala to elicit no biological activity. A comparative observation was made by Lewis et al. (7) who conducted an alanine-scan where each amino acid in SRIF-14 was systematically replaced with an Ala residue to understand the role of each amino acid in the peptide. From this study, only the replacement of Trp and Lys amino acids had no biological activity when replaced with Ala residues, highlighting their importance. This study confirmed that the β-turn region was essential for high affinity binding to hSSTR1-5 which encompasses Phe-Trp-Lys-Thr residues.

The loss of biological activity when the Lys residue is removed is a complication for the pursuit of oral delivery as peptide permeability across a lipid membrane is greatly reduced by ionisable residues like Lys. From studies reported in Chapter 3, it was found that replacement of the Lys residue to Ala can improve permeability by 100-fold but this same change in structure makes the pasireotide analogues inactive (Table 4.2). The important role of the Lys residue in binding can be seen in Figure 4.2, where the Lys residue fits into the binding pocket to form an interaction with Asp.

It is possible to create lipophilic and orally available peptides as seen by Hill et al. (25) who synthesised a peptide containing solely lipophilic Leu resides (i.e. *cyclo*[Leu-Leu-Leu-Leu-Leu-Leu]) which had 4-17% oral bioavailability. However, there remains the fact that an orally bioavailable peptide without biological activity remains not useful and the problem of how to make bioactive peptides orally bioavailable while retaining their bioavailability is key (21).

4.4.2. Benzyl groups in the pasireotide backbone supports biological activity.

There have been published reports that found that the presence of Phe residues in somatostatin, specifically the Phe residues highlighted blue in Figure 4.1, stabilise the peptide in its active form via hydrophobic interactions between benzyl rings (10, 26).

The importance of these Phe residues for the biological activity of pasireotide can also be seen through peptide A5. This analogue describes a previously identified permeable scaffold in the form of naturally derived Phepropepsin A that merges the pharmacophore of pasireotide within it. A study exploring the effect of stereochemistry on Phepropeptin C permeability and solubility was conducted by Schwochert et al. (27) where it was found that the natural L-Pro in the structure was more cell permeable than its D-Pro isomer. The incorporation of solely the pharmacophore of pasireotide was not sufficient in creating an active analogue. This suggests that the pharmacophore of pasireotide relies on more than just two amino acids in the structure and that the surrounding amino acids are important to achieving peptide shape in the binding site. Again, this supports the need of these stabilising benzyl rings to support the peptide and its active conformer.

The reinsertion of a benzyl group on the Tyr5 residue as seen in the original pasireotide structure, resulted in an increase in peptide potency. The change from the original Thr residue in somatostatin to a Tyr residue was supported by a study from Veber et al. (28) who found that the hydroxyl group of tyrosine was seen to have a 10-fold increase in potency which was correlated to the increase in hydrophobicity. This was later changed by Bruns et al. (13) to a Tyr(Bzl) group for the peptide pasireotide. It was not explicitly stated what the function of the benzyl group was in the initial design of pasireotide, but the results here suggest that the benzyl group provides further support of the active conformer of the peptide by further hydrophobicity. In comparison to peptide A1, peptide A2 has a 20-fold increase in activity due to the reintroduction of the benzyl group at position 5 and this single addition to peptide A1 significantly improves the *in vitro* activity. Given that the Phe-Phe interactions in the endogenous peptide are key to stabilising the peptide, perhaps the addition of the Bzl group to Tyr5 is additionally stabilising the structure in its active conformer. The Bzl group additionally aids in peptide

permeability, and further research into how this protecting group can both improve permeability and activity requires further exploration.

4.4.3. Stereochemistry of peptide backbone effects *in vitro* potency

The pharmacophore for pasireotide, D-Trp-Lys, has long been established to be essential for peptide potency (2). This was explained by the D-Trp residue stabilising the β-turn formation between residues Phe-Trp-Lys-Thr in the original peptide. From this discovery, all subsequent somatostatin analogues contain this D-Trp-Lys motif, including pasireotide. The loss in potency between the synthesised peptides compared to pasireotide is large, approximately 1000-fold. This is not unexpected as pasireotide contains additional motifs on its amino acids that can influence its potency that were removed from the pasireotide analogues for ease of synthesis. What was surprising was that the D-Trp-Lys motif was not exclusively required for potent biological activity. Figure 4.12 compares the activity of peptides A1, B1, C1, D1, E1 and F1. These six analogues all have a Lys residue in position 4 but vary in L- and D- patterns for both Trp and Lys residues. The results show that peptides B1, C1, D1 and E1 all demonstrate similar activities with IC_{50} values ranging from 1.8-4.5 μM. Peptides B1 and C1, and peptides D1 and E1 are enantiomers of each other. Studies into the effect of enantiomers on peptide activity have revealed that one peptide within the pair end to have better activity than the other (29, 30). However, the results here suggest that these mirror image peptide pairs are forming similar shapes at the binding site to one another.

Within the peptides in Figure 4.12, only peptides A1 and B1 retain the pasireotide pharmacophore of D-Trp-Lys. These two peptides however have very different IC_{50} values, with peptide A1 being 0.7 μM and peptide B1 being 2.4 μM. This suggests that the presence of the D-Trp-Lys motif is not enough to secure potent biological activity but that the shape of the peptide at the receptor plays a significant role. The increase in peptide potency for peptide A1 in comparison to peptides B1, C1, D1 and E1 is not necessarily surprising as this peptide is most like commercial pasireotide, however what is surprising is the activity of analogue F1 which has a 4-fold improvement in IC_{50} from A1. Peptide F1 differs from peptide A1 by having D-amino acids in position 4, 5, and 6 (D-Lys4-D-Tyr5-D-Phe6). This configuration differs from the previously established pharmacophore of D-Trp-Lys and instead introduces an additional D-amino acid at position 4 (D-Trp-D-Lys).

From this result, it can be assumed that the conformation peptide F1 was forming due to these two amino acids in their D-form was promoting better potency by allowing a better fit into the binding pocket of the receptor. However, it is important to note that peptide D1 also has this D-Trp-D-Lys pattern but not the same potency in bioactivity. In fact, the only difference between peptides D1 and F1 is the Tyr residue in position 5 but these peptides have a 24-fold difference in potency. All these differences in activity between difference patterns of L- and D-amino acids suggest that activity prediction cannot solely be made from amino acid presence but moreover the shape that peptides are forming in solution. In fact, a paper from Wang et al. (31) suggests that peptides with D-amino acid modification rarely exhibit effective biological activity, however this has found not to be the case in this study. Two peptides with identical amino acid content but single stereoisomer change can have large differences in activity.

4.5. Conclusion

The peptide analogues that we designed and synthesised to be more orally bioavailable were tested *in vitro* for bioactivity and the extent of activity lost due to modifications was measured. A cAMP-based activity assay was initially developed to measure IC_{50} values for each peptide using HEK293 cells and CHO-k1 cells each stably expressing hSSTR2 and hSST5 receptors. Due to incorrect DNA plasmids, the receptors were folding incorrectly resulting in unusable cells and new HEK293 cells stably expressing hSSTR5 were obtained. Following this, a new robust *in vitro* assay was successfully developed and optimised for HEK293 cells expressing hSSTR5 receptors. The BRET activity assay uses transient transfection of fluorescently tagged G proteins to measure ligand binding. From this assay, it was determined that the Lys residue in the peptide backbone is essential for the activity of pasireotide analogues. In addition, the peptides surrounding the pharmacophore play an important role in peptide bioactivity by stabilising the active conformation and the removal of key stabilising amino acids like Phe2 and Phe6 impacts peptide activity. It was found that stereochemistry influences the potency of bioactivity, and the established pharmacophore of D-Trp-Lys is not obligatory for peptide activity. Other stereochemical backbones can also elicit high potency demonstrating that understanding peptide conformations and how they can improve potency is important for furthering activity. This study shows that modifying the parent peptide pasireotide has consequences on bioactivity. However, a reduction in potency is acceptable should the peptide demonstrate sufficient absorption into the bloodstream. The findings of this study can be used to further the understanding of how lipophilicity and stereochemistry interplays with activity.

4.6. References

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Chapter 5 : *In vivo* pharmacokinetics of pasireotide analogues

5.1. Introduction

Peptide drugs have garnered considerable attention in the pharmaceutical industry owing to their specificity, potency, and reduced off-target effects compared to conventional small molecule drugs (1-4). However, their oral therapeutic potential is hindered by poor pharmacokinetic properties such as absorption, leading to poor distribution, susceptibility to metabolism, and rapid excretion. Prior to uptake, orally delivered peptides must survive the harsh acidic environment of the stomach, as well as avoid proteolytic degradation by digestive enzymes in the gut, before uptake across the gastrointestinal membrane and reaching the systemic circulation (1, 2). Once in the blood, an orally absorbed peptide can distribute to multiple tissues and to its intended site of action.

Currently there are several *in vitro* models that can be used to measure aspects of oral delivery, including the PAMPA assays used in this thesis, Caco-2 cell uptake assays or Ussing chamber ex-vivo tissue penetration assays to explore drug permeability. While these assays provide useful indications about a drug's absorption potential, they cannot mimic the exact *in vivo* conditions that drug candidates face in the body, primarily due to different sink conditions, lack of enzymatic degradation and efflux transporters. PAMPA assays are a simplistic bilayer model typically used to screen large amounts of drug candidates in a relatively quick and cost-effective manner. However, the PAMPA assay cannot factor in paracellular transport or the effect of active transporters and efflux on drug candidates and is solely used to predict transcellular membrane absorption. Even then, the membrane used (phospholipid and oil) is not comparative to an *in vivo* cell membrane. Caco-2 assays introduce an additional level of complexity in the form of a single cell layer which mimics the GI membrane lining with more complex heterogeneous cocultures used to generate the mucosal barrier of the GI tract (5). However, despite this more complex system, Caco-2 studies still lack the fluid, flow, peristalsis and cell turnover that is present in *in vivo* conditions (6, 7). Ussing chambers use ex vivo epithelial tissues to measure the movement of drug across an excised biological membrane (6, 7).

This method, while using an intact intestinal barrier, is not conducive to large scale screening of drug candidates. As such, while *in vitro* assays remain essential to screen drug candidates for absorption, the ultimate study to determine drug oral potential remains *in vivo* bioavailability studies.

Poor oral absorption of peptides has long been established in the literature as the major hurdle to oral therapeutic development, with majority of peptides reporting less than 1% oral bioavailability (8). An example of this is leuprolide acetate, a typical linear and ionisable peptide, which has a reported oral bioavailability of 0.074% when delivered in a simple aqueous solution (9). Currently there are many strategies being explored to improve peptide absorption. Earlier chapters of this thesis have shown how stereochemistry (in the form of D-amino acids) and lipophilicity can generate pasireotide analogues with improved passive *in vitro* membrane absorption. Other strategies to improve oral bioavailability of peptides include utilising lipid-based formulations and additional compounds like permeation enhancers. Following will be a discussion of peptides reported in the literature in which *in vivo* absorption is measurable.

There are several reports in the literature of peptides with good gut absorption and oral bioavailability (10-15). Many of these peptides are structurally similar to cyclosporin A (*cyclo*[Abu-Sar-N(Me)Leu-Val-N(Me)Leu-Ala-D-Ala-N(Me)Leu-N(Me)Leu-N(Me)Val-N(Me)Bmt(E)]) as they share common features such as being highly lipophilic and often containing N-methylation or several Leu residues in their structures (13, 14, 16). The following three peptides are all backbone cyclic hexapeptides (like the analogues in this thesis) but each exhibit different oral bioavailability. Biron et al. (10) designed an analogue of the Veber-Hirschmann peptide (17) (peptide A3 in this thesis) to contain three N-Methyl groups in the structure (*cyclo*[Pro-Phe-N(Me)D-Trp-N(Me)Lys-Thr-N(Me)Phe]). This peptide has an oral bioavailability of 9.9% in *in vivo* rat studies (10). In a similar fashion, White et al. (16) reported a permeable cyclic hexapeptide with structure *cyclo*[Leu-N(Me)D-Leu-N(Me)Leu-Leu-D-Pro-N(Me)Tyr] that has an oral bioavailability of 28% in rats. Vorherr et al. (15) reports a peptide with oral bioavailability of 39% and a structure of *cyclo*[Abu-Leu-N(Me)D-Leu-N(Me)Leu-Leu-D-Pro]. Given all three of these peptides share similar structural features it is important to note that there is an almost 4-fold difference in oral bioavailability between the most and least absorbed peptides. Hill

et al. (12) also reports of an orally bioavailable peptide (*cyclo*[Leu-Leu-Leu-Leu-Leu-Leu]) which has an oral bioavailability of 17% but this structure does not have any N-methylation. This suggests that it is not solely the N-methylation that is driving oral absorption. Bockus et al. (13) also reports a cyclic hexapeptide (*cyclo*[D-Pro-S,S Sta-N(Me)D-Leu-N(Me)Leu-S,S-Sta-N(Me)Leu]) with an oral bioavailability of 21%. Again, this peptide is highly lipophilic with many Leu residues, however this study additionally discusses the importance of stereochemistry on absorption. While not compared *in vivo*, this study reports the differences between *in vitro* permeability values between stereoisomer peptide pairs. In a similar way, cyclosporin has many different variations yet none demonstrate the same level of oral bioavailability to cyclosporin A (18). This corroborates with the *in vitro* studies explored in Chapter 3 of this thesis.

Peptides have also been reported in the literature to have improved oral bioavailability with the aid of formulation (e.g. lipid-based formulations) or additional agents that can assist absorption such as permeation enhancers (19-22). The most recognisable example of this is for the peptide CsA which is used in two lipid-based formulations, marketed as Neoral® and Sandimmune®, that form nano emulsions upon oral ingestion (23). Prior to its formulation in a lipid emulsion, CsA did not demonstrate promising oral bioavailability in humans. The first time a single dose of solid CsA was administered in humans, it was not absorbed, and no pharmacologically active levels of drug were found in the blood plasma (24). Currently, CsA can achieve oral bioavailability of ~40% in humans. Studies have also shown that lipophilic ion pairing of lipophilic salts with ionisable peptides in conjunction with lipid-based formulations can greatly improve oral bioavailability. Hintzen et al. (9) found that the peptide leuprorelin can have an improved oral bioavailability of 0.483% in self-micro emulsifying drug delivery systems (SMEDDS) compared to 0.074% when delivered as a solution. This can be further improved to1.276% by lipophilic ion pairing an oleate ion to leuprorelin in addition to the SMEDDS formulation.

Coformulation of peptides with permeation enhancers has also been shown to improve oral bioavailability of peptides. The most discernible example of this is the peptide semaglutide which has been co-formulated with the permeation enhancer SNAC. Studies found that the semaglutide-SNAC formulation has an oral bioavailability of 1.22% in dog models and a relative oral bioavailability of 0.4-1% via gastric absorption in humans (25-27). Other examples of permeation enhancers aiding oral absorption include a study by McCartney et al. (28) who found that peptide formulated with sodium laurate had a relative bioavailability of 8.9%. Enabling formulation development is an important factor to consider in the pursuit of oral peptide delivery and has been shown in many cases to improve oral bioavailability.

The parent peptide in this thesis, pasireotide, has not been extensively studied as an oral candidate and there is limited literature around its' oral delivery potential. However, there have been many studies for its pharmacokinetic behaviour as a subcutaneous injection. *In vivo* studies of pasireotide have shown that this peptide is well absorbed subcutaneously and has a prolonged duration of action in comparison to endogenous somatostatin, as measured by effective inhibition of growth hormone 6 hours post drug administration (29, 30). Studies by Bruns et al. (30) found that when administered as a single subcutaneous bolus injection, there was low systemic clearance suggesting that pasireotide does not undergo extensive hepatic metabolism. The distribution, and the terminal elimination half-lives for pasireotide was 0.74 and 23h, respectively (30).

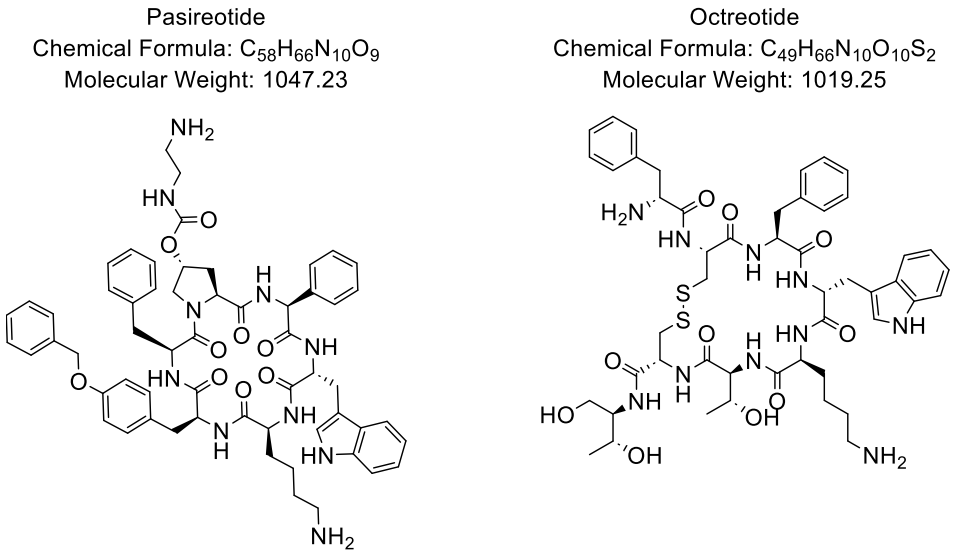


Figure 5.1 Chemical structure and molecular weight of somatostatin analogues, pasireotide and octreotide.

Unlike pasireotide, the somatostatin analogue octreotide has undergone much study into its oral delivery potential using a range of techniques and formulations Figure 5.1). Studies by Drewe et al. (31) found that the oral absorption of octreotide could be increased by 23-fold in rats and 8-fold in humans when co-administered with polyoxyethylene (24)-cholesterol-ether. Polyoxyethylene (24)-cholesterol-ether is a non-ionic surfactant that is likely to form micelles in solution (32). In a similar fashion, Parmentier et al. (33) revealed a 4-fold increase in bioavailability in rats for a liposomal formulation containing 25% of a tetraether lipid. These examples demonstrate the utility of colloidal formation in aiding peptide absorption. Octreotide for oral delivery has also been explored using lipophilic ion pairs. Bonengel et al. (34) reported a 17.9-fold improvement in oral bioavailability for octreotide when formulated as a lipophilic salt in lipid-based formulation. This study used a range of lipophilic salts but found octreotide-deoxycholate to have the highest absorption. Octreotide is currently in various stages of clinical trials for oral delivery. As of 2021, octreotide was in phase I trials for a formulation delivery device called RaniPill™ (35). This delivery system involves an enteric-coated pill that injects directly into the wall of the jejunum. The results of these trials suggest that oral octreotide was able to reach 70% oral bioavailability (35). Octreotide is also in phase III clinical trials for a formulation comprising of permeation enhancer, organic enzyme inhibitor and an enteric coated capsule (Peptelligence®) (35). Finally, octreotide is currently on the market in an oral form marketed under the brand name MYCAPSSA®. This formulation contains Transient Permeability Enhancer® which is an oily suspension of soluble hydrophilic microparticles of octreotide and polyvinyl pyrrolidone dispersed in an oil mix of glycerol monocaprylate and glycerol tricaprylate. The results of the clinical trials of this formulation reports an oral bioavailability of ~1% (35, 36).

This chapter will explore the oral bioavailability of select novel synthesised pasireotide analogues, compare their pharmacokinetic properties to commercial octreotide, and determine the bioavailability factors and clearance behaviour of each analogue. This thesis has previously explored the effect of D-amino acids and increasing lipophilicity on peptide oral delivery using backbone cyclic peptide scaffolds. Therefore, here the aims of this study are to investigate the *in vivo* absorption of pasireotide analogues in an industry standard oral formulation, investigate the intravenous pharmacokinetic profiles of each peptide analogues and determine the effect of formulation on the oral absorption of peptides.

5.2. Methods

5.2.1. Formulation preparation and administration to Sprague Dawley rats

All *in vivo* studies were conducted at the Monash Institute of Pharmaceutical Sciences and used established procedures in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All surgical and experimental procedures were reviewed and approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee.

The pharmacokinetics of each test compound were studied in male Sprague Dawley rats aged 7-8 weeks. Animals were fasted overnight with access to food reinstated 4 hours post-dose. All animals had access to water ad libitum throughout both the pre- and post-dosing period. Samples of arterial blood, total faeces and urine were collected up to 24 hr post-dose. Samples of arterial blood at 200 µL per aliquot was collected using the Culex ABS system (BASi, West Lafayette, IN, USA) into borosilicate vials at 4°C containing heparin as the anticoagulant. Blood samples were centrifuged, and supernatant plasma was removed. Plasma and urine samples were stored frozen at -80°C until analysis by LC/MS. All rats were euthanized by administering a lethal IV dose of pentobarbitone (0.4 mL per rat) at the end of the sampling period.

For IV studies, the test compound was administered intravenously as a 10 min constant rate infusion via an indwelling jugular vein cannula (n = 3 rats per compound) at 1mg/kg. Formulation concentration did not exceed 0.3mg/mL and volume dosed was dependant on body weight of the rats. All IV formulations consisted of 1% (v/v) ethanol and 5% (w/v) Kolliphor EL in DPBS and were filter sterilised prior to administration. The sole exception to this was peptide D4 which was dissolved in 1.24% (v/v), 5% (w/v) and DPBS.

All oral test compounds were administered orally by gavage at 3mL/kg (n = 3 rats per compound). Each oral formulation was made to a peptide concentration of 3.3mg/mL. Standard oral formulation was a uniform, fine, off-white suspension consisting of 0.5% (w/v) hydroxypropyl methylcellulose, 0.5% (v/v) benzyl alcohol and 0.4% (v/v) polysorbate 80 in Milli-Q water. The self-emulsifying drug delivery system (SEDDS) formulation was a cloudy, faintly blue, solution consisting of 1.5% (v/v) absolute ethanol, 4% (w/v) Maisine cc, 5% (w/v) Kolliphor RH40 and 1% (w/v) propylene glycol in milli Q water. Each oral formulation was dosed at 10mg/kg with an average of 0.8mL of formulation given to each rat. Plasma samples were analysed using a validated analytical assay (LC-MS/MS) with PAS as internal standard. The validation parameters of those methods are described in Chapter 2 (page 43).

5.2.2. Pharmacokinetic Data-Analysis

Data were plotted as drug concentration (ng/mL) vs. time (mins). Peak plasma concentrations (C_{max}), the time of peak plasma concentration occurrence (T_{max}), the area the plasma concentration vs. time profiles ($AUC_{0-\infty}$), and elimination half-life ($t_{1/2}$) were calculated using PK solver (Version 2.0, add-in program for Microsoft Excel) (37) with non-compartmental analysis of plasma data. Total blood volume of each rat was calculated using equation reported by Lee et al. (38) (Equation 3). Equation 4 shows the calculation of absolute bioavailability (F%)

Equation 3 Total blood volume of a rat. V is the blood volume of the rat. M is the mass of the rat.

$$V = 0.06 \times M + 0.77$$

Equation 4 Equation for the calculation of oral bioavailability.

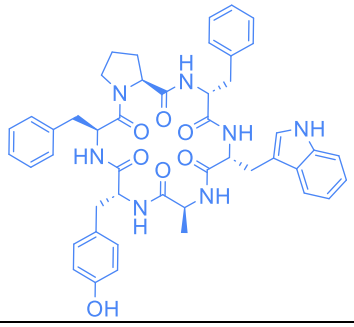
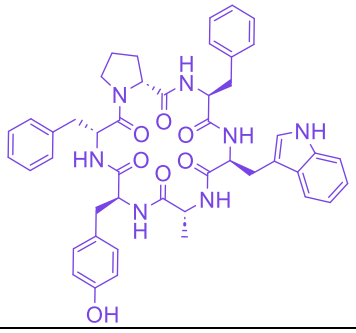
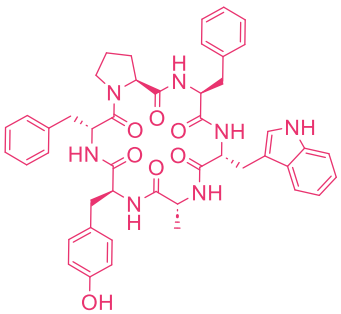
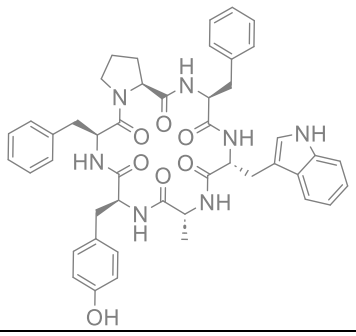
$$F\ (\%) = 100\ \times \frac{AUC_{po}}{dose_{po}} \times \frac{dose_{iv}}{AUC_{iv}}$$

5.3. Results

5.3.1. Selection of peptide analogues for *in vivo* studies

Based on results obtained from Chapter 3 of this thesis, four synthesised pasireotide analogues were chosen for further *in vivo* investigation. These peptides were tested orally and intravenously to calculate their oral bioavailability and pharmacokinetic properties. Peptide B2 was selected as the peptide with the highest likelihood of good oral absorption. This was determined by its good *in vitro* aqueous solubility (103.6 μM) and permeability (-6.63 cm/s) from the PAMPA assay. Peptide C2, the enantiomer of peptide B2, was selected as a comparison to peptide B2. This peptide demonstrated comparable aqueous solubility (97.6 μM) and *in vitro* permeability (-6.75 cm/s) values to peptide B2 but was highly susceptible to metabolic degradation with 98% loss in peptide concentration after incubation with porcine pancreatin extract ($t_{1/2}$ 3.9 mins). Peptide D2 was chosen due to its good *in vitro* permeability (-6.11 cm/s), and its poor aqueous solubility (29.9 μM). Peptide D2 would highlight any effects that solubility has on *in vivo* absorption rates in comparison to peptide B2. The final peptide chosen was peptide D4 which had moderate aqueous solubility (66.5 μM) but poor *in vitro* permeability (-8.90 cm/s). This peptide was chosen to demonstrate whether there is any correlation between *in vitro* PAMPA permeability studies and *in vivo* absorption rates. A summary of the structures and *in vitro* data obtained for these four selected peptides can be found in Table 5.1.

Table 5.1 Summary of in vitro permeability, solubility, and stability results obtained from Chapter 3.

Pasireotide analogues	**PAMPA permeability, $LogP_{app}$, cm/s**	**Measured solubility, 5% DMSO in PBS, max conc 200 µM**	**Enzymatic stability in SIF using pancreatin, $t_{1/2}$, mins**
B2 	-6.63 ± 0.10	103.6 ± 18.6	Stable (over 4 hrs, >20% degradation)
C2 	-6.75 ± 0.22	97.6 ± 15.0	3.9
D2 	-6.11 ± 0.07	29.9 ± 8.2	Stable (over 4 hrs, >20% degradation)
D4 	-8.90 ± 0.20	66.5 ± 3.6	Stable (over 4 hrs, >20% degradation))

5.3.2. *In vivo* absorption of octreotide

To serve as a comparison to the pharmacokinetic data obtained from the synthesised pasireotide analogues, the pharmacokinetic profile of commercial octreotide was used. Octreotide was chosen in replacement of pasireotide due to limitations obtaining sufficient material of pasireotide while octreotide is readily available. Like pasireotide, octreotide is an ionisable cyclic peptide analogue of endogenous somatostatin (Figure 5.1). The mean concentration of octreotide in plasma concentration versus time profile is depicted in Figure 5.2 and normalised to 2 mg/kg to permit cross-comparison. A summary of the pharmacokinetic parameters of octreotide is provided in Table 5.2.

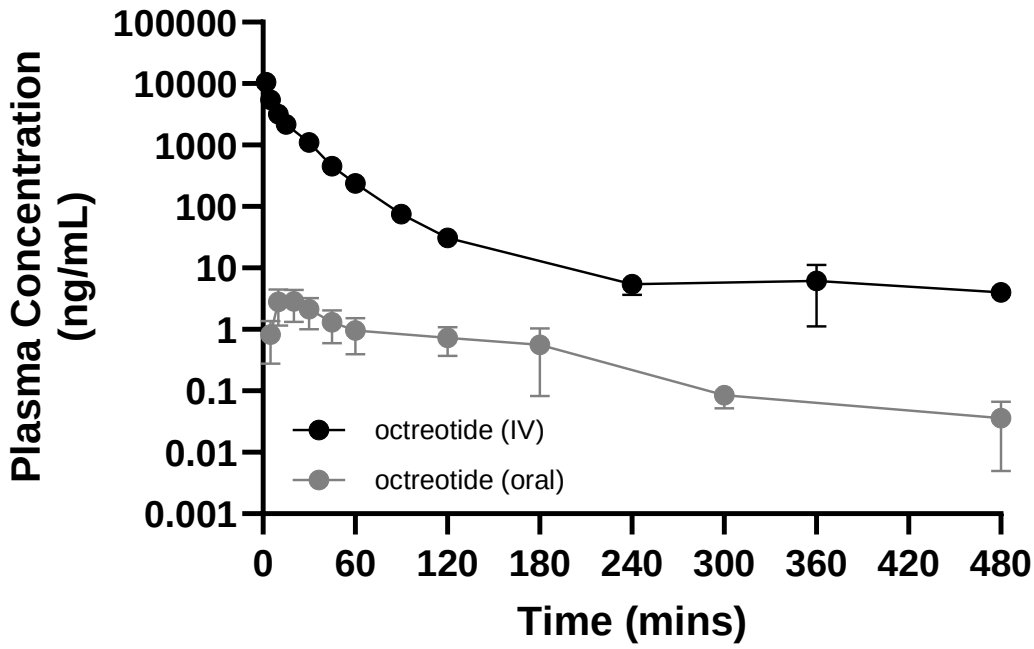


Figure 5.2 Mean plasma concentration-time curve of octreotide in oral solution (grey) vs intravenous (black) administration [mean ± SEM, n=4]. IV drug were dosed at 0.1 mg/kg and oral drug were dosed at 2 mg/kg. Doses were normalised to 2 mg/kg in this graph to allow direct comparison.

Table 5.2 Pharmacokinetic parameters for octreotide after intravenous administration [mean ± SEM, n=4] and oral administration in solution [mean ± SEM, n=4] to fasted male Sprague Dawley rats.

	IV	**Oral (solution)**
Dose (mg/kg)	0.1	2
C_{max} (ng/mL)	529.68 ± 13.99	2.95 ± 0.80
T_{max} (min)	2 ± 0	12.50 ± 2.50
$t_{1/2}$ (min)	72.61± 5.71	75 ± 9.18
$AUC_{0-\infty}$ ((ng/mL)*min)	6067.00 ± 308.00	250.86 ± 68.66
V_d (L)	0.73 ± 0.38	-
Cl (mL/min)	4.98 ± 0.26	-
Absolute bioavailability (%)	-	0.21

From the intravenous profile of octreotide, the calculated drug half-life is 1.2 hours with rapid clearance of 5 mL/min, presumably via glomerular filtration. The results of the oral administered groups show that $AUC_{0-\infty}$ and C_{max} for orally delivered octreotide was low, with only 2.95 ng/mL of peptide detectable in the plasma. The absolute oral bioavailability of octreotide was 0.21%, illustrating that octreotide is not well absorbed into the systemic circulation. This aligns with studies by Drewe et al. who reports an oral bioavailability of 0.3% for octreotide in rats (31).

5.3.3. *In vivo* oral performance of pasireotide analogues

Peptide analogues B2, C2, D2 and D4 were tested *in vivo* in both intravenous and oral administrations. All intravenous injections were dosed at 1 mg/kg. All peptides were formulated as oral suspension formulations, with peptide B2 additionally being formulated into a simple lipid-based formulation. All oral formulations were dosed at 10 mg/kg and all peptide plasma concentration vs time profiles are reported as mean ± SEM (n=3) unless otherwise specified with data normalised to 10 mg/kg to allow direct comparison. The pharmacokinetic properties of each peptide are also tabulated.

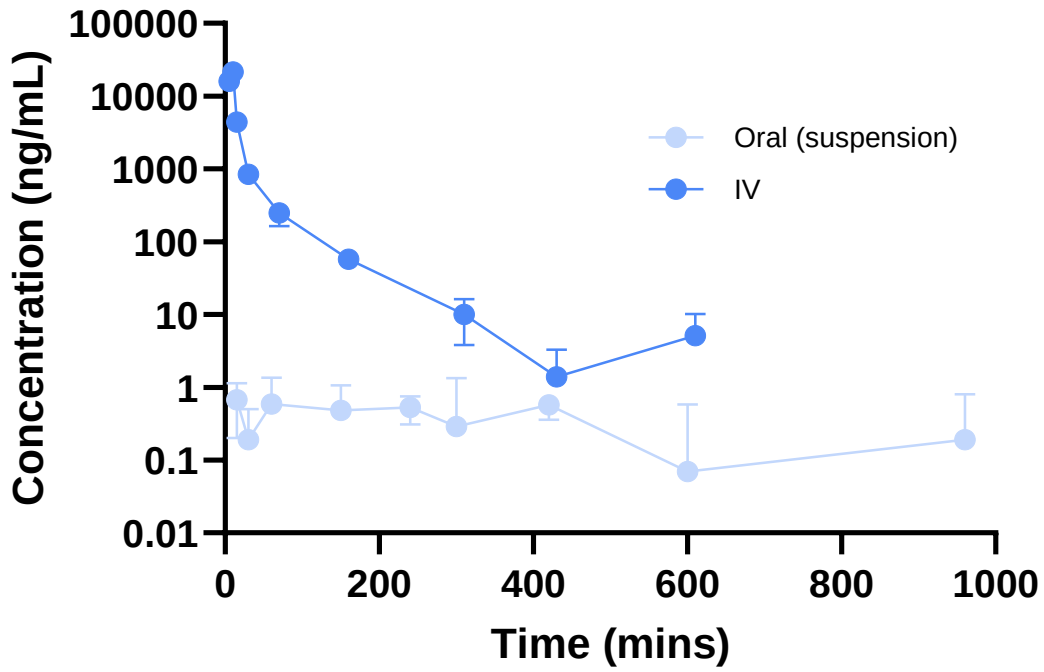


Figure 5.3 Mean plasma concentration-time curve of peptide B2 in oral solution vs intravenous administration [mean ± SEM, n=3]. Doses were normalised to 10 mg/kg in this graph to allow direct comparison.

Table 5.3 Pharmacokinetic parameters for peptide B2 after intravenous administration [mean ± SEM, n=3] and oral administration in suspension [mean ± SEM, n=3] to fasted male Sprague Dawley rats.

	IV	**Oral**
Dose (mg/kg)	1	10
C_{max} (ng/mL)	2139.11 ± 138.33	1.25 ± 0.39
T_{max} (min)	10 ± 0	260 ± 106
$t_{1/2}$ (min)	84.54 ± 15.77	486.74 ± 93.93
$AUC_{0-\infty}$ ((ng/mL)*min)	27988.73 ± 1641.86	1095.38 ± 538.75
V_d (L)	1.12 ± 0.16	-
Cl (mL/min)	9.35 ± 0.52	-
Absolute bioavailability (%)	-	0.39
C_{urine} (0-24hrs post oral administration) (ng/mL)	2164.01 ± 1111.83	2.95 ± 1.28

The intravenous profile of peptide B2 shows that the drug has an elimination half-life of just over 1.5 hours with very rapid clearance of 9.35 mL/min when compared to octreotide. When administered in an oral suspension, the $AUC_{0-\infty}$ and C_{max} for B2 was low, with maximal concentrations of 1.25 ng/mL of peptide detectable in the plasma. The absolute oral bioavailability of peptide B2 was 0.39%. Given that octreotide oral bioavailability was measured at 0.21%, this is a 2-fold improvement in bioavailability for a peptide in the same family of somatostatin analogues.

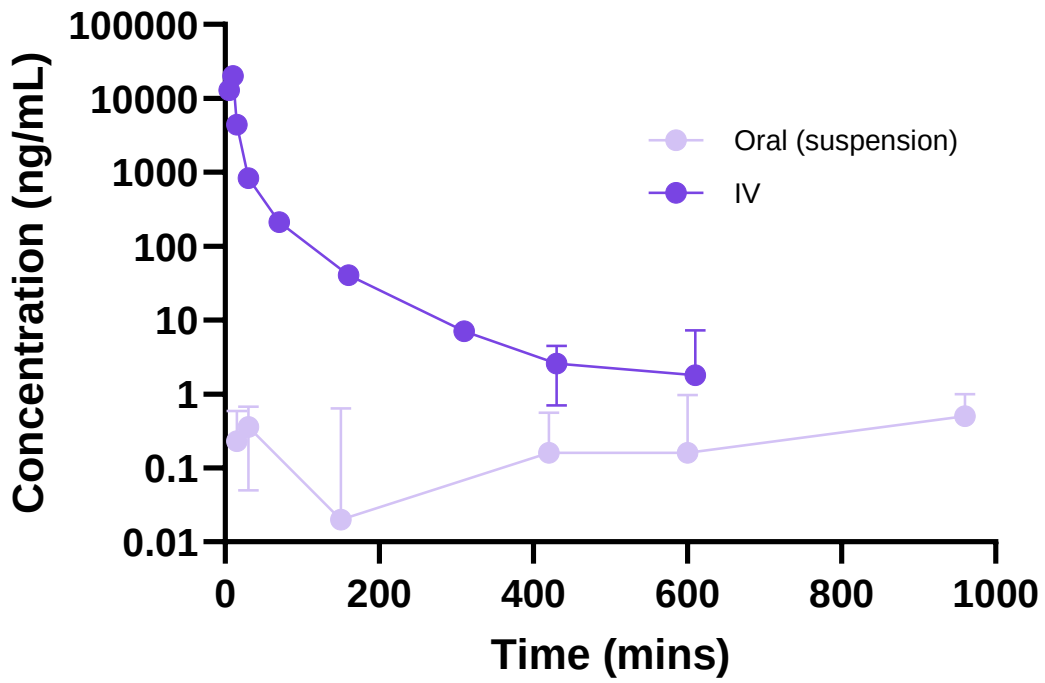


Table 5.4 Pharmacokinetic parameters for peptide C2 after intravenous administration [mean ± SEM, n=3] and oral administration in suspension [mean ± SEM, n=3] to fasted male Sprague Dawley rats.

Figure 5.4 Mean plasma concentration-time curve of peptide C2 in oral solution vs intravenous administration [mean ± SEM, n=3]. IV drug were dosed at 1 mg/kg and oral drug were dosed at 10 mg/kg. Doses were normalised to 10 mg/kg in this graph to allow direct comparison.

	IV	Oral
Dose (mg/kg)	1	10
C_{max} (ng/mL)	2013.86 ± 113.33	0.64 ± 0.23
T_{max} (min)	10 ± 0	660 ± 159
$t_{1/2}$ (min)	129.95 ± 51.95	144.70*
$AUC_{0-\infty}$ ((ng/mL)*min)	25289.47 ± 1506.70	896.39*
V_d (L)	2.19 ± 1.02	-
Cl (mL/min)	11.16 ± 0.70	-
Absolute bioavailability (%)	-	0.35
C_{urine} (0-24hrs post oral administration) (ng/mL)	5600.50 ± 379.38	8.31 ± 4.11

*n=1 as only 1 replicate provided an elimination phase

Changing the structure of the peptide to its enantiomer (peptide C2) showed a slightly longer plasma circulation time with a half-life of 2 hours, however clearance is still rapid at a rate of 11.16 mL/min. The concentration of peptide in the urine is higher than the maximum concentration detected in the plasma, and 96.8% of the total concentration of peptide in urine was detected within 7 hrs post IV dosing. This suggests that peptide is cleared quickly from the plasma into the urine. The results of the *in vivo* study show that $AUC_{0-\infty}$ and C_{max} for orally delivered peptide C2 in suspension

formulation was low, with only 0.64 ng/mL of peptide detectable in the plasma The absolute oral bioavailability of peptide C2 was 0.35%. Like peptide B2, this is a modest improvement in comparison to octreotide bioavailability but considering he susceptibility of peptide C2 to enzymatic degradation from *in vitro* studies, the comparable bioavailability to peptide B2 was an unexpected and positive result.

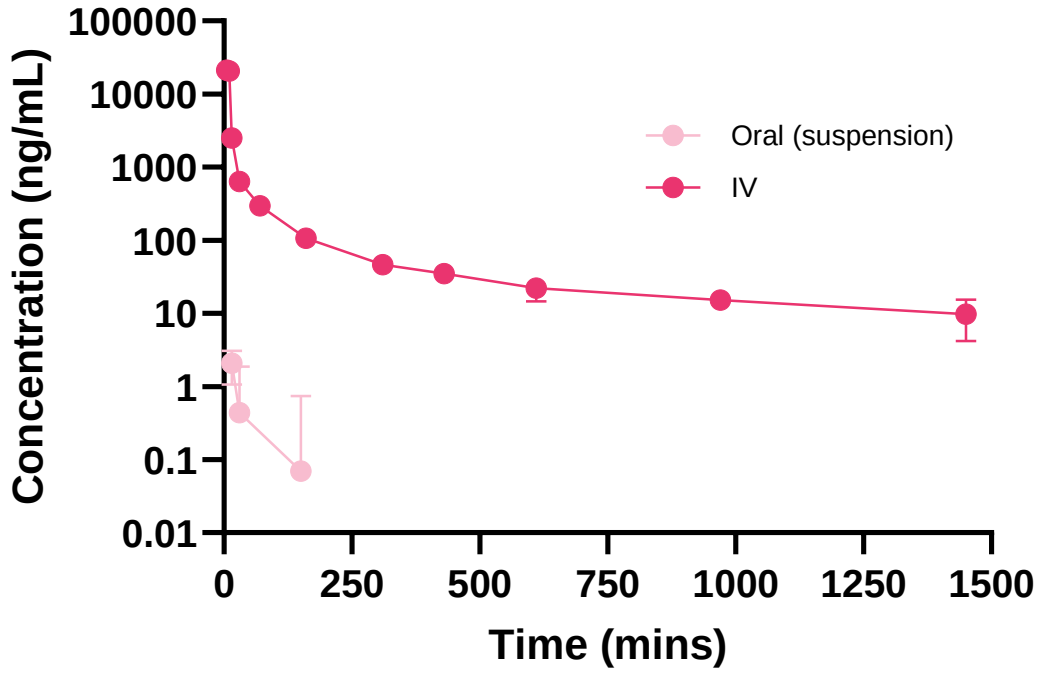


Figure 5.5 Mean plasma concentration-time curve of peptide D2 in oral solution vs intravenous administration [mean ± SEM, n=3]. IV drug were dosed at 1 mg/kg and oral drug were dosed at 10 mg/kg. Doses were normalised to 10 mg/kg in this graph to allow direct comparison.

Table 5.5 Pharmacokinetic parameters for peptide D2 after intravenous administration [mean ± SEM, n=3] and oral administration in suspension [mean ± SEM, n=3] to fasted male Sprague Dawley rats.

	IV	**Oral ***
Dose (mg/kg)	1	10
C_{max} (ng/mL)	2222.44 ± 113.53	2.64 ± 0.12
T_{max} (min)	6.67 ± 1.67	15 ± 0
$t_{1/2}$ (min)	404.65 ± 44.79	-
$AUC_{0-\infty}$ ((ng/mL)*min)	31646.31 ± 1515.09	-
V_d (L)	4.64 ± 0.61	-
Cl (mL/min)	7.93 ± 0.36	-
Absolute bioavailability (%)	-	-
C_{urine} (0-24hrs post oral administration) (ng/mL)	2403.35 ± 844.79	36.52 ± 15.54

* Peptide was only detectable in the plasma at the first three time points, therefore a pharmacokinetic profile following oral administration could not be accurately calculated.

Peptide D2 is another lipophilic peptide that demonstrated good permeability but poor solubility from the *in vitro* data. It was unknown how these physicochemical characteristics would impact oral absorption. The intravenous profile of peptide D2 shows an increase in half-life to over 6.5 hours, with a reduction in clearance rate of 7.93 mL/min. When administered in an oral suspension, the absorption of peptide D2 was low, with maximal concentrations of 2.64 ng/mL of peptide detectable in the plasma. The absolute oral bioavailability of peptide D2 could not be calculated as plasma levels were only detectable for the first three data points. Therefore, a pharmacokinetic profile following oral administration could not be accurately calculated. It is likely that this peptide did not solubilise rapidly enough from the suspension formulation to be absorbed in any appreciable quantity.

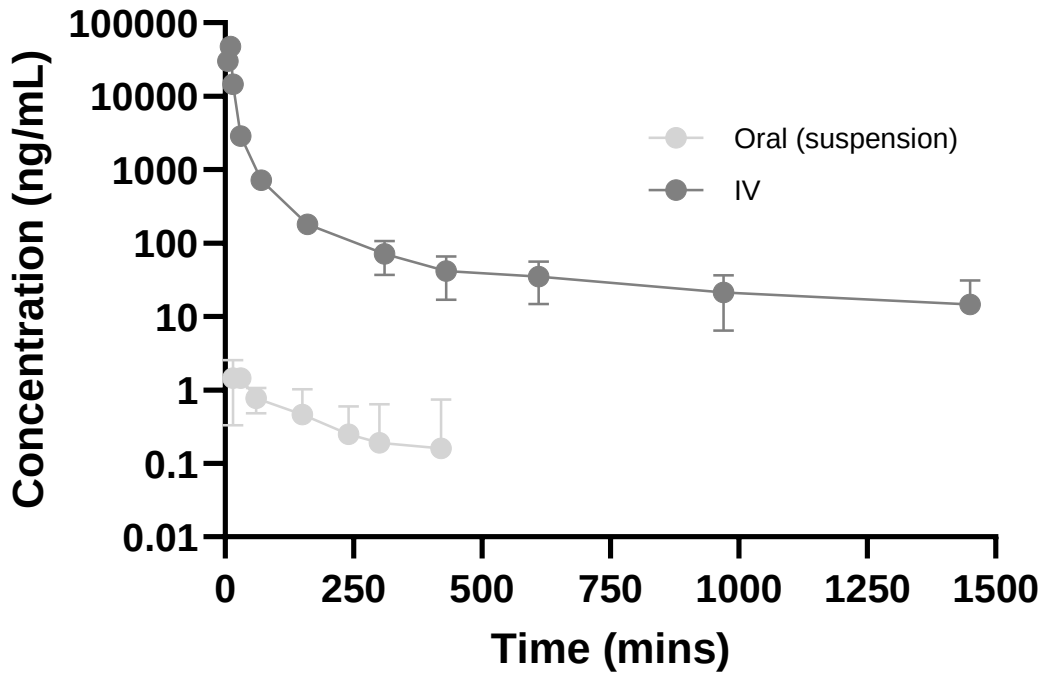


Figure 5.6 Mean plasma concentration-time curve of peptide D4 in oral solution vs intravenous administration [mean ± SEM, n=3]. IV drug were dosed at 1 mg/kg and oral drug were dosed at 10 mg/kg. Doses were normalised to 10 mg/kg in this graph to allow direct comparison.

Table 5.6 Pharmacokinetic parameters for peptide D4 after intravenous administration [mean ± SEM, n=3] and oral administration in suspension [mean ± SEM, n=3] to fasted male Sprague Dawley rats.

	IV	**Oral**
Dose (mg/kg)	1	10
C_{max} (ng/mL)	4725.67 ± 75.81	1.29 ± 0.43
T_{max} (min)	10 ± 0	25 ± 5
$t_{1/2}$ (min)	629.95 ± 141.30	402.93 ± 118.90
$AUC_{0-\infty}$ ((ng/mL)*min)	73557.52 ± 2220.12	467.50 ± 180.15
V_d (L)	3.54 ± 0.70	-
Cl (mL/min)	3.95 ± 0.12	-
Absolute bioavailability (%)	-	0.06
C_{urine} (0-24hrs post oral administration) (ng/mL)	1546.55 ± 299.64	20.18 ± 15.77

Lastly, we evaluated the oral uptake of moderately soluble but poorly permeable peptide D4 to understand how *in vitro* permeability may impact observed oral uptake. Peptide D4 had an increased elimination half-life of 10.5 hours and a clearance rate of 3.95 mL/min. Oral dosed groups showed that $AUC_{0-\infty}$ and C_{max} for orally delivered peptide D4 in suspension formulation was low, with only 1.29 ng/mL of peptide detectable in the plasma. The absolute oral bioavailability of peptide D4 was 0.06% and is the pasireotide analogue with the poorest absorption. Compared to commercial octreotide, peptide D4 is 3.5-fold less absorbed. This supports the data found in Chapter 3 which showed that peptide D4 has both moderately poor *in vitro* solubility and poor permeability.

5.3.4. *In vivo* performance of peptide in lipid-based formulation

To explore the effect of formulation, specifically lipid-based formulations, on the *in vivo* absorption of peptide into the bloodstream, peptide B2 was chosen to be formulated in a simple self-emulsifying drug delivery system (Appendix D, page 166). This formulation mimics that of the commercial Neoral®, the formulation that catapulted cyclosporin A to 40% oral bioavailability (18).

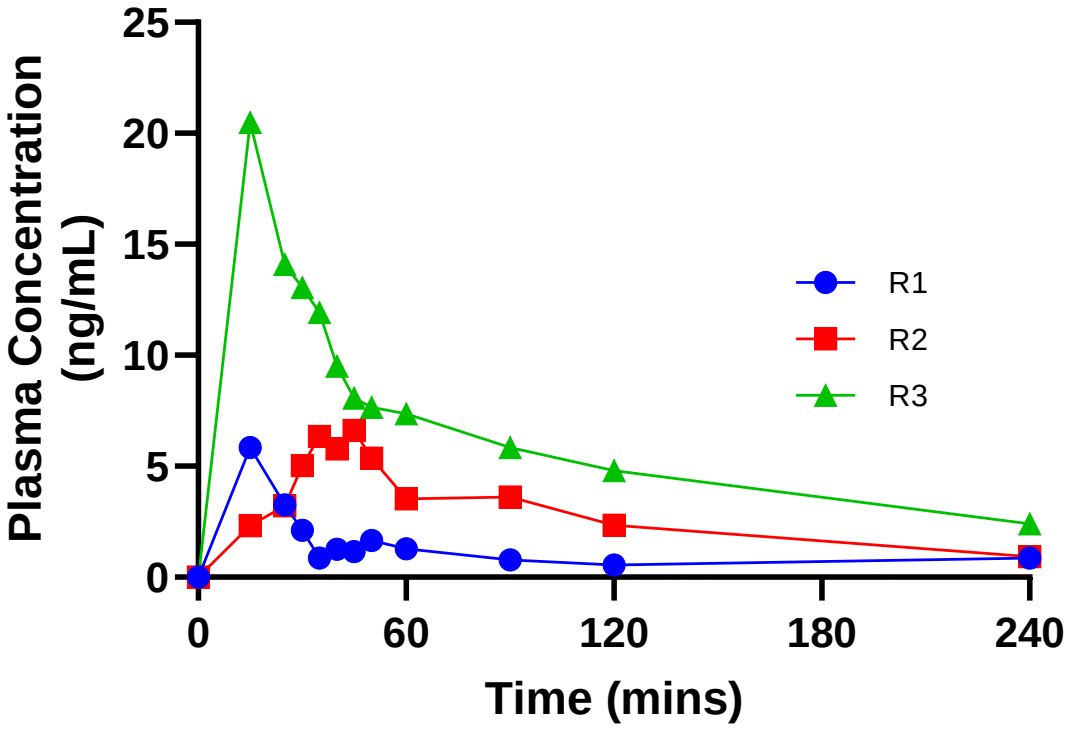


Figure 5.7 Individual rat (R1-3) peptide in plasma concentration vs time curves of peptide B2 delivered orally using SEDDS formulations. Dose administered to each rat was 10 mg/kg.

Figure 5.7 plots the plasma concentration vs time curves of the three individually dosed rats. From this data, rat 3 had a higher concentration of peptide in the plasma than rats 1 and 2, this may have been due to issues with gavaging the formulation. As a result, the following data uses the singular results of rat 3 to show the maximum absorption potential of peptide B2 in conjunction with lipid-based formulation, understanding that further work is required to confirm these preliminary findings.

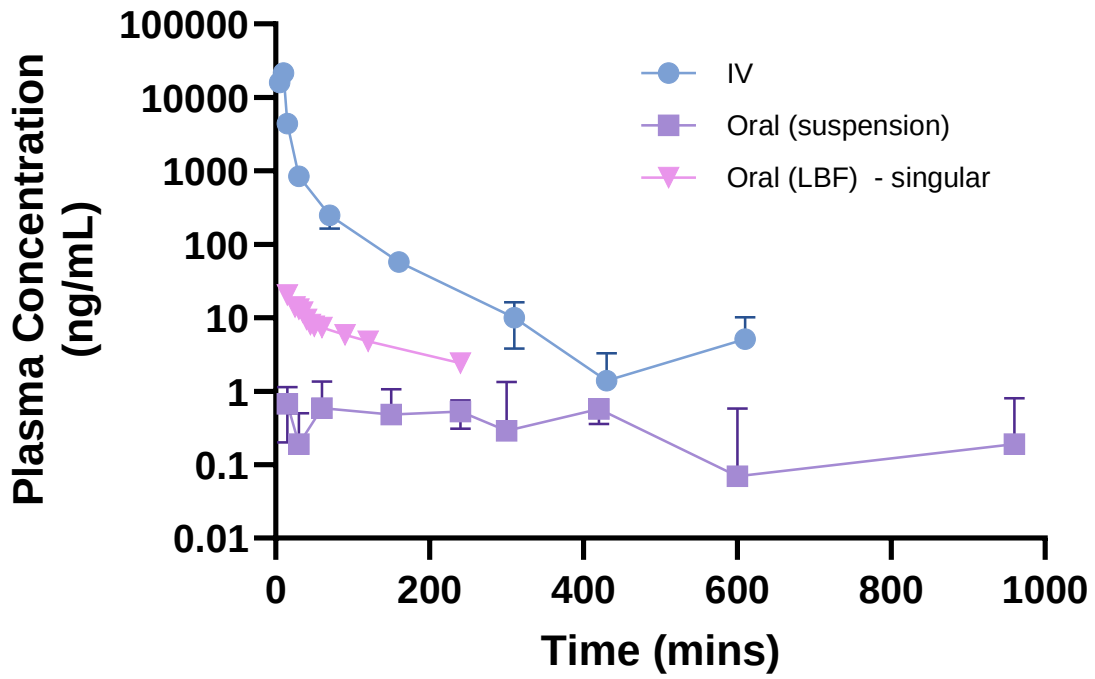


Figure 5.8 Mean plasma concentration-time curve of peptide B2 administered intravenously (blue) vs oral suspension (purple), oral LBF (pink). Doses were normalised to 10 mg/kg.

Table 5.7 Pharmacokinetic parameters for peptide B2 after oral administration in suspension [mean ± SEM, n=3] and SEDDS vehicles [n=1] to fasted male Sprague Dawley rats.

	Oral (suspension)	**Oral (SEDDS)**
C_{max} (ng/mL)	1.25 ± 0.39	20.48
T_{max} (min)	260 ± 106	15
$t_{1/2}$ (min)	486.74 ± 93.93	117.66
$AUC_{0-\infty}$ ((ng/mL)*min)	1095.38 ± 538.75	2134.58
Absolute bioavailability (%)	0.39	0.76
C_{urine} (0-24hrs) (ng/mL)	2.95 ± 1.28	23.17

The mean plasma concentration versus time profiles for peptide B2 following intravenous, and oral suspension and solution administration is depicted in Figure 5.8 and normalised to 10 mg/kg to permit cross-comparison. A summary of the pharmacokinetic parameters for oral administration is provided in Table 5.7. The results of the *in vivo* study show that $AUC_{0-\infty}$ and C_{max} for orally delivered peptide B2 in the lipid-based formulation was higher than oral delivery in suspension formulation. The absolute bioavailability of peptide B2 in lipid-based formulation doubled to 0.76%, demonstrating that lipid-based formulations indeed aid in oral absorption.

5.3.5. Comparison of Peptide Analogue IV Profiles

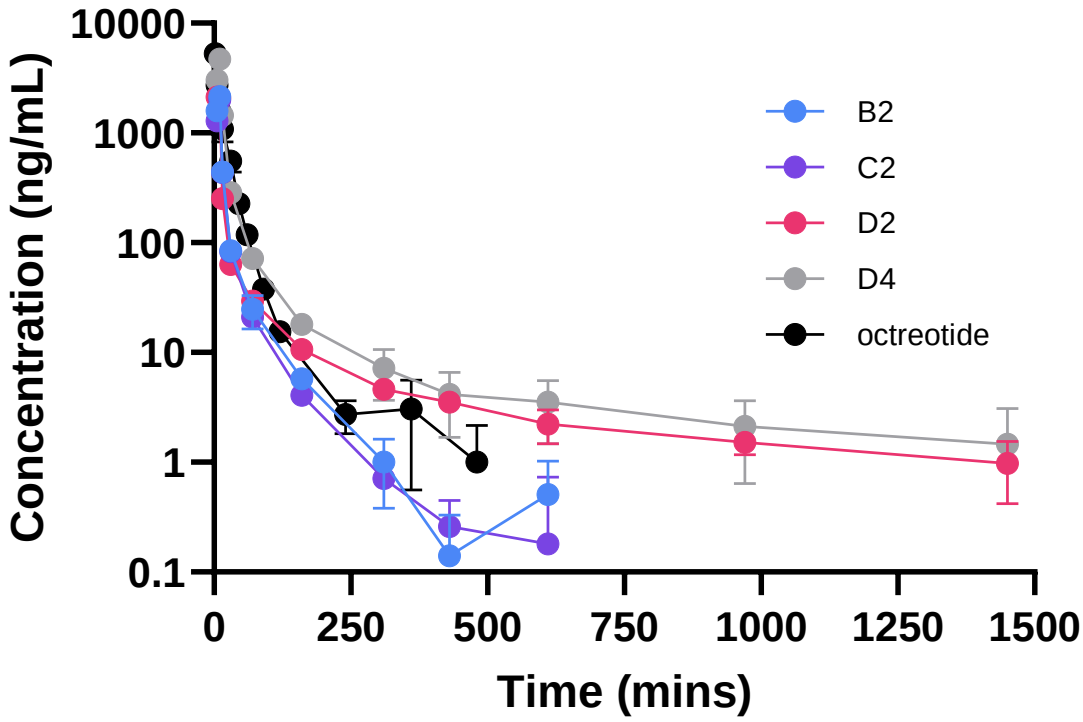


Figure 5.9 Comparison of mean plasma concentration-time curve of synthesised peptides B2, C2, D2 and D4, and commercial octreotide. Each peptide was administered intravenously. Doses were normalised to 1 mg/kg to allow comparison.

A closer look at the intravenous profiles of the pasireotide analogues reveals that these peptides are cleared quickly from the plasma. A direct comparison of the IV plots can be found in Figure 5.9. Peptide B2 had an elimination half-life of 1.4 hrs, peptide C2 of 2.2 hrs, peptide D2 of 6.7 hrs and peptide D4 of 10.5 hrs. Oral drugs should have a half-life of 12-24 hours for once daily dosing (39), however most peptides like somatostatin, leuprolide and oxytocin have half-lives of less than 8 hours (1, 4.5 and 6.75 hrs, respectively) (40).

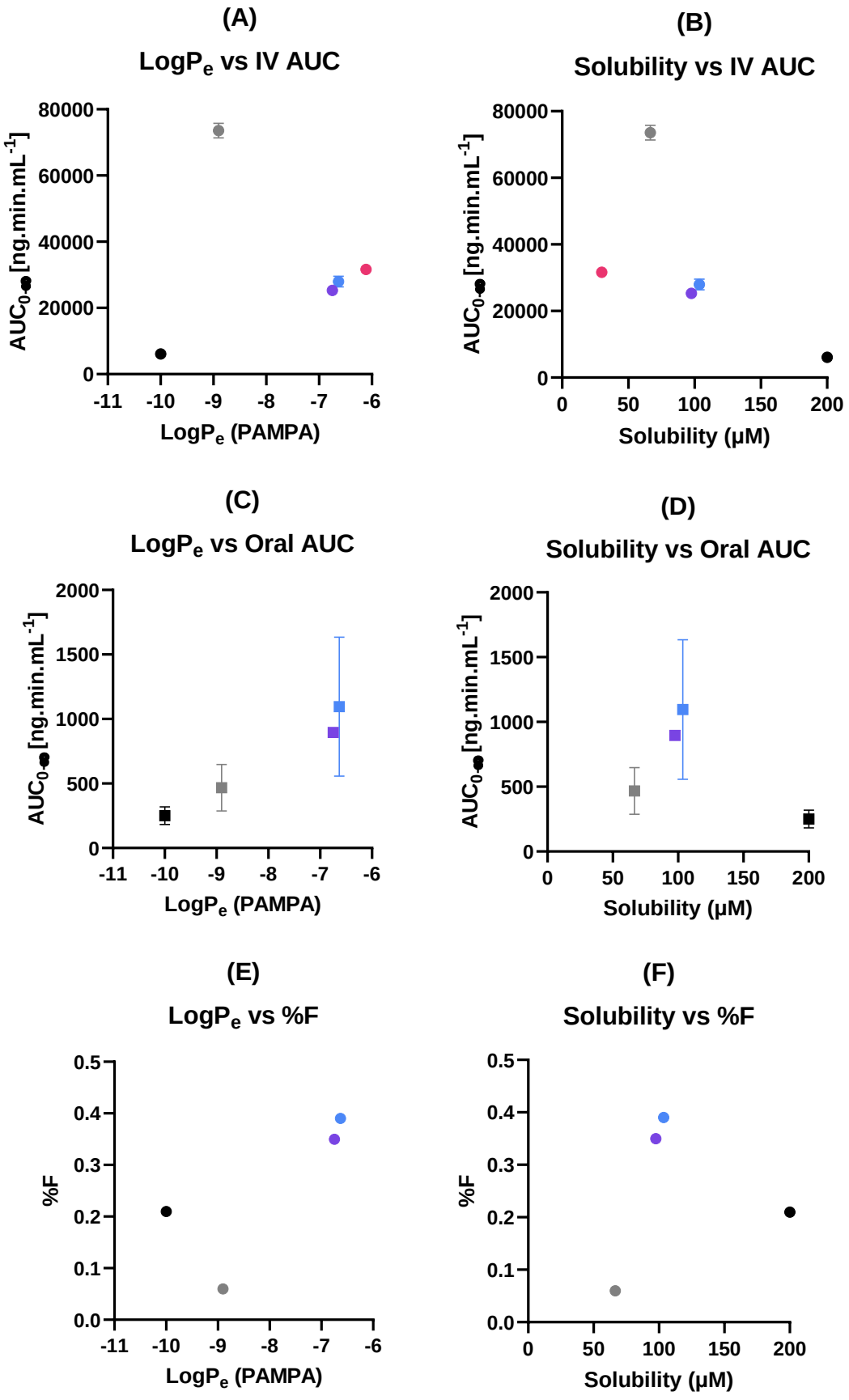


Figure 5.10 Summary of $AUC_{0-\infty}$ and %F vs $LogP_e$ and Solubility data for octreotide (black) and each peptide analogue (B2- blue, C2- purple, D2- pink and D4- grey) after oral and IV administration. Data is represented by mean ± SEM, n=4 animals compared to mean PAMPA and solubility data generated in Chapter 3.

Comparing the exposure data ($AUC_{0-\infty}$) for each peptide to the permeability and solubility data generated in Chapter 3 shows that there is no clear correlation between the oral absorption of these pasireotide analogues and changes in solubility. There is a minor trend towards improved exposure with increases in permeability that can be seen in graph C in Figure 5.10, however the data is too variable to have any correlative power. Comparing the oral bioavailability data (%F) for each peptide to *in vitro* permeability and solubility, again there is no clear correlation between oral bioavailability and either physicochemical property. However, if the octreotide data point is excluded, there are minor positive correlations between both permeability and solubility of the more lipophilic peptide analogues (peptides B2, C2 and D4). It is important to remember that octreotide retains its Lys residue unlike the other pasireotide analogues, which is a major structural difference between the synthesised peptides and octreotide. This structural difference appears to contribute to the outlier in the trends.

5.4. Discussion

5.4.1. Oral Pasireotide Analogues are Rapidly Cleared from Blood Plasma

The four synthesised peptides stratify into two groups with similar IV profiles with longer and shorter half-lives, peptides B2 and C2, and peptides D2 and D4 (Figure 5.9). From a structural perspective, this is not unexpected as peptides B2 and C2 are closely related in structure being enantiomeric pairs. Peptides D2 and D4 differ by a single stereoisomer (D-Phe6 and L-Phe6, respectively) and largely contain the same structural backbone. From the IV profiles peptide B2 and C2 are rapidly cleared from the plasma. However, the elimination half-lives of peptides D2 and D4 are longer, 6.7 hours and 10.5 hours, respectively, suggesting that those structural changes influenced clearance from plasma.

In vitro studies from Chapter 3 found that peptide C2 is highly susceptible to degradation from gastrointestinal enzymes yet the peptide was stable in the presence of plasma enzymes. These *in vivo* studies found peptide C2 to have the highest clearance rate among the four synthesised analogues. Peptide D2 had comparable *in vitro* permeability to peptide B2 yet the absolute bioavailability dropped by over 50%. From the *in vitro* studies, peptide D2 had a lower aqueous solubility to peptide B2 (29.9 μM and

103.6 µM, respectively). It is likely this has also impacted the *in vivo* absorption of peptide D2 seen in this study. Peptide D4 had poor *in vitro* permeability, and this is reflected in the *in vivo* study where peptide D4 has an oral bioavailability of 0.06%. Peptide D2 had an oral bioavailability of 0.17% and peptide D4 is less than half this value. Again, this highlights the difference a single stereoisomer can make to peptide absorption.

From the IV pharmacokinetic profiles, peptides B2, C2 and D2 all demonstrated rapid clearance rates from the plasma. Peptide B2, C2, D2 and D4 had clearance rates of 9.35, 11.16, 7.93 and 3.95 mL/min respectively. Peptides can be cleared from the plasma in two ways, renal clearance, and hepatic clearance (41). Hydrophilic peptides are cleared primarily through renal clearance (42), however, it is unlikely that the synthesised pasireotide analogues are hydrophilic enough for renal clearance and run the risk of reabsorption back into systemic circulation (43). It is more likely that these peptides are cleared via hepatic clearance, similar to lipophilic peptide cyclosporin A (44). Early pharmacokinetic studies of pasireotide by Bruns et al. (30) suggested that this peptide did not undergo extensive hepatic metabolism. However, pasireotide retains its Lys residue, unlike the pasireotide analogues in this study which replace Lys with more lipophilic residues.

5.4.2. Oral Pasireotide Analogues are Poorly Absorbed Across the Gut Wall

All the synthesised pasireotide analogues showed low absorption when orally delivered via suspension formulation. The oral bioavailability values for the four synthesised peptides were all less than 1%. This is not uncommon for oral peptides as majority of peptides in the literature have an oral bioavailability of less than 1% (8, 12, 41, 45). Peptide B2, the peptide with the best *in vitro* properties for oral bioavailability, also had the best absolute bioavailability of the peptide analogues with a %F of 0.39%. This peptide has an oral bioavailability 2-fold better than commercial octreotide, likely due to its lipophilicity and good solubility and permeability. The next best performing peptide was peptide C2 with an oral bioavailability of 0.35%. Again, this peptide had a better absorption than octreotide and comparable absorption to peptide B2. This is not surprising given they are enantiomer pairs. Peptide D2 and D4 had an oral bioavailability of 0.17% and 0.06%, respectively. Both peptides demonstrated lower oral bioavailability than octreotide. Little of each peptide was found in the urine post oral administration

(B2- 2.95 ng/mL, C2- 8.31 ng/mL, D2- 36.52 ng/mL, D4- 20.18 ng/mL) suggesting that majority of the unabsorbed peptide was excreted through the faeces.

For comparison, commercially available octreotide had an oral bioavailability of 0.19% which falls in the middle of the synthesised peptides' bioavailability. Here, octreotide was tested orally and intravenously as a comparator peptide for *in vivo* studies of the selected pasireotide analogues. Both pasireotide and octreotide contain ionisable residues, one Lys in the case of pasireotide and two Lys in the case of octreotide. These peptides are both somatostatin analogues and share many structural similarities, hence octreotide was deemed an appropriate substitution. Given octreotide is an ionisable peptide, and therefore hydrophilic in nature, it is likely that any absorption detected in the plasma is due to paracellular absorption rather than the target transcellular pathway (31, 46, 47). Early studies by Jaehde et al. (47) into the absorption pathway of octreotide found through confocal laser scanning microscopy that octreotide passes the epithelial monolayer primarily via the paracellular route without significant contribution of carrier mediated transport. The paracellular route is limiting to the amount of peptide that can be absorbed over a given period as the tight junctions between cells are only ~0.1% of the intestinal membrane and peptide is more likely to be cleared from the intestinal lumen than rapidly absorbed via paracellular routes (6). Given the oral bioavailability of peptides B2 and C2 surpass that of octreotide, it is likely that these peptides are being absorbed through the transcellular route rather than the paracellular pathway. This is further supported by the higher lipophilicities of these analogues which is more conducive to transcellular absorption (48-50).

Peptide structure and conformation play an important role in oral absorption. As previously discussed, there are several cases where the successful oral delivery of cyclic, lipophilic hexapeptides have yielded bioavailability as high as 28% from *in vivo* studies (16). Taking this example from White et al. (16), a cyclic hexapeptide, *cyclo*[Leu-N(Me)D-Leu-N(Me)Leu-Leu-D-Pro-N(Me)Tyr], was able to overcome issues around oral peptide delivery and record an oral bioavailability close to cyclosporin A (29% from the study by White et al.). Comparing the peptide from White et al. to synthesised peptide B2 with the highest oral bioavailability from this study, the two peptides are structurally not vastly different from one another. Peptide B2 has a structure of *cyclo*[Pro-D-Phe-D-Trp-Ala-D-Tyr-Phe], both comprising of six amino acids, backbone cyclisation, and having majority lipophilic amino acids. Yet, the peptide generated by White et al. has a significantly better oral bioavailability to peptide B2. It is possible that the amino acid composition is contributing to the high bioavailability value of 28% in the study by White et al., as a peptide comprising majority of Leu residues is more lipophilic than peptide B2. However, studies by Bockus et al. (13) show that peptides mostly comprised of Leu residues are not solely sufficient in producing orally bioavailable peptides. In fact, Bockus et al. (13) highlights the importance of stereochemistry and shape on bioavailability which is also seen in this study. The peptides in series D have a different stereochemical backbone to peptide B2, however chemically, these peptides are not too dissimilar as they are made of the same amino acids, yet each have widely different bioavailability. Peptides D4 (*cyclo*[PFwaYF])and B2 (*cyclo*[PfwAyF]) differ at 3 stereochemical centres yet peptide B2 has 6.5-fold better absorption. Similar observations can be made between peptides B2 and D2 but to a smaller extent. Structurally, peptides B2 (*cyclo*[PfwAyF]) and D2 (*cyclo*[PFwaYf]) differ by 4 stereochemical centres. Both peptides demonstrated good *in vitro* permeability yet there is a 2-fold difference in their oral absorptions. This is yet another example of the importance of stereochemistry for oral absorption.

5.4.3. Formulation is Key For *in vivo* Drug Performance

All selected pasireotide analogues demonstrated poor oral bioavailability *in vivo* when delivered as suspension formulations. This is not necessarily surprising as suspension formulations show that the drug is not fully solubilised in the formulation and remain as solid particles dispersed in the solvent. Given the aqueous vehicle of the suspension formulation, this suggests that the peptides are not fully soluble in the aqueous environment and therefore will likely not be fully soluble in the gut.

It is well established in the literature that enabling formulations can aid in the oral absorption of peptides, whether this be in the form of lipid-based formulations or permeation enhancers (9, 51-55). Cyclosporin A is currently sold on the market in several oral forms (Neoral® and Sandimmune®) but both are formulated in lipid-based formulations. This study explored the effect of a simple self-emulsifying system on the oral bioavailability of peptide B2. The formulation used was based on the formulation of Neoral®, a self-emulsifying drug delivery system (SEDDS). The results of this study found that the use of this formulation increased the oral bioavailability of peptide B2 by up to 2-fold of the concentrations found in the plasma post oral dosing of the suspension formulation. All the plasma concentrations for the oral SEDDS dosed rats were higher than their suspension formulation counterparts. In suspension formulation, peptide B2 had a %F of 0.39% and in the lipid-based formulation this value increased to up to 0.76% across the three individual rats observed. This suggests that the implementation of SEDDS formulation increases the absorption of peptides, however, the variable plasma concentrations within the three rat replicates suggests that further replicates are required to reach definitive conclusions about the extent of absorption the SEDDS formulation can provide.

Studies by Li et al. (21) have shown that lipid-based formulations can improve peptide absorption when co-formulated with lipophilic ion-paired drugs. From this study, without the use of a lipophilic ion pair, octreotide had an AUC of 4.18 ng*hr/mL when delivered orally in solution. Li et al. reports that octreotide without the presence of a lipophilic salt has an AUC of 25.8 ng*hr/mL in long chain lipid-based formulations. This is already a 6-fold improvement in absorption with a lipid-based formulation alone. Li et al. reports that this value increases to 446.1 ng*hr/mL when octreotide is ion paired with a docusate ion. This is a further 17.2-fold improvement in absorption or a 100-fold increase from neat octreotide in solution.

The improved oral bioavailability for peptide B2 using lipid-based formulation is a promising sign for further oral pasireotide analogue development in the future. It is noteworthy to mention that although peptide B2 bioavailability (0.76%) is modest, it is comparable to oral octreotide currently being sold on the market with an oral bioavailability of ~0.7% when co-formulated with Transient Permeation Enhancer® (20, 36). Therefore, although the absolute oral bioavailability of these pasireotide analogues is still low, there is potential for these formulations to translate to a useable therapeutic. These studies show that systematic structural changes to pasireotide can positively (and negatively) impact absolute bioavailability but further exploration into predicting which way these properties will be impacted in required.

5.5. Conclusion

Four synthesised pasireotide peptides, each displaying different *in vitro* physicochemical properties were tested *in vivo* for gut absorption. Each oral peptide was formulated in a suspension formulation and compared against its IV control. Select peptide B2 was additionally tested in an oral lipid-based formulation. The pharmacokinetic results revealed that all four pasireotide analogues are rapidly cleared from the plasma and into the urine. Each peptide, irrespective of backbone stereochemistry spent little time in the systemic circulation and did not distribute into tissues. The oral absorption studies found that in a simple suspension formulation, very little of the peptide can be found in the blood plasma and therefore very little absorption has occurred. This is not unlike the absorption behaviour of many of the therapeutic peptides. The use of lipid-based formulations in conjunction with peptide B2 was found to double the oral bioavailability of the peptide, suggesting that this method could be beneficial for future studies exploring new lipophilic peptides and their absorption potential for oral peptide delivery.

5.6. References

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Chapter 6 : Concluding Remarks and Future Directions

The aim of this thesis was to explore the factors that are important in developing orally available peptides. These factors include the effect of peptide structure on permeability, metabolic stability, bioactivity and *in vivo* absorption. Throughout this thesis, we have investigated the effects of increasing lipophilicity and changing backbone stereochemistry to improve the poor oral absorption of peptide therapeutics. This was achieved by designing and synthesising pasireotide analogues, each that addressed a unique hypothesis.

Linear peptides are intrinsically polar in nature due to the ionisable terminus (amino and carboxylic acid groups). Additionally, the polar amide bond has the potential for hydrogen bonding with polar solvents. Ionisable moieties can be essential for bioactivity as they form specific interactions with their binding sites. This is what contributes to peptide drug specificity and potency. However, due to peptides being highly soluble, they are unlikely to cross biological membranes through the passive transcellular route. This route is the most common absorption route of small, lipophilic drug molecules. It is important to target this passive pathway as it allows larger drug payloads to cross the membrane, as opposed to alternate absorption routes like paracellular absorption.

This thesis describes the synthesis of a series of head to tail cyclic pasireotide analogues for improved membrane permeability. Head to tail cyclisation of peptides reduces the flexibility of the peptide and aids oral peptide delivery on two fronts: increasing lipophilicity of the peptide by eliminating the presence of the terminal ionisable group and increasing the enzymatic stability of the peptide by reducing metabolism by exopeptidases and some endopeptidases. Therefore, with the goal of increasing peptide permeability in mind, the *in vitro* permeability of these analogues was measured in PAMPA assays as a surrogate for passive transmembrane transport. In addition, any consequences to peptide solubility, enzymatic stability, and peptide bioactivity were examined.

Chapter 3 of this thesis explored how changes in lipophilicity can influence *in vitro* permeability and solubility. Assessing the data solely from a passive permeability perspective, the increase of peptide lipophilicity directly correlates with a positive improvement in artificial membrane permeability. This was highlighted in the analogues where Lys4 residues were substituted with Ala4, Nle4 and Gly(2-pent)4 residues (i.e. peptides B2, B3, B4, B6 and B7 within series B). These peptide analogues exhibited increasing permeability with increases in side chain lipophilicity as shown with Ala<Nle<Gly(2-pent). However, increases in lipophilicity had negative consequences on the aqueous solubility of the peptides. This is problematic, as a drug must be in solution prior to being absorbed across the gastrointestinal wall. It is important to note that the *in vitro* PAMPA assay does not take this lack of aqueous solubility into account when calculating the permeability rate. The lower the concentration of dissolved peptide in the aqueous medium, the higher the apparent permeability of this peptide. This skews the permeability ratio, as it appears there is a smaller concentration difference across the membrane due to the undissolved peptide. This was seen with peptide B4, where the highly lipophilic peptide was unable to be fully dissolved in 5% DMSO in PBS solution. However much of peptide B4 that was able to be dissolved in solution could permeate across the simulated biological membrane. Therefore, it is important to consider peptide solubility in conjunction with the PAMPA assay as just the permeability values alone do not paint the whole picture. In fact, a balance must be struck between increasing the lipophilicity of a peptide and the subsequent decrease in its aqueous solubility. A way to address this could be to find alternative solvents that can dissolve lipophilic peptides such as lipidic solvents to make lipid-based formulations. Cyclosporin A was first delivered in olive oil as the vehicle to orally deliver the peptide (1). In addition, the orally bioavailable peptide reported by White et al. (2) used olive oil as the formulation vehicle for their oral *in vivo* study and saw an oral bioavailability of 28%. Future work to evaluate whether higher solubility (and subsequent *in vivo* absorption) could have been achieved for peptides like peptide B4 in lipidic excipients would be valuable. If a peptide demonstrates good lipid solubility, this opens the door to shift peptide design strategies to produce more lipophilic constructs with tandem enabling formulation.

Chapter 3 also explored how stereochemistry can influence *in vitro* solubility, permeability and metabolic stability of the pasireotide analogues. This work found that a single change to a chiral centre of our model peptide backbone can increase peptide solubility by over 6-fold. An example of this can be seen when comparing peptides B6 (D-Ala4) and B2 (L-Ala4), which had measured solubility values of 15.3 μM and 103.6 μM, respectively. Likewise, a similar trend could be observed when measuring *in vitro* permeability. There was a 100-fold negative shift in permeability ($logP_e$ -6.11 and -8.9 cm/s, respectively) between peptides D2 and D4 which were also stereoisomers. These examples highlight how a seemingly small change in stereochemistry can have large impacts on physicochemical properties. The influence of stereochemistry on *in vitro* enzymatic stability was also examined. Only one peptide series (series C) demonstrated susceptibility to gastrointestinal enzymatic degradation. Peptide series C is the mirror enantiomer of peptide series B, and while all the peptides within series B showed no significant degradation under the same enzymatic degradation conditions as series C, the series C peptides demonstrated high metabolism (e.g. peptide C2 has a half-life of 3.9 mins). This again, highlights the importance of peptide conformation in solution. It is likely that the conformations peptide series C are forming allow more labile moieties to be exposed to the environment than the conformation peptide series B forms. These studies all show the importance of stereochemistry as it appears to affect all the factors that contribute towards oral peptide delivery, including solubility, permeability, enzymatic stability and bioactivity.

By observing the results of this thesis in its entirety, rather than as individual physicochemical factors, we can see that across the board, peptide conformation plays an important role in many aspects of oral delivery. We observed that singular changes to peptide backbone stereochemistry could drastically change *in vitro* permeability and similarly mirror enantiomers of peptides there could be the difference between an enzymatically stable or unstable peptide. While not directly measured, these changes are likely due to the formulation of different peptide conformations in solution. Therefore, a robust method to visualise the conformations of structures in various biological conditions is required. Current methods used to explore peptide conformation include nuclear magnetic resonance spectroscopy (NMR) studies and computational modelling

(3, 4). NMR is a technique that can be used to determine molecular structure, however from a membrane permeability standpoint, lipidic solvents are not commonly used in NMR but rather deuterated chloroform to mimic a hydrophobic environment (5). The use of deuterated chloroform reduces the amount of solvent interference for proper measurements to be taken. However, chloroform does not provide the solvent environment to replicate the conformations that would be seen within the lipidic formulations, therefore the usefulness of NMR for peptide conformations in hydrophobic conditions remains limited. The same can be said about computational modelling of peptide conformations. Currently, computational models lack the complexity to simulate intricate environments such as the interface between gut lumen and intestine lining. However, as computational modelling continues to progress, this could be a useful method of determining peptide conformations (6).

The bioactivity assays explored in Chapter 4 revealed that the Lys residue is integral to the activity of the peptide. This was not surprising as it is common for peptides to contain ionisable amino acids like Lys, Arg, Asp or Glu that interact with the binding pocket of the receptor. Pasireotide and a fraction of the synthesised pasireotide analogues contain a Lys4 residue. However, the presence of ionisable residues poses an issue for oral peptide delivery as shown in Chapter 3. Despite the efforts to decrease the influence of the Lys residue by increasing the lipophilicity of the surrounding amino acid residues, these analogues did not demonstrate an adequate *in vitro* permeability measurement that would likely correlate with good *in vivo* absorption. This again shows how ionisation, and subsequent improvement in solubility, is diametrically opposed to passive permeability across a membrane and that a balance must be struck for oral peptide delivery to occur. This issue was briefly addressed with peptide B5, which used the literature reported prodrug, $COCF_3$ (7). Unfortunately, this functional group was not sufficiently labile to be a prodrug as it did not cleave under the *in vitro* enzymatic conditions used. Therefore, further investigation into alternative prodrugs that form salt bridges to mask ionisable side chains and cleave under enzymatic conditions is still required. There are several studies from the literature that report successful use of prodrugs for peptide permeability. For example, a study by Schumacher-Klinger et al. (8) found that masking of the arginine side chain with a carbamate and the aspartic acid with

an ester of cyclic RGD peptides was shown to improve cell permeation. In a similar study, Huo et al. (9) found that masking of a lysine residue with methyl groups improves the permeation of cyclic pentapeptides. These strategies could be employed in future studies to probe the utility of prodrug peptides as therapeutic agents.

The *in vitro* activity assays reported in Chapter 4 also revealed that a Tyr(Bzl)5 group in the peptide structure of pasireotide analogues aided in increasing peptide potency. The original structure of pasireotide has a Tyr(Bzl) group in position 5. This benzyl group was removed from the Tyr along with the replacement of Hyp in position 1 and Phg in position 2 with Pro and Phe, respectively to form peptide A1. This peptide had a 400-fold loss in activity. The simple reintroduction of the benzyl group to Tyr5 reintroduced activity by 18-fold. While it is not completely understood why the benzyl group reintroduced activity to such as extent given the Tyr residue is not part of the peptide's pharmacophore, it can be assumed that the benzyl group is stabilising the active confirmation in some way. It has been reported that the Phe residues in somatostatin perform a similar function, in that they stabilise the biologically active conformer through hydrophobic bonding but do not interact themselves with the receptor (10, 11). Given the improvement in bioactivity with the inclusion of Tyr(Bzl), any further work in optimising pasireotide analogues for oral delivery should consider retaining this moiety as it benefits not only the activity of the peptide but also its lipophilicity.

Chapter 5 of this thesis explored the *in vivo* absorption and pharmacokinetics of pasireotide analogues. There were some minor correlations between *in vitro* permeability and *in vivo* bioavailability. For example, peptide B2 demonstrated high *in vitro* permeability and had the best oral bioavailability of the tested analogues. In addition, peptide D4, which was not permeable from *in vitro* studies, had the lowest bioavailability of the tested analogues. Peptide C2, which had poor *in vitro* gastrointestinal stability, had the largest clearance of the analogues. However, all the tested analogues had rapid elimination from the plasma, suggesting that these peptides would not be ideal for once-a-day oral peptide delivery. Rapid elimination could be overlooked should the peptide demonstrate good oral absorption and high enough potency. However, given the low oral bioavailability of even the best performing analogue was less than 1%, and coupled with its rapid elimination, further modifications must be

made before pasireotide could be an appropriate oral therapeutic. Although there were some minor correlations between the *in vitro* properties that influence *in vivo* absorption, these correlations were not supported enough to solely rely on *in vitro* prediction to determine *in vivo* absorption. Rather, *in vivo* absorption studies remain the only way to truly measure the likelihood of good oral bioavailability.

Unlocking peptide absorption across a membrane remains one of the key barriers to achieving oral peptide delivery. This thesis supports the notion of increasing lipophilicity to facilitate uptake, however, removing key bioactive moieties like ionisable residues is unlikely to be the answer. Unless the peptide already has a hydrophobic pharmacophore, which would be a good peptide lead for oral delivery, alternative methods worth exploring include hydrophobic prodrugs that will be cleaved once the peptide has been orally absorbed. While this thesis briefly attempted this strategy, there are other hydrophobic prodrugs that can be further explored (7). N-methylation is another strategy that may prove useful in peptide absorption (12, 13). Cyclosporin A contains N-methylated amino acids, and reverse engineering cyclosporin A to determine which structural changes will lead to a nonabsorbable peptide might provide key information about how this peptide lends itself so well to oral delivery. A point to consider, however, is how N-methylation may impact peptide conformation in aqueous solution. As supported in this thesis, peptide shape remains an important consideration for oral delivery and if this aspect can be understood, oral peptide delivery will be more easily accessible. Researchers should not shy away from lipophilic peptides as further exploration into how peptides may be incorporated into lipid-based formulations remains a possibility for exploration.

While the goal of this thesis was to improve peptide permeability through systematic changes to lipophilicity and stereochemistry, each facet of oral peptide delivery cannot be considered in isolation. In fact, this thesis reports that each parameter that broadly dictates uptake (solubility, ionisation, tertiary structure) has direct consequences on others (permeability, oral absorption and activity). Take increasing lipophilicity for example; while aiding in membrane permeability, lipophilicity has a direct impact on aqueous solubility- another factor of great importance for oral delivery. The same goes for removing ionisable moieties, which aids in membrane

permeability but has severe consequences on peptide bioactivity. Each facet of oral peptide delivery cannot be considered individually and must be approached in a multifaceted manner.

Thus, oral peptide design remains a balancing act, preserving activity while enhancing solubilisation and permeability. By capitalising on enabling formulation technologies to circumvent solubility and permeability limitations, and using prodrug strategies to protect an active pharmacophore, we may be able to develop the oral peptides of the future. This thesis has laid some of that groundwork, showing how systematic changes to structure can improve uptake in some areas, yet detracts in others. In the context of pasireotide analogues, the preservation of Lys residues maintains peptide activity, while increased lipophilicity improved permeability (with an enabling lipid-based formulation allowing us to bypass associated solubility issues). *In vivo*, these constructs showed comparable absolute bioavailability levels to oral octreotide, but as was observed, further improvements are possible. The future of oral peptide delivery involves cutting edge peptide design and synthesis, using biodegradable prodrug strategies like those recently reported by RisenPharma, and N-methylation like Chugai and Roche's Luna 18 cyclic peptide demonstrate how these structural changes can translate to early clinical development (14). Coupling cutting edge medicinal chemistry to the latest technology in enabling formulations also proved successful for Merck with their MK-0616 peptide, formulated with the permeation enhancer sodium caprate entering phase III clinical trials (15). Molecular dynamics exploration into understanding how transient permeation enhancers interact with lipid bilayers, peptide drugs and intestinal bile salts under fed and fasted conditions are also being investigated to further the understanding of how co-formulation can push the barriers of oral delivery (16-18). These very recent examples show that oral cyclic peptide delivery is possible but remains a complex problem that is being solved by multidisciplinary teams.

6.1. References

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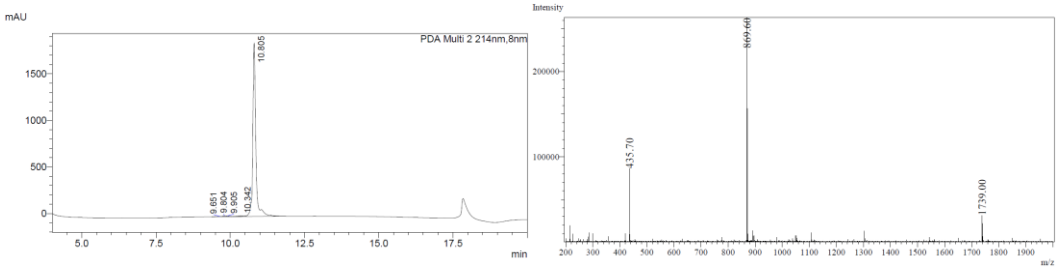
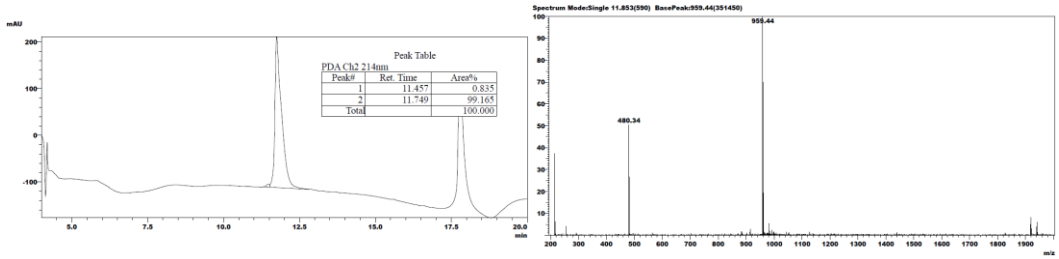
Appendix

Appendix A: Analytical LC/MS Chromatograms

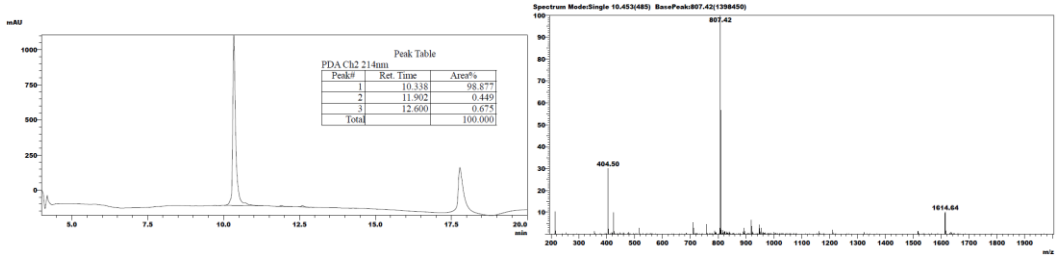
A1



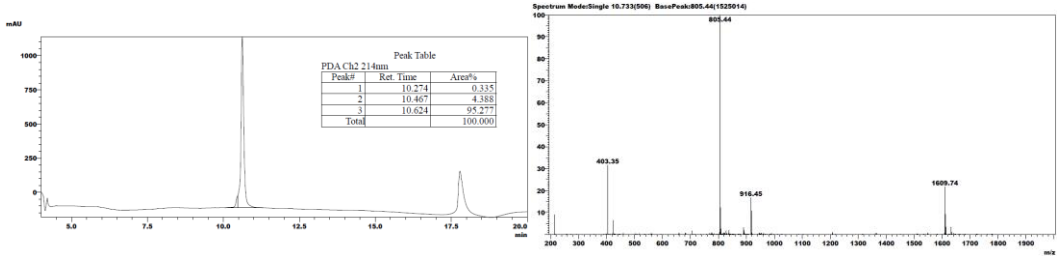
A2



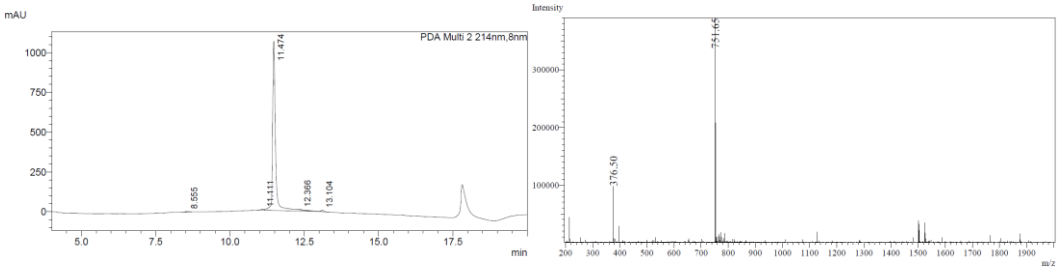
A3



A4



A5



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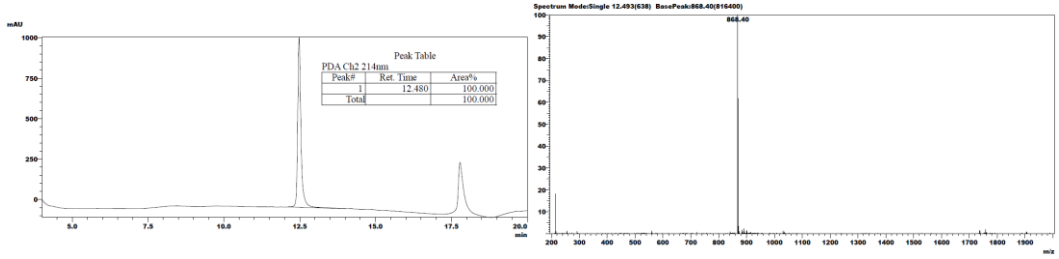

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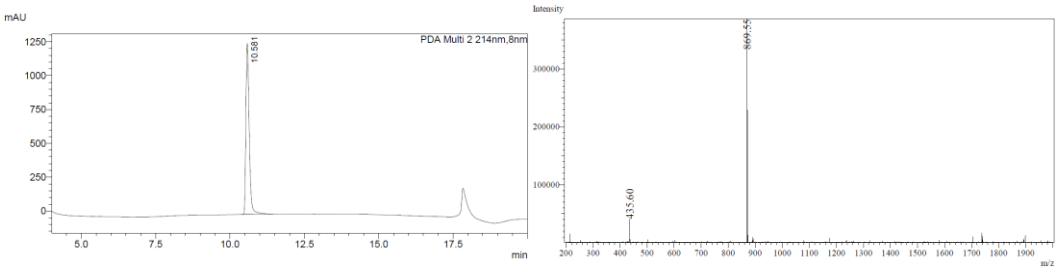

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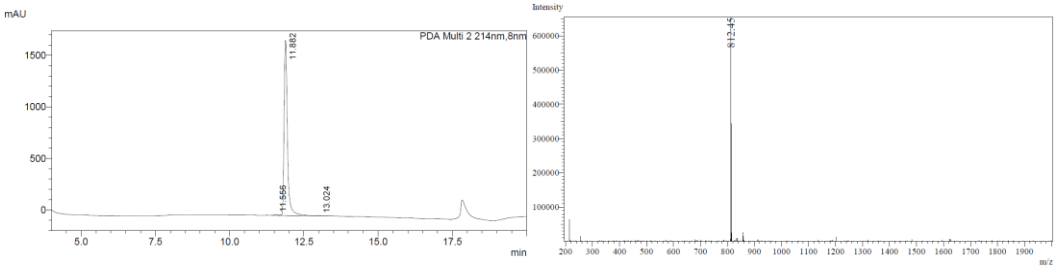

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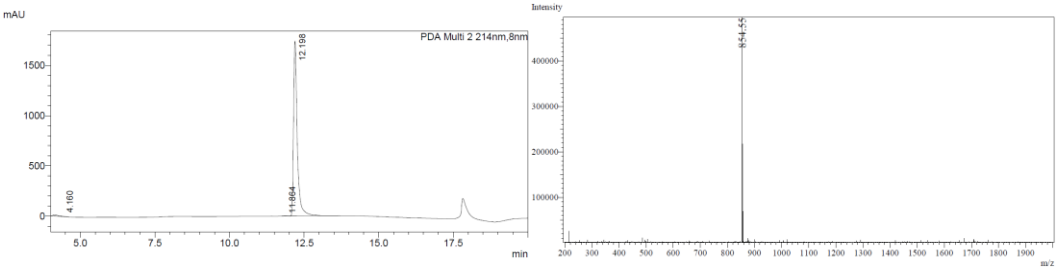

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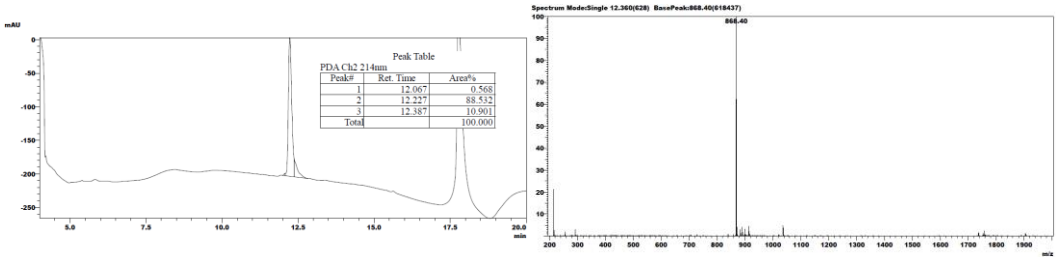

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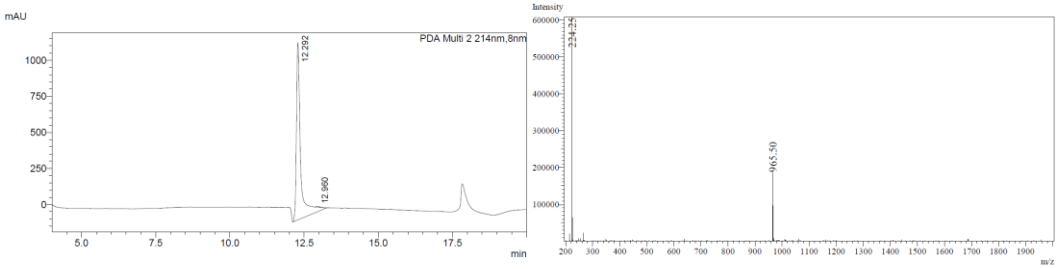

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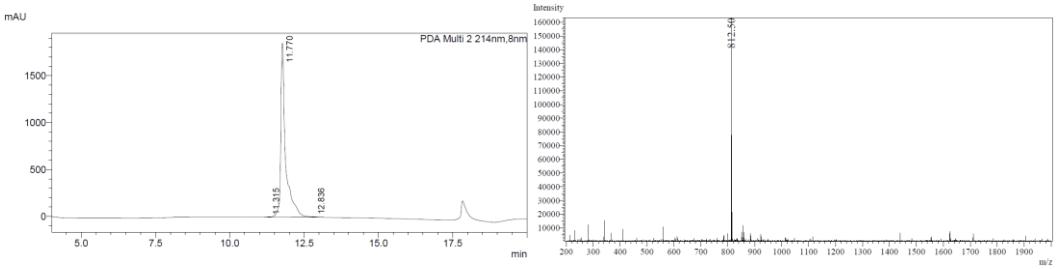

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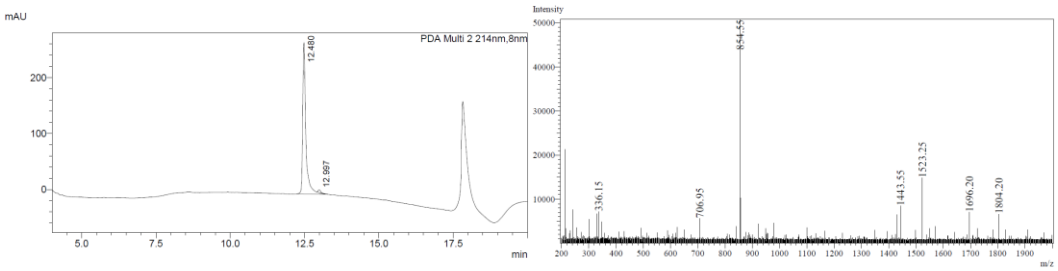

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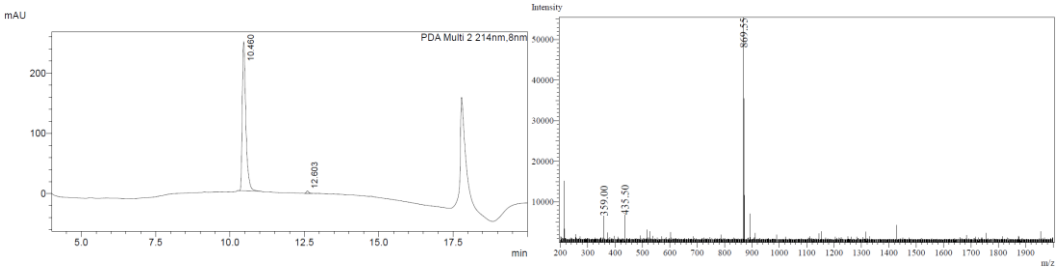

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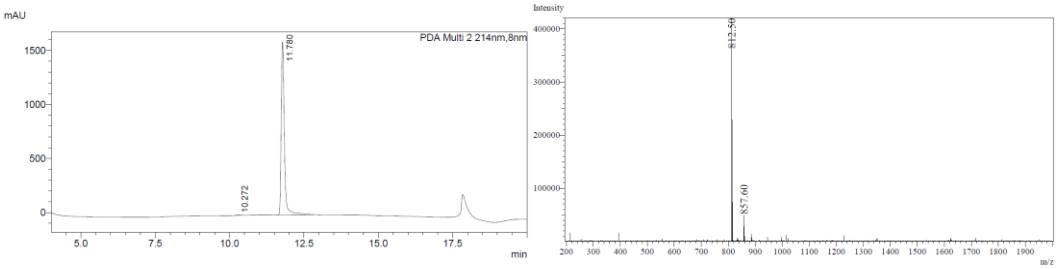

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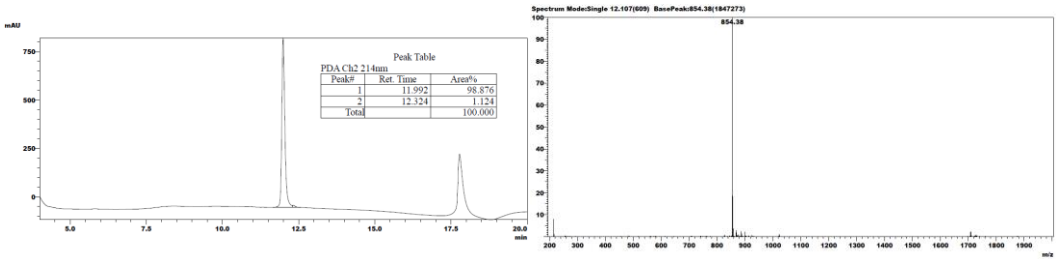

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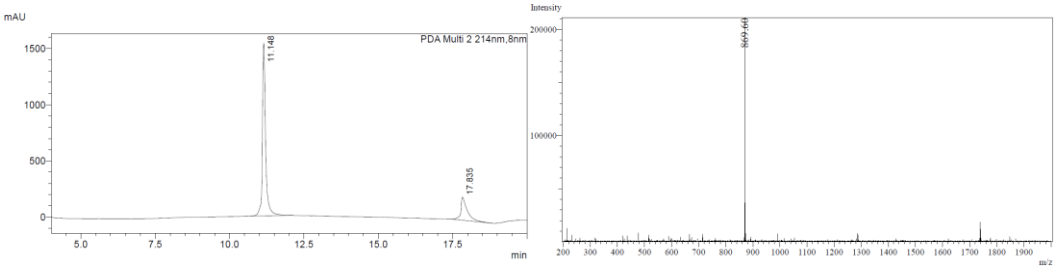

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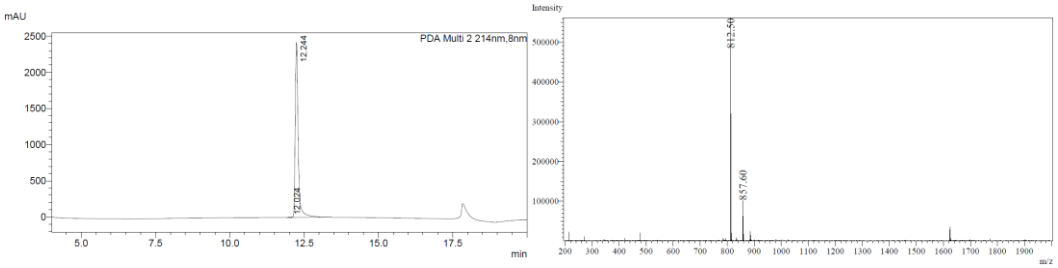

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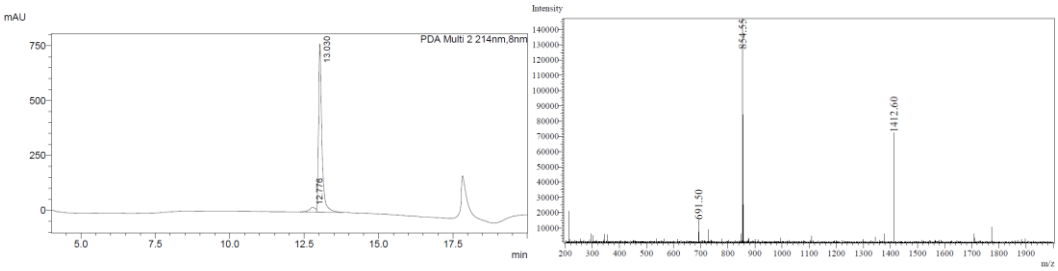

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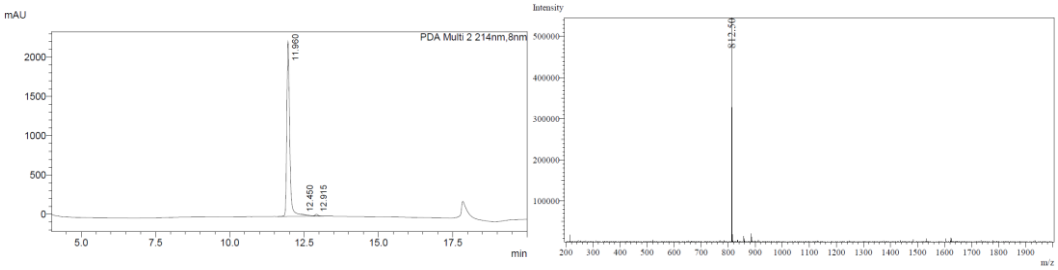

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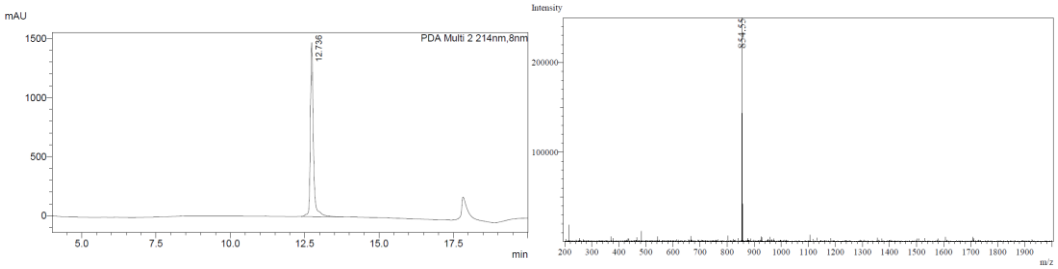

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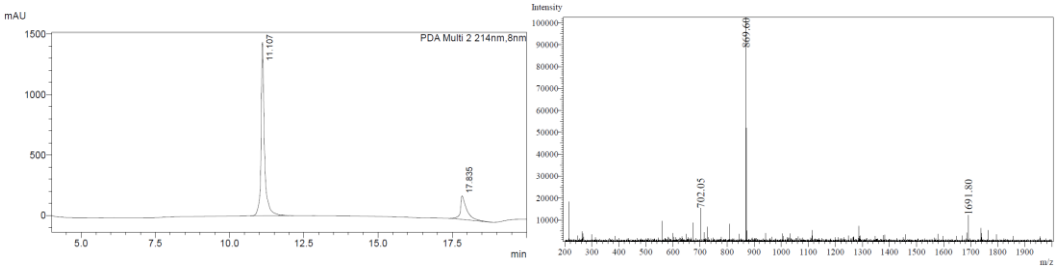

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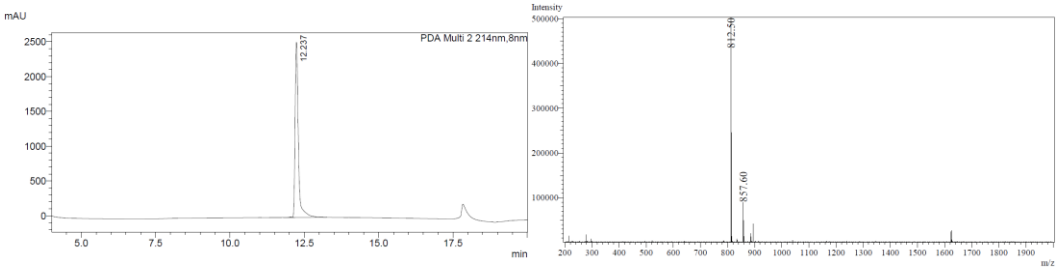

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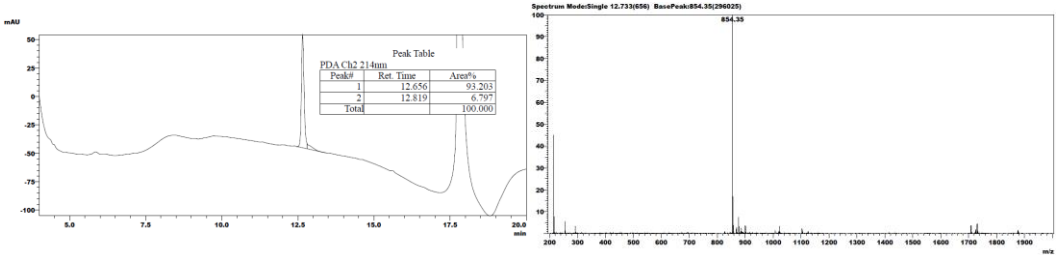

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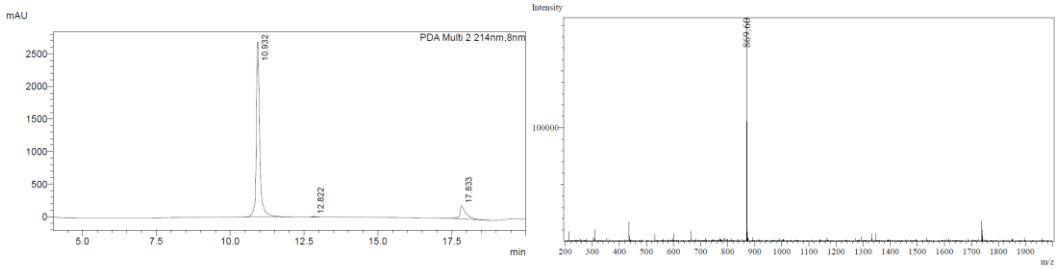

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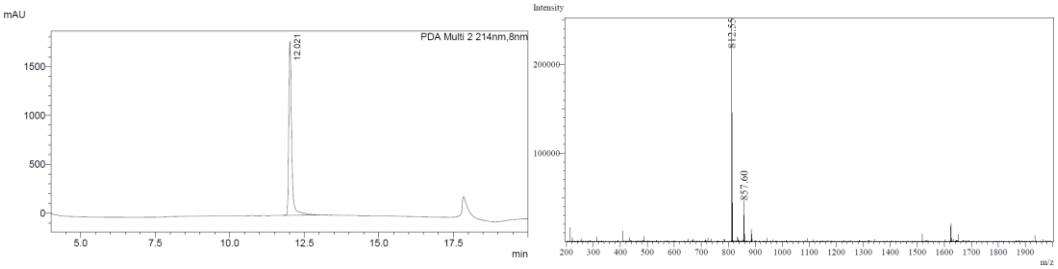

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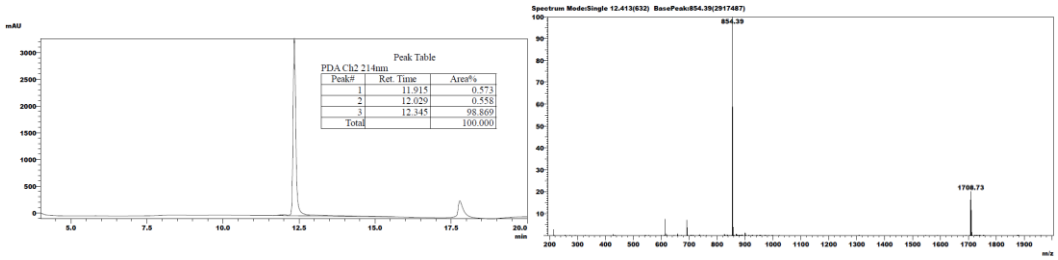


Appendix B: Bioanalytical Methods for Synthesised Peptides on LC-MS/MS

Column	Phenomenex Luna® Omega 3µm PS C18 100Å LC column 50 x 2.1mm
Guard Column	Phenomenex C18 4 x 2.0mm
Column Temp	40
Sample Temp	Room temperature
Injection Volume	2µL
Flow Rate	0.5 ml/min
Internal Standard (pasireotide)	
Retention time IS	1.567
m/z	811.30>224.30
Collision Energy	-45

Capillary KV	4.00
Interface Temp	300°C
DL Temp	250°C
Nebulizing Gas Flow	3 L/hr
Heating Gas Flow	10 L/min
Drying Gas Flow	10L/hr

Linear Gradient Elution Table				
Time (minutes)		**% Solvent A** (0.1% formic acid in H_2O)	**% Solvent B** (0.1% formic acid in ACN)	**Flow Rate (ml/minute)**
	0	90	10	0.5
	0.5	90	10	0.5
	2.5	2	98	0.5
Wash Step	2.9	2	98	0.5
	3.0	90	10	0.5
	3.5	90	10	0.5

Peptide	**Retention time (mins)**	**m/z**	**Collision Energy (CE)**
Somatostatin	1.519	546.80>120.20	-33
Octreotide	1.466	510.30>120.10	-28
B1	1.731	869.30>217.20	-55
B2	2.183	812.20>217.20	-51
B3	2.271	854.30>217.05	-53
B5	2.255	965.20>245.20	-49
C1	1.732	869.30>245.15	-48
C2	2.183	812.20>217.20	-48
C3	2.268	854.30>217.30	-55
D2	2.276	812.20>217.00	-50
D3	2.409	854.20>245.25	-44
D4	2.211	812.20>217.05	-53

Appendix C: Analytical Method Validation for Peptides B2, C2, D2 and D4

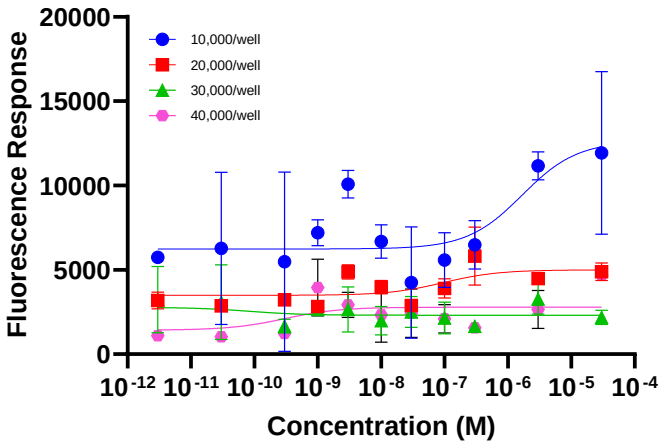
	THEORETICAL CONCENTRATION (ng/mL, average ± SEM)	MEASURED CONCENTRATION (ng/mL, average ± SEM)	ACCURACY (%, average ± SEM)	PRESCISION (%RSD, average ± SEM)
B2	10	7.12 ± 0.32	99.71 ± 0.03	10.19 ± 5.94
	50	39.71 ± 3.67	99.79 ± 0.07	7.35 ± 3.81
	500	424.61 ± 44.28	99.85 ± 0.09	8.79 ± 4.95
C2	10	9.52 ± 0.18	99.95 ± 0.02	8.52 ± 1.79
	50	47.63 ± 0.14	99.95 ± 0.003	11.54 ± 3.23
	500	617.61 ± 9.94	100.24 ± 0.02	9.35 ± 1.11
D2	10	10.93 ± 2.63	100.09 ± 0.26	6.76 ± 0.64
	50	56.46 ± 15.87	100.13 ± 0.32	9.37 ± 2.50
	500	504.92 ± 6.49	100.01 ± 0.01	6.48 ± 2.16
D4	10	9.37 ± 0.36	93.72 ± 3.60	13.74 ± 9.38
	50	47.14 ± 1.72	94.29 ± 3.44	10.83 ± 7.43
	500	536.91 ± 0.13	107.38 ± 0.03	8.73 ± 5.70

Appendix D: Image of Oral LBF containing peptide B2 used for *in vivo* studies

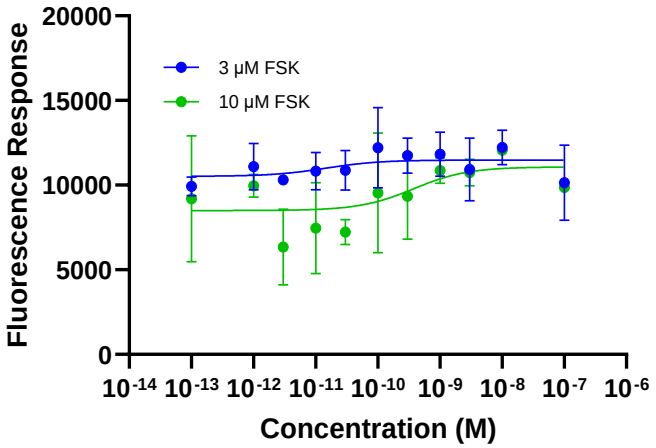
Appendix E: Preliminary *in vitro* permeabilities of pasireotide analogues

Peptide Analogue	**PAMPA logP_e (n=1)**
cyc [Pro-Phe-D-Trp-Ala-Tyr-D-Phe]	-9.26
cyc [Pro-Phe-D-Trp-D-Ala-Tyr-Phe]	-9.29
cyc [Pro-D-Phe-D-Trp-Ala-Tyr-Phe]	-6.94
cyc [D-Pro-Phe-D-Trp-Ala-Tyr-Phe]	-9.20
cyc [Pro-Phe-D-Trp-Ala-Tyr-Phe]	-9.17
cyc [Pro-Phe-D-Trp-D-Ala-Tyr-D-Phe]	-6.13
cyc [Pro-D-Phe-D-Trp-Ala-Tyr-D-Phe]	-8.53
cyc [Pro-Phe-D-Trp-Ala-D-Tyr-Phe]	-8.43
cyc [Pro-Phe-D-Trp-D-Ala-D-Tyr-Phe]	-7.72
cyc [Pro-D-Phe-D-Trp-Ala-D-Tyr-Phe]	-6.06
cyc [Pro-D-Phe-D-Trp-D-Ala-Tyr-Phe]	-9.34
cyc [Pro-Phe-D-Trp-Ala-D-Tyr-D-Phe]	-8.34
cyc [Pro-D-Phe-D-Trp-D-Ala-Tyr-D-Phe]	-6.50
cyc [Pro-Phe-D-Trp-D-Ala-D-Tyr-D-Phe]	-6.45
cyc [Pro-D-Phe-D-Trp-D-Ala-D-Tyr-Phe]	-9.55
cyc [Pro-D-Phe-D-Trp-Ala-D-Tyr-D-Phe]	-8.20
cyc [Pro-D-Phe-D-Trp-D-Ala-D-Tyr-D-Phe]	-9.04
cyc [D-Pro-Phe-D-Trp-Ala-Tyr-D-Phe]	-8.22
cyc [D-Pro-D-Phe-Trp-D-Ala-Tyr-Phe]	-8.39
cyc [D-Pro-Phe-Trp-D-Ala-D-Tyr-Phe]	-8.01
cyc [Pro-D-Phe-Trp-Ala-D-Tyr-D-Phe]	-8.45
cyc [Pro-D-Phe-D-Trp-Lys-Tyr-Phe]	< -10
cyc [Pro-Phe-D-Trp-D-Lys-Tyr-D-Phe]	< -10
cyc [Pro-D-Phe-D-Trp-Lys-D-Tyr-Phe]	< -10
cyc [Pro-Phe-Trp-Ala-Tyr-Phe]	-8.33
cyc [D-Pro-Phe-Trp-D-Ala-D-Tyr-D-Phe]	-6.99
cyc [D-Pro-D-Phe-Trp-Ala-D-Tyr-Phe]	-6.40
cyc [D-Pro-Phe-Trp-D-Ala-Tyr-D-Phe]	-6.35
cyc [D-Pro-Phe-Trp-Ala-D-Tyr-Phe]	-7.07
cyc [D-Pro-D-Phe-Trp-Ala-Tyr-Phe]	-6.93
cyc [Pro-Phe-D-Trp-Lys-Tyr-Phe]	< -10

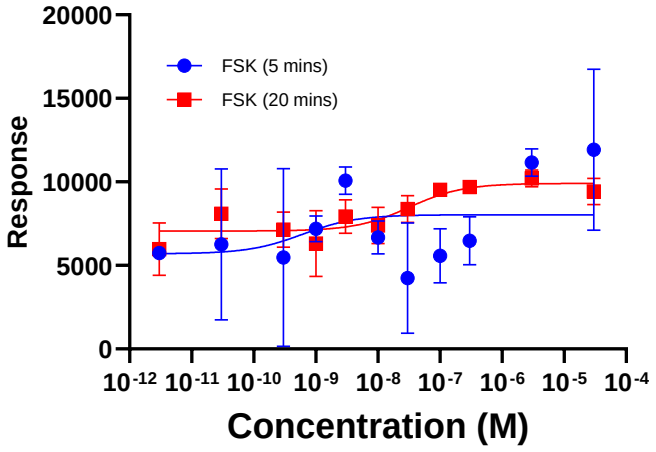
Appendix F: Optimisation Strategies used for the cAMP Activity Assay



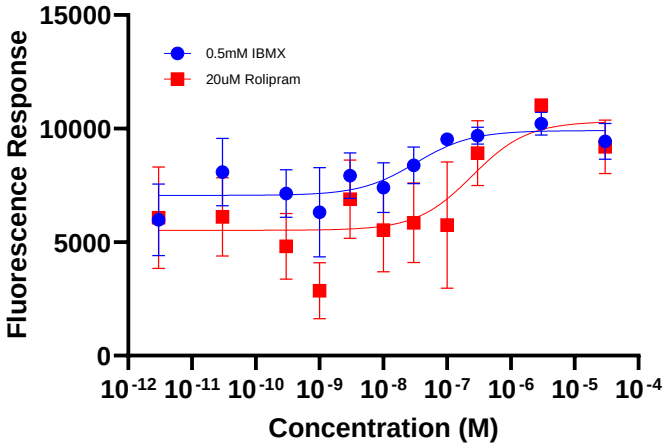
S 1. Dose-response curves of different cell densities on cAMP concentration. Ligand was octreotide using HEK293 cells stably expressing hSSTR5 receptors. Data presented as mean ± SD, n=3.



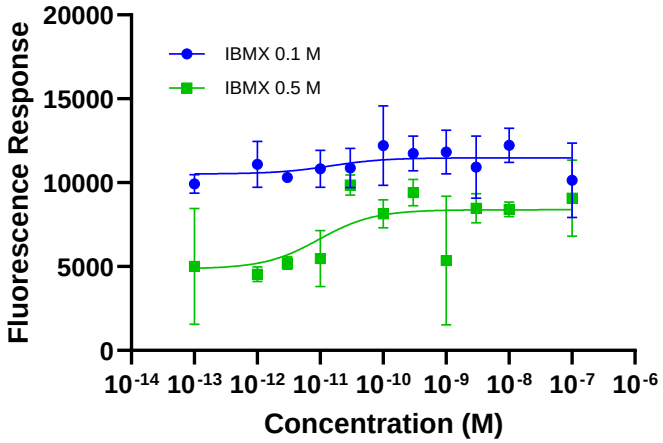
S 2. Dose-response curves of different forskolin concentrations on cAMP concentration. Ligand was octreotide using HEK293 cells stably expressing hSSTR5 receptors. Data presented as mean ± SD, n=3.



S 3. Dose-response curves of different forskolin stimulation time on cAMP concentration. Ligand was octreotide using HEK293 cells stably expressing hSSTR5 receptors. Data presented as mean ± SD, n=3.

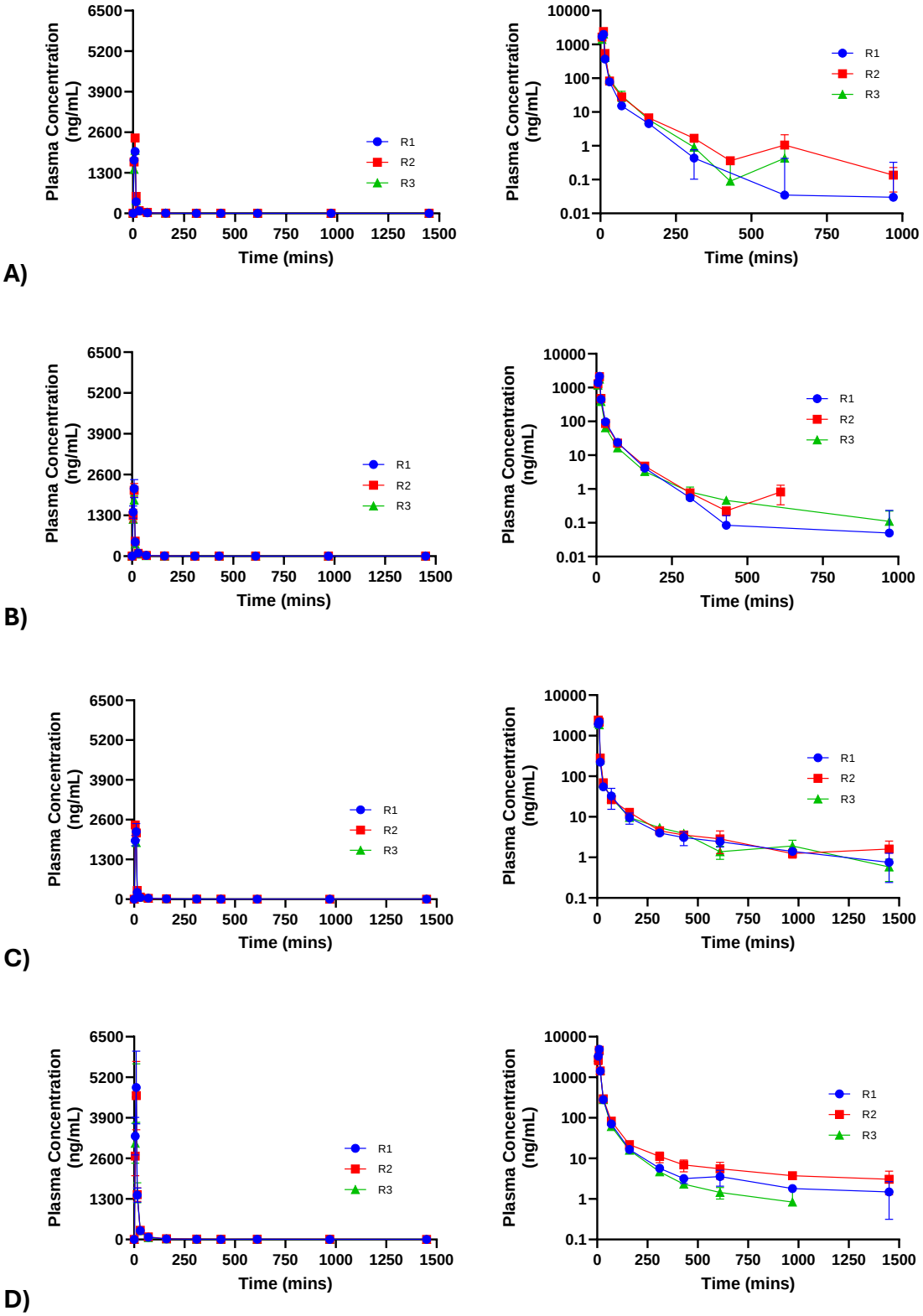


S 4. Dose-response curves of different phosphodiesterase inhibitors on cAMP concentration. Ligand was octreotide using HEK293 cells stably expressing hSSTR5 receptors. Data presented as mean ± SD, n=3.

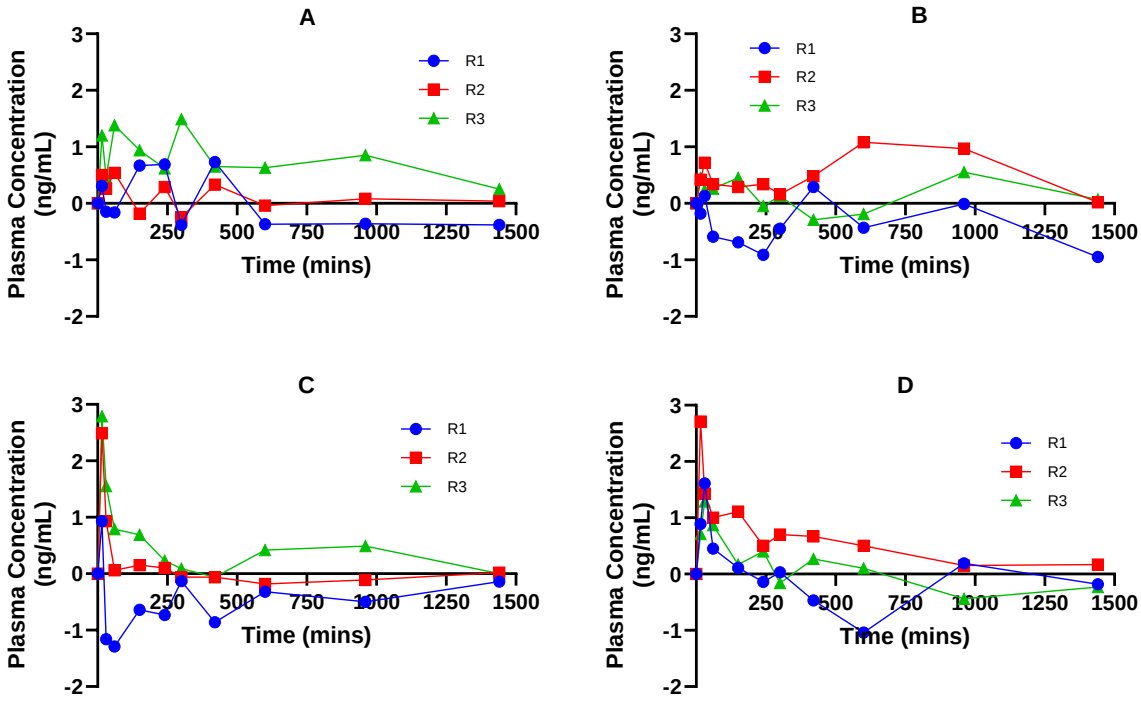


S 5. Dose-response curves of different concentrations of phosphodiesterase inhibitor IBMX on cAMP concentration. Ligand was octreotide using HEK293 cells stably expressing hSSTR5 receptors. Data presented as mean ± SD, n=3.

Appendix G: Individual rat peptide plasma concentration vs time profiles



S 6. IV Individual rat plasma time concentrations on a linear scale (left) and log scale (right). A) peptide B2, B) peptide C2, C) peptide D2, D) peptide D4.



S 7. Individual rat plasma time concentrations of oral suspension. A) peptide B2, B) peptide C2, C) peptide D2, D) peptide D4

Appendix H: Full List of References

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