The Ghrelin Receptor and its Appetite for Highly Potent Ghrelin Analogues

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Abstract

The growth hormone secretagogue receptor-1a (GHS-R1a) is expressed in many human tissues, most notably the hypothalamus, and causes an increase in appetite upon activation by its endogenous ligand, ghrelin. GHS-R1a is differentially expressed in malignant compared to benign tumours. Therefore, there is an interest in developing GHS-R1a-targeted peptides as novel drugs to modulate signaling for diseases such as cancer cachexia and obesity and to image the receptor for disease diagnosis and following progression.

Chapter 2 discusses a fluorescently labelled ghrelin analogue for imaging GHS-R1a in ex vivo biopsy analysis and in vivo distribution studies. The analogue was created through side-chain cyclization resulting in an improved affinity and stability compared to natural ghrelin. This stapled peptide was used as a cancer cell-specific fluorescent stain.

G7039, a peptidomimetic ghrelin agonist (IC$_{50}$ 5.2 nM/EC$_{50}$ 0.18 nM), underwent structure-activity relationship studies to generate improved ligands and positron emission tomography (PET) agents. The first generation peptidomimetic (Chapter 3) [1-Nal$^4$,Lys$^5$(4-fluorobenzoyl-4-FB)]G7039 (IC$_{50}$ 69 nM/EC$_{50}$ 1.1 nM) was radiolabelled with $^{18}$F in a radiochemical yield of 48%, radio purity of ≥ 99%, and molar activity of ≥ 34 GBq/μmol. Despite success in radiolabelling, its solubility (cLogP = 8.76) and binding affinity needed improvement.

The second generation peptidomimetic (Chapter 4) [Tyr$^4$,Lys$^5$(2-fluoropropionyl (2-FP))]G7039 (IC$_{50}$ 0.28 nM/EC$_{50}$ 0.12 nM) had improved binding and lipophilicity (cLogP = 4.36). Labeling of this ligand was low yielding, however, a unique H-bond interaction was identified with molecular docking.

The third generation (Chapter 5) required a modified prosthetic group (2-FP to ammonium methyltrifluoroborate-AMBF$_3$) to radiolabel in higher yields, resulting in [Tyr$^4$,Lys$^5$(AMBF$_3$)]G7039 (IC$_{50}$ 0.85 nM). This compound was radiolabelled in a single step and with improved radiochemical data.

Finally, in Chapter 6 a homobivalent G7039 ligand (IC$_{50high}$ 0.43 nM:IC$_{50low}$ 0.42 pM /EC$_{50}$ 1.8-2.1 nM) was found to bind to GHS-R1a homo-oligomer and was designed to study GHS-R1a homo-oligomerization. Differential signalling with the GHS-R1a
homo-oligomer was observed by measuring cellular signals such as β-arrestin, ERK, and gene reporters. A successful series of GHS-R1a targeting probes have been synthesized and characterized with applications driven towards imaging and therapy.
Keywords

Molecular imaging, prostate cancer, ovarian cancer, positron emission tomography, imaging agents, growth hormone secretagogues, GHS-R1a agonists, GHS-R1a, ghrelin, oligomerization
Summary for Lay Audience

Within health care there is a growing need to better diagnose and monitor human diseases to have the best possible outcome for the patient. Improvements in disease diagnosis and monitoring is due to new and emerging biomarkers (human proteins) that can be targeted by fluorescent and/or radioactive pharmaceuticals. These various pharmaceuticals can also allow us to study a biomarker from a molecular pharmacology standpoint (i.e. signalling and regulation). The pharmaceuticals that are designed to image human diseases can also have the dual purpose in treating the human disease by targeting and regulating the biomarkers.

This thesis describes the development of chemical tools that target a very attractive G-protein coupled receptor (a specific family of human proteins): the growth hormone secretagogue receptor-1a (GHS-R1a). This is a protein that is found on the cellular membrane of human tissues such as the heart, lungs, and brain. GHS-R1a is an attractive protein target because of its wide range of physiological functions in the human body including metabolism, cardiac output, depression, and neuroprotection. This protein is also regulated differently when there is disease present. Within malignant prostate, ovarian, lung, and uterine cancer the GHS-R1a has greater expression. The upregulation or downregulation of GHS-R1a can change the outcome of human diseases, leading to worse or better prognosis. GHS-R1a is also highly promiscuous as it couples (hetero-oligomerizes) with many other GPCRs (i.e. dopamine, melanocortin), as well as it has been shown to couple (homo-olgiomerize) with itself. When it couples with itself or other GPCRs there are changes to the normal human cellular events leading to different physiological outcomes in humans. This can alter the human disease to make the prognosis either worse or better.

Overall in my PhD I was able to develop several different styles of drug molecules targeting GHS-R1a. Each of these drugs was modified in such a way as to have applications in either imaging or therapy. This work opens the doors to further drug development to study this protein in human physiology and disease.
Co-Authorship Statement

Chapter 2 (performed 95% of the experimental work in this chapter) is published in Peptide Science where I am the first author, and only student author on this paper. I performed all the peptide chemistry and evaluation of the peptide-based probes. Help was provided by Dr. Trevor Shepherd (current affiliation: Department of Anatomy and Cell Biology, University of Western Ontario) with cell line development and characterization. I also performed all of the fluorescent imaging. The various authors were involved in the editing of the manuscript.


Chapter 3 (performed 45% of the experimental work in this chapter) is a published article in European Journal of Medicinal Chemistry, where I’m second author. This was a continuation from the M.Sc. thesis of Milan Fowkes (current affiliation: Department of Orthopedics Rheumatology and Musculoskeletal Sciences, Oxford University, England). Milan Fowkes performed the structure activity relationship studies for the various classes of peptidomimetics, including the characterization of the peptidomimetics by NMR and HPLC-MS. He also performed the competitive binding assays on the various peptidomimetics. I worked on the improvement of radiosynthesis techniques for the lead compound, further synthesis of peptides and small molecules, cell line development and evaluation, compound stability assay, cellular uptake, cell preparation for animal work, radiosynthesis for animal work, and participated in the writing of the paper (sections involving the radiosynthesis of the tracer, as well as the evaluation of the tracer both in vitro and in vivo).


Chapter 4 (performed 90% of the experimental work in this chapter) is a manuscript that is in preparation. All of the peptides in this chapter were developed and evaluated by me (synthesis, purification, and characterization of the peptides, as well as
the binding affinity and BRET assay). Pierre Thibeault (current affiliation: Department of Physiology and Pharmacology, University of Western Ontario) was involved with construct development in the laboratory of Dr. Rithwik Ramachandran (current affiliation: Department of Physiology and Pharmacology, University of Western Ontario). Dr. Jinqiang Hou (current affiliation: Department of Chemistry, Lakehead University, Thunder Bay) performed molecular docking experiments for this project.


**Chapter 5** (performed 95% of the experimental work in this chapter) is a manuscript that is in preparation. It is in collaboration with Dr. David Perrin (current affiliation: University of British Columbia Chemistry Department). He provided the AMBF3 prosthetic group as well as guidance in labelling this group. I performed chemical synthesis, radiosynthesis, cellular uptake and evaluation of the lead tracer in this project.

**Chapter 6** (performed 50% of the experimental work in this chapter) is a manuscript that is in preparation. All of the peptides were synthesized and evaluated for binding affinity by Dr. Jinqiang Hou (current affiliation: Department of Chemistry, Lakehead University, Thunder Bay). Pierre Thibeault (current affiliation: Department of Physiology and Pharmacology, University of Western Ontario) was involved with construct development/signalling assays in the laboratory of Dr. Rithwik Ramachandran (current affiliation: Department of Physiology and Pharmacology, University of Western Ontario). I performed a majority of the cell line development, signalling assays (BRET, homo-oligomer assay, gene reporter, and western blotting), as well as construct development.
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I will begin by thanking Dr. Len Luyt for his guidance and mentorship throughout my PhD. He was always able to help no matter how busy his schedule was. I also want to acknowledge his support in sending me to a wide variety of conferences that enhanced my knowledge in the fields of organic and peptide chemistry, as well as molecular imaging.

My first acknowledgement is to my Dad for supporting me and my decision to pursue graduate work. He was always there when I need support after many tough days in the lab. He gave me my most valuable lessons in life; to never to back down from a challenge. He was always encouraging and pushing me to be the best at my craft and I wouldn’t be graduating my PhD today without him in my life.

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Next, I want to thank my lab mates for their guidance and participation in my various projects that allowed me to have such a wonderful time as a PhD student with the challenging projects that I took on.

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I would like to personally thank Dr. Mark Milne for assistance in the lab with both experimental design and writing of my manuscripts. Hopefully I can collaborate with Mark in the future.

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I have created bonds and memories with so many over the past 5 years and this is a chapter in my young life thus far that I won’t forget and will be forever grateful to those that have been here for me. I feel like this acknowledge list can be a thesis in itself, but I will refrain from writing more and get on to writing Chapter 1.
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List of Abbreviations, Symbols, and Nomenclature

AMBF$_3$: ammonium methyltrifluoroborate

$[^{18}\text{F}]$FBA: 4-$[^{18}\text{F}]$-fluorobenzoic acid

$[^{18}\text{F}]$FDHT: $[^{18}\text{F}]$fluorodihydrotestosterone

$[^{18}\text{F}]$NFP: 4-nitrophenyl 2-$[^{18}\text{F}]$fluoropropionate

$[^{18}\text{F}]$FPBZA: $[^{18}\text{F}]$N-(2-diethylaminoethyl)-4-[2-(2-fluoroethoxy)ethoxy]ethoxy benzamide

$[^{18}\text{F}]$SFB: N-succinimidyl-4-$[^{18}\text{F}]$fluorobenzoate

1H-NMR: proton-nuclear magnetic resonance

1-Nal: 1-naphthylalanine

2-FP: 2-fluoropropionyl

2-FPA: 2-fluoropropionic acid

2-Nal: 2-naphthylalanine

4-$[^{19}\text{F}]$FBA: fluorobenzoic acid

4-FB: 4-fluorobenzyol

64Cu-CB-TE2A-AR06: 64Cu-4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo(6.6.2)hexadecane-PEG4-D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH$_2$

3D: three dimensional

2-FPA: 2-fluoropropionic acid

μPET: micro positron emission tomography

$[^{18}\text{F}]$-FDG: $^{18}\text{F}$-fluorodeoxyglucose

ACN: acetonitrile

AEEA: aminoethanolethylamine

Alloc: allyloxy carbonyl
BSA: Bovine Serum Albumin
BOC: t-butoxycarbonyl
BPH: benign prostatic hyperplasia
BRET: bioluminescent resonance energy transfer
Bq/cc: Becquerel per milliliter
CA: contrast agents
CB-TE2A: cross-bridged-tetraazamacrocycle 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo [6.6.2]hexadecane
CHO cells: Chinese hamster ovary cells
Cl: chemical ionization
CL: cytoplasmic loop
cpm: counts per minute
CRE: cyclic AMP response element
CSP: chemical shift perturbation
CT: computed tomography
Cy5: cyanine 5
Cy7.5: cyanine 7.5
c-zone: central zone
CXCR4: C-X-C chemokine receptor 4
D-2-Nal: D-2-naphthylalanine
d: days
d.c.: decay corrected
DAPI: 4’,6-diamino-2-phenylindole
DCM: dichloromethane
DIPEA: N,N-diisopropylethylamine
DMEM: dulbecco’s modified eagles medium
DMF: dimethylformamide
DMSO: dimethylsulfoxide
DOTA: 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
Dpr: diaminopropionic acid
DRE: digital rectal exam
DW-MRI: diffusion weighted MRI
e−: electron
EC50: half-maximal effective concentration
EDC: N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide
EDTA: ethylenediaminetetraacetic acid
EF: enkephalin
EL: extracellular loop
EOS: end of synthesis
ERK1/2: extracellular signal-regulated kinases-1/2 (p44/42)
pERK1/2: phosphorylated extracellular signal-regulated kinases-1/2 (p-p44/42)
ESI+: electrospray ionization
equiv: equivalents
eV: electronvolt
FBS: fetal bovine serum
FMOC: fluorenylmethyloxycarbonyl
G418: geneticin
GBq/μmol: gigabecquerel per micromole

Gd: gadolinium

GH: growth hormone

GHRP: growth hormone releasing peptide

GHS: growth hormone secretagogues

GHS-R1a: growth hormone secretagogue receptor-1a – ghrelin receptor

GHS-R1b: growth hormone secretagogue receptor-1b

GOAT: ghrelin-O-acyl transferase

GPCR: G-protein coupled receptor

GRPr: gastrin releasing peptide receptor

h: hours

HATU: 1-{Bis(dimethylamino)methylene}-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate

HCTU: (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate)

HEK293: human embryonic kidney 293 cells

HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

HPLC: high-performance liquid chromatography

HeyA8: high grade ovarian serious adenocarcinoma

HRMS: high resolution mass spectrometry

IC₅₀: half maximal inhibitory concentration

Inp: isonipecotic acid

Kₐ: dissociation binding constant

kDa: kiloDaltons
Kᵢ: binding affinity
LC: liquid chromatography
m: minutes
αMSH: α-melanocyte-stimulating hormone
MC1R: melanocortin-1 receptor
MeOTf: methyl trifluoromethansulfonate
m/z: mass over charge ratio
MBq: megabecquerel
MRI: magnetic resonance imaging
MSH: melanocyte-stimulating hormone
MHz: megahertz
Mtt: methyl trityl
mV: megavolts
n: number
NFAT: nuclear factor of activated T-cells
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NHS: N-hydroxysuccinimide
nM: nanomolar
Nle: norleucine
NMR: nuclear magnetic resonance
OVCAR3: human ovarian carcinoma cells-3
OVCAR8: human ovarian carcinoma cells-8
PBS: phosphate buffered saline
PCa: prostate cancer
PEG: polyethylene glycol
Pen-Strep: Penicillin-Streptomycin
PET: positron emission tomography
PIN: prostate intraepithelial neoplasia
pM: picomolar
PIN: prostatic interneoplasia
PSMA: prostate specific membrane antigen
p-zone: peripheral zone
RP: reverse phase
RPMI 1640: roswell park memorial institute
rpm: rotations per minute
SPECT: single-photon emission computed tomography
SPPS: solid-phase peptide synthesis
SUV: standard uptake value
SUVR: relative standard uptake value
SRE: serum response element
SRF: serum response factor
TBMe: tert-butyl methyl ether
TFA: trifluoroacetic acid
Thi: D-2-thienyalanine
TIPS: triisopropylsilane
TM: transmembrane
TRUS: trans-rectal ultrasound

uHPLC: ultra-high-performance liquid chromatography

V: voltage

$V_e$: neutrino

$\beta^+$: beta positron

VOI: volume of interest
Chapter 1

1 Introduction

1.1 Peptides as Molecular Imaging Tools

Abnormal expression of proteins is largely due to dysregulated or mutated genes that can be seen across a wide variety of diseases such as cancer and cardiovascular disease. These dysregulated proteins are considered hallmark biomarkers to track the progression of and treat human diseases. Tracking and monitoring these biomarkers are the reason in the development of molecular probes to monitor these biomarkers in real time. Molecular imaging is a wide and ever-growing field that has allowed many researchers and clinicians to be able to better understand the complexity of living systems. It has allowed for non-invasive techniques to diagnose and treat patients in a time sensitive manner. This field is enabling researchers and clinicians to visualize biochemical processes in both normal and disease states. This is performed in real time on tissues, living cells, and whole organisms. There are many chemical classes of molecular imaging agents such as small molecules, peptides, aptamers, engineered proteins and nanoparticles. However, the focus within the thesis will be the development of peptide molecular imaging agents to target a specific cell surface receptor. Molecular imaging is a multidisciplinary field that involves many scientific fields such as biophysics, biology, bioengineering, molecular biology, chemistry, and clinical sciences. All of these disciplines work together in designing, testing, and validating a molecular imaging probe. However, chemistry has been a key discipline for many years in the field of molecular imaging, and in many cases has been the rate-limiting step, due to complications in chemical synthesis with challenges associated with chemical purity, yields and complex synthetic routes. Once a compound begins the crucial stages of a clinical trial it can also fail to make it as a clinically used pharmaceutical due to the complications that may occur in human physiology such as off target effects and toxicity. This is also another rate-limiting step in the development of novel pharmaceuticals.

Molecular imaging is widely used in the detection and treatment of cancer and has many advantages in this area. Molecular imaging techniques can perform whole body
readouts in an intact system, which is more reliable than *in vitro* or *ex vitro* studies\(^3\). This helps decrease the work load and accelerate the diagnosis and treatment plan for patients\(^3\). By doing so the patient can have a better overall all prognosis. Molecular imaging of cancer patients also allows for longitudinal studies being performed on the same patient to assess the progression of the diseases state and validate the success of the selected treatment plan\(^3\). Therefore, the detection of molecular markers such as cell surface receptors can allow for an earlier diagnosis, earlier treatment, and an overall better prognosis for the patient\(^3\). All imaging probes have some commonalities in that they must be biocompatible, have target specificity, high disease-to-normal-tissue signal ratio, and rapid clearance. There are three main components to most imaging agents; a targeting moiety, a linker, and an imaging moiety (Figure 1.1).

**Figure 1.1 - Typical three component imaging agents.** The targeting moiety is specific to the biomarker, the linker provides space between the targeting moiety and the imaging moiety, and the imaging moiety allows for visualization of the biomarker.

The targeting moiety is selective to only one target and typically needs to bind with a high affinity (high k\(_i\)). The targeting moiety should also possess a high selectivity, a strong metabolic stability, and potentially membrane permeability if the target of interest is an intracellular protein. The linker provides enough space between the targeting and imaging moiety to ensure the imaging moiety does not interfere with binding to the receptor.

The development of a novel molecular imaging agent has a number of key steps in order to have a successful probe in the end. These key steps are highlighted in Figure 1.2 below. These steps are guidelines that help direct the research in a uniform fashion towards the end goal, however in some cases these steps are altered due changes in
project design such that the imaging agent might target another biomarker more selective then the original choice. First (step one Figure 1.2) a disease state must be chosen, such as a certain class of cancer. The disease state that is being targeted in this thesis is prostate cancer (PCa). Cancer is only one type of disease that can be targeted by the use of molecular imaging agents; however, this general scheme can be used in the development of imaging agents used in the detection and monitoring of other diseases such as cardiovascular disease. A biological target that is highly expressed must be identified within the disease state chosen (step two Figure 1.2). Identification of this biomarker is done through various techniques such as genotyping a population of people with a common disease. This is usually followed up by pathology of the tissue identifying the biomarker with an antibody or chemical stain. Typically, a target that may show differential expression between disease states (i.e. malignant vs. benign) is preferred along with a target that is overexpressed within the chosen disease state (step three Figure 1.2). Choosing a target that is unexpressed would not be beneficial since the expression is far too low for most imaging techniques. An imaging technique that satisfies the signaling target must be decided upon before devising a synthetic route for the molecular agent as the addition of the signalling entity is incorporated in the chemical design of all the probes being studied. For instance using fluoride-18 (^{18}\text{F}) in conjunction with positron emission tomography (PET)\(^2\). There are several imaging modalities that exist such as single-photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI), however the focus of this thesis is utilizing PET in conjunction with the radioisotope ^{18}\text{F}. The major reason for this choice is the wide spread use of both this imaging technique and isotope among healthcare. It will be discussed later that one of the most widely used PET imaging agents is fluorinated glucose. The next stage is the design and chemical synthesis of a molecular imaging probe (step four Figure 1.2). This is because chemical synthesis is time consuming and requires careful planning to create a target that will have a high affinity for the receptor\(^4\). This requires the consideration that the imaging probe has a binding property with a fast on-rate (k_{on}) and a slow off-rate (k_{off})\(^3\). Also, the imaging probe will need to have appropriate pharmacodynamic and pharmacokinetic properties for in vivo use. Pharmacokinetics is the effect the body has on the chemical probe such as absorption, distribution, metabolism, and excretion\(^5\). These
properties can be controlled based on the chemical structure of the probe, including the linker, and the signalling entity. In the case of using peptides as imaging agents the chemical nature of the amino acids can directly affect the pharmacokinetic properties. However, pharmacodynamics refers to the chemical probes’ effects on the body such as adverse effects and off target effects. Again the chemical nature of the probe greatly dictates the pharmacodynamics. *In vitro* assays help confirm the effectiveness of the probe on the target under non-radioactive conditions before moving into animal studies (step five Figure 1.2). These types of assays involve competitive radioactive probes to assess the binding of novel chemical probes, and this competitor itself can change the chemical and structural properties of the novel chemical probe and the target itself. *In vitro* studies are able to directly assess product performance on an isolated target. However directly assessing an isolated target with success does not directly translate to the same success in the initial animal models. This is because the isolated target is most likely a purified protein or an overexpressed protein in a given cell line or membrane fragmentation preparation. Typically experiments on a chemical probe switch between step four to five (Figure 1.2) until an optimal molecular probe is identified with specificity and selectivity for the given target. When designing a molecular imaging probe, a site for labeling must be considered at this stage (step six Figure 1.2) making it easier for a synthetic scheme to label the molecular probe with the appropriate radionuclide such as $^{18}$F. Incorporation of the imaging modality after the design of the core pharmacophore can leads to changes in both selectivity and specificity for the given target leading to further optimization and synthetic challenges such as the location to attached the imaging component. Once the compound can be successfully labelled with high yield, purity, and reproducibility the study can progress into an appropriate animal model for the disease being studied (step seven Figure 1.2). *In vivo* studies are considered the gold standard in assessing the pharmacokinetics and pharmacodynamics of the probe. Increasing the animal number within all desired control groups (step eight Figure 1.2) ensures that the images are reproducible among many trials. This sometimes requires the study to go back to step four (Figure 1.2) to improve the pharmacokinetics and pharmacodynamics, which in turn will improve the PET images in order to have a higher level of confidence when identifying cancerous tissue in the image. Then finally
clinical studies (step nine Figure 1.2) with a lead compound will begin if there has been success at steps one through eight. Clinical studies pose their own challenges as now the molecular imaging agent will have been challenged against human immunity and a potential increase in off target effects due to the larger number of biomarkers, they regulate human pharmacology and physiology.

Figure 1.2 - The nine critical steps in designing a successful imaging probe. These steps are just a guideline in designing a successful imaging agent. As these steps progress changes can occur with both the imaging agent and the target of interest

1.2 Prostate Cancer
Within the scope of this thesis the disease state of interest is prostate carcinoma (PCa). The prostate is a major male reproductive organ that is involved in the addition of nutrients and fluid to the sperm. It takes the shape of a walnut that is divided into right and left lobes and is located on the ventral side of the rectum below the bladder. Cell growth in the prostate is modulated by testosterone, regardless whether the cells are cancerous or healthy. There are three zones of the prostate that include the peripheral zone (p-zone), the central zone (c-zone), and the transitional zone (t-zone). It is known that about 75% of malignant tumours originate in the p-zone, and typically benign masses are found in the t-zone. PCa is the most common cancer among Canadian men. The statistic as of 2018 is that one in seven men will be diagnosed with PCa in their lifetime. PCa acts similar to that of all cancers as it has uncontrolled growth, abnormal structure,
and has the ability to metastasize. PCa can persist for many years without showing noticeable symptoms, hence the importance in the development of imaging agents for earlier detection. According to Prostate Cancer Canada (PCC) there are five major risk factors that males should be aware of in order to ensure early diagnosis of the carcinoma. It is recommended that men over the age of 40 should be tested for prostate specific antigen (PSA) levels to establish a baseline and monitor fluctuations as the years pass. PSA is a protein produced in the prostate gland that is secreted into the seminal fluid. There is free PSA and complex PSA. Free PSA moves freely in the blood stream, and complex PSA is associated with other blood proteins such as human serum albumin. The PSA test is a blood test that measures the amount of PSA found in blood protein and above 4 ng/mL is considered higher than normal. Free PSA is usually associated with benign prostatic hyperplasia (BPH). However, this relatively non-invasive test cannot stage the progression of the cancer, and also this is not a definitive diagnosis, and therefore further testing is required. Elevated PSA levels are also associated with prostate inflammation, and this is highly treatable with pharmaceuticals and diet. If there are elevated PSA levels, then a digital rectal exam (DRE) is a next logical step. This is when the physician examines the size and shape of the prostate and feels for lumps or hard areas within and around the prostate. Hard areas are signs of tumour growth, whereas soft tissue enlargement is usually indicative of inflammation. If cancer tissue is suspected, then a trans-rectal ultrasound (TRUS)-guided biopsy is performed to obtain a small segment of prostate tissue. Pathology of the tissue can be examined to determine whether or not it is cancerous. This is then scored from one to five depending how much of the tissue is cancerous. This is known as the Gleason score, where a score of one indicates mostly normal prostate tissue is observed, and a score of five is when growth patterns of the prostate tissue is highly abnormal and considered to be an aggressive form of prostate cancer. However, all of these techniques have sampling errors, especially with the biopsy, leading to missed and late diagnoses. This is why there is a need for non-invasive imaging methods to improve the disease identification through the detection of a specific overexpressed biomarker in the disease. If this can be done at earlier stages, this leads to improvement in patient prognosis and survival.
1.3 Imaging Modalities

The typical imaging modality used to detect prostate cancer is magnetic resonance imaging (MRI), and this is performed before a biopsy\textsuperscript{8-9}. This technique is performed as a whole-body scan to detect if the cancer has metastasized to other tissues. MRI uses a magnetic field and radio waves in order to create a detailed image of organs and tissue in a biological system\textsuperscript{10}. The magnetic field temporality realigns the hydrogen atoms of water within a tissue and the radio waves allow these aligned atoms to create signals that are transferred to a series of detectors to reconstruct an image\textsuperscript{10}. The advantages to MRI are that there is no ionizing radiation and the images have a high spatial resolution (less than 1 mm) without compromising the image. However, there are still signal-to-noise and contrast-to-noise issues with MRI that need to be improved. There have been attempts to use stronger magnets and dedicated computational software to help with contrast and noise, but in the past 30 years it is the contrast agents (CA) that have been able to enhance the image to provide a better diagnosis and treatment plan\textsuperscript{4}. MRI contrast agents such as gadolinium (Gd) are used to alter the longitudinal ($T_1$) and transverse ($T_2$) relaxation rates of the surrounding water protons in a tissue. An MRI CA must be a biocompatible magnetic material to be able to alter these relaxation rates, and CAs are classified based on their magnetic properties and relaxation mechanisms. This type of technology is used to show the invasiveness of the cancer and whether other tissues may also need to be biopsied. However, MRI shows limited lesion detection in the central gland of the prostate making it difficult to delineate between benign and malignant tumours\textsuperscript{11-13}. Computed tomography (CT) can also be used to localize and identify a tumour, however it lacks accuracy, sensitivity, and specificity when identifying is the tissue is cancerous or benign\textsuperscript{14}.

1.4 Fluorine-18 Positron Emission Tomography

Positron emission tomography (PET) is another non-invasive imaging modality with decent spatial resolution (5 to 7 mm), however it has superior sensitivity (100 to 1000 fM) compared to other imaging techniques. This sensitivity is important as very little of the imaging pharmaceutical is used in order to have a detectable signal in the affected tissue. This imaging method allows for the detection of a pharmacological process in patients using a positron emitting radioisotope\textsuperscript{15}. Fluorine-18 ($^{18}$F) is a
radioactive isotope with a half-life of 109 min, and a Beta\(^+\) (\(\beta^+\)) emission. It is created by a cyclotron by bombarding water that is enriched with the oxygen-18 (\(^{18}\text{O}\)) isotope with high energy protons (18 Mega-electronvolts). The desired radionuclide \(^{18}\text{F}\) is then created with a highly unstable nuclease with 9 protons and 9 neutrons in the nuclease and 9 electrons. There is a production of two gamma (\(\gamma\)) photons of 511 keV in 180\(^\circ\) direction from each other after an annihilation event occurs between the radionuclide and the electrons in surrounding tissues (releasing the \(\beta^+\))\(^{15}\). The half-life of \(^{18}\text{F}\) being two hours is beneficial as the decay rate is long enough to perform a lengthier radiosynthesis route, but short enough lived that the radiation does not persist too long in a biological system reducing radiation exposure for patients. PET has the capability of detecting two coincident gamma emissions simultaneously (\textbf{Figure 1.3})\(^{15}\).

\textbf{Figure 1.3} – Beta Positron Annihilation with Electron in Body Tissue to Release Gamma Photons of 511 KeV

The images acquired from a clinical PET camera have spatial and temporal resolution of 5-7 mm and second-minutes respectively\(^{15}\). PET also has a sensitivity of 10^{-12} to 10^{-13} M, meaning that a very low concentration of the radiolabelled imaging probe can be used\(^{15}\). All of these factors above make PET a very good imaging modality for the clinician and patient.

Currently the most common clinical PET probe is fluorodeoxyglucose ([\(^{18}\text{F}\)]FDG). The reason for using glucose in the detection of carcinoma is that cancerous
cells metabolize glucose at a much faster rate than normal cells, which creates a different contrast on the PET image, and this is known as the Warburg effect\textsuperscript{16}. Since glucose passes through the body by renal excretion, it causes a buildup of the tracer in the bladder\textsuperscript{16}. With the bladder in close proximity to the prostate, there are difficulties associated with visualizing the disease tissue\textsuperscript{16}. The current PET imaging agents are unable to delineate between malignant and benign PCa. This is why there is a need for more specific PET imaging agents in order to improve the accuracy in diagnosis of PCa within patients.

1.5 Peptides as Molecular Imaging Agents

The major advantage to using peptides as imaging agents is that they show a high binding affinity and specificity with cell receptors both \textit{in vitro} and \textit{in vivo}\textsuperscript{17}. The other advantage to peptides as imaging agents is that they show good pharmacokinetic characteristics such as absorption and distribution in an \textit{in vivo} scenario when targeting cell receptors that are overexpressed in tumour cells\textsuperscript{18}. Peptides are biologically synthesized in many different organs in the body but have multiple functions else were controlling human pharmacology and physiology\textsuperscript{18}. Peptides have a particular target of interest within a given tissue in order to elicit their response. Peptides are able to also target proteins/peptides in cancerous tissue leading the progression or regression of disease\textsuperscript{18}. A majority of these peptide targets are overexpressed receptors called G-protein coupled receptors (GPCRs). Peptide based radiopharmaceuticals have been in the clinic for over 2 decades and have showed promising results in the diagnosis of cancer\textsuperscript{18}. The first peptide that showed success was \textsuperscript{111}In-DTPA\textsuperscript{O}-octreotide a somatostatin (SST) analog\textsuperscript{18}. This receptor is overexpressed in neuroendocrine tumours\textsuperscript{18}. This success has led many to develop peptides as imaging agents over these past decades.

Major success has also been seen when it comes to imaging the melanocortin-1 receptor (MC1R) that is expressed in melanocytes. MC1R aids in the synthesis of epidermal melanin pigments, as well as aids in the photoprotective response via DNA-repair pathways and antioxidant defences. However, MC1R is highly overexpressed in primary melanomas and their metastasis leading to the development of peptide imaging probes\textsuperscript{19}. The development stemmed from the natural ligand for the MC1R: \(\alpha\)-melanocyte-stimulating hormone (\(\alpha\)MSH). In 2014 a \textsuperscript{18}F-labelled benzamide derivative,
[^18F]N-(2-diethylaminoethyl)-4-[2-(2-fluoroethoxy) ethoxy]ethoxy]benzamide ([^18F]FPBZA) was developed by Shih-Yen Wu and co-workers to specifically target primary melanomas and their metastasis in mice bearing B16F0 melanoma (non-metastatic) or A375 amelanotic melanoma (metastatic) tumor tissue^20. More recently Chengcheng Zang and co-workers developed a lactam bridge-cyclized αMSH (Ac-Nle (norleucine)^4-cyclo(5, 10) [Asp^5-Hid DPhe^2-Arg-Trp-Lys^10]-NH₂) analogue tagged with gallium-68 (^68Ga) via a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelator. They made a couple variants of this analogue and each of their variants showed successful single-photon emission computed tomography (SPECT) images in mice bearing non-metastatic and metastatic tumor tissue^21. These are just a few of the peptide-based imaging probes that have been developed in order to image disease in a non-invasive fashion^19. Currently there are over 150 peptides that are in clinical trials for the purpose of both diagnosing and treating disease^22-23.

In particular within this thesis, the peptides being developed are small therefore allowing them to have many advantages. Small peptides are easy to synthesize, and easier to radiolabel. They are amenable to chemical and molecular modifications and have the ability to be conjugated to metal chelators or other imaging components on either the N or C terminus of the peptide. They tend to have higher receptor binding affinities, higher tumor penetration, and higher retention in the target tissue than small molecules. They have favourable pharmacodynamics, rapid clearance from off target tissues, and the rate and route of excretion can be modified by changing the chemical structure. They tend to have fewer side effects, they are highly non-immunogenic, and, in some cases, they can be used for more than one biological target of interest^18-19. These are the main reasons for using lower molecular weight peptides as imaging agents.

1.6 Modifying Peptides for Improved In Vivo Stability and Target Affinity

Peptides are natural molecules that can have several disadvantages in terms of their oral bioavailability and lower metabolic stability. In terms of imaging agents, bioavailability is not always a concern as the typical route of administration will be intravenous (IV). However metabolic stability for imaging agents is highly important as the probe must be able to make it to the target tissue in order to bind to its receptor. Structural modifications are needed in order to inhibit or reduce enzymatic degradation.
Exopeptidases are one of the major enzymes that hydrolyze the C and N termini of linear peptides. One approach to prevent this degradation is to synthesize a peptide to have the C terminus as an amide and perform an N terminal acetylation although this may cause a reduction in binding in some cases\textsuperscript{24}. Within the scope of this thesis all peptides have a C terminus that is amidated in order to slow the degradation by exopeptidases. Hydrolyzing peptide bonds is caused by endopeptidases that recognize the natural L-amino acid within the peptide sequence. One particular way to prevent this is to replace L-amino acids with D-amino acids or other unnatural amino acids. For instance, the use of D-amino acids was highly used in the peptidomimetics designed within Chapters 3 to 5. The main choice was unnatural aromatic D-amino acids (D-1-naphthylalanine, D-2-naphthylalanine) as they prove to be involved in a series of hydrophobic interactions within the binding pocket of GHS-R1a as determined by MD simulations. Other modifications are the use of the unnatural beta and gamma amino acid substitution in comparison to the standard alpha amino acids\textsuperscript{25}. This allows the amino acid side chains to arrange in very specific three-dimensional conformations forming helical or pleated sheet secondary structural motifs. These are some of the modifications that can enhance \textit{in vivo} stability while only making minor changes to the natural peptide sequence in order to maintain affinity towards the target\textsuperscript{25} (\textbf{Figure 1.4}).

![Figure 1.4 - Stereochemistry of natural and unnatural amino acids](image)

Pseudo peptides is another way to enhance the stability of peptides, since they contain chemical modifications within the backbone of the peptides, therefore resulting in improved resistance against peptidases. Examples of this are peptoids, aza-peptides, and several amide bond surrogates (\textbf{Figure 1.5})\textsuperscript{25}. Peptoids or N-substituted glycine can
increase the peptide stability as well as the cell permeability if we are considering a target that may be cytoplasmic or nuclear. Attachment of the peptide side chain to the backbone nitrogen this will cause the elimination of the polar N-H bond, driving up lipophilicity to enhance cell permeability even further. Aza-peptides refers to the replacement of one or more alpha carbons with nitrogen\textsuperscript{25}. This causes a lost in stereogenicity and reduces the flexibility by switching the rotatable alpha carbon-C(O) bond with a rigid alpha nitrogen-C(O) bond. When an aza residue is placed in the \(i + 1\) or \(i + 2\) position this can cause the induction of beta turns changing the conformation and enhancing peptide stability overall. Amide bond surrogate examples are thioamides, esters, alkenes, and fluoroalkenes and these modifications will mimic the geometric structure of a peptide bond but lead to an increase in peptide stability in most cases\textsuperscript{26-27}. However, based on the number of atoms and valence arrangement, thioamides most accurately mimic that of a natural peptide bond. The drawback to thioamides is that sulphur is a poorer H-bond acceptor in comparison to oxygen, but the nitrogen compensates for this loss in thioamides. Ester substitution can destabilize secondary structure of a peptide since they are unable to perform H-bond donation acting as poorer H-bond acceptors. Esters also have the tendency to undergo hydrolysis. Alkene surrogates can mimic bond rigidity, angle and length even though they lack a heteroatom for non-covalent interactions\textsuperscript{26-27}. Alkenes can also undergo isomerization, oxidation, and chemical liability \textit{in vivo} even though they have been incorporated into previous natural peptides and proteins to enhance stability (e.g. C-X-C chemokine receptor 4 CXCR4 and the tripeptide RGD). 1, 2, 4-Oxadiazole, 1,3,4-oxadiazole, and 1,2,4-triazole can be used as heterocyclic moieties mimicking amide bonds\textsuperscript{30-31}. All of these modifications are highly dependent on the overall structure of the natural peptide, the target of interest and the intent of the chemical probe (e.g. imaging versus therapeutic). However, they all come with challenges and disadvantages that must be considered when performing structure activity relationships (SARs) on natural peptide sequences\textsuperscript{30-31}. This process is always the limiting step but is highly necessary in order to advance a lead peptide drug candidate towards clinical trials. This approach can be used whether the intended chemical probe is to act as an agonist, antagonist, inverse agonist (induces opposite response to that of an agonists), or partial agonist (partial efficacy towards the receptor) and this can be a way to alter the chemical
probe towards these specific pharmacological molecules. The focus for chemical modifications within this thesis will be using unnatural amino acids, D-amino acids and a backbone bridging technique (lactam bridge) in order to enhance the lead peptides as possible future drug candidates in a clinical trial. The lactam bridge stabilization will be discussed further in section 1.13.

![Peptide Bond Surrogates]

**Figure 1.5 - Backbone modifications to help increase in vivo stability of natural peptides**

1.7 Peptide Design and Structure Activity Relationship

The peptides that are being designed in this project are based on a discovery in 1977 by Bowers et al. The group was interested in Met (methionine in the 5th position of this peptide)-enkephalin (EF) analogues in order to understand structure activity relationship (SAR) in relation to pituitary hormone secretion. The natural structure of enkephalin is a pentapeptide and its function is in the regulation of nociception in humans by binding to the delta and mu opioid receptors. The naturally occurring enkephalin derivatives differ by either a Met or Leu on the C-terminus of the pentapeptide (Figure 1.6).
Bowers et al.\textsuperscript{32-34} set out to design enkephalin analogues that would also be involved in the release of growth hormone (GH) from the pituitary gland in the female rats. They discovered early on through a series of structure activity relationship studies that position-2 in their analogues was highly conserved for very select amino acids in order to still maintain GH release. Two of their first analogues (H-Tyr-DTrp-Gly-Phe-Met-NH\textsubscript{2} and H-Tyr-DPhe-Gly-Phe-Met-NH\textsubscript{2}) they discovered that an aromatic residue was required in the second position (DTrp and DPhe) in order for \textit{in vitro} GH-releasing activity\textsuperscript{32-34}. Seven other analogues they developed without an aromatic amino acid in the second position didn’t have any GH-releasing activity. It was also discovered that the L amino acids of Phe and Tyr did not cause any GH-releasing activity. Upon the discovery of these enkephalin analogues Bowers \textit{et al} created a hexamer H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH\textsubscript{2} (GHRP-6). This then became the first synthetic peptide to release GH both \textit{in vivo} and \textit{in vitro}\textsuperscript{32-34}. From this point on many researchers began to develop peptides that had the ability to release GH. These classes of compounds then became known as growth hormone secretagogues (GHSs)\textsuperscript{35}. They are in their own chemical class based on the function of secreting GH from the pituitary instead of the alternative route from GH releasing hormone (GHRH)\textsuperscript{35}. These various GHSs encompassed peptides: GHRP-1\textsuperscript{36}, hexarelin\textsuperscript{37}, KP-102 (later designated GHRP-2)\textsuperscript{38}, peptidomimetics (e.g. G7039\textsuperscript{39}, [1-
Nal\(^4\)G7039\(^{40}\)and ipamorelin\(^{41}\) and small-molecules (e.g. L-692,429\(^{42-44}\)and MK-0677\(^{42-44}\)). The structure of these representative GHSs can be seen in Figure 1.7.

**Figure 1.7 - Chemical structures of the peptides, peptidomimetics, and small molecules that cause GH-releasing activity in rats and/or humans**

It wasn’t until 1996 that the growth hormone secretagogue receptor-1a (GHS-R1a) was discovered by the group of R. Smith at Merck Sharp and Dohme Research Laboratory in the United States\(^{45-47}\). Their discovery was made possible by various
expression cloning methods (i.e. point mutations) and they were the first to identify that GHS-R1a interacted with these various GHSs causing GH-releasing activity. This receptor belongs to the rhodopsin-like family of GPCRs\textsuperscript{48}. Its seven transmembrane (TM) alpha helical domains consist of 366 amino acids having an approximate mass of 41 KDa (\textbf{Figure 1.9})\textsuperscript{46-47}. GHS-R1a has a known splice variant (GHS-R1b) which only has five TMs consisting of 289 amino acids. To date this splice variant has no known biological activity\textsuperscript{44-48}. GHS-R1a is expressed in a wide variety of tissues and organs including the hypothalamus, pituitary gland, thyroid gland, stomach, intestines, pancreases, spleen, ventricular myocardium, aorta, lung, adrenal gland, kidney, testis, adipose tissue, ovaries and lymphocytes\textsuperscript{48}. Because it is widely spread in so many tissues and organs it has several human physiological outcomes that are highlighted in \textbf{Figure 1.8}\textsuperscript{49}.

\textbf{Figure 1.8 - Physiological effects of GHS-R1a in humans (modified from 47, copyright permission number 11855562)
Figure 1.9 - Homology model of GHS-R1a. This model is based on sequence alignment with variations of the neurotensin receptor with various ligands docked in its pocket. The 7-alpha helical domains (in red) span the cellular membrane (in green).

In 1999 Kojima et al isolated the natural ligand ghrelin from a rat’s stomach\textsuperscript{50}. Before this date GHS-R1a was considered to be an orphaned GPCR. Human ghrelin is a 28-amino acid peptide that is derived from a 117-amino acid sequence (preproghrelin) and through post-translational modifications, terminating with the octanoylation of Ser-3 by ghrelin O-acyl transferase (GOAT) the active form is produced\textsuperscript{50}. Without this fatty acid chain ghrelin is unable to bind to its receptor. This is the only known instance of a peptide being post-translationally modified with a fatty acid, and it is required for its binding to and biologically activating GHS-R1a\textsuperscript{47} (Figure 1.10).
Merck then continued to investigate the interaction between ghrelin and GHS-R1a and learned that the first 5 N-terminal amino acids of ghrelin were essential in binding to and activating the receptor\textsuperscript{51}. They discovered this through the development of truncated versions of the ghrelin peptide by measuring the EC\textsubscript{50} (half-maximal effective concentration) in HEK293T cells that transiently overexpressed GHS-R1a. The EC\textsubscript{50} was calculated by monitoring the intracellular calcium release from the endoplasmic reticulum downstream of the G\textsubscript{aq}\textsuperscript{51}. These truncated versions can be seen in Figure 1.11. Since then many ghrelin derivatives have been developed and tested that have similar properties to that of the natural ligand. Some of these include small molecules that have been developed by Merck\textsuperscript{52}, Bristol-Myers Squibb\textsuperscript{53}, Sumitomo Pharmaceuticals and Abbott Laboratories\textsuperscript{54}. Also, many have worked on the development of peptidomimetic and pseudo peptide agonists developed by Novo Nordisk (DK). When developing novel ligands targeting this receptor certain criteria must be carefully considered\textsuperscript{55-65}. Deciding the nature of the ligand (e.g. agonist, antagonist, partial agonist, inverse agonist) is the first major step in beginning an extensive structure activity relationship (SAR) study. The structural nature of the ligand must be decided before beginning synthesis. This could be
choosing the development of small molecules, peptides, peptidomimetics, proteins, or antibodies as the initial starting framework. Then in terms of having these ligands be suitable imaging agents one must choose the location and method of radioisotope incorporation into the structure of the ligand.

![Chemical structures](image)

**Figure 1.11** - Derivatizes of ghrelin developed by Merck indicated that the first 5 amino acids are essential to binding to and activating GHS-R1a

### 1.8 Molecular Imaging Agents Designed to be either Agonists or Antagonists

A peptide agonist has been the most frequently used radiolabelled molecular imaging tool when it comes to targeting overexpressed proteins in cancerous tissue. Agonists become internalized when binding to the receptor, allowing them to accumulate and be retained in the targeted cells. This became a well-established concept in the 1980s being studied with the somatostatin receptors that has been shown to be upregulated in a variety of neuroendocrine tumours. For examples an indium-111 labelled somatostatin derivative developed by Reubi et al. showed specificity in targeting the highly expressed somatostatin receptors within neuroendocrine tumours in a mouse model. Upon binding of an agonist such as this somatostatin derivative to its given target a receptor–radioligand complex forms and this becomes internalized into the tumour cell. This allows for an increase in radioactivity in the tumour tissue compared to other organs such as the liver, lungs and spleen. As the radioligand accumulates in the tumour tissue there is a contrast enhancement allowing the in vivo target to be visualized by PET. This is one of the main reasons why most decided on using agonists versus antagonists. This is because when an antagonist binds to a receptor it has a purpose is to block the natural ligand from interacting with its natural target.

Others in the field of imaging probe design focus on using an antagonist as the target entity to be tagged with a radionuclide. For example, Ginj et al. showed that a somatostatin derivative designed to be an antagonist showed improved in vivo results
(less buildup of the radiotracer in noncancerous tissues) then a variety of radiolabelled agonists\textsuperscript{70}. Another example when using an antagonist as an imaging probe was Wadas et al. and their comparison between the antagonist $^{64}$Cu-CB-TE2A-sst2-ANT and the agonist $^{64}$Cu-CB-TE2A-Y3-TATE, when targeting the gastrin-releasing peptide receptor (GRPr) that is overexpressed in prostate cancer (PCa). Their results indicated a reduction in kidney and intestine uptake while maintaining tumour uptake of the imaging probe. The preference between using an agonist versus an antagonist is receptor dependent, and therefore when designing an imaging probe both agonists and antagonist peptides for the same target may be beneficial to design at the chemical synthesis phase\textsuperscript{71-72}.

### 1.9 Designing Agonist Imaging Probes Through Structure Activity Relationships.

Within this thesis it was decided to generate growth hormone secretagogues (GHSs) as imaging agents allowing for a rapid synthesis by either manual or automated fluorenymethyloxycarbonyl solid phase peptide synthesis (Fmoc-SPPS). Synthesising small molecules in most cases requires lengthier synthetic procedures. Small molecules also typically are designed to consist mostly of the pharmacophore in order to interact with the receptor. Slight changes to a small molecule pharmacophore can drastically reduce its binding towards a given target. Peptides tend to have a non-pharmacophore portion thereby allowing for the attachment of a desired radionuclide to form the imaging agent without interfering with receptor binding. Lastly there has been a large number of previous studies that probed the interaction between GHS-R1a and growth hormone releasing peptides (GHRPs), pseudo-peptides and peptidomimetic GHSs. This gives us a great starting scaffold to do further SAR studies as well as to modify the peptides in the appropriate way to generate imaging probes\textsuperscript{73-78}.

Rationale of these agonists was based on Feighner et al.\textsuperscript{77} who utilized site directed mutagenesis\textsuperscript{79} and molecular modelling to determine the key residues in GHS-R1a that interacted with specific ligands (GHRP-6, MK-0677, and L-692585) (Figure 1.7)\textsuperscript{77}. The activation of GHS-R1a signalling by these various ligands was measured by an aequorin bioluminescence assay detecting the changes in intracellular calcium ion levels.

A homology model based on the helical impression of bacteriorhodopsin and a 2-dimensional sequence alignment of repeated motifs across many GPCRs (neurotensin,
somatostatin-2, angiotensin-2, beta-2-adrenergic, human/swine/rat GHS-Rs) was used to determine the number of amino acids that are needed to allow for a successful ligand interaction with GHS-R1a. **Figure 1.12** below is the 366 amino acid GHS-R1a discovered by homology modeling, with the indicated mutated residues in red.

**Figure 1.12** - The GHS-R1a with mutated sites in red performed by Feighner et al\(^\text{36}\). Adapted from 36.

It was from here that a larger number of residues in the receptor were identified as key for the binding and activation by a variety of synthetic ligands. For instance, E124 in transmembrane 3 (TM3) forms a salt bridge with the primary ammonium cation on the N terminus of a peptide ligand or an amino group that is on a small molecule ligand\(^\text{77}\). This interaction was confirmed by site directed mutation from E124 to Q124. Residue M213 was crucial to the binding of L-692585 (ghrelin small molecule)\(^\text{80}\), and when mutated to D213 binding and activity was lost. D99 (TM2) was also key as the functional activity decreased by half when mutated to N99 (again with L-692585)\(^\text{77}\). Residue C116
was also key for the binding, since all activity was lost when mutated to A116 (TM2) (proven with various ligands listed in Figure 1.7). This Cys116 residue forms a disulfide interaction with Cys198 that is crucial in the stabilization of the receptor in an active conformation. Also, residues S123 and Q120 were shown to have a high importance in the binding of ligands to GHS-R1a. This was confirmed by using \( ^{35}\text{S}\)-MK-067781 and mutating S123 to A123 and Q120 to H120 in TM377.

Several follow up studies utilized computational methods to further investigate the interaction of GHS-R1a and various ligands. In 2001 Huang et al.78 identified 6 key pharmacophoric regions in GHS-R1a by using six peptide-based and 4 non-peptide-based ligands. They first identified the low-energy conformers for each of the ligands and used a mutated version of GHRP-6 as a negative control ([Val\(^3\)]GHRP-6). The computer program that was designed by Huang et al. (DistComp)\(^82\) was used to identify the 3-dimensional pharmacophore and its functional groups that are required to activate GHS-R1a. The key functional groups identified were: two aromatic rings, a proton acceptor, a protonated amine and a small hydrophobic group. Once this pharmacophore region with the key residues was identified it was further screened against a larger database of compounds in order to find potential hit ligands. This resulted in the identification of a hit ligand agonist benzothiazepin GH secretagogue (Figure 1.13) with an EC\(_{50}\) of 1 nM. This was a conformation that the computational method developed by Huang et al. was a valid way to identify hit ligands that target GHS-R1a\(^78\).

Figure 1.13 - Benzothiazepin identified to have 1 nM potency when targeting GHS-R1a

The protonated C-terminal amine (previously identified by Feighner et al.) and aromatic groups in benzothiazepin was the establishment in the necessary molecular framework in designing peptide base agonist that targeted GHS-R1a. Hansen et al. also confirmed through the design of ipamorelin (Figure 1.7) derivatives that there is a need for C-terminal aromatic groups in order to maintain binding towards GHS-R1a\(^59\).
Pedretti et al. then conducted a fragment based computational study of GHS-R1a in order to further study its interaction with various ligands. The 7TM GHS-R1a was divided into 15 fragments and these fragments were modelled against rhodopsin. The active core of the ghrelin peptide (Gly-Ser-Ser(n-octanoyl)-Phe-NH₂ in Figure 1.11) along with 35 literature peptidomimetic GHSs was docked against this fragmentation-based model for each of the 15 fragments. Additional sub-pockets in the binding domain of GHS-R1a were identified. There was the identification of a polar sub-pocket bordered by TM2 and TM3 and a non-polar/aromatic pocket surrounded by TM5 and TM6. Since these binding pockets are too far apart for a single ligand to interact at both sites it was determined that there is a cooperative effect between the various binding modes. The receptor goes into an active state when GHSs bind to the aromatic sub-pocket by way of a non-competitive mechanism through partial agonism\textsuperscript{79}.

Martin-Pastor et al. and Jörg Großauer et al. conducted a \textsuperscript{1}H-NMR study on the interaction between the ghrelin ligand with/without the octanoyl chain and GHS-R1a. This was an \textit{in vitro} NMR study using both human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells stably transfected with GHS-R1a. 2-dimensional total correlation spectroscopy (2D-TOCSY), chemical shift perturbations (CSP) and slow conformational exchange (SCE) were used to better understand the interaction between GHS-R1a and its natural ligand. There were a number of residues affected by the interaction between the octanoylated version of human ghrelin and GHS-R1a, most notably the octanoyl chain itself, indicating its importance for binding and activating the receptor\textsuperscript{81-82}.

More recently Hou et al. investigated the interaction between G7039 (agonists developed by Genentech in Figure 1.7) and GHS-R1a by means of computational studies; homology modelling, molecular docking, molecular dynamics simulations, and binding free energy calculation. This investigation included altering the amino acid structure of G7039 and its consequences on binding. Glu124 in TM3 was a key residue identified in agonist binding and activation of GHS-R1a. One of the binding models within this work indicated that pronated amine on the N-terminus of G7039 forms a salt bridge with the negatively charged residue Glu124. Other interactions identified was a cation- \( \pi \) interaction between the charged amine of G7039 and Phe279, a hydrophobic
interaction between the Phe of G7039 and Phe286 and a π-π interaction between the aromatic (D2Nal) residue of G7039 and the amide of Asn305. These interactions indicate that the first 4 positions are highly vulnerable to change and altering them could lead to a loss in binding. This was showed by the removal of these groups in Hou et al.\textsuperscript{83}

The extensive literature that has been reported on various ligands and their interaction with GHS-R1a sets the ground work to further optimizing these ghrelin peptidomimetics in order to chemically convert them into useable imaging agents.

1.10 Radiolabelling Strategy

One final important step in designing an appropriate molecular probe is to determine if the addition of radionuclide can be done either in a direct or indirect method, as this can determine the success of the potential imaging agent for industrial production\textsuperscript{3}. A direct labeling approach is usually favored, as it requires fewer steps allowing for a more efficient synthesis and a less radiochemical decay. This method is done when the precursor chemical molecule is synthesized with a designated site for substitution, elimination, or insertion of the radionuclide of interest (e.g. the chelation of a metal). This also requires the radionuclide to be non-reactive with other sites on the molecule\textsuperscript{3}. In contrast, a prosthetic group labeling approach is when a precursor is synthesized that will first be reacted to the radionuclide, and then can be coupled or reacted to the molecular probe via a linker on the targeting entity. This method is required when the ligand may have too many other reactive sites towards the radionuclide that was chosen. Prosthetic group labelling requires more steps and can be less efficient. However, this approach is used a lot of the time, as to design a molecular probe for direct labeling requires for the reactivity of the molecule to be changed for efficient synthesis, and this could cause changes in binding affinity for the target\textsuperscript{3}. Both labelling approaches can be seen in Figure 1.14 below. Within the scope of the thesis both a prosthetic group and direct labelling will be used in order to design the lead imaging candidates.
1.11 Development of Heterobivalent and Homobivalent Ligands Targeting the GHS-R1a Homo-Oligomer

G-protein-coupled receptors (GPCRs) until very recently were thought of as monomers that are expressed on the surface of both neuronal and non-neuronal cells. However, GPCRs have been shown by many to exist as oligomers impacting both their pharmacology and physiology. However, GPCR oligomerization in terms of neurotransmission is more accepted by the scientific community and therefore is more well studied. This is because neurotransmitter receptors cannot be considered as a single unit, but a multimolecular subunits of proteins that lie on the plasma membrane. This idea began in the mid-1970s and was shown with the use of radioligand binding assays that there was a negative cooperative interaction with the Beta-adrenergic receptor binding sites. Initially it was thought that the ligand was binding to two separate monomeric entities; uncoupled or coupled G protein. Then in the 1980s this was further backed up with the use of Western blotting and/or coimmunoprecipitation with the Beta-adrenergic and muscarinic acetylcholine receptors in both transfected cells and tissues samples isolated from the brain. Techniques such as fluorescence and bioluminescence resonance energy transfer (FRET and BRET) have become other accepted methods to indicate oligomerization of GPCRs in cells. However, FRET and BRET are common techniques to show receptor homo-oligomerization over hetero-oligomerization. When a ligand interacts with a GPCR oligomer there can be the occurrence of two independent binding sites, with different affinities for the ligand. Franco et al devised a receptor-oligomer model that allowed for the quantification of cooperativity and termed this the: “oligomer cooperativity index.” This allows for the measurement in the extent of...
binding from one orthosteric center to another in the GPCR oligomer complex. We then can consider this as a measure of intermolecular cross-talk that occurs in a receptor oligomer. We often see a biphasic binding curve when measuring the affinity of a ligand towards a receptor oligomer. A biphasic curve is due to conformational changes in one receptor when the ligand binds altering the state of a neighbouring receptor, and this is considered a cooperative mechanism in receptor oligomerization. Cooperativity can either be negative or positive during oligomerization of GPCRs. So far, many have targeted GPCR hetero-oligomers with two separate ligands. This approach has a major limitation when it comes to designing a novel drug for a receptor oligomer in terms of drug dosing and formulation. Knowing that this is a possible mechanism in which a ligand can bind and change the active state of a GPCR oligomer, one can develop novel heterobivalent and homobivalent ligands that would modulate the signalling in a differential fashion when compared to the monomeric receptor. By selectively connecting two ligands together by a given linker length one can create a signal ligand that will have the same effect as the two ligands separately. The chemical nature of the linker can also improve pharmacokinetic and pharmacodynamic properties of a chemical probe, such as enhancing solubility, metabolic stability and enhanced affinity. There have been many examples in the literature of successful attempts at designing bivalent-based ligands. Most recently Lensing and co-workers developed a bivalent ligand that targeted the human melanocortin-4 receptor oligomer (hMC4R). This led to a biased signalling stimulating cyclic adenosine monophosphate (cAMP, adenylyl cyclase pathway) signalling, but minimal activation of β-arrestin signalling.

GHS-R1a has potential therapeutic application towards a wide range of diseases such as obesity, diabetes, cancer, etc. GHS-R exists in two isoforms: GHS-R1a and GHS-R1b. GHS-R1a is activated by its endogenous ligand, ghrelin, to induce growth hormone release, while GHS-R1b is functionally inactive and has no binding affinity for ghrelin. Along with numerous other GPCRs, GHS-R1a has been shown to form a homo-oligomer. In 2005, Holst et al. were the first to propose the homo-oligomerization of GHS-R1a. In this study it was shown that, in the presence of the endogenous agonist ghrelin, and co-administration of other agonists (GHRPs) acted as a neutral, positive, or negative modulators of the GHS-R1a receptor in COS-7 cells transfected with GHS-
Further evidence of GHS-R1a homo-oligomerization was provided by Jiang et al. using a HEK293 derived cell line co-transfected with GHS-R1a-green fluorescent protein (GFP) and GHS-R1a-Renilla luciferase (rluc). The BRET (bioluminescence resonance energy transfer) study showed a hyperbolic function of GHS-R1a-GFP/GHS-R1a-rluc ratio, suggesting oligomerization rather than molecular crowding. Kern et al. observed a FRET signal when CLIP-GHS-R1a and SNAP-GHS-R1a are overexpressed in HEK293 cell through time-resolved (Tr) FRET measurement, again suggesting the formation of the GHS-R1a homo-oligomer. Leung et al., also confirmed homo-oligomeric GHS-R1a in HEK293 cell using BRET and co-immunoprecipitation. A visual representation of the GHS-R1a homo-oligomer docked with the novel homobivalent ligand developed in Chapter 6 can be seen in Figure 1.15. In addition, evidence suggested that the GHS-R1a receptor has the ability to not only oligomerize with itself but also with other GPCRs, including GHS-R1b receptor, prostaglandin E2 receptor (EP3-1), prostacyclin receptor (IP), thromboxane A2 receptor (TPα), Somatostatin receptor 5 (SST5), dopamine receptors (D1 and D2), melanocortin 3 receptor (MC3), and serotonin 2C receptor (5-HT2C). The first of these interactions to be observed was with the D1- and D2-like dopamine receptors as both of these receptors are coexpressed in a number of sites (e.g. brain regions that are linked to food intake and reward behaviours). Kern et al. in 2012 was able to demonstrate that when there is a lack of ghrelin circulation in the brain, GHS-R1a will oligomerize with D2R leading to anorexigenic effects. Another example in which GHS-R1a oligomerizes with another GPCRs and changes the downstream signalling is with MC3R. Rediger et al. demonstrated that in the arcuate nuclease GHS-R1a is coexpressed in neurons with MC3R by the use of FRET. Typical signalling by means of MC3R is the Gαs pathway causing an accumulation of cAMP. However, when oligomerization occurs with GHS-R1a cAMP accumulation increases by 2-fold, and GHS-R1a constitutive activity along with ghrelin induced activity cause a decrease of intracellular calcium by 40%. And when the MC3R receptor is downregulated, this leads to an increase in GHS-R1a signalling explaining an increase in fat mass within a MC3R−/− knockout model. These are just a few of the examples in which GHS-R1a can oligomerize with another receptor and change the downstream signalling events leading to different physiological outcomes.
Rational design of a bivalent ligand is further discussed in section 6.2.1. Ligands that are capable of targeting these receptor oligomers holds great promise as a new class of therapeutics, but equally important, are a powerful chemical biology tool for understanding the function, mechanism, and disease significance of GPCR oligomers, for which there is limited knowledge to date.

Figure 1.15 - Visual representation of the GHS-R1a homo-oligomer docked with the homobivalent ligand designed in Chapter 6. This is not the actual interface that occurs between the GHS-R1a homo-oligomer

1.12 Tagging Molecular Probes with Fluorescent Moieties

Fluorescently tagged ligands can help us better understand the physiological and pathological mechanisms of model cell systems. Cells by their inherent nature are complex, and without the appropriate molecular probes it is difficult to interrogate the complex cellular structures and gain a deeper insight into molecular interactions, enzyme activity, and the many other cellular processes that are occurring every second. The ability for using labelled peptides with platforms such as fluorescence energy transfer (FRET), bioluminescence resonance energy transfer (BRET), flow cytometry, and fluorescent microscopy (in vitro, in vivo, and ex vivo) allows us to probe the key cellular processes involved in human physiology and disease.

Fluorophores work by absorbing light in the ultraviolet or visible range and re-emit part of their energy as radiation (fluorescence). A longer wavelength is seen within
the emitted radiation then the excitation light and this is called the Stokes shift. The emission wavelength depends on the wavelength of the excitation light\textsuperscript{106}. Fluorescence is highly controlled based on the chemical structure of the fluorophore and many of these fluorophores are commercially available. For the purpose of this thesis a sulfo-cyanine5 dye is used and is commercially available as a pre-activated N-Hydroxysuccinimide (NHS) ester in order to couple onto a primary amine of a Lys located on the C-terminal portion of a ghrelin ligand in Chapter 2.

Conjugating a fluorophore to a peptide is similar to designing a radiolabelled peptide probe. The location of the fluorophore addition to the biomolecule is carefully considered as to not affect the binding properties of the peptide towards the target. The decision to use a certain fluorophore depends on the experimental conditions for the particular \textit{in vitro, in vivo}, or \textit{ex vivo} application. For example, fluorophores within the visible range have an emission between 400 nm and 700 nm (e.g. 7 amino-4-methylcoumarin (AMC), fluorescein isothiocyanate (FITC), and 5-carboxytetramethylrhodamine (TAMRA)). These fluorophores are typically seen for uses in cellular applications such as confocal microscopy experiments. However, in the case of \textit{in vivo} applications fluorophores in the emission range of 650 nm to 900 nm (near-infrared – NIR) are required as they will produce less autofluorescence with body components such as water and hemoglobin. There are a number of commercially available dyes in the NIR range such as the Cyanine dyes, Alexa Fluor dyes, and IR dyes that can be used \textit{in vivo}. All of these commercially available dyes have been chemically modified to allow for the conjugation to a target peptide in a variety of ways, including click chemistry and amide bond formation\textsuperscript{107}.

This is the method to design an imaging agent that is almost identical to the PET probe (same targeting entity), however it differs by only its imaging modality, providing a dual-purpose ligand towards the same target.

There are many past examples of using peptides conjugated to fluorophores in both \textit{in vitro} and \textit{in vivo} applications. There has been a series of somatostatin derivatives that have been conjugated to NIR dyes in order to perform \textit{in vivo} imaging of mice bearing cells that overexpress the somatostatin receptor-2 (SSTR-2). One of these derivatives is the conjugation between octreotate and indotricarbocyanine (ITCC) NIR
dye that was able to show high levels of fluorescence in the mouse tumours even after 24 hours post intravenous (IV) injection\textsuperscript{107-108}.

Within our own group there has been a recent publication using a NIR inulin optical probe for measuring the glomerular filtration rate (GFR). Inulin is a polysaccharide that is considered the clinical gold standard for measuring GFR in order to assess the filtering capacity of the kidney and renal function helping to indicate the occurrence of chronic kidney disease (CKD). Inulin was conjugated to a Cyanine-7.5 dye and used to measure the GFR in a pig model non-invasively\textsuperscript{109}.

Within this current work (Chapter 2) I report the design of a ghrelin analogue conjugated to a sulfo-cyanine-5 dye that has been shown thus far to image both tissue in an \textit{in vitro} and \textit{ex vivo} scenario. However, since sulfo-cyanine-5 is a NIR dye with an emission maximum at 669 nm, this probe can be used for optical imaging in an \textit{in vivo} scenario.

This imaging tool presented in Chapter 2 has the potential to be used in the clinic as a marker for GHS-R1a (\textbf{Figure 1.16}). In addition, it could serve as a probe to screen for ghrelin expression in a broad array of cancer cell lines and tumour tissues, as well as for quantification using flow cytometry techniques.
Figure 1.16 - Stabilized fluorescent ghrelin analogue developed in chapter 2 targeting and visualizing GHS-R1a in an in vitro scenario. DAPI stained blue nucleus, Sulf-Cy5 tagged G(1-20) analogue in red. Cells are OVCAR8 stably transfected with GHS-R1a.

1.13 Stapling Peptides to Enhance Both Stability and Binding Towards a Target of Interest

Ghrelin is a peptide that is mainly α-helical. This was determined by 2D NMR indicating Pro-7 to Pro-21 being helical, and molecular dynamics simulations showing the same helical region from Pro-7 to Gly-13. The α-helicity of ghrelin is stabilized upon binding to GHS-R1a. The first five amino acids are essential in binding to GHS-R1a, shown by the fact that ghrelin(1-5) still retains its affinity to GHS-R1a. Shorter eight amino acid ghrelin derivatives have been developed for the purpose of molecular imaging and were determined to have similar GHS-R1a affinity compared to ghrelin.

Ghrelin is therefore an ideal candidate for modification in designing a molecular imaging probe.

The use of natural peptides in various in vitro and in vivo assays is difficult because they tend to have lower stability, as well as a high cost of production (usually a longer amino acid sequence). Shortening a natural peptide is a way to overcome this particular problem. Stabilizing H-bonds seen within physiological conditions of natural
peptides is drastically reduced when the peptide becomes truncated, as there is a loss in alpha helical stabilizing amino acids. These additional physiological interactions also facilitate complete binding towards a target of interest\textsuperscript{115-116}, as confirmation is vital for ligand recognition\textsuperscript{116}. Many biological processes rely on a secondary helical structure\textsuperscript{112,116}. However, there is a high energy cost that is required to generate a secondary structure limiting the stability of truncated natural peptides\textsuperscript{116}.

A possible way to overcome this high energetic barrier is by pre-folding, or stapling peptides. The truncated peptides are then able to adopt a desired secondary structure to increase both stability and biological activity\textsuperscript{112,116}. When choosing location for cyclization it is often by means of the positions at \(i\) and either \(i+4\) or \(i+7\) as these are positioned on the same face of the helical secondary structure. The four common methods for constraining a peptide to promote \(\alpha\)-helix formation include a lactam bridge, a hydrocarbon bridge, a metal-ion clip, or a hydrogen bond surrogate\textsuperscript{115-116}. These variations in cyclization can be visualized in Figure 1.17.

![Figure 1.17 - 4 common methods of stapling. (A) Lactam bridge, (B) hydrocarbon bridge, (C) metal ion clip, and (D) hydrogen bond surrogate (permission granted by author)\textsuperscript{117}](image)

These various stapling strategies described above have successfully produced cyclic peptides with improved target affinity. However, both the hydrocarbon and lactam cyclization method show a greater level of \textit{in vivo} stability\textsuperscript{115-118}. The lactam bridge is a more appropriate method of cyclization for use in a clinical setting due to its natural amide bond composition\textsuperscript{119}. This is because when this amide bond becomes cleaved it reveals two natural amino acid side chains. There must be careful consideration for the location of the staple as to not block the face of the helix that is important in binding.
towards the target of interest. As well as the amino acids altered to incorporate the staple can also affect secondary structure and binding of the ligand towards the given target. ‘Staple scanning’ is the preferred method for identifying a peptide sequence that will exhibit optimal binding, secondary structure, and stability of the modified ligand. A staple scan is performed by modifying each residue not essential for binding and measuring the peptide’s new affinity and secondary structure. The staple is moved along the peptide through the modification of the amino acids in the $i$ and either $i+4$ or $i+7$ positions in order to find its optimal location. This method will allow for the best staple candidate to be identified and further tested. It has been shown that using a glutamic acid residue in the $i$ position and a lysine residue in the $i+4$ or $i+7$ position provides peptides with maximum helicity and reversing the placement of these amino acids decreases helicity (Figure 1.18). This alteration is thought because reversing the order could change the dipole direction of the amide bonds with respect to the dipole direction of the helix.

Figure 1.18 - Synthesis route to forming a lactam bridge with Glu in the $i$ position and Lys in the $i+4$ position

Within Chapter 2 of this thesis a staple scan is reported for ghrelin(1-20) analogues starting in position four and working the staple all the way to position 19, in order to identify the optimal location for the staple based on alpha helicity and binding to GHS-R1a (Figure 1.19). This optimization provided a cyclic ghrelin analogue with greater binding affinity towards GHS-R1a as well as having improved stability.
Figure 1.19 - Stabilizing ghrelin(1-20) analogues with a lactam bridge (i,i+4 or i,i+7) in order to improve the secondary alpha-helical structure and maintain binding towards GHS-R1a.

1.14 Scope of this Thesis

GHS-R1a is expressed in many human tissues (most notably the hypothalamus) and causes an increase in appetite upon activation by its endogenous ligand; ghrelin. GHS-R1a is differentially expressed in malignant compared to benign tumours (e.g. prostate). Therefore, there is an interest in developing GHS-R1a-targeted peptides as novel drugs to modulate signaling (e.g. cancer cachexia and obesity) and image receptor (e.g. disease diagnosis and progression). The focus of this thesis is the development of ghrelin analogues that act as agonists when targeting GHS-R1a.

The first of these analogues was the development of a stabilized ghrelin 1 to 20 analogue by means of a lactam bridge (Chapter 2). The lead analogue with a sulfo-cyanine-5 (Cy5) conjugated to the C-terminal lysine (cyclo-12, 16(H-GSXFLSP)EHQR[EQQPK]ESKK(Sulfo-Cy5)-NH₂) has an [θ]_222/[θ]_208 ratio of 0.84, and an IC₅₀ of 1.0 nM. This probe was highly successful in imaging GHS-R1a by fluorescent microscopy in both cell lines and tissue samples from different disease states.
(cancer, cardiovascular disease, and stroke) associated with the overexpression of GHS-R1a. This probe has the potential to image the receptor in an *in vivo* scenario due to the high emission maximum of sulfo-Cy5.

Chapters 3 to 6 focussed on the development of ghrelin peptidomimetic analogues with applications driven to both therapy and imaging. The initial compound G7039, a peptidomimetic ghrelin agonist (IC$_{50}$ 5.2 nM/EC$_{50}$ 0.18 nM) underwent structure-activity relationship (SAR) studies to generate ligands and positron emission tomography (PET) agents to study/image the GHS-R1a. The first generation peptidomimetic (Chapter 3) [1-Nal$^4$,Lys$^5$ (4-fluorobenzoyl-4-FB)]G7039 (IC$_{50}$ 69 nM/EC$_{50}$ 1.1 nM) was radiolabelled with $^{18}$F in a decay-corrected radiochemical yield of 48%, radio purity of $\geq 99\%$, and molar activity of $\geq 34$ GBq/μmol. Despite its success as a PET imaging agent, its solubility (cLogP = 8.27) and binding affinity (IC$_{50}$ = 69 nM) needed improvement.

The second generation peptidomimetic (Chapter 4) [Tyr$^4$,Lys$^5$(2-fluoropropionyl (2-FP))G7039 (IC$_{50}$ 0.28 nM/EC$_{50}$ 0.12 nM) had improved binding (IC$_{50}$ = 0.28 nM) and lipophilicity (cLogP = 4.36). Labeling of this ligand was low yielding, however two peptides from this project were identified as partial agonists.

The third generation (Chapter 5) required a modified prosthetic group (2-FP to ammonium methyltrifluoroborate-AMBF$_3$) in order to radiolabel in higher yields, resulting in [Tyr$^4$,Lys$^5$(AMBF$_3$)]G7039 (IC$_{50}$ 0.85 nM). This compound was radiolabelled in a single step, with a decay-corrected radiochemical yield of 28%, radiopurity of $\geq 99\%$, and molar activity $\geq 15$ GBq/μmol. Currently this lead compound is ready to be evaluated in an *in vivo* scenario.

Finally, the fourth generation (Chapter 6), a homobivalent G7039 ligand (IC$_{50.high}^{50}$ 0.43 nM:IC$_{50.low}^{50}$ 0.42 pM /EC$_{50}$ 1.8-2.1 nM) was found to bind to the GHS-R1a homo-oligomer and was designed to study GHS-R1a homo/hetero-oligomerization. This homobivalent ligand when targeting the GHS-R1a is able to change the downstream signalling compared to the natural signalling mechanisms seen when activating the monomeric version of GHS-R1a. Four generations of unique peptidomimetics eliciting differences in receptor binding and activation have been synthesized with potential applications towards imaging and therapy (*Figure 1.20*).
Overall several ghrelin analogues have been developed in this thesis that have applications both driven towards imaging and therapy.

Figure 1.20 - The various chemical tools developed within this thesis that all have a heavy appetite for GHS-R1a
1.15 References


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Chapter 2

Stapled Ghrelin Peptides as Fluorescent Imaging Probes

2.1 Introduction

Ghrelin was first discovered in 1999 by Kojima et al., when it was identified to be the endogenous ligand of the growth hormone secretagogue receptor type 1a (GHS-R1a)\textsuperscript{1,2}. It was originally reported that GHS-R1a was found within the pituitary and hypothalamus\textsuperscript{2}. GHS-R1a is a seven trans-membrane α-helical G-protein coupled receptor with an extracellular binding domain, and its activation has been shown to increase cellular proliferation in prostate cancer through the ERK1/2 MAPK pathway\textsuperscript{3–6}. The overexpression of GHS-R1a increases the motile phenotype in astrocytomas, in which increased GHS-R1a expression is correlated with a higher tumour grade\textsuperscript{7}. Overexpression of GHS-R1a has more recently been demonstrated in some ovarian epithelial carcinomas, a disease with poor prognosis due to the late stages in which it is usually diagnosed\textsuperscript{8,9}.

Human ghrelin is a 28-amino acid peptide that is derived from a 117-amino acid sequence (preproghrelin) through post-translational modifications, terminating with the octanoylation of Ser-3 by ghrelin O-acyl transferase (GOAT) to produce the active form\textsuperscript{3,10}. This is the only known instance of a peptide being post-translationally modified with a fatty acid, and it is required for its binding to and activating GHS-R1a\textsuperscript{1,11,12}. Ghrelin is a peptide that is mainly α-helical, with 2D NMR studies showing Pro-7 to Pro-21 being helical, and molecular dynamics simulations showing that there is a helical region from Pro-7 to Gly-13\textsuperscript{13,14}. The α-helicity of ghrelin is stabilized by its natural environment in addition to its binding to GHS-R1a\textsuperscript{15}. The first five amino acids have been shown to be key in binding to GHS-R1a, as ghrelin(1-5) still retains its affinity to the receptor\textsuperscript{16}. Recently, ghrelin derivatives have been developed for the purpose of molecular imaging and were determined to have similar GHS-R1a affinity as that of the endogenous ligand, even when truncated to eight amino acids length\textsuperscript{16,17}. This makes it

an ideal candidate for modification for the purpose of imaging GHS-R1a using fluorescence microscopy\textsuperscript{18,19}.

Using natural peptides within \textit{in vitro/in vivo} assays is challenging due to the typically low stability of a linear peptide, as well as a high cost of production\textsuperscript{20}. To increase the stability of ghrelin and attempt to decrease production costs, the amino acid sequence can be shortened. However, truncated peptides are unlikely to retain their secondary structure in physiological conditions due to the decrease in stabilizing hydrogen bonding from the backbone. These interactions also facilitate complete binding\textsuperscript{21,22}, as conformation is vital for ligand recognition\textsuperscript{15}. Helical structures play an important role in many biological processes\textsuperscript{15,22}; however, the energy required to generate this conformation is very high, which limits the structural stability of truncated peptides\textsuperscript{23}.

Pre-folding, or stapling the peptide assists in overcoming this energy barrier. This enables the truncated peptides to adopt the desired secondary structure, potentially increasing its biological activity and reducing susceptibility to hydrolysis\textsuperscript{15,25}. Cyclization typically occurs at positions \textit{i} and either \textit{i}+4 or \textit{i}+7 as they are positioned on the same face of a helix \textsuperscript{22,24}. There are four commonly used methods of constraining a peptide and promoting \textit{α}-helix formation: a lactam bridge, a hydrocarbon bridge, a metal-ion clip, or a hydrogen bond surrogate\textsuperscript{20,22}.

All of these stapling strategies have successfully produced cyclic peptides with increased target affinity. Peptides constrained with hydrocarbon and lactam bridges typically having greater stability \textit{in vivo}\textsuperscript{21,22}. The lactam bridge is predicted to be more appropriate for use in therapeutics due to its natural amide bond composition\textsuperscript{25}. The location of a staple is an important consideration in constrained peptides, as improper choice may block one face of the peptide\textsuperscript{23}. ‘Staple scanning’ is the preferred method for identifying a peptide sequence that will exhibit optimal agonist properties\textsuperscript{21}. This is done by manipulating each residue not essential for binding, and measuring the peptide’s affinity and secondary structure\textsuperscript{21}. This method will allow for the best staple candidate to be identified and further tested. The use of a glutamic acid residue in the \textit{i} position and a lysine residue in the \textit{i}+4 or \textit{i}+7 position appear to give peptides maximum helicity, as reversing the placement of these amino acids results in a decrease in helicity\textsuperscript{26}. 
The aim of this project was to create an improved fluorescently-tagged ghrelin peptide as a novel GHS-R1a detection tool, while gaining a better understanding of the secondary structure of ghrelin as the natural ligand for GHS-R1a. Only the first 20 amino acids of ghrelin were used in this study, as these amino acids demonstrated α-helical character based on 2D NMR studies\textsuperscript{13,14}, while the 8 amino acids at the C-terminus of ghrelin are predicted to be a random coil. In an attempt to recover α-helicity in the truncated peptide, lactam bridges were created in the $i$, $i+4$ and the $i$, $i+7$ positions with a staple scan utilizing amino acids 4 to 19 of ghrelin(1-20) being performed. Each peptide in this library was analyzed by circular dichroism (CD) spectroscopy and competitive receptor binding assay in order to determine α-helical character and binding affinity to GHS-R1a, respectively.

2.2 Results and Discussion

GHS-R1a has been shown to have increased expression in 32% of patient samples from ovarian cancer tissue\textsuperscript{27,28}. We propose that an improved ghrelin imaging probe, for fluorescence imaging of ovarian cancer cells or tissue samples through the use of chemistaining or confocal microscopy, can be developed through the creation of stapled ghrelin analogues. Previous studies have shown that truncated fluorescently labelled ghrelin analogs, where the fluorescent dye is attached through a Lys\textsuperscript{19} ε-amine of a modified ghrelin(1-19), provides a fluorescent probe with comparable GHS-R1a affinity to that of natural ghrelin(1-28) and was demonstrated to bind to GHS-R1a in cardiac tissues\textsuperscript{18,19}. This imaging probe was also used to distinguish between prostate cancer and benign hyperplasia in \textit{ex vivo} prostate tissue\textsuperscript{29} and for \textit{in vivo} biodistribution analysis\textsuperscript{30}. There is also a report indicating that a DOTA chelator attached to Lys\textsuperscript{19} can be used to form a gallium-68 radiolabelled ghrelin analogue for PET imaging\textsuperscript{31}. These previous analogues have reduced binding affinity towards GHS-R1a.

To establish a baseline and enable measurement of the effect synthetic staples have on α-helicity and binding to GHS-R1a, the natural ghrelin(1-20) sequence was synthesized by standard Fmoc solid-phase peptide synthesis (SPPS). In order to incorporate the unnatural n-octanyl side chain at position three, diaminopropionic acid was used. This allows for the coupling of octanoic acid via an amide linkage, which offers increased stability and ease of synthesis over the natural ester linkage (Figure 2.1).
Figure 2.1 - Depiction of ghrelin(1-20) indicating the region that is predicted to be coiled or part of an α-helix. X indicates the unnatural amino acid, diaminopropionic acid, which was used in order to attach the n-octanyl side chain at the third position. The dashed line shows the region of the sequence in which i, i+4 or i, i+7 staples were systematically placed.

The lactam bridge in the ghrelin(1-20) derivatives was formed utilizing glutamic acid in position i and lysine in position i+4 or i+7. To synthesize the stapled peptides, the linear peptide was first synthesized by SPPS, incorporating allylester-protected glutamic acid and alloxycarbonyl-protected lysine residues in the i and i+4 or i+7 positions respectively. These protecting groups were used because they can be selectively deprotected using a palladium (0) catalyst under basic conditions, allowing for lactam bridge formation without removing any other protecting groups or cleaving the peptide from the resin. After completing global deprotection and cleaving the peptide from the resin, the peptides were purified and characterized by LC-MS. Both i, i+4 (Table 2.1) and i, i+7 (Table 2.2) staple libraries were synthesized to observe any staple-related differences in helicity and binding when compared to the linear sequence (1).
Table 2.1 - Sequences of ghrelin(1-20) containing \((i, i+4)\) staples. Mean residue ellipticity at 222 nm ([\(\theta\])_{222}) and ratios of mean residue ellipticities at 222 and 208 nm ([\(\theta\])_{222}/[\(\theta\])_{208}) at 20°C. Competitive binding affinity data (IC\(_{50}\)) was determined using HEK293 cells expressing GHS-R1a and [\(^{125}\)I]ghrelin

<table>
<thead>
<tr>
<th>Sequence</th>
<th>[(\theta)](_{222}) (x 10(^5)) deg cm(^{-2}) dmol(^{-1}) Water</th>
<th>40% TFE/Water</th>
<th>[(\theta)](<em>{222}/\theta)(</em>{208}) Water</th>
<th>40% TFE/Water</th>
<th>IC(_{50}) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.46</td>
<td>0.13</td>
<td>-1.33</td>
<td>-0.04</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
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<td>-3.56</td>
<td>0.11</td>
<td>0.56</td>
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<tr>
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<td>0.79</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>6</td>
<td>-2.41</td>
<td>-6.19</td>
<td>0.51</td>
<td>0.86</td>
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<tr>
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<td>0.89</td>
<td>0.95</td>
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<td>13</td>
<td>-7.04</td>
<td>-13.99</td>
<td>0.71</td>
<td>0.94</td>
<td>10.9</td>
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Table 2.2: Sequences of ghrelin(1-20) containing (i, i+7) staples. Mean residue ellipticity at 222 nm ([θ]_{222}) and ratios of mean residue ellipticities at 222 and 208 nm ([θ]_{222}/[θ]_{208}) at 20°C are included to demonstrate helicity. Competitive binding affinity data (IC_{50}) was determined using HEK293 cells expressing GHS-R1a and [^{125}I]ghrelin.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>[θ]_{222} (× 10^4) deg cm⁻² dmol⁻¹</th>
<th>40% TFE/Water</th>
<th>[θ]<em>{222}/[θ]</em>{208}</th>
<th>IC_{50} (nM)</th>
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<tbody>
<tr>
<td>1</td>
<td>H-GSFLPQHQRVKRKESKK-NH₂</td>
<td>2.46 0.13</td>
<td>−1.33 −0.04</td>
<td>25</td>
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<tr>
<td>14</td>
<td>cyclo-4, 11OH-GSFLPQHQRVKRKESKK-NH₂</td>
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<tr>
<td>16</td>
<td>cyclo-6, 13OH-GSFLPQHQRVKRKESKK-NH₂</td>
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<td>&gt;1000</td>
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<tr>
<td>17</td>
<td>cyclo-7, 14OH-GSFLPQHQRVKRKESKK-NH₂</td>
<td>1.38 −0.49</td>
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<tr>
<td>18</td>
<td>cyclo-8, 15OH-GSFLPQHQRVKRKESKK-NH₂</td>
<td>−1.93 −4.49</td>
<td>0.48 0.69</td>
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</tr>
<tr>
<td>19</td>
<td>cyclo-9, 16OH-GSFLPQHQRVKRKESKK-NH₂</td>
<td>−2.20 −4.42</td>
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<td>cyclo-12, 19OH-GSFLPQHQRVKRKESKK-NH₂</td>
<td>−2.02 −4.43</td>
<td>0.48 0.69</td>
<td>29.0</td>
</tr>
</tbody>
</table>

To determine the degree of α-helicity, all compounds were analyzed by CD spectroscopy in either deionized water or 40% TFE, which is used to stabilize the α-helix to observe the maximum possible helicity for each peptide. To analyze the degree of α-helicity induced by these staples, the mean residue ellipticity at 222 nm ([θ]_{222}) and the ratio of the mean residue ellipticities at 222 and 208 nm ([θ]_{222}/[θ]_{208}) were utilized. The unstapled peptide (1) has the CD spectra characteristic of a random coil, with a maximum at 220 nm and a minimum at 190 nm. This was expected due to the lack of stabilizing factors. When the i, i+4 staples are introduced, the α-helicity increases for all sequences, with greater helicity (represented by a smaller [θ]_{222} value and a [θ]_{222}/[θ]_{208} ratio nearing 1.00) observed in peptides with the staple introduced near the C-terminus. Compounds 7, 9, and 10 exhibited the greatest helicity in both deionized water and 40% TFE. The observed differences in helicity could be due to differences in amino acid sequence. The region in between the glutamic acid and the lysine in compounds 9 and 10 is rich in glutamine, a helix-stabilizing residue, which may decrease the energy barrier for helix formation.

The increase in helicity induced by the i, i+7 staples is not as great relative to the increase generated by the i, i+4 staples. This could be due to the increased distance between the residues that generate the staple, which could be increasing the strain on the
cyclic peptide and discouraging helix formation. There is currently little agreement in the literature about the most effective ring size of the staple, as the effectiveness of stapling is very case dependent; it is possible that in this case, the ring size of the \( i+7 \) staple is too great. Regardless, compounds 18, 19 and 22 all show relatively large increases in helicity in both water and 40% TFE. Notably, compound 16 does not exhibit any appreciable increase in helicity; the proline residue adjacent to the glutamic acid could cause this. Proline is a helix destabilizing residue, as it causes more strain within the backbone, and therefore could have countered the helix-promoting effects of the staple.

Figure 2.2 - CD spectra of linear unstapled sequence of ghrelin (1-20) (1) and the \((i, i+4)\) (10) and \((i, i+7)\) (18) peptides that showed the greatest increase in helicity

The peptides were tested in 25% human serum, to determine if the cyclization provides improved stability to enzymatic hydrolysis. The linear sequence (1) saw a 40% degradation of the peptide within the first four hours, while both 10 and 18 were stable for 24 hours. This indicates that the presence of the staple has improved the stability of this truncated sequence, which plausibly is due to the increase in helicity.

The unstapled [Dpr\(^3\)-octanoyl]ghrelin(1-20) (1) peptide was used as a reference in order to compare how well the introduced staples improved the binding affinity of the
truncated sequence. To test the affinity of these peptides towards GHS-R1a, a competitive binding assay was performed with both unstapled and stapled peptides, with [125I] human ghrelin as the competitor. The IC50 for the \(i, i+4\) and \(i, i+7\) staples can be seen in Table 2.1 and Table 2.2, respectively. The greatest binding affinity is observed in peptides with staples close to the C-terminus. In general, binding affinity is lower than the linear peptide when the staple is within the first 7 amino acids, and greater when in the 8th position or further. These results correlate to those of the CD spectra, as the binding improves with increased \(\alpha\)-helicity. This relates to earlier findings that have noted that the first 5-8 amino acids are critical for receptor binding\(^{17}\). Therefore, modifying these positions to incorporate a lactam bridge would interfere with receptor recognition.

As with the helicity data, the binding affinities of the \(i, i+7\) staples were lower than that of the \(i, i+4\) staples and the unstapled ghrelin(1-20) (1), with the exception of 18 and 20. This could be due to the decrease in helicity relative to the \(i, i+4\) staples; it is also possible that the staple could be blocking the binding surface or forcing the peptide into a conformation that is incompatible with the receptor binding site.

Based on the helicity data and the IC50 values, one compound from the \(i, i+4\) (10) and \(i, i+7\) (18) staple libraries was chosen (Table 2.3). These compounds exhibited the optimal combination of \(\alpha\)-helicity and receptor binding.

Table 2.3 - Summary of IC50 (nM) and \(|\theta|_{222}/|\theta|_{208}\) values (in deionized water) for the best compounds from the \(i, i+4\) and \(i, i+7\) staple libraries. The values of the unstapled sequence are included for reference

| Compound | \(|\theta|_{222}/|\theta|_{208}| \) | IC50 (nM) |
|----------|---------------------------------|----------|
| 1        | 1.33                            | 25       |
| 10       | 0.88                            | 7.9      |
| 18       | 0.48                            | 10.3     |

To identify the effect of staple orientation on the binding of the peptides to GHS-R1a, a helical wheel projection was used. This will model the spatial location of the \(i, i+4\) and \(i, i+7\) staples in peptides 10 and 18, and will facilitate comparison by showing the positioning of amino acid residues, assuming that the ghrelin(8-20) region forms a perfect helix (Figure 2.3). While it is likely that this region is not perfectly helical, this is the
portion of natural ghrelin that is predicted to be helical\textsuperscript{13,14}. Both the $i, i+4$ and $i, i+7$ staples fall on the same face of the helix, indicating that the positioning of the staple may be imperative for binding.

![Helical wheel projection of ghrelin(8-20).](image)

**Figure 2.3** - Helical wheel projection of ghrelin(8-20). Indicated on the image are the locations of the staple in compound 10 ($i, i+4$) and 18 ($i, i+7$). This projection assumes that ghrelin(8-20) forms a perfect helix.

### 2.2.1 Modifying the best cyclic peptides for use in imaging GHS-R1a using confocal microscopy

The lead peptides identified from the staple scan (10 and 18) were labelled with a Sulfo-Cyanine 5 dye (Cy5) by forming an amide bond with the side chain of Lys\textsuperscript{20} (Table 2.4). This allowed for the dye to be as removed as possible from the binding areas of the peptide, as well as allowing for facile attachment of the dye. Sulfo-Cy5 was chosen due to its favourable properties such as its high solubility in water and high extinction coefficient (271000 M\textsuperscript{-1} cm\textsuperscript{-1} at 646 nm).

**Table 2.4** - Sequences of linear, ($i, i+4$), and ($i, i+7$) stapled ghrelin(1-20) peptides with a sulfo-cyanine5 dye attached to Lys\textsuperscript{19}. Mean residue ellipticity at 222 nm.
([θ]_{222}) and ratios of mean residue ellipticities at 222 and 208 nm ([θ]_{222}/[θ]_{208}) at 20°C are included to demonstrate helicity. Binding affinity data (IC_{50}) is also included.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>[θ]_{222} (× 10^5) deg cm⁻² dmol⁻¹</th>
<th>[θ]<em>{222}/[θ]</em>{208}</th>
<th>IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water 40% TFE/Water</td>
<td>Water 40% TFE/Water</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>-1.10 -13.83</td>
<td>0.22 0.70</td>
<td>11</td>
</tr>
<tr>
<td>24</td>
<td>-7.27 -17.52</td>
<td>0.86 0.95</td>
<td>1.0</td>
</tr>
<tr>
<td>25</td>
<td>-0.94 -8.88</td>
<td>0.19 0.59</td>
<td>6.2</td>
</tr>
</tbody>
</table>

These labelled peptides were analyzed to determine whether the Cy5 dye had an effect on secondary structure and ability to bind GHS-R1a. Interestingly, the α-helicity of the linear peptide with Cy5 (23) was greater than that of 1. This was unexpected, as the addition of Cy5 to the lead i, i+4 and i, i+7 sequences (24 and 25, respectively), did not elicit the same increase in helicity (Figure 2.4).

![Figure 2.4 - CD spectra of linear unstapled ghrelin(1-20) (1), the lead (i, i+4) stapled sequence (10), and the lead (i, i+7) stapled sequence (18) compared to their respective sequences containing the Sulfo-Cy5 dye (23-25)](image)

However, this increase in helicity of the Cy5-labelled linear peptide did not correlate with a significant change in binding to GHS-R1a. All labelled sequences had similar, or slightly improved, binding to GHS-R1a compared to the unlabelled sequences.
Considering the improved stability data for 10 and 18, this suggests that these probes have both improved GHS-R1a affinity and stability as compared to linear dye-ghrelin entities.

These compounds were then tested in a group of selected ovarian cancer cell lines. These cell lines were previously assessed for their relative GHS-R1a expression level by confocal microscopy using established linear dye-ghrelin probes (Figure 2.S1). Two cell lines (HEYA8 and OvCar3) express detectable endogenous GHS-R1a, while one cell line (OvCar8) had nearly undetectable expression. Thus, OvCar8 cells were stably transfected to express GFP-tagged GHS-R1a to generate a high expression line. The OvCar8-GHS-R1a cell line also served as a positive control to verify that these peptides were specifically binding to GHS-R1a and no other targets at the cell surface. Compounds 23-25 were incubated with the cells in order to identify if the binding assay results positively correlated with the ability of the peptides to bind to GHS-R1a in situ (Figure 2.5).

![Confocal microscopy of compounds 23-25 incubated with various ovarian cancer cell lines expressing GHS-R1a. One cell line (OvCar8) does not](image-url)
express GHS-R1a, one cell line (OvCar8-GHS-R1a+) was stably transfected with GHS-R1a, and two cell lines (HEYA8 and OvCar3) have naturally high expression of GHS-R1a. Cy5 signal can be seen in red and DAPI can be seen in blue

The $i, i+4$ sequence (24) exhibited increased binding to GHS-R1a in vitro compared to the linear sequence (23) and the $i, i+7$ sequence (25). This was expected, as 23 and 25 have a lower IC$_{50}$ value than 24. This difference is observed with the endogenous GHS-R1a expression in HEYA8 and OvCar3 cells, but most evident within the OvCar8 cells-GHS-R1a overexpressing cell line when compared with parental OvCar8 cells. Also compounds 23, 24, and 25 were co-incubated with a 10x concentration of compound 1 in each of the cell lines presented in Figure 5. This blocking study confirmed specificity towards GHS-R1a (Figure 2.S2 and 2.S3).

### 2.3 Conclusions

A staple scan of ghrelin(1-20) utilizing both $i, i+4$ and $i, i+7$ lactam bridge staples was performed and two peptides (10 and 18) were identified with superior qualities, in terms of increased helical content and greater binding affinity to GHS-R1a compared to the linear sequence. Modification of the lead sequences and the linear sequence with a Sulfo-Cy5 dye was then done in order to identify a better tool for fluorescence imaging of GHS-R1a. The dye-labelled version of the $i, i+4$ stapled peptide (24) had greatly increased affinity for GHS-R1a with similar helical content. Compound 24 was shown to bind to GHS-R1a in vitro when expressed by ovarian cancer cell lines that are stably-transfected with GHS-R1a, as well as cells with elevated endogenous expression. This potentially allows 24 to act as a specific and sensitive imaging tool for the identification and quantification of GHS-R1a among ovarian cancer cell lines, and potentially tumours. We propose that compound 24 could be further tested as an improved stain to aid in differentiating between malignant and benign tissue. This imaging tool therefore has the potential to be used in the clinic as a marker for GHS-R1a, a biomarker of aggressive malignancy within ovarian, prostate, and other cancer tissues$^{29}$. In addition, compound 24 could serve as a probe to screen for GHS-R1a expression in a broad array of cancer cell lines and tumour tissues, as well as quantification using flow cytometry techniques.
2.4 Acknowledgements

We would like to thank the Natural Science and Engineering Research Council (NSERC) of Canada and the Canadian Institutes of Health Research (CIHR) for funding through a Collaborative Health Research Project grant. Thanks to the Molecular Imaging Collaborative Program, University of Western Ontario. We also thank Ms. Becky McGirr for assistance with cell preparation and Dr. Jinqiang Hou for graphical support.

2.5 Materials and Methods

2.5.1 General Information

All reagents were obtained from the commercial suppliers and used without further purification. Fmoc-protected amino acids, HCTU, HATU, and Rink amide MBHA resin (4-(2’,4’-dimethoxyphenyl)-(9-fluorenylmethoxycarbonyl)-aminomethyl)-phenoxy-acetamidonorleucyl-4-methyl benzhydrylamine resin) were obtained from ChemImpex. Tetrais(triphenylphosphine)palladium(0), and phenylsilane were obtained from Sigma-Aldrich. All solvents were obtained from Fischer Thermo-Scientific. Sulfo-Cyanine-5 NHS Ester was obtained from Lumiprobe.

2.5.2 Solid-Phase Peptide Synthesis

Fmoc-based solid-phase synthesis was carried out manually and automatically using a Biotage® Syro Wave™ automated peptide synthesizer. Synthesis was done on a 0.1 mmol scale with 0.52 mmol/g Fmoc-Rink amide MBHA resin and a 3-fold excess of the protected amino acids. The resin was allowed to swell in DCM (2.0 mL, 15 min) and then solvent was removed. The resin was then rinsed with DMF (1.0 mL, 1 min); after removal of the solvent, Fmoc deprotection was performed by adding a solution of 20% piperidine/DMF (1.5 mL) to the resin and vortexing for 5 minutes. The resin was then washed three times with DMF (2.0 mL, vortex 30 sec) and the resin was vortexed for 15 min with 20% piperidine/DMF (1.5 mL). The resin was then washed with DMF six times to remove any unreacted products (2.0 mL, vortex 30 sec). A Kaiser test was performed after the Fmoc removal to verify the presence of a free primary amino group. The desired Fmoc-protected amino acid (0.3 mmol) and the coupling reagent HCTU (0.3 mmol) was dissolved in DMF (1.5 mL) and added to the resin. The mixture was vortexed for 30 seconds and then DIPEA (0.6 mmol) was added to the mixture and was vortexed for 1 hour. This deprotection/amino acid coupling cycle was repeated until the desired amino
acid sequence was obtained. After the final amino acid was coupled, the resin was washed 3 times with DMF (2.0 mL, vortex 30 sec) and then 3 times with DCM (2.0 mL, vortex 30 sec), dried under vacuum, and stored in the freezer at -20°C. The removal of the final Fmoc protecting group on the N-terminus was achieved using the previously described procedure.

To determine if the correct peptide has been synthesized, a microcleave was performed before full cleavage from the resin. A small amount of resin (<5 mg) was added to a small peptide vessel and a solution of 95% trifluoroacetic acid/2.5% triisopropylsilane/2.5% water (500 μL) was added. The mixture was vortexed for 3-4 hours, and the resulting liquid was precipitated with cold tert-butyl methyl ether (TBME) (2 mL). The precipitate was centrifuged (3000 xg for 10 min), and the mother liquor was decanted. The pellet was dissolved in water (2 mL), frozen, and lyophilized. If the correct peptide was synthesized as determined by analytical HPLC/MS, then a full cleave was performed. The procedure for full cleaves identical to the microcleave procedure: a solution of 95% trifluoroacetic acid/2.5% triisopropylsilane/2.5% water (3 mL) was added to the resin and vortexed for 4-6 hours. The solution was drained, precipitated in TBMe (20 mL) and centrifuged (3000 xg for 10 min). The mother liquor was decanted, and the pellet was dissolved in water (20 mL) and lyophilized to obtain the crude fully deprotected peptide.

2.5.3 Kaiser Test
To qualitatively determine if there is free amine present (i.e. after a Fmoc-deprotection, amino acid coupling, or alloc/allylester deprotection), a Kaiser test was performed. A small amount of resin (<5 mg) was placed in a small test tube. To the test tube, solutions of Phenol:EtOH (8:2 v/v), 0.001M KCN:pyridine (2.98 v/v) and ninhydrin in EtOH (5% w/v) were added. The test tube was heated to 70°C and the presence of a free amine was indicated by the resin beads turning blue. The lack of free amine was indicated by the presence of clear/yellow resin beads.

2.5.4 Deprotection of Allyloxycarbonyl (Alloc) and Allylester (OAll) Protecting Groups
Selective deprotection of the allyloxycarbonyl and the allylester protecting groups was accomplished by adding DCM (4.5 mL) to the resin-bound peptide and shaking gently for 10 minutes. After addition of phenylsilane (24 equiv.), the peptide vessel was
flushed with nitrogen and allowed to react for 5 minutes. Then, tetrakis(triphenylphosphine) palladium (0) (0.1 equiv.) was added to the mixture and the peptide vessel was again flushed with nitrogen, and the reaction was allowed to proceed for 10 minutes. Next, the peptide-resin was washed with DCM four times (1.5 mL), followed by a series of washings with 1.5 mL of DCM, DMF, MeOH, DMF, and DCM (30 sec each).

2.5.5 Lactam Bridge Formation
After the resin-bound peptide was selectively alloc- and allylester-deprotected, HATU (3 equiv.) was dissolved in DMF (1.5 mL) and allowed to vortex for 30 seconds. DIPEA (6 equiv.) was then added to the peptide vessel in order to form lactam bridge and the reaction was vortexed for 2 hours. Final washings with DMF and DCM (2.0 mL, 3 x 30 sec each) were performed to remove any residual reactants.

2.5.6 4-Methyltrityl (Mtt) Deprotection
Selective deprotection of the 4-methyltrityl (Mtt) protecting group was accomplished by swelling the resin in DCM (3 mL) for 15 minutes. The DCM was then drained and a cocktail of 95% DCM, 4% TIPS, and 1% TFA was added to the resin (2 mL). The resin was then vortexed for 2 minutes followed by a thorough rinse with DCM. This addition of the cocktail and vortexing was done 10 times, or until the yellow colour was no longer seen.

2.5.7 Coupling of Octanoic Acid
After the Mtt protecting group has been removed, octanoic acid was coupled to the modified serine residue in the third position. This was accomplished by adding octanoic acid (0.3 mmol) and the coupling reagent HATU (0.3 mmol) dissolved in DMF (1.5 mL) to the resin. This mixture was vortexed for 2 hours, and the resin was then washed 3 times with DMF (2.0 mL, vortex 30 sec) and 3 times with DCM (2.0 mL, vortex 30 sec), then dried under vacuum.

2.5.8 Purification of RP-HPLC/ESI-MS
Peptides were analyzed using a reverse-phase analytical HPLC column (Agilent Zorbax SB-C8 column 4.6 x 150 mm, 3.5 μm). This system was equipped with a Waters 600 136 controller, Waters Prep degasser, and Waters Masslynx software (version 4.1). Employed mobile phases were 0.1% TFA in deionized water (eluent A) and 0.1% TFA in
acetonitrile (eluent B). The flow rate was set at 1.5 mLmin⁻¹ over 15 minutes. The column eluate was monitored using a Waters 2998 Photodiode array detector set at 220 nm, 254 nm and 400 nm. Peptides were purified using a reverse-phase preparative HPLC column (Agilent Zorbax SB-C18 column 21.2 x 150 mm, 5 μm) on the same system mentioned above. The detection method and eluents were the same as mentioned above for the analytical system and the flow rate was set at 20 mLmin⁻¹. The collected fractions were then lyophilized to a solid and analyzed by ESI-MS. Purity of final products was determined by analytical RP-HPLC (200-800 nm).

2.5.9 Circular Dichroism (CD) Spectroscopy
CD spectra were obtained on a Jasco J-810 spectropolarimeter and recorded in the range of 180-260 nm. Peptide solutions were prepared with a 0.1 M phosphate buffer solution to a concentration of 0.5 mM. The measurements were performed in quartz cuvettes with a path length of 1 mm and a scanning speed of 10-50 nm/min. Five individual data points were averaged by the instrument to obtain reported CD spectra. The measurements were carried out at 20°C. A blank solution of 0.1 M phosphate buffer solution was run before every measurement in order to correct for any UV interference from the buffer.

2.5.10 Receptor Ligand Binding Assay
Competitive ligand binding assays were performed in triplicate using HEK293/GHS-R1a cells and [¹²⁵I]Ghrelin as the competitive ligand, as previously described¹⁷–¹⁹. Receptor binding affinities were calculated as IC₅₀ values as previously described¹⁷–¹⁹. In brief HEK293T cells were transiently transfected with GHS-R1a for 48 hours by means of calcium phosphate. Post transfection cells were harvested by membrane fragmentation at 50,000 g and stored at -80°C at 2 million cells per vial. The day of the experiment cells were thawed, and a complete vial was used per binding assay.

2.5.11 Transfection of OvCar8 with GFP-GHS-R1a
The OvCar8 human ovarian cancer cell line (ATCC) was maintained in RPMI medium (Wisent) supplemented with 10% fetal bovine serum (FBS; Wisent). Cells were seeded into 6-well tissue culture plates (Sarstedt) at a density of 1.5 x 10⁵ cells per 35-mm well. The following day, the culture media were removed and replaced with OptiMEM (Invitrogen) containing 10% FBS, and cells were transfected with 0.5 μg
pCMV6-GHS-R1a-EGFP plasmid using LipofectAMINE 3000 (Invitrogen) as per manufacturer’s instructions. Forty-eight hours later, cells were trypsinized and expanded into 4 separate 100-mm plates containing RPMI/10% FBS media. Stable clones were selected in 400 μg/ml G418 (Wisent) and maintained (in the same concentration of G418 for 7 to 10 days). GFP-positive colonies were visualized by indirect fluorescence using a Leica DMI 4000B inverted microscope, isolated using cloning rings, and transferred to 24-well plates containing RPMI/10% FBS.

2.5.12 Confocal Microscopy

OvCar3, HEYA8, OvCar8 and OvCar8 cells stably transected with GHS-R1a (OvCar-GHS-R1a) were trypsinized and seeded onto coverslips at a density of 75000 cells/well. The following day, media were removed and replaced with serum-free RPMI containing 0.1 μM of compounds 23, 24, and 25 and incubated at 37°C for 1 hour. Cells were then washed three times with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde in PBS and mounted onto slides containing Pro-Long Gold Antifade mounting medium with DAPI. Images were acquired using an Olympus FluoView FV 1000 confocal microscope.

Blocking experiments were conducted using OvCar8-GHS-R1a cells. Cells were suspended in serum-free RPMI and incubated with 0.1 μM of compounds 23, 24, and 25, together with a 10-fold excess of compound 1, at 37 °C for one hour. Cells were washed 3 times with PBS, reseeded onto coverslips, cultured in RPMI and allowed to adhere. Cells were washed with PBS, fixed with 4% formaldehyde in PBS and mounted onto slides. Images were then acquired as described above.

2.6 References


### Chapter 2 Supplementary Information

#### Peptide Characterization Tables

**Supplementary Table 2.1 - Characterization of synthesized unstapled truncated Ghrelin(1-20) (Where X is modified serine residue with an octanyl side chain)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>[M+3H]$^{3+}$ Expected m/z</th>
<th>Observed m/z</th>
<th>Purity (%)</th>
<th>Yield (%)</th>
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<td>GSXFLSPEHQRVQQR KESKK-NH$_2$</td>
<td>620.8769</td>
<td>620.8730</td>
<td>&gt;99</td>
<td>15</td>
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**Supplementary Table 2.2 - Characterization of synthesized $i, i+4$ lactam stapled truncated Ghrelin 1-20 (Where X is modified serine residue with an octanyl side chain)**

<table>
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<th>Compound</th>
<th>Sequence</th>
<th>[M+3H]$^{3+}$ Expected m/z</th>
<th>Observed m/z</th>
<th>Purity (%)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>H-(cyclo-4, 8)-GSX[ELSPK]HQRVQQRKESKK-NH$_2$</td>
<td>815.1706</td>
<td>815.8046</td>
<td>&gt;99</td>
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<tr>
<td>3</td>
<td>H-(cyclo-5, 9)-GSXF[ESPEK]QRVQQRKESKK-NH$_2$</td>
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<td>824.3851</td>
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</tr>
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<td>H-(cyclo-6, 10)-GSXFL[EPEHK]RVQQRKESKK-NH$_2$</td>
<td>836.5114</td>
<td>836.0917</td>
<td>&gt;99</td>
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<tr>
<td>5</td>
<td>H-(cyclo-7, 11)-GSXFLS[EEHQK]VQQRKESKK-NH$_2$</td>
<td>823.8233</td>
<td>823.4539</td>
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<td>6</td>
<td>H-(cyclo-8, 12)-GSXFLSP[EHQRK]QQRKESKK-NH$_2$</td>
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<td>H-(cyclo-9, 13)-GSXFLSPE[EQRVK]QQRKESKK-NH$_2$</td>
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<td>819.4630</td>
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<td>8</td>
<td>H-(cyclo-10, 14)-GSXFLSPEH[ERVVK]RQESKK-NH$_2$</td>
<td>822.8333</td>
<td>822.3869</td>
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<tr>
<td>9</td>
<td>H-(cyclo-11, 15)-GSXFLSPEHQ[EVQQK]KESKK-NH$_2$</td>
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<td>H-(cyclo-7, 14)-GSXFLS[EEHQQ]KKESKK-NH₂</td>
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<td>826.1233</td>
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Supplementary Table 2.3 - Characterization of synthesized $i, i+7$ lactam stapled truncated Ghrelin 1-20 (Where X is modified serine residue with an octanyl side chain)
| 22 | H-(cyclo-12, 19)-GSXFLSPEHQR[EQQRKESK]K-NH₂ | 831.4466 | 832.1008 | >95 | 13 |
**IC₅₀** competition binding curves for compounds 1-22 using HEK 293 cells in a competitive binding assay with [¹²⁵I]human ghrelin

**Compound 1**

![Graph of Compound 1 with IC₅₀ at 25 nM]

**Compound 2**

![Graph of Compound 2 with IC₅₀ >1000 nM]
Compound 3

% Radioligand Bound

Log Concentration (M)

984 nM

Compound 4

% Radioligand Bound

Log Concentration (M)

667 nM
Compound 5

% Radioligand Bound

Log Concentration (M)

>1000 nM

Compound 6

% Radioligand Bound

Log Concentration (M)

14.7 nM
Compound 7

% Radioligand Bound

23.4 nM

Log Concentration (M)

Compound 8

% Radioligand Bound

10.6 nM

Log Concentration (M)
Compound 9

Log Concentration (M) vs. % Radioligand Bound

- Compound 9 at 37.3 nM

Compound 10

Log Concentration (M) vs. % Radioligand Bound

- Compound 10 at 7.85 nM
Compound 11

% Radioligand Bound

8.16 nM

Log Concentration (M)

Compound 12

% Radioligand Bound

11.8 nM

Log Concentration (M)
Compound 13

% Radioligand Bound

Log Concentration (M)

10.9 nM

Compound 14

% Radioligand Bound

Log Concentration (M)

>1000 nM
Compound 15

Compound 16
Compound 17

Log Concentration (M) % Radioligand Bound

10^-12 10^-10 10^-8 10^-6 10^-4

80 100

48.3 nM

Compound 18

Log Concentration (M) % Radioligand Bound

10^-12 10^-10 10^-8 10^-6 10^-4

0 20 40 60 80 100 120 140

10.3 nM
Compound 19

[Graph showing concentration-response curve]

Log Concentration (M)

% Radioligand Bound

106 nM

Compound 20

[Graph showing concentration-response curve]

Log Concentration (M)

% Radioligand Bound

7.41 nM
Compound 21

**% Radioligand Bound**

Log Concentration (M)

91.9 nM

Compound 22

**% Radioligand Bound**

Log Concentration (M)

29.0 nM
Compound 23

% Radioligand Bound

Log Concentration (M)

Compound 24

% Radioligand Bound

Log Concentration (M)
Compound 25

Log Concentration (M) vs. % Radioligand Bound

Log Concentration (M):
- $10^{-12}$
- $10^{-10}$
- $10^{-8}$
- $10^{-6}$
- $10^{-4}$

% Radioligand Bound:
- 0
- 20
- 40
- 60
- 80
- 100
- 120

6.2 nM
HPLC and MS traces for compounds 1-22

**Compound 1**

**LC:**

**MS:**

\[ [M+4H]^4+ \]

620.8730
Compound 2

LC:

MS:

[M+3H]$^{3+}$
815.8046
Compound 3

LC:

MS:
Compound 4

LC:

MS:

\[ [M+3H]^{3+} \]
836.0917
**Compound 5**

LS:

MS:

\[[M+3H]^3+\]
823.4539
Compound 6

LC:

MS:

[M+3H]^{3+}
831.7017
Compound 7

LC:

MS:

$[\text{M+3H}]^{3+}$

819.4630
Compound 8

LC:

MS:

[M+3H]^{3+}
822.3869
Compound 9

LC:

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MS:

[M+3H]^{3+}

803.6989
Compound 10

LC:

MS:

$[M+3H]^{3+}$
832.1008
Compound 12

LC:

MS:

[M+3H]^3+

836.0917
Compound 13

LC:

MS:

[M+3H]^3+
813.0776
Compound 14

LC:

MS:

[M+3H]^{3+}
806.6921
Compound 15

LC:

MS:

\[ [\text{M+3H}]^{3+} \]
836.9563
Compound 16

LC;

MS:

[\text{[M+4H]}^{4+}]

678.2451
**Compound 17**

**LC:**

**MS:**

\[ [M+4H]^{4+} \]

678.4536
Compound 18

LC:

MS:

\[[M+3H]^3+\]  
845.6032
Compound 19

**LC:**

**MS:**

[M+3H]^3+  
819.2634
Compound 20

LC:

MS:

$[M+3H]^{3+}$

822.1901
Compound 21

LC:

MS:

$[M+3H]^{3+}$
826.7796
**Compound 22**

**LC:**

![LC chromatogram](image)

**MS:**

![MS spectrum](image)

**[M+3H]^3+** 832.1005
Compound 23

LC:

MS:

[M+3H]^{3+}
Compound 24

LC:

MS:

$[M+3H]^{3+}$
Compound 25

LC:

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MS:

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<td>12.00E+00</td>
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</tr>
</tbody>
</table>

[m+3H]^3+
Additional Confocal Images

Supplementary Figure 2.1 - (top) Confocal microscopy images incubated with the Cy5-Ghrelin(1-19) in 5 different ovarian cancer cell lines; (middle) Confocal microscopy images incubated with the Cy5-Ghrelin(1-19) and blocked with 10x concentrated Ghrelin(1-19) in 5 different ovarian cancer cell lines; (bottom) Confocal microscopy images incubated with the scrambled-Cy5-Ghrelin(1-19) in 5 different ovarian cancer cell lines
Supplementary Figure 2.2 - (a) Confocal microscopy of compound 25 incubated with compound 1 in the Ovcar8 GHS-R1a<sup>+/+</sup> cell line; (b) Confocal microscopy of compound 24 incubated with compound 1 in the Ovcar8 GHS-R1a<sup>+/+</sup> cell line; (c) Confocal microscopy of compound 23 incubated with compound 1 in the Ovcar8 GHS-R1a<sup>+/+</sup> cell line; (d) Confocal microscopy of compound 25 incubated with compound 1 in the Ovcar8 cell line; (e) Confocal microscopy of compound 24 incubated with compound 1 in the Ovcar8 cell line; (f) Confocal microscopy of compound 23 incubated with compound 1 in the Ovcar8 cell line.
Supplementary Figure 2.3 - (a) Confocal microscopy of compound 25 incubated with compound 1 in the Ovcar8 GHS-R1α+/+ cell line; (b) Confocal microscopy of compound 24 incubated with compound 1 in the Ovcar8 GHS-R1α+/+ cell line; (c) Confocal microscopy of compound 23 incubated with compound 1 in the Ovcar8 GHS-R1α+/+ cell line; (d) Confocal microscopy of compound 25 incubated with compound 1 in the Ovcar8 cell line; (e) Confocal microscopy of compound 24 incubated with compound 1 in the Ovcar8 cell line; (f) Confocal microscopy of compound 23 incubated with compound 1 in the Ovcar8 cell line
Chapter 3

Peptidomimetic growth hormone secretagogue derivatives for positron emission tomography imaging of GHS-R1a

3.1 Introduction

Growth hormone secretagogues (GHSs) are a class of compounds that stimulate the secretion of growth hormone (GH) by acting on the growth hormone secretagogue receptor type-1a (GHS-R1a). This receptor is predominantly found in the hypothalamus and pituitary gland and its secretion occurs through a route disparate from that of growth hormone-releasing hormone (GHRH). The first GHSs to show GH release in vitro were a series of Met-enkephalin analogues described by Bowers et al in 1980. Further development of these analogues led to a hexamer that released GH in vivo in a number of animals, including humans. This peptide was later termed growth hormone-releasing peptide-6 (GHRP-6), and a number of GHSs were subsequently synthesised in order to find alternatives to recombinant human GH therapy. These encompassed peptides (e.g. GHRP-1, hexarelin, KP-102 (later designated GHRP-2), peptidomimetics (e.g. G7039, [1-Nal]4G7039 and ipamorelin) and small-molecules (e.g. L-692,429 and MK-0677). The amino acid sequences of the peptidic GHSs and the structures of the peptidomimetic and small-molecule GHSs are shown in Figure 3.1. The endogenous ligand for GHS-R1a is ghrelin, a 28-mer peptide with an n-octanoyl group on the Ser side-chain, which was discovered in 1999 and exhibits a multitude of biological activities, such as the regulation of food intake and glucose metabolism.

---

GHS-R1α is expressed in a number of human malignancies including prostatic carcinoma cell lines and tissues, breast carcinoma tumours and cell lines, testicular tumours, and malignant ovarian cysts and tumours. In addition, it has an elevated level of expression in tissues from patients with atherosclerosis and in biopsies from those with chronic heart failure. GHS-R1α thus represents a potential target for molecular imaging of carcinoma and cardiovascular disease.

Among the imaging modalities most commonly utilised in the clinic, positron emission tomography (PET) combines a high spatial resolution with high sensitivity. A number of recent publications have thus described the development of PET radiotracers for visualising the GHS-R1α receptor in disease. For example, Hou and co-workers synthesized the first picomolar quinazolinone binder of GHS-R1α and successfully radiolabelled two nanomolar compounds in high radiochemical purity. In another study on small-molecule derivatives, in vivo imaging in mice with a \([^{11}C]\)radiotracer revealed a higher specific uptake in the pancreas compared to other organs. However, these studies and others have primarily focused on either small-molecule quinazolinones or ghrelin-
derived compounds\textsuperscript{29,30,33}. To the best of our knowledge, a growth hormone secretagogue-based PET probe has not been reported to date. We reasoned that modification of peptidic and peptidomimetic GHSs should result in a clinically translatable PET agent with high target specificity, \textit{in vivo} stability and favourable pharmacokinetic properties. Fluorine-18 was our preferred choice of radioisotope as it is a small innocuous unit that can be easily installed in the absence of a chelator using the $[^{18}\text{F}]$fluorobenzoyl ($[^{18}\text{F}]$FB) prosthetic group. This modification can be initially trialled with $4-[^{19}\text{F}]$fluorobenzoic acid ($[^{19}\text{F}]$FBA), in order to determine the optimal location for the $^{18}$F-radioisotope without significantly affecting peptide/peptidomimetic binding to GHS-R1a.

We describe herein the design, synthesis and biological evaluation of 4-fluorobenzoylated derivatives of GHSs with peptidic (GHRP-1, GHRP-2 and GHRP-6) and peptidomimetic (G7039, [1-Nal\textsuperscript{4}]G7039 and ipamorelin) structures; the determination of their IC\textsubscript{50} values for GHS-R1a; the identification of the lead peptide [1-Nal\textsuperscript{4},Lys\textsuperscript{5}(4-FB)]G7039 with nanomolar IC\textsubscript{50} and EC\textsubscript{50} values; and the $^{18}$F-radiolabelling of its precursor [1-Nal\textsuperscript{4}]G7039 to furnish the peptidomimetic growth hormone secretagogue [1-Nal\textsuperscript{4},Lys\textsuperscript{5}(4-$[^{18}\text{F}]$-FB)]G7039, which could be applied to PET imaging of carcinoma and cardiovascular disease \textit{via} targeting of GHS-R1a.

3.2 Results and Discussion

3.2.1 Design strategy for peptidic and peptidomimetic growth hormone secretagogues
Initially, we sought to determine the location for $^{18}$F insertion into the peptidic and peptidomimetic GHSs without significantly affecting receptor binding affinity. A study of the literature revealed that Huang and co-workers had designed a 3D pharmacophore for GHS activity using peptidic and non-peptidic compounds without inclusion of the lysine side-chain\textsuperscript{34}. This led to the synthesis of a benzothiazepin compound with low nanomolar \textit{in vitro} efficacy for GHS-R1a\textsuperscript{34}. In addition, we recently reported a GHS-R1a homology model based on neurotensin and opioid receptors with docking of the peptidomimetic G7039\textsuperscript{35}. The lysine residue was found to be unimportant for G7039-GHS-R1a binding owing to an unfavourable interaction energy between its side-chain amino group and polar Glu\textsuperscript{197}/Arg\textsuperscript{199} residues\textsuperscript{35}. This led us to select the lysine side-chain for $^{18}$F-radioisotope insertion in the peptidic (GHRP-1, GHRP-2 and GHRP-6)
and peptidomimetic (G7039, [1-Nal\textsuperscript{4}]G7039 and ipamorelin) GHSs using the $[^{18}\text{F}]$FB prosthetic group \textit{via} the pre-activated N-succinimidyl-4-$[^{18}\text{F}]$fluorobenzoate ($[^{18}\text{F}]$SFB)$^{36}$. This modification was first tested by coupling a non-radioactive $[^{18}\text{F}]$FB mimic ($[^{19}\text{F}]$FBA) to the lysine side-chain of each individual GHS class using the orthogonal allyloxycarbonyl (Alloc) protecting group by standard Fmoc-solid phase peptide synthesis (SPPS). Any change in binding affinity for GHS-R1a (expressed in terms of the IC\textsubscript{50}) was determined experimentally through a competitive receptor-ligand binding assay. Each parent growth hormone secretagogue (Figure 3.1) would be modified in turn until an IC\textsubscript{50} $< 100$ nM was achieved.

\subsection*{3.2.2 \textit{Synthesis and characterisation of peptidic and peptidomimetic growth hormone secretagogues}}

The peptidic and peptidomimetic GHSs were synthesised through manual or automated Fmoc-SPPS and purified by reverse-phase (RP)-HPLC. Amino acids sequences for these GHSs are shown in the supporting information (Table 3.1S1), with characterisation data listed in Table 3.1.

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Peptide/Peptidomimetic & HRMS (calculated) [M + H]\textsuperscript{+} & HRMS (found) [M + H]\textsuperscript{+} & \% Purity & \% Yield \\
\hline
Ipamorelin            & 712.3935 & 712.3959 & $> 97$ & 19 \\
[Lys\textsuperscript{5}(4-FB)]ipamorelin & 834.4103 & 834.4133 & $> 99$ & 15 \\
[Lys\textsuperscript{5}(AEEA-4-FB)]ipamorelin & 979.4842 & 979.4868 & $> 98$ & 8 \\
[D-2-Thi\textsuperscript{4}, Lys\textsuperscript{5}(4-FB)]ipamorelin & 840.3667 & 840.3693 & $> 98$ & 4 \\
[Inp\textsuperscript{1}, Lys\textsuperscript{5}(4-FB)]ipamorelin & 860.4259 & 860.4284 & $> 96$ & 6 \\
[Inp\textsuperscript{1}, D-2-Nal\textsuperscript{4}, Lys\textsuperscript{5}(4-FB)]ipamorelin & 910.4416 & 910.4400 & $> 98$ & 10 \\
[Inp\textsuperscript{1}, D-2-Thi\textsuperscript{4}, Lys\textsuperscript{5}(4-FB)]ipamorelin & 866.3824 & 866.3850 & $> 99$ & 13 \\
\hline
\end{tabular}
\caption{HRMS data, purities and yields for synthesised peptides and peptidomimetics. All amino acids are designated by the standard three-letter code. 4-FB, 4-fluorobenzoyl; AEEA, 2-(2-(2-aminoethoxy)ethoxy)acetic acid; D-2-Nal, D-2-naphthylalanine; D-2-Thi, D-2-thienylalanine; Dpr, 2,3-diaminopropionic acid; Inp, isonipecotic acid. *Characterisation data reported previously\textsuperscript{35}}
\end{table}
3.2.3 Structure-activity relationships of peptidic and peptidomimetic growth hormone secretagogues

The IC\textsubscript{50} values of the peptidic and peptidomimetic GHSs were determined through receptor-ligand binding assays utilising human embryonic kidney 293 (HEK293)/GHS-R1a cells and [\textsuperscript{125}I]ghrelin as the competitive radioligand. In order to ascertain whether overexpression of the receptor in HEK293 cells had been achieved, the endogenous ligand ghrelin was assayed first. An IC\textsubscript{50} value of 7.63 nM was obtained (Figure 3.S1), indicating a satisfactory level of receptor expression and maintaining consistency with previously reported values\textsuperscript{37}.

The initial peptidomimetic that was chosen for \textsuperscript{19}F-fluorobenzoylation was the pentapeptide ipamorelin (H-Aib-His-D-2-Nal-D-Phe-Lys-NH\textsubscript{2}, compound 1, Table 3.2). This is because it displayed a nanomolar binding affinity in previous work using HEK293/GHS-R1a cells (\(K_i = 240\) nM)\textsuperscript{38}.

<table>
<thead>
<tr>
<th>Compound</th>
<th>\text{M + Na}\textsuperscript{+}</th>
<th>\text{M + Na}\textsuperscript{+}</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHRP-1</td>
<td>955.4943</td>
<td>955.4964</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>GHRP-2</td>
<td>840.4204</td>
<td>840.4173</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>GHRP-6</td>
<td>873.4524</td>
<td>873.4531</td>
<td>&gt; 97</td>
</tr>
<tr>
<td>[Lys\textsuperscript{6}(4-FB)]GHRP-6</td>
<td>1017.4511</td>
<td>1017.4522</td>
<td>&gt; 96</td>
</tr>
<tr>
<td>[Dpr\textsuperscript{6}]GHRP-6</td>
<td>831.4055</td>
<td>831.4070</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>[Dpr\textsuperscript{6}(4-FB)]GHRP-6</td>
<td>953.4223</td>
<td>953.4237</td>
<td>&gt; 98</td>
</tr>
<tr>
<td>G7039\textsuperscript{*}</td>
<td>798.4343\textsuperscript{*}</td>
<td>798.4339\textsuperscript{*}</td>
<td>&gt; 99\textsuperscript{*}</td>
</tr>
<tr>
<td>[Lys\textsuperscript{5}(4-FB)]G7039</td>
<td>920.4511</td>
<td>920.4529</td>
<td>&gt; 97</td>
</tr>
<tr>
<td>[1-Nal\textsuperscript{4}]G7039</td>
<td>848.4499</td>
<td>848.4501</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>[1-Nal\textsuperscript{4}, Lys\textsuperscript{5}(4-FB)]G7039</td>
<td>970.4667</td>
<td>970.4693</td>
<td>&gt; 96</td>
</tr>
</tbody>
</table>
Table 3.2 - IC₅₀ values of ipamorelin and a series of derivatives thereof. The listed IC₅₀ values were determined in triplicate using HEK293/GHS-R1a cells. For corresponding displacement curves, the reader is referred to the supporting information.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>IC₅₀, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ipamorelin</td>
<td>483</td>
</tr>
<tr>
<td>2</td>
<td>[Lys⁵(4-FB)]ipamorelin</td>
<td>170</td>
</tr>
<tr>
<td>3</td>
<td>[Lys⁵(AEEA-4-FB)]ipamorelin</td>
<td>474</td>
</tr>
<tr>
<td>4</td>
<td>[D-2-Thi⁴, Lys⁵(4-FB)]ipamorelin</td>
<td>161</td>
</tr>
<tr>
<td>5</td>
<td>[Inp¹, Lys⁵(4-FB)]ipamorelin</td>
<td>688</td>
</tr>
<tr>
<td>6</td>
<td>[Inp¹, D-2-Nal⁴, Lys⁵(4-FB)]ipamorelin</td>
<td>1920</td>
</tr>
<tr>
<td>7</td>
<td>[Inp¹, D-2-Thi⁴, Lys⁵(4-FB)]ipamorelin</td>
<td>1170</td>
</tr>
</tbody>
</table>

Introduction of a fluorobenzoyl moiety into the lysine side-chain caused the IC₅₀ value of ipamorelin to decrease from 483 nM to 170 nM (compound 2, [Lys⁵(4-FB)]ipamorelin). This could be a consequence of the fluorobenzoyl group strengthening hydrophobic interactions with aromatic residues (e.g. Phe222 and Phe226) in the non-polar sub-pocket described in the open GHS-R1a homology model of Pedretti and co-workers.³⁹ Ipamorelin also possesses the same three C-terminal residues as G7039 (the only difference being D-Phe⁴ as opposed to Phe⁴, respectively) and thus the additional fluorobenzene ring could also be interacting with residues in one of the hydrophobic subpockets (e.g. Phe286) detailed by Hou et al.³⁵. Extension of the lysine side-chain through a short mini-PEG linker (2-(2-(2-aminoethoxy)ethoxy)acetic acid, AEEA) prior to 4-fluorobenzoyl group coupling furnished [Lys⁵(AEEA-4-FB)]ipamorelin, compound 3. This had a higher IC₅₀ value compared to compound 2 (cf. 474 nM for [Lys⁵(AEEA-4-FB)]ipamorelin to 170 nM for [Lys⁵(4-FB)]ipamorelin). This may result from the additional flexibility afforded by the mini-PEG linker, which could cause a reduction in important hydrophobic interactions of the 4-fluorobenzoyl moiety when compared to the less flexible compound 2. Replacement of the D-Phe residue with D-2-thienylalanine (D-2-Thi) was expected to improve binding affinity. This strategy was based on a study by Hansen and co-workers on dipeptide ipamorelin derivatives, where this alteration caused the EC₅₀ value to improve by an order of magnitude.⁴⁰ Unfortunately, this modification...
furnished a compound with comparable affinity to the [Lys<sup>5</sup>-(4-FB)]ipamorelin analogue 2 with an IC<sub>50</sub> = 161 nM ([D-2-Thi<sup>4</sup>,Lys<sup>5</sup>-(4-FB)]ipamorelin, compound 4).

The primary amine at the N-terminus of ipamorelin was then exchanged for the secondary amine isonipecotic acid (Inp) so as to make the subsequent compound ([Inp<sup>1</sup>,Lys<sup>5</sup>-(4-FB)]ipamorelin, compound 5) similar to the peptidomimetic G7039, known to have an EC<sub>50</sub> = 0.18 nM<sup>10</sup>. This led to a rise in the IC<sub>50</sub> value (cf. [Lys<sup>5</sup>-(4-FB)]ipamorelin, IC<sub>50</sub> = 170 nM, compound 2 to [Inp<sup>1</sup>,Lys<sup>5</sup>-(4-FB)]ipamorelin, IC<sub>50</sub> = 688 nM, compound 5).

In order to probe the significance of the D-Phe residue in sub-pocket binding, this residue was replaced with D-2-Nal in [Inp<sup>1</sup>,Lys<sup>5</sup>-(4-FB)]ipamorelin (5) to give [Inp<sup>1</sup>,D-2-Nal<sup>4</sup>,Lys<sup>5</sup>-(4-FB)]ipamorelin (IC<sub>50</sub> = 1920 nM, compound 6). This caused an approximately three-fold reduction in binding affinity for GHS-R1a compared to the parent compound 5. This suggests that D-Phe may be taking part in π-π or hydrophobic interactions with Phe119 in TM3<sup>39,41</sup>, playing the same role as it does in the active tetra-peptide core of ghrelin<sup>42</sup>. The synthesis of compound 7 ([Inp<sup>1</sup>,D-2-Thi<sup>4</sup>,Lys<sup>5</sup>-(4-FB)]ipamorelin) and its corresponding IC<sub>50</sub> value (1170 nM) could be seen as an approximately two-fold reduction in binding affinity for GHS-R1a from switching D-2-Thi for D-Phe in compound 5 ([Inp<sup>1</sup>,Lys<sup>5</sup>-(4-FB)]ipamorelin); or an approximately seven-fold decrease in binding resulting from insertion of an unfavourable secondary amine (Inp) into compound 4 ([D-2-Thi<sup>4</sup>,Lys<sup>5</sup>-(4-FB)]ipamorelin). In summary, the various synthesised derivatives of ipamorelin did not yield the desired IC<sub>50</sub> of < 100 nM, despite some improvements to its initial IC<sub>50</sub> value.

Next, our attention turned to the peptidic GHSs (GHRP-1, GHRP-2 and GHRP-6) due to the low nanomolar K<sub>i</sub> values that were obtained for GHRP-2 and GHRP-6 (1.4 nM and 3.3 nM, respectively) using HEK293/GHS-R1a cells<sup>38</sup>. The IC<sub>50</sub> values of GHRP-1, GHRP-2 and GHRP-6 are shown in Table 3.3 (compounds 8-10, respectively).
Table 3.3 - IC₅₀ values of peptidic GHSs and their derivatives. The listed IC₅₀ values were determined in triplicate using HEK293/GHS-R1a cells. For corresponding displacement curves, the reader is referred to the supporting information.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>IC₅₀, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>GHRP-1</td>
<td>181</td>
</tr>
<tr>
<td>9</td>
<td>GHRP-2</td>
<td>449</td>
</tr>
<tr>
<td>10</td>
<td>GHRP-6</td>
<td>73</td>
</tr>
<tr>
<td>11</td>
<td>[Lys⁶(4-FB)]GHRP-6</td>
<td>384</td>
</tr>
<tr>
<td>12</td>
<td>[Dpr⁶]GHRP-6</td>
<td>397</td>
</tr>
<tr>
<td>13</td>
<td>[Dpr⁶(4-FB)]GHRP-6</td>
<td>1060</td>
</tr>
</tbody>
</table>

It was found that GHRP-6, (H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂) had the strongest binding to GHS-R1a (IC₅₀ = 73 nM, compound 10) compared to GHRP-1 (IC₅₀ = 181 nM, compound 8) and GHRP-2 (IC₅₀ = 449 nM, compound 9). This peptide was therefore transformed into the fluorobenzoylated analogue [Lys⁶(4-FB)]GHRP-6 (IC₅₀ = 384 nM, compound 11). Unfortunately, this alteration was not tolerated by GHS-R1a in spite of prior success with ipamorelin (cf. compound 11, IC₅₀ = 384 nM to compound 2, IC₅₀ = 170 nM). One possible explanation for this is that the lysine residue of GHRP-6 is involved in binding to polar sub-pocket residues (e.g. Arg199).³⁹

Endeavouring to increase affinity for GHS-R1a, the lysine of GHRP-6 was replaced with 2,3-diaminopropionic acid (Dpr) to furnish [Dpr⁶]GHRP-6 (IC₅₀ = 397 nM, compound 12). This increase in IC₅₀ could be a product of the shorter side-chain of Dpr which could be decreasing lysine’s charge-transfer interactions with Arg199 in the polar sub-pocket. This would also explain the greater loss in binding affinity after the attachment of the 4-fluorobenzoyl group ([Dpr⁶(4-FB)]GHRP-6, IC₅₀ = 1060 nM, compound 13). Not only does the lysine side-chain appear to play an important role in receptor binding for GHRP-6 (a result contrary to that obtained for ipamorelin), but the length of the alkyl chain also appears to be essential. Further derivatisation of this class of peptides was not pursued owing to the high initial IC₅₀ values of GHRP-1 (181 nM, compound 8) and GHRP-2 (449 nM, compound 9) and the assumption that the lysine residue in these peptides was also important for receptor interaction.
Finally, two Genentech 5-mers G7039 (H-Inp-D-2-Nal-D-2-Nal-Phe-Lys-NH₂) and [1-Nal⁴]G7039 (H-Inp-D-2-Nal-D-2-Nal-1-Nal-Lys-NH₂) were investigated as a consequence of their reported low nanomolar in vitro efficacies (EC₅₀ = 0.18 nM for G7039 and 0.10 nM for [1-Nal⁴]G7039, respectively) and our recent computational study on the docking of G7039 to GHS-R1a, which suggested that the lysine residue was not critical for receptor binding. The parent compounds G7039 (compound 14) and [1-Nal⁴]G7039 (compound 16) and their fluorobenzoyl congeners (compounds 15 and 17, respectively) can be viewed in Table 3.4.

Table 3.4 - IC₅₀ values of Genentech peptidomimetics and their derivatives. The listed IC₅₀ values were determined in triplicate using HEK293/GHS-R1a cells. For corresponding displacement curves, the reader is referred to the supporting information. *Literature data

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>IC₅₀, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>G7039*</td>
<td>5.2*</td>
</tr>
<tr>
<td>15</td>
<td>[Lys⁵(4-FB)]G7039</td>
<td>242</td>
</tr>
<tr>
<td>16</td>
<td>[1-Nal⁴]G7039</td>
<td>28</td>
</tr>
<tr>
<td>17</td>
<td>[1-Nal⁴, Lys⁵(4-FB)]G7039</td>
<td>69</td>
</tr>
</tbody>
</table>

Attaching G7039 to para-fluorobenzoic acid had the effect of increasing the half-maximal inhibitory concentration (5.2 nM to 242 nM for [Lys⁵(4-FB)]G7039, compound 15), whilst a smaller increase in the IC₅₀ value was observed for [1-Nal⁴]G7039 (28 nM to 69 nM for [1-Nal⁴,Lys⁵(4-FB)]G7039, compound 17). The smaller increase observed for [1-Nal⁴]G7039 compared to G7039 indicates that the introduction of an additional aromatic functionality (fluorobenzene) along with the already present 1-Nal⁴ residue is simply strengthening hydrophobic sub-pocket binding, whereas for G7039 this modification is less favourable; perhaps due to the reduction of salt-bridge interactions with Glu197 in the polar sub-pocket. In spite of the increase in IC₅₀ value for [1-Nal⁴,Lys⁵(4-FB)]G7039, this peptidomimetic represents the first lead compound to be synthesised with the required nanomolar IC₅₀ value (< 100 nM) making it the most suitable candidate for further study of physical and biochemical characteristics prior to [¹⁸F]-radiolabelling with [¹⁸F]SFB.
3.2.4 **Determination of lipophilicity of peptidic and peptidomimetic growth hormone secretagogues**

The log\(P\) value of [1-Nal\(^4\),Lys\(^5\)(4-FB)]G7039 as well as the other peptidic and peptidomimetic GHSs were computed using ACD/Log\(P\) software (Table 3.S2). Inserting the 4-fluorobenzoyl moiety into any peptidomimetic parent compound resulted in an increase in hydrophobicity for all derivatives (+2.25). Most of the parent compounds exhibited a favourable log\(P\) value in the 1-3 unit range, except for G7039 (5.28 ± 0.82) and [1-Nal\(^4\)]G7039 (6.51 ± 0.82). This is virtue of the smaller size of these peptidomimetics compared to GHRP-1, GHRP-2 and GHRP-6, and the prevalence of more hydrophobic aromatic residues compared to ipamorelin (D-2-Nal and 1-Nal).

Regrettably, the lead compound [1-Nal\(^4\),Lys\(^5\)(4-FB)]G7039 had a log\(P\) value of 8.76 ± 0.88, suggesting a lack of suitability for *in vivo* PET imaging due to potential solubility issues. Nevertheless, [1-Nal\(^4\),Lys\(^5\)(4-FB)]G7039 possesses a considerably lower IC\(_{50}\) value compared to the next best compound [D-2-Thi\(^4\), Lys\(^5\)(4-FB)]ipamorelin (cf. 69 nM to 161 nM) and was thus investigated in further *in vitro* assays.

3.2.5 **Determination of efficacy of lead peptidomimetic [1-Nal\(^4\),Lys\(^5\)(4-FB)]G7039**

The EC\(_{50}\) value of [1-Nal\(^4\),Lys\(^5\)(4-FB)]G7039 was calculated in terms of the release of intracellular calcium ions (Figure 3.2). As expected, the control ligand ghrelin had a low *in vitro* potency of 1.6 nM, whilst the EC\(_{50}\) value of [1-Nal\(^4\),Lys\(^5\)(4-FB)]G7039 was determined as 1.1 nM. This low nanomolar efficacy signifies that the final compound remains a potent GHS-R1a agonist despite blocking of the lysine side-chain with the 4-fluorobenzoyl group.
3.2.6 Stability of lead peptidomimetic [1-Nal⁴,Lys⁵(4-FB)]G7039 in human serum

The lead peptidomimetic agonist [1-Nal⁴,Lys⁵(4-FB)]G7039 was tested for stability in human serum in order to assess its biological half-life. This serum half-life was found to be 718 minutes, indicating a good potential time-frame for in vivo PET imaging in small animal models of carcinoma or cardiovascular disease. The [1-Nal⁴]G7039 lead precursor was thus carried forward for radiolabelling with [¹⁸F]SFB.

3.2.7 Synthesis of [¹⁸F]SFB and [¹⁸F]-radiolabelling of the lead precursor [1-Nal⁴]G7039

The synthesis of the [¹⁸F]SFB prosthetic group is shown in scheme 1. Acid catalysed esterification of the 4-dimethylaminobenzoic acid starting material 18 furnished the intermediate t-butyl ester 19. Methylation with trifluoromethanesulfonate (MeOTf) gave the triflate salt 20 in a yield of 38% and ≥ 95% purity by UHPLC. Having synthesized the precursor salt 20, nucleophilic aromatic substitution with the [¹⁸F⁻] anion (acquired from the PET cyclotron by irradiation of [¹⁸O]H₂O) led to the formation of the radioactive compound 21.
Scheme 3.1 - Synthesis of the $[^{18}\text{F}]$$SFB$ prosthetic group and subsequent $^{18}\text{F}$-radiolabelling of $[1\text{-Nal}^4]\text{G7039}$. Reagents and Conditions: a) i) trifluoroacetic anhydride, THF, 0°C ii) tBuOH, room temperature, 2 hrs; b) MeOTf, N$_2$, 0 °C, 1 hour; c) $^{18}\text{F}$, K$_2$CO$_3$, Kryptofix 2.2.2, DMSO, 120 °C, 10 minutes; d) 6M HCl, 120 °C, 10 minutes; e) NHS, EDC, MeCN, room temperature, 15-20 minutes; f) $[1\text{-Nal}^4]\text{G7039}$, N,N-diisopropylethylamine (DIPEA), MeCN/H$_2$O (1:1 ratio), 65 °C, 15-20 minutes.

Acid-catalysed de-esterification furnished $[^{18}\text{F}]\text{FBA}$ (22) with an average d.c. (decay-corrected) radiochemical yield of 82%. The product was confirmed by coinjection with $[^{19}\text{F}]\text{FBA}$ (see Figure 3.S3 for representative radiochromatogram). Compound 22 was then coupled to $N$-hydroxysuccinimide (NHS) using the coupling reagent $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide (EDC) which led to the formation of the $[^{18}\text{F}]$$SFB$ prosthetic group 23. The crude compound was purified by reverse-phase HPLC to give an average decay-corrected radiochemical yield of 71% and radiochemical purity $\geq 99\%$ (see Figure 3.S4 for representative radiochromatogram). The pure $[^{18}\text{F}]$$SFB$ was added to the peptide precursor $[1\text{-Nal}^4]\text{G7039}$ in a mixture of MeCN/H$_2$O using a small quantity of the hindered base DIPEA. After 15-20 minutes, the crude $[1\text{-Nal}^4,\text{Lys}^5(4-[^{18}\text{F}]\text{-FB})]\text{G7039}$ lead compound was obtained and purified by
semi-preparative HPLC. The final radiolabelled peptide [1-Nal\textsuperscript{4},Lys\textsuperscript{5}(4-[\textsuperscript{18}F]-FB)]G7039 (24) was obtained in an average decay-corrected radiochemical yield of 81%. For the entire synthesis, an overall average d.c. radiochemical yield of 48% was achieved (n = 3), an average molar activity of > 34 GBq/μmol and a radiochemical purity of ≥ 99%. Figure 3 displays a series of stacked chromatograms including the peptidomimetic precursor [1-Nal\textsuperscript{4}]G7039 (16), the cold standard [1-Nal\textsuperscript{4},Lys\textsuperscript{5}(4-FB)]G7039 (17) as well as the radiolabelled [1-Nal\textsuperscript{4},Lys\textsuperscript{5}(4-[\textsuperscript{18}F]-FB)]G7039 peptidomimetic (24).

![Stacked HPLC Chromatograms](image)

**Figure 3.3 - Stacked HPLC Chromatograms for [1-Nal\textsuperscript{4}]G7039, [1-Nal\textsuperscript{4},Lys\textsuperscript{5}(4-FB)]G7039 (both λ = 254 nm) and [1-Nal\textsuperscript{4}, Lys\textsuperscript{5}(4-[\textsuperscript{18}F]-FB)]G7039**

The retention times of the cold standard and “hot” peptide are almost identical ([1-Nal\textsuperscript{4}, Lys\textsuperscript{5}(4-FB)]G7039, t\textsubscript{R} = 6.70 mins compared to t\textsubscript{R} = 6.65 mins for the “hot” peptide), indicating that the precursor [1-Nal\textsuperscript{4}]G7039 has been selectively radiolabelled at the amino group of the lysine side-chain. Further evidence for successful coupling of [\textsuperscript{18}F]SFB to [1-Nal\textsuperscript{4}]G7039 via the lysine side-chain was provided by a co-injection of a pre-mixed solution of the cold standard and “hot” peptidomimetic (**Figure 3.4**).
Once again, this showed very similar retention times between the two peptidomimetic species ([1-Nal⁴, Lys⁵(4-FB)]G7039 \( t_R = 6.26 \) mins and [1-Nal⁴, Lys⁵(4-[¹⁸F]-FB)]G7039 \( t_R = 6.17 \) mins) lending further credence to regioselective \(^{18}\)F-fluorobenzoylation at the lysine side-chain.

3.3 Conclusions

In this study, several families of peptidic (GHRP-1, GHRP-2 and GHRP-6) and peptidomimetic (ipamorelin, G7039, [1-Nal⁴]G7039) GHS-R1a agonists were derivatised through 4-fluorobenzoylation of the lysine side-chain. This led to a range of half-maximal inhibitory concentrations ranging from 69 nM ([1-Nal⁴, Lys⁵(4-FB)]G7039) to 1920 nM ([Inp¹, D-2-Nal⁴, Lys⁵(4-FB)]ipamorelin). The peptidomimetic [1-Nal⁴]G7039 was identified as the most suitable candidate for \(^{18}\)F-radiolabelling, as its \(^{19}\)F-congener ([1-Nal⁴, Lys⁵(4-FB)]G7039, compound 17) had a nanomolar binding affinity (IC\(_{50} = 69\) nM), high \textit{in vitro} potency (EC\(_{50} = 1.1\) nM) and good serum stability (t\(_{1/2} = 718\) mins). This is most likely due to the presence of a core framework of aromatic amino acids that are involved in strong hydrophobic interactions with hydrophobic pockets of the GHS-R1a. Prosthetic group radiolabelling of [1-Nal⁴]G7039 with \(^{18}\)FSFB delivered the radiolabelled peptidomimetic in an overall average decay-corrected radiochemical yield.
of 48%, a radio-purity ≥ 99% and an average molar activity of > 34 GBq/μmol. This compound could potentially be used as a PET probe for the detection of diseases that are characterised by overexpression of GHS-R1a.

3.4 Materials and Methods

3.4.1 General information

All starting peptide reagents (resin, amino acids, coupling agents, and bases) were obtained from commercial suppliers and used without further purification. Peptides were either synthesised manually or through the use of a Biotage SyroWave automated peptide synthesizer. Peptide vessels were shaken using an IKA Vibra VXR basic shaker with centrifugation performed on a Beckman Coulter Allegra X-30R or Fisher GS-6R centrifuge. In order to aid peptide dissolution, sonication of solutions was accomplished via a Branson 2510R-MTH or Fisher F5-14 ultrasonic cleaner. A Fisher 2052 Isotemp machine was used to heat test tubes in the Kaiser Test. Peptides were lyophilised using a Labconco FreeZone Freeze Dry System. Accurate weighing was carried out on a Mettler-Toledo XP6 microbalance. UV traces were obtained with a Waters 2487 UV/Vis Dual λ Absorbance Detector (170-900 nm) and low-resolution mass spectra with a Micromass Quattro micro API mass spectrometer (ESI-LC-MS). Peptide purification was achieved through HPLC (MeCN + 0.1% TFA, H2O + 0.1% TFA solvent system). All peptides and small molecules obtained had a purity ≥ 95% as determined by HPLC or UHPLC analysis. A RP preparative C-18 column (SunFire OBD, 19 x 150 mm or Agilent Zorbax 21.2 x 150 mm) was used for preparative HPLC, whilst a C-18 RP column (SunFire, 4.6 x 150 mm or Agilent Zorbax, 4.6 x 150 mm) was used for analytical HPLC. Accurate mass spectrometry (HRMS) was carried out on a Finnigan MAT 8400 mass spectrometer (EI) for small molecules and on a Micromass LCT mass spectrometer (ESI-TOF) for peptides. 1H NMR and 13C NMR spectroscopy were performed on a Mercury VX 400 machine at 400 and 100 MHz respectively. Chemical shifts are referenced to residual solvent, reported in ppm on a δ scale and all coupling constants quoted in hertz (Hz).

3.4.2 Manual Fmoc-SPPS

Rink amide MBHA resin (192 mg, 0.1 mmol, 1.0 equiv., 0.52 mmol g⁻¹ loading) was vortexed in DCM (2.0 ml, 1 min.), allowed to swell (15 mins) and solvent removed. This was followed by addition of DMF (2.0 ml), vortexing (1 min.) and removal of
solvent. Deprotection of the Fmoc group was then performed. A solution of 20% piperidine/DMF (1.5 ml, v/v) was added to the resin and the subsequent mixture vortexed (2 mins) and solvent removed. This was then repeated a second time with vortexing for 15 minutes. After solvent had been removed, the resin was washed of any unreacted by-products with DMF six times (2.0 ml, vortex 30 s). The desired amino acid or small molecule (0.3 mmol, 3.0 equiv.) and coupling reagent (HCTU, 0.12 g, 0.3 mmol, 3.0 equiv.) were then dissolved in DMF (1.5 ml) and added to the deprotected resin. After vortexing (30 s), DIPEA (111 μl, 0.6 mmol, 6.0 equiv.) was added and the final mixture vortexed (1-2 hrs). The resin was then washed with DMF (2.0 ml, 30 s vortex) a final four times. The deprotection/coupling cycle was then repeated unless the final amino acid in the sequence had been added, in which case the peptide was washed with DCM five times (2.0 ml, 30 s vortex) after washing with DMF and stored in a refrigerator. Removal of the N-terminal Fmoc-group was carried out in the same fashion as the deprotection cycle described previously, with resin washing occurring six times with DMF (2.0 ml, 30 s vortex) and four times with DCM (2.0 ml, 30 s vortex). Successful synthesis of the desired peptide was then ascertained via a microcleave prior to full cleavage of the peptide from the solid-support. This was carried out as follows: a solution of 95% TFA: 2.5% (iPr)3SiH: 2.5% H2O (300 μl) was added to a small number of resin beads (< 5 mg) and the subsequent mixture vortexed (3 hrs). The clear liquid was then evaporated under a stream of N2. Analytical HPLC was then performed to determine whether the correct peptide had been synthesised. If the correct peptide had been obtained, a full cleavage was performed using a mixture of 95% TFA: 2.5% (iPr)3SiH: 2.5% H2O (2.0 ml) for 5-7 hrs. The subsequent solution was cooled in an ice-bath alongside tert-butyl methyl ether (TBMe, 40 ml). After 10 minutes, TBMe (20 ml) was added to the peptide solution, leading to the formation of a white precipitate. The precipitate was cooled further (10 mins) and then centrifuged (7 mins). Decanting of the supernatant was followed by addition of a second aliquot of TBMe (20 ml), vortexing (30 s) and final centrifugation (7 mins). After decanting, a white solid was obtained. This was then freeze-dried (20 mins) to furnish crude peptide. Preparative HPLC was then used to purify the product peptide.
3.4.3 Deprotection of the Alloc protecting group
The resin-bound peptide was vortexed in DCM (4.5 ml, 30 s) and allowed to swell (10 mins). Deprotection was carried out under a blanket of N₂. The swollen resin-bound peptide was stirred (5 mins) before addition of PhSiH₃ (296 μl, 2.4 mmol, 24.0 equiv.). Further stirring (5 mins) ensued prior to treating with Pd(PPh₃)₄ (12 mg, 0.01 mmol, 0.1 equiv.). After 5 minutes, the solution was vortexed (5 mins), solvent removed and the brown-coloured resin washed four times with DCM (2.0 ml, 30 s vortex). The procedure was then repeated ab initio, with final resin washing occurring in the following order: DCM, DMF, MeOH, DMF and DCM (all 2.0 ml, 30 s vortex).

3.4.4 Kaiser Test
A small number of resin beads (< 5 mg) were taken and treated with phenol: EtOH (200 μl, 8:2 v/v), 0.001 M KCN (aq.): pyridine (200 μl, 2:98 v/v, 0.001 M aqueous KCN) and ninhydrin in EtOH (200 μl, 5% w/v), respectively. Tentagel resin (< 5 mg) was used as a control. Both test tubes were heated to 70 °C. The presence of free amine was indicated by blue resin beads whilst yellow or clear resin beads indicated the presence of protected amino groups.

3.4.5 Synthesis of peptidic- and peptidomimetic growth hormone secretagogues
All peptidomimetics were synthesised by the same general procedure described previously unless otherwise noted.

H-Aib-His-D-2-Nal-D-Phe-Lys-NH₂: Ipamorelin (1)
The product was purified by preparative HPLC (5-80% MeCN + 0.1% TFA). This furnished a white powder (20.5 mg, 19%): 1H-NMR (400 MHz, CD₃OD); D-2-Nal, D-Phe, His: δ 7.99 (s, 1H, ArH), 7.80-7.76 (m, 1H, ArH), 7.71 (d, J = 8.6 Hz, 2H, ArH), 7.56 (s, 1H, ArH), 7.44-7.37 (m, 2H, ArH), 7.32-7.17 (m, 6H, ArH), 6.92 (s, 1H, His H₆), 4.58 (m, 3H, H₂), 3.30-3.21 (m, 1H, D-2-Nal H₆), 3.15-3.00 (m, 2H, H₂), 2.96-2.72 (m, 3H, H₂), Lys: 4.11 (dd, J = 9.7, 4.2 Hz, 2H, H₂), 2.96-2.72 (m, 2H, H₂), 1.75-1.64 (m, 1H, H₆), 1.54-1.40 (m, 3H, H₂), 1.05-0.93 (m, 2H, H₇), Aib: 1.48 (s, 3H, CH₃) 1.44 (s, 3H, CH₃) ppm. ESI-LC-MS m/z 356.9 [M + 2H]²⁺; HRMS (ESI-MS) calcd. for C₃₈H₅₀N₉O₅ [M + H]⁺ 712.3935, found 712.3959.
H-Aib-His-D-2-Nal-D-Phe-Lys(4-FB)-NH$_2$: [Lys$^5$(4-FB)]ipamorelin (2)

Purification by preparative HPLC (20-70% MeCN + 0.1% TFA) yielded a white powder (15.4 mg, 15%): $^1$H-NMR (400 MHz, CD$_3$OD); D-2-Nal, D-Phe, His, 4-FB: $\delta$

7.91 (s, 1H, ArH), 7.85-7.77 (m, 3H, ArH, 2F-ArH), 7.70 (d, $J = 7.9$ Hz, 2H, ArH), 7.54 (s, 1H, ArH), 7.45-7.40 (m, 2H, ArH), 7.29-7.19 (m, 6H, ArH), 7.14-7.07 (m, 2H, 2F-ArH), 6.93 (s, 1H, His H$_\beta$), 4.65-4.52 (m, 3H, H$_\alpha$), 3.30-3.27 (m, 1H, H$_\beta$), 3.23 (d, $J = 4.0$ Hz, 1H, H$_\gamma$), 3.10 (dd, $J = 13.5$, 7.8 Hz, 1H, H$_\beta$), 3.04-2.74 (m, 3H, H$_\beta$),

Lys: 4.12 (dd, $J = 9.8$, 4.2 Hz, 1H, H$_\alpha$), 3.04-2.74 (m, 2H, H$_\alpha$), 1.77-1.67 (m, 1H, H$_\beta$), 1.58-1.42 (m, 3H, H$_\beta$, 2H$_\alpha$), 1.14-1.03 (m, 2H, H$_2$), Aib: 1.50 (s, 3H, CH$_3$), 1.46 (s, 3H, CH$_3$) ppm. ESI-LC-MS m/z 418.0 [M + 2H]$^2^+$; HRMS (ESI-MS) calcd. for C$_{45}$H$_{53}$F$_{10}$O$_6$ [M + H]$^+$ 834.4103, found 834.4133.

H-Aib-His-D-2-Nal-D-Phe-Lys(AEEA-4-FB)-NH$_2$: [Lys$^5$(AEEA-4-FB)]ipamorelin (3)

Peptide purification by preparative HPLC (20-60% MeCN + 0.1% TFA) delivered an off-white powder (9.2 mg, 8%): $^1$H-NMR (400 MHz, CD$_3$OD); His, D-2-Nal, D-Phe, 4-FB: $\delta$

7.93 (s, 1H, ArH), 7.86-7.77 (m, 3H, ArH, 2F-ArH), 7.71 (d, $J = 8.0$ Hz, 2H, ArH), 7.55 (s, 1H, ArH), 7.46-7.41 (m, 2H, ArH), 7.31-7.18 (m, 6H, ArH), 7.17-7.10 (m, 2H, F-ArH), 6.94 (s, 1H, His H$_\delta$), 4.65-4.58 (m, 2H, H$_\alpha$), 4.55 (t, $J = 7.5$ Hz, 1H, H$_\alpha$), 3.16-2.76 (m, 6H, H$_\beta$), Lys: 4.10 (dd, $J = 9.7$, 4.2 Hz, 1H, H$_\alpha$), 3.16-2.76 (m, 2H, H$_\delta$), 1.72-1.62 (m, 1H, H$_\beta$), 1.52-1.43 (m, 1H, H$_\gamma$), 1.41-1.30 (m, 2H, H$_\alpha$), 1.07-0.97 (m, 2H, H$_\gamma$), AEEA linker: 3.93 (s, 2H, NHCOCH$_2$O), 3.65-3.61 (m, 6H, CH$_3$), 3.53 (t, $J = 5.6$ Hz, 2H, CH$_2$), Aib: 1.50 (s, 3H, CH$_3$), 1.47 (s, 3H, CH$_3$) ppm. ESI-LC-MS m/z 490.4 [M + 2H]$^2^+$; HRMS (ESI-MS) calcd. for C$_{53}$H$_{64}$FN$_{10}$O$_9$ [M + H]$^+$ 979.4842, found 979.4868.

H-Aib-His-D-2-Nal-D-2-Thi-Lys(4-FB)-NH$_2$: [D-2-Thi$^4$,Lys$^5$(4-FB)]ipamorelin (4)

The title peptide was synthesized by automated peptide synthesis and purified by preparative HPLC (20-80% MeCN + 0.1% TFA). This furnished a white solid (4.20 mg, 4%): $^1$H-NMR (400 MHz, CD$_3$)$_2$SO); $\delta$

8.83 (s, 1H, ArH), 8.59 (d, $J = 8.1$ Hz, 1H, NH), 8.42 (t, $J = 5.6$ Hz, 1H, NH), 8.29 (d, $J = 9.3$ Hz, 1H, NH), 8.20 (d, $J = 8.2$ Hz, 1H, NH), 8.09 (d, $J = 8.9$ Hz, 1H, NH), 7.99 (s, 2H, NH), 7.86-7.81 (m, 2H, F-ArH), 7.79-7.75 (m, 1H, ArH), 7.72 (dd, $J = 8.7$, 3.7 Hz, 2H, ArH), 7.65 (s, 1H, ArH), 7.41-7.37 (m, 2H, ArH), 7.35-7.31 (m, 2H, ArH, NH), 7.26 (dd, $J = 4.8$, 1.5 Hz, 1H, Thi-
H-\textit{Inp}-\textit{His-D-2-Nal-D-Phe-Lys(4-FB)}-\textit{NH}_2: \textit{[Inp}^1,\textit{Lys}^5(4-FB)]ipamorelin (5)

The title peptide was synthesized via automated peptide synthesis and purified by preparative HPLC (20-70% MeCN + 0.1% TFA). The title compound was acquired as a white solid (6.30 mg, 6%): \textsuperscript{1}H-NMR (400 MHz, (CD\textsubscript{3})\textsubscript{2}SO); δ 8.79 (s, 1H, His H\textsub{o}), 8.72 (s, 1H, NH), 8.44 (dd, J = 10.8, 6.4 Hz, 2H, NH), 8.19 (d, J = 7.8 Hz, 1H, NH), 8.17-8.10 (m, 2H, NH), 7.87-7.81 (m, 2H, F-ArH), 7.80-7.76 (m, 1H, ArH), 7.71 (d, J = 7.9 Hz, 2H, ArH), 7.63 (s, 1H, ArH), 7.42-7.35 (m, 2H, ArH), 7.33-7.29 (m, 2H, NH), 7.22-7.18 (m, 6H, ArH), 7.17-7.11 (m, 2H, F-ArH), 7.06 (s, 1H, NH), 6.96 (s, 1H, His H\text{o}), 4.64-4.56 (m, 1H, H\text{o}), 4.55-4.44 (m, 2H, H\text{o}), 4.15-4.07 (m, 1H, Lys-H\text{o}), 3.19-3.07 (m, 5H, CH\textsubscript{2}), 2.94 (dd, J = 13.6, 5.9 Hz, 1H, CH\textsubscript{2}), 2.87-2.67 (m, 5H, CH\textsubscript{2}), 2.58-2.48 (m, 1H, CH\textsubscript{2}), 2.38-2.29 (m, 1H, H\text{o}), 1.67-1.34 (m, 8H, CH\textsubscript{2}), 1.15-1.00 (m, 2H, CH\textsubscript{2}), ppm. ESI-LC-MS m/z 430.9 [M + 2H]\textsuperscript{2+}; HRMS (ESI-MS) calcd. for C\textsubscript{47}H\textsubscript{55}FN\textsubscript{6}O\textsubscript{6} [M + H]\textsuperscript{+} 860.4259 found 860.4284.

H-\textit{Inp}-\textit{His-D-2-Nal-D-2-Nal-Lys(4-FB)}-\textit{NH}_2: \textit{[Inp}^1,\textit{D-2-Nal}^4,\textit{Lys}^5(4-FB)]ipamorelin (6)

The title peptide was made by automated peptide synthesis and purified by preparative HPLC (20-80% MeCN + 0.1% TFA). This delivered a white solid (11.3 mg, 10%): \textsuperscript{1}H-NMR (400 MHz, (CD\textsubscript{3})\textsubscript{2}SO); δ 8.77 (s, 1H, His H\text{o}), 8.51 (d, J = 7.5 Hz, 2H, NH), 8.36 (t, J = 5.5 Hz, 1H, NH), 8.19-8.10 (m, 2H, NH), 8.05 (d, J = 8.7 Hz, 1H, NH), 7.85-7.79 (m, 3H, 2F-ArH, ArH), 7.79-7.74 (m, 3H, ArH), 7.73 (s, 1H, ArH), 7.70 (s, 1H, ArH), 7.68 (d, J = 2.4 Hz, 2H, ArH), 7.59 (s, 1H, ArH), 7.43-7.35 (m, 5H, ArH), 7.31-7.27 (m, 2H, NH), 7.20-7.13 (m, 2H, F-ArH), 7.05 (s, 1H, NH), 6.93 (s, 1H, His H\text{o}), 4.67-4.60 (m, 2H, H\text{o}), 4.52-4.44 (m, 1H, H\text{o}), 4.16-4.08 (m, 1H, Lys-H\text{o}), 3.17-2.93 (m, 7H, CH\textsubscript{2}), 2.88-2.66 (m, 4H, CH\textsubscript{2}), 2.53-2.47 (m, 1H, CH\textsubscript{2}), 2.34-2.25 (m, 1H, H\text{o}), 1.63-1.34 (m, 6H, CH\textsubscript{2}), 1.32-1.23 (m, 2H, CH\textsubscript{2}), 1.07-0.95 (m, 2H,
The title peptide was prepared by automated peptide synthesis and purified by preparative HPLC (20-80% MeCN + 0.1% TFA). This furnished a white powder (14.0 mg, 13%): \( ^1H\)NMR (400 MHz, CD\(_3\)OD); \textbf{His, D-2-Nal, D-2-Thi, 4-FB}: \( \delta \) 8.23 (s, 1H, His \( \varepsilon \)), 7.86-7.81 (m, 2H, F-ArH), 7.80-7.77 (m, 1H, ArH), 7.73 (dd, \( J = 8.8, 3.3 \) Hz, 2H, ArH), 7.59 (s, 1H, ArH), 7.45-7.39 (m, 2H, ArH), 7.27 (dd, \( J = 8.5, 1.6 \) Hz, 1H, ArH), 7.22 (dd, \( J = 5.0, 1.2 \) Hz, 1H, Thi-ArH), 7.15-7.08 (m, 2H, F-ArH), 6.92 (s, 1H, His \( \delta \)), 6.91-6.85 (m, 2H, Thi-ArH), 4.61 (dd, \( J = 10.3, 4.4 \) Hz, 1H, \( \alpha \)), 4.53-4.47 (m, 2H, \( \alpha \)), 4.22 (dd, \( J = 9.8, 4.2 \) Hz, 1H, Lys-H\( \alpha \)), 3.39-3.29 (m, 6H, \( CH_2 \)), 2.53-2.44 (m, 1H, Inp-\( \alpha \)), 1.87-1.50 (m, 8H, \( CH_2 \)), 1.35-1.22 (m, 2H, \( CH_2 \)), ppm. ESI-LC-MS \( m/z \) 433.8 [M + 2H]\(^{2+}\); HRMS (ESI-MS) calcd. for C\(_{45}\)H\(_{53}\)FN\(_9\)O\(_6\)S [M + H]\(^+\) 866.3824 found 866.3850.

\textbf{H-Ala-His-D-2-Nal-Ala-Trp-D-Phe-Lys-NH\(_2\): GHRP-1 (8)}

The product was purified by preparative HPLC (15-80% MeCN + 0.1% TFA). This yielded a white powder (26.4 mg, 19%): \( ^1H\)NMR (400 MHz, CD\(_3\)OD); \textbf{His, D-2-Nal, Trp, D-Phe}: \( \delta \) 8.41 (s, 1H, His \( \varepsilon \)), 7.81-7.71 (m, 3H, ArH), 7.65 (s, 1H, ArH), 7.49 (d, \( J = 7.8 \) Hz, 1H, ArH), 7.46-7.40 (m, 2H, ArH), 7.35 (dd, \( J = 8.4, 1.7 \) Hz, 1H, ArH), 7.31 (d, \( J = 8.1 \) Hz, 1H, ArH), 7.24-7.13 (m, 3H, ArH), 7.10-7.04 (m, 4H, ArH), 7.00 (t, \( J = 7.5 \) Hz, 1H, ArH), 6.89 (d, \( J = 0.8 \) Hz, 1H, His \( \delta \)), 4.67-4.60 (m, 2H, \( \alpha \)), 4.38 (t, \( J = 7.4 \) Hz, 1H, \( \alpha \)), 4.32 (t, \( J = 7.7 \) Hz, 1H, \( \alpha \)), 3.25-2.68 (m, 8H, \( \beta \)), \textbf{Ala, Ala}: 4.17-4.10 (m, 1H, \( \beta \)), 3.94 (q, \( J = 7.0 \) Hz, 1H, \( \alpha \)), 1.35 (d, \( J = 7.1 \) Hz, 3H, \( CH_3 \)), 1.10-1.01 (m, 3H, \( CH_3 \)), \textbf{Lys}: 4.17-4.10 (m, 1H, \( \alpha \)), 3.25-2.68 (m, 2H, \( \beta \)), 1.81-1.70 (m, 1H, \( \beta \)), 1.58-1.45 (3H, \( \beta \)), 1.10-1.01 (m, 2H, \( \beta \)), ppm. ESI-LC-MS \( m/z \) 478.5 [M + 2H]\(^{2+}\); HRMS (ESI-MS) calcd. for C\(_{51}\)H\(_{63}\)N\(_{12}\)O\(_7\) [M + H]\(^+\) 955.4943 found 955.4964.
H-D-Ala-D-2-Nal-Ala-Trp-D-Phe-Lys-NH$_2$: GHRP-2 (9)

Purification by preparative HPLC (25-70% MeCN + 0.1% TFA) delivered the title peptide as a white powder (19.7 mg, 17%): $^1$H-NMR (400 MHz, CD$_3$OD); D-2-Nal, Trp, D-Phe: $\delta$ 7.76 (d, $J$ = 9.2 Hz, 2H, ArH), 7.73 (s, 1H, ArH), 7.65 (s, 1H, ArH), 7.51-7.47 (m, 1H, ArH), 7.44-7.37 (m, 2H, ArH), 7.35 (dd, $J$ = 8.4, 1.7 Hz, 1H, ArH), 7.25-7.14 (m, 4H, ArH), 7.09-7.01 (m, 4H, ArH), 7.00 (s, 1H, ArH), 4.77 (dd, $J$ = 10.9, 4.6 Hz, 1H, H$_a$), 4.49 (t, $J$ = 6.8 Hz, 1H, H$_a$), 4.36 (t, $J$ = 7.6 Hz, 1H, H$_a$), 3.11 (t, $J$ = 6.7 Hz, 2H, H$_\beta$), 3.07 (d, $J$ = 4.5 Hz, 1H, H$_\beta$), 2.88-2.70 (m, 3H, H$_\beta$), D-Ala, Ala: 4.23 (q, $J$ = 7.1 Hz, 1H, H$_a$), 3.79 (q, $J$ = 7.0 Hz, 1H, H$_a$), 1.27 (d, $J$ = 7.2 Hz, 3H, CH$_3$), 1.00 (d, $J$ = 7.1 Hz, 3H, CH$_3$), Lys: 4.16 (dd, $J$ = 10.3, 4.1 Hz, 1H, H$_a$), 2.88-2.70 (m, 2H, H$_\delta$), 1.82-1.72 (m, 1H, H$_\beta$), 1.56-1.43 (m, 3H, H$_\beta$, 2H$_\delta$), 1.07-0.96 (m, 2H, H$_\gamma$) ppm. ESI-LC-MS m/z 409.9 [M + 2H]$^2^+$$;\text{HRMS (ESI-MS) calcd. for C}_{45}\text{H}_{55}\text{N}_{10}\text{O}_{6}\text{Na} [M + Na]^+$ 840.4204 found 840.4173.

H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH$_2$: GHRP-6 (10)

Purification by preparative HPLC (15-80% MeCN + 0.1% TFA) gave a white powder (19.7 mg, 14%): $^1$H-NMR (400 MHz, CD$_3$OD); His, D-Trp, Trp, D-Phe: $\delta$ 8.46 (s, 1H, His H$_a$), 7.54 (d, $J$ = 7.8 Hz, 1H, ArH), 7.46 (d, $J$ = 7.8 Hz, 1H, ArH), 7.28 (t, $J$ = 7.7 Hz, 2H, ArH), 7.23-7.13 (m, 4H, ArH), 7.12-6.92 (m, 8H, 7ArH, His H$_\delta$), 4.47 (t, $J$ = 7.4 Hz, 2H, Trp H$_\alpha$), 4.35 (t, $J$ = 6.7 Hz, 1H, H$_a$), 4.28 (t, $J$ = 7.9 Hz, 1H, H$_a$), 3.22-3.14 (m, 3H, H$_\beta$), 3.14-3.02 (m, 3H, H$_\beta$), 2.83 (d, $J$ = 8.6 Hz, 2H, H$_\beta$), Lys: 4.03 (dd, $J$ = 10.3, 3.9 Hz, 1H, H$_a$), 2.75 (t, $J$ = 6.8 Hz, 2H, H$_a$), 1.76-1.63 (m, 1H, H$_\beta$), 1.51-1.39 (m, 3H, H$_\beta$, 2H$_\delta$), 0.97-0.88 (m, 2H, H$_\gamma$), Ala: 3.91 (q, $J$ = 7.2 Hz, 1H, H$_a$), 0.86 (d, $J$ = 7.3 Hz, 3H, CH$_3$) ppm. ESI-LC-MS m/z 437.4 [M + 2H]$^2^+$$;\text{HRMS (ESI-MS) calcd. for C}_{46}\text{H}_{57}\text{N}_{12}\text{O}_6 [M + H]^+$ 873.4524 found 873.4531.

H-His-D-Trp-Ala-Trp-D-Phe-Lys(4-FB)-NH$_2$: [Lys$^4$(4-FB)]GHRP-6 (11)

The product was purified by preparative HPLC (15-80% MeCN + 0.1% TFA). This yielded a white powder (9.60 mg, 7%): $^1$H-NMR (400 MHz, CD$_3$OD); His, D-Trp, Trp, D-Phe, 4-FB: $\delta$ 8.50 (s, 1H, His H$_a$), 7.88-7.80 (m, 2H, F-ArH), 7.57 (d, $J$ = 7.9 Hz, 1H, ArH), 7.49 (d, $J$ = 7.8 Hz, 1H, ArH), 7.30 (t, $J$ = 8.2 Hz, 2H, ArH), 7.26-6.95 (m, 14H, 13ArH, His H$_\delta$), 4.53-4.46 (m, 2H, H$_a$), 4.37-4.30 (m, 2H, H$_a$), 3.26-3.04 (m, 6H, H$_\beta$), 2.87 (d, $J$ = 8.0 Hz, 2H, H$_\beta$), Lys: 4.04 (dd, $J$ = 10.2, 4.0 Hz, 1H, H$_a$),
3.26-3.04 (m, 2H, H\textsubscript{ε}), 1.81-1.70 (m, 1H, H\textsubscript{δ}), 1.58-1.43 (m, 3H, 2H\textsubscript{δ}, H\textsubscript{ε}), 1.06-0.97 (m, 2H, H\textsubscript{δ}), Ala: 3.94 (q, J = 7.3 Hz, 1H, H\textsubscript{α}), 0.87 (d, J = 7.3 Hz, 3H, CH\textsubscript{3}) ppm. ESI-LC-MS m/z 498.4 [M + 2H]\textsuperscript{2+}; HRMS (ESI-MS) calcd. for C\textsubscript{33}H\textsubscript{39}FN\textsubscript{12}O\textsubscript{7}Na [M + Na]\textsuperscript{+} 1017.4511 found 1017.4522.

**H-His-D-Trp-Ala-Trp-D-Phe-Dpr-NH\textsubscript{2}: [Dpr\textsuperscript{6}]GHRP-6 (12)**

Purification by preparative HPLC (15-80% MeCN + 0.1% TFA) furnished the title peptide as a white powder (4.7 mg, 3%): \textsuperscript{1}H-NMR (400 MHz, CD\textsubscript{3}OD); 8.49 (s, 1H His H\textsubscript{ε}), 7.55 (d, J = 7.9 Hz, 1H, ArH), 7.46 (d, J = 7.9 Hz, 1H, ArH), 7.30 (t, J = 8.6 Hz, 2H, ArH), 7.21-7.12 (m, 3H, ArH), 7.11-7.04 (m, 3H, ArH), 6.89 (d, J = 1.0 Hz, 1H, His H\textsubscript{δ}), 4.62 (t, J = 6.8 Hz, 1H, H\textsubscript{α}), 4.52 (t, J = 7.9 Hz, 1H, Ala-H\textsubscript{α}), 4.44-3.11 (m, 3H, CH\textsubscript{3}), 3.12-2.92 (m, 5H, CH\textsubscript{2}), 2.89-2.76 (m, 2H, CH\textsubscript{2}), 0.94 (d, J = 7.3 Hz, 3H, Ala-CH\textsubscript{3}) ppm. ESI-LC-MS m/z 831.4 [M + H]\textsuperscript{+}; HRMS (ESI-MS) calcd. for C\textsubscript{43}H\textsubscript{51}FN\textsubscript{12}O\textsubscript{6} [M + H]\textsuperscript{+} 831.4055 found 831.4070.

**H-His-D-Trp-Ala-Trp-D-Phe-Dpr(4-FB)-NH\textsubscript{2}: [Dpr\textsuperscript{6}(4-FB)]GHRP-6 (13)**

Preparative HPLC (25-70% MeCN + 0.1% TFA) gave the title compound as a white solid (19.8 mg, 14%): \textsuperscript{1}H-NMR (400 MHz, CD\textsubscript{3}OD); His, D-Trp, Trp, D-Phe, 4-FB: δ 8.58 (d, J = 1.3 Hz, 1H, His H\textsubscript{ε}), 7.82-7.75 (m, 2H, F-ArH), 7.52 (d, J = 7.9 Hz, 1H, ArH), 7.40 (d, J = 7.9 Hz, 1H, ArH), 7.27 (dd, J = 8.6, 1.5 Hz, 2H, ArH), 7.15-7.07 (m, 5H, ArH), 7.06-6.89 (m, 3H, 2ArH, His H\textsubscript{δ}), 4.54-4.45 (m, 1H, H\textsubscript{α}), 4.44-3.40 (m, 3H, H\textsubscript{α}), 3.22-2.98 (m, 6H, CH\textsubscript{2}), 2.93-2.74 (m, 2H, H\textsubscript{β}), Dpr: 4.54-4.45 (m, 1H, H\textsubscript{α}), 3.67 (dd, J = 13.8, 5.6 Hz, 1H, H\textsubscript{β}), 3.54 (dd, J = 13.8, 7.8 Hz, 1H, H\textsubscript{β}), Ala: 3.93 (q, J = 7.3 Hz, 1H, H\textsubscript{α}), 0.86 (d, J = 7.3 Hz, 3H, CH\textsubscript{3}) ppm. ESI-LC-MS m/z 477.4 [M + 2H]\textsuperscript{2+}; HRMS (ESI-MS) calcd. for C\textsubscript{50}H\textsubscript{54}FN\textsubscript{12}O\textsubscript{7} [M + H]\textsuperscript{+} 953.4223 found 953.4237.

**H-Inp-D-2-Nal-D-2-Nal-Phe-Lys-NH\textsubscript{2}: G7039* (14)**

Purification of the peptide proceeded through preparative HPLC (25-80% MeCN + 0.1% TFA). The title compound was obtained as a white powder (6.70 mg, 7%): \textsuperscript{1}H-NMR (400 MHz, CD\textsubscript{3}OD); 7.80-7.76 (m, 1H, ArH), 7.74 (dd, J = 6.1, 2.2 Hz, 2H, ArH), 7.70 (dd, J = 8.5, 3.5 Hz, 3H, ArH), 7.56 (s, 1H, ArH), 7.50 (s, 1H, ArH), 7.46-7.35 (m,
4H, ArH), 7.26 (dd, J = 8.5, 1.5 Hz, 1H, ArH), 7.20-7.10 (m, 6H, ArH), 4.66-4.56 (m, 2H, Hα), 4.50 (dd, J = 9.3, 5.4 Hz, 1H, Hα), 4.30 (dd, J = 9.5, 4.7 Hz, 1H, Lys-Hα), 3.20-2.80 (m, 10H, CH₂), 2.78-2.64 (m, 2H, CH₂), 2.33-2.24 (m, 1H, Inp-Hα), 1.92-1.82 (m, 1H, CH₂), 1.74-1.65 (m, 1H, CH₂), 1.64-1.44 (m, 5H, CH₂), 1.44-1.24 (m, 3H, CH₂), ppm. ESI-LC-MS m/z 399.8 [M + 2H]^2+; HRMS (ESI-MS) calcd. for C₄₇H₅₆N₇O₅ [M + H]^+ 798.4343 found 798.4339. HRMS data and yield has been reported previously[35], whereas the ¹H-NMR data has not.

**H-Inp-D-2-Nal-D-2-Nal-Phe-Lys(4-FB)-NH₂: [Lys⁵(4-FB)]G7039 (15)**

Preparative HPLC (35-80% MeCN + 0.1% TFA) furnished the title compound as a white solid (5.90 mg, 6%): ¹H-NMR (400 MHz, (CD₃)₂SO); δ 8.50 (d, J = 8.2 Hz, 1H, NH), 8.45 (t, J = 5.5 Hz, 1H, NH), 8.39 (s, 1H, NH), 8.15-8.09 (m, 2H, NH), 8.04 (d, J = 8.4 Hz, 1H, NH), 7.86-7.82 (m, 2H, F-ArH), 7.82-7.76 (m, 2H, ArH), 7.75-7.66 (m, 4H, ArH), 7.58 (s, 1H, ArH), 7.48 (s, 1H, ArH), 7.45-7.38 (m, 4H, ArH), 7.31 (dd, J = 8.5, 1.5 Hz, 1H, ArH), 7.28 (s, 1H, ArH), 7.26 (s, 1H, NH), 7.24-7.17 (m, 5H, ArH), 7.15-7.08 (m, 2H, ArH), 7.04 (s, 1H, NH), 4.66-4.60 (m, 1H, Hα), 4.60-4.53 (m, 2H, Hα), 4.20-4.14 (m, 1H, Lys-Hα), 3.25-3.14 (m, 2H, CH₂), 3.13-3.02 (m, 3H, CH₂), 2.98-2.91 (m, 1H, CH₂), 2.90-2.83 (m, 1H, CH₂), 2.81-2.56 (m, 5H, CH₂), 2.30-2.22 (m, 1H, Inp-Hα), 1.75-1.65 (m, 1H, CH₂), 1.62-1.55 (m, 1H, CH₂), 1.54-1.43 (m, 6H, CH₂), 1.38-1.19 (m, 2H, CH₂), ppm. ESI-LC-MS m/z 452.5 [M + 4H - F]^2+; HRMS (ESI-MS) calcd. for C₅₄H₅₉FN₇O₆ [M + H]^+ 920.4511 found 920.4529.


Peptide purification by preparative HPLC (25-80% MeCN + 0.1% TFA) delivered a white powder (24.9 mg, 23%): ¹H-NMR (400 MHz, CD₃OD); δ 8.12 (d, J = 8.4 Hz, 1H, ArH), 7.84 (d, J = 7.8 Hz, 1H, ArH), 7.78-7.72 (m, 2H, ArH), 7.68 (d, J = 8.5 Hz, 3H, ArH), 7.62-7.46 (m, 5H, ArH), 7.43-7.35 (m, 5H, ArH), 7.30 (d, J = 6.4 Hz, 1H, ArH), 7.27-7.21 (m, 2H, ArH), 7.02 (dd, J = 8.4, 1.5 Hz, 1H, ArH), 4.69 (dd, J = 9.4, 5.4 Hz, 1H, Hα), 4.65-4.55 (m, 2H, Hα), 4.32 (dd, J = 9.4, 4.8 Hz, 1H, Lys-Hα), 3.65 (dd, J = 14.4, 5.4 Hz, 1H, CH₂), 3.20-3.10 (m, 2H, CH₂), 3.05-2.94 (m, 3H, CH₂), 2.92-2.80 (m, 5H, CH₂), 2.76-2.62 (m, 2H, CH₂), 2.30-2.23 (m, 1H, Inp-Hα),
1.92-1.81 (m, 1H, CH$_2$), 1.75-1.29 (m, 9H, CH$_2$), ppm. ESI-LC-MS m/z 424.8 [M + 2H]$^2^+$; HRMS (ESI-MS) calcd. for C$_{51}$H$_{58}$N$_7$O$_5$ [M + H]$^+$ 848.4499, found 848.4501.

**H-Inp-D-2-Nal-D-2-Nal-Lys(4-FB)-NH$_2$: [1-Nal$^6$,Lys$^6$(4-FB)]G7039 (17)**

The product was purified by preparative HPLC (25-90% MeCN + 0.1% TFA) which yielded a white powder (9.80 mg, 9%): $^1$H-NMR (400 MHz, (CD$_3$)$_2$SO); δ 8.64 (d, $J$ = 8.3 Hz, 1H, NH), 8.46 (t, $J$ = 5.5 Hz, 1H, NH), 8.33 (s, 1H, NH), 8.25 (d, $J$ = 8.4 Hz, 1H, ArH), 8.13 (d, $J$ = 8.0 Hz, 1H, NH), 8.09 (d, $J$ = 7.5 Hz, 1H, NH), 8.03 (d, $J$ = 8.5 Hz, 1H, NH), 8.01-7.97 (m, 1H, NH), 7.89 (d, $J$ = 8.2 Hz, 1H, ArH), 7.86-7.80 (m, 2H, F-ArH), 7.79-7.72 (m, 3H, ArH), 7.71-7.66 (m, 2H, ArH), 7.58-7.50 (m, 4H, ArH), 7.44 (d, $J$ = 6.9 Hz, 1H, ArH), 7.42-7.34 (m, 5H, ArH), 7.31-7.25 (m, 2H, ArH), 7.21-7.15 (m, 3H, 2F-ArH, ArH), 7.07 (s, 1H, NH), 7.02 (d, $J$ = 8.4 Hz, 1H, ArH), 4.72-4.65 (m, 1H, H$_\alpha$), 4.62-4.50 (m, 2H, H$_\alpha$), 4.19 (dd, $J$ = 13.3, 8.4 Hz, 1H, Lys-H$_\alpha$), 3.61 (dd, $J$ = 14.3, 3.9, 1H, CH$_2$), 3.24-3.00 (m, 5H, CH$_2$), 2.97-2.89 (m, 1H, CH$_2$), 2.82-2.49 (m, 5H, CH$_2$), 2.29-2.19 (m, 1H, Imp-H$_\alpha$), 1.78-1.66 (m, 1H, CH$_2$), 1.66-1.54 (m, 1H, CH$_2$), 1.53-1.40 (m, 5H, CH$_2$), 1.40-1.16 (m, 2H, CH$_2$), ppm. ESI-LC-MS m/z 477.4 [M + 4H - F]$^2^+$; HRMS (ESI-MS) calcd. for C$_{58}$H$_{61}$FN$_7$O$_6$ [M + H]$^+$ 970.4667 found 970.4693.

**3.4.6 Synthesis of 4-(tert-butoxycarbonyl)-N,N,N-trimethylbenzenammonium triflate (20)**

4-dimethylaminobenzoic acid (1.00 g, 6.05 mmol, 1.0 equiv.) was added to THF (50 ml) and the resultant mixture stirred and cooled to 0°C. After 15 minutes, trifluoroacetic anhydride (1.85 ml, 13.3 mmol, 2.2 equiv.) was added dropwise and the consequent blue solution stirred (35 mins). Addition of $^1$BuOH (11.4 ml, 119 mmol, 19.7 equiv.) was followed by further stirring at room temperature (2 hrs). The solution was then poured into saturated NaHCO$_3$ (250 ml) and extracted with DCM (3x 100 ml, 3x 50 ml). The combined organic layers were dried (MgSO$_4$), filtered by gravity and residual solvent removed by rotary evaporation. This delivered a black oil which was eluted through a silica pad (DCM, 60 ml) and solvent removed by concentration in vacuo. The final yellow oil (1.12 g, crude) was then re-dissolved in DCM (dry, 30 ml) and cooled to 0°C under a blanket of N$_2$. This was followed by the addition of MeOTf (0.86 ml, 7.61 mmol, 1.5 equiv.) and stirring at 0°C (1 hr). The reaction mixture was then poured into an
ice-cold solution of Et₂O (200 ml) which caused instant precipitation of the product salt as a white crystalline solid (740 mg, 38%): ¹H-NMR (400 MHz, CD₃COCD₃); δ 8.24 (d, J = 9.3 Hz, 2H), 8.18 (d, J = 9.3 Hz, 2H), 3.91 (s, 9H, NMe₃), 1.60 (s, 9H, OC(CH₃)₃); ¹³C-NMR (100 MHz, CD₃COCD₃); δ 205.5 (CO) 163.6 (ArC), 133.7 (ArC), 131.0 (ArC), 120.9 (ArC), 81.7, 56.9, 27.3 (CH₃CO) ppm. ESI-MS m/z 165.1 [C₁₀H₁₃O₂]⁺; HRMS (ESI-MS) calcd for C₁₄H₂₂NO₂ [M-CF₃O₂S]⁺ 236.1651, found 236.1658.

3.4.7 Receptor-ligand binding assay

Competitive binding assays were carried out using HEK293/GHS-R1a cells with [¹²⁵I]ghrelin as the competitive radioligand. A solution of binding buffer (50 ml, pH 7.4) was made up by adding 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, 0.3 g, 25 mM), MgCl₂ (0.051 g, 5 mM), CaCl₂ (7.4 x 10⁻³ g, 1 mM), EDTA (0.015 g, 2.5 mM) and BSA (0.2 g, 0.4%) to distilled water. pH adjusted with 5 M HCl. The resultant solution was filtered through a 0.22 µm syringe filter and kept on ice.

An aliquot of frozen cells (HEK293T cells transiently transfected with GHS-R1a by means of calcium phosphate transfection for 48 hours before being harvested and fragmented by 50,000 g centrifugation and frozen to 2 million cells per vial in 10 % DMSO in FBS) was thawed to room temperature (1.5 x 10⁶ cells/pellet), centrifuged (3000 rpm, 10 mins, room temperature) and the subsequent cell pellet re-suspended in binding buffer (2 ml) and placed on ice. A stock solution of peptide/peptidomimetic was diluted in binding buffer to acquire final concentrations between 10⁻⁵ to 10⁻¹¹ M and added (30 µl) to these cells (50 µl) in triplicate. [¹²⁵I]-ghrelin (10 µl) was added to binding buffer (3 ml) and vortexed. An aliquot of this [¹²⁵I]-ghrelin solution (20 µl) was counted on a Cobra II Auto-Gamma gamma counter and initial volume adjusted to obtain 14000-17000 counts per minute (cpm) per 20 µl aliquot. [¹²⁵I]-ghrelin (20 µl) was added to each peptide/peptidomimetic-cell mixture, to give a final volume of 300 µl in binding buffer. Each solution concentration was vortexed, followed by agitation (550 rpm, 20 mins, 37 ºC). After 20 minutes, samples were spun (13 000 rpm, 5 mins, 4 ºC) and placed on ice. The supernatant was removed and the cell pellet rinsed with ice-cold Tris-HCl (200 µl, 50 mM, pH 7.4). The samples
were spun again (13,000 rpm, 5 mins, 4 °C), cooled on ice, supernatant removed and cell pellet counted using a Cobra II Auto-Gamma gamma counter.

All binding assays were performed in triplicate for each concentration of peptide/peptidomimetic. Binding buffer (300 µl) and \([^{125}\text{I}]\)-ghrelin (20 µl) alone were used as background controls. \([^{125}\text{I}]\)-ghrelin (20 µl) and cells (50 µl) in binding buffer (230 µl) were used to calculate the percentage of bound \([^{125}\text{I}]\)-ghrelin displaced by each concentration of peptide/peptidomimetic.

3.4.8 Computation of partition coefficients (LogP) for peptides and peptidomimetics
The \(n\)-octanol/water partition coefficients for all peptides and peptidomimetics were calculated using ACD/LogP prediction software from ACD/ChemSketch (Freeware) 2017.2.1.

3.4.9 Calcium flux dose-response assay
This assay was used to determine the EC\(_{50}\) value for \([1-\text{Nal}^4,\text{Lys}^5(4-\text{FB})]\)G7039 in terms of intracellular Ca\(^{2+}\) release, with ghrelin used as the control ligand. The peptidomimetic compound was dissolved in DMSO (1 ml) at a concentration of 250 µM and solubility tested in Hank’s balanced salt solution (HBSS): 1.26 mM CaCl\(_2\), 0.493 mM MgCl\(_2\)·6H\(_2\)O, 0.407 mM MgSO\(_4\)·7H\(_2\)O, 5.33 mM KCl, 0.441 mM KH\(_2\)PO\(_4\), 4.17 mM NaHCO\(_3\), 137.9 mM NaCl, 0.338 mM Na\(_2\)HPO\(_4\), 5.56 mM D-Glucose, 20 mM HEPES, pH 7.4 with a final concentration of 1.2 % DMSO. Following successful solubility, human recombinant GHS-R1a calcium-optimised stable cell lines (chem-1 cells) were loaded with a fluorescent calcium dye (Fluo-8 NW from ABD Bioquest 21080) and calcium flux detected in response to the agonist by a Molecular Devices FLIPR\(^{\text{TETRA}}\) instrument in a modified HBSS buffer (20 mM HEPES, 2.5 mM Probenecid, pH 7.4). Fluorescence values underwent baseline correction and the percentage activation was normalised to the maximum response (\(E_{\text{max}}\)) of the control ligand ghrelin. The agonist assay was performed for a total of 180 seconds and each assay concentration was performed in duplicate.

3.4.10 Serum stability study
A Waters Oasis HLB plate was used for both collection and extraction. The peptidomimetic \([1-\text{Nal}^4,\text{Lys}^5(4-\text{FB})]\)G7039 was incubated in a solution of 25% human serum in PBS for 24 hours. Aliquots were removed at 0, 20, 60, 240, 420, and 1440
minutes, with each time-point performed in triplicate. These aliquots were then mixed with 4% (v/v) ammonium hydroxide (NH₄OH) in water to denature peptidomimetic-serum (albumin) interactions. [1-Nal⁴,Lys⁵(4-FB)]G7039 was then extracted from human serum using 20% MeOH/2% HCO₂H in water and quantified by UHPLC-MS. The amount was then expressed as the percentage of the area under the curve (AUC) in the UV chromatogram relative to time 0, plotted against time (minutes) and data fitted to a curve of exponential decay to determine the serum half-life (t₁/₂).

3.4.11 Radiochemistry

General information
All reagents and solvents used for radiosynthesis were purchased from Sigma-Aldrich unless otherwise specified. ¹⁸F-fluoride was produced via the ¹⁸O(p,n) ¹⁸F reaction in a GE PETtrace 880 cyclotron (Lawson Health Research Institute, London, Ontario, Canada). A Biotage V-10 evaporator was used to remove solvent. A Waters HPLC with a 1525 binary pump system (solvent A = MeCN + 0.1% TFA, solvent B = H₂O + 0.1% TFA) and two detectors (Waters 2487 Dual λ absorbance detector set at 254 nm and a radioactive flow count detector) were utilised for product analysis and purification. An analytical column (Agilent Eclipse XDB-C18, 4.6 × 150 mm, 5 μm) was used for determining radiochemical purity and molar activity of [1-Nal⁴,Lys⁵(4-[¹⁸F]-FB)]G7039. A semi-preparative column (Agilent Zorbax SB-C18, 9.4 × 150 mm, 5 μm) was used for the purification of [¹⁸F]SFB and [1-Nal⁴,Lys⁵(4-[¹⁸F]-FB)]G7039.

Synthesis of [¹⁸F]FBA
Scheme 1 shows the synthetic pathway to [¹⁸F]FBA. Aqueous [¹⁸F]fluoride was trapped on a Waters Sep-Pak Accell Plus Light (46 mg) QMA Carbonate cartridge pre-activated with MilliQ water (10 ml). The initial radioactivity of the cartridge was measured using a dose calibrator (980-1300 MBq) A solution of acetonitrile/H₂O (1 ml, 25:75, v/v) containing potassium carbonate (2.1 mg, 0.015 mmol, 2.6 eq.) and 4,7,13,16,21,24-Hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (6.0 mg, 0.016 mmol, 2.8 eq.) was used to elute [¹⁸F]fluoride into the reaction vial. The solvent was removed azeotropically under vacuum at 50 °C. [¹⁸F]fluoride was then dried a further two times under the aforementioned conditions by adding anhydrous acetonitrile (1 ml). 4-Tert-
butoxycarbonyl)-N,N,N-trimethylbenzenammonium triflate (2.0 mg, 0.0057 mmol, 1.0 eq.) in anhydrous DMSO (400 μl) was added to dried [\(^{18}\)F]F\(^-\) and the subsequent solution heated for 10 mins at 120 °C. Aqueous HCl (1 ml, 6 M) was added and the resultant mixture heated further (10 mins, 120 °C). Water (2 ml) was added to dilute the reaction mixture containing crude [\(^{18}\)F]FBA. This solution was then trapped on two inter-connected Waters Sep-Pak C-18 light cartridges sequentially pre-treated with ethanol (15 ml) and water (15 ml). Elution of product then occurred using anhydrous acetonitrile (800 μl) to deliver the title compound [\(^{18}\)F]FBA in an average d.c. radiochemical yield of 82% (n = 3). This compound was used in the next step without further purification. To confirm that the correct product had been acquired, a co-injection of 4-[\(^{19}\)F]-FBA and [\(^{18}\)F]-FBA in water was performed (20 μl, 30-70% MeCN + 0.1% TFA gradient system, see Figure 4.5S3 supporting information).

**Synthesis of [\(^{18}\)F]SFB**

[\(^{18}\)F]FBA was trapped onto two inter-connected Waters Sep-Pak C-18 light cartridges sequentially pre-treated with ethanol (15 ml) and water (15 ml) and eluted with acetonitrile (800 μl) into a vial charged with NHS (29 mg, 0.25 mmol.) and EDC (71 mg, 0.46 mmol.). The resultant solution was allowed to stand at room temperature for 15-20 minutes before being diluted with water + 0.1% TFA (1.2 ml) and purified by semi-preparative HPLC (30-70% MeCN + 0.1% TFA gradient system). After solvent removal (V-10 evaporator, 36 °C) dry [\(^{18}\)F]SFB was obtained in an average d.c. radiochemical yield of 71% (n = 3) and ≥ 99% radiochemical purity.

**Synthesis of [1-Nal\(^4\), Lys\(^5\)(4-[\(^{18}\)F]-FB)]G7039**

A solution of [1-Nal\(^4\)]G7039 (2.0 mg, 1.86 x 10\(^{-3}\) mmol.) in acetonitrile (100 μl) and H\(_2\)O (100 μl) was added to the [\(^{18}\)F]SFB, followed by DIPEA (10 μl) and the resultant solution heated to 65 °C for 15-20 minutes. Crude [1-Nal\(^4\), Lys\(^5\)(4-[\(^{18}\)F]-FB)]G7039 began to precipitate out of solution as a white solid. The solvent was subsequently removed and the solid re-dissolved stepwise with DMF (300 μl), H\(_2\)O (500 μl) and MeCN (200 μl). This solution was purified by preparative HPLC (30-70% MeCN + 0.1% TFA gradient system) and delivered the title [\(^{18}\)F]radiolabelled peptidomimetic [1-Nal\(^4\),Lys\(^5\)(4-[\(^{18}\)F]-FB)]G7039 in an average d.c. radiochemical yield of 81% (n = 3).
an overall average d.c. radiochemical yield of 48% (n = 3), a radiochemical purity ≥ 99% and an average molar activity of 34 GBq/μmol (n = 3).

3.5 Acknowledgements
Special thanks go to Rebecca McGirr (Lawson Health Research Institute) for the preparation of HEK293/GHS-R1a cells for use in the receptor-ligand binding assays. We would also like to thank Prostate Cancer Canada and the Canadian Institutes of Health Research (CIHR) for funding this study.

3.6 Appendix A. Supplementary data
Amino acid sequences, lipophilicity data, HPLC chromatograms, $^1$H-NMR spectra and displacement curves for compounds 1-17, the radio-chromatogram of $[^{18}F]$SFB, the displacement curve for the endogenous ligand ghrelin and the UHPLC chromatogram, $^1$H-NMR- and $^{13}$C-NMR spectra of 4-(tert-butoxycarbonyl)-N,N,N-trimethylbenzenammonium triflate (20).

3.7 Conflicts of interest
Some of the peptidomimetic compounds in this work are the subject of the patent application “Peptidomimetics for Imaging the Ghrelin Receptor”, U.S. Patent WO2016/191865 A1, December 8th 2016.

3.8 References


Supplementary Information

Supplementary Table 3.1 - Synthesised peptides and peptidomimetics. 4-FB, 4-fluorobenzoyl; AEEA, 2-(2-(2-aminoethoxy)ethoxy)acetic acid; D-2-Nal, D-2-naphthylalanine; D-2-Thi, D-2-thienylalanine; Dpr, 2,3-diaminopropionic acid; Inp, isonipecotic acid

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<th>Peptide/Peptidomimetic</th>
<th>Amino Acid Sequence</th>
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<tr>
<td>[Lys⁷(4-FB)]ipamorelin</td>
<td>H-Aib-His-D-2-Nal-D-Phe-Lys(4-FB)-NH₂</td>
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<tr>
<td>GHRP-1</td>
<td>H-Ala-His-D-2-Nal-Ala-Trp-D-Phe-Lys-NH₂</td>
</tr>
<tr>
<td>GHRP-2</td>
<td>H-D-Ala-D-2-Nal-Ala-Trp-D-Phe-Lys-NH₂</td>
</tr>
<tr>
<td>GHRP-6</td>
<td>H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂</td>
</tr>
<tr>
<td>[Lys⁷(4-FB)]GHRP-6</td>
<td>H-His-D-Trp-Ala-Trp-D-Phe-Lys(4-FB)-NH₂</td>
</tr>
<tr>
<td>[Dpr⁹]GHRP-6</td>
<td>H-His-D-Trp-Ala-Trp-D-Phe-Dpr-NH₂</td>
</tr>
<tr>
<td>[Dpr⁹(4-FB)]GHRP-6</td>
<td>H-His-D-Trp-Ala-Trp-D-Phe-Dpr(4-FB)-NH₂</td>
</tr>
<tr>
<td>G7039</td>
<td>H-Inp-D-2-Nal-D-2-Nal-Phe-Lys-NH₂</td>
</tr>
<tr>
<td>[Lys⁷(4-FB)]G7039</td>
<td>H-Inp-D-2-Nal-D-2-Nal-Phe-Lys(4-FB)-NH₂</td>
</tr>
<tr>
<td>[1-Nal⁴, Lys⁴(4-FB)]G7039</td>
<td>H-Inp-D-2-Nal-D-2-Nal-1-Nal-Lys(4-FB)-NH₂</td>
</tr>
</tbody>
</table>
Supplementary Figure 3.1 - Displacement curve for the endogenous ligand ghrelin

Supplementary Table 3.2 - LogP values for peptides and peptidomimetics. These values were computed using ACD/LogP prediction software from ACD/Labs

<table>
<thead>
<tr>
<th>Peptidomimetic</th>
<th>LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipamorelin</td>
<td>1.72 ± 0.85</td>
</tr>
<tr>
<td>[Lys⁵(4-FB)]Ipamorelin</td>
<td>3.97 ± 0.89</td>
</tr>
<tr>
<td>[Lys⁵(AEEA-4-FB)]Ipamorelin</td>
<td>2.80 ± 0.93</td>
</tr>
<tr>
<td>[D-2-Thi⁴,Lys⁵(4-FB)]Ipamorelin</td>
<td>3.65 ± 0.90</td>
</tr>
<tr>
<td>[Inp¹,Lys⁵(4-FB)]Ipamorelin</td>
<td>3.92 ± 0.89</td>
</tr>
<tr>
<td>[Inp¹,D-2-Nal⁴,Lys⁵(4-FB)]Ipamorelin</td>
<td>5.15 ± 0.89</td>
</tr>
<tr>
<td>[Inp¹,D-2-Thi⁴,Lys⁵(4-FB)]Ipamorelin</td>
<td>3.60 ± 0.89</td>
</tr>
<tr>
<td>GHRP-1</td>
<td>2.60 ± 0.90</td>
</tr>
<tr>
<td>GHRP-2</td>
<td>3.41 ± 0.86</td>
</tr>
<tr>
<td>GHRP-6</td>
<td>1.51 ± 0.88</td>
</tr>
<tr>
<td>[Lys⁵(4-FB)]GHRP-6</td>
<td>3.76 ± 0.92</td>
</tr>
<tr>
<td>[Dpr⁶]GHRP-6</td>
<td>1.61 ± 0.89</td>
</tr>
<tr>
<td>[Dpr⁶(4-FB)]GHRP-6</td>
<td>3.86 ± 0.93</td>
</tr>
<tr>
<td>G7039</td>
<td>5.28 ± 0.82</td>
</tr>
<tr>
<td>[Lys⁵(4-FB)]G7039</td>
<td>7.53 ± 0.88</td>
</tr>
<tr>
<td>[1-Nal⁴]G7039</td>
<td>6.51 ± 0.82</td>
</tr>
<tr>
<td>[1-Nal⁴,Lys⁵(4-FB)]G7039</td>
<td>8.76 ± 0.88</td>
</tr>
</tbody>
</table>
Supplementary Figure 3.2 - Decay-curve for the lead peptidomimetic [1-Nal\textsuperscript{4},Lys\textsuperscript{5}(4-FB)]G7039 in human serum. The amount of peptidomimetic is expressed as the percentage of the area under the curve (AUC) from time $= 0$ ($T_0$) in the UV chromatogram. Each time point was performed in triplicate.
Supplementary Figure 3.3 - Radiochromatogram of $^{19}$F-FBA and $^{18}$F-FBA co-injection
Supplementary Figure 3.4 - Radiochromatogram of [$^{18}$F]SFB
Peptidic and peptidomimetic HPLC chromatograms

Ipamorelin: H-Aib-His-D-2-Nal-D-Phe-Lys-NH₂ (1)

[Lys⁵(4-FB)]ipamorelin: H-Aib-His-D-2-Nal-D-Phe-Lys(4-FB)-NH₂ (2)
[Lys\(^5\)(AEEA-4-FB)]ipamorelin: H-Aib-His-D-2-Nal-D-Phe-Lys(AEEA-4-FB)-NH\(_2\) (3)

[D-2-Thi\(^4\),Lys\(^5\)(4-FB)]ipamorelin: H-Aib-His-D-2-Nal-D-2-Thi-Lys(4-FB)-NH\(_2\) (4)
[Inp$^1$,Lys$^5$(4-FB)]ipamorelin: H-Inp-His-D-2-Nal-D-Phe-Lys(4-FB)-NH$_2$ (5)

[Inp$^1$,D-2-Nal$^4$,Lys$^5$(4-FB)]ipamorelin: H-Inp-His-D-2-Nal-D-2-Nal-Lys(4-FB)-NH$_2$ (6)
[Inp$^1$,D-2-Thi$^4$,Lys$^5$(4-FB)]ipamorelin: H-Inp-His-D-2-Nal-D-2-Thi-Lys(4-FB)-NH$_2$ (7)

GHRP-1: H-Ala-His-D-2-Nal-Ala-Trp-D-Phe-Lys-NH$_2$ (8)
GHRP-2: H-D-Ala-D-2-Nal-Ala-Trp-D-Phe-Lys-NH₂ (9)

GHRP-6: H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂ (10)
[Lys⁶(4-FB)]GHRP-6: H-His-D-Trp-Ala-Trp-D-Phe-Lys(4-FB)-NH₂ (11)

[Dpr⁶]GHRP-6: H-His-D-Trp-Ala-Trp-D-Phe-Dpr-NH₂ (12)
[Dpr⁶(4-FB)]GHRP-6: H-His-D-Trp-Ala-Trp-D-Phe-Dpr(4-FB)-NH₂ (13)

G7039: H-Inp-D-2-Nal-D-2-Nal-Phe-Lys-NH₂ (14)
[Lys⁵(4-FB)]G7039: H-Inp-D-2-Nal-D-2-Nal-Phe-Lys(4-FB)-NH₂ (15)

[1\textsuperscript{-}Nal\textsuperscript{4},Lys\textsuperscript{5}(4-FB)]G7039: H-Inp-D-2-Nal-D-2-Nal-1-Nal-Lys(4-FB)-NH\textsubscript{2} (17)
Peptidic- and peptidomimetic $^1$H-NMR spectra

Ipamorelin: H-Aib-His-D-2-Nal-D-Phe-Lys-NH$_2$ (1)
[Lys\textsuperscript{5}(4-FB)]ipamorelin: H-Aib-His-D-2-Nal-D-Phe-Lys(4-FB)-NH\textsubscript{2} (2)
[Lys⁵(AEEA-4-FB)]ipamorelin: H-Aib-His-D-2-Nal-D-Phe-Lys(AEEA-4-FB)-NH₂ (3)
[D-2-Thi\textsuperscript{4},Lys\textsuperscript{5}(4-FB)]ipamorelin: H-Aib-His-D-2-Nal-D-2-Thi-Lys(4-FB)-NH\textsubscript{2} (4)
[Inp$^1$, Lys$^5$(4-FB)]ipamorelin: H-Inp-His-D-2-Nal-D-Phe-Lys(4-FB)-NH$_2$ (5)
[Inp$^1$,D-$2$-Nal$^4$,Lys$^5$(4-FB)]ipamorelin: H-Inp-His-D-2-Nal-D-2-Nal-Lys(4-FB)-NH$_2$ (6)
[Inp\(^1\),D-2-Thi\(^4\),Lys\(^5\)(4-FB)]ipamorelin: H-Inp-His-D-2-Nal-D-2-Thi-Lys(4-FB)-NH\(_2\) (7)
GHRP-1: H-Ala-His-D-2-Nal-Ala-Trp-D-Phe-Lys-NH₂ (8)
GHRP-2: H-D-Ala-D-2-Nal-Ala-Trp-D-Phe-Lys-NH₂ (9)
GHRP-6: H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH$_2$ (10)
[Lys<sup>6</sup>(4-FB)]GHRP-6: H-His-D-Trp-Ala-Trp-D-Phe-Lys(4-FB)-NH<sub>2</sub> (11)
[Dpr$^6$]GHRP-6: H-His-D-Trp-Ala-Trp-D-Phe-Dpr-NH$_2$ (12)
[Dpr$^6$(4-FB)]GHRP-6: H-His-D-Trp-Ala-Trp-D-Phe-Dpr(4-FB)-NH$_2$ (13)
G7039: H-Inp-D-2-Nal-D-2-Nal-Phe-Lys-NH$_2$ (14)
[Lys$^5$(4-FB)]G7039: H-Inp-D-2-Nal-D-2-Nal-Phe-Lys(4-FB)-NH$_2$ (15)
Peptidic- and peptidomimetic displacement curves

Ipamorelin (1)

[Lys\(^{5}\)(4-FB)]ipamorelin (2)
[Lys$^5$(AEEA-4-FB)]ipamorelin (3)

---

[D-2-Thi$^3$, Lys$^5$(4-FB)]ipamorelin (4)
[Inp\textsuperscript{1}, Lys\textsuperscript{5}(4-FB)]ipamorelin (5)

![Graph showing the concentration of the ligand vs. % bound radioligand.]

[Inp\textsuperscript{1}, D-2-Nal\textsuperscript{4}, Lys\textsuperscript{5}(4-FB)]ipamorelin (6)

![Graph showing the concentration of the ligand vs. % bound radioligand.]

[Inp$^1$,D-2-Thi$^4$,Lys$^5$(4-FB)]ipamorelin (7)

GHRP-1 (8)
GHRP-2 (9)

% Bound Radioligand

log[peptide]

GHRP-6 (10)

% Bound Radioligand

log[peptide]
[Lys\textsuperscript{6}(4-FB)]GHRP-6 (11)

![Graph showing percentage bound radioligand vs. log([peptidomimetic])]  

[Dpr\textsuperscript{6}]GHRP-6 (12)
Please note that this data has already been published\textsuperscript{1}, but is presented here for completeness.
[Lys\(^5\)(4-FB)]G7039 (15)

[1-Nal\(^4\)]G7039 (16)
[1-Nal\textsuperscript{4},Lys\textsuperscript{5}(4-FB)]G7039 (17)
**UHPLC chromatogram of 4-(tert-butoxycarbonyl)-N,N,N-trimethylbenzenammonium triflate (20)**

![UHPLC Chromatogram](image)

**1H-NMR spectrum of 4-(tert-butoxycarbonyl)-N,N,N-trimethylbenzenammonium triflate (20)**

![1H-NMR Spectrum](image)
$^{13}$C-NMR spectrum of 4-(tert-butoxycarbonyl)-N,N,N-trimethylbenzenammonium triflate (20)

References

Chapter 4

Single Amino Acid Replacement in G7039 Leads to a 70-Fold Increase in Binding Towards GHS-R1a

4.1 Introduction

Ghrelin is a hormone produced mainly by the stomach that was first discovered in 1999 by Kojima et al. Ghrelin actions are mediated via activation of the G Protein Coupled Receptor (GPCR) growth hormone secretagogue receptor type 1a (GHS-R1a)\(^1,2\). It was originally reported that GHS-R1a was found within the pituitary gland and hypothalamus\(^2\) but recent work has revealed a more widespread expression in other tissues, including other regions of the brain and heart\(^3,4\). GHS-R1a is also reported to be differentially expressed in certain cancer cells. For example, GHS-R1a activation increases cellular proliferation in prostate cancer through activation of the p44/42 MAPK signalling pathway\(^5-7\). Overexpression of GHS-R1a increases the motile phenotype in astrocytomas, in which increased GHS-R1a expression is correlated with a higher tumor grade\(^8\). Overexpression of GHS-R1a has more recently been demonstrated in some ovarian epithelial carcinomas, a disease with poor prognosis due to the late stages in which this cancer is usually diagnosed\(^9-14\).

A number of synthetic peptides acting as GHS-R1a agonists have been described. In 1977 Bowers et al, while undertaking structure-activity relationship (SAR) on enkephalin analogues, discovered that some of these derivatives were involved in the release of growth hormone (GH) from the pituitary gland in the female rats\(^15-17\). The hexamer H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH\(_2\) was identified as the first synthetic peptide to release GH both \textit{in vitro} and \textit{in vivo} through the activation of GHS-R1a. From this point on, researchers began to develop peptides that had the ability to release growth hormone through the activation of GHS-R1a, generating the class of compounds known as growth hormone secretagogues (GHSs)\(^18\). They are uniquely classified based on their function of secreting GH from the pituitary by activation of GHS-R1a, instead of the

through secondary release stimulated by the action of GH releasing hormone from the hypothalamus\textsuperscript{18-19}.

GHSs allow for a more rapid synthesis (5 to 6-mer peptides) compared to the full-length ghrelin peptide and act as a small molecule when targeting the receptor\textsuperscript{20}. Our development of these agonists was guided by work from Feighner \textit{et al.} who utilized site directed mutagenesis to determine the key residues in GHS-R1a that interacted with the GHSs (GHRP-6, MK-0677, and L-692,585) they studied\textsuperscript{20}.

The starting peptidomimetic for this project was G7039 (H-Inp-D2Nal=D2Nal-1Nal-Lys-NH\textsubscript{2} or [1-Nal\textsuperscript{4}]G7039). Previously, we reported on the evaluation of a fluorine-containing G7039 peptidomimetic ([1-Nal\textsuperscript{4}, Lys\textsuperscript{5} (4-fluorobenzoyl-4-FB)]G7039) as a positron emission tomography (PET) tracer, through addition of a 4-fluorobenzoic acid (4-FBA) group on the C-terminal lysine, allowing for radiolabeling with fluorine-18 (\textsuperscript{18}F) (\textbf{panel B Figure 4.1})\textsuperscript{21}.

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{figure4_1.png}
\caption{(A) Starting peptidomimetic ([1-Nal\textsuperscript{4}]G7039) to perform SAR. (B) Previously reported fluorine-containing G7039 peptidomimetic ([1-Nal\textsuperscript{4}, Lys\textsuperscript{5} (4-FB)]G7039)\textsuperscript{21}. (C) Current peptidomimetics being studied with changes in the} \end{figure}
fourth position to either Ser and/or Phe, and Tyr (indicated by red X), and modification of prosthetic group from 4-FB to 2-FP (seen in blue)

This led to a compound that had moderate affinity for GHS-R1a (IC$_{50}$ = 69 nM compared to ghrelin IC$_{50}$ = 3 to 5 nM) in addition to high lipophilicity (cLogP of 6.51 ± 0.82)$^{21}$. This lead candidate was assessed in a healthy mouse model (C57BL/6) and showed significant uptake in off target tissues (e.g. liver, spleen, kidneys, and lungs), as expected due to the high lipophilicity$^{22}$. However, it was noted that the first 3 amino acids were important for binding and activating the receptor, therefore, to improve receptor affinity, modifications of only the fourth position were proposed, leaving the C-terminal lysine free for the attachment of a different prosthetic group$^{21}$. The main goal of this current work was to evaluate different amino acids in the fourth position in order to reduce the cLogP and improve the binding affinity towards GHS-R1a. An additional goal was to switch from the 4-FB group to a 2-fluoropropionyl group (2-FP), appended to the side-chain of the C-terminal lysine. This change in prosthetic group was expected to decrease the hydrophobicity, but still provide a facile radiolabelling route to the synthesis of a PET probe$^{23}$. These proposed structural modifications can be seen in panel C, Figure 4.1. In total seven G7039 analogues were synthesized and evaluated for GHS-R1a affinity by a competitive binding assay (IC$_{50}$), a β-arrestin-1 and β-arrestin-2 recruitment assay (EC$_{50}$), and a computational docking study. This led to the discovery of novel peptides with high affinity and potency that have potential to be used as therapeutics and/or imaging agents when targeting GHS-R1a.

4.2 Results and Discussion

As an initial step, the previously used prosthetic group 4-FB located off the ε-amine of the C-terminal lysine, was replaced with the smaller 2-FP group to give compound 2. This analogue maintained moderate GHS-R1a affinity while maintaining relatively high cLogP value (Table 4.1). Further structure activity relationship studies were undertaken to change the fourth position in order to improve both the affinity and cLogP of this class of peptidomimetics.

| Table 4.1 - Peptidomimetics with calculated binding numbers (IC$_{50}$) and predicted lipophilicity (cLogP). Only the fourth position was changed and evaluated with the |
exception of compound 3 and 4. The 2-fluoropropionic acid group was added to Lys 5 in all cases except compounds 1 and 7.

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>Peptide Sequence</th>
<th>IC_{50} (nM)</th>
<th>cLogP (ACD/Labs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H-Inp-D2Nal-D2Nal-Phe-Lys-NH_{2}</td>
<td>5.2</td>
<td>5.28 ± 0.82</td>
</tr>
<tr>
<td>2</td>
<td>H-Inp-D2Nal-D2Nal-Phe-Lys(2-FP)-NH_{2}</td>
<td>19</td>
<td>4.34 ± 0.90</td>
</tr>
<tr>
<td>3</td>
<td>H-Inp-D2Nal-D2Nal-Ser-Phe-Lys(2-FP)-NH_{2}</td>
<td>62.9</td>
<td>4.33 ± 0.90</td>
</tr>
<tr>
<td>4</td>
<td>H-Inp-D2Nal-D2Nal-Phe-Ser-Lys(2-FP)-NH_{2}</td>
<td>4.35</td>
<td>4.33 ± 0.90</td>
</tr>
<tr>
<td>5</td>
<td>H-Inp-D2Nal-D2Nal-Tyr-Lys(2-FP)-NH_{2}</td>
<td>0.28</td>
<td>2.77 ± 0.87</td>
</tr>
<tr>
<td>6</td>
<td>H-Inp-D2Nal-D2Nal-Ser-Lys(2-FP)-NH_{2}</td>
<td>60.3</td>
<td>2.77 ± 0.87</td>
</tr>
<tr>
<td>7</td>
<td>H-Inp-D2Nal-D2Nal-Tyr-Lys-NH_{2}</td>
<td>0.70</td>
<td>2.69 ± 0.89</td>
</tr>
</tbody>
</table>

All peptides in Table 4.1 were synthesized using Fmoc solid-phase peptide chemistry, purified using reverse-phase high performance liquid chromatography (RP-HPLC) and analyzed by ESI-MS. To allow the addition of the fluorine-containing entity, peptides required an orthogonal Alloc protecting group on the C-terminal lysine to allow for selective deprotection and coupling to the 2-fluoropropionic acid (2-FPA) prosthetic group. Purity for each peptide was greater than 98% as determined by UHPLC (Table 4.S1).

To test the affinity of these peptides towards GHS-R1a, a competitive binding assay was performed with [125I] human ghrelin as the competitor (Table 4.1). When a non-aromatic amino acid was present in the fourth position, affinity towards GHS-R1a is diminished. Compound 3 and 6, with Ser in position 4, have IC_{50} values of 62.9 nM and 60.3 nM respectively. If an aromatic group is in the fourth position, as per Phe for compound 4, the affinity improves to 4.35 nM. Moreover, when incorporating Tyr in the fourth position the binding substantially improves to 0.28 nM (compound 5). The 2-FP group was removed from compound 5 in order to confirm that the increase in binding was due to the Tyr in the 4th position. With no 2-FP prosthetic group compound 7 was found to maintain this sub-nanomolar affinity with an IC_{50} of 0.70 nM.

As modifications were made to the C-terminal amino acids of the peptidomimetic, it was noted that when Phe remains in either the fourth or fifth position (compound 4 and
respectively in Table 4.1) the cLogP remains above 4. However, when the fourth position is a Tyr or Ser, cLogP drops below 3. This lowered cLogP is desirable and could help with improving pharmacokinetic behavior in vivo. Tyrosine in the fourth position was the optimal amino acid change, dramatically decreasing the cLogP (comparing compounds 1-4 vs. compound 5 and 7). The addition or absence of the 2-FP prosthetic group doesn’t drastically change the cLogP values (compound 5 vs. 7).

Next, we wanted to assess the potency of the peptides through monitoring the recruitment of β-arrestin-1 and β-arrestin-2 to GHS-R1a to obtain EC\textsubscript{50} values for the peptides. Both of these intracellular proteins are normally recruited by the activation of GHS-R1a leading to its internalization and recyclization. In all cases, compounds 1 through 7 were agonists for the receptor, with EC\textsubscript{50} values in the sub-picomolar range. Based on the concentration-effect curves (Figure 4.2), some compounds were more potent and efficacious than others. From Table 4.2 the most potent peptide is compound 2 (EC\textsubscript{50} of 15.8 pM and 72.8 pM for β-arrestin-1 and β-arrestin-2, respectively), while compounds 3 (EC\textsubscript{50} of 202.4 pM and 178.1 pM for β-Arrestin-1 and β-Arrestin-2 respectively) and 7 (EC\textsubscript{50} of 178.7 pM and 262.5 pM for β-Arrestin-1 and β-Arrestin-2 respectively) were the least potent. The E\textsubscript{max} for compound 3 is 0.0780 and 0.1106 for β-Arrestin-1 and β-Arrestin-2 respectively and the E\textsubscript{max} for compound 6 is 0.0740 and 0.1295 for β-Arrestin-1 and β-Arrestin-2 respectively. Compound 3 and 6 have a 1.5 – 2x reduction in E\textsubscript{max} compared to the other peptides in this series. This indicates that compounds 3 and 6 are acting as a partial agonist when binding to GHS-R1a.
Figure 4.2 - β-Arrestin-1 and β-Arrestin-2 recruitment concentration curves measuring the EC$_{50}$ of the 7 peptides in HEK293T cells co-transfected with GHS-R1a-eYFP and GHS-R1a-Rluc constructs. Data are shown as net BRET (eYFP/rluc)
Table 4.2 - Potency measured by the recruitment of β-Arrestin-1 and β-Arrestin-2 upon binding of an agonist to GHS-R1a

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>Peptide Sequence</th>
<th>β-Arr-1 EC₅₀ (pM)</th>
<th>β-Arr-1 Eₘₐₓ (Net BRET Ratio)</th>
<th>β-Arr-2 EC₅₀ (pM)</th>
<th>β-Arr-2 Eₘₐₓ (Net BRET Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H-Inp-D2Nal-D2Nal-Phe-Lys-NH₂</td>
<td>59.9</td>
<td>0.1159</td>
<td>61.7</td>
<td>0.1889</td>
</tr>
<tr>
<td>2</td>
<td>H-Inp-D2Nal-D2Nal-Phe-Lys(2-FP)-NH₂</td>
<td>15.8</td>
<td>0.1072</td>
<td>72.8</td>
<td>0.1819</td>
</tr>
<tr>
<td>3</td>
<td>H-Inp-D2Nal-D2Nal-Ser-Phe-Lys(2-FP)-NH₂</td>
<td>202.4</td>
<td>0.0780</td>
<td>178.1</td>
<td>0.1106</td>
</tr>
<tr>
<td>4</td>
<td>H-Inp-D2Nal-D2Nal-Phe-Ser-Lys(2-FP)-NH₂</td>
<td>90.9</td>
<td>0.1307</td>
<td>81.7</td>
<td>0.1748</td>
</tr>
<tr>
<td>5</td>
<td>H-Inp-D2Nal-D2Nal-Tyr-Lys(2-FP)-NH₂</td>
<td>125.7</td>
<td>0.1194</td>
<td>121.1</td>
<td>0.1616</td>
</tr>
<tr>
<td>6</td>
<td>H-Inp-D2Nal-D2Nal-Ser-Lys(2-FP)-NH₂</td>
<td>135.6</td>
<td>0.0740</td>
<td>207.8</td>
<td>0.1295</td>
</tr>
<tr>
<td>7</td>
<td>H-Inp-D2Nal-D2Nal-Tyr-Lys-NH₂</td>
<td>178.7</td>
<td>0.1176</td>
<td>262.5</td>
<td>0.1687</td>
</tr>
</tbody>
</table>

To understand the molecular basis for the experimental observation that Tyr incorporation in the fourth position improves the binding affinity for this series, a molecular docking study was performed on compound 7 using a homology model for GHS-R1a recently developed by our lab²³. Our previous molecular dynamics (MD) simulation revealed that the N-terminal end of compound 1 (G7039) is situated at the bottom of the orthosteric site engaging in a salt bridge interaction with Glu124, and the 2nd and 3rd residue D2Nal binds to the other two hydrophobic sub-pockets (Figure 4.3A)²³. The molecular docking study on compound 7 suggested a very similar binding pattern except that the fourth residue Tyr is H-bonding with Arg283 (Figure 4.3B). Our previous model also showed that the 4th residue Phe of G-7039 is surrounded by hydrophobic residues suggesting switching it to a hydrophilic residue Ser would reduce the binding affinity, which is consistent with the experimental data (compound 2 vs 6). The computational studies showed that the aromatic ring of the Tyr retains the interaction with the surrounding hydrophobic residues such as Phe 286 in the receptor (Figure 4.3).
and the additional H-bonding interaction with Arg 283 contributes to the improved binding affinity.

![Figure 4.3 - The structure of compound 1 (G7039) in complex with GHS-R1α obtained from our previous computational study. Compound 1 is situated at the bottom of orthosteric site engaging a salt bridge interaction with Glu124, and the 2nd and 3rd residues D2Nal binds to the other two hydrophobic sub-pockets. (B) The structure of compound 7 in complex with GHS-R1α from molecular docking study suggested a very similar binding pattern except that the 4th residue Tyr is H-bonding with Arg283.](image)

4.3 Conclusion

The goal of the current work was to improve the binding affinity and decrease lipophilicity of a previously reported GHS-R1α targeting peptidomimetic. Changes were made to both the fourth position and prosthetic group conjugated from the C-terminal Lys of ([1-Nal⁴, Lys⁵ (4-FB)]G7039), followed by assessing the binding affinity, cLogP, potency, and molecular docking to confirm the outcomes of the amino acid changes. In total, seven novel ghrelin agonists were synthesized and assessed for their binding affinity and potency towards GHS-R1α. Compound 5 showed the greatest binding affinity ($IC_{50} = 0.28 \text{ nM}$) with Tyr in the fourth position and it was noted that an aromatic amino acid is required in the fourth position in order to retain binding affinity towards the receptor. Compound 3 and 6 were identified as partial agonists and this class of molecule can be useful in terms of regulating the GHS-R1α signalling in a differential
fashion. Molecular docking confirmed an additional H-bond interaction with Tyr in compound 5 explaining the increase in binding. Compound 5 could be used as an imaging agent since there is an available fluorine atom (the 2-FP moiety on Lys five) that can be replaced by fluorine-18 through established prosthetic radiochemistry methods\textsuperscript{24}. Compound 5 is currently the most potent fluorine-containing peptidomimetic reported for GHS-R1a (based upon β-arrestin recruitment) and uniquely has the potential to be used as an imaging agent for the diagnosis of human diseases associated with GHS-R1a.

4.4 Materials and Methods

4.4.1 General Information

All reagents were obtained from the commercial suppliers and used without any further purification. All Fmoc-protected amino acids were obtained from ChemImpex. HCTU, HATU, and Rink Amide MBHA Resin (4-(2',4'-dimethoxyphenyl)-(9-fluorenylmethoxycarbonyl)-aminomethyl)-phenoxy-acetamidonorleucyl-4-methyl benzhydrylamine resin) were obtained from ChemImpex. Tetrakis(triphenylphosphine)palladium(0), and phenylsilane. All other solvents were obtained from Fischer Thermo-Scientific.

4.4.2 Solid-Phase Peptide Synthesis

Automated Fmoc-based solid-phase synthesis was carried out using a Biotage\textsuperscript{®} Syro Wave\textsuperscript{™} automated peptide synthesizer. Synthesis was done on a 0.1 mmol scale with 0.52 mmol/g Fmoc-Rink amide MBHA resin and a 3-fold excess of the protected amino acids. The resin was allowed to swell in DCM (2.0 mL, 15 min) and then solvent was removed. The resin was then rinsed with DMF (1.0 mL, 1min), and after removal of the solvent, deprotection of the Fmoc group was performed by adding a solution of 20% piperidine/DMF (1.5 mL) to the resin and was allowed to vortex for 5 minutes. The resin was then washed three times with DMF (2.0 mL, vortex 30 sec) and then the resin was vortexed for 15 min with 20% piperidine/DMF (1.5 mL). The resin was then washed with DMF six times to remove any unreacted products (2.0 mL, vortex 30 sec). A Kaiser test was performed after the Fmoc removal to verify the presence of a free primary amino group. The desired Fmoc-protected amino acid (0.3 mmol) and the coupling reagent HCTU (0.3 mmol) was dissolved in DMF (1.5 mL) and added to the resin. The mixture was allowed to vortex for 30 seconds and then DIPEA (0.6 mmol) was added to the
mixture and was allowed to vortex for 1 hour. This deprotection/amino acid coupling cycle was repeated until the desired amino acid sequence was obtained. After the final amino acid was coupled, the resin was washed 3 times with DMF (2.0 mL, vortex 30 sec) and then 3 times with DCM (2.0 mL, vortex 30 sec) and then dried under vacuum and stored in the freezer. The removal of the final Fmoc protecting group on the N-terminus is achieved using the same procedure for the other deprotections as previously described. To determine if the correct peptide has been synthesized, a microcleave was performed before full cleavage off the resin. A small amount of resin (<5 mg) is added to a small peptide vessel and 95% trifluoroacetic acid/2.5% triisopropylsilane/2.5% water (500 μL) was added. The mixture was allowed to vortex for 3-4 hours, and the resulting liquid was precipitated with cold tert-butyl methyl ether (TBMe) (2 mL). The precipitate was centrifuged, the mother liquor was decanted off, the pellet was dissolved in water (2 mL), frozen and then lyophilized. If the correct peptide was synthesized as determined by analytical UHPLC/ESI-MS, then a full cleavage of all of the resin was performed. The procedure for full cleaves is the same as a microcleave, a solution of 95% trifluoroacetic acid/2.5% triisopropylsilane/2.5% water (3 mL) was added to the resin and vortexed for 4-6 hours. The solution is drained, precipitated in TBMe (20 mL) and centrifuged. The mother liquor is decanted, the pellet is dissolved in water (20 mL) and lyophilized to obtain the crude fully deprotected peptide.

4.4.3 Kaiser Test
To qualitatively test if there is presence of a free amine present (after a Fmoc-deprotection, amino acid coupling, or alloc deprotection), a Kaiser test is performed. A small amount of resin (<5 mg) is taken and placed in a small test tube. To the test tube, solutions of: Phenol:EtOH (8:2 v/v), 0.001M KCN: pyridine (2:98 v/v) and ninhydrin in EtOH (5% w/v) were added. The test tube was heated to 70°C and the presence of a free amine was indicated by the resin beads turning blue. The indication of no free amine was determined by the presence of clear/yellow resin beads.

4.4.4 Deprotection of Allyloxycarbonyl (Alloc)
Selective deprotection of the allyloxycarbonyl protecting group was accomplished by adding DCM (4.5 mL) to the resin-bound peptide and shaking gently for 10 minutes. After addition of phenylsilane (24 equiv.), the peptide vessel was flushed with nitrogen
and allowed to react for 5 minutes. Then, tetrakis(triphenylphosphine) palladium (0) (0.1 equiv.) was added to the mixture and the peptide vessel was again flushed with nitrogen, and the reaction was allowed to proceed for 10 minutes. Next, the peptide-resin was washed with DCM (4 x 30 s), followed by a series of washings with DCM, DMF, MeOH, DMF, DCM (30 s each).

4.4.5 Coupling of 2-Fluoropropionic Acid Group
Following removal of Alloc, 2-fluoropropionic acid (2-FPA) is coupled onto Lys adding 2-FP (0.3 mmol) and the coupling reagent HATU (0.3 mmol) dissolved in DMF (1.5 mL) and then added to the resin. This mixture was allowed to vortex for 2 hours, and the resin was then washed 3 times with DMF (2.0 mL, vortex 30 sec) and then 3 times with DCM (2.0 mL, vortex 30 sec) and then dried under vacuum.

4.4.6 Purification of RP-HPLC/ESI-MS
Peptides were analyzed using a reverse-phase analytical HPLC column (Agilent Zorbax SB-C8 column 4.6 x 150 mm, 3.5 μm). This system was equipped with a Waters 600 136 controller, Waters Prep degasser, and Waters Masslynx software (version 4.1). Employed mobile phases were 0.1% TFA in deionized water (eluent A) and 0.1% TFA in acetonitrile (eluent B). The flow rate was set at 1.5mLmin⁻¹ over 15 minutes. The column eluate was monitored using a Waters 2998 Photodiode array detector set at 220 nm, 254 nm and 400 nm. Peptides were purified using a reverse-phase preparative HPLC column (Agilent Zorbax SB-C18 column 21.2 x 150 mm, 5 μm) on the same system mentioned above. The detection method and eluents were the same was mentioned above for the analytical system and the flow rate was set at 20 mLmin⁻¹. The collected fractions were then lyophilized to a solid. For analytical UHPLC-MS, studies were performed on a Waters, Inc. Acquity UHPLC H-Class system, combined with a Xevo QTof mass spectrometer (ESI+, cone voltage = 30 V). A Waters Acquity UHPLC BEH C18 2.1 x 50 mm, 1.7 μm column was used with a gradient solvent system consisting of 0.1% formic acid in acetonitrile and 0.1% formic acid in water.

4.4.7 Receptor Ligand Binding Assay
Competitive ligand binding assays were performed in triplicate using HEK293/GHS-R1a cells and [¹²⁵I]Ghrelin as the competitive ligand, as previously
described\textsuperscript{21}. Receptor binding affinities were calculated as IC\textsubscript{50} values as previously described\textsuperscript{21}.

4.4.8 Molecular cloning and constructs
YFP tagged human GHS-R1a from Addgene (hGHS-R1a) was made by replacing PAR4 in the previously published PAR4-YFP (Ramachandran et al., 2017) construct\textsuperscript{25} with GHS-R1a using restriction enzymes digestion with EcoRI and Xhol and ligation. Constructs was verified by direct sequencing (University of Western Ontario, Robarts DNA sequencing facility).

4.4.9 \(\beta\)-Arrestin Recruitment Assay
Bioluminescent resonance energy transfer was measured between c-terminally YFP tagged GHS-R1a and Renilla Luciferase (Rluc) tagged \(\beta\)-arrestin 1 (\(\beta\)-Arr-1) or \(\beta\)-arrestin 2 (\(\beta\)-Arr-2), following 20 minutes of receptor activation in the presence of agonist compounds in HEK293 cells. HEK293 cells are maintained in Dulbecco’s Modified Eagle’s medium (DMEM) from Sigma-Aldrich (D5796) with added 5% sodium pyruvate, 5% penicillin streptomycin, and 10% fetal bovine serum (FBS) at 37\(^\circ\)C with 5% CO\textsubscript{2}. Cells were cultured in a T-75 flask to 95% confluency then lifted by trypsin-ETDA (0.25%). Once cells were counted and spun down at 1000 rpm they were resuspended and then re-plated in 10 cm culture dishes at 1 million cells per plate. Seeding was then accomplished for 24 hrs at 37\(^\circ\)C with 5% CO\textsubscript{2}. GHS-R1a-YFP (2 \(\mu\)g) and \(\beta\)-arrestin-1-rluc/ -2-luc (0.2 \(\mu\)g; a kind gift from Dr. Michel Bouvier) were transiently transfected for 24 hours. At 24 hours transfection, cells were re-plated into white 96-well culture plates (Brand Tech.) and cultured for an additional 24 hours. Interactions between GHS-R1a and \(\beta\)-arrestin-1/2 were detected by measuring BRET ratios (eYFP/rluc) at 20 min of agonist stimulation, following the addition of 5 \(\mu\)M coelenterazine (Nanolight Technology, Pinetop, AZ) 10 min prior to quantification on a Mithras fluorescence plate reader (Berthold) using the appropriate filters. All drugs being tested were dissolved in Hanks’ Balanced Salt Solution (HBSS) with 0.1% DMSO. The highest drug concentration of 10\textsuperscript{-5} M was first prepared, and each full log concentration was prepared by serial dilution thereafter. The control well contained just HBSS with 0.1% DMSO. The serum media was removed from the plate before then addition of the
drug at varying concentrations (10^{-5} M to 10^{-17} M). Data are shown as net BRET ratios (eYFP/rluc – baseline ratio in no agonist control). Concentration effect curves were calculated using 3 parameter non-linear regression curve fitting, as this was most appropriate based on the R squared value (Prism 7).

4.4.10 Molecular docking studies
The homology model of GHS-R1a previously developed by our lab was used to study the interactions between compound 7 and GHS-R1a. The structure of compound 7 was generated from modification of compound 1, thus both compound 1 and 7 have identical conformation. To investigate the effort of Tyr replacement in the fourth position on the binding pattern, the residues of GHS-R1a (Phe289, Arg283, Phe279) surrounding the Tyr in the fourth position were set to be flexible while the conformation of the compound 7 remains unchanged during the docking calculation. Docking studies were performed using the AUTODOCK 4.2 program. The dimensions of the active site box were chosen to be large enough to encompass the entire orthosteric site in the receptor. Docking calculations were carried out using the Local Search Algorithm. The docking experiment was consisted of 200 independent runs. Default values were used for other parameters. All the calculations were performed on high-performance computing (HPC) facilities within the Compute Canada network. The graphics were generated using DS Visualizer (BIOVIA).

4.5 References


Peptides. *Biochemical and Biophysical Research Communications*, 287(1), 142–146.


27. SHARCNET (www.sharcnet.ca) is a consortium of colleges, universities and research institutes operating a network of high-performance computer clusters across south western, central and northern Ontario.
Chapter 4 Supplementary Information

Half-Maximal Inhibitory Concentration (IC$_{50}$) Curves

*Compound 1 previously published in reference 21*

**Compound 2**

![Graph showing concentration vs. % radioligand bound for Compound 2 with IC$_{50}$ of 19 nM.]

**Compound 3**

![Graph showing concentration vs. % radioligand bound for Compound 3 with IC$_{50}$ of 62.9 nM.]

Compound 4

Concentration (M)

% Radioligand Bound

4.35 nM

Compound 5

Concentration (M)

% Radioligand Bound

0.283 nM
Compound 6

![Graph showing the binding affinity of Compound 6]

- % Radioligand Bound
- Concentration (M)
- 60.3 nM

Compound 7

![Graph showing the binding affinity of Compound 7]

- % Radioligand Bound
- Concentration (M)
- 0.70 nM
## UHPLC-MS Traces

### Supplementary Table 4.1 – Analytical Data for Compounds 1 to 7

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### Compound 1

![UHPLC-MS Trace for Compound 1](image)
Compound 2

Compound 3
Compound 4

Compound 5
Compound 6

Compound 7
Chapter 5
Trifluoroborate Containing Peptidomimetic GHS-R1a Agonist for PET Imaging of Prostate Cancer

5.1 Introduction
Growth hormone secretagogues (GHSs) are a class of peptides and small molecules that stimulate the secretion of growth hormone (GH) by acting on the growth hormone secretagogue receptor type-1a (GHS-R1a). This receptor is predominantly expressed in the hypothalamus and pituitary gland. When GHS-R1a is activated it causes the release of GH by a route that is different the normal route, the growth hormone-releasing hormone (GHRH). Bowers and co-workers were the first to develop a series of GHSs based on the natural ligands Met-enkephalin and Leu-enkephalin and performed in vitro analysis to prove that these analogues could still cause GH release. Most of these ligands had the initial purpose to treat patients who have GH abnormalities. Examples of some of these compounds include the growth hormone-releasing peptide-6 (GHRP-6), GHRP-1, hexarelin, GHRP-2, peptidomimetics (e.g. G7039, [1-Nal]G7039 and ipamorelin) and small-molecules (e.g. L-692,429 and MK-0677). These various GHSs can be visualized in Figure 5.1. Human ghrelin is the natural ligand for GHS-R1a first identified by Kojima et al. in 1999. Ghrelin is a 28-amino acid peptide with an octanoylation on Ser-3. This octanoylation is performed by ghrelin O-acyl transferase (GOAT) to produce the active form of the ligand. The activation of GHS-R1a has a multitude of biological activities, such as the regulation of food intake, glucose metabolism, cardiovascular and neuroprotective effects.
Figure 5.1 - Peptidic, peptidomimetic and small-molecule growth hormone secretagogues that have been designed to target GHS-R1a leading to its activation. All amino acids are designated by the standard three-letter code (D2Nal, D2-naphthylalanine; Inp, isonipecotic acid; D-Trp(2-Me); D-2-methyltryptophan)

The GHS-R1a is over expressed and dysregulated in a number of human malignancies, including prostate cancer\textsuperscript{18-19}, breast cancer\textsuperscript{21}, testicular cancer\textsuperscript{22}, ovarian and lung cancer\textsuperscript{23}. The expression of this receptor continues to appear in several other cancers as more examples become apparent in the literature and cBioPortal. It also has been shown to be upregulated in a number of other human diseases such as protective effects in atherosclerosis and chronic heart failure\textsuperscript{24-25}. This makes GHS-R1a a highly favourable target in terms of designing novel imaging agents in order to perform
diagnosis and monitor the variety of human diseases in which it is overexpressed. The imaging modality within this work that was chosen is positron emission tomography (PET). This is because PET combines a relatively high spatial resolution (5 to 7 mm) with high sensitivity (in the femtomolar range) and is among the widely used imaging modalities in the clinic today\textsuperscript{26}. A number of recent publications describe PET radiotracers to monitor GHS-R1a in human disease\textsuperscript{27-30}. Hou et al. synthesized radiolabelled quinazolinone derivatives as a small molecule capable of targeting GHS-R1a\textsuperscript{30}. Also, a [\textsuperscript{11}C]radiotracer revealed a higher specific uptake in the pancreas compared to other organs in a mouse model were the tumour cells over expressed GHS-R1a\textsuperscript{28}. However, these studies\textsuperscript{27-29} and others\textsuperscript{30-31} have primarily focused on either small-molecule quinazolinones\textsuperscript{27-31} or ghrelin-derived compounds\textsuperscript{29,30,31}. Peptidic and peptidomimetic GHSs would result in a clinically translatable PET agent because of their high target specificity, \textit{in vivo} stability and favourable pharmacokinetic properties.

Fluorine-18 (\textsuperscript{18}F) was our preferred choice of radioisotope as it is a small innocuous unit that can be easily installed in the absence of a bulky chelator that potentially can reduce the affinity towards a given target. Also, \textsuperscript{18}F can be easily installed to the desired ligand in a direct labelling manor.

Perrin and co-workers synthesized a novel prosthetic group for \textsuperscript{18}F chemistry that is able to directly label a peptide in one step with no HPLC purification, based upon the precursor alkyne containing ammonium methyltrifluoroborate (AMBF\textsubscript{3} – seen in blue in Figure 5.2). This prosthetic group can easily be installed onto a lysine azide of a desired peptide by copper catalyzed click chemistry in high purity and yields. Once on the peptide, direct labelling is accomplished in buffered conditions (aqueous) with high radiochemical purity (>98\%), highly specific molar activity (40 – 111 GBq/\textmu mol), and high radiochemical yields (20 to 35\%). This is data that has been collected over 20 trials\textsuperscript{32}. This particular prosthetic group has been proven to be a beneficial and efficient way to label biologically relevant peptides in the past such as a bombesin derivative for prostate cancer imaging and a Des-Ar(10)\textsuperscript{33}Kallidin derivative for imaging Bradykinin B\textsubscript{1} receptor expression. In the cases of imaging gastrin-releasing peptide receptor (GRPR)-expressing pancreatic and PC-3 prostate cancer tumours in xenograft models with the radiofluorinated bombesin derivative there was excellent tumour visualization and low
background uptake in other organs. This exact same result was seen when visualizing the Bradykinin B1 receptor expression in a xenograft model\textsuperscript{33-34}.

The peptidomimetic G7039 (Figure 5.1) has undergone substantial structure-activity relationship (SAR) studies in order to both improve binding towards the receptor, as well as enhance the hydrophilicity\textsuperscript{35}. Fowkes and co-workers made several changes with respect to the prosthetic group as well as changes to the fourth position of the peptide. The first three amino acids (piperidine-4-carboxylic acid (Inp), D-2-naphthylalanine (D2Nal), and D-1-naphthylalanine (D1Nal)) are highly vulnerable to change and have been shown necessary to bind and activate GHS-R1a\textsuperscript{35}. A follow up study by Lalonde and co-workers (Chapter 4) determined that Tyr in the fourth position caused an 18-fold increase in binding towards GHS-R1a compared to the parent G7039 molecule (5.2 nM versus 0.70 nM measure by a competitive radioligand assay (half maximal inhibitory concentration-IC\textsubscript{50})). This increase in binding is believed to be due to an additional H-bond interaction with Arg 283 in the binding pocket of the GHS-R1a homology model (determined by docking analysis). The addition of a 2-fluoropropionic acid (2-FPA) group to the C-terminal lysine of ([Tyr\textsuperscript{4}, Lys\textsuperscript{5}(2-fluoropropionyl-2-FP)]G7039) caused a slight improvement in binding to 0.28 nM and provided a potential site to radiolabel the peptide with \textsuperscript{18}F in order to have the respective PET imaging probe. However, the 2-FP group proved to be a lengthy four-step labelling with very low yields. This is because the exchange of a leaving group on the two position of 2-FP for \textsuperscript{18}F caused an elimination product over the desired substitution product.

By conjugating AMBF\textsubscript{3} to a lysine azide group of ([Tyr\textsuperscript{4}, Lys\textsuperscript{5}(N\textsubscript{3})]G7039) the peptidomimetic ([Tyr\textsuperscript{4}, Lys\textsuperscript{5}(AMBF\textsubscript{3})]G7039) was synthesized. This peptide still maintained binding towards the receptor but can now be labelled in a single step by isotopic exchange (IEX) of \textsuperscript{19}F for \textsuperscript{18}F. This work provides a method to prepare a sub-nanomolar PET agent for the GHS-R1a with rapid and high yielding radiochemistry in order to image the receptor by PET.

5.2 Results and Discussion

The azide peptide ([Tyr\textsuperscript{4}, Lys\textsuperscript{5}(N\textsubscript{3})]G7039) was synthesized using fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide chemistry, purified using reverse-phase high performance liquid chromatography (RP-HPLC) and analyzed by
electrospray ionization-mass spectrometry (ESI-MS). In a single step, AMBF$_3$ was conjugated onto the lysine azide utilizing copper click chemistry in an 89% chemical yield to furnish ([Tyr$^4$, Lys$^5$(AMBF$_3$)]G7039) (Scheme 5.1), followed by purification by RP-HPLC and analysis by ESI-MS.

![Chemical synthesis diagram](image)

**Scheme 5.1 - Chemical synthesis ([Tyr$^4$, Lys$^5$(AMBF$_3$)]G7039) using copper click chemistry. The Tyr in position four is highlighted in pink and is a significant contributor to improved GHS-R1a affinity. AMBF$_3$ is highlighted in blue and is the prosthetic group required for IEX.**

Previously the peptide ([1-Nal$^4$, Lys$^5$(4-fluorobenzoyl - 4-FB)]G7039) determined to have an IC$_{50}$ of 69 nM and a cLogP of 8.76 ± 0.88$^{35}$. The lower binding affinity and high cLogP of the peptide led to poorer in vivo PET images in a cancer mouse model$^{35}$. In Chapter 4 Lalonde and co-workers improved on both the binding affinity (IC$_{50}$ of 0.28 nM) and cLogP (2.77 ± 0.87) by altering the Tyr in the fourth position and the 4-FB prosthetic group to 2-FP ([Tyr$^4$, Lys$^5$(2-FP)]G7039). This prosthetic group proved to be low yielding during radiosynthesis with $^{18}$F. Finally, in this current work the prosthetic group was changed one additional time to AMBF$_3$. The addition of the AMBF$_3$ caused no drastic change in binding affinity with a determined IC$_{50}$ value of 0.85 nM (Figure 5.2). This was done by a competitive radioligand assay with $^{125}$I-human ghrelin as the competitor. It can also be noted that the cLogP of this compound was calculated to be 2.54 ± 0.85, which in terms of hydrophobicity is in an acceptable range to have good
pharmacokinetics. This lead compound has improved on both the binding and cLogP from the first generation\textsuperscript{35}.

Figure 5.2 - IC\textsubscript{50} curve for ([Tyr\textsuperscript{4}, Lys\textsuperscript{5}(AMBF\textsubscript{3})]G7039) as determined by competitive radioligand assay

([Tyr\textsuperscript{4}, Lys\textsuperscript{5}(AMBF\textsubscript{3})]G7039 was then radiolabelled with \textsuperscript{18}F by isotopic exchange (IEX) with \textsuperscript{19}F. This was done in a pyridazine-HCL buffer followed by purification with a C-18 Sep-Pak. Over the course of four trials a decay corrected radiochemical yield was between 17 and 35\%, a radiochemical purity >99\%, and a molar activity of 12 to 15 GBq/\(\mu\)mol. The retention times of the cold standard and “hot” peptide are almost identical ([Tyr\textsuperscript{4}, Lys\textsuperscript{5}(AMB\textsuperscript{19}F\textsubscript{3})], \(t\text{R} = 5.50\) mins compared to \(t\text{R} = 5.48\) mins for the “hot” peptide ([Tyr\textsuperscript{4}, Lys\textsuperscript{5}(AMB\textsuperscript{18}F\textsubscript{3})]), indicating that the precursor ([Tyr\textsuperscript{4}, Lys\textsuperscript{5}(AMB\textsuperscript{19}F\textsubscript{3})]) has been selectively radiolabelled by IEX (Figure 5.3). In terms of the radiolabelling performed with ([1-Nal\textsuperscript{4}, Lys\textsuperscript{5}(4-FB)]G7039) this is a drastic improvement in the number of steps to the final product (four versus one step). Radiosynthetic data is similar in terms of purity, yield, and molar activity\textsuperscript{35}. 
Figure 5.3 - (A) Synthesis scheme for $^{18}$F labelling; (B) Overlaid HPLC chromatograms for ([Tyr$^4$, Lys$^5$(AMB$^{18}$F$_3$)] and ([Tyr$^4$, Lys$^5$(AMB$^{19}$F$_3$)] (both $\lambda = 254$ nm); (C) Radiochemical yields, purity and molar activity

To demonstrate that ([Tyr$^4$, Lys$^5$(AMB$^{18}$F$_3$)] was still able to bind to GHS-R1a after being radiolabelled, a radioligand cellular assay was performed with LNCaP cells that stably express GHS-R1a. As a control, blocking the radioligand signal coming from Tyr$^4$, Lys$^5$(AMB$^{18}$F$_3$) was performed with a 10 mM concentration of Tyr$^4$, Lys$^5$(AMB$^{19}$F$_3$). There was on average 10% cellular uptake of the PET agent, which was significantly different than the blocking study (< 1% uptake). This demonstrates that the imaging agent is targeting the receptor specifically when using the ([Tyr$^4$, Lys$^5$(AMB$^{18}$F$_3$)] PET probe (Figure 5.4).
5.3 Conclusion

This work has highlighted the development of a sub-nanomolar PET probe with excellent radiochemical data that specifically targets GHS-R1a. The ([Tyr$^4$, Lys$^5$(AMB$^{18}$F$_3$)] analogue of G7039 in terms of the changes made to the fourth position (Phe to Tyr) and the addition of the AMBF$_3$ improved both binding affinity (0.85 nM) and cLogP ($2.54 \pm 0.85$). The new prosthetic group allowed for a single step radiosynthesis route with no HPLC purification. Cellular uptake was demonstrated (10 to 12%), compared to almost none when blocked with ([Tyr$^4$, Lys$^5$(AMB$^{19}$F$_3$)]). Over the course of four trials a decay corrected radiochemical yield was between 17 and 35%, a radiochemical purity $>99\%$, and a radiochemical molar activity of 12 to 15 GBq/μmol. This demonstrates consistency in radiolabelling with $^{18}$F and will facilitate the use of this PET agent for in vivo imaging experiments. This tracer has the advantage of being labelled in only one step with no HPLC purification required (32 min to end of synthesis) making it very attractive to be driven towards a clinical setting.
5.4 Materials and Methods

5.4.1 General Information

All reagents were obtained from commercial suppliers and used without further purification. Peptides were either synthesised manually or through the use of a Biotage SyroWave automated peptide synthesizer. Peptide vessels were shaken using an IKA VibraX VXR basic shaker with centrifugation performed on a Beckman Coulter Allegra X-30R or Fisher GS-6R centrifuge. In order to aid peptide dissolution, sonication of solutions was accomplished via a Bransonic 2510R-MTH or Fisher F5-14 ultrasonic cleaner. A Fisher 2052 Isotemp machine was used to heat test tubes in the Kaiser Test. Peptides were lyophilised using a Labconco FreeZone Freeze Dry System. Accurate weighing was carried out on a Mettler-Toledo XP6 microbalance. UV traces were obtained with a Waters 2487 UV/Vis Dual λ Absorbance Detector (170-900 nm) and low-resolution mass spectra with a Micromass Quattro micro API mass spectrometer (ESI-LC-MS). Peptide purification was achieved through HPLC (MeCN + 0.1% TFA, H₂O + 0.1% TFA solvent system). All peptides obtained had a purity ≥ 95% as determined by HPLC or UHPLC analysis. A RP preparative C-18 column (SunFire OBD, 19 x 150 mm or Agilent Zorbax 21.2 x 150 mm) was used for preparative HPLC, whilst a C-18 RP column (SunFire, 4.6 x 150 mm or Agilent Zorbax, 4.6 x 150 mm) was used for analytical HPLC.

5.4.2 Solid-Phase Peptide Synthesis

Fmoc-based solid-phase synthesis was carried out manually and automatically using a Biotage® Syro Wave™ automated peptide synthesizer. Synthesis was done on a 0.1 mmol scale with 0.52 mmol/g Fmoc-Rink amide MBHA resin and a 3-fold excess of the protected amino acids. The resin was allowed to swell in DCM (2.0 mL, 15 min) and then solvent was removed. The resin was then rinsed with DMF (1.0 mL, 1 min); after removal of the solvent, Fmoc deprotection was performed by adding a solution of 20% piperidine/DMF (1.5 mL) to the resin and vortexing for 5 minutes. The resin was then washed with three times with DMF (2.0 mL, vortex 30 sec) and the resin was vortexed for 15 min with 20% piperidine/DMF (1.5 mL). The resin was then washed with DMF six times to remove any unreacted products (2.0 mL, vortex 30 sec). A Kaiser test was performed after the Fmoc removal to verify the presence of a free primary amino group.
The desired Fmoc-protected amino acid (0.3 mmol) and the coupling reagent HCTU (0.3 mmol) was dissolved in DMF (1.5 mL) and added to the resin. The mixture was vortexed for 30 seconds and then DIPEA (0.6 mmol) was added to the mixture and was vortexed for 1 hour. This deprotection/amino acid coupling cycle was repeated until the desired amino acid sequence was obtained. After the final amino acid was coupled, the resin was washed 3 times with DMF (2.0 mL, vortex 30 sec) and then 3 times with DCM (2.0 mL, vortex 30 sec), dried under vacuum, and stored in the freezer at -20°C. The removal of the final Fmoc protecting group on the N-terminus was achieved using the previously described procedure.

To determine if the correct peptide has been synthesized, a microcleave was performed before full cleavage from the resin. A small amount of resin (<5 mg) was added to a small peptide vessel and a solution of 95% trifluoroacetic acid/2.5% triisopropylsilane/2.5% water (500 μL) was added. The mixture was vortexed for 3-4 hours, and the resulting liquid was precipitated with cold tert-butyl methyl ether (TBME) (2 mL). The precipitate was centrifuged (3000 xg for 10 min), the mother liquor was decanted. The pellet was dissolved in water (2 mL), frozen, and lyophilized. If the correct peptide was synthesized as determined by analytical HPLC/MS, then a full cleave was performed. The procedure for full cleaves identical to the microcleave procedure: a solution of 95% trifluoroacetic acid/2.5% triisopropylsilane/2.5% water (3 mL) was added to the resin and vortexed for 4-6 hours. The solution was drained, precipitated in TBMe (20 mL) and centrifuged (3000 xg for 10 min). The mother liquor was decanted, and the pellet was dissolved in water (20 mL) and lyophilized to obtain the crude fully deprotected peptide.

5.4.3 Synthesis of ([Tyr4, Lys5(AMB19F3)] AMBF₃ was conjugated to the C-terminal lysine of the peptide by copper click chemistry as previously described³².

5.4.4 General Radiochemistry Information

All reagents and solvents used for radiosynthesis were purchased from Sigma-Aldrich unless otherwise specified. ¹⁸F-fluoride was produced via the ¹⁸O(p,n) ¹⁸F reaction in a GE PETtrace 880 cyclotron (Lawson Health Research Institute, London, Ontario, Canada). A Biotage V-10 evaporator was used to remove solvent. A Waters
HPLC with a 1525 binary pump system (solvent A = MeCN + 0.1% TFA, solvent B = H2O + 0.1% TFA) and two detectors (Waters 2487 Dual λ absorbance detector set at 254 nm and a radioactive flow count detector) were utilised for product analysis and purification. An analytical column (Agilent Eclipse XDB-C18, 4.6 × 150 mm, 5 μm) was used for determining radiochemical purity and molar activity of ([Tyr4, Lys5(AMB18F3)].

5.4.5 Radiosynthesis of ([Tyr4, Lys5(AMB18F3)]

The micro QMA (a gift from Dr. Neil Vasdev) cartridge and reaction vessel setup was the same as previously described. However, the cartridge was preconditioned with 6 mL of 1 M HCl, 6 mL of brine, and finally 6 mL of milli-Q water. Once the fluoride-18 was trapped on the micro QMA it was eluted into the plastic vessel (a 15 mL falcon tube cut at the 4 mL mark capped with a rubber septum) with 20 uL of pyridazine-HCl buffer (pH=2) and then azeotropically dried under nitrogen for 10 min at 110°C with an ascarite filled syringe as the vent needle. The peptide ([Tyr4, Lys5(AMB19F3)]) was then added directly to the vessel at a 10 mM concentration in 10 uL of pyridazine-HCl buffer (pH=2). The vent ascarite filled syringe (a 5 mL syringe fitted with a 20-gauge needle with the plunger removed and packed with glass wool then filled to the 4 mL with ascarite) was removed and the reaction proceeded for 8 min at 110°C. After the reaction was complete it was quenched with 1 mL of 5% (v/v) NH₄OH and directly loaded onto a C-18 light Sep-Pak cartridge precondition with 10 mL of EtOH and 10 mL of water. The cartridge was washed with 4 mL of water (2X), followed by elution of the final product with 1 mL of 1:1 ethanol: water solution. A 20:80 water:acetonitrile HPLC gradient was used to determine radiochemical purity and molar activity. Over the course of four trials the decay corrected radiochemical yield was between 17 and 35%, a radiochemical purity >99% for each trial, and a radiochemical molar activity of 12 to 15 GBq/μmol. The retention times of the cold standard and “hot” peptide are almost identical ([Tyr4, Lys5(AMB19F3)], tR = 5.50 mins compared to tR = 5.48 mins for the “hot” peptide ([Tyr4, Lys5(AMB18F3)]), indicating that the precursor ([Tyr4, Lys5(AMB19F3)]) has been selectively radiolabelled by IEX.

5.4.6 Purification of RP-HPLC/ESI-MS

([Tyr4, Lys5(AMB19F3)] and ([Tyr4, Lys5(N3)]) was analyzed using a reverse-phase analytical HPLC column (Agilent Zorbax SB-C8 column 4.6 x 150 mm, 3.5 μm). This
system was equipped with a Waters 600 136 controller, Waters Prep degasser, and Waters Masslynx software (version 4.1). Employed mobile phases were 0.1% TFA in deionized water (eluent A) and 0.1% TFA in acetonitrile (eluent B). The flow rate was set at 1.5 mL/min over 15 minutes. The column eluate was monitored using a Waters 2998 Photodiode array detector set at 220 nm, 254 nm and 400 nm. Peptides were purified using a reverse-phase preparative HPLC column (Agilent Zorbax SB-C18 column 21.2 x 150 mm, 5 μm) on the same system mentioned above. The detection method and eluents were the same as mentioned above for the analytical system and the flow rate was set at 20 mL/min. The collected fractions were then lyophilized to a solid and analyzed by ESI-MS. Purity of final products was determined by analytical RP-HPLC (200-800 nm).

5.4.7 Receptor Ligand Binding Assay

Competitive ligand binding assays were performed in triplicate using HEK293/GHS-R1a cells and [125I]Ghrelin as the competitive ligand, as previously described. Receptor binding affinities were calculated as IC50 values as previously described.

5.4.8 Molecular cloning and constructs

YFP tagged human GHS-R1a from Addgene (hGHS-R1a) was made by replacing PAR4 in the previously published PAR4-YFP (Ramachandran et al., 2017) construct with GHS-R1a using restriction enzymes digestion with EcoRI and Xhol and ligation. Constructs was verified by direct sequencing (University of Western Ontario, Robarts DNA sequencing facility).

5.4.9 Stable Transfection of LNCaP with GHS-R1a

The LNCaP (metastatic prostate cancer derived from left supraclavicular lymph node) cell line (ATCC) was maintained in RPMI medium (Wisent) supplemented with 10% fetal bovine serum (FBS; Wisent). Cells were plated at a density of 1 million cells in a 10 cm dish for 24 hrs. After 24 hrs 6 μg pcDNA-GHS-R1a-eYFP was added along with Fugene-6 (Promega) in a 1:3 ratio as per the protocol provided by Promega. After 48 hours cells were rinsed with PBS (3x) and replaced with media containing 300 μg/mL of G418 (Wisent). Cells were then kept under selective pressure for 7 to 10 days. Cells were then sorted (by FLOW cytometry identify the YFP tagged GHS-R1a) for single cell populations at the London Regional Flow Cytometry Facility (LRFCF) by manager Dr.
Kristin Chadwick. A single cell population was collected and re-sorted by the same method mentioned above. This single cell clonal population was confirmed by confocal microscopy identifying the tagged GHS-R1a in yellow. Confocal microscopy confirmed 90-95% of cells were expressing GHS-R1a with high cellular membrane expression (Figure 5.5.1).

5.4.10 Cellular Uptake

LNCaP cells stably transfected with GHS-R1a were plated at 150,000 cells per well in a 6 well plate for 48 hours. Post 48 hours the cells were first rinsed (3X) and then mechanically lifted in 1x HBSS and treated with 0.1 MBq of ([Tyr⁴, Lys⁵(AMB¹⁸F₃)]) per well for 20 min at 37°C and 5% CO₂. After the incubation the cells were transferred to 1.5 mL Eppendorf tubes and spun down at 13,000 G for 5 min. The supernatant was removed from cell pellet and collected in an additional 1.5 mL Eppendorf tubes. The cell pellet was washed with 1x HBSS and spun again at 13 000 Gs for 5 min. The supernatants were combined. The rinse fluid and cell pellet were counted on a gamma counter to measure a percent uptake value (counts in rinse versus counts in pellets). The same procedure was performed for blocking with 10 x concentration of ([Tyr⁴, Lys⁵(AMB¹⁰F₃)]). This whole experiment was performed in triplicate.

5.5 References


Chapter 5 Supplementary Information

HPLC-MS Traces

[Tyr^4, Lys^5(N_3)] (Found - [M+H]^+ - 841.18, Calculated - [M+H]^+ - 841.17)
[Tyr$^4$, Lys$^5$(AMB$^{19}$F$_3$)] (Found - [M+H]$^+$ - 1006.30, Calculated - [M+H]$^+$ - 1006.31)
Supplementary Figure 5.1 - Confocal microscopy images of stable transfect LNCaP with GHS-R1a-eYFP indicating the receptor is highly expressed and localized to the cell membrane
Chapter 6

Targeting the GHS-R1a Homo-Oligomer: Bivalent Ligands with Exceptional Binding Affinity and Potency

6.1 Introduction

The growth hormone secretagogue receptor type 1a (GHS-R1a) is a G-protein coupled receptor (GPCR) that belongs to the rhodopsin-like family of GPCRs (Class A)\(^1\). It is primarily expressed in the pituitary gland and hypothalamus but has also shown expression in a wide range of other tissues such as the heart, lungs, and brain\(^2\). The natural ligand for this receptor, ghrelin, is a 28-amino acid peptide with an octanoylation on Ser-3, and this hydrocarbon tail is needed in order to bind the GHS-R1a causing an activation. Activation of this receptor has been identified in controlling a large number of human physiological functions including glucose metabolism, insulin release, gut motility, and many more\(^2\). However, its activation has been shown to be problematic in human disease such as causing an increase of cellular proliferation in prostate cancer through the p44/42 mitogen-activated protein kinase (MAPK) pathway\(^3\)–\(^6\). The overexpression of GHS-R1a increases the motile phenotype in astrocytomas, in which increased GHS-R1a expression is correlated with a higher tumour grade\(^7\). Overexpression of GHS-R1a has more recently been demonstrated in some ovarian epithelial carcinomas, a disease with poor prognosis due to the late stages in which it is usually diagnosed\(^8\)–\(^12\). Interestingly, activating this receptor with potent agonists (i.e. anamorelin) has also been identified to be beneficial in human disease, as is the case of cancer cachexia\(^13\). This makes GHS-R1a an attractive target in the development of ligands that can control its dysregulation within human diseases.

GPCRs constitute the largest group of membrane receptors in eukaryotes and play a role in an incredible number of diversified functions in the human body. Significant progress has been made in understanding GPCR structures and modes of function. Recent years have seen an explosion in the number of studies showing that GPCRs not only exist as isolated entities but also may interact with themselves and/or other membrane protein to form homo-oligomers and hetero-oligomers respectively\(^14\)–\(^16\). Most evidence indicates that oligomers may be a predominant species, with the potential for formation of higher-order oligomers. GPCR oligomerization represents functional importance such as
enabling cross-talk, inducing activation of alternative signalling pathways, influencing pharmacology, influencing receptor internalization and is critical for receptor trafficking and function\textsuperscript{14,15,17,18}. Therefore, GPCR oligomers offer attractive potential therapeutic targets for drug development.

GHS-R exists in two distinct isoforms: GHS-R1a and GHS-R1b. GHS-R1a as mentioned previously is activated by ghrelin, to induce growth hormone release,\textsuperscript{17} however GHS-R1b is functionally inactive and does not bind ghrelin\textsuperscript{24-25}. GHS-R1a has potential therapeutic application towards a range of diseases such as obesity, diabetes, and cancer\textsuperscript{19-23}. Along with numerous other GPCRs, GHS-R1a has been proven to form a homo-oligomer. The first example of GHS-R1a homo-oligomerization was described in 2005 by Holst \textit{et al.} Holst and co-workers used transfected COS-7 cells with GHS-R1a and in the presence of the endogenous agonist ghrelin, along with the co-administration of other agonists (small molecules L 692,429 and MK-0677, as well as the peptide growth hormone releasing peptide-6 (GHRP-6)) there was a neutral, positive, or negative modulation in signalling\textsuperscript{26}. Jiang and co-workers confirmed this oligomerization in a human embryonic kidney (HEK293) derived cell line co-transfected with GHS-R1a-green fluorescent protein (GFP) and GHS-R1a-\textit{Renilla} luciferase (rluc)\textsuperscript{26}. Upon stimulation with human ghrelin a fluorescence resonance energy transfer (FRET) signal was observed. A time-resolved (Tr) FRET measurement was also observed by Kern \textit{et al.} using a CLIP-GHS-R1a and SNAP-GHS-R1a HEK293 derived cell line\textsuperscript{28}. The GHS-R1a homo-oligomerization was also observed by Leung \textit{et al.} using bioluminescence resonance energy transfer (BRET) and co-immunoprecipitation\textsuperscript{28}.

GHS-R1a has the ability to not only oligomerize with itself but also with other GPCRs, including GHS-R1b receptor,\textsuperscript{27} prostaglandin E2 receptor (EP\textsubscript{3}-1),\textsuperscript{29} prostacyclin receptor (IP),\textsuperscript{29} thromboxane A2 receptor (TP\textsubscript{a}),\textsuperscript{29} Somatostatin receptor 5(SST5),\textsuperscript{30} dopamine receptors (D\textsubscript{1} and D\textsubscript{2}),\textsuperscript{27,31} melanocortin 3 receptor (MC\textsubscript{3}),\textsuperscript{32} and serotonin 2C receptor (5-HT\textsubscript{2C})\textsuperscript{33}. Kern \textit{et al.} was one of the first to describe the oligomerization between the D1- and D2-like dopamine receptors and GHS-R1a. These receptors are coexpressed in a number of brain regions linked to food intake and reward behaviours. They demonstrated when there was a lack of ghrelin circulation in the brain the oligomerization between D1- and D2-like dopamine receptors and GHS-R1a leads to
anorexigenic effects. Rediger et al. demonstrated an additional example for the oligomerization between GHS-R1a and MC3R. GHS-R1a and MC3R are coexpressed in the arcuate nuclease of neurons as observed by FRET. The stimulation of MC3R leads to the accumulation of cAMP by means of Goαs. cAMP increases by 2-fold when MC3R is stimulated and coupled with GHS-R1a, however GHS-R1a constitutive activity along with ghrelin induced activity decreases intracellular calcium by 40%. However, when the MC3R receptor is antagonized, there is an increase in GHS-R1a signalling underlying an increase in fat mass within a MC3R−/− knockout mouse model. These are examples in which GHS-R1a hetero-oligomerization change the downstream signalling events leading to different physiological outcomes.

The existence of multiple GHS-R1a oligomerization states significantly increases the pharmacological diversity of the GHS-R1a receptor, which is expected to result in enhanced specificity of GHS-R1a-targeted drugs. On the other hand, GHS-R1a has a broad spectrum of functions and is associated with a wide range of diseases. Therefore, targeting the GHS-R1a monomer may suffer from low specificity as it will not only affect the biological function of interest, but will also affect the other systems where the receptor is involved. The ability to target the specific GHS-R1a homo- or hetero-oligomer is more desirable as it is likely to lead to a more selective pharmacotherapy.

Jiang et al. showed that the co-administration of human ghrelin and dopamine caused a 4-fold increase in cAMP signalling enhancing positive physiological outcomes associated with mood, learning and memory. This was only seen in the presence D1R and GHS-R1a oligomerization. More recently Schellekens et al. demonstrated the positive effects of the oligomerization between the 5-hydroxytryptamine receptor 2C (5-HT2C – serotonin receptor). Oligomerization between GHS-R1a and 5-HT2C blocks ghrelin’s orexigenic effects as well as attenuation of ghrelin-induced food intake after the administration of human ghrelin and/or Lorcaserin (treatment for obesity). Both of these studies indicate how a combined treatment plan could be necessary to combat human diseases such as obesity, mood, memory, and learning.

A powerful approach to pharmacologically targeting GPCR oligomers is the use of bivalent ligands consisting of two pharmacophores linked by a spacer with appropriate length. Bivalent ligands are capable of binding to both receptors in the oligomer
simultaneously, resulting in dramatically enhanced binding affinity due to the total binding energy of two pharmacophores. However negative cooperativity can lead to reduction in binding and efficacy when targeting GPCR oligomers. A number of bivalent ligands have been recently developed targeting other GPCR homo- and hetero-oligomers, but none has been developed for GHS-R1a. In the present study, we reported the design, synthesis, binding affinity and signalling responses from bivalent ligands targeting GHS-R1a. Through a rational structure-based design approach, we discovered a bivalent ligand based on peptidomimetic G7039 that possesses exceptionally high binding affinity and is able to bind to oligomeric GHS-R1a in HEK293 cells. Compound 6 not only displayed exceptional binding, but also caused an increase in β-arrestin recruitment thus changing the signalling of p44/42 MAPK pathway. However, signalling mechanism changes were evident when studies were conducted using G protein (Gq, Gq/11, Gi/o, and G12/13) signaling couple gene reporter assays. The molecular basis for cellular distribution of, and more importantly, the functional relevance in disease of the GHS-R1a homo-oligomer formation have been elusive. The bivalent ligand developed herein presents a powerful tool to enlighten how the homo-oligomer functions and has the potential to advance a promising novel therapeutic approach for homo-oligomer related pathological condition through fine-tuning GHS-R1a receptor-mediated activity.

6.2 Results and Discussion

6.2.1 Rationalizing the Design of our Bivalent Ligand

Bivalent ligands intended to simultaneously bind to both receptors in the oligomer consist of two recognition elements linked by a spacer with optimal length. A number of studies have described that bivalent can be rationally designed if some important aspects such as suitable pharmacophore, spacer length, and attachment point are considered. The peptidomimetic G7039, discovered by Genentech Inc from extensive medicinal chemistry studies on GHRP-6 and G-7502, was used as the pharmacophore due to its high binding affinity (IC50 = 5.2 nM as determined in our lab) and being readily synthesized via solid phase synthesis (Figure 6.1A).

Theoretically, bivalent ligands successfully bridging two binding sites within the homo-oligomer should exhibit extremely high binding affinity as the result of the total binding free energy of two pharmacophores. Indeed, a number of bivalent ligands with
improved binding affinities as compared to their parent compounds have been reported\textsuperscript{43}. Notably, the bivalent ligands mentioned in the literature are small molecule-based pharmacophores with spacer lengths ranging from about 6 to 20 atoms. It has been proposed that bivalent ligands with short spacer lengths are not able to extend sufficiently out of the transmembrane domain to enable the binding to its neighboring receptor\textsuperscript{44}. It is more likely that these ligands bind to different sites (i.e., orthosteric site and allosteric site) on a single receptor as suggested by molecular modelling (therefore have been coined the name bitopic ligands)\textsuperscript{45}. Moreover, Vagner et al. proposed that the enhanced binding affinities of bivalent ligands with short linkers (<20Å) relative to monomers was mainly due to the ‘statistical binding effect’, that is, the binding of one pharmacophore of a bivalent ligand to one receptor leads to an increased local concentration of the ligand, resulting in the increased statistical probability of binding the other receptor within the oligomer\textsuperscript{46-47}. The ‘statistical binding effect’ suggests a ‘dual-acting phenotype’, rather than a true bivalent binding\textsuperscript{48}. The crystal structure of GPCR oligomers as well as modeling studies estimated the distance between two adjacent binding sites to be in a drastically wide range ranging from 25 to 100Å, in the case of loosely packed receptors\textsuperscript{48}.

![Figure 6.1 - Structure-based approach for the design of bivalent ligands. (A) Structure of G7039 and the (B) G7039-GHS-R1a complex model previously developed in our lab indicated that the (C) C-terminus is the suitable attachment point](image)

With the previous literature in mind, we set out to design a bivalent ligand with the appropriate linker length in order to target the GHS-R1a homo-oligomer. In order to better guide our attempt at the appropriate linker length we generated a homo-oligomer model based on the G7039-GHS-R1a complex model previously developed in our lab\textsuperscript{49} and the crystal structure of the beta-1 adrenergic receptor oligomer (PDBID: 4GPO\textsuperscript{50}).
Beta-1-adrenergic and GHS-R1a share a relatively high sequence similarity of about 38%. Our homo-oligomer model of GHS-R1a in complex with G7039 showed a minimum distance of 50Å between the C-terminus attachment points of G7039 (Figure 6.1C). However, the model indicated that a longer spacer length should be required to enable a bivalent ligand interaction with the oligomer for a number of reasons. First, the amides on the C-terminus are not facing straight towards each other due to the binding pattern of the ligand in the orthosteric site of the receptor. Second, the ligand is buried in the binding pocket to some extent and an additional spacer length will be needed to extend the ligand to the extracellular side. A third reason supporting the need for a longer linker is because the receptor binding pocket is partially blocked by the N-terminus of GHS-R1a. An estimation of the distance allowing the ligand to be accessible from extracellular side and at the same time get away with the N-terminus suggested a spacer length between 50-74Å is required to crosslink both receptors. We next considered the location to attach the linker between the two G7039 peptides. The G7039-GHS-R1a complex model shows that the positively charged N-terminus serves as an anchor point for binding via a charge-charge interaction with Glu124 on the GHS-R1a, while the C-terminus is accessible from the extracellular side. Based on the model, the C-terminus is determined to be the suitable attachment points for linking two pharmacophores with a spacer (Figure 6.1B).

Furthermore, the composition of the spacer regarding hydrophilicity, flexibility, and the ease of synthesis was also considered. We decided to use PEG (polyethylene glycol) units as this would avoid cumulative increase in hydrophobicity. The Fmoc-AEEA (Fmoc-8-amino-3,6-dioxaoctanoic acid) was selected as it can be treated as an amino acid that is readily added on to the peptide via solid phase synthesis. Each of the AEEA unit consists of 9 atoms and is about 10Å. We concluded that 5-7 AEEA units will be necessary to enable a bivalent-binding mode, while a ligand containing a spacer shorter than that should not be able to bridge two binding sites. The ligands bearing 1-4 AEEA units were synthesized as control agents to investigate more thoroughly how the spacer length affects the binding affinity. Additionally, the ligand with 8 AEEA units was also synthesized.
6.2.2 Synthesis

The bivalent ligands based on G7039 were synthesized using standard Fmoc peptide chemistry on a solid support. They were synthesized with amino acids bearing standard protecting groups, with the exception of the C-terminus Lys, that contained the orthogonal protecting group allyloxycarbonyl (Alloc). The first G7039 unit with additional Alloc protected Lys at the C-terminus was synthesized on solid support using automated peptide synthesis. The selective deprotection of the lysine Alloc protecting group was carried out and the resulting free amine at the Lys side chain allows for the ligation of individual spacer (Fmoc-AEEA) with different lengths.

The second G7039 pharmacophore was then coupled with the deprotected AEEA using automated peptide synthesis. After both G7039 pharmacophores were added to the solid support, all protecting groups were cleaved from resin (Scheme 6.1).

![Scheme 6.1 - Solid phase synthesis of peptidomimetics. a: Fmoc-Lys(Alloc)-OH; b: (1) piperidine/DMF, (2)Fmoc-amino acid; c: TPP palladium; d: Fmoc-AEEA; e: piperidine/DMF; f: Fmoc-amino acid; e: total deprotection from the resin using 95% TFA, 2.5% TIPS, and 2.5% H2O](image)

6.2.3 Radioligand binding evaluation

The bivalent ligands with a spacer length ranging from 13-76 atoms (17-92 Å) were synthesized. Their binding profiles (IC50) were determined by competitive radioligand ([125I]-ghrelin) binding assay using GHS-R1a transfected HEK293 cells as receptor source (Table 6.1, Figure 2). Compound 1 with the shortest spacer showed slightly increased binding affinity (1.6 nM) as compared to that of the parent ligand G7039 (5.2 nM). When the spacer length was increased to 22 atoms (28 Å), the binding
affinity increased to 0.55 nM (Compound 2). Further elongation of the spacer to 31 atoms (38 Å) resulted in a slightly increased binding affinity for bivalent compound 3 (0.32 nM). After this point, further extension of the spacer to 49 atoms (60 Å) resulted in comparable affinities for compounds 3, 4 and 5. All the binding displayed monophasic curves. However, the binding characteristics changed dramatically by the extension of the spacer to 58 atoms (70 Å) for compound 6, a biphasic competition curve with two individual values for $IC_{50}^{high}$ and $IC_{50}^{low}$ differing by about 2 orders of magnitude was observed indicating two binding states (Figure 6.2F). The high-affinity binding was determined to be 0.43 nM with a high-affinity population of 45%, while the affinity for the low-affinity binding was determined to be 0.00042 nM. The biphasic competition curve can be explained by the coexistence of different receptor subpopulations, which has been observed looking at other GPCRs52-54. Our data suggests that GHS-R1 also exists as two different receptor subpopulations, that is monomer and homo-oligomer. The low-affinity binding corresponded to a monovalent-binding mode to the receptor, while the high-affinity binding represented to a bivalent receptor bridging mode of compound 6 to the GHS-R1 homo-oligomer. Interestingly, the ligands (compound 7, compound 8) with longer spacer length showed comparable binding affinities (0.31 nM and 0.22 nM, respectively) but displayed monophasic binding curves rather than biphasic ones, suggesting that ligands bearing a spacer that is too long (> 67 atoms/82 Å) are not effective at bridging two receptors in the homo-oligomer. To factor out possible contributions of the spacer to binding affinity, monovalent ligand compound 10 containing a spacer with 58 atoms (70 Å) was synthesized as control. The $IC_{50}$ was determined to be 1.09 nM, suggesting that the spacer does not have appreciable contribution to the binding affinity (Table 6.2). This was also proven by a monophasic binding curve indicating that compound 10 does not target the homo-oligomer.
Table 6.1 - Binding affinities for bivalent ligands determined by $[125$I]-ghrelin displacement

Bivalent ligands

<table>
<thead>
<tr>
<th>Compound n</th>
<th>Spacer Length (atoms)</th>
<th>Spacer Length (Å)</th>
<th>IC$_{50}$ (mean ± SE)</th>
<th>Curve shapes</th>
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<td>1</td>
<td>13</td>
<td>17</td>
<td>1.60 ± 0.57 nM</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>22</td>
<td>28</td>
<td>0.55 ± 0.28 nM</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>31</td>
<td>38</td>
<td>0.32 ± 0.01 nM</td>
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<td>4</td>
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<td>40</td>
<td>49</td>
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<td>49</td>
<td>60</td>
<td>0.23 ± 0.02 nM</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>58</td>
<td>70</td>
<td>IC$<em>{50}^{low}$ =0.43 ± 0.30 nM IC$</em>{50}^{high}$ =0.42 ± 0.53 pM</td>
</tr>
<tr>
<td>7</td>
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<td>82</td>
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<td>76</td>
<td>92</td>
<td>0.22 ± 0.13 nM</td>
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</tbody>
</table>
Table 6.2 - Binding affinities for monovalent ligands determined by $^{125}\text{I}$-ghrelin displacement

Monovalent ligands

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<tr>
<th>Ligands (Compound Number)</th>
<th>n</th>
<th>Spacer Length (atoms)</th>
<th>Spacer Length (Å)</th>
<th>IC$_{50}$ (mean ± SE)</th>
<th>Curve shapes</th>
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<tr>
<td>G7039 (9)</td>
<td></td>
<td></td>
<td></td>
<td>3.10 ± 2.97 nM</td>
<td>Monophasic</td>
</tr>
<tr>
<td>(10)</td>
<td>6</td>
<td>58</td>
<td>70</td>
<td>1.09 ± 0.04 nM</td>
<td>Monophasic</td>
</tr>
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</table>
Vagner et al. noted that bivalent ligands with shorter spacer (<20Å) exhibited dual acting binding mode rather than bivalent binding mode due to the ‘statistical effect’. We suggest that the bivalent ligands compound 1, compound 2, compound 3, compound 4, and compound 5 engage GHS-R1a receptor in a dual acting mode and the enhanced binding affinities of these bivalent ligands is due to the ‘statistical effect’. Among these ligands, the effect became more significant when the spacer is long enough to extend the second pharmacophore to the extracellular site and enable an increase in local concentration. With an optimal spacer length of 58 atoms/70 Å, the bivalent ligand compound 6 is capable of bridging two receptors in the oligomer, leading to a markedly boosted binding affinity ($IC_{50}^{high}$). However, due to the coexistence of monomer subpopulation in the cells, a monovalent binding mode is inevitable, leading to a higher binding affinity ($IC_{50}^{low}$). With an even longer spacer, the bivalent ligand is not able to...
bridge both receptors in the oligomer due to the entropy cost, associated with restricting the second pharmacophore to the second binding site, being too great.

Figure 6.3 - β-Arrestin 1 and 2 recruitment measuring the potency of the ligands (EC_{50}) values. (A) β-Arrestin 1 recruitment indicating an entire log shift in potency increase for the bivalent ligand. (B) β-Arrestin 2 recruitment indicating an entire log shift in potency increase for the bivalent ligand. The bivalent ligand has an increase in net BRET ratio compared to the other ligands indicating that the bivalent ligand is more of a full agonist. (C) Measured EC_{50} values with the bivalent ligand being the most potent in the series (n=3 for both β-Arrestin 1 and 2 recruitment). *P<0.05 compared to G7039

From this point forward, four ligands were evaluated in terms of differences seen in downstream signalling with the GHS-R1a homo-oligomer versus the GHS-R1a monomeric receptor. The lead bivalent ligand (6) was compared to the controls consisting of human ghrelin, G7039 (9) and G7039-L (10). We used a bioluminescence resonance energy transfer (BRET) assay to monitor β-arrestin recruitment to GHS-R1a. This was accomplished by transiently transfecting HEK293 cells with an eYFP tagged GHS-R1a (GHSR-1a-eYFP) and a Renilla luciferase (rluc) tagged β-arrestin1 (β-Arrestin 1-rluc) or β-arrestin 2 (β-Arrestin 1-rluc) construct in a 10 to 1 ratio, respectively. Upon addition of the various ligands (Figure 6.3 panel C) at a concentration range from 1x10^{-5} M to 1x10^{-13} M a net BRET ratio was measured to assess agonist stimulated arrestin recruitment to GHS-R1a. Previously it has been shown that when GHS-R1a is activated by an agonist
such as human ghrelin it causes the recruitment of both β-arrestin-1 and β-arrestin-255-57. It has been demonstrated that GHS-R1a forms a transient complex with β-arrestin-2 on the cell membrane provided by the stabilization of phosphate acceptor sites from the tail of the receptor. This was proven to be destabilized by mutagenesis experiments with the conserved residues (Pro 148, Ala 144 and Leu 149) seen within this region. This interaction with β-arrestin-2 is crucial in the induction of Ras Homolog Family Member A (RhoA) signalling and the remodelling of actin filaments58. We show that the bivalent ligand (6) has greater potency than ghrelin for activation of β-arrestin-1 and β-arrestin-2 recruitment with EC$_{50}$ values in the low nanomolar range (EC$_{50}$ = 1.07 nM for β-Arr-1 and EC$_{50}$ = 3.09 nM for β-Arr-2). G7039-L (10), and the natural ligand for the receptor, ghrelin, have 2-80x fold reduction in the recruitment of β-arrestin-1 and β-arrestin-2 (Figure 6.3). This increase in potency is possibly due to the activation of a homo-oligomer receptor compared to a monomer. The homo-oligomeric complex is causing an increase in the localization of β-arrestin-1 and β-arrestin-2 to GHS-R1a at the membrane due GPCR oligomerization when targeting with bivalent based ligands47-48. It can also be noted that compound 9 has a significant increase in potency (pM) for β-arrestin-1 and β-arrestin-2 recruitment compared to all other compounds. This is an interesting observation seen as this compound is only targeting the monomeric GHS-R1a.

To further assess whether or not this bivalent ligand (6) was targeting a homo-oligomer, an assay was designed using transiently transfected cells with the BRET pair GHS-R1a-eYFP (2 µg) and GHS-R1a-rluc (2 µg). This particular assay has been used in the past to show GHS-R1a homo-oligomerization26-28. 48-hour post transfection cells were treated with the various ligands from a 1x10$^{-5}$ M to 1x10$^{-15}$ M concentration range and the net BRET signal monitored receptor oligomerization.
Figure 6.4 – Homo-oligomer assay indicating that the bivalent ligand is able to induce a homo-oligomer formation with GHS-R1a causing a net increase in BRET signal that is 3x greater compared to the control ligands. (A) Net BRET ratio measured over a concentration range. (B) Net BRET ratio a given high and low concentration. The control ligands have little to no increase in net BRET signal at both a high and low concentration (n=3)

The data in Figure 6.4 panel A indicates that the bivalent ligand (6) targets the GHS-R1a homo-oligomer as there is an increasing net BRET signal, with a 4-log shift lower concentration (1x10^{-10}M vs. 1x10^{-6}M) compared to the control ligands. This bivalent ligand may also be involved in stabilizing the GHS-R1a oligomers. An increase in net BRET signal is only seen when higher concentrations (1x10^{-6}M to 1x10^{-5}M) of the control ligands are used and may be indicative of a signaling dependent clustering of the receptors in membrane microdomains or vesicles. Panel B in Figure 6.4 is an additional representation of a given high (1x10^{-6}M) and low (1x10^{-11}M) concentration again showing the bivalent ligand (6) is able to target the GHS-R1a oligomer. This assay indicated that 6 is able to target the receptor homo-oligomer, and possible stabilize its formation. The control ligands are only able to bind the monomer receptor as they do not show an increase in the net BRET signal at the wide range of concentrations assessed.

Changes in intracellular calcium were then measured using LNCaP cells stably expressing GHS-R1a-eYFP. A leftward shift in the concentration effect curve was observed with compound 6 compared to ghrelin indicating that compound 6 is more potent than ghrelin (EC_{50} of 12 nM versus 121 nM). The E_{max} is comparable between both
ghrelin and compound 6, reaching 30% of the maximum calcium signal obtained with calcium ionophore (A23187) treatment (Figure 6.5).

![Graph showing calcium signal](image)

**Figure 6.5 - Changes in intracellular calcium measured in LNCaP cells stably transfected with GHS-R1a. This is a percent calcium signal obtained with the treatment of calcium ionophore (n=3)**

A series of experiments were designed to investigate whether activation of GHS-R1a homo-oligomer causes differential downstream signalling compared to when activating the GHS-R1a monomer. The downstream signals measured were the phosphorylation of extracellular signal-regulated kinases-1 and -2 (ERK-1/-2; a.k.a. p44/42), and gene reporters nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), serum response element (SRE), serum response factor (SRF), nuclear factor of activated T-cells (NFAT), and cyclic AMP response element (CRE). GHS-R1a mediated phosphorylation of ERK-1/-2 (p-p44/42) involves both the interaction with β-arrestin and G protein components. GHS-R1a activation signalling occurs through a series of G-protein components including Gq, Gq/11, Gi/o, and G12/13.

![Graph showing pERK/ERK ratio](image)

**Figure 6.6 - (A) pERK/ERK ratio when treated with ghrelin and bivalent ligand from 0 to 30 min. (B) Representative blot from total data graphed in A. Ghrelin**
induced a higher level of phosphorylation of ERK 1 and 2 compared to the bivalent at 10- and 20-min incubation. This was done in HEK293T cells that transiently expressed GHS-R1a-eYFP (eYFP used as loading control – data not shown) (n=3, p<0.05 for 10- and 20-min time points)

The bivalent ligand (6) caused less phosphorylation at 10- and 20-min. incubation periods compared to ghrelin. The signal intensity for the pERK/tERK ratio steadily increases to a maximum at 20 min followed by a steady decrease till 30 min (P<0.05). These results are the first indication that GHS-R1a homo-oligomer modulates downstream signalling differently compared to when it is activated as a monomer. Even though there was a greater increase in β-arrestin recruitment, it was believed that the activation of the oligomer would have caused an increase in ERK-1/-2 phosphorylation. However, since compound 6 is more involved in β-arrestin recruitment we expect a decrease in G-protein recruitment leading to a decrease in ERK phosphorylation (Figure 6.6). Additional ERK phosphorylation was followed up by assessing the other ligands at various concentrations for a 20 min incubation period (Figure 6.7).

![Figure 6.7](image)

**Figure 6.7** - pERK/tERK ratio measured for all 4 ligands from a 1 to 100 nM concentration for a 20 min incubation period. Background (0 nM) was removed. (B) Representative western blot indicating there is an increase in pERK/tERK for ghrelin compared to compound 6. 0 nM concentration is vehicle control with HBSS and 0.1% DMSO. Only significance for the 100 nM concentration was seen (indicated by *) (n=3, P<0.05 for 100 nM concentration)

At a concentration of 100 nM there was significantly less phosphorylation (2x to 3x less) of ERK 1 and 2 with the bivalent ligand (6) compared to the other three monovalent ligands (P<0.05). No significance was seen with the lower concentrations. It must be noted that for all western blots eYFP was probed for, to ensure equivalent
receptor expression levels in cells from which lysates were obtained. Data does not change when the ratio of (pERK/tERK)/(eYFP) is assessed (data not shown).

Next, we investigated the source of ERK activation downstream of GHS-R1a activation to further investigate our finding that the bivalent ligand (6) activated ERK to lesser magnitude than that found with ghrelin. ERK activation can occur downstream of both Gq/11 and β-arrestin signalling pathways. Pre-incubation with with Gq/11 specific inhibitor, YM254890 (100 nM), was sufficiently able to inhibit ERK activation at 20 minutes following ghrelin stimulation (100 nM; Figure 6.8). Thus, ERK activation occurs downstream of Gq/11 activation following GHS-R1a agonism with ghrelin.

**Figure 6.8** - (A) Stimulation of ERK (p-p44/42) phosphorylation with or without pre-incubation with Gq/11 inhibitor YM254890 (100 nM) and stimulation with ghrelin (100 nM). Data are shown as ratio of pERK/tERK. (B) Representative western blot indicating pERK is completely inhibited in the presence of YM254890. Significance was only observed between unstimulated and ghrelin treated vehicle control (indicated by *) (n=3, p<0.05)

Finally, gene reporters were assessed to test the hypothesis that the GHS-R1a homo-oligomer leads to differential downstream signalling. The gene reporters that were studied have known upstream G-protein partners. SRE leads to mitogen activated protein kinase (MAPK) response and is downstream of Gq/11, Gs, and Gi. SRF is downstream of Gq/11, and G12/13. CRE is downstream of Gs. NFAT is downstream of Gq/11. Finally, NF-κB is downstream of many pathways (Figure 6.9). The intention with using these gene reporters was to better understand how the GHS-R1a homo-oligomer modulates G-protein partners when activated.
Figure 6.9 - Gene reporters assessed at a 100 nM concentration for ghrelin and the bivalent ligand. CRE and NFAT did not show any signal for all treatments. HBSS with 0.1% DMSO and Serum are control conditions. No significance was seen between Ghrelin vs. the Bivalent only trends were observed; (n=3)

The bivalent ligand (6) showed differential signalling compared to the other three ligands, with 6 causing the greatest activation with SRF and SRE. Ghrelin was least potent in activating SRF and SRE. This was an expected result as the activation of GHS-R1a is known to come from both $G_{q/11}$ and $G_{12/13}$. However, receptor oligomerization led to an increase in the activation of these G-proteins when targeted with this highly potent homobivalent agonist. This was reversed for NF-κB, as the bivalent ligand (6) began to slow this signalling pathway, where ghrelin caused an activation of NF-κB. CRE and NFAT did not showing any activation with any of the four ligands. The lack of CRE activation is downstream of $G_s$ and this data is not unexpected since GHS-R1a is not reported to couple to this G protein. The lack of NFAT activation was however somewhat unexpected since GHS-R1a coupling to $G_{\alpha_{q/11}}$ has been reported and NFAT gene
reported activity occurs downstream of Gaq/11. This discrepancy could stem from the time after agonist treatment at which these assays were done and represent a limitation of using gene reporters to probe signaling pathways. 1x HBSS was used as the negative control and showed no activation of any of the gene reporters. DMEM media containing 10% FBS was used as a positive control causing an upregulation of SRF, SRE, and NF-κB as these are serum response genes. The reduction in NF-κB is highly beneficial in terms of being able to agonize GHS-R1a in diseases such as cancer cachexia, without upregulating other pathways involved in inflammation\textsuperscript{13}. This is the first known instant of targeting the GHS-R1a homo-oligomer with a homobivalent agonist resulting in changes in downstream signalling events.

6.3 Conclusions
We report herein the development and characterization of a novel ligand targeting the GHS-R1a homo-oligomer. The ligand that was chosen was the established ghrelin peptidomimetic G7039, as the initial goal was to determine the spacer length between the two linked G7039 molecules in order to target the oligomerized GHS-R1a with high affinity and potency. An optimal spacer length of 58 atoms was determined in order for compound 6 to target the GHS-R1a homo-oligomer. The discovery of the spacer length sets the ground work to synthesize additional ghrelin ligands such as heterobivalent ligands (e.g. agonist linked to antagonist) and other homobivalent ligands (e.g. antagonist linked to antagonist). Also, based on the previous design of imaging agents derived from G7039, this high affinity probe can easily be converted into a PET imaging probe\textsuperscript{54}. The linker region of compound 6 is also a potential sight to conjugate various fluorescent dyes and imaging moieties for various modalities such as PET, SPECT and confocal microscopy.

The second major outcome of this paper is when you target and activate the GHS-R1a homo-oligomer using compound 6 there is the presence of differential downstream signalling. The bivalent ligand (6) caused 2 to 80-fold increase (versus human ghrelin and G7039-L) in β-arrestin-1 and β-arrestin-2 recruitment. This correlates to a large increase (versus human ghrelin and G7039) in binding (2000 to 11,000-fold increase) when 6 targets the GHS-R1a homo-oligomer. Interestingly G7039 is 50-fold more potent in β-arrestin-1 and β-arrestin-2 recruitment when targeting the GHS-R1a monomer. However,
even though compound 6 has the greatest affinity and potency, it caused a decrease in the phosphorylation of ERK-1/-2 compared to when ghrelin bound the monomeric GHS-R1a. GHS-R1a oligomerization is an explanation for this decrease in phosphorylation of ERK 1 and 2 as well as the increase observed in β-arrestin recruitment when targeting the homo-oligomer with 6.

β-arrestin commonly performs the role of desensitizing activated GPCRs leading to the cessation of G-protein signalling. This hypothesis is supported by the observation of inhibited ERK phosphorylation in the presence of Gq/11 inhibitor and stimulation with ghrelin. Thus, increasing β-arrestin recruitment may be contributing to increased desentization of Gq/11 signalling and the downstream ERK activation pathway.

Compound 6 also caused an increase in SRF and SRE gene reporters compared to ghrelin. Interestingly this pattern was reversed for NF-κB as ghrelin caused a greater increase in NF-κB compared to compound 6. NF-κB is an inflammation biomarker, therefore 6 has the potential to treat diseases associated with lower levels of GHS-R1a activation (cancer cachexia) but not cause a drastic increase in inflammatory markers leading to further side effects13, 38-40.

This is the first ever demonstration of the development and assessment of a ghrelin homobivalent peptide ligand that targets the GHS-R1a homo-oligomer with the greatest known affinity for a peptide targeting this receptor. It was also discovered that the receptor homo-oligomer displays differential signalling mechanisms when looking at specific downstream genes and proteins.

6.4 Materials and Methods

6.4.1 General Information

All reagents were obtained from Sigma-Aldrich, Fisher Scientific, Ark Pharm Inc or Oakwood Chemicals. The purity of the compounds was analyzed using analytical UHPLC. These results are summarized in Table S2 and Figure S4, with all compounds determined to have ≥95% purity. Acquity UHPLC H-Class system (Waters, Inc.) combined with a Xevo QTof mass spectrometer was used for analytical UHPLC-MS work. Waters Acquity UHPLC BEH C18 column (2.1 x 50 mm, 1.7 μm) was used. High resolution mass spectra were obtained on a Thermo Scientific DFS (Double Focusing
Sector) mass spectrometer. [\textsuperscript{125}I]-Ghrelin for binding assay was purchased from Perkin Elmer.

6.4.2 General Fmoc Synthesis of bivalent ligands

Automated peptide synthesis was carried out using Fmoc-based solid-phase peptide chemistry. Peptides were synthesized at a 0.1 mmol scale on Rink Amide MBHA resin (0.52 mmol/g) using a glass peptide reaction vessel. The resin was initially swelled with \textit{N,N}-dimethylformamide (DMF), followed by Fmoc deprotection using 2 mL of 20\% piperidine in \textit{N,N}-dimethylformamide (DMF) for two cycles (10 minutes, 5 minutes). Amino acids were preactivated by combining 3 eq. of Fmoc-protected amino acid, 3 eq. of O-(6-chlorobenzotriazol-1-yl)-\textit{N,N,N',N’}-tetramethyluronium hexafluorophosphate (HCTU) and 6 eq. of \textit{N,N}-diisopropylethylamine (DIPEA) in 2 mL of DMF. The mixture was added to the resin and vortexed for 60 minutes. These cycles were repeated until all N-terminal amino acids were coupled to the resin. Allyloxycarbonyl deprotection of diaminopropionic acid was performed under inert atmospheric \textit{N}_2 conditions. DCM was dried over sieves for 24 hours before adding 1 mL to the resin. Two equivalents of phenylsilane in 1 mL dry DCM was then added to the peptide resin followed by 0.045 eq. of tetrakis(triphenylphosphine) palladium (0) in 1 mL dry DCM. The peptide vessel was removed from inert conditions and allowed to react for 30 minutes. 4 eq. of HCTU and 6 eq. of DIPEA in DMF were used. The reaction mixture was left to couple overnight. Full deprotection of the synthesized peptide was performed by adding a 2 mL mixture of 95\% trifluoroacetic acid (TFA), 2.5\% triisopropylsilane (TIS) and 2.5\% water to the resin and allowed it to mix for 5 hours. The cleaved peptide was precipitated from solution using ice-cold tert-butyl methyl ether (TBME) and centrifuged (3000 rpm, 10 minutes) resulting in a crude peptide pellet. The supernatant was decanted and the resulting peptide pellet was dissolved in 20\% acetonitrile in water, frozen at -78 °C and lyophilized to a white crude powder. Purification was performed using preparative HPLC-MS and purity of the resulting peptides were analyzed using analytical UHPLC. These results are summarized in Table 4, with all compounds determined to have ≥95\% purity.

Table 6.3 - Analytical data of bivalent and monovalent ligands
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### 6.4.3 Kaiser Test

To qualitatively determine if there is free amine present (i.e. after a Fmoc-deprotection, amino acid coupling, or alloc deprotection), a Kaiser test was performed. A small amount of resin (<5 mg) was placed in a small test tube. To the test tube, solutions of Phenol:EtOH (8:2 v/v), 0.001M KCN:pyridine (2:98 v/v) and ninhydrin in EtOH (5% w/v) were added. The test tube was heated to 70°C and the presence of a free amine was indicated by the resin beads turning blue. The lack of free amine was indicated by the presence of clear/yellow resin beads.

### 6.4.4 Deprotection of Allyloxycarbonyl (Alloc) Protecting Group

Selective deprotection of the allyloxycarbonyl protecting group was accomplished by adding DCM (4.5 mL) to the resin-bound peptide and shaking gently for 10 minutes. After addition of phenylsilane (24 equiv.), the peptide vessel was flushed with nitrogen and allowed to react for 5 minutes. Then, tetrais(triphenylphosphine) palladium (0) (0.1 equiv.) was added to the mixture and the peptide vessel was again flushed with nitrogen, and the reaction was allowed to proceed for 10 minutes. Next, the peptide-resin was washed with DCM four times (1.5 mL), followed by a series of washings with 1.5 mL of DCM, DMF, MeOH, DMF, and DCM (30 sec each).
6.4.5 Purification of RP-HPLC/ESI-MS
Peptides were analyzed using a reverse-phase analytical HPLC column (Agilent Zorbax SB-C8 column 4.6 x 150 mm, 3.5 μm). This system was equipped with a Waters 600 136 controller, Waters Prep degasser, and Waters Masslynx software (version 4.1). Employed mobile phases were 0.1% TFA in deionized water (eluent A) and 0.1% TFA in acetonitrile (eluent B). The flow rate was set at 1.5 mL min\(^{-1}\) over 15 minutes. The column eluate was monitored using a Waters 2998 Photodiode array detector set at 220 nm, 254 nm and 400 nm. Peptides were purified using a reverse-phase preparative HPLC column (Agilent Zorbax SB-C18 column 21.2 x 150 mm, 5 μm) on the same system mentioned above. The detection method and eluents were the same was mentioned above for the analytical system and the flow rate was set at 20 mL min\(^{-1}\). The collected fractions were then lyophilized to a solid. For analytical UHPLC-MS, studies were performed on a Waters, Inc. Acquity UHPLC H-Class system, combined with a Xevo QTof mass spectrometer (ESI+, cone voltage = 30 V). A Waters Acquity UHPLC BEH C18 2.1 x 50 mm, 1.7 μm column was used with a gradient solvent system consisting of 0.1% formic acid in acetonitrile and 0.1% formic acid in water.

6.4.6 Coupling of Fmoc AEEA Linker
After the Alloc protecting group has been removed, Fmoc-AEEA (1 to 8 linkers added) was coupled to additional C-terminal Lys residue of G7039. This was accomplished by adding Fmoc-AEEA (0.3 mmol) and the coupling reagent HATU (0.3 mmol) dissolved in DMF (1.5 mL) to the resin. This mixture was vortexed for 2 hours, and the resin was then washed 3 times with DMF (2.0 mL, vortex 30 sec) and 3 times with DCM (2.0 mL, vortex 30 sec), then dried under vacuum.

6.4.7 Competitive Binding Assay (IC\(_{50}\))
The affinity for GHS-R1a was determined using a radioligand competitive binding assay. Assays were performed using GHS-R1a transfected HEK293 cells as receptor source and human \([^{125}\text{I}]\)-ghrelin(1-28) (Abcam) as the radioligand. Human ghrelin(1-28) was used as reference to ensure the validity of the results. A suspension of membrane from HEK293/GHS-R1a cells (50,000 cells per assay tube) were incubated with ghrelin(1-8) peptide analogues (at concentrations of 10\(^{-5}\) M, 10\(^{-6}\) M, 10\(^{-7}\) M, 10\(^{-8}\) M, 10\(^{-9}\) M, 10\(^{-10}\) M and 10\(^{-11}\) M) and \([^{125}\text{I}]\)-ghrelin (15 pM per assay tube) in binding buffer
(25 mM HEPES, 5 mM magnesium chloride, 1 mM calcium chloride, 2.5 mM EDTA, and 0.4% BSA, pH 7.4). The resulting suspension was incubated for 20 minutes under shaking (550 rpm) at 37 °C. Unbound \(^{125}\text{I}\)-ghrelin was removed and the amount of \(^{125}\text{I}\)-ghrelin bound to the membranes was measured by gamma counter. IC\(_{50}\) values were determined by nonlinear regression analysis. All binding assays were performed in duplicate or triplicate.

### 6.4.8 Molecular cloning and constructs

YFP tagged human GHS-R1a from Addgene (hGHS-R1a) was made by replacing PAR4 in the previously published PAR4-YFP (Ramachandran et al., 2017) construct\(^6\) with GHS-R1a using restriction enzymes digestion with EcoRI and Xhol and ligation. Constructs was verified by direct sequencing (University of Western Ontario, Robarts DNA sequencing facility).

### 6.4.9 Bioluminescence Resonance Energy Transfer (BRET): \(\beta\)-Arrestin 1/2 Recruitment Assay

HEK293T cells were transfected with BRET pair GHS-R1a-eYFP (2 μg) and \(\beta\)-arrestin-1-rluc or \(\beta\)-arrestin-2-rluc (0.2 μg) using calcium phosphate in tissue culture treated white 96-well plates. 48 hours after transfection, cells were treated with the agonists being tested, Renilla luciferase substrate (h-coelenterazine) was added to each well (5 μM final) and incubated (37 °C, 20 min), before measuring BRET ratios on a Berthold Mithras LB 940. Responses are expressed as net emission of eYFP/rluc (calculated by subtracting HBSS baseline eYFP/rluc ratio from agonist eYFP/rluc ratio) and normalized to a positive control. Experiments were performed in triplicate with 3 separate cultures of cells and data was fitted with a non-linear regression analysis four-parameter dose-response curve using GraphPad Prism 6 to determine EC\(_{50}\) values, as this fitting provided the greatest R squared value.

### 6.4.10 Homodimer Assay

HEK293T cells were transfected with BRET pair GHS-R1a-eYFP (2 μg) and GHS-R1a-rluc (2 μg) using calcium phosphate in tissue culture treated white 96-well plates. 48 hours after transfection, cells were treated with the agonists being tested, Renilla luciferase substrate (h-coelenterazine) was added to each well (5 μM final) and incubated (37 °C, 20 min), before measuring BRET ratios on a Berthold Mithras LB 940.
Increase in a net BRET signal indicated that each of the differentially tagged receptors were in close proximity.

**6.4.11 Gene Reporter Assay**

HEK293T cells are maintained in Dulbecco’s Modified Eagle’s medium (DMEM) from Sigma-Aldrich (D5796) with added 5% sodium pyruvate, 5% penicillin streptomycin, and 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. Cells were cultured in a T-75 flask to 95% confluency then lifted by trypsin-ETDA (0.25%). Once cells were counted and spun down at 1000 rpm they were resuspended and then re-plated in 10 cm culture dishes at 1 million cells per plate. Seeding was then accomplished for 24 hrs at 37°C with 5% CO₂. After 24 hrs cells were transiently transfected with 2 ug of GHS-R1a-eYFP and 2 ug of either NFAT/SRE/SRF/NF-κB/CRE-nluc by calcium phosphate transfection method for 24 hrs at 37°C with 5% CO₂. Following the 24 hr transfection cells were rinsed 3x with PBS and then 11 mL of serum containing media was added. Plates were then allowed to sit at 37°C with 5% CO₂ for 4 hrs before being lifted by pipette and re-plated in a 96-well white wall and white bottom plate. Cells then were maintained at 37°C with 5% CO₂ for an additional 24 hrs before reporter assay was performed. All drugs being tested were dissolved in Hanks’ Balanced Salt Solution (HBSS) with 0.1% DMSO. The highest drug concentration of 10⁻⁵ M was first prepared, and each full log concentration was prepared by serial dilution thereafter. The control well contained just HBSS with 0.1% DMSO. The serum media was removed from the plate before then addition of the drug at varying concentrations (10⁻⁵ M to 10⁻¹⁷ M). The drugs were then incubated in the plate for 4 hrs at 37°C with 5% CO₂. After 4 hours Nano-Glo luciferase (Promega) substrate is added to have a final concentration of 10⁻⁸ M and then placed at 37°C with 5% CO₂ for 10 mins. The plate is then read by a Berthold Mithras LB 940 plater reader at a λ max of 460 nm. The HBSS control is then removed as the background to all other signals and a dose response plot is used to quantify the signal from the genes being up or down regulated. The entire experiment described above was completed in triplicates for each drug and gene being studied.
6.4.12 Western Blotting
HEK293T cells are maintained in Dulbecco’s Modified Eagle’s medium (DMEM) from Sigma-Aldrich (D5796) with added 5% sodium pyruvate, 5% penicillin streptomycin, and 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. Cells were cultured in a T-75 flask to 95% confluency then lifted by trypsin-ETDA (0.25%). Once cells were counted and spun down at 1000 rpm they were resuspended and then re-plated in 6-well culture dishes at 500 000 cells per well. Seeding was then accomplished for 24 hrs at 37°C with 5% CO₂. After 24 hrs cells were transiently transfected with 0.33 ug of GHS-R1a-eYFP per well. Following 48 hours of transfection cells were rinsed 3x with PBS and 1 mL of serum free media was added to each well. The ligands were then added to have final concentrations of 1 nM, 10 nM, 100 nM, and no ligand as for the control. Plates were incubated at 37°C with 5% CO₂ for 0 min, 5 min, 10 min, 20 min, and 30 min. After the incubation period media was removed and cells were lysed and spun down at 17 G to collect total protein. SDS gel electrophoresis was ran in a 4 to 12% gradient gel for 1 hour, followed by transferring to the PVDF membrane for 90 min. pERK was measured with a specific primary antibody for 24 hours at 4°C, followed by stripping the blot and measuring tERK with an additional specific antibody for 24 hours at 4°C. Then the blot was stripped a third time and a specific primary antibody for eYFP was incubated for 24 hours at 4°C. The ratio of pERK to tERK was calculated and graphed to show difference in phosphorylation. All antibodies were purchased from Cell Signalling. GHS-R1a stimulated ERK signalling in HEK-293 +/- YM254890: Cells were cultured and prepared as described above. Either YM254890 (100 nM; FUJIFILM Wako Pure Chemical Corporation) or DMSO vehicle control (0.001%) were added to wells for 30 minutes prior to agonist stimulation (ghrelin, 100 nM). Cells were incubated with ghrelin for 20 minutes. Lysates were prepared, processed and quantified as described above.

6.4.13 Stable Transfection of LNCaP cells with GHS-R1a-eYFP.
The LNCaP (metastatic prostate cancer derived from left supraclavicular lymph node) cell line (ATCC) was maintained in RPMI medium (Wisent) supplemented with 10% fetal bovine serum (FBS; Wisent). Cells were plated at a density of 1 million cells in a 10 cm dish for 24 hrs. After 24 hrs 6 μg pcDNA-GHS-R1a-eYFP was added along with Fugene-6 (Promega) in a 1:3 ratio as per the protocol provided by Promega. After 48
hours cells were rinsed with PBS (3x) and replaced with serum media containing 300 µg/mL of G418 (Wisent). Cells were then kept under selective pressure for 7 to 10 days. Cells were then sorted (by FLOW cytometry identify the YFP tagged GHS-R1a) for single cell populations at the London Regional Flow Cytometry Facility (LRFCF) by manager Dr. Kristin Chadwick. A single cell population was collected and re-sorted by the same method mentioned above. This single cell clonal population was confirmed by confocal microscopy identifying the tagged GHS-R1a in yellow. Confocal microscopy confirmed 90-95% of cells were expressing GHS-R1a with high cellular membrane expression.

6.4.14 Calcium Assay
LNCaP cells stably transfected with GHS-R1a-eYFP (maintained in RPMI with 10% FBS, 1% Pen-Strep, and 300 µg/mL G418 – Wisent) were grown to 75 to 85% confluency in a T-75 flask. Cells are lifted from the T-75 flask using 5ml of 1 mM EDTA in PBS (Wisent). Cell-PBS-EDTA suspension is spun at 1.0 x 10^3 rpm to pellet cells and PBS-EDTA is removed. Cells are then suspended in 500μl Fluo-4 NW (no wash, NW) dye solution and assay buffer (1x HBSS, 20mM HEPES) and incubated (r.t., 30 min.) on rocking platform. Fluo-4 NW cell suspension is then increased to volume required for assay with HBSS containing calcium chloride and magnesium chloride. Cells are aliquot into cuvettes (2ml/cuvette) containing a magnetic stir-bar to keep cells in suspension for assay. Individual cuvettes are loaded into a Photon Technologies Institute (PTI) spectrophotometer. Time-based assay parameters are assigned through PTI software as follows: excitation 480nm, emission 530nm with 8nm capture window, and 5000 secs duration. Individual cuvettes emission is collected for approximately 10 secs to obtain baseline emission. Agonist is pipette into cuvette and cellular response is measured. As a control 3-5 untreated cuvettes are treated with 3μM ionomycin calcium salt in DMSO to give the maximum response. Each agonist concentration is expressed as a percentage of average maximum response.

6.5 References


Chapter 6 Supplementary Information

UPLC and ESI-MS tracers

Compound 1

![Graph showing HPLC and ESI-MS data for Compound 1]
Compound 2

JQ-5-21-3-c23-20170123-05-95

3: Diode Array
Range: 7.569e+1

1: TOF MS ES+

JQ-5-21-3-c23-20170123-05-95 1082 (1.535) Cm (1076:1114)

1: TOF MS ES+

JQ-5-21-3-c23-20170123-05-95 1082 (1.535) Cm (1076:1114)

1: TOF MS ES+

500.0591

500.3142

500.5694

541.0862

541.6023

666.4235

667.0944

667.4381

667.7820

999.6514

101.0844

186.9762

470.5632

500.0591

541.0862

541.6023

666.4235

667.0944

667.4381

667.7820

999.6514

m/z

%
### Compound 3

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**Diagram:**

- X-axis: Time (0.00 to 3.00)
- Y-axis: AU (0.0 to 6.0e+1)

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**M/z Values:**

- m/z: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300
- AU: 0.0 to 6.0e+1

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**Notes:**

- Range: 7.819e+1
- JQ-5-21-2-c7-20170202-05-95
- 1: TOF MS ES+
- 2: 2.13e3
- 3: Diode Array
Compound 4
Compound 5

JQ-5-19-2-c10-20170123-05-95-2

3: Diode Array
Range: 9.085e+1

1: TOF MS ES+
6.13e3

1051 (1.493) Cm (1046:1100)

608.7670
609.2831
611.2400
811.7061
812.3923
1217.0791

m/z

101.0653

0 200 400 600 800 1000 1200 1400

0 2.0e+1 4.0e+1 6.0e+1

%
Compound 8

JQ-5-27-2-F11-20170303-05-95

3: Diode Array
Range: 9.954e+1

JQ-5-27-2-F11-20170303-05-95 1241 (1.756) Cm (1226:1250)
1: TOF MS ES+
315

100

JQ-5-27-2-F11-20170303-05-95 1241 (1.756) Cm (1226:1250)
1: TOF MS ES+
315

100
Compound 10

JQ-5-27-4-F11-20170307-05-95

3: Diode Array
Range: 8.925e+1

1: TOF MS ES+
1.74e3

m/z

0.0 2.0e+1 4.0e+1 6.0e+1

JQ-5-27-4-F11-20170307-05-95 1136 (1.610) Cm (1126:1154)

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599.8250
600.1664
600.5081
600.8497
899.2583
899.7524
900.2657
900.2657

0.0e+0 2.0e+1 4.0e+1 6.0e+1

JQ-5-27-4-F11-20170307-05-95

599.4990
599.8250
600.1664
600.5081
600.8497
899.2583
899.7524
900.2657

0.0e+0 2.0e+1 4.0e+1 6.0e+1
Supplementary Figure 6.1 - pERK and tERK blots at 0, 5, 10, 20, and 30 min intervals with the treatment of Ghrelin versus Compound 6 (n=3)

Supplementary Figure 6.2 - pERK and tERK blots for various concentrations of Compound 6, Compound 9, Compound 10, and Ghrelin for a 20 min incubation (n=3)
Supplementary Figure 6.3 - pERK and tERK blots with either DMSO vehicle control or YM254890 with/without ghrelin stimulation for 20 min incubation (n=3)
Supplementary Figure 6.4 - Confocal microscopy images of stable transfect LNCaP with GHS-R1a-eYFP indicating the receptor is highly expressed and localized to the cell membrane.
7.1 Conclusion

The focus of this work was the development of chemical tools, both fluorescent and PET probes, that target the growth hormone secretagogue receptor-1a (GHS-R1a). All of these chemical tools acted as agonists when targeting GHS-R1a leading to an activation in signalling. This receptor is an attractive GPCR target because of its wide range of physiological functions in the human body (e.g. metabolism, cardiac output, depression, neuroprotection)\(^1\-^2\). GHS-R1a is also highly dysregulated within certain disease states. It is over and differentially expressed in malignant prostate cancer versus benign, as well as upregulated in a variety of other cancers such as ovarian, lung, uterine\(^3\-^4\). GHS-R1a is also highly promiscuous, as it hetero-oligomerizes with many other GPCRs such as the dopamine and melanocortin receptors. More recently, some have shown how GHS-R1a homo-oligomerizes with itself. However, within the scope of this work, the development of a ghrelin homobivalent ligand was used to target the GHS-R1a oligomer to elucidate the changes in the signaling cascade as a result of the oligomer. When GHS-R1a oligomerizes with itself or other GPCRs there are changes to the downstream signalling (e.g. changes in G-protein activation) leading to different physiological outcomes\(^5\-^8\).

The natural ligand for the GHS-R1a is ghrelin, a 28-amino acid peptide that is derived from a 117- amino acid sequence (preproghrelin), through post-translational modifications, terminating with the octanoylation of Ser-3 by ghrelin O-acyl transferase (GOAT) producing the active form of the ligand\(^3\). This is the only known instance of a peptide being post-translationally modified with a fatty acid, and this modification is required for its binding to, and activation of GHS-R1a\(^1\-^2\). However, the natural ligand is highly susceptible to degradation by enzymes, due to its natural amide backbone and the 28 natural amino acids that make up the structure of the peptide. Also, the ester linkage for the attachment of the octanoyl chain to Ser in position three is susceptible to esterase hydrolysis. Therefore, this is changed to an amide linkage to improve stability but still maintain binding to GHS-R1a. There is a need to develop GHS-R1a targeting probes that have a structure with a greater level
of stability but still maintain binding towards the receptor. There is a greater interest in developing imaging-based probes to monitor the changes in GHS-R1a receptor expression in the presence or absence of diseases such as cancer, cardiovascular, and metabolic imbalance. The design of chemical probes driven towards therapy is also a consideration, as down regulation of this receptor is seen in diseases such as cancer cachexia (muscle wilting after chemotherapy).\(^4\)

There are many styles of chemical probes that can be developed (e.g. small molecules, proteins, antibodies), however, peptides have several advantages. Peptides are easily synthesized by Fmoc solid phase peptide synthesis, and because of the ease of this synthesis technique they can be chemically changed without difficulty (e.g. D-amino acids, amidated C-terminus, backbone stabilization). Peptides also have a greater affinity towards their target of interest, since most known targets only have one natural peptide ligand that binds to them and changes its state of activation. Peptides have a natural structure, their degradation by proteases can be readily determined, therefore this structure can be altered to improve their stability and efficacy as drug molecules.

The first Chapter of this thesis focused on the development of a stabilized ghrelin fluorescent probe that was able to bind to the receptor with nanomolar affinity. With a lactam bridge stabilization in the backbone of a ghrelin 1 to 20 analogue (as determined by a staple scan), the peptide probe was fully stable over a 24-hour period when incubated with 25% human serum. This probe was demonstrated in an \textit{in vitro} situation identifying receptor mediated binding in cells that artificially had over expression of the receptor, as well as in cells that had endogenous levels of GHS-R1a. This peptide probe was also able to detect changes in GHS-R1a expression in a mouse model of stroke by immunofluorescent imaging (ghrelin expression upregulated after stroke due to its neuroprotective effects). This work is not part of the immediate thesis but is an ongoing collaboration with Dr. Rithwik Ramachandran from the Department of Physiology and Pharmacology at Western University. By evaluating this probe in a mouse model of stroke, we can further validate the probe in a more realistic scenario of detecting the GHS-R1a dysregulated expression in an \textit{ex vivo} situation. This chemical tool will be highly effective as an imaging probe in both
an *in vitro* and *ex vivo* situation as most known antibodies for this receptor have proven to have inadequate specificity.

The second section of this thesis, encompassing Chapters 3-5, was a “roller coaster” in terms of probe synthesis and design (structure activity relationship studies – SAR studies) due to many challenges associated with receptor affinity, radiochemistry, and lipophilicity. This second section consists of three individual chapters and was driven towards applications in an *in vivo* imaging scenario. This research focussed on modifying a G7039 peptidomimetic first developed by Genentech. This peptide was originally developed to be used as a growth hormone drug, however due to its relatively small size (mimicking a small molecule class of drug) the conversion into an imaging agent was highly feasible. Prior work highlighted in Chapter 3 looked at the simple addition of 4-fluorobenzoic acid group onto the C-terminal portion and a fourth position changed from a Phe to a 1-naphthylalanine (1-Nal). This proved to be ineffective when it came to imaging by PET (compound 17 in Chapter 3). It had a substantially lower affinity, a higher cLogP, and a lengthy radiosynthesis route. However, this project included an extensive SAR study that indicated which positions of this short 5-mer peptide could be altered without negatively affecting the binding towards GHS-R1a. This allowed for a follow up project that would focus on only altering the fourth position of the 5-mer peptide, as well as changing the prosthetic group to improve lipophilicity, binding, and the radiosynthetic route.

These findings led into the second part of the project (Chapter 4) that had the goals of reducing the cLogP of the compound, increasing the binding affinity to GHS-R1a, and improving the radiosynthesis route. Converting the fluoride-19 (¹⁹F) group to a fluoride-18 (¹⁸F) group on the 4-fluorobenzoic acid prosthetic group in Chapter 3 was too lengthy of a radiosynthetic route. In the end, two of these tasks were accomplished, and further opportunities for subsequent structural modifications (further changes to the radiosynthetic prosthetic group) were considered. The fourth position of G7039 was modified from a Phe to a Tyr, and the prosthetic group chosen was a 2-fluoropropanoic acid group on the C-terminal lysine. These changes resulted in a compound with an affinity that was sub-nanomolar and a reduced cLogP was
observed. This was thought to be the compound that would be labelled and provide decent PET images in an in vivo scenario. This was not the case as the 2-fluoropropionic acid labelling with $^{18}$F proved to be low yielding and thus had a similar issue to that found when using the 4-fluorobenzoic acid prosthetic group. However, this new peptidomimetic proved to have low nanomolar affinity and picomolar activity as measured by β-arrestin recruitment. This is believed to be due to the Tyr in the fourth position forming a new H-bond interaction with Arg 283 in the binding pocket of the receptor (determined by molecular docking). When Ser was placed in the fourth position binding and activity decreased. A lowered $E_{\text{max}}$ was identified for compounds 3 and 6, indicating that they act as a partial agonists when targeting GHS-R1a. Compound 5 in Chapter 4 still has the potential to be labelled with $^{18}$F, however it has a greater potential as a drug therapeutic for diseases where GHS-R1a activation is lowered.

The final stage of this peptidomimetic work was to see if it was possible to change the prosthetic group of the C-terminal Lys a third time while leaving the Tyr in the fourth position (Chapter 5). An alkyne containing ammonium methyltrifluoroborate (AMBF$_3$) was chosen as the new prosthetic group for its ease in conjugation to a peptide as well as an efficient radiosynthetic route. This particular prosthetic group is easily installed onto the previous peptide (compound 5 in Chapter 4) by copper click chemistry using a Lys azide on the C-terminus instead of the previous standard Lys group in the fifth position. Once installed the binding affinity was found to still be sub-nanomolar, however the cLogP was three-fold less than the original G7039 peptidomimetic. This drastic decrease in cLogP will help with the past issues of the tracer being retained in lipophilic organs such as the liver. This tracer can now be directly labelled in one step with no HPLC purification and great radiochemical data. The “hot” $^{18}$F compound displayed a 10 to 12% uptake in cells expressing GHS-R1a. This chemical tool can now be used as a PET tracer to identify the over expression of GHS-R1a in an in vivo cancer mouse model.

Despite the challenges (affinity, lipophilicity, and radiosynthetic route), the three iterations of peptidomimetics each had a positive outcome for its intended purpose. The Chapter 3 outcome helped guide further SAR studies. Chapter 4 confirmed that
only select positions of this class of ghrelin peptidomimetic can be altered in order to maintain affinity and potency when targeting GHS-R1a (confirmed by molecular docking). Chapter 5 finally was the improved avenue towards a simplistic and efficient radiosynthetic route for a novel and improved PET tracer. Each of these unique peptidomimetic ghrelin derivates all have applications towards imaging the dysregulated GHS-R1a by PET. The three generations of peptidomimetics are depicted in Figure 7.1.

Figure 7.1 - Three generations of ghrelin peptidomimetics with changes to the fourth position (highlighted in green from structures A to C) from a naphthylalanine group (structures A) to a tyrosine (structure A and C), and the use of 3 different prosthetic groups (highlighted in red from structures A through C) from generation 1 through 3 conjugated on to the C-terminal lysine.

The final project was the study of GHS-R1a homo-oligomerization, but more so the design of chemical tools to target this homo-oligomer. This project was the most challenging due to the complexity of the assays involved in studying this oligomerization. GHS-R1a has a wide functioning role in human diseases such as obesity, diabetes, stress, anxiety, and depression. Because of its broad range of function in the human body it has been shown that GHS-R1a preferentially oligomerizes with other GPCRs that have similar physiological effects. This makes it very difficult to target GHS-R1a with potent agonists and antagonists with high
efficacy. However, by having a greater understanding of receptor oligomerization one could anticipate designing specific drugs in terms of pharmacological treatment while minimizing side effects. Jiang et al. in 2006 was able to first observe a GHS-R1a homo-oligomer in HEK293 cells that were over expressing GHS-R1a-Rluc and GHS-R1a-GFP by a BRET signal that gave a hyperbolic shape. GHS-R1a has been shown to oligomerize with other GPCRs such as the dopamine receptors (D1R and D2R), the serotonin receptor (5-HT$_2$C), the melanocortin receptor (MC3R), and the cannabinoid receptors (CB1 and CB2)$^7$. GHS-R1a cross talk has widely had a focus driven towards the neuroendocrine system in human physiology, however little work has focussed on the implications of self-oligomerization. The dimerization with other receptors has been shown to either attenuate or cause an augmentation of the downstream cascade of signalling events associated with GPCR activation. Examples of such oligomerization is between GHS-R1a and melanocortin-3 receptor (homeostatic control of food intake) and this oligomerization causes an attenuation of signalling when GHS-R1a is activated by ghrelin and simultaneously associated with melanocortin-3 receptor$^9$. This leads to a reduction in signalling events such as the slowing down of appetite response, whereas when ghrelin acts alone on its receptor it causes an increase in appetite.

Many in the field have targeted GPCR hetero-oligomers with two separate ligands. This approach has a major limitation when it comes to designing a novel drug for a receptor oligomer in terms of drug dosing and formulation. Knowing that this is a possible mechanism in which a ligand can bind and change the active state of a GPCR oligomer, one can develop novel heterobivalent and homobivalent ligands that would modulate the signalling in a differential fashion when compared to the monomer. There is still limited knowledge about the GHS-R1a homo-oligomer and until our work, there are no known homobivalent ligands that can target this homooligomer simultaneously and stabilize its formation. Our studies showed that the homobivalent ligand (compound 6 in Chapter 6) possesses exceptionally high binding affinity and can bind to both receptors within the GHS-R1a homo-oligomer in HEK293 cells. This is indicated by a bivalent binding curve. In addition, we demonstrated that this chemical probe is a strong agonist by its activation of β-
arrestin-1 and β-arrestin-2 recruitment in the low nanomolar range. The monomeric version of the heterobivalent ligand; G7039-L (10), the unmodified G7039 peptidomimetic (9), and the natural ligand for the receptor; ghrelin, are 2-15x less potent then homobivalent ligand based on EC_{50} values for β-arrestin-1 and β-arrestin-2 recruitment. We determined that there was 3-fold increase in homo-oligomerization BRET response with the homobivalent ligand (6) compared to ghrelin, G7039 (9), and G7039-L (10). GHS-R1a activation triggers several signaling pathways that can be harnessed for therapeutic response in disease states (e.g. inflammation, diabetes, and hypotensive effects), leading to the potential for the homobivalent ligand (6) to be used as a therapeutic agent. Targeting the homo-oligomer system with a novel ghrelin homobivalent ligand (6) has caused a 12,000-fold increase in binding compared to ghrelin targeting the monomer receptor, however there is a reduction in the MAPK and NF-κB signalling that is partially due to this receptor oligomerization. Being that NF-κB is a typical protein involved in the inflammation signalling cascade, this is a promising agonist that could be used in the treatment of diseases lacking GHS-R1a signalling (i.e. cancer cachexia) but being able to slow down inflammatory signalling responses. Ligands that are capable of targeting these receptor oligomers holds great promise as a new class of therapeutics, but equally important, are a powerful chemical biology tool for understanding the function, mechanism, and disease significance of GPCR oligomers, for which there is limited knowledge to date.

Overall in my PhD I was able to develop several different styles of chemical probes targeting GHS-R1a with different degrees of affinity and potency. Each of these probes was modified in such away to have applications in the direction of either imaging or therapy and opens the door to further probe development to study this receptor in human physiology and disease.

7.2 References
hypothalamus that functions in growth hormone release. Science, 273(277), 974-977.


CURRICULUM VITAE

Education/Academic Experience
PhD Candidate – Organic Chemistry & Molecular Imaging  September 2014-present
University of Western Ontario
London, ON

- Supervisor: Prof. Len G. Luyt
  Project: Peptidomimetic Growth Hormone Secretagogue Agonists and Stapled Ghrelin-Mimic Peptides as PET and Fluorescent Imaging Agents for Cancer

Honours Bachelor of Science (Cum Laude)  September 2010-April 2014
Laurentian University
Sudbury, ON

- Honours Specialization in Biochemistry
- Supervisor: Prof. Joy Gray Munro
  Project: AFM Studies of Protein Absorption on Magnesium Alloy AZ91

Awards (Total Value of Awards - $118,450)
1. Canadian Society of Chemistry Travel Award, Western University, Department of Chemistry – for the 26th American Peptide Symposium (2019) - $1000
2. Molecular Imaging Travel Award – for the 26th American Peptide Symposium (2019) - $300
3. Japan Society for the Promotion of Science Postdoctoral Fellowship (through NSERC), University of Tokyo (November 30th, 2019 to November 30th, 2021) – $108,000
4. 35th European Peptide Symposium 2018 (Oral Presentation – 1st place Dr. Bert L. Schram Award in Young Investigator Oral Symposium) - $1125
5. Molecular Imaging Travel Award – for the 35th European Peptide Symposium (2018) - $400
6. 35th European Peptide Symposium Travel Award (2018) – $225
8. Molecular Imaging Travel Award – for the 25th American Peptide Symposium (2017) - $400
9. 25th American Peptide Symposium 2017 (Poster Presentation – 1st place Poster Award) - $550
10. Imaging Applications in Prostate Cancer Workshop 2017. (Poster Presentation – 1st place award) - $200
11. Molecular Imaging Travel Award – for 99th Canadian Chemistry Conference and Exhibition (2015) - $500
12. 99th Canadian Chemistry Conference and Exhibition 2015 (Oral Presentation – 1st place award Biological and Medicinal Division) - $250
13. Cum Laude (Graduated with Distinction), Laurentian University, Department of Chemistry and Biochemistry (2010-2014)
14. Entrance and Merit Scholarship, Laurentian University, Department of Chemistry and Biochemistry (2010-2012) - $3500

Publications

Completed Projects/Manuscripts in Progress

Conference Presentations


8. Lalonde, T.; Shaw, G.; Shepherd, T.G.; Luyt, L.G.; Stapled ghrelin (1-20) analogues targeting GHSR-1a for use in cancer diagnostics. 100th Canadian Chemistry Conference and Exhibition. Toronto, ON. May 28th-June 1st, 2017. (Oral Presentation)


Fallona Family Interdisciplinary Showcase. London, ON. December 8th, 2015. (Poster Presentation)


Skills and Endorsements

- NMR (2D included)
- Western Blotting
- Cell Culture
- Molecular Biology
- Organic Chemistry
- Fluorescence Microscopy
- Confocal Microscopy
- Cell Based Assays (cell viability, IC_{50}, EC_{50}, reporter gene, BRET)
- AFM
- Radiochemistry
- Peptide Synthesis
- Surface Chemistry

Professional Services

1. Wrote JSPS postdoctoral fellowship for the 2018 grant application. This project is in collaboration with Dr. Hiroaki Suga at the University of Tokyo.
2. Wrote CIHR postdoctoral fellowship for the 2018 grant application. This project is in collaboration with Dr. Wenshe Liu at Texas A and M University.
3. Assisted in the writing and contributed to the work involved in Dr. Len Luyts’ 2018 CIHR research grant application.
4. Assisted in reviewing a Manuscript for Dr. Len Luyt. (Dr. Len Luyt was one of the primary reviewers).

Teaching Experience

1. Integrated Science 1001X Teachers Assistant (Winter 2019) – 35 students
2. Medicinal Chemistry 3393 Teachers Assistant (Winter 2019) – 280 students
3. Organic Chemistry 3373 Teachers Assistant (Fall 2018) – 65
4. Integrated Science 1001X Teachers Assistant (Winter 2018) – 35 students
5. Medicinal Chemistry 3393 Teachers Assistant (Winter 2018) – 160 students
6. Organic Chemistry 3373 Teachers Assistant (Fall 2017) – 65 students
7. Integrated Science 1001X Teachers Assistant (Winter 2017) – 32 students
8. Organic Chemistry 3373 Teachers Assistant (Fall 2016) – 70 students
10. Organic Chemistry 2213/2223 Teachers Assistant (Fall 2014 to Winter 2016) – 1200 students

Committees and Extra Circular
3. Molecular Imaging Journal Club at Western University. (2014 to present).

**Invited Lecture**

**Student Mentoring**

**Collaborations/Additional Projects**
1. Dr. Rithwik Ramachandran: bivalent ghrelin analogues targeting the GHS-R1a homodimer
2. Dr. Jeffrey Keillor: Fluorescent integrated stapled ghrelin (1-20) analogues for the targeting of GHSR-1a in cancer
3. Dr. David Perrin: $^{18}$F labeling of peptidomimetics using organotrifluoroborates
4. Dr. Alfonso AbizaidBucio: Cyanine-5 ghrelin analogues for imaging
5. Dr. Mario Perello: FITC ghrelin analogues for neuroimaging
6. Dr. Luba Sominsky: Cyanine-5 ghrelin analogues for imaging
7. Dr. Savita Dhanvantari: Cyanine-5 ghrelin analogues and $^{18}$F labelled ghrelin peptidomimetics for cardiac imaging

**Volunteer Experience**
3. Let’s Talk Science UWO. London, ON, Canada. (September 2015 to 2017)
5. Volunteer with Neuroradiologist at University Hospital in London, ON, Canada. (2015 to 2019)