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Abstract

The melanocortin system regulates a variety of physiological functions in humans, including skin pigmentation, steroid genesis, energy homeostasis, exocrine secretion, sexual function, analgesia, inflammation, immunomodulation, temperature control, cardiovascular regulation, and neuromuscular regeneration. Five different melanocortin receptors, belonging to the family of G protein-coupled receptors (GPCRs), as well as their endogenous peptide ligands make up the melanocortin system and are responsible to mediate several divers functions through intracellular signaling. The human melanocortin 4 receptor (hMC4R) via its endogenous agonist α-melanocyte stimulating hormone (α-MSH) and its endogenous inverse agonist agouti related peptide (AgRP) is involved in the regulation of energy homeostasis. Hence this signalling system is of great interest for the development of anti-obesity drugs as well as therapeutics for the prevention and treatment of anorexia and cachexia. Very recently, the cardioprotective effect of biased signaling ligands targeting the MC4R has also sparked great interest for the treatment of heart disease, the most common co-morbidity of obesity.

For this project, the active sequence of α-MSH, which binds to the hMC4R and activates the receptor, was inserted into four different, nature-derived peptide scaffolds, tachyplesin-1, protegrin-4, arenicin-3 and gomesin. These peptide scaffolds are animal derived antimicrobial peptides, and have been selected based on their structure which should be able to target and activate hMC4R due to their similarity to the endogenous ligand α-MSH. In this thesis I pharmacologically analyzed 14 novel MSH-peptide analogues that have been previously generated through ‘molecular grafting’ using solid phase peptide synthesis. By applying bioluminescence resonance energy transfer (BRET) and second messenger quantification assays, I determined their potency and efficacy for β-arrestin-2 recruitment as well as the production of cyclic adenosine monophosphate (cAMP) at the hMC4R .

Animal-derived, cysteine rich, antimicrobial scaffolds show great potential to target not only hMC4R and the melanocortin system but are a new and exceptional niche when it comes to the development of novel drugs targeting GPCRs.

Zusammenfassung

Das Melanocortin-System beeinflusst eine Vielzahl von Körperfunktionen im menschlichen Körper zu welchen Hautpigmentierung, Steroidbildung, Energiehomöostase, exokrine Sekretion, Sexualfunktionen, Schmerzlinderung, Inflammation, Immunmodulation, Temperaturkontrolle, Herz-Kreislauf-Regulierung und neuromuskuläre Regeneration gehören. Fünf verschiedene Melanocortin-Rezeptoren, die zur Familie der G-Protein-gekoppelten Rezeptoren (GPCRs) gehören, sowie ihre endogenen Liganden sind Teile des Melanocortin-Systems und für die Vermittlung dessen Funktionen durch intrazelluläre Signalübertragung verantwortlich. Der humane Melanocortin-4-Rezeptor (hMC4R) und sein endogener Agonist α-Melanozyten-stimulierendes Hormon (α-MSH) sowie endogener inverser Agonist Agouti Related Peptide (AgRP) regulieren die Energiehomöostase im menschlichen Körper und sind daher von großem Interesse für die Entwicklung von Medikamenten gegen Fettleibigkeit und der Vorbeugung sowie Behandlung von Anorexie und Kachexie relevant. In jüngster Zeit gewann auch die kardioprotektive Wirkung von „biased signaling“ des MC4R an Interesse für die Behandlung von Herzerkrankungen, der häufigsten Begleiterkrankung von Fettleibigkeit.

Für dieses Projekt wurde die aktive Sequenz von α-MSH, die an hMC4R bindet und den Rezeptor aktiviert, in vier verschiedene Peptidgerüste eingefügt welche auch als Scaffolds bezeichnet werden. Tachyplesin-1, Protegrin-4, Arenicin-3 und Gomesin bei denen es sich um aus der Natur – genauer gesagt aus dem Tierreich stammende, antimikrobielle Peptide handelt wurden hierfür verwendet. Diese Scaffolds ähneln dem endogenen Liganden in ihrer Struktur und sollten in der Lage sein, hMC4R zu aktivieren. Insgesamt 14 neuartige lineare Peptidanaloga wurden für dieses Projekt zuvor mittels Solid Phase Peptide Synthese und Verwendung der Fluorenylmethoxycarbonyl Schutzgruppe (Fmoc) synthetisiert um pharmakologisch mit Hilfe von Biolumineszenz-Resonanzenergietransfer (BRET) und zyklischem Adenosinmonophosphat (cAMP) Assay untersucht zu werden, um die β-Arrestin-2-Rekrutierung sowie die Produktion von zyklischem Adenosinmonophosphat (cAMP) am hMC4R zu bestimmen.

Von Tieren gewonnene, cysteinreiche, anti-mikrobielle Scaffolds zeigen großes Potential für die Entwicklung neuer Wirkstoffe, welche MC4R und das Melanocortin

System erreichen und beeinflussen können. Weiters eröffnet diese Art von Peptiden eine neue, vielversprechende Nische für die Entwicklung von neuen Medikamenten.

Acknowledgements

First of all, I want to thank Assoc. Prof. Priv.-Doz. Dr. Christian W. Gruber for giving me the opportunity to work on this project, supervising me throughout the process and taking time to always respond rapidly with constructive criticism to ensure constant progress. Special thanks to Edin Muratspahić PhD who took his time despite having an overflowing agenda and explained to me everything I had to know in the laboratory as well as trusting me to work with his materials. Thank you also for your positive energy, making everybody in the office laugh.

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Table of content

1 Introduction

For thousands of years nature has been a source of medicinal agents which humans relied on for the treatment of diverse diseases [1]. Receptors, enzymes, ion channels and transporters are the main classes of drug targets available for the treatment of diseases [2] with pharmaceuticals on the market ranging from natural products and their derivates to synthetic molecules [3]. Looking at these categories, natural products are an especially great source of leads for the development of drugs and persist to be the source of many active pharmaceutical ingredients (APIs) in medicinal use. Up until today, most compounds and medicines are sourced or based on structures from plants, microbes and animals and therefore also include synthetic or semi-synthetic molecules [4]. From 1981 to 2019, a total of 1881 new drugs where approved, 35% of which are unaltered natural products, botanical drugs, natural drug derivates and mimics of natural products and therefore are under the umbrella of nature derived molecules/ natural products [5].

Natural products show great diversity in structure and their biological effects such as hormonal and enzyme controlling activity, cell communication and host defense, which makes them interesting for pharmacological research [6]. They often present greater therapeutic properties such as higher selectivity, potency and stability *in vivo* than their human counterparts [7] as well as superiority when compared to purely synthetic compounds by taking advantage of active transport mechanisms in the body, made possible through their resemblance of biosynthetic intermediates or endogenous metabolites [8].

Among natural products are very different types of molecules such as polycyclic compounds, followed by simple compounds, steroids, penicillin derivates, macrocyclic molecules, nucleotides, peptides, sugars, nucleosides and those of other nature [3]. The molecules explored in this thesis are nature derived peptides, These types of peptides can be sourced from the plant and animal kingdom as well as fungi and bacteria [9].

1.1 Peptides in drug discovery

Up until today, over 7000 naturally occurring peptides where identified and carry functions as hormones, neurotransmitters, growth factors, ion channel ligands or anti-infectives in the human body [10]. Endogenous peptides have a pivotal role for functions in the human body and the understanding of deficit or excess of specific peptides can now be connected to the development of various diseases [6]. A big success for pharmacological research has been the synthesis of the peptides hormones oxytocin, vasopressin. The evolvement of peptide synthesis from solution-phase chemistry, taking up months to years of time, to solid-phase peptide synthesis in 1963 together with advanced purification methods where important steps for the pharmaceutical industry influencing and facilitating the way we are able to work nowadays [7].

In general, peptides are molecules usually containing fewer than 50 amino acids, linked by peptide bonds and are distinguished from small molecule chemicals and larger proteins by their size [11]. Currently used peptide drugs can be either categorized as traditional small molecules with a molecular weight of <500 Da (0,5 kDa) or much larger biologics typically >5000 Da (5 kDA) [11]. Small molecules are organic compounds able to penetrate cells easily enabled through their size and then can affect molecular pathways by targeting proteins [12]. Generally small molecules are oral bioavailable but might lack target selectivity, leading to a greater potential of the manifestation of unwanted side effects. Larger protein drugs, often need to be applied via injections, an application form historically suffering from poorer patient compliance. They can reach over 100 kDa and possess a are more complex secondary and tertiary structure than smaller peptides, which has to be maintained in different circumstances and are likely not oral bioavailable because of their metabolic instability [11]. Furthermore many protein therapeutics require posttranslational modifications to become biologically active [13]. Peptides and peptide drugs are characterized by properties such as their affinity for a biological target, their ability to bind to a specific receptor for a certain period of time to allow a sufficient effect target behavior (dissociation rate) and their ability to change the behavior of this target (efficacy) [14]. Another important aspect not only for endogenous receptor ligands but also drug development is the selectivity a ligand displays to a certain receptor over other targets. Selectivity allows to determine the manifestation of side effects a certain molecule might be able to induce. Endogenous

hormones, growth factors, neurotransmitters and signalling molecules as well as immunologic and defense agents typically show great binding specificity *in vivo* since these molecules have been established and modified throughout evolution until they were naturally optimized and able to bind to their target. [11]. From a pharmacological viewpoint, peptides are often potent and possess a safe mode of action which makes these molecules not only great inspiration and building bricks for the development of novel therapeutic but also potentially comprising enhanced pharmacological profiles [10]. Nature derived peptides are one form of peptides and stem from sources such as bacteria, fungi, plants, and animals and often display certain similarities with human endogenous peptide ligands. They can provide favorable therapeutic properties like higher selectivity, potency and in vivo stability when compared to the endogenous human ligands [7].

1.2 Cysteine rich peptides

Cysteine rich peptides (CRPs) can be found in both, the plant and animal kingdom. They usually contain less than 100 residues including several cysteine residues with the ability to form disulfide bonds. In many cases the N and C terminal of CRPs merge, and together with the disulfide bonds confers the peptide with its extraordinary properties including withstanding high temperature and changes in pH [15]. Not only play disulfide bonds a role in structure and function of a peptide, they possess constraint and very well defined 3D structures which can also result in increased potency, selectivity and permeability [16]. CRPs share a similar size, the conserved N-terminal region comprising a secretion peptide signal and a C-terminal domain rich in cysteines containing between 4-16 cysteine residues. Computational analyses of several plant genomes have previously reported CRPs making up to about 2% of expressed genes in certain plant species and mediate and regulate functions including growth and reproduction and defense [17]. Snakins are one class of AMPs and currently regarded as the most prevalent plant host defense peptides [18]. Further different AMP families are thionins, defensins, lipid transfer proteins, cyclotides, hevein-like proteins and knottin-type peptides [19,20].

Cyclotides are a big group of CRPs with many different functions, one of them being a defense strategy for the plant expressing it. Cyclotides usually comprise 28-37 residues in a characteristic arrangement which contains three intramolecular disulfide bonds at C1-C4, C2-C5 and C3-C6 as well as a head-to-tail cyclized backbone structure also referred to as cyclic cysteine knot (CCK) [15]. Cyclic peptides are extraordinary stable and resistant to several ways of proteolytic degradation making cyclotides a promising scaffold for molecular grafting with the potential to overcome challenges such as serum and gastrointestinal stability, oral activity and membrane permeability as well as other requirements therapeutics a facing [21].

Such cysteine rich peptides can also be used by some animals as their venom which is a very useful source of peptide leads since by nature they have to act fast, and need to be stable thereby making a promising starting point for further drug development [11]. For survival, as protection and predation, animals of many different species have developed the strategy of producing toxins, poisons and potential remedies which consist of a mixture of molecules including enzymes, proteins, and peptides, which are

not only able to harm prey, predators and humans but can also be investigated and exploited by humankind for drug development. One historical anecdote is the following, reported from Appian, a Roman historian stating that a doctor saved a very badly injured man bleeding strongly from a sword wound on his leg, by administration of a small amount of steppe viper (*Vipera ursinii*) venom, to stop the bleeding and thereby saving the man's life. Nowadays we do not have to work on the principal of trial and error anymore since drug discovery mostly begins with the screening of compound libraries which include a diversity of natural products with the potential to become new lead molecules [22].

1.2.1 Anti-microbial peptides

Antimicrobial peptides (AMPs) are gene encoded, widely distributed peptides in the plant and animal kingdom belonging to the group of CRPs. AMPs are conferred with chemical, thermal as well as proteolytic stability due to their formation of disulfide bonds making them interesting for pharmaceutical research [17,23,24].

In the first place, the purpose of AMPs is to neutralize a broad range of microorganisms like bacteria, fungi, and viruses thereby defending the living organism producing these peptides some of which even capable of exerting a cytotoxic effect on cancer cells. Over 800 AMPs are up to this date isolated and described and have been sourced from bacteria, fungi, protozoans, insects, fishes, amphibians, reptiles, aves, mammals and plants [25]. The classification of AMPs can be based on their source, activity, structure as well as the class of amino acid-rich AMPS seen in **Figure 1**. In total 2600 natural AMPs have been discovered with roughly one third being peptides produced by invertebrates. Usually their polypeptide chain varies from 15 to 40 amino acid residues with a typical mass below 25-30kDa but also shorter sequences of 5-10 amino acids as well as peptides with up to the size of 150 amino acid residues have been discovered. [26,27].

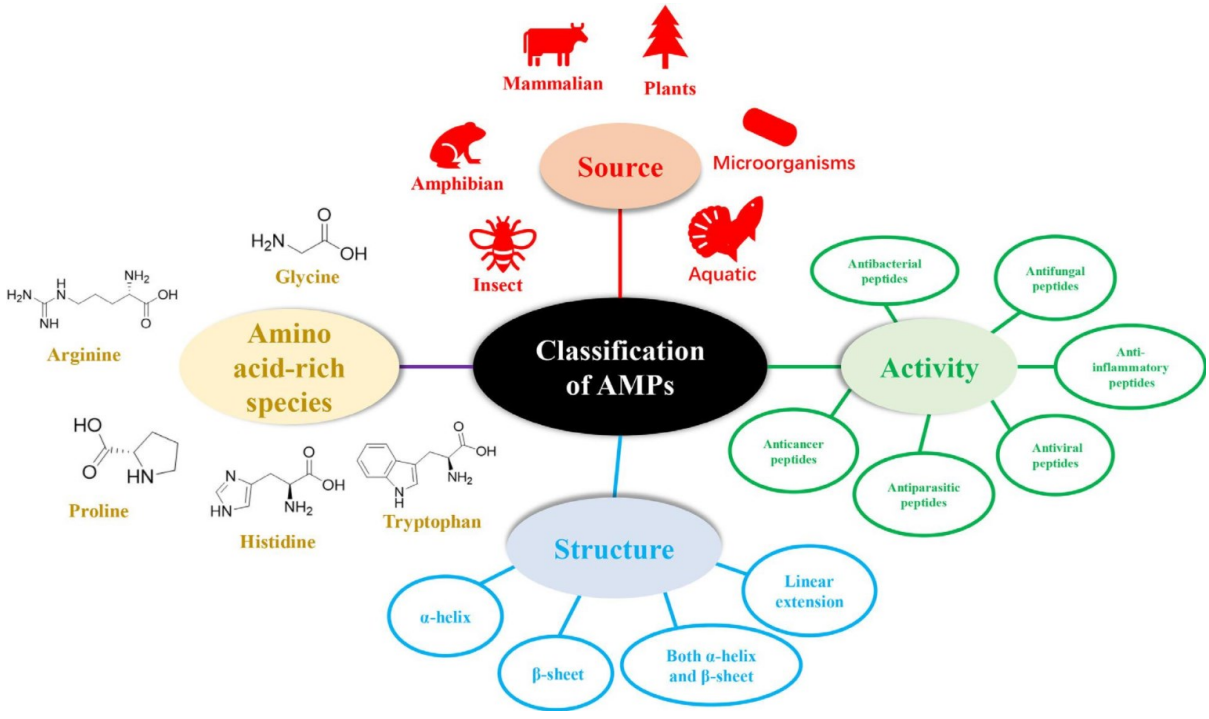


Figure 1. Classification of anti-microbial peptides [28].

AMPs can be, as recognized for now, divided in four main classes which are peptides with either α-helical, β-sheet, αβ, or non- αβ elements as seen in **Figure 2** [29].

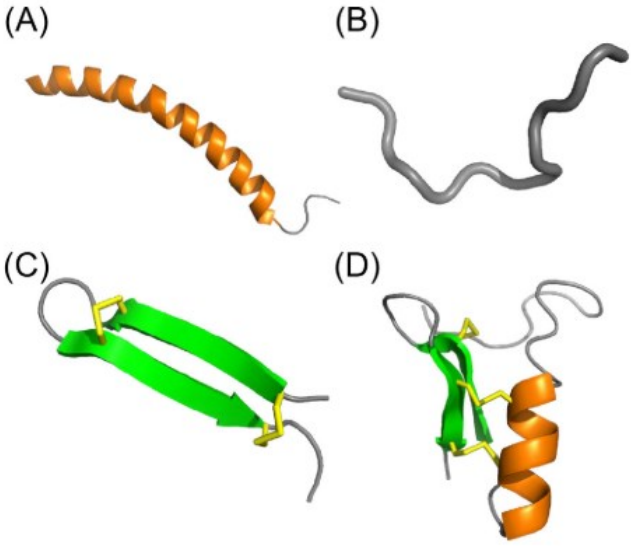


Figure 2. The four common classes of naturally occurring AMPs are shown as cartoon representing nuclear magnetic resonance (NMR) solution structures to highlight secondary structure elements. (A) LL-37 in typical α-helical conformation; (B) Extended AMPs like indolicidin usually don't adopt well defined 3D conformations; (C) peptides containing a β-sheet are typically stabilized by disulfide bonds such as the spider derived gomesin; (D) The insect CSαβ defensin phormicin for example contains both elements [29].

Additionally, there is a growing pool of more complex topologies which include post-translational modifications. The α-helices and β-sheet AMPs are further divided into four classes: linear α-helical peptides, linear extended structures which do not contain α or β elements but typically are rich in one amino acid such as Gly, Arg, Trp or Pro, furthermore β-sheet containing peptides often comprising one or more disulfide bonds and peptides involving both α and β elements [27,29]. While all peptides adopting a β-hairpin structure exert broad spectrum anti-microbial activity, those with CSαβ motif show a less abundant activity spectrum like insect defensins, that are mainly active against Gram-positive bacteria whereas some plant defensins, drosomycin and heliomycin are more peculiar and only active against fungi [30]. The most common shorter AMPs include cationic amino acids such as lysine and arginine, conferring these molecules with their usual net positive charge. About 50% of the sequence include the amino acids leucin, isoleucine, valine, phenylalanine and tryptophan which are hydrophobic and enable the AMP to interact and penetrate membranes of cells [31].

AMPs are especially interesting for the development of antibiotics since resistance to anti-micro biotic agents is increasing and new molecules need to be developed to overcome limitations of present drugs. Previously, natural occurring AMPs have been engineered into new molecules to at least partially defeat practical challenges. Rational for the investigation into these peptides was the theory of the evolutionary properties of action these molecules comprise and the potential to obstruct bacteria from the development of resistance [32].

The protective role of insect defensins which target the membrane of Gram-positive bacteria is exerted through a channel-forming mechanism. Insect defensins have been used as templates for the design of therapeutics like the engineering of the carboxyl-terminal β-sheet of navidefensin2-2 derived from *Nasonia vitripennis* because it confers structural similarity to naturally occurring microbicidal β-hairpin peptides. The findings of this experiment show the anti-infective potential of the β-sheet subdomain of insect defensin proteins [33].

In general cysteine rich peptides comprising the family of anti-microbial peptides have been of great interest for stable protein engineering and have been successfully used for stable peptide grafting. The cysteine residues of these molecules form disulfide bridges conferring them with stability against high temperature, proteolysis and high pH. Furthermore, when engineering novel proteins and peptides, CRPs allow for substitution in their inter-cysteine loops, making it possible to exchange non-cysteine residues with high tolerance [15].

The human body itself produces host defense peptides such as a variety of α-defensins some of which are expressed in leukocytes and neutrophils and possess a β-sheet structure with 3 disulfide bonds. α-Melanocyte stimulating hormone, which will be thoroughly discussed in chapter **1.4 The melanocortin system.** This peptide is expressed primarily expressed in the pituitary gland but also present in defense cells and periphery sites including the skin and its anti-microbial properties. Its anti-microbial activity through membrane permeabilization have been suggested additional to several other features which overlap with those of AMPs such as being a linear short peptide consisting of 13 amino acids, its positive charge which in its bioactive form adopts an alpha helical form and its “age” being an ancient peptide that existed when the innate immunity of organisms was the only defense available [34].

Through genome database mining, using conserved sequence patterns as well as motifs of known sequences it is possible to detect structures with related sequences. For example has the 6-cysteine pattern which is typical for defensins, used for the identification of novel human and murine β-defensin genes [6].

1.2.2 Peptide synthesis and molecular grafting

Three different approaches can be used for the construction of peptides and proteins, first the stepwise synthesis, in which one amino acid at a time is added onto the next. Second fragments of individual peptide strands which have initially also been constructed step by step, can be assembled, purified, and covalently linked. Third, also called the directed assembly uses stepwise constructed peptide strands, purifies them and then noncovalently drives them to associate into protein-like structures [35]. Open chain and cyclic peptides are synthesized from C- to N-terminus whereas open chain peptides are often synthesized using a Fmoc chemistry approach. Fmoc (9-fluorenylmethoxycarbonyl) but also Boc (tert-butyloxycarbonyl) are protecting groups used in solid phase peptide synthesis (SPPS). The process includes a resin, onto which the first amino acid is loaded, and a mixture is added to allow coupling of a further amino acid. Then the Fmoc protecting group is cleaved with a base like piperidine before coupling onto the next residue [36].

The name “grafting” stems from horticulture where a new organism can be created through fusion of the scion of one plant to the stock of another and thereby featuring the desired properties of both plants. Molecular grafting is an approach to stabilize bioactive peptides and potential drug leads [37]. The process comprises the insertion of a bioactive peptide sequence (epitope) into a molecule with favorable biopharmaceutical properties (scaffold) as seen in **Figure 3** **Figure 3.** The unstable epitope contains the active sequence to target a receptor; 2 the sunflower trypsin inhibitor-1 scaffold which comprises a disulfide bond, is used as a scaffold by “cutting” a part from the loop of and 3 grafting the epitope into the remaining framework thereby generating a novel peptide [39].[37]. For drug design many these scaffolds can be sourced of, or inspired by nature and feature biologically relevant molecular scaffolds [38]. Grafting with CRP for example has led to success for possible treatments of multiple diseases such as cancer, diabetes, multiple sclerosis and are promising for the treatment for infectious diseases [15].

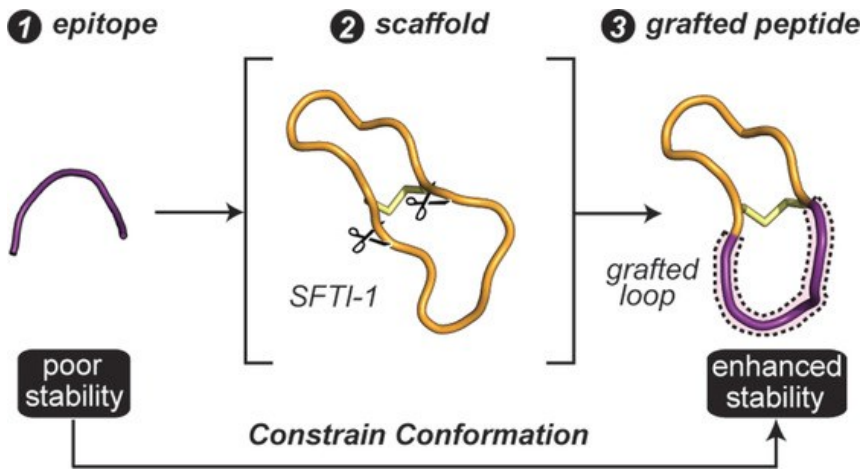


Figure 3. The unstable epitope contains the active sequence to target a receptor; 2 the sunflower trypsin inhibitor-1 scaffold which comprises a disulfide bond, is used as a scaffold by "cutting" a part from the loop of and 3 grafting the epitope into the remaining framework thereby generating a novel peptide [39].

A good example of CRPs which have been used as a grafting template is the prototype cysteine rich and also cyclic peptide kalata B1, extracted from the African plant Oldenlandia affinis which has long been used by African women during child birth as a native drug called kalata-kalata [40]. As a cyclotide, kalata B1 confers a head-to-tail cyclized backbone and cysteine knot core and possesses thermal, chemical and enzymatic stability [41]. These properties make it a very stable framework for grafting which lead to the cyclotide being used as a scaffold for the development of many drugs including the treatment of multiple sclerosis (MS), which was achieved by inserting peptide sequences of myelin oligodendrocyte glycoprotein (MOG) as an epitope into the kalata B1 scaffold, creating a peptide displaying ability to prevent MS development in a mouse model of MS [42].

Another example for peptide grafting studies is the sunflower trypsin inhibitor SFTI-1 derived from sunflower seeds. It consists of 14-amino acids comprising two short anti-parallel β-strands including one disulfide bond and belongs to the Bowman Birk Inhibitor (BBI) class of proteins, which have been studied for their anti-inflammatory and anti-cancer properties. With this knowledge in mind SFTI-1 showed great therapeutic potential [16]. Some of its specific properties are the ability to inhibit trypsin at a low to nanomolar range, its stability even in acyclic form conserving its trypsin inhibitory effect in human serum. Additionally, it is a candidate for the development of small and stable binders to two receptors, which then can be used to diagnose and

treat tumors with heterogenous expression of multiple receptors [43]. SFTI-1 grafting has been extended to a very valuable class of drug targets currently attracting significant attention, the G-protein-coupled receptors (GPCRs). For example has the SFTI-1scaffold been used to graft ligands for the somatostatin receptors, melanocortin receptors and the bradykinin B_1 receptor all of which belong to the GPCR family [44].

1.3 G-protein coupled receptors

Comprising approximately 830 members, GPCRs are the largest membrane protein family in humans and eukaryotes [7,45,42]. Located at the surface of the cell these type of receptors are responsible for the regulation of a large variety of cellular processes via signal transduction. Approximately half of all GPCRs have sensory functions such as mediating olfaction, taste, perception of light, and pheromone signaling. The other, non-sensory GPCRs mediate their signaling through their ligands ranging from small molecules over peptides to proteins [46].

GPCRs can be, based on the similarities of their amino acid sequence, grouped into six categories for distinction: class A also called rhodopsin-like receptors, make up over 80% of all human GPCRs; Class B the secretin-like receptors and adhesion receptors; Class C or metabotropic glutamate receptors; Class D or pheromone receptors; Class E or cAMP receptors which are only found in non-mammalian species and class F also known as frizzled/smoothened receptors [47,48,49] . Class A receptors are the most abundant class of GPCRs in humans with over 719 receptors half of which are involved in smell or vision while the other half are targets for disulfide ligands such as hormones and neurotransmitters [50].

The characteristics for GPCRs include an extracellular N-terminus, an intracellular C-terminus as well as three interhelical loops on both sides of the cell membrane leading to a total of seven transmembrane α-helices spanning across the cell membrane, giving receptors of this family the name seven transmembrane (TM) receptors. GPCRs hold orthosteric binding sites for their ligands such as peptides as well as allosteric binding sites where ligands can bind and modulate receptor activity [47].

As a visual reference for the most abundant human GPCR, the structure of a class A GPCR, more specifically the human melanocortin 4 receptor can be seen in **Figure 4**, showing the receptors placement in the cell membrane and scheme of the 7 TM domains. Characteristic for a class A GPCR is a short and disordered N-terminal even though exceptions such as the glycoprotein hormone receptor with a well ordered and larger N-terminal domain region exist [50].

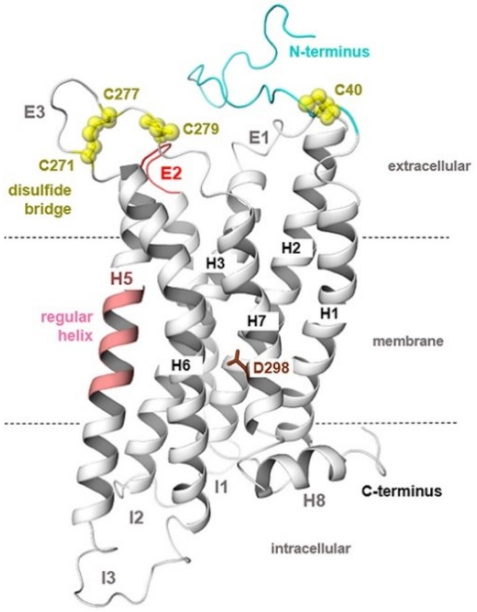


Figure 4. Three-dimensional model of the human melanocortin 4 receptor, a class A GPCR in its inactive state. Characteristic for this receptor is the bulk in the fifth transmembrane domain caused by highly conserved proline which in the MCRs is substituted by methionine. The second extracellular loop in the MCRs is shorter compared to other GPCRs [51].

Because of their multifold range of influence in physiological processes and their druggable sites accessible at the cell surface, GPCRs have long been an interesting target for the development of new drugs [52]. The proportion of human protein drug targets in major families as well as the proportion of small-molecule drugs targeting major families can be seen in **Figure 5**.

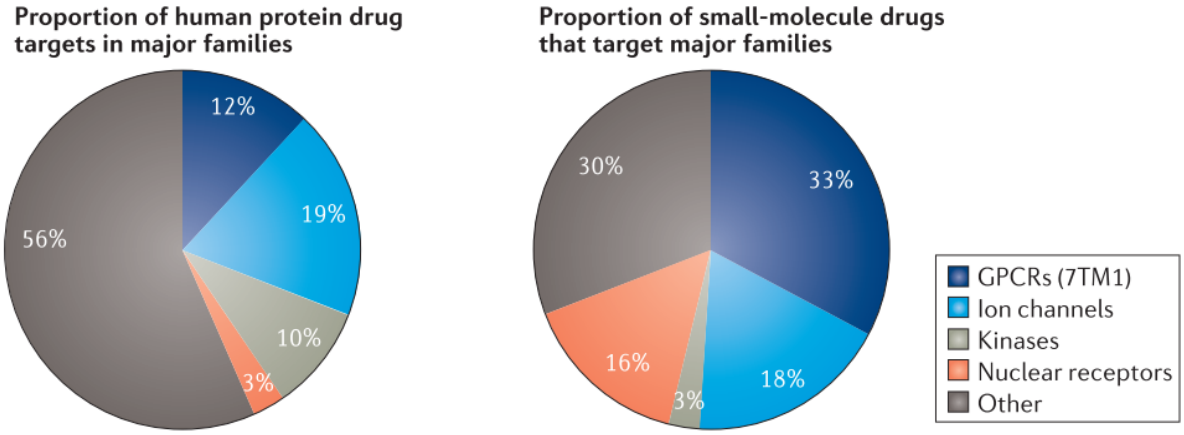


Figure 5. Major protein families as drug targets in humans. Shows the distribution of drug targets by gene family on the left and by the fraction of drugs which target these families on the right side [53].

Ligands of GPCRs can be small hormones and neurotransmitters and proteins such as chemokines which can bind to the receptor and thereby leading to structural changes [54]. These ligands are very diverse with each one of them comprising a certain characteristic of activating or deactivating its target receptor which is referred to as efficacy. In agreement with the ligand's efficacy, they can be classified as full agonists and partial agonists activating the receptor fully or partially and antagonists or inverse agonists deactivating their target [55]. A way to distinguish whether a receptor is the target of a peptide or a non- peptide principal-component analysis can be performed and reveal differences such as the size of the receptors binding cavities. The sequence and structure characteristics of GPCRs can be determined, and has in several analyses lead to the knowledge that the β-sheet, a long ECL2 and large binding cavity are able to facilitate binding of large proteins or peptides [56]. Important for the functionality and ability to transmit signals through the membrane of the cell of GPCRs is their ability to change in shape and transition between different conformations. An enormous breakthrough was the determination of GPCRs structure through crystallography leading to progress in the understanding of the structural basis for dynamic signalling processes [54].

1.3.1 GPCR signalling

The activation of GPCRs is an allosteric process which couples the binding of an agonist to G-protein recruitment with the outward movement of the transmembrane helix 6 (TM6), a process which yet has to be fully understood [57]. Upon activation of a GPCR, signalling can be initiated through heterotrimeric G-proteins which consist of Gα, Gβ and Gγ subunits and can belong to one of the following G-protein families: G_s is stimulatory, $G_{i/o}$ is inhibitory [58], $G_{q/11}$ activates β-isoforms of the phospholipase C which downstream mobilizes intracellular Ca^{2+} [59] and $G_{12/13}$ specifically targeting RhoGEFs which are structural domains comprising guanine nucleotide exchange factors (GEFs) able to activate and regulate small GTPases in the Rho family [60]. Additionally, there are G-protein independent pathways able to induce physiological changes such as G-protein coupled receptor kinase (GRK)-mediated phosphorylation as well as arrestin coupling. In inactive state, the Gα subunit is bound to guanosine diphosphate (GDP) and associated with the Gβγ dimer forming an inactive heterotrimer. After receptor activation and a change of conformation, GDP can dissociate from the Gα subunit allowing guanosine triphosphate (GTP) to bind and the dissociation of the Gα subunit from Gβγ subunit as seen in **Figure 6**. Gα subunits target the effectors adenylyl cyclase, cGMP, phosphodiesterase, phospholipase C and RhoGEFs in contrast to Gβγ subunits which possess the ability to recruit GRKs to the membrane and regulate G-protein coupled inwardly rectifying potassium channels, voltage dependent Ca^{2+} channels, adenylyl cyclase, phospholipase C, phosphoinositide 3 kinase and mitogen-activated protein kinases [58].

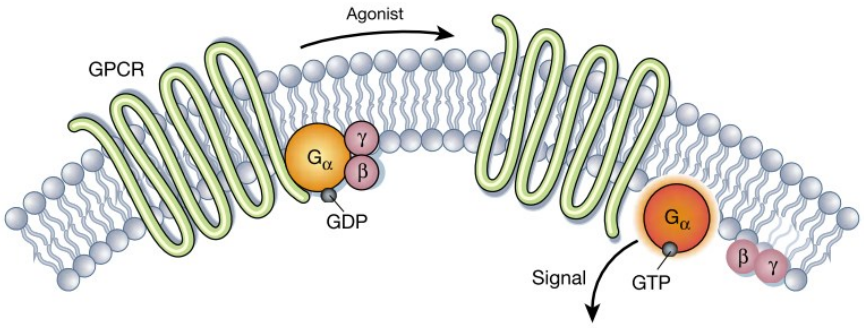


Figure 6. Scheme of general GPCR signal transduction upon agonist stimulation. In unstimulated state the Gα subunit is bound to GDP and Gβγ subunits. When stimulated by an agonist, Gα dissociates from the receptor and Gβγ subunits and exchanges GDP for GTP leading to further signals [61].

Signalling patterns can besides the different G proteins also be regulated by other proteins such as arrestins (β-arrestin 1 and 2 isoforms), PDZ-domain-containing scaffolds and non-PDZ-scaffolds such as A kinase anchor proteins (AKAPs) which initiate or control their own distant signalling pattern. Arrestins serve a negative regulatory effect in GPCR signalling through hindering the receptor in its active state from binding to the heterotrimeric G-proteins thereby desensitizing signalling and furthermore targeting GPCRs occupied by a ligand for endocytosis [37,49]. Because of their presence in all cells, β-arrestins can function as desensitizers of most GPCRs with the exception of rhodopsin and not only serve in termination of GPCR signaling, but can also internalize the receptor and are crucial for receptor degradation and recycling [62].

Ligands for GPCRs can either be agonists, able to promote or stabilize the conformational changes which result after activation of the heterotrimeric G proteins and generation of second messengers, and the counterpart are antagonists, which are blocking the activation [63]. Nowadays it is also known, that an agonist can not only cause tissue activation but possesses the ability to show multiple behaviors on its target receptor. Furthermore, the stimulus-response cascades can be biased towards a certain pathways over the other which leads to agonist functional selectivity further explained in **Figure 7Fehler! Verweisquelle konnte nicht gefunden werden.** and can be a great advantage in the development of agonist therapeutics [14,63].

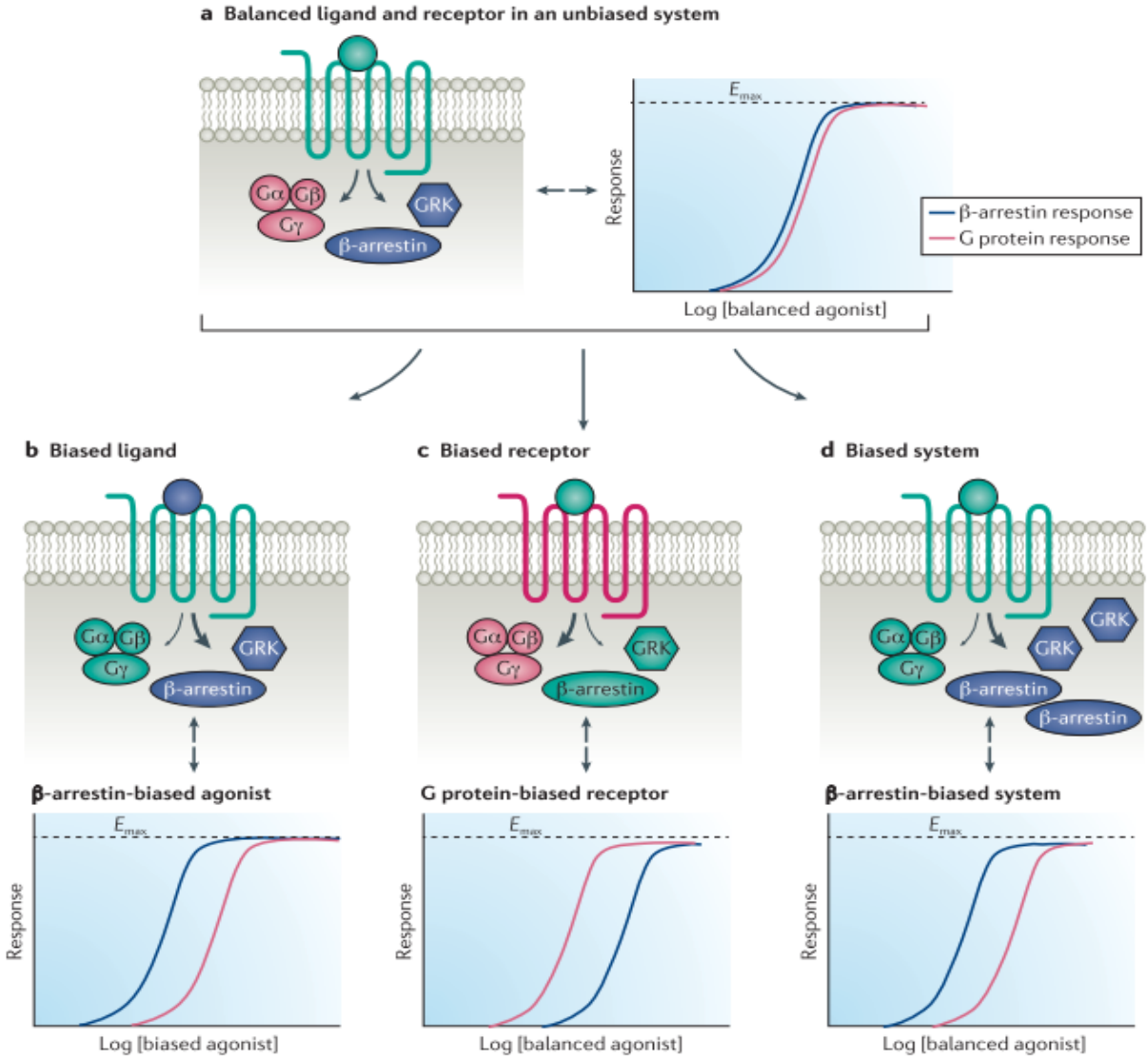


Figure 7. The three general mechanisms of biased signalling: **a.** The balanced agonist binds to a balanced receptor in an unbiased system. It is possible that equivalent potencies for two different pathways such as G protein and β-arrestin are displayed under the same assay conditions. **b:** The complex of biased ligand-receptor and effector generates a distinct conformation, that signals preferentially through one certain pathway rather than the other e.g. β-arrestin biased relative to G protein biased. Under the same assay conditions, a β-arrestin bias may display a left shift in potency relative to the G protein pathway. **c:** Biased receptors such as receptors with a G protein bias can lack C-terminal phosphorylation sites which are necessary for the recruitment of β-arrestin leading to the preferred signalling through G protein even though being stimulated by a balanced ligand displaying a left shift in potency of one pathway relative to the other which under same assay conditions may not be observed at an unbiased receptor. **d:** Differential expression of signalling effectors or other cofactors for example higher expression of the G protein-coupled receptor kinase (GRK) and/ or isoforms of β-arrestin can lead to a system bias towards β-arrestin pathway as shown in the figure. E_{max} is the maximal effect a ligand can produce [64].

Besides being activated by agonists, it has to be mentioned that GPCRs are capable of transitioning into an active state and are able to couple to G proteins which is referred to as basal activity [50].

1.3.2 GPCRs as drug targets

Besides ion channels, kinases, nuclear hormone receptors or proteases, GPCRs are one of the protein families that are used as main drug targets with ~27% of the global marketed drugs functioning on this receptor family. About 475 drugs or ca. 34% of all by the US Food and Drug Administration (FDA) approved drugs are together able to act on 108 different GPCRs [52]. The number of identified peptides targeting GPCRs has risen since the 1980s. However, with the recognition of nature-derived peptides as GPCR ligands and their potential in drug development between 2015 and 2018 there has been a significant increase in newly identified ligands [9].

The two endogenous peptides oxytocin and vasopressin are targeting class A GPCRs and where the first chemically synthesized peptides clinically used in the 1950s. With drugs of class A GPCRs mainly being agonists the evaluation of oxytocin and vasopressin accommodated the basis for the examination of properties endogenous ligands and aid to the strategies of drug development [65]. Biased agonism can be a very interesting research approach for drug development as well. For example, agonistic drugs such as morphine and fentanyl targeting the μ-opioid receptor provide analgesia but are limited by their addictive potential and negative side effects. At the same time an antagonists of the very same receptor, namely naloxone can be used for the treatment of substance abuse and opioid overdose. While studying the β-arrestin signalling on the μ-opioid receptor it has been suggested, that a bias towards the G-protein signalling is likely to increase the analgesic effect while at the same time showing less side effects showing us that each individual signaling pathway can lead to a certain result and needs to be investigated [64].

Some of the problems occurring when developing a drug able to target a GPCR is the way a molecule can bind to the receptor. So is for example the orthosteric binding site of the muscarinic M_1 receptor which is a target for agonists potentially able to alleviate cognitive decline during neurodegeneration almost identical to the binding site of the other four related muscarinic receptors which all are targeted by the endogenous ligand acetylcholine. Because of their similarity receptor subgroups can be activated leading to dose-limiting effects such as gastrointestinal disturbance and cardiovascular effects [50]. Naturally occurring GPCRs ligands can be sources from plants, animals fungi and bacteria and a graph of the distribution of these sources can be seen in

Figure 8. However, the development of drugs from natural peptides is limited due to their pharmacological properties such as short half-life, easy being degraded, shortfall of bioavailability, poor permeability and so on. For drug development there is a huge demand for the development addressing these drawbacks before a peptide can become an efficient drug [65].

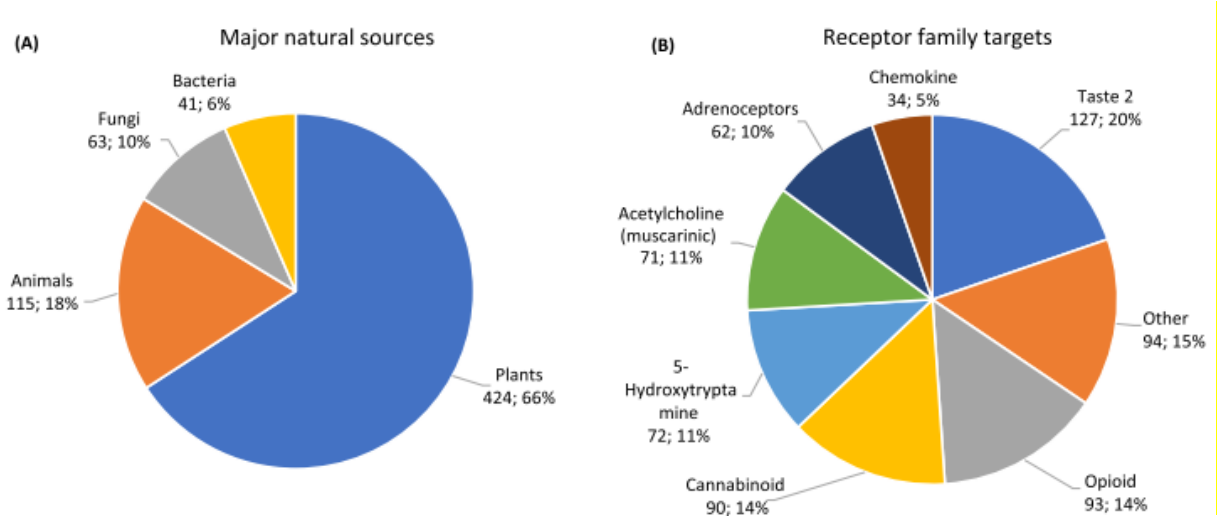


Figure 8. The diversity of naturally occurring GPCR ligands (A) plants are the most common source of nature-derived GPCR ligands. (B) The leading GPCR targets are taste 2, aminergic, opioid and cannabinoid receptors. Modified after [9].

For the prediction and identification of compounds with potential drug-like properties, able to target GPCRs, computational approaches have become essential. 3D structures for diverse GPCRs have been solved leading to the “golden age” of structural biology for GPCRs. In crystal structure a GPCR structure can be shown in inactive state for example bound to an antagonist. In general the arrangement of the TM domains, location of the orthosteric- and allosteric binding site as well as the biased ligand binding sites or homo- or hetero- oligomerization of receptors and the structural changes upon activation/ inactivation can be revealed using this technique leading an abundancy of chemical data accessible for research [66].

1.4 The melanocortin system

The melanocortin system is formed by a family of five related seven trans membrane G-protein coupled melanocortin receptors (MC1R- MC5R) and its mediating peptides, adrenocorticotropin (ACTH) and α-, β- and γ—melanocyte stimulating hormones (MSH). Melanocortins are produced by the gene encoding pro-opiomelanocortin (POMC) together with β-endorphins and exhibit diverse functions as hormones and neuropeptides. MSHs mediate through a family of five class A GPCRs, MC1R-MC5R [67], while additionally being modulated by the two ancillary proteins mahogany and syndecan-3. Their physiological functions include skin pigmentation, steroid genesis, energy homeostasis, exocrine secretion, sexual function, analgesia, inflammation, immunomodulation, temperature control, cardiovascular regulation, and neuromuscular regeneration [68]. Several analogues of melanocortins have been synthesized in the past with the aim to improve pharmacological characteristics such as receptor selectivity, potency, proteolytic stability, bioavailability, duration of action, biodistribution ,especially across the blood brain barrier, and for the obtainment of selective antagonists [69]. Novel α-MSH peptide ligands as well as the native peptide exert antimicrobial activity against bacteria and yeasts and have also been investigated for their potential to treat infections in humans showing the versatility of how endogenous receptor ligands could be modified and used as very distinct inspirations for further research [70].

Uniquely the melanocortin system also contains two endogenous melanocortin antagonists which regulate melanocortin signaling: agouti-signaling protein (ASIP) and agouti related peptide (AgRP), also referred to as agouti related protein [67]. While ASIP is a potent antagonist at MC1R and MC4R, AgRP which is mainly produced in the arcuate nucleus of the hypothalamus and the adrenal gland, acts as a high-affinity antagonist of the MC3R and MC4R [67,71,72]. The human AgRP consist of 132 amino acids, a cysteine rich C-terminal domain, an acidic middle part and the signaling sequence. Since its function is to antagonize MC3R and MC4R it elicits an increase in food intake upon injection into the intracerebroventricular region and may be useful in the development of treatments for disorders in which negative energy balance leads to low body weight such as anorexia nervosa and cachexia. At the mouse MC4R AgRP has an antagonistic potency of 0.8nM [73].

Another part of the melanocortin system are the melanocortin receptor accessory proteins (MRAPs) possessing modulatory functions such as the regulation of melanocortin mRNA expression and functional expression of the MCRs. In the case of MC2R certain MRAPs are assumed to have an influence on the receptor sensitivity but could additionally influence and promote the presence of MC2R in the membrane. Same might be the case for the functions regulated by the other MCRs [72].

In the human body five melanocortin receptors are expressed which each one of them being linked to the generation of cyclic adenosine monophosphate (cAMP) through the stimulatory G protein (G_s) and adenylyl cyclase. The five receptor subtypes are responsible for individual functions and have distinct affinity for their endogenous agonists and antagonists [68].

MC1R is involved in pigment regulation leading to the association of several MC1R variant alleles with red hair, fair skin, and increased skin cancer risk. MC1R is mainly expressed in melanocytes and stimulated by α-MSH or ACTH which results in increased melanocyte proliferation, dendricity, melanosome transfer and an elevation of the ratio between the darker eumelanin pigment over the red/ yellow pheomelanin pigment [74].

MC2R is expressed in high quantities in the adrenal cortex and is activated specifically by ACTH. MC2R controls the steroid genesis [75]. Mutations of MC2R make up to 25% of familial glucocorticoid deficiencies (FDG), an autosomal disorder presenting itself with high plasma ACTH levels but cortisol deficiency eventually leading to recurrent infections or death of hypoglycemia [72].

MC3R influences body composition, natriuresis, immune function and circadian rhythm. It is expressed in the hypothalamus and limbic region of the brain as well as peripheral tissue. When observing MC3R knockout mice, they displayed reduced lean body mass, increased fat mass and accelerated diet-induced obesity [76].

MC4R holds a key position in the regulation of energy homeostasis and thereby influences body weight. The receptor is expressed in the hypothalamus of the brain and is validated as a therapeutic target to treat obesity based on pharmacological and human genetic evidence [77]. Furthermore it has been revealed that MC4R has a critical role in regulating the structure and functionality in the hippocampus of the brain [78].

MC5R is the last MCR to be cloned and characterized. The receptor is widely expressed in the central nervous system and several peripheral organ systems in humans and is suggested to play a key role in governing immune reaction and inflammatory response and seems to be pivotal regulation sexual behavior, thermoregulation and exocrine secretion according to evidence. Selective MC5R agonists have demonstrated positive effects in immune-mediated diseases, metabolic endocrinopathies and conditions lite glomerular diseases, dry eyes, skin and mouth [79].

All of the five melanocortin receptors show unusually short extracellular and cytoplasmatic tails with the MC2R being the smallest of hundreds of identified GPCRs [72] . The five MCRs and their main characteristics are summarized in **Table 1**.

Table 1. The five melanocortin receptors and their characteristics modified from [80].

Receptor	Expression site	Known function	Agonists	Antagonists
MC1R	Melanocytes	Pigmentation (ratio eumelanin: pheomelanin)	α-MSH, β-MSH, ACTH > γ-MSH	Agouti
MC2R	Adrenal cortex	Adrenocortical steroidgenesis	ACTH	/
MC3R	CNS, GI tract, kidney	Energy homeostasis, natriuresis	γ-MSH, α-MSH, β-MSH, ACTH	AgRP
MC4R	CNS	Energyhomeostasis, erectile function	α-MSH, β-MSH, ACTH, > γ-MSH	AgRP, Agouti
MC5R	Exocrine cells	Synthesis and secretion of exocrine gland products	α-MSH, β-MSH, ACTH, > γ-MSH	/

Melanocortin based drugs are currently being considered or developed for the treatment of many pathologies such as skin cancer and other cutaneous disorders, anorexia, cachexia, obesity, erectile dysfunction, inflammatory diseases, pain and nerve injury [68]. Quite recently, three peptides have been approved by the FDA to target receptors of the melanocortin system: Afamelanotide, used for erythropoietic protoporphyria, a gene defect leading to photosensitivity of the skin, has been

approved in October 2019. Bremelanotide was approved in June 2019 for the treatment of hypoactive sexual desire disorder and targets melanocortin receptors. Last but not leas setmelanotide used as obesity treatment in certain cases by targeting MC4R has been approved in November 2020 and will be further discussed in chapter 1.3.3 [81]. With the aim to improve selectivity for one MCR, potency, proteolytic stability, bioavailability, duration of action as well as biodistribution, several ligand analogs have previously been synthesized and pharmacologically characterized [69]. One of the main goals for the development of therapeutic agents to target the MCRs but also GPCRs in general is to identify a drug, able to produce a desired effect while only leading to minimal side effects which accomplishable for example through the synthesis of receptor subtype specific agonists or antagonists [82].

1.4.1 The Melanocortin 4 Receptor

The MC4R is a seven-transmembrane domain receptor belonging to the melanocortin receptors which are under the class A/ rhodopsin receptor subcategory. In humans MC4R plays a major role in energy homeostasis, more specifically the regulation of body weight through modulation of energy input and expenditure thereby generating a balance leading to neither weight gain, nor loss [71]. Imperative for this balance is the adequate function and expression of MC4R in the central nervous system (CNS) with loss of function of the receptor through gene mutations being the root of the, to this date, most frequent cause of monogenic overweight and /or obesity [45,83], effecting about 1-5% of severe obese individuals. The regulation of bodyweight through MC4R has been recognized as sensitive to quantitative variation in MC4R expression which was observed in MC4R knockout mice. While mice with loss of only one MC4R allele show an intermediate obesity phenotype, MC4R knockouts established severe obesity [71]. Obesity-associated MC4R mutations are generally sorted into functional classes like the loss-of-function mutations which is caused by defective expression (class I mutants), cases in which the trafficking of the receptor is disrupted (class II mutants) and those with decreased binding affinity (class III mutants), or a defective coupling of the receptor to the stimulatory G-protein ($G\alpha_s$) which normally activates the main signalling pathway of MC4R (class IV mutants) [84]. But there is one more mutation possible, the gain-of-function which in the case of MC4R has recently been associated with leanness through its increased receptor activation [85]. Additionally the activation of MC4R can be influenced by accessory proteins for example the melanocortin receptor accessory protein 2 (MRAP2) [71].

Together with MC4R the MC3R is expressed in the (CNS) [68] and both receptors are working together closely in establishing energy homeostasis. While the main function of MC4R is to control feeding behavior through modulation of hunger and appetite, MC3R acts mainly as a regulator for feeding efficiency [86]. The melanocortin system has one unique characteristic because it bears an endogenous inverse agonist/ antagonist. Agouti-related peptide (AgRP) which to the current day is the only one of its kind described for GPCRs besides agouti-signalling protein for MC1R. AgRP is secreted by AgRP-expressing neurons localized in the ARC when leptin levels are low, thereby inhibiting MC4R and enhancing the sensation of appetite [45].

Agonists of the MC4R contain a conserved tetrapeptide sequence His-Phe-Arg-Trp which is the minimal sequence required for receptor activation [87–89]. Using these tetrapeptide sequence, many approaches to develop potent agonists have been already performed in the past. Melanotan, [Nle^4, $_D$-Phe^7]-α-MSH (NDP-α-MSH) is one of them and was synthesized over 40 years ago by Sawyer et al. This peptide is resistant to degradation by serum enzymes and possesses increased biological activity with a potency 26 times as high as α-MSH with the data being determined in the frog skin bioassay as well as activation of mouse melanoma adenylate cyclase [90]. Furthermore the efficacy of melanotan is in the nanomolar range for all the MCRs except MC2R [91].

MC4R shows the same typical features as other GPCRs comprising seven trans membranes which are connected by intra- and extracellular loops. The N-terminus is the extracellular and the C-terminal the intracellular tail and in between there are highly conserved amino acids in each helix which are responsible for the common structural properties namely kinks and bulges but also intramolecular interactions, essential for its intrinsically encoded signal transduction. MC4R but also the other MCRs differ from other GPCRs in its relatively short second extracellular loop (ECL2) with MC4R containing only four amino acids as well as the absence of the highly conserved disulfide bridge between ECL2 and trans membrane helix 3 (TMH3) [51]. The sequence of the hMC4R can be seen in the **Appendix** at page 89.

Very recently, the crystal structure of the receptor was published and provided a great insight into the inactive state of the receptor bound to its antagonist SHU9119 which can be seen in **Figure 9**. The study shows the importance of calcium ions which mediate and/or support antagonist binding [45]. Furthermore, the active human MC4R structure, bound to setmelanotide, a cyclic peptide agonist was determined in complex with the Gs heterotrimer at a resolution of 3 Å using cryo-EM revealed that MC4R differs from previously reported class-A GPCR-G-protein complexes [92].

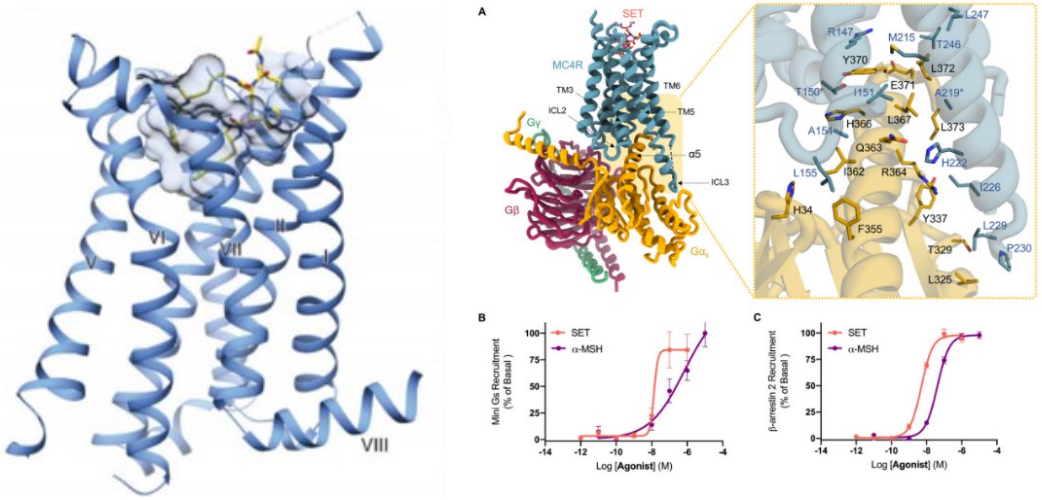


Figure 9. Structure of MC4R in inactive state bound to the antagonist SHU9119 revealing the importance of calcium ions for antagonist binding [85] and the even more recently revealed structure of the activated receptor bound to setmelanotide [92].

1.4.2 MC4R signalling

The human body can maintain a rather constant weight through signals from the periphery which reflect fat stores and nutritional state including nutrients and peptides which are produced by white adipose tissue, pancreas and the gut. Leptin, insulin, ghrelin, glucagon-like peptide-1, peptide YY as well as cholecystokinin are signals which in the hypothalamus induce an appropriate change in both appetite and energy expenditure according to the bodies feeding state to ensure homeostasis of body weight [69].

After the detection of receptor mutations in obese patients the function of MC4R in body weight regulation through the leptin-melanocortin pathway became more evident and the gates for more research have been opened [93].

Two neural populations in the arcuate nucleus, the proopiomelanocytes/ cocaine and amphetamine regulated transcript (POMC/CART) and neuropeptide Y/ agouti related peptide (NPY/AgRP), are modulated by signals from adipose tissue or gut and produce either MSHs or AgRP which are able to either activate or block MC4R [69]. While α-MSH induces negative energy balance therefore inducing catabolism, AgRP binds selectively to neural MCRs and antagonizes the endogenous agonist, but also has the ability to block ligand-independent signalling, thereby increasing food intake and thereby representing the anabolic arm of the melanocortin system [86].

A scheme of the melanocortin system and its involved components can be seen in **Figure 10**.

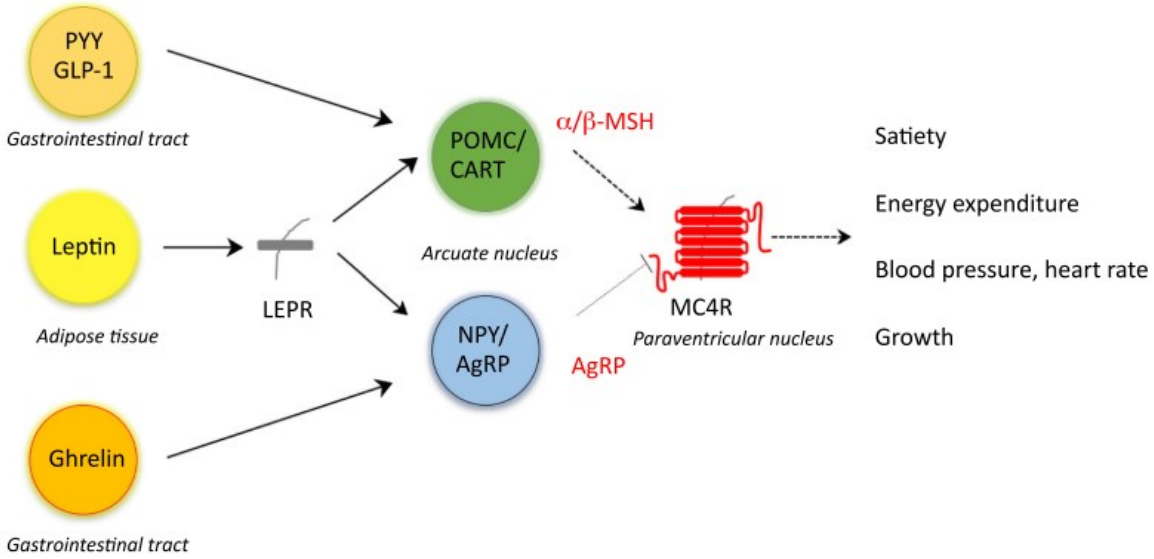


Figure 10. Signals from the periphery of the human body such as leptin, ghrelin, peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) have an effect on the neurons in the hypothalamic arcuate nucleus (ARC). Leptin receptors in ARC get activated by leptin secreted from the adipose tissue, leading to the activation of proopiomelanocortin (POMC)-expressing neurons which then leads to the secretion of melanocyte stimulating hormone (MSH). MSH can then activate the MC4R expressed in the paraventricular nucleus (PVN) leading to its effects in satiety, energy expenditure, blood pressure and heart rate as well as growth. Leptin at the same time also inhibits Agouti-related peptide (AgRP)-expressing neurons localized in the ARC. AgRP is able to inhibit MC4R signalling [93].

The Gs/ cAMP signalling pathway and subsequent activation of protein kinase A (PKA) appears to be the major signalling pathway of MC4R [93] which can be seen in the over 200-fold more potent mediation of G_s signalling than recruitment of β-arrestin induced by the receptors native ligand α-MSH. Nonetheless it has to be noted, that results of EC_{50} values are highly dependent on the assay that has been used [84]. Additionally MC4R can mediate a high level of ligand-independent (basal/ constitutive signalling) or ligand-dependent signalling [45]. A scheme for the MC4R signaling pathway can be seen in **Figure 11**.

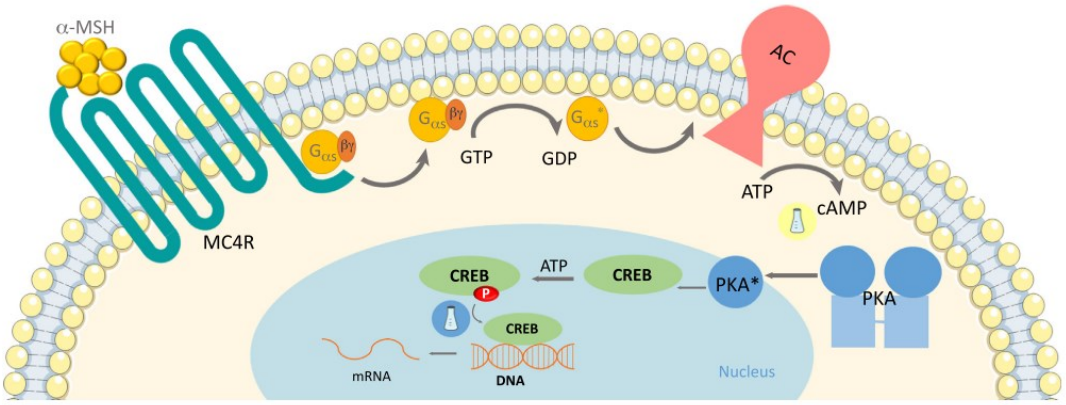


Figure 11. Scheme for the cAMP signaling cascade for the melanocortin regulation of gene expression. Upon stimulation of the MC4R by α-MSH, the α-subunit of the stimulatory heterotrimeric G protein ($G_{\alpha s}$) activates adenylyl cyclase enzymes converting ATP into cAMP. cAMP can then activate protein kinase A (PKA) leading to the dissociation of the catalytic subunit of the PKA complex. The active catalytic subunit of PKA can translocate into the nucleus and phosphorylate transcription factor CREB (cAMP response element binding protein). CREB-P can bind to cAMP response elements (CREs) which is an upstream consensus DNA sequence and facilitate the transcription of a gene by interaction with the core transcriptional machinery [88].

A table with the established EC_{50} values of α-MSH and the more potent NDP- α-MSH for cAMP signaling as well as β-arrestin 2 recruitment can be seen in **Table 2**.

Table 2. MC4R wildtype EC_{50} values for cAMP signaling and β-arrestin 2 recruitment measured on HEK293 cells modified after [94].

MC4R wildtype	**cAMP via G_s signaling**		**β-arrestin 2 recruitment**	
	α-MSH	NDP- α-MSH	α-MSH	NDP- α-MSH
EC_{50} [nM]	10.12 +/- 3.78	3.98 +/- 2.17	154.48 +/- 0.03	106.48 +/- 0.00

As for the peptide ligands, the secondary structure of elements from peptide hormones that stimulate GPCRs show a reverse turn or β-turn which occurs at the fourth residue of the peptide where the direction of the chain is reversed. This turn is stabilized through a hydrogen bond between a carbonyl group of the first residue and the amide of the third peptide bond. In studies with α-MSH it was suggested that the termina residues 1-3, 5, 10 and 12 play a role in potency while the remaining show no significance in potency or affinity of the endogenous peptide to MC4R. When looking at the endogenous ligand of MC4R α-MSH the replacement of the $_L$Phe residue with

$_{D}$Phe at position 7 of the peptide, its effect as an agonist was potentiated, creating NDP-MSH. Further modifications to target the MC4R have been made to also produce SHU9119, the first high affinity antagonist of the same receptor contributing enormously to the research and development of MC4R targeting peptides [87]

1.4.3 MC4R as therapeutic target

Acute fluctuation between energy intake and energy expenditure of a mammal during a short period of time is usually well tolerated and mitigated. However the continued imbalance of these two factors can not only lead to overweight and obesity but additionally the associated comorbidities of excess bodyweight/ bodyfat [88]. With the prevalence of obesity rising across all sectors of society, diseases such as hypertension, type 2 diabetes and eating disorders are increasing as well [95]. This epidemic has brought attention to the need of new and better treatments for overweight and obesity, with one target being the MC4R known for its function of attuning food intake in humans [82].

The role of the melanocortin system, particularly the MC4R, in feeding behavior, has made it an attractive target for experiments and the development of anti-obesity agents [68]. MC4R regulates energy homeostasis in humans and therefore is a favored monogenic target for the treatment when imbalance occurs [88]. POMC mutations, ACTH deficiency and mutations in leptin (LEP) or leptin receptor (LEPR) whose products are upstream of MC4R, lead to early onset obesity in humans manifesting in excess body fat and often restraint perception of satiety [96]. Leptin deficiency, a rare disease that can be treated with administration of recombinant human leptin, effects the human body weight and body composition by deposition of fat mass. Leptin's function in the body is the stimulation of neurons in the arcuate nucleus which leads to the expression of POMC yielding the melanocortin peptides targeting MC3R and MC4R as well as inhibiting neurons expressing melanocortin antagonists agouti related protein and NPY [97].

In 1997, Huszar et al generated mice lacking expression of MC4R and reported in 1997 that the animals showed maturity onset obesity syndrome associated with hyperphagia, hyperinsulinemia and hyperglycemia occurring in the absence of MC4R [98].

In 2012, Eliasen et al used cyclotides which have been suggested as scaffolds to stabilize pharmaceutically active compounds and aimed to develop an MC4R agonist by inserting the His-Phe-Arg-Trp sequence into the very well-studied cyclotide kalata B1 [99].

The application of first generation MC4R agonists resulted in suppressed food intake and thereby induced weight loss but also lead to a significant increase in blood pressure and heart rate in rodents, primates, and humans. The synthetic cyclic peptide setmelanotide binds to the human MC4R with high affinity (inhibitory constant [Ki] = 2.1 nmol/L) and a 50% effective concentration [EC50] = 0,27nM. In a cell model has been shown to not only be more potent than the endogenous ligand α-MSH but also can disproportionally rescue signalling of some strongly impaired MC4R mutants [71]. Setmelanotide, formerly known as RM-493 or BIM-22493 and now known under the name IMCIVREE™ (Rhythm Pharmaceuticals), is a second-generation MC4R agonist which was developed for the treatment of ultrarare genetic disorders leading to obesity from either proopiomelanocortin (POMC), proprotein convertase subtilisin/ kexin type 1 (PCSK1) or leptin receptor (LEPR) deficiency [100]. The peptide has effectively shown to induce weight loss (average 0.6kg/ week) in obese patients with MC4R deficiency while not leading to cardiovascular adverse effects but only mild adverse events. Furthermore it was applied to regulate hunger and satiety of obese patients exhibiting POMC deficiency or LEPR defects with its additional capability of activating nuclear factor of activated T cell (NFAT) signaling and thereby restoring this signaling pathway in chosen variants of the MC4R [96,101]. The structure of the synthetic cyclic peptide which is labeled as a first-in-class MC4R agonist and possesses 20-fold selectivity over the MC3 receptor potentially allowing it to avoid cardiovascular side effects can be seen in **Figure 12** [65].

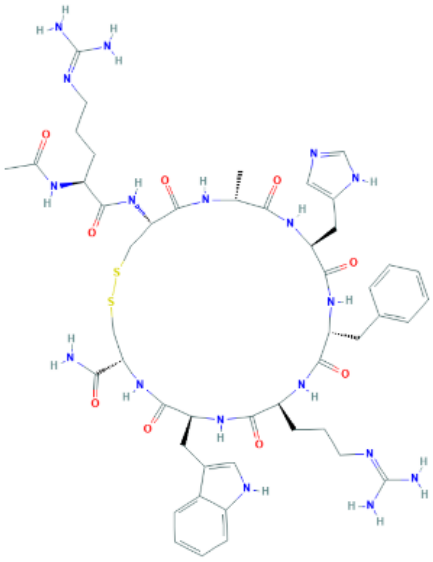


Figure 12. Structure of the synthetic cyclic peptide Setmelanotide with the molecular formular $C_{49}H_{68}N_{18}O_9S_2$ consisting of eight amino acids [71,102].

In the phase III trials of setmelanotide in which ten participants enrolled in the POMC trial and eleven enrolled in the LEPR trial, after one year eight participants of the POMC group and five of the LEPR group achieved at least 10% weight loss over one year and a mean reduction in hunger score of -27.1% in the POMC group and -43.7% in the LEPR group. The most common reported side effects include reaction of the injection site, hyperpigmentation which was reported in all ten participants of the POMC group, nausea and vomiting [101]. In animal models of diet- induced obese mice, rats, dogs and monkeys it has additionally shown to restore insulin sensitivity. The weight reducing properties are possibly due to a chaperone effect, whereby the pharmaceutical can rescue mutant forms of the receptor by restoring their expression at the cell surface [71]. Important to note is that even though setmelanotide is under priority review at the FDA for certain forms of syndromic obesity, there is no approved drug that selectively targets the MC4R for the treatment of common dietary obesity.

One of the challenges for the development of MCR ligands is their similarity, rendering the development of subtype specific molecules difficult. This problem is solved for the endogenous ligands by nature because of the distinct tissue distribution of the MCRs allowing the native ligands themselves not having to be selective towards only one specific melanocortin receptor, making the endogenous ligands such as α-MSH not a good candidate for a drug. Furthermore are these ligands unsuitable for the application as drugs due to their short half-life *in vivo* [65,103].

1.4.4 Cysteine rich anti-microbial peptides to target MC4R

For potential drug candidates plasma stability is an important parameter and certain functional groups of a compound such as esters, amides, lactones, lactams, carbamides, sulfonamides and peptide mimetics are more susceptible to hydrolysis by enzymes of plasma than others [104]. Antimicrobial peptides (AMPs) are very diverse and widespread through the animal as well as the plant kingdom [105]. Some of the β-hairpin AMPs show an intrinsic disorder and need to be in contact with a lipophilic membrane target to take on an ordered confirmation. Some AMPs adopt a favored structure but show changes between being in solution and contact with cell membrane surfaces. Under physiological pH AMPs are often cationic and form amphipathic structures, which mimic those of phospholipids, allowing AMPs to interact with the lipid membranes of bacteria [106]. On this matter it is important to note that the endogenous agonist of the MC4R α-MSH itself has AMP activity against bacteria and yeasts. The C-terminal sequence of this peptide Lys-Pro-Val have been shown to inhibit growth of the yeast *Candida albicans* as well as *Staphylococcus aureus,* a gram-positive bacterium. The N-terminal region of α-MSH containing the His-Phe-Arg-Trp motif, showed anti-microbial activity against *Cryptococcus neoformans* [70].

To understand the structure-activity relationship of antimicrobial peptides with membrane destructive effects in general, the molecular interaction between the AMP and the lipids of the membrane can be investigated using solid state nuclear magnetic resonance (NMR) spectroscopy [107]. The therapeutic potential of AMPs can be assessed using cell selectivity testing and the antimicrobial/activity/toxicity index (ATI). The ATI can be improved by increasing the antimicrobial activity, decreasing hemolytic activity or both which will lead to a higher index number [104].

For this project four different antimicrobial scaffolds with the potential to target MC4R have been used for the grafting by inserting different variations of the His-Phe-Arg-Trp motif into the former animal derived AMPs. Varieties of the active sequence, needed to activate the receptor have been established in previously used frameworks to target MC4R with the original establishment of the minimal needed sequence to target MC4R established using the frog skin bioassay in 1987 by Hruby et al [88,89,99]. The four scaffolds used in this study are tachyplesin-1, protegrin-4, arenicin-3 and gomesin all

of which are animal derived peptides with antimicrobial properties in their natural structure.

Tachyplesin originally isolated from the Japanese horseshoe crab *Tachypleus tridentatus* exhibits broad spectrum and potent antimicrobial activity against bacteria and fungi by forming a complex with bacterial lipopolysaccharide [108]. Its ability to lyse membranes of potential pathological bacteria through its amphipathic design unfortunately also makes it highly hemolytic against mammalian erythrocytes. Tachyplesin-1 is a cationic peptide comprising six cationic residues with two cross strand disulfide bonds forming its β-hairpin structure [104,107]. In the hemocytes of related horseshoe crabs, several isoforms were identified of this 17-19 amino acids containing AMP. They possess an arginine α-amide at the carboxy-terminal and share the same motif, consisting of three tandem repeats of the tetramer: hydrophobic residue, cysteine, hydrophobic residue, arginine [109].

Protegrin was first isolated from porcine neutrophils and showed antimicrobial properties against Gram-positive and Gram-negative bacteria as well as yeasts. In solution these peptides adopt a β-sheet structure which includes a β-turn. Their structure is maintained by disulfide bonds which confer to the pattern Cys^{6}-Cys^{15} and Cys^{8}- Cys^{13}. Out of all the protegrins, PG-4 differs in the β-turn sequence (RGWI instead of RRRF) which is believed to be the reason for its decreased potency. In general data on the structure-activity relationships of protegrins suggests, that their anti-microbial activity is mainly driven by the overall structure including their amphiphilicity, charge and shape rather than the presence of specific amino acids in its sequence [110].

Arenicin was first discovered and isolated from the marine lugworm *Arenicola marina* and contains nine hydrophobic residues, four positively charged arginine residues as well as two disulfide bonds between Cys^{3} -Cys^{20} and Cys^{7} -Cys^{16}, stabilizing its amphipathic β-hairpin structure. Arenicin shows high protein binding to serum components and was therefore discussed to be hemolytic and cytotoxic besides its good activity against a variety of Gram-negative bacteria [111].

Gomesin is the first AMP that has been isolated from spiders. While there have been numerous structural characterizations and structures revealed of toxins from spider venoms, it took longer to characterize the peptide with anti-microbial activities from spiders [30]. The 18-residue long cationic AMP confers a β-hairpin structure similar to

the one of tachyplesin and was originally isolated from the hemocytes of the tarantula *Acanthoscurria gomesiana*, native in Brazil. The peptide forms two disulfide bonds between Cys2 and Cys15 as well as Cys6 to Cys11. Gomesin is cytotoxic for clinically relevant microbes, such as Gram-positive and Gram-negative bacteria, fungi and parasites but also shows *in vitro* and *in vivo* anti-cancer activities against several murine and human cancers. Its activity is exerted by permeabilizing cell membranes [112]. Its broad activity against bacteria, filamentous fungi and yeast is exhibited at concentrations often below 10µM and additionally it was found to affect viability of *Leishmania amazonensis*, a parasite [113].

The antimicrobial activity and therefore the cytotoxicity of these AMPs seems to be very much dependent of the presence of the disulfide bridges. For example when gomesin disulfide bridges where reduced and alkylated, a pronounced decrease in activity against M. luteus and E. coli was observed. Same can be said for tachyplesin analogs with alanine replacing cysteine residues unable to form disulfide bridges as well as chemically protected SH groups where kept from cyclization. It has been suggested that the disulfide bonds are especially important for the bioactivity in media containing salt comparable to physiological conditions [113].

1.5 Aims of the thesis

Previous studies have identified the potential of nature derived peptides as GPCR ligands, a family of receptors highly investigated and researched for the development of novel peptide drugs [6,11,52]. Nature derived peptides such as SFTI-1 and kalata B1 have previously shown to be able to target MCRs when a variation of the minimal required sequence for receptor activation (HFRW) was inserted into these scaffolds. The analog compound of STFI-1 exerted it's highest potency at MC1R and interestingly also seemed to be highly selective over other MCRs tested, leading to the understanding, that SFTI-1 can be used to target specific GPCRs [103]. Kalata B1, a cyclotide has also been used as scaffold to target the MCRs with one of the analogues kB1(GHFRWG;23-28) showed even higher affinity for the human MC4R (hMC4R) than its endogenous agonist α-MSH but lacks in potency [99].

Aim 1.: Activity screening of 14 novel linear peptide ligands using BRET kinetics assay for β-arrestin-2 recruitment

Based on their size and structure, four different animal derived, cysteine rich, anti-microbial, β-hairpin structured peptide scaffolds have previously been chosen and used as scaffolds for the synthesis of novel MC4R peptide ligands using solid phase peptide synthesis performed by Edin Muratspahić. In theory these peptides should be able to bind and activate the MC4R as well as recruit β-arrestin-2 . As a first screening of activity on MC4R, all peptides are screened using the bioluminescence energy transfer (BRET) assay for β-arrestin-2 recruitment. The results will be measured as BRET ratio occurring between the MC4R tagged with enhanced green fluorescent protein (EGFP) and nano luciferase (Nluc) which is coupled to β-arrestin-2. First a kinetics approach will be performed using one concentration of each novel ligand to see if they are able to activate MC4R and potentially recruit β-arrestin-2 within a certain period of time. Results will be compared and the follow- up screening chosen upon the data collected.

Aim 2.: Follow-up exploration of β-arrestin recruitment ability on MC4R

β-Arrestin recruitment at the MC4R has recently been explored on gain-of-function receptor variants leading to the association of lower BMI, lower odds of obesity, type 2 diabetes and coronary artery disease with this variant [114]. For further investigation of

the ability to recruit β-arrestin-2, the peptides showing activity on MC4R in the kinetics assay will undergo further screening using BRET assay in a concentration-dependent manner. With the results from this assay, further insights such as EC50 values for β-arrestin-2 recruitment can be calculated.

Aim 3.: Analysis of cyclic adenosine mono phosphate (cAMP) production

After decoupling of the $G_{\alpha s}$ subunit from βγ-subunit, the adenylyl cyclase located in the cell membrane is activated and cAMP is produced in the cell leading to further cell response.

For the evaluation of the ability to activate the $G_{\alpha s}$ signaling pathway, all 14 novel peptide ligands will be tested for their ability to produce cAMP upon MC4R binding and activation of the receptor. After production of native cAMP by the cell, the homogenous time resolved fluorescence (HTRF) remaining can be measured, after cell produced cAMP binds to an antibody and displaces a recombinant luciferase tag, thereby decreasing detectable light emission. The results can then then compared to the control NDP-α-MSH, and EC_{50} values calculated, leading to insight about the agonistic and stimulatory activity of the novel peptide ligands on MC4R.

2 Materials and Methods

2.1 Materials

2.1.1 Cell culture and pharmacological assays

BRET kit: Nano Glo Luciferase assay System (Promega), cAMP Gs dynamic kit (Cisbio), Dulbecco's modified Eagle Medium (DMEM; Thermo Fisher Scientific), DMEM phenol red free (Thermo Fisher Scientific), Ethylenediaminetetraacetic acid solution (SIGMA), fetal bovine serum (FBS; Sigma-Aldrich), Hank's Buffered Salt Solution (HBSS; Thermo Fisher Scientific), jetPRIME transfection reagent (Polyplus-transfection SA), jetPRIME transfection buffer (Polyplus-transfection SA), kanamycin (Sigma-Aldrich), NucleoBond®Xtra kit (MACHEREY-NAGEL), penicillin-streptomycin (Sigma-Aldrich).

2.1.2 Peptides synthesis and preparation

Human MC4R plasmid pcDNA3.1 and new pEGFP-N1 vector from Origene.

As control for the pharmacological assays, (Nle^4 D-Phe^7)-α-MSH from BACHEM Art. No. 4003800 0001; Lot No. 107614 was used.

Previously grafted novel peptides were dissolved in 500 µL dH_2O and their extinction measured in a 1:10 dilution using the Nano Drop. Concentration of each peptide was calculated using the absorption coefficient of the scaffold (ε) being used and mean extinction measurement:

Mean peptide extinction / ε * 10 = [M]

2.1.3 Instruments for pharmacological assays

AllegraX-12 centrifuge (Beckman Coulter), FlexStation 3 (Molecular Devices), HERA safe KS18 (Thermo Scientific), Nano Drop 2000 Spectrophotometer (Thermo Fisher Scientific), Sigma 3-16 centrifuge (SIGMA), Voyager adjustable tip spacing pipette (Integra).

2.1.4 Software for measurement and analysis

Results were initially processed in SOFTMAX PRO GxP software (MOLECULAR DEVICES) and further analyzed using GraphPadPrism (GraphPad Software), concentration of plasmids was determined using Nano Drop 2000 Software (Thermo Fisher Scientific).

2.2 Methods

2.2.1 Novel MC4R peptide ligand synthesis

Novel peptide ligands for the MC4R have been synthesized by Edin Muratspahić according to previously established protocols [115–117]. Linear peptide analogs are synthesized from C- to N-terminus often using Fmoc chemistry approach. Fmoc (9-fluorenylmethoxycarbonyl) but also Boc (tert-butyloxycarbonyl) are protecting groups which are used in solid phase peptide synthesis (SPPS). At the start of this process, amino acids are loaded onto a resin, allowing them to couple one by one while being protected by a Fmoc protecting group which then has to be cleaved with a base like piperidine before coupling onto the next residue. For the grafting process, a sequence of amino acids, the epitope, that is unstable on its own is inserted into a more stable scaffold with valuable pharmacological properties. In this case animal derived, cysteine rich anti-microbial peptides have been used as scaffolds to carry the variations of the HFRW motif capable of activating MC4R.

2.2.2 Preparation of plasmids

Preparation of the EGFP labeled hMC4R plasmid has been performed by Xaver Kozisek. Human MC4R plasmids tagged with enhanced green fluorescent protein (EGFP) label where isolated from bacterial glycerol stocks which have previously been prepared in the laboratory. The pre-culture was growing under constant swaying at 37°C with the addition of 50 µg/mL kanamycin. Subsequently, the pre-culture was transferred with the addition of 50 µg/mL of kanamycin into sterile 200 mL LB and incubated overnight at 37°C under constant swaying. Afterwards, the bacterial culture needed to be centrifuged for 20 minutes at 5000g at 3°C allowing for the isolation of DNA for which a Midi Prep was performed according to the Plasmid DNA purification user manual from NucleoBond®Xtra Midi (MACHEREY-NAGEL). Plasmids where then eluted in 500 µL trisaminomethane buffer (TRIS) using the NucleoBond Finalizers. Concentrations where measured using Nano Drop 2000 spectrophotometer at 280 nm wavelength.

2.2.3 Cell culture and transfection

Work with cells was performed under aseptic conditions. HEK293 cells where thawed and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, penicillin and streptomycin in an incubator at 37°C and 5% CO_2. For the transfection only cells below passage number 35 where used.

For the cAMP- and BRET-assay transfection buffer and plasmid DNA where mixed with transfection reagent and incubated at room temperature for 10 min according to manufacture protocol before being added to the cells. The transfection of one well in a 6-well plate required 200 µL of transfection buffer, 2 µg of plasmids and 4 µL transfection reagent where mixed. Accordingly, for a 10 cm plate 500 µL transfection buffer, 10 µg plasmids and 20 µL transfection reagent have been used.

2.2.4 Analysis of β-arrestin-2 recruitment through BRET assay

BRET kinetics was measured at concentration of 10 µM of each novel peptide ligand over the time span of 35 min.

For BRET kinetics assay and BRET concentration response curve HEK 293 cells where transiently transfected when about 80% confluent. For each well of a 6-well plate 200 µL of transfection buffer, 2 µg of DNA in ratio 1:10 → 1.8 µg hMC4R DNA with EGFP (acceptor) label and 0.2 µg β-arrestin-2 DNA labeled with Nluc (donor) where mixed with 4 µL of transfection reagent. For the 10 cm plate 500 µL transfection buffer, 9 µg EGFP hMC4R and 1 µg β-arrestin-2 were mixed with 20 µL of transfection reagent. After incubating for 10 min at room temperature, the reagents were carefully pipetted onto the cells and incubated overnight. The next day cells got transferred into clear, phenol-free DMEM containing FBS and antibiotics, counted and seeded into a 96-well-clear bottom plate, each well containing of 50.000 cells/ 100µL medium providing enough wells to measure each novel peptide to be measured in triplicated. Cells are then incubated overnight. Before the assay, the medium was removed, and cells starved in 100 µL phenol-free DMEM without FBS and antibiotics in the incubator for one hour. During incubation, ligands where prepared using HBSS buffer. For the

BRET kinetics assay the final concentration of the ligands was 10 µM. For the additional concentration response evaluation, eight concentrations in logarithmic scale where prepared starting at 10 µM leading to 1 pM. The substrate furimazine was diluted in ratio 1:50 in HBSS and added as a first step into the assay. 50 µL of furimazine were added manually to each well and incubated in the preheated plate reader of FlexStation3 at 37°C for 5 min to allow oxidation of the substrate. Afterwards, 50 µL ligands at a concentration of 10 µM were added automatically by the FlexStation3, leading to a total reaction volume of 200 µL to perform the BRET kinetics assay. A 5 min baseline was generated while total measurement continued for further 35 min. In the concentration response approach, the same protocol was used and the substrate added first, then the ligands were added manually in triplicates of each concentration and incubated for 5 minutes at 37°C before measuring BRET ratio at a wavelength of 460 nm for furimazine and 510 nm for EGFP. A scheme for the BRET assay can be seen in **Figure 13**.

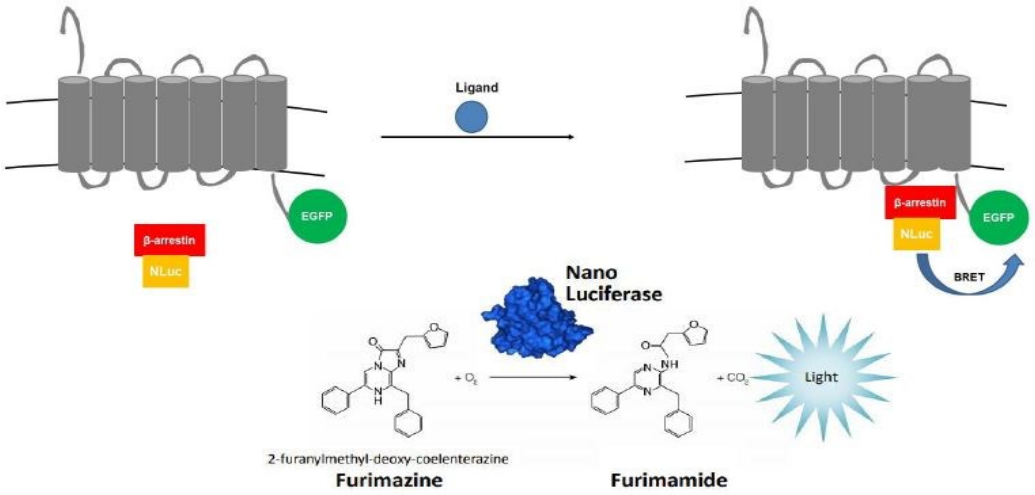


Figure 13. β-arrestin recruitment BRET-assay. The receptor is labeled with an acceptor, in this case enhanced green fluorescent protein (EGFP) which can be excited if near the nano luciferase enzyme (donor) which is bound to β-arrestin. Upon ligand binding the receptor can recruit β-arrestin which brings NLuc and EGFP in proximity allowing energy transfer through nonradiative dipole-dipole coupling resulting in fluorescence emission measurable at a specific wavelength that can be measured and compared to a control ligand [118,119] . Courtesy from Edin M. modified after [120].

After measurement of the HTRF ratio data is analyzed using GraphPad Prism. Mean EC_{50} [nM] and SD [nM] is calculated and results are getting restrained for NDP-α-MSH = 0 -100 and novel peptide ligands = 0 – no restrain.

2.2.5 Analysis of cAMP production through cAMP assay

HEK293 cells for the cAMP-assay were seeded in 6-well plates overnight and transfected at approximately 80% confluency using 200 µL transfection buffer, 2 µg EGFP hMC4R and 4 µL transfection reagent per well, then incubated overnight. For the assay stimulation buffer for Gi/Gs (5x stock) was prepared in a 1:5 ratio with dH_2O and IMBX (final concentration 0.5 mM). 0,8 mL stimulation buffer where mixed with 3,2 mL dH_2O and 8 µL IBMX (phosphodiesterase-inhibitor blocks degradation of cAMP through phosphodiesterase). 30 µL of each ligand concentration were prepared in 2x working solutions to achieve final concentrations of 10 µM to 0,0003 µM in a semi-logarithmic manner. Cells are washed in 37°C EDTA (ethylenediaminetetraacetic acid solution), centrifuged and after removal of supernatant resuspended in 1 mL stimulation buffer. Next, 10.000 cells/ 5µL were pipetted in each well of a 384-well plate then 5 µL of the ligands are pipetted onto the cells creating triplicates of each concentration per peptide to be tested. The plate was centrifuged at 200 rpm for 1 min and incubated at 37°C for 30 min. cAMP-d2 (acceptor) and anti-cAMP cryptate (AB) (donor) (monoclonal cAMP europium cryptate labeled antibody (europium donor) of the Gs kit were diluted in ratio 1:20 in lysis buffer and 5 µL, starting with d2, added into each well of the plate. After centrifuging and incubation for at least 60 min at RT HTRF was measured using the FlexStation3 at wavelength 665 (LM1) to 620 (LM2). Results can then be analyzed using GraphPad Prism and EC_{50} [nM] and SD [nM] calculated. Results get restrained for NDP-α-MSH = 0 -100 and novel peptide ligands = 0 – no restrain. A scheme of the mechanism used to carry out the cAMP assay can be seen in **Figure 14**.

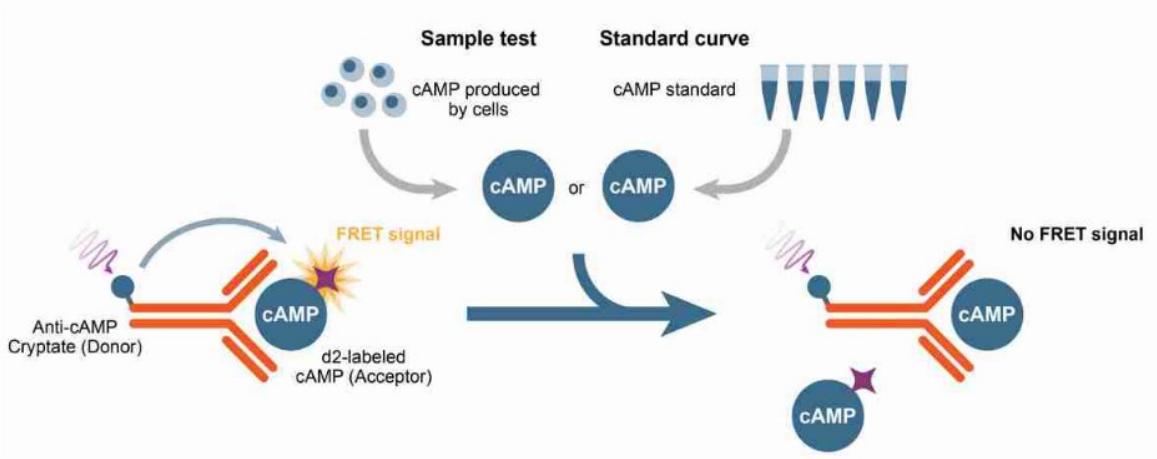


Figure 14. cAMP assay includes anti-cAMP cryptate and d2-labeled cAMP as detection reagents. Upon receptor activation, cAMP is produced intracellularly and detected through the competition of intracellular cAMP and the d2-labeled cAMP for the anti-cAMP antibody. Increase in intracellular cAMP levels thereby reduces the fluorescent signal, here FRET signal, decrease in intracellular cAMP leads to a higher FRET signal [121].

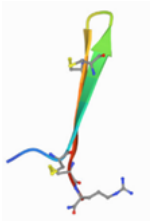
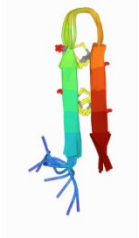
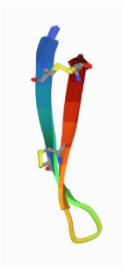
3 Results

3.1 Anti-microbial scaffolds to target MC4R

Based on similarity in structure, four scaffolds have been chosen to synthesize novel peptide ligands to target the MC4R using solid phase peptide synthesis which has been performed by Edin Muratspahić. The motif for the activation of the receptor has been established based on the minimal sequence able to activate MC4R which consist of only four amino acids: His-Phe-Arg-Trp (HFRW). For this project, variations of this motif have been used (P)Hf(H/R)W and inserted between second and third cysteine residues of the scaffolds with the exception of thearenicin-3 scaffold due to its longer sequence.

For pharmacological optimization and consideration of previous literature, was not only phenylalanine substituted with D-phenylalanine for enhanced potency, but also prolin used in some variations potentially leading to stabilization and enhanced selectivity properties [122]. The animal derived cysteine rich AMP scaffolds have been chosen based on their structure comprising a β-sheet structure in their native form which can be of interest when reestablishing disulfide bonds for structural stability. For this project all peptides are linear analogues with their sequence and name shown in **Table 3**.

Table 3. Anti-microbial scaffolds, structure, including native and grafted sequences

Scaffold	Structure	Name	Sequence
Tachyplesin-1		native	**KWCFRVCYRGICYRRCR-NH_2**
		TaLP1	KWCFRVC**PHfHW**CYRRCR-NH_2
		TaLP2	KWCFRVC**HfHW**CYRRCR-NH_2
		TaLP3	KWCFRVC**HfRW**CYRRCR-NH_2
Protegrin-4		native	**RGGRLCYCRGWICFCVGR-NH_2**
		PrLP1	RGGRLCYC**PHfHW**CFCVGR-NH_2
		PrLP2	RGGRLCYC**HfHW**CFCVGR-NH_2
		PrLP3	RGGRLCYC**HfRW**CFCVGR-NH_2
Arenicin-3		native	**GFCWYVCVYRNGVRVCYRRCN-NH_2**
		ArLP1	GFCWYVC**PHfHW**CYRRCN-NH_2
		ArLP2	GFCWYVCVY**PHfHW**VCYRRCN-NH_2
		ArLP3	GFCWYVCV**PHfHW**RVCYRRCN-NH_2
		ArLP4	GFCWYVCVY**HfHW**RVCYRRCN-NH_2
		ArLP5	GFCWYVCVY**HfRW**RVCYRRCN-NH_2
Gomesin		native	**ZCRRLCYKQRCVTYCRGR-NH_2**
		GoLP1	ZCRRLC**PHfHW**CVTYCRGR-NH_2
		GoLP2	ZCRRLC**HfHW**CVTYCRGR-NH_2
		GoLP3	ZCRRLC**HfRW**CVTYCRGR-NH_2

Four different AMP scaffolds have been used for the grafting of 14 novel MC4R peptide ligands. TaLP = Tachyplesin-1 like peptide, PrLP = Protegrin-4 like peptide, ArLP = Arenicin-3 like peptide, GoLP = Gomesin like peptide.

The previously established yields from the synthesis as well as concentrations measured using the Nano Drop 2000 Spectrophotometer after peptides got dissolved in 500 µL dH_2O are shown in **Table 4**.

Table 4. Native scaffold names new label including yield and concentration TaLP = tachyplesin like peptide, PrLP = protegrin like peptide, ArLP = arenicin like peptide, GoLP = gomesin like peptide

Nr.	Scaffold	Name	Yield [mg]	[M]
1	**Tachyplesin-1**	TaLP1	1.52	852.96 µM
2		TaLP2	3.14	812.40 µM
3		TaLP3	1.19	588.37 µM
4	**Protegrin-4**	PrLP1	5.51	675.91 µM
5		PrLP2	6.88	895.98 µM
6		PrLP3	6.15	845.12 µM
7	**Arenicin-3**	ArLP1	2.58	559.47 µM
8		ArLP2	n.d.	245.58 µM
9		ArLP3	7.38	674.19 µM
10		ArLP4	n.d.	196.64 µM
11		ArLP5	2.73	537.95 µM
12	**Gomesin**	GoLP1	n.d.	1167.14 µM
13		GoLP2	8.66	1219.57 µM
14		GoLP3	n.d.	498.59 µM

3.2 Cloning, labeling and cell culture

The preparation of hMC4R in the laboratory consisted of re-cloning, sequencing and fluorescence microscopy of the human MC4R and has been previously performed in the laboratory by Xaver Kozisek. A scheme of the vectors used for recloning can be seen in

Figure 15. After recloning, untransfected HEK293 where compared to the transfected HEK293 cells to determine fluorescence. A figure of untransfected HEK293 cells compared to HEK293 cells transfected containing the EGFP tag can be seen in the **Appendix** at page 89.

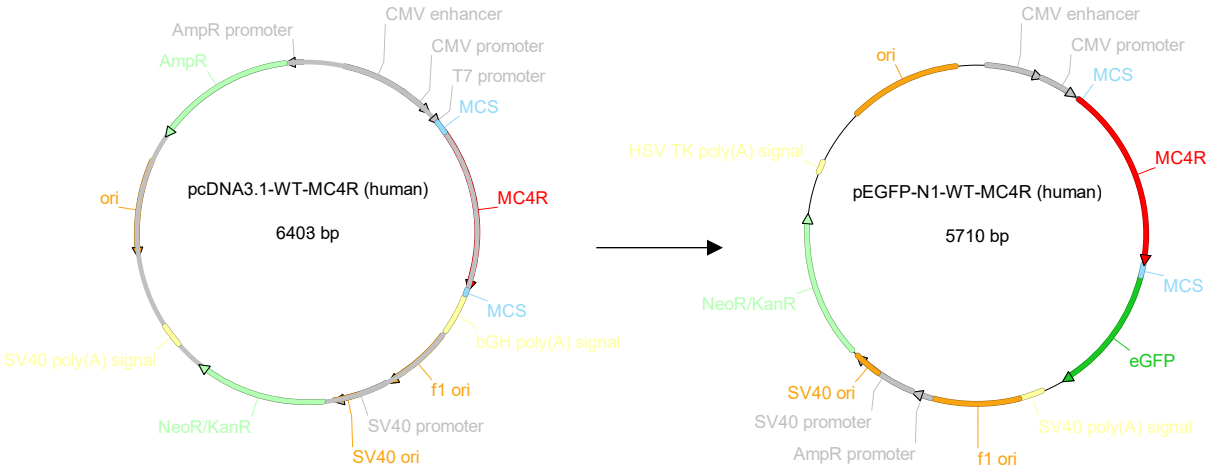


Figure 15. Re-cloning of hMC4R into a new vector: pcDNA3.1 to pEGFP-N1 containing the EGFP label. Courtesy from Xaver Kozisek.

Human MC4R plasmids tagged with enhanced green fluorescent protein (EGFP) label where isolated from bacterial glycerol stocks previously prepared in the laboratory then isolated according to the user manual of the NucleoBond®Xtra Midi (MACHEREY-NAGEL). Concentration of hMC4R plasmids were measured using Nano Drop 2000 spectrophotometer at 280 nm wavelength.

First batch: 1120 µg/µL

Second batch: 788 µg/µL

β-Arrestin-2 plasmids have been previously prepared in the laboratory.

3.3 Pharmacological analysis of novel peptide ligands using BRET assay

The aim of the BRET kinetics assay was to identify if a scaffold or single peptides able to activate MC4R and evaluate peptides for further screening to establish their ability to recruit β-arrestin-2 for the comparison with NDP-α-MSH which is used as control.

3.3.1 Identification of activity and lead peptides for β-arrestin recruitment using BRET kinetics assay

14 linear analogues grafted from four different scaffolds tachyplesin-1, protegrin-4, arenicin-3 and gomesin have been initially analyzed for their ability to recruit β-arrestin-2 using BRET kinetics measurement at a concentration of 10μM over the time span of 42 min at wavelengths of 460 nm for furimazine and 510 nm for EGFP. As control NDP-α-MSH was used to verify function of the assays as well as comparison of the ligands ability to recruit β-arrestin-2.

The BRET assay is based on the measurement of energy transfer between the EGFP tagged MC4R receptor and nano-luciferase labeled β-arrestin-2 which is enabled when both fluorescent molecules come in proximity. Results are shown as BRET ratio (EGFP/ Nluc), indicative of β-arrestin recruitment to MC4R upon ligand binding.

TaLP1 and TaLP2 both increase BRET ratio after 311 sec (ca. 5 min) immediately after the ligands where added automatically by the FlexStation3. From the start, TaLP1 and TaLP2 show lower β-arrestin-2 recruitment ability then the control NDP-α-MSH.

TaLP3 and PrLP1 both increase BRET ratio after 311 sec (ca. 5 min) immediately after the ligands where added automatically by the FlexStation3. While TaLP3 shows about the same capability of β-arrestin-2 recruitment, PrLP1 seems to reaches a higher value than NDP-α-MSH.

Both PrLP2 and PrLP3 increase BRET ratio after 311 sec (ca. 5 min) immediately after the ligands where added automatically by the FlexStation3. PrLP2 as well as PrLP3

show less capability of recruiting β-arrestin-2 than NDP-α-MSH with values further reducing over the remaining timespan.

ArLP1 and ArLP2 both increase BRET ratio after 311 sec (ca. 5 min) immediately after the ligands where added automatically by the FlexStation3. Both ArLP1 and ArLP2 exhibit greater β-arrestin-2 recruitment than NDP-α-MSH over this timespan with ArLP2 even seemingly increasing the ratio over the remaining timespan.

ArLP3 and ArLP4 both gradually increase BRET ratio after 311 sec (ca. 5 min) when added automatically by the FlexStation3. Both peptides do not reach same values as NDP-α-MSH until approximately 1000-1500 sec (16- 25 min).

ArLP5 and GoLP1 both increase BRET ratio after 311 sec (ca. 5 min) immediately after the ligands where added automatically by the FlexStation3. BRET ratio of tested ArLP5 gradually increases after the addition of the ligand, but does not reach the level of NDP-α-MSH. GoLP1 increases the BRET ratio faster and exhibits values similar and above NDP-α-MSH over the remaining timespan.

GoLP2 and GoLP3 both increase BRET ratio after 311 sec (ca. 5 min) immediately after the ligands where added automatically by the FlexStation3. Both GoLP2 and GoLP3 show less capability of recruiting β-arrestin-2 than NDP-α-MSH.

In **Figure 16** - **Figure 22** results of BRET kinetics measurement can be seen. Dots show the lowest BRET ratio measured, error bars reach up to the highest measured value. All kinetics assays have been performed in triplicates at three independent times generating graphs of n=3 if not otherwise stated.

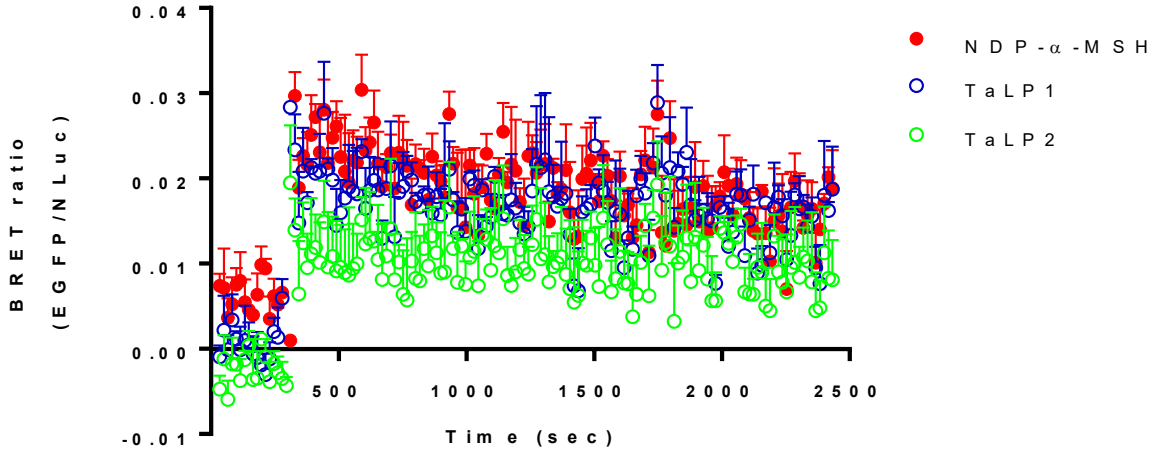


Figure 16. BRET kinetics assay of 10μM TaLP1 and TaLP2 tested on 1,8μg MC4R with EGFP label and 0,2μg β-arrestin-2. Demonstrated is the recruitment of β-arrestin-2 compared to NDP-α-MSH over a time span of 2500 sec (42 min) on the x-axis, indicated by the BRET ratio on the y-axis.

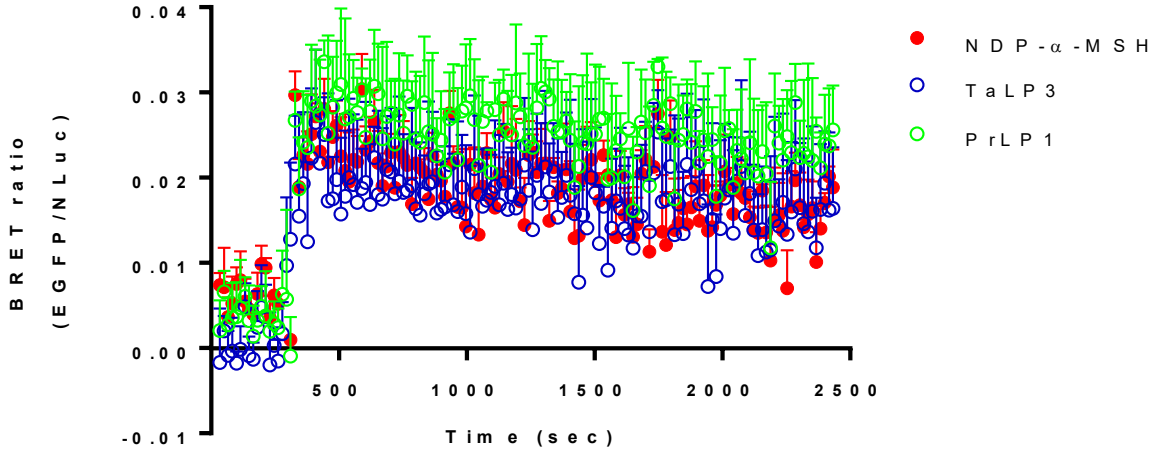


Figure 17. BRET kinetics assay of 10μM TaLP3 and PrLP1 tested on 1,8μg MC4R with EGFP label and 0,2μg β-arrestin-2. Demonstrated is the recruitment of β-arrestin-2 compared to NDP-α-MSH over a time span of 2500 sec (42 min) on the x-axis, indicated by the BRET ratio on the y-axis.

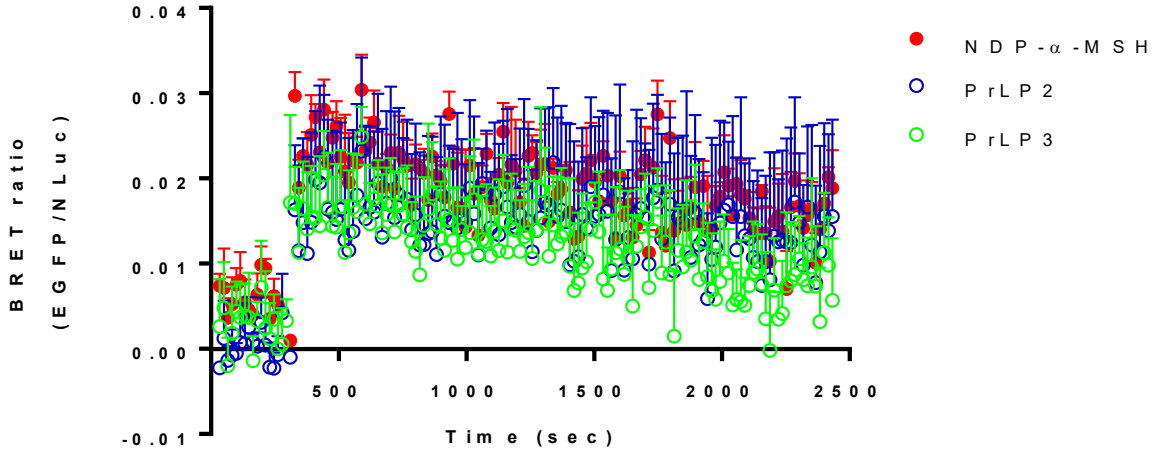


Figure 18. BRET kinetics assay of 10µM PrLP2 and PrLP3 tested on 1,8µg MC4R with EGFP label and 0,2µg β-arrestin-2. Demonstrated is the recruitment of β-arrestin-2 compared to NDP-α-MSH over a time span of 2500 sec (42 min) on the x-axis, indicated by the BRET ratio on the y-axis.

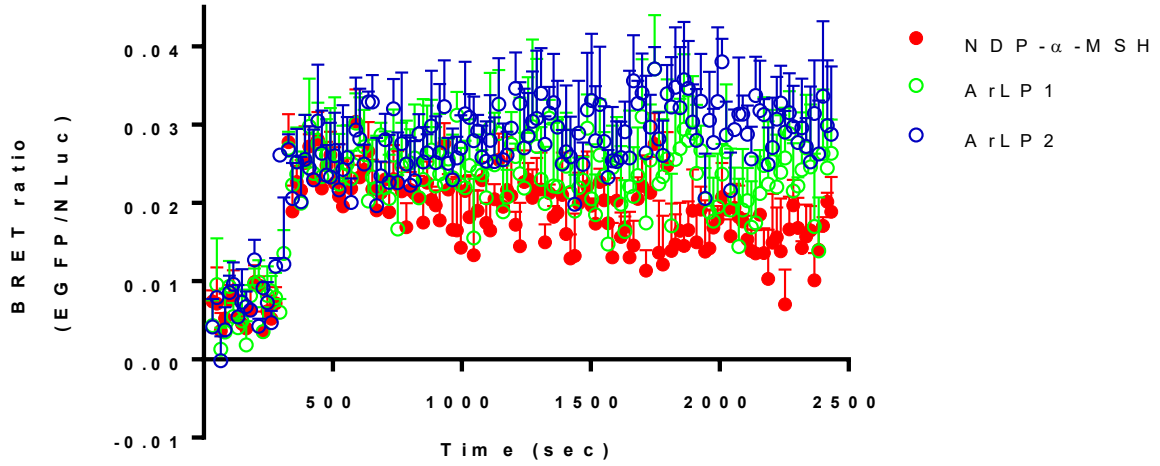


Figure 19. BRET kinetics assay of 10µM ArLP1 and ArLP2 tested on 1,8µg MC4R with EGFP label and 0,2µg β-arrestin-2. Demonstrated is the recruitment of β-arrestin-2 compared to NDP-α-MSH over a time span of 2500 sec (42 min) on the x-axis, indicated by the BRET ratio on the y-axis.

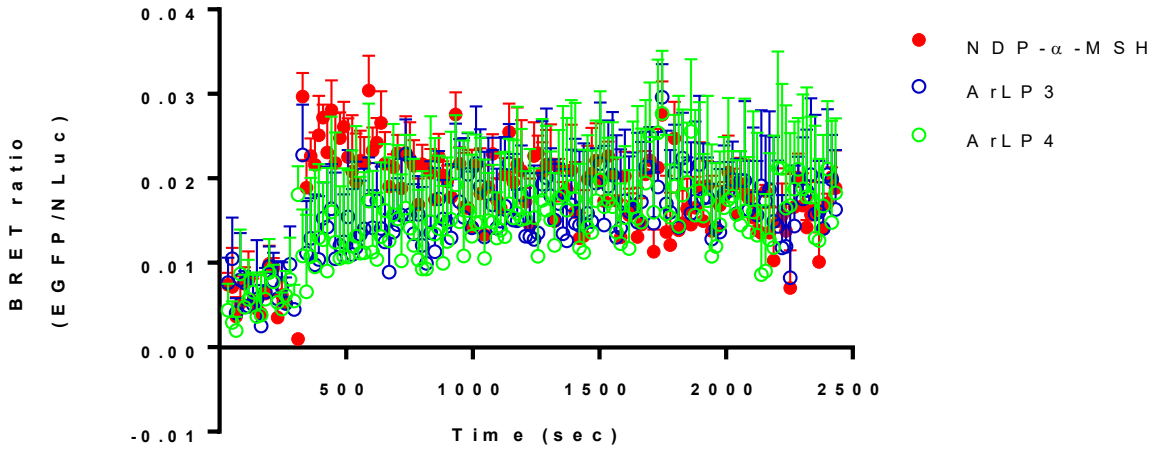


Figure 20. BRET kinetics assay of 10µM ArLP3 and ArLP4 tested on 1,8µg MC4R with EGFP label and 0,2µg β-arrestin-2. Demonstrated is the recruitment of β-arrestin-2 compared to NDP-α-MSH over a time span of 2500 sec (42 min) on the x-axis, indicated by the BRET ratio on the y-axis.

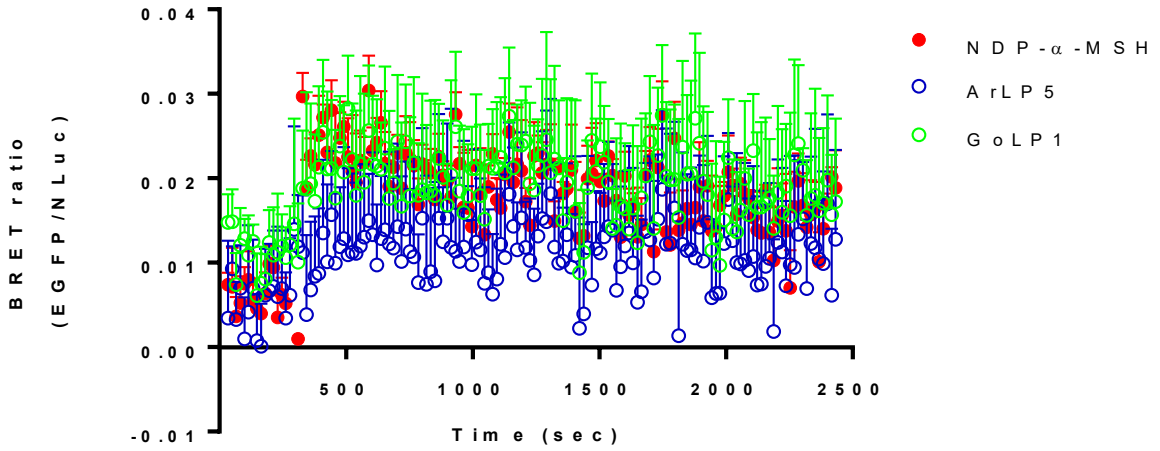


Figure 21. BRET kinetics assay of 10µM ArLP5 and GoLP1 tested on 1,8µg MC4R with EGFP label and 0,2µg β-arrestin-2. Demonstrated is the recruitment of β-arrestin-2 compared to NDP-α-MSH over a time span of 2500 sec (42 min) on the x-axis, indicated by the BRET ratio on the y-axis.

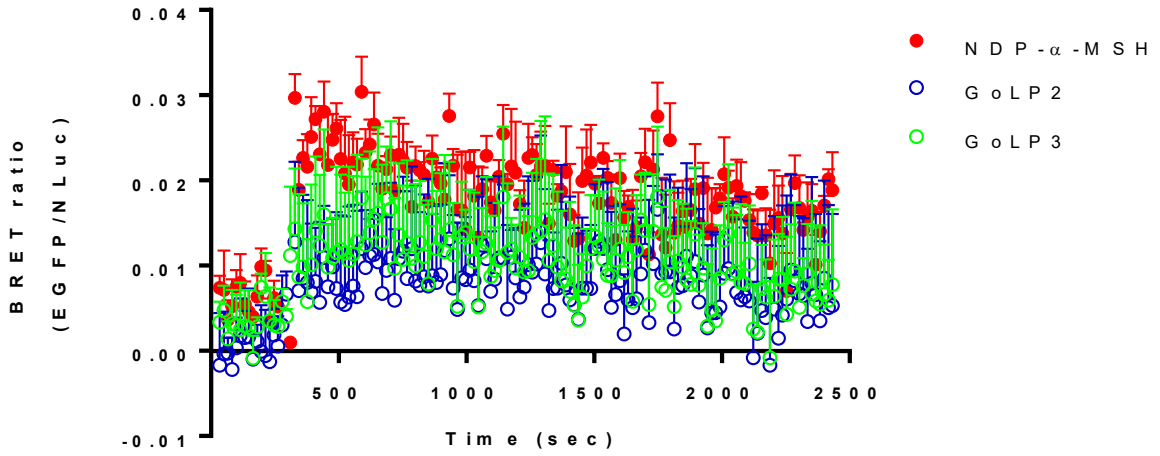


Figure 22. BRET kinetics assay of 10µM GoLP2 and GoLP3 tested on 1,8µg MC4R with EGFP label and 0,2µg β-arrestin-2. Demonstrated is the recruitment of β-arrestin-2 compared to NDP-α-MSH over a time span of 2500 sec (42 min) on the x-axis, indicated by the BRET ratio on the y-axis.

All of the 14 peptide ligands where able to show activity at the MC4R and are shown in relation to NDP-α-MSH, an analogue of the endogenous ligand, which has been set to NDP-α-MSH = 1 in **Figure 23** for comparison and selection for further screening.

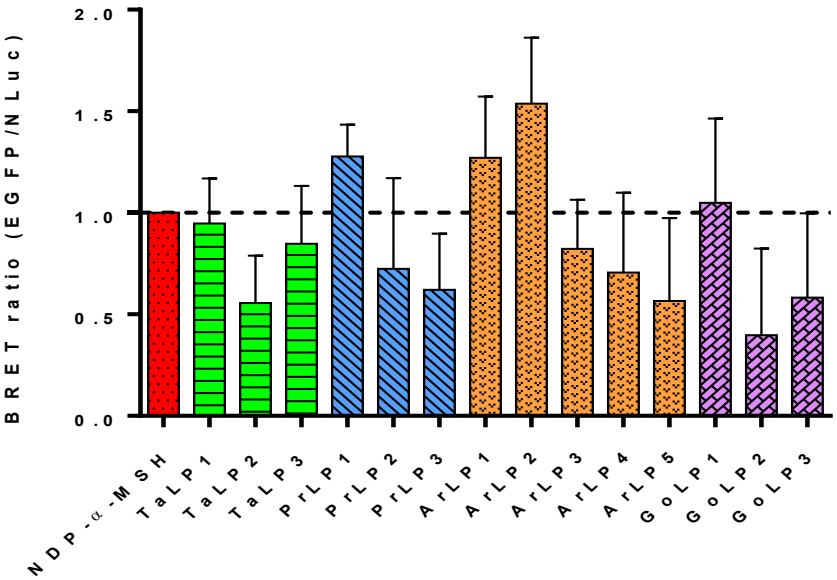


Figure 23. Histogram showing the 14 novel peptides on the x-axis and their mean ligand induced values of β-arrestin-2 recruitment shown as BRET ratio on the y-axis, measured in the BRET kinetics assay over a time span of approximately 42 minutes. Baseline BRET ratio was

measured for approximately 5 min then 10µL of each ligand was added automatically by the FlexStation3. NDP-α-MSH has been set to 1 for comparison. Each assay has been repeated three individual times leading to n=3

While peptides of the tachyplesin-1 scaffold did not reach the recruitment values of NDP-α-MSH, PrLP1 of the protegrin-4 scaffold can exceed the BRET ratio values of NDP-α-MSH with a value of 1.3 [±0.2]. BRET ratio of both ArLP1=1.3 and ArLP2=1.5 surpass the control in recruitment of β-arrestin-2 in the kinetics assay. Furthermore GoLP1 of the gomesin scaffold can reach up to the control with a BRET ratio of 1.0. With the results of this assay seen in **Table 5**, lead peptides have been chosen for further screening including at least one novel peptide ligand of each scaffold.

Table 5. Results of BRET kinetics β-arrestin-2 recruitment assay with **NDP-α-MSH = 1.** All values are mean of three independent measurements leading to n=3 unless otherwise stated.

Peptide	Mean ± SD
TaLP1	0.9 ± 0.4
TaLP2	0.6 ± 0.4
TaLP3	0.8 ± 0.5
PrLP1	1.3 ± 0.2
PrLP2	0.7 ± 0.8
PrLP3	0.6 ± 0.5
ArLP1	1.3 ± 0.5
ArLP2	1.5 ± 0.6
ArLP3	0.8 ± 0.4
ArLP4	0.7 ± 0.7
ArLP5	0.6 ± 0.7
GoLP1	1.0 ± 0.7
GpLP2	0.1 ± 0.7
GpLP3	0.6 ± 0.8

3.3.2 Pharmacological characterization of six novel MC4R peptide ligands using BRET based concentration response assay

The previous BRET kinetics assay allowed all 14 novel peptide ligands to be screened for their ability to recruit β-arrestin-2. Six peptides have been, according to their BRET ratio results, chosen to be tested in a concentration dependent manner, including at least one peptide of each scaffold.

BRET concentration response of TaLP3 and PrLP1 in concentrations 10 µM- 0.000001 µM (1 pM) follows a similar curve as the control NDP-α-MSH with higher mean EC_{50} levels calculated. At a concentration of 0.01 nM (log -11) TaLP3 has an outlier while PrLP1 has outliers at concentrations of 10 µM and 1µM, contributing to a high SD.

ArLp1 and ArLP2 where compared to NDP-α-MSH and there seemed to be a problem with the concentration response. Not only does the control and peptides have high standard deviation, also ArLP2 does not follow the concentration response curve. No EC_{50} values could be calculated.

For ArLP4 and GoLp1 it can be seen that the concentration response curve of both peptides follows the scheme of the control but with higher EC_{50} values and high SD values.

TaLP3, PrLP1, AeLp1,2 and 4 as well as GoLp1 where tested in eight concentrations: 10 µM – 1 pM (10 µM, 1 µM, 0.1 µM, 0.01 µM, 0.001 µM, 0.0001 µM 0.00001 µM, 0.000001 µM) then mean EC_{50} [nM] and SD [nM] calculated using GraphPad Prism. The results and can be seen in **Figure 24** - **Figure 26**.

Dots represent the lowest ligand induced BRET (% max NDP-α-MSH) error bars show SD between lowest and highest BRET values.

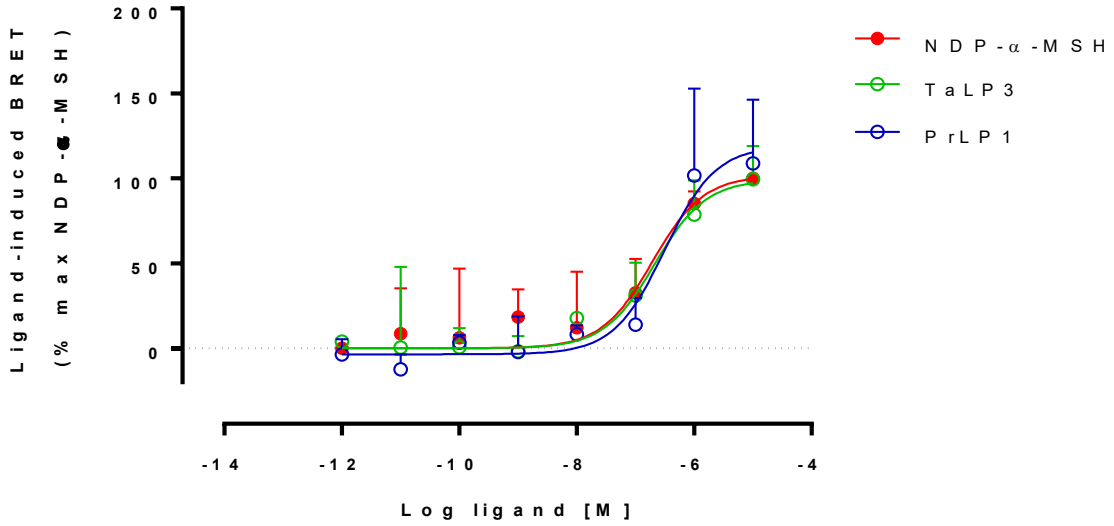


Figure 24. BRET concentration response curve of TaLP3 and PrLP1 in concentrations 10 µM-0.000001 µM (1 pM) in logarithmic manner. Ligands were added manually in triplicates of each concentration and incubated for 5 minutes at 37°C before measuring BRET ratio. The x-axis is showing the concentration of the novel peptide ligand and the control NDP-α-MSH; y-axis is showing the ligand induced BRET signal in % max NDP-α-MSH. The dose response curve of TaLP3 and PrLP1 look similar to the control NDP-α-MSH and lead to EC_{50} values above the control.

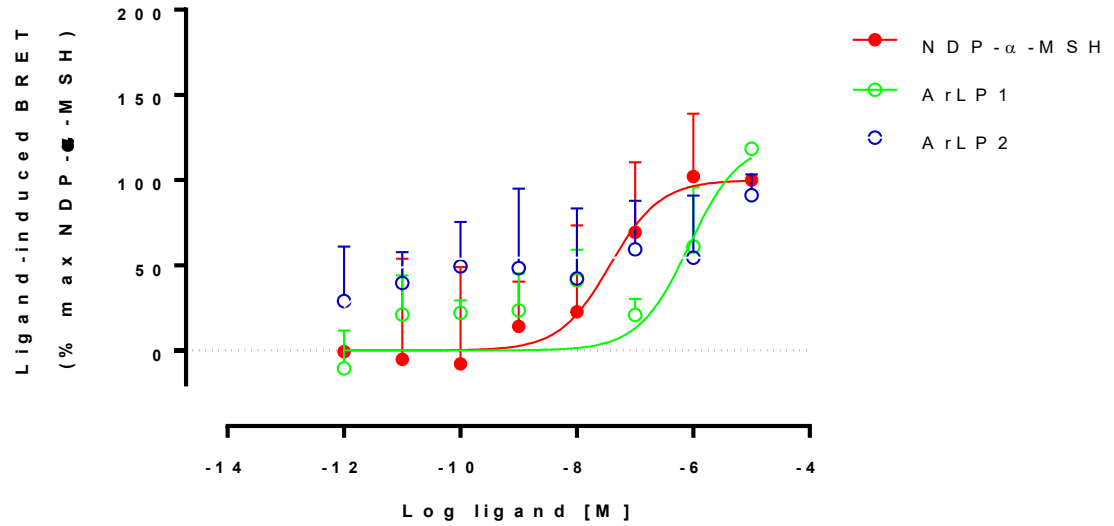


Figure 25. BRET concentration response curve of ArLP1 and ArLP2 in concentrations 10 µM-0.000001 µM (1 pM) in logarithmic manner. Ligands were added manually in triplicates of each concentration and incubated for 5 minutes at 37°C before measuring BRET ratio. The x-axis is showing the concentration of the novel peptide ligand and the control NDP-α-MSH; y-axis is showing the ligand induced BRET signal in % max NDP-α-MSH. For ArLP1 a dose response curve is noticeable but the EC_{50} value cannot be calculated due to high standard derivation. As for ArLP2 there is no clear if the assay has worked for this peptide.

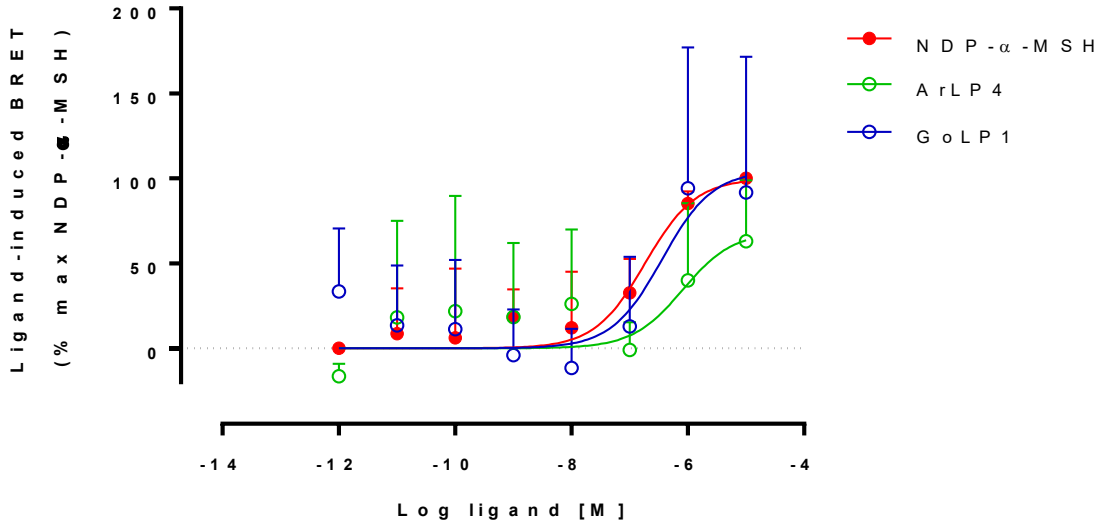


Figure 26. BRET concentration response curve of ArLP4 and GoLP1 in concentrations 10 µM-0.000001 µM (1 pM) in logarithmic manner. Ligands were added manually in triplicates of each concentration and incubated for 5 minutes at 37°C before measuring BRET ratio. The x-axis is showing the concentration of the novel peptide ligand and the control NDP-α-MSH; y-axis is showing the ligand induced BRET signal in % max NDP-α-MSH. Dose response curve of ArLP4 does not allow for the calculation of EC_{50} values and indicates a possible problem with the assay for this peptide.

Table 6. Results of BRET based concentration response assay. All values represent the mean of 3 independent measurements unless otherwise stated.

	Mean EC_{50} ± SD [nM]
NDP-α-MSH	**193.3 ± 162.2**
ArLP1*	**222.9 ± 212.2**
ArLP2*	**399.3 ± 173.3**
NDP-α-MSH	**97.3 ± 97.1**
ArLP1	**n.a.**
ArLP2	**n.a.**
NDP-α-MSH	**193.3 ± 162.2**
ArLP4	**n.a.**
GoLP1*	**480.1 ± 337.9**

*n=2

3.4 Pharmacological characteristic of 14 novel MC4R peptide ligands using cAMP assay

Through this assay, the cAMP production of the cell upon ligand binding can be detected and EC_{50} values calculated. When MC4R is activated by one of the novel peptide ligands, cAMP is produced intracellularly and can be detected through the competition of intracellular cAMP and the d2-labeled cAMP for the anti-cAMP antibody. An increase in intracellular cAMP levels reduces the fluorescent signal by displacing d2-labeled cAMP. The concentration response curve and EC_{50} values for cAMP generation of all 14 peptide ligands was determined by performing cAMP-assay in a concentration dependent manner (10 concentrations 10 µM; 3 µM; 1 µM; 0.3 µM; 0.1 µM; 0.03 µM; 0.01 µM; 0.003 µM; 0.001 µM; 0.0003 µM) and the HTRF ratio evaluated using the FlexStation3 at wavelength 665 (LM1) to 620 (LM2). As control, NDP-α-MSH, a more potent analogue of the endogenous MC4R ligand α-MSH when it comes to $G_{\alpha s}$ signaling was used.

When comparing novel peptides of the tachyplesin-1 scaffold, all of them are able to reach an EC_{50} value in the nanomolar range in the cAMP assay with a curve comparable to NDP-α-MSH. From this scaffold TaLP3 shows the highest potency with EC_{50} = 16.7 nM.

Within the protegrin-4 scaffold PrLP1 shows the highest potency with an EC_{50} = 3.7 nM.

From the arenicin-3 scaffold ArLP1 with an EC_{50} = 1.0 nM shows the highest potency.

GoLP1 grafted using the gomesin scaffold shows an EC_{50} = 26.0 nM and therefore also makes a potent peptide for the activation of MC4R and G_s signaling compared to the other 2 candidates grafted from gomesin.

The results of the concentration response assay can be seen in **Figure 27 -Figure 30Fehler! Verweisquelle konnte nicht gefunden werden.** results of EC_{50} values and SD are shown in **Table 7**. Dots represent the lowest cAMP accumulation (% max NDP-α-MSH), error bars show the SD and highest cAMP accumulation.

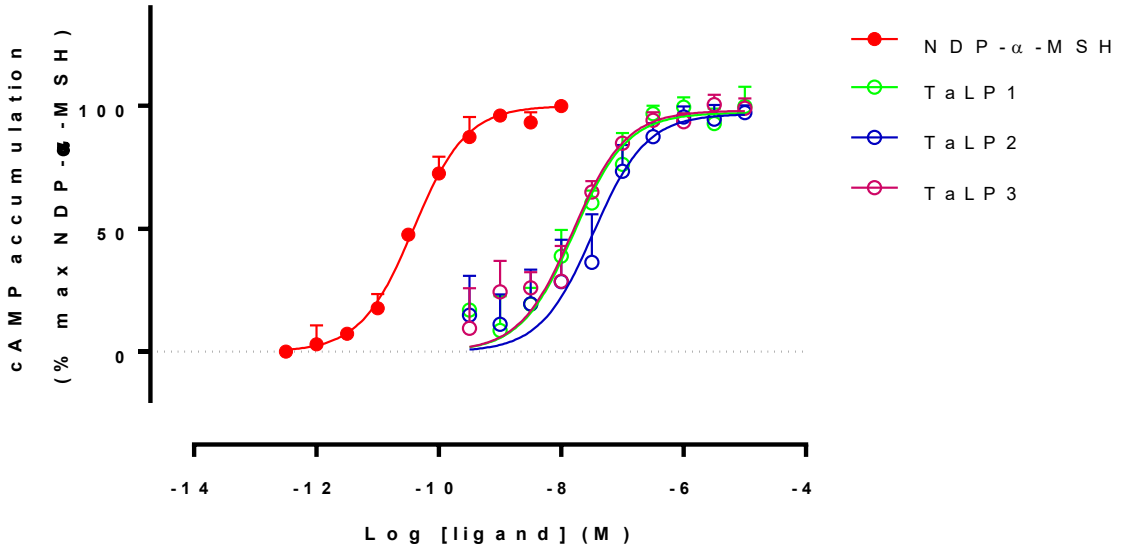


Figure 27. cAMP dose response curve of the three novel tachyplesin-1 like peptide ligands at concentrations from 10 µM to 3 nM in semi logarithmic manner. The control NDP-α-MSH was measured at concentrations from 0.01 µM to 0.3 pM. The x-axis shows the concentration of the ligand and control NDP-α-MSH; y-axis shows the accumulation of cAMP in % max. NDP-α-MSH which has been measured in n=3 and used as reference for all graphs.

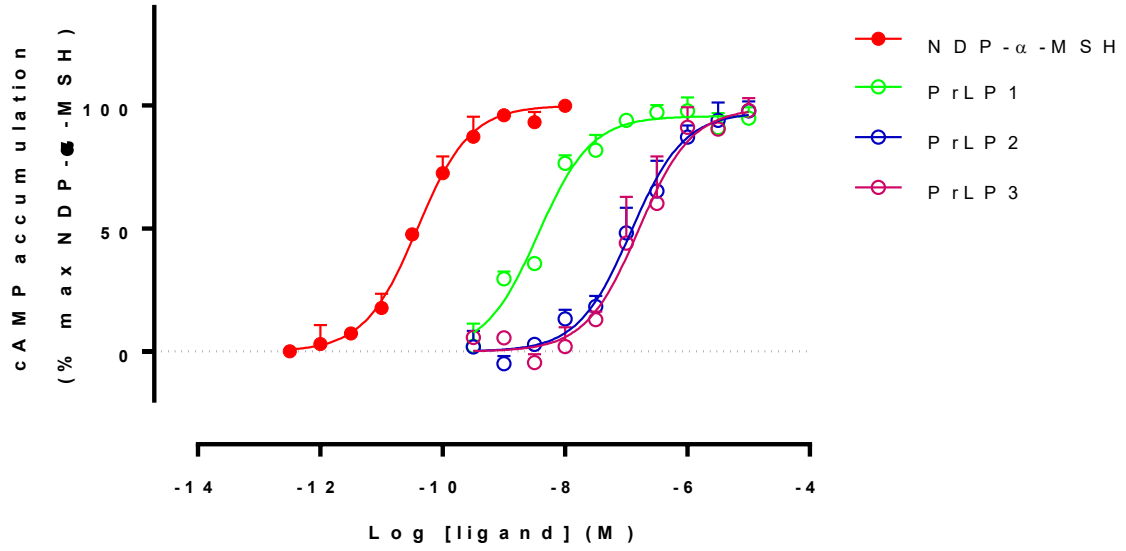


Figure 28. cAMP concentration response curve of the three novel protegrin-4 like peptide ligands at concentrations from 10 µM to 3 nM in semi logarithmic manner. The control NDP-α-MSH was measured at concentrations from 0.01 µM to 0.3 pM. The x-axis shows the concentration of the ligand and control NDP-α-MSH; y-axis shows the accumulation of cAMP in % max. NDP-α-MSH which has been measured in n=3 and used as reference for all graphs.

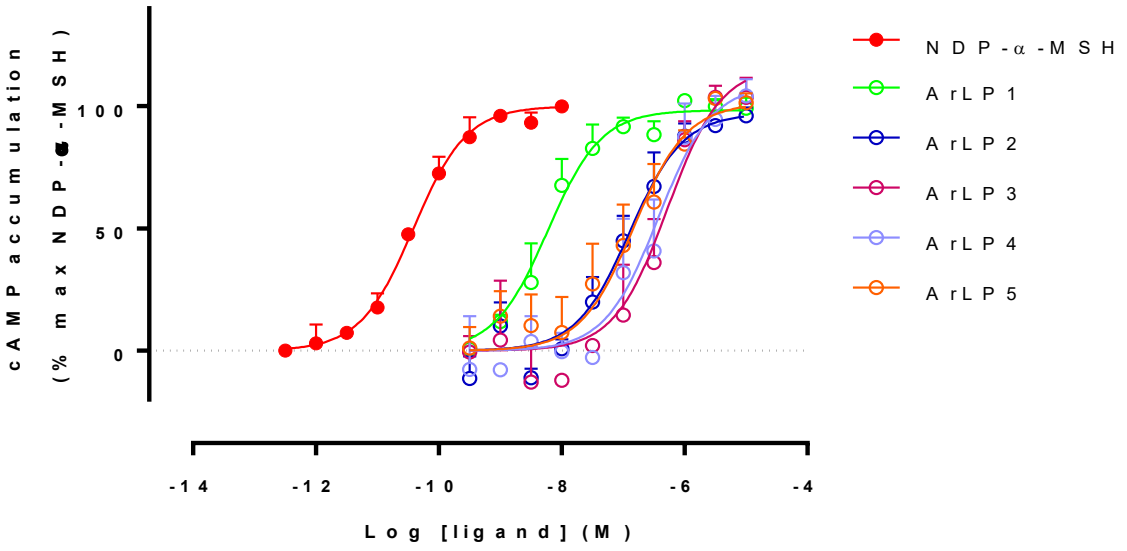


Figure 29. cAMP concentration response curve of the five novel Arenicin-3 like peptide ligands at concentrations from 10 µM to 3 nM in semi logarithmic manner. The control NDP-α-MSH was measured at concentrations from 0.01 µM to 0.3 pM. The x-axis shows the concentration of the ligand and control NDP-α-MSH; y-axis shows the accumulation of cAMP in % max. NDP-α-MSH which has been measured in n=3 and used as reference for all graphs.

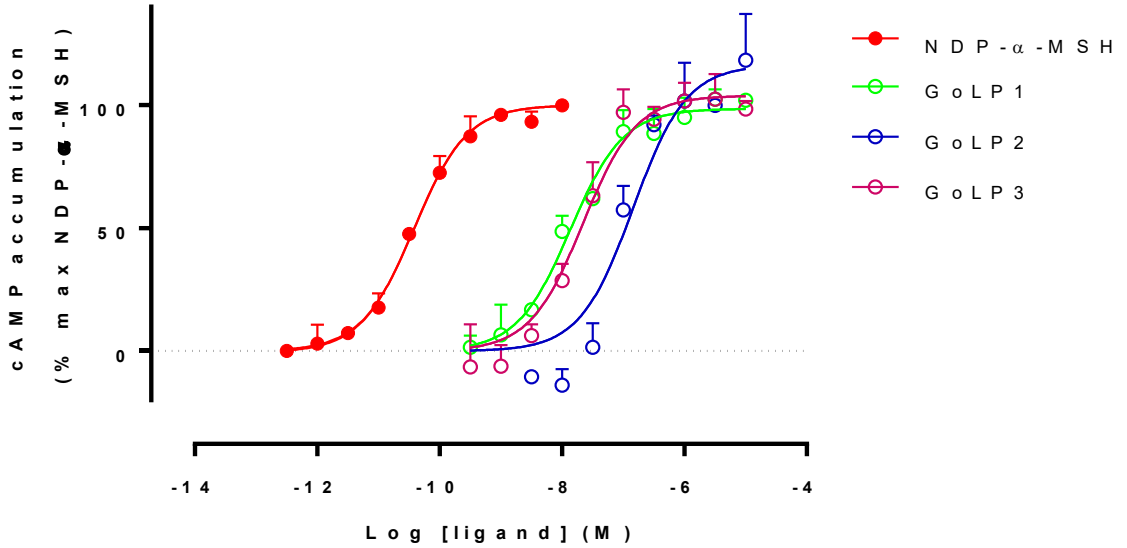


Figure 30. cAMP concentration response curve of the three novel gomesin like peptide ligands at concentrations from 10 µM to 3 nM in semi logarithmic manner. The control NDP-α-MSH was measured at concentrations from 0.01 µM to 0.3 pM. The x-axis shows the concentration of the ligand and control NDP-α-MSH; y-axis shows the accumulation of cAMP in % max. NDP-α-MSH which has been measured in n=3 and used as reference for all graphs.

Table 7. All results of cAMP assay EC_{50} values and SD for all 14 novel peptide ligands. All values represent a mean of 3 independent measurements unless otherwise stated.

	Mean EC_{50} ± SD [nM]
NDP-α-MSH	**0.04 ± 0.02**
TaLP1	**20.3 ± 13.6**
TaLP2	**48.0 ± 40.1**
TaLP3	**16.7 ± 11.4**
PrLP1	**3.7 ± 0.7**
PrLP2	**151.4 ± 96.5**
PrLP3*	**208.3 ± 152.5**
ArLP1*	**1.0 ± 0.1**
ArLP2*	**17.8 ± 4.5**
ArLP3*	**1004.9 ± 263.2**
ArLP4*	**819.6 ± 3.1**
ArLP5	**261.0 ± 213.5**
GoLP1*	**26.0 ± 15.7**
GoLP2*	**149.4 ± 67.3**
GoLP3	**39.1 ± 23.5**

*n=2

4 Discussion

More and more peptides and peptide therapeutics are entering clinical trials since they have gained a wide range of applications in biotechnology and medicine. Reason being their safety, tolerability as well as efficacy profile in humans [10]. As off now natural products currently represent a niche for the development of new GPCR drugs with great potential. Considering GPCRs are today's most exploited drug target it is pursued to search for ligands targeting this receptor family that can easily and abundantly be sourced. Such is the case for natural products from plants, animals, fungi and bacteria [9].

14 novel MC4R ligand peptides have been grafted by inserting different variations of the active sequence of melanocortins able to target MC4R into antimicrobial peptide scaffolds which in their natural form are stable and due to their β-sheet structure likely to bind to GPCRs.

4.1 BRET based peptide activity evaluation on hMC4R and β-arrestin-2 recruitment investigation

For a general activity assessment all 14 novel peptides have been tested using BRET based β-arrestin-2 recruitment kinetics assay allowing us to see if the ligands are active and the needed incubation time. The recruitment of β-arrestin is a marker for normal function of the MC4R and it has been established, that even though most MC4R variants are loss-of function, some gain-of function variants exists and can be associated with significantly lower BMI, overall lower odds of obesity, type 2 diabetes mellitus and reduced risk of coronary artery disease. These variants and their protective characteristics stem from the variants signaling bias towards β-arrestin and increased mitogen-activated protein pathway activation [114]. Additionally, it has been shown that biased signalling more specifically a β-arrestin bias leading to a greater recruitment of β-arrestin than the activation of other signalling pathways, on the MC4R has a cardioprotective effect. This finding can lead to the development of drugs potentially reducing comorbidities of overweight and obesity [84]. Through the kinetics assay it was determined, that all of the 14 novel peptide ligands are active at the

hMC4R. The control NDP-α-MSH usually shows its peak in β-arrestin-2 recruitment almost immediately after the ligand has been added then slightly and evenly the curve is being reduced, suggesting less and less β-arrestin-2 being bound to MC4R. The 14 novel peptide ligands in general followed the immediate increase in BRET signal after being added to the assay, and followed the curve of the control on either higher or lower BRET ratio values. Only one peptide, ArLP2 seemed to increase its β-arrestin-2 recruitment over time showing higher BRET ratio over time. The kinetics assay of the 14 novel peptide ligands therefore allowed for the distinction between peptides with potential better β-arrestin-2 recruitment from such with seemingly lower recruitment. The peptide ligands with higher BRET ratio in than the control have been further tested in a concentration dependent manner which included at least one peptide of each scaffold. The increase in signal almost directly after adding addition of the ligands into the assay allowed the assumption that measurement of BRET signal for the concentration response is appropriate after 5 minutes of incubation when performing the dose dependent assay.

For the BRET dose response assay, TaLP3, PrLP1, AeLp1,2 and 4 as well as GoLp1 where tested in eight concentrations. For some peptides the assay did not work well, and strong variations of EC_{50} values of the control NDP-α-MSH as well as high standard derivation suggest that some parts of the assay did not work as planned. It has been observed, that some wells of the measurement plate especially first column and first row of the plate regularly led to outlying data and made interpretation of these data points difficult. Since the dose response assay did not work out as planned and some of the results are not allowing for the calculation of EC_{50} values, it is difficult to determine whether or not a significant β-arrestin-2 recruitment could be present and no bias ratios have been calculated. The bias for a certain pathway would be able to be identified comparing EC_{50} values of β-arrestin-2 pathway of the endogenous ligand to the one of the novel ligand.

4.2 Evaluation of cAMP production on all novel peptide ligands

The cAMP assay was performed for all of the 14 novel peptide ligands, allowing a comparison of the four different scaffolds used. Some peptides have been determined as more potent than others. PrLP1 one of the peptides grafted with the protegrin-4

scaffold showed an EC_{50} = 3.7 [nM]. The reference peptide, NDP-α-MSH used as control in the assay reached an EC_{50} of 0.4 [nM]. PrLP1 was thereby more effective than the other protegrin-4 analogues but not much as NDP-α-MSH. Furthermore ArLP1 grafted from the Arenicin-3 scaffold surpassed the other novel peptide ligands with its EC_{50} = 1.0 [nM] thereby coming even closer to NDP-α-MSH. Both of these novel lead peptides transcended EC_{50} = 4.7 [nM] measured in previous literature for the endogenous ligand of MC4R α-MSH [77]. For further comparison, setmelanotide has to bind and activate mutated MC4R with over 20-fold higher potency than the endogenous agonists [123]. In a cell based cAMP accumulation assay with transiently transfected HEK293 cells, the potency of this peptide lies in nanomolar range with EC_{50} = 0.27 nM with a part of the efficacy of setmelanotide potentially being due to its chaperone effect, rescuing mutant forms of the receptor by restoring the receptor expression at cell surface [71].

The novel peptide ligands show full agonistic activity MC4R by leading to the accumulation of cAMP in transfected HEK293 cells, confirming that the scaffolds used are able to target MC4R. It also has to be taken into account that factors such as the MRAPs have not been co-transfected with MC4R into the HEK293 cells and could potentially lead to different outcomes in in activity which could be assessed in the future.

For the cell based assays performed for this project it has to be mentioned, that HEK293 cells have been used for their accessibility as well as certain other properties. HEK293 do not express MCRs and also don’t respond to NDP-α-MSH. However it has to be taken into account that this cell system also brings limitations by not resembling the repertoire of proteins of the hypothalamic neurons.

4.3 Impact and outlook of AMP scaffolds to target MCR / MC4R

The structure of AMPs has the potential to bring advantages such as stability and oral bioavailability due to their structure and inclusion of cysteines forming disulfide bonds in their natural form. Since all 14 novel peptide ligands for MC4R have been established to be full agonists by activating the G_s signaling pathway similarly to NDP-α-MSH, the nature/animal derived cysteine rich, AMP scaffolds and peptides show that

research in this niche is promising for the development of novel peptide drugs. Future research targets of the established lead peptides could be the assessment of their cytotoxic properties in linear analogue state and examination of stability which determines whether or not the novel peptide ligands could potentially be turned into oral bioactive therapeutics in the future. Reestablishment of the disulfide bonds originally present in the AMP scaffolds can lead to insights of cytotoxicity and stability of the cyclized form of these peptides as well as potential increased or decreased pharmacological aspects and their usability. The possibility of co-transfection with MRAPs and the usage of Ca^{2+} as cofactor for ligand binding which been determined very recently could also be included in further assessments [85]. At the time writing these concluding words, new insights have been gained about the structure-based design of MC4R ligands using the SHU-9119-hMC4R peptide, a well-known antagonist of MC4R. More specifically options for the development of more selective ligands such as the side chain in SHU-9119 featuring Nle^4, $DNal(2')^7$ and Trp^9 residues as well as an amide linkage between Asp^5 and Lys^{10} side chain being alterations which can be made and have the property of engaging in a selectivity switch that can be taken into account for further development of the novel peptides tested in this study [124]. In general the established peptides can be used as templates for further optimization in their pharmacological properties thereby contributing to the research of animal-derived, cysteine rich antimicrobial scaffolds in the development of new GPCR drug candidates with less side effects due to higher selectivity and oral bioavailability for easy administration.

5 Conclusion

Peptides derived from nature are an abundant source of potential GPCR ligands with an important role in the development of novel drugs for the treatment of various diseases. Stable peptide frameworks, containing cysteines and disulfide bonds are of special interest and can be used as scaffolds targeting GPCRs while being resistant to the degradation under physiological conditions due to their structure [99].

The melanocortin system in humans consists of five GPCRs the MCRs and their endogenous agonists and antagonist, regulating a wide range of physiological functions such as food intake, energy homeostasis, skin pigmentation, sexual function and inflammation which are mediated through five MCR subtypes which are structurally similar making it a challenge to develop subtype specific targeting molecules. Even though highly potent MCR ligands such as melanotan, setmelanotide (RM493) and bremelanotan have been established, they are not selective towards one receptor subtype which is one of the goals for the development of save therapeutics with minimal unwanted side effects [103]. Furthermore, the development of ligands leading for biased signaling, especially on the MC4R is currently a favorable research area not only for the treatment of weight imbalance but also in preventing the emerging co-morbidities, as has been suggested by Lotta et al, evaluating Gain-of-Function MC4R variants exhibiting a signaling bias potentially capable to protect against obesity [114].

Even though some of the grafted peptides initially showed potential in higher β-arrestin-2 recruitment when compared to NDP-α-MSH in the BRET kinetics assay, the strong variations of data when performing the concentration response assay was not able to give constructive results about the ability of these peptides when it comes to this signaling pathway. However we are able to conclude that all of the 14 novel peptide ligands are able to target MC4R and induce G_s signaling thereby seem to be full agonists. This project allows us to prove that animal-derived cysteine rich AMP-peptide ligands are a promising and versatile source of scaffolds to target the MC4R, potentially more receptors in the melanocortin system and GPCRs.

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7 List of Abbreviations

AB	antibody
AgRP	agouti related peptide
API	active pharmaceutical ingredient
ASIP	agouti-signaling protein
ATI	antimicrobial/activity/toxicity index
BMI	body mass index
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
CRP	cysteine rich peptide
d2	d2-labeled cAMP (red acceptor)
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle Medium
EDTA	ethylenediaminetetraacetic acid solution
EGFP	enhanced green fluorescent protein
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
HBSS	Hank's buffered salt solution
HEK	human embryonic kidney
HTRF	homogenous time resolved fluorescence
IBMX	3-isobutyl-1-methylxanthine
MCR	melanocortin receptor
MSH	melanocyte stimulating hormone
NDP-α-MSH	[Nle^4, D-Phe^7]-α-melanocyte stimulating hormone

NLuc	nano luciferase
NMR	nuclear magnetic resonance
POMC	proopiomelanocortin
PPI	protein-protein interaction
RPM	rounds per minute (centrifuge)
RT	room temperature
TRIS	trisaminomethane
SPSS	solid phase peptide synthesis

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10 Appendix

Supplementary figure 1: Sequence of the human wild type MC4R [125].

Supplementary figure 2: Fluorescence microscopy of un-transfected and transfected HEK-293 cells

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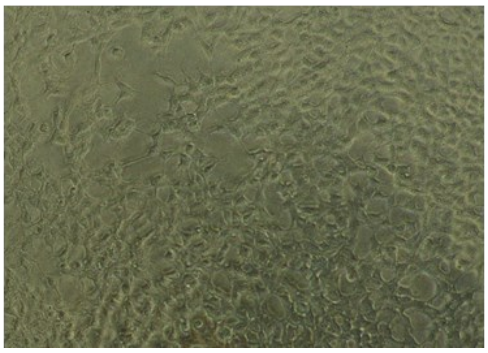
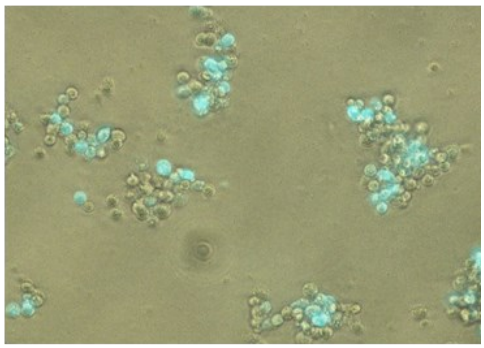
Supplementary figure 1: Sequence of the human wild type MC4R [125].

A)



B)



Supplementary figure 2: Image using fluorescence microscopy at 10-fold magnification of A) untransfected HEK-293 cells and B) transfected HEK-293 cells showing fluorescence response of the EGFP tag.