FUNCTIONAL MODULATION OF THE PATTERN RECOGNITION FORMYL PEPTIDE RECEPTORS IN NEUTROPHILS

Lipopeptides – allosteric modulators of G-protein-coupled receptors

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Functional Modulation of the Pattern Recognition Formyl Peptide Receptors in Neutrophils – Lipopeptide – allosteric modulators of G-protein couple receptors

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An expert is a person who has made all the mistakes that can be made in a very narrow field.

Niels Bohr

ABSTRACT

G-protein-coupled receptors (GPCRs), which are the largest class of cell-surface receptors, are involved in a range of physiologic processes and pathologies, making this a highly interesting group of proteins as targets for drug development. Studies of these receptors have uncovered novel receptor biology concepts, including biased signaling, functional selectivity, and allosteric modulation. "Tailor-made" lipopeptides (pepducins and lipopeptoids) represent novel and promising classes of receptor-specific allosteric modulators. In this thesis, immunomodulating lipopeptides that interact with a group of pattern recognition receptors, formyl peptide receptors (FPRs), which play key roles in host defense against microbial infections, tissue homeostasis, and the initiation and resolution of inflammation, are generated and functionally characterized. The FPRs are expressed in both human and murine white blood cells, and novel allosteric lipopeptide modulators that selectively interact with human and murine receptors are described. We show that the targeted receptor is not always the one that might be expected. This receptor hijacking process raises questions about the precise mechanisms of action of these lipopeptides and of these types of molecules acting as a molecular pattern that is recognized by the receptor group studied. Fundamental differences are also revealed by the receptor-ligand recognition profiles, between mice and men. This represents important knowledge needed for the development and use of animal models for human diseases.
In summary, the results presented in this thesis not only highlight the value of the different lipopeptides as tools for modulating receptor activities in human and murine immune cells, but also provide new insights into the allosteric modulation concept.

Keywords: Human, mouse, neutrophil, reactive oxygen species, formyl peptide receptor, pepducin, G-protein-coupled receptor, lipopeptoid, pattern recognition receptor

POPULÄRVETENSKAPLIG SAMMANFATTNING

Receptorer som för sin signalering är beroende av ett s.k. G-protein utgör den största gruppen av igenkänningsstukturer som uttryck på våra cellers yta. Dessa receptorer är av stor betydelse för styrningen av många fysiologiska processer och ett fel i signalsystemet (för stark eller för svag signal, eller signalering vid fel tillfälle) riskerar att leda till sjukdom. Detta gör att kunskaper om denna grupp av receptorer har kunnat (och kommer att kunna) användas för att utveckla läkemedel mot många typer av sjukdomar, och forskningen inom området är både omfattande och expanderande. När den här gruppen av receptor beskrevs var uppfattningen att det fanns två aktivitetslägen, antingen på eller av, men senare forskning visar att styrsystemen är mycket mer komplexa och begrepp som funktionell selektivitet, ”biased” signalering, full/partiell/invers agonism, och allosterisk modulering, används idag för att beskriva komplexiteten. Den grupp av lipopeptider som kallas pepduciner och som flera av delarbetena i denna avhandling beskriver, är en grupp av modulatorer. Normalt aktiveras en receptor genom att en informationsbärare binder till delar av receptorn som är tillgängliga från cellens utsida, och denna bindning ändrar receptors funktion så att information förs vidare till det signalerande G-proteinet på insidan av cellens membran. Pepduciner, som består av en kort kedja av aminosyror (peptid) ihopkopplade med en så kalla fettsyra, har förmåga att ta sig in i celler och binda till delar av receptorer som är tillgängliga från insidan och de kan antingen aktivera samma signaler som en informationsbärare som kommer utifrån (positiv allosterisk modulering), eller hindra signalen att gå fram trots att informationsbäraren bundit till receptorn (negativ allosterisk modulering). En pepducin påverkar inte funktionen av vilken receptor som helst, utan bara de receptorer som någonstans på insidan av cellens membran själv har en peptidkedja som innehåller samma aminosyror som pepducinen och de skall dessutom finnas i samma inbördes ordning. Detta avhandlingsarbete visar att det inte alltid fungerar så; flera pepduciner och andra lipopeptider känner igen en speciell receptor (formyl peptide receptor 2; FPR2) trots att den kedja av aminosyror som förmodats vara avgörande för funktion, saknas i denna receptor.

I *arbete I* i avhandlingen undersöks effekter av pepduciner med en palmitinsyra (fettsyra) kopplad till en kedja aminosyror som är identisk eller nästan identisk med en bit (den tredje intracellulära loopen) som finns på en av de delar av receptorn FPR2 som exponeras på insidan av vita blodkroppars membran. I detta arbete (och i ett av forskargruppen tidigare publicerat arbete) visas att det finns en klar koppling mellan pepducinens aminosyrasekvens och den som finns i den receptor som aktiveras, men dessutom kan dessa pepduciner döda bakterier. Även denna funktion var helt beroende av att både fettsyra och peptidkedja, men basen för de två olika funktionerna (aktivering av vita blodkroppar - avdödning av bakterier) skiljer sig åt. De resultat som presenteras väcker frågan om det skulle vara möjligt att i framtiden kombinera de bakteriedödande och de immunomodulerande egenskaperna i en klass av nya antibakteriella läkemedel.

I *arbete II* i avhandlingen undersöks effekter av en pepducin med samma fettsyra som i de tidigare undersökta pepducinerna men med en kedja aminosyror som hämtats från en annan receptor, den med FPR2 närbesläktade FPR1. Aminosyrasekvensen är identisk med det tredje intracellulära loop i FPR1. I motsats till den tidigare beskrivna pepducinen som aktiverade vita blodkroppar, så hämmade FPR1-pepducinen cellernas funktion, men det var inte FPR1 funktionen som hämmades, utan den här pepducinen "kidnappade" FPR2. Dessa resultat väcker frågor om själva pepducinkonceptet och dessa frågor får ytterligare näring av att den här pepducinen påverkar extracellulära signalmolekylers förmåga att binda till receptorn på cellernas utsida.

I *arbete III* studeras vita blodkroppar från försöksdjur, allt i avsikt att undersöka funktionslikheter/skillnader mellan mus och människa som båda är utrustade med de receptorer som beskrivits i arbete I och II, FPR1 (som i mus fått heta Fpr1) och FPR2 (som i mus fått heta Fpr2). Ett relativt stort antal agonister (aktiverare) och antagonister (hämmare) har beskrivits och dessa påverkar selektivt FPR1 respektive FPR2, eller båda receptorerna. Mycket hur fungerar dessa receptor ligander i relation till motsvarande musreceptorer? Genom att använda vita blodkroppar som isolerats från benmärg tagen från normal friska möss och från djur som saknar arvsanlaget för Fpr2, identifierades ett par specifika agonister för Fpr1 respektive Fpr2, och tillgången till en dess specifika agonister gjorde det möjligt att identifiera receptorspecifika antagonister för dessa musreceptorerna. De absolut bästa FPR1 och FPR2 antagonisterna hade inga hämmande effekter på motsvarande musreceptorer, men en mindre potent FPR1 antagonist visas fungera också för att hämma funktionen av Fpr1. En ny potent och selektiv Fpr2 hämmare (Lau-(Lys-βNSpe)$_6$-NH$_2$ introduceras. Denna hämmare tillhör en grupp av molekyler som brukar kallas lipopeptoider, och har en fettsyra kopplat till en kedja som är uppbyggd av kemiskt modifierade, "onormala", aminosyror.

I *arbetet IV* studerades effekter på Fpr1/Fpr2 (musreceptorerna) av pepduciner med peptiddelarna hämtade från motsvarande humana receptorer, och det omvända. De två "humana pepducinerna" (beskrivna i arbetena I och II) är strukturellt väldigt lika (olika aminosyror på två ställen i peptiderna) men skiljer i funktion; den ena hämmar och den andra aktiverar, men båda är selektiva för FPR2. Båda dessa pepduciner modulerar också selektivt Fpr2 men båda aktiverar musreceptorn. Mus Fpr1 pepducinen har en peptidkedja som är mycket lik motsvarande humana variant (skiljer i en aminosyra) och de har också samma effekter; aktiverar funktionen av Fpr2 och hämmar den av FPR2. Den pepducin som är hämtad från Fpr2 hämmar funktionen av denna receptor med den hämmar också funktionen av FPR2 trots att det är ganska stora skillnader mellan dessa receptorer i delar av respektive receptor som finns på insidan av de vita blodkropparnas membran. Sammanfattningsvis fungerar pepduciner som bra verktyg som kan användas för att modulera receptorfunktion, men det är uppenbart att det inte bara finns *en* mekanism för hur de fungerar.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I

Winther M, Gabl M, Oprea TI, Jönsson B, Boulay F, Bylund J, Dahlgren C, Forsman H
Antibacterial activity of pepducins, allosterical modulators of formyl peptide receptor signaling
Antimicrobial Agents and Chemotherapy (2014) 58:2985-2988

II

Winther M, Gabl M, Welin A, Dahlgren C, Forsman H
A neutrophil inhibitory pepducin derived from FPR1 expected to target FPR1 signaling hijacks the closely related FPR2 instead
FEBS Letters (2015) 589:1832-1839

III

Skovbakke SL, **Winther M**, Gabl M, Holdfeldt A, Linden S, Wang JM, Dahlgren C, Franzyk H, Forsman H
The peptidomimetic Lau-(Lys-βNSpe)$_6$-NH_2 antagonizes formyl peptide receptor 2 expressed in mouse neutrophils
Biochemical Pharmacology (2016) 119:56-65

IV

Winther M, Rajabkhani Z, Holdfeldt A, Gabl M, Bylund J, Dahlgren C, Forsman H
Pepducins from formyl peptide receptors allosterically modulate the function of the same receptor in human and murine neutrophils, but with an outcome (positive or negative) that is not dependent upon the origin of their receptors
In manuscript

CONTENT

Abbreviation x

Introduction to receptor biology 1

G-protein-coupled receptors 3
- Basic concepts underlying orthosteric ligand regulation of GPCR activity 5
- Allosteric modulators of GPCR 6
- G-protein-dependent signalling downstream GPCRs 7
- Biased signalling and β-arrestin binding downstream of activated GPCRs 9

Pepducins – a novel concept for the regulation of GPCR function 11
- Activating and inhibiting pepducins 12
- Receptor-independent effects mediated by pepducins 14

Innate immunity and the role of neutrophils 15
- The neutrophil granulocyte 16

Functions of neutrophils expressing GPCRs 19

Formyl peptide receptors 21
- FPR signaling and regulation in neutrophils 22
- Conventional FPR agonists 23
- Conventional and allosteric FPR antagonists 26
- FPR2-derived pepducins activate FPR2 27
- Bacterial killing and immunomodulation 28
- Receptor hijacking: FPR2-interacting pepducins with amino acid sequences derived from other GPCRs 29

Modulation of Fpr functions in mice neutrophils 33
- Fprs in mice neutrophils 33
- Ligand recognition differences between the receptors in mouse and man 34
- Allosteric modulators of Fpr function 36

Future perspectives 38

Acknowledgement 40

References 40

Abbreviation

Boc-FLFLF • Butyloxycarbonyl- phenylalanine-leucyl phenylalanine-leucyl phenylalanine
Boc-MLF • Butyloxycarbonyl- -methionyl-leucyl-phenylalanine
CXCR4 • Chemokine receptor type 4
DAG • diacylglycerol
ERK1/2 • Extracellular signal-regulating kinase
fMIFL • N-formyl-methionyl-isoleucine-phenylalanine-leucyl
fMLF • N-formyl-methionyl-leucyl-phenylalanine
FPR • Human Formyl Peptide Receptor
Fpr • Mice Formyl Peptide Receptor
GEFs • Guanine-nucleotide exchange factors
GPCR • G-protein-coupled receptor
IP_3 • Inositol 1,4,5-triphosphate
LXA_4 • Lipoxin A_4
NAM • Negative allosteric modulator
PAM • Positive allosteric modulator
PAR • Protease-activated receptor
PI3K • Phosphoinositide 3-kinase
PKC • Protein kinase C
$PLC\beta_2$ • Phospholipase $C\beta_2$
PMN • Polymorphonuclear leukocyte
PSM • Phenol-soluble modulin
PTX • Pertussis toxin
O_2^- • Superoxide anions
ROS • Reactive oxygen species
SAA • Serum amyloid A

Introduction to Receptor Biology

All living cells must be able to sense the environment and respond to changes that affect survival, reproduction, and cell-to-cell communication. Proteins whose main function is to sense and transmit signals that lead to regulation of cellular responses to chemical/physical changes in the environment are referred to as receptors (the unit that receives a signal/message). Such proteins are carried by all living cells and are expressed either inside the cell or on the cell surface. Cells have an elaborate repertoire of receptors, with collective specificities for a multitude of stimuli, including nutrients, growth factors, hormones, and toxins. Stimulation of these receptors activates signal transduction mechanisms, which produce chemical signals to mediate a wide range of cellular responses. These responses regulate many different cellular activities, such as cytoskeletal alterations, metabolism, and gene expression. Intracellular receptors are located in the cytoplasm, nucleus or vacuoles of the cell and are activated by signaling molecules that are generated intracellularly or molecules that can pass through cell membranes.

While receptors that are expressed on the surface of a cell can be connected to the membrane in different ways (e.g., membrane associated/anchored or integral to the membrane), they have in common that they all recognize external signals and transfer the information about the presence/absence of a particular signal to an intracellular signal, which is transferred to second messengers and ultimately, to effector functions related to the receptor involved and the precise signal received. The cell-surface receptors that are involved in the signaling processes that occur in multicellular organisms can be divided into three general categories: receptors with ion channel activities; receptors with enzymatic activities; and receptors that rely on a G-protein for signal transduction, the so-called G-protein-coupled receptors (GPCRs). The last category is the largest receptor family, encoding approximately 950 GPCR members in the human genome, and will be the focus of this thesis.

G-PROTEIN-COUPLED RECEPTORS

Knowledge about the family of receptors now known as GPCRs was initially obtained from studies of the light-sensitive receptor rhodopsin, which was the first GPCR to be studied in detail. The possibility to obtain large quantities of a highly enriched and stable protein from the bovine retina supported these studies, which resulted in the publication of the primary sequence of rhodopsin in 1983 [1]. Around 10 years later, the two-dimensional crystal structure of bovine rhodopsin was obtained, and the three-dimensional crystal structure was obtained in Year 2000 [2, 3]. GPCRs have in common that they are membrane-spanning proteins that traverse seven times the membrane in which they are expressed, placing the N- and C-termini on different sides of the membrane [1, 4]. The importance for signaling of GTP/GDP-binding heterotrimeric proteins (large G-proteins) was known before this group of proteins was proposed to act as intermediate transducers of the second messenger signals generated by GPCRs [5, 6]. The β-adrenergic receptor was the first receptor in the family to be cloned, and the basic functions of this receptor has since then been used as the prototypic GPCR [7]. We now know that even if the family members in the GPCR superfamily share common structural features, e.g., seven α-helical transmembrane domains and alternating cytoplasmic and extracellular loops, there is significant diversity among these receptors. GPCRs regulate a vast number of basic biological functions, as well as physiological processes, ranging from vision and smell to neurologic, cardiovascular, and reproductive functions. GPCRs currently constitute major targets for drug development and indeed, more than 40% of drugs that are currently on the market target GPCRs [8-10]. The importance of GPCRs is evidenced by the Nobel Prizes awarded to researchers describing the role of G-proteins in signal transduction (Gilman and Rodbell, 1994), the biological activities of GPCR-binding neurotransmitters (Kandel, Carlsson and, Greenard, 2000), the description of olfactory GPCRs (Axel and Buck, 2004), and the GPCR structure-function relationships (Lefkowitz and Kobilka, 2012).

Several classification systems have been used to categorize this superfamily of receptors. The first and most frequently used classification system is based on sequence

homology, dividing the GPCRs into the following six classes: A, rhodopsin-like; B, secretin receptor family); C, metabotropic glutamate; D, fungal mating pheromone receptors: E. cyclic AMP receptors; and F, frizzled. Of these, none of the receptors that belong to the D and E classes has been found in vertebrates. An alternative classification system is the GRAFS system introduced by Fredriksson *et al* in 2003, which is based on the GPCR phylogenetic tree. This system divides vertebrate GPCRs into five subgroups, overlapping the A–F nomenclature: glutamate; rhodopsin; adhesion; frizzled/taste; and secretin (Figure 1) [11-14]. The GPCRs are known to recognize and respond to many different types of ligand, from photons, neurotransmitters, and hormones to inflammatory mediators belonging to different chemical groups [15, 16]. Despite the diverse range of GPCR ligands, many receptors

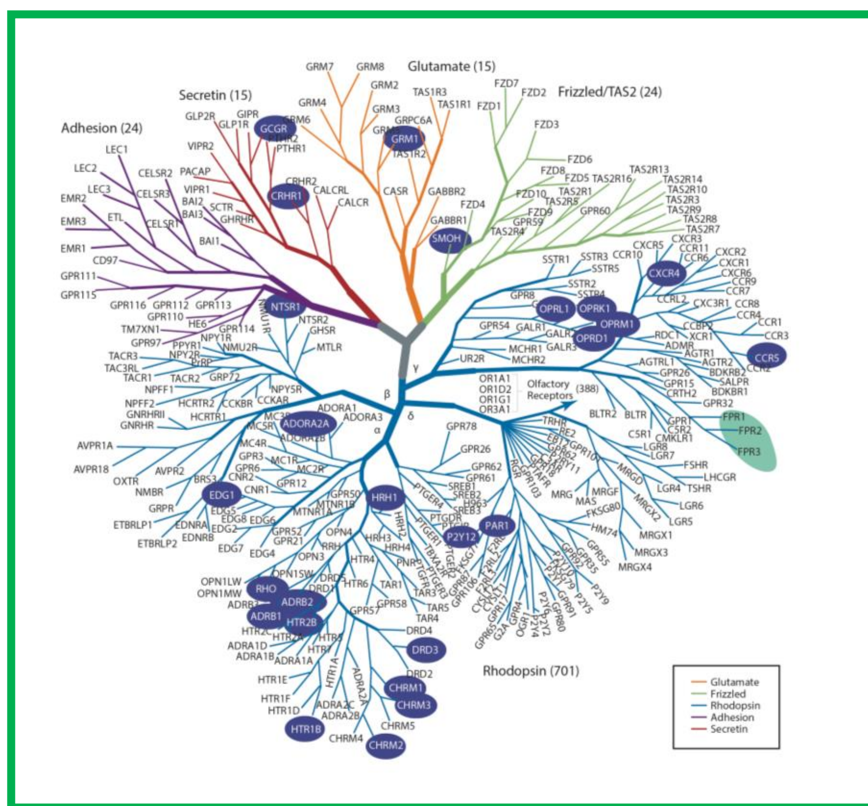

Figure 1. Phylogenetic tree of the human GPCR superfamily constructed using sequence similarities within the seven-transmembrane regions. The GPCRs are listed according to the gene name used in the UniProt database. Family members with known structure are indicated by the blue circles within the tree. The turquoise circle highlights the three FPR family members (FPR1, FPR2, FPR3). Adapted from [17].

share structural similarities (e.g., seven transmembrane domains) and use similar/identical G-protein-dependent intracellular signaling pathways to regulate different cell functions [14, 16].

Basic concepts underlying orthosteric ligand regulation of GPCR activity

In the classic two-state model that describes the interaction between a ligand and its receptor, the conformation of a receptor can vary between two different states that are in equilibrium [18]. In one conformational state, the signaling is turned off, whereas in the other state the signaling is on. Depending on the direction of the equilibrium (towards 'on' or 'off'), the basic activities of receptors may vary. Ligand binding will then change the conformation of the receptor and thereby alter its activation state. GPCR ligands that bind to sites for natural ligands, so called orthosteric sites that are situated on parts of the receptor that are exposed on the extracellular side of the membrane, are accordingly termed orthosteric ligands. Based on the physiologic effect that is induced, this type of ligand stabilizes the receptor in a conformational state whereby the signaling is switched off (inverse agonist), partially on (a

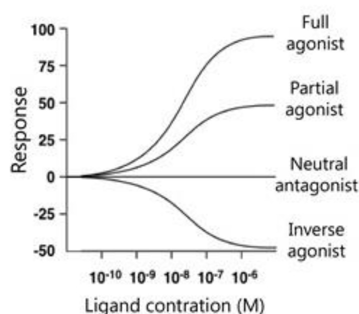

Figure 2. Hypothetical dose-response curves induced by different types of receptor-targeting ligands. A full agonist elicits the maximal response following receptor occupation and activation, whereas a partial agonist is unable to elicit the maximal response through the same receptor and inverse agonist binds to the same receptor-binding site as the agonist but reverses the constitutive activity of the receptor, thereby exerting pharmacologic effects opposite to those of the agonist. A neutral antagonist is a drug that binds to the same site as the agonist and blocks the effect of an agonist. Adapted from https://en.wikipedia.org/wiki/Inverse_agonist#/media/File:Inverse_agonist_3.svg under the Creative Commons licence CC BY-SA 4.0.

partial agonist), fully on (full agonist) or has no effect on the conformation but blocks the binding of other ligands (antagonist) (Figure 2) [19-22].

It is clear from more recent work conducted on GPCRs, that the classical two-state model for how signaling is turned on and off is inadequate for describing the dynamic systems that regulate receptor function. This is clearly illustrated by the effects observed for allosteric and biased ligands. The ternary complex model for GPCR activation, which describes a receptor that moves laterally in the cell membrane to couple physically with a trimeric G-protein after activation by an agonist, only accounts for part of the complexity of GPCR signaling system. Recent theories have revised the ternary complex model to reflect that a receptor may exist in many active conformation states [23, 24]. A criticism of this revisionism is that not all of these potential conformations may be physiologically relevant.

Allosteric modulators of GPCR

GPCRs are protein structures that transmit chemical signals across the cell membrane. Accordingly, agonist binding to the extracellular domains of a receptor induces a conformational change in those parts of the receptor that are located on the cytosolic side of the membrane, which leads to activation of the G-protein. In addition to endogenous (natural) ligand binding to the orthosteric binding site, GPCRs may expose allosteric (Greek for "other site") binding sites that are topographically distinct from the orthosteric site [25]. Ligands that interact with an allosteric binding site are called allosteric modulators, and while they may functionally resemble agonists, antagonists or inverse agonists, they may also modulate basic functions induced by ligands that interact with the orthosteric binding site of the receptor [20, 26]. Depending on its effects on an orthosteric agonist (increasing or decreasing activity), an allosteric modulator can be classified as a positive allosteric modulator (PAM) or a negative allosteric modulator (NAM) [27-30]. Allosteric ligands modulate the receptor in two ways: 1) affinity modulation with conformational change to the receptor; and 2) modulation of efficacy by changing the intracellular signaling capacity (Figure 3).

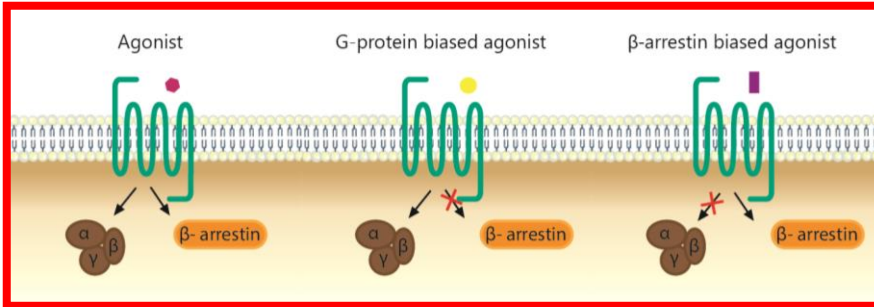

Figure 3. Signaling directed by a G-protein and/or β-arrestin. A conventional/orthosteric/natural GPCR agonist triggers activation of the G-protein- and β-arrestin-mediated signaling pathways (left panel). A biased GPCR agonist triggers selectively or primarily activation of the G-protein-mediated signaling pathway (middle panel) or the β-arrestin-mediated signaling pathway (right panel).

In GPCR-based drug discovery, the recent identification of allosteric modulators for certain GPCRs represents a major breakthrough. Traditionally, GPCR-based drug screening programs have identified drug candidates that target the orthosteric binding sites, making it difficult to achieve high selectivity for specific GPCR subtypes, given that these sites are often highly conserved across members of the single GPCR subfamily. Furthermore, ligands that bind at orthosteric sites for some GPCRs, such as peptide or protein receptors, have other physicochemical and pharmacokinetic properties that are incompatible with scaffolds, which are useful for small molecule drug discovery. Thus, the development of selective allosteric modulators for a specific receptor serves as an alternative approach. *In vivo*, these agents can also have the specific advantage of modulating exclusively receptor activity when the orthosteric agonist is present to occupy the receptor, thereby maintaining spatial and temporal control of receptor signaling [20, 30-32]. A classic example of a PAM is the benzodiazepines used for modulating GABA receptors, providing an effective and safe approach to the treatment of anxiety and sleep disorders [33].

G-protein-dependent signalling downstream GPCRs

GPCRs generally signal through coupling to heterotrimeric guanine nucleotide-binding proteins (G-proteins) that are composed of an α-subunit and a heteromeric βγ-complex. However, there are exceptions to this (see discussion of biased signaling below). There are four main α-subunits ($G\alpha_S$, $G\alpha_{12}$, $G\alpha_q$ and $G\alpha_{i/0}$) and they can be combined with at least 5 different β-subunits and 12 different γ-subunits. The activity

of a G-protein is regulated by GDP/GTP exchange in the α-subunit, which is inactive in its GDP-bound form and active when it is separated from the βγ-complex in the GTP-bound form. Upon ligand binding, the conformational change of the agonist-occupied receptor initiates the GDP/GTP exchange, which results in dissociation of the βγ-complex from the α-subunit [34-36]. It is now well established that not only the α-subunit, but also the βγ -complex is active in signaling. Activation of phospholipase C (PLC) is an early signal initiated by the activated α and βγ complexes that secondarily generates further downstream messengers produced during hydrolysis of the lipid phosphatidyl inositol bisphosphate (PIP_2), giving rise to diacylglycerol (DAG) and Inositol 1,4,5-triphosphate (IP_3). IP_3 triggers the release of Ca^{2+} from intracellular storage organelles and DAG activates protein kinase C (PKC), which is a kinase that is associated, for example, with activation of the superoxide-generating NADPH-oxidase in neutrophils. The phosphoinositide 3-kinase (PI3K) is activated together with other kinases, such as the extracellular signal-regulating kinase (ERK1/2), p38 MAP kinase, and the guanine-nucleotide exchange factors (GEFs). GEFs regulate small G-proteins of the Rho family (Rho, Rac, Cdc42), which are key regulators of several cellular functions [37-40] (for more information see review [41]).

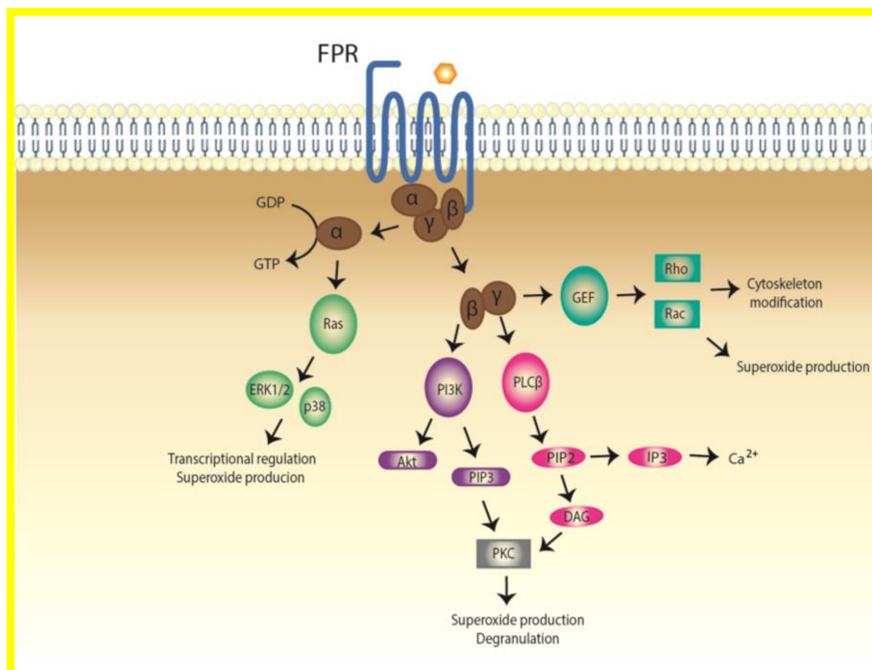

Figure 4. Schematic of the main G-protein-mediated signaling pathways downstream of FPR. Agonist binding to FPR results in dissociation of the heterotrimeric G-protein complex into Gα-GTP and the Gβγ-subunits, leading to activation of downstream signaling cascades and effector functions, including ROS production, degranulation, and transcriptional responses

Biased signalling and β-arrestin binding downstream of activated GPCRs

The classic two-state model for receptor activation was challenged when the concept of biased signaling was introduced around 10 years ago [42-44]. Recent research has demonstrated that the binding of different agonists to the same receptor induces conformational changes in the cytoplasmic signaling domains of the occupied receptor, which triggers signals cascades with or without the direct involvement of a G-protein (Figure 3) [45]. The recruitment and binding of β-arrestins to an activated GPCR blocks G-protein binding; this was initially described as the mechanism for the termination of signaling, but we now know that β-arrestin is an endocytic adaptor protein with its own signaling properties that are independent of any G-protein [46, 47]. To date, only a few receptors have been shown to possess this β-arrestin-mediated biased signaling characteristic, and the precise mechanisms and biological consequences of biased signaling have not yet been clarified, even though this is currently one of the most intensively studied topics in the field of GPCR signaling [48-51].

PEPDUCINS – A NOVEL CONCEPT FOR THE REGULATION OF GPCR FUNCTION

The findings that GPCRs constitute a large protein family of interest for drug development (approximately 40% of drugs currently on the market are GPCR-based) and that allosteric modulators are promising drug candidates inspired Covic and colleagues to introduce a novel concept for GPCR modulation. They showed that a group of membrane-penetrating lipopeptides, named pepducins, could be used to modulate allosterically GPCR signaling [17, 52]. The N-terminal lipid part (usually palmitate) of a pepducin makes the molecule membrane-permeable, while the peptide portion, with sequence identical to that of one of the intracellular loops or the tail of a GPCR, determines receptor preference and selectivity. It has been suggested that the lipid group anchors the pepducin to the membrane, a process that is rapidly and efficiently followed by flipping of the peptide part, such that the peptide sequence becomes exposed on the inner side of the plasma membrane. A direct modulatory effect is then achieved through allosteric modulation of receptor signaling, with the

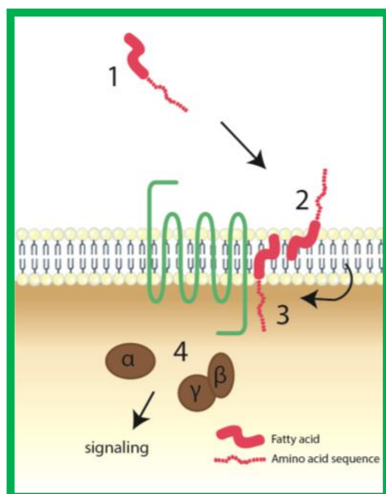

Figure 5. Proposed mechanism for pepducin activities. A pepducin is a fatty acid-conjugated peptide with a peptide sequence identical to one of the intracellular domains of a GPCR (see 1). The fatty acid anchors the pepducin to the cell membrane and the peptide part flips and translocates across the membrane (see 2-3). Once inside the membrane, the peptide part of the pepducin interferes with the signaling domains of the receptor and either activates or inhibits receptor function (see 4).

outcome of either inhibition or direct activation of the cognate GPCRs from which the peptide sequence is derived (Figure 5) [17, 52-54].

The conceptual difference between pepducins and orthosteric ligands is that the functions of the targeted receptor are regulated from the outside of the cell by orthosteric ligands and from the inside of the cell by pepducins. For orthosteric ligands, receptor selectivity is achieved through precise fitting of the ligand to a defined and unique three-dimensional binding pocket that is available in the targeted receptor, whereas the pepducin relies on amino acid sequence identity between the pepducin and the targeted receptor. Several criteria must be fulfilled for the pepducin concept to be valid. One of these criteria is that there should be a difference in activity between the fatty acid-linked peptide and the non-lipidated peptide. In this respect, it is clear that in order for pepducins to be active, the presence of the hydrophobic fatty acid is essential (this applies to all pepducins described), and the fatty acid possibly facilitates plasma membrane passage [53, 55-58]. However, this does not conclusively prove that pepducins initiate signaling through interactions with domains that are facing the cytosol, and it is a much more challenging task to prove rather than merely show that the peptide can pass through the membrane. Another criterion is that the pepducin should be able to trigger a response in cells that express the targeted receptor only. However, this is also the case for orthosteric extracellular receptor agonists and it is by no means a unique property of pepducins. Moreover, according to the pepducin concept, the functional activities of pepducins should not be affected by conventional antagonists, and their binding should not be affected by conventional receptor agonists or antagonists.

Activating and inhibiting pepducins

The pepducin concept has prompted the design of different palmitoylated peptide sequences derived from a number of GPCRs, among which the pepducins derived from the protease-activated receptor (PAR1) are the most studied. A 19-amino acid pepducin (P1pal-19) derived from the third intracellular loop of PAR1 selectively induced a Ca^{2+} response in PAR1-expressing cells, and the response was identical to that induced by a conventional PAR1 agonist [52]. More importantly, that previous study shows that a PAR1 antagonist that inhibits the conventional PAR1 agonist has no effect on the P1pal-19-induced Ca^{2+} response and that a mutant PAR1 receptor with deletion of the entire C-terminal tail responds to a conventional agonist but does not respond to the pepducin. This suggested that the C-tail of PAR1 was required for pepducin binding, and the data obtained represented a proof of principle for receptor-modulating pepducins [52]. Pepducins with amino acid sequences identical to other intracellular domains of PAR1 or those of other members of the PAR family

(PAR1, PAR2, and PAR4) have been identified and shown to exert either receptor-activating or receptor-inhibiting functions [53, 57, 59-61]. The precise mechanism of action has been studied using a PAR1 pepducin and a FRET-based assay to determine binding, and the results indicate that the pepducin is located close to the inner leaflet of the plasma membrane [58].

A library screen using pepducins derived from the intracellular domains of chemokine receptor type 4 (CXCR4) identified several activating pepducins with amino acid sequences identical to those in the first intracellular loop of the receptor. One of these pepducins, ATI2341, has been shown to induce a CXCR4-dependent Ca^{2+} increase, chemotactic migration, and mobilization of white blood cells from the bone marrow [62]. A bioluminescence resonance energy transfer (BRET) assay system has been used to determine pepducin-induced recruitment to CXCR4 of different G-proteins ($G\alpha_i$, $G\alpha_{13}$) and β-arrestin, respectively, and it has been shown that this CXCR4 pepducin is a biased CXCR4 agonist, promoting the engagement and activation of $G\alpha_i$ but not of $G\alpha_{13}$ or β-arrestin [56]. In addition, a pepducin derived from the second intracellular loop of the sphingosine-1-phosphate receptor (S1P3 or EGD3) has been shown to induce cellular responses similar to those induced by the conventional agonist [63].

Receptor-specific pepducins have also been identified for the adrenergic receptor and the formyl peptide receptor 2 (FPR2) [64, 65]. Pharmacokinetic, pharmacodynamic, and bio-distribution studies have shown that pepducins are widely distributed throughout the body, with the exception of the brain, and possess drug-like properties that make them appropriate for use in vivo [66]. The beneficial effects of pepducins have been observed in several mice disease models [58, 59, 67-69]. Overall, the pepducin concept has been shown to be valid as a means to activate or inhibit functional responses mediated by a wide range of receptors (Table 1), which suggests that this type of allosterically modulating lipopeptide may be a valuable tool for basic GPCR research and for de-orphanizing receptors for which no agonists have yet been identified.

Table 1. List of selected pepducins.[1]

Ligand	Loop[2]	Derived[3]	Effect	Reference
P1pal12	i3	PAR1	Antagonist	[52, 53, 67, 70]
P1pal19	i3	PAR1	Agonist	[52, 61]
P2pal8S	i3	PAR2	Antagonist	[71]
P4pal10	i3	PAR4	Antagonist	[72, 73]
P4pal-i1	i1	PAR4	Antagonist	[60, 74]
F2pal10	i3	FPR2	Agonist	[75]
x1/2pal-i3	i3	CXCR1/CXCR2	Antagonist	[59] [76]
x4pal-i1	i1	CXCR4	Antagonist	[59]
ATI-2341	i3	CXCR4	Agonist	[59] [62]
KRX-725	i2	S1P3	Agonist	[63]
SMOi2-1	i2	SMO	Antagonist	[77]

[1]Adapted from [59].
[2]The intracellular (i) loops are numbered from the N-terminal domain
[3]The receptor from which the sequence is derived

Receptor-independent effects mediated by pepducins

Pepducins were introduced as a novel type of GPCR modulator with high selectivity for their receptors. However, receptor-independent effects have also been observed for pepducins. Using a screening approach to search for β2AR pepducins, Carr et al [64, 78] identified a number of receptor-dependent pepducins, as well as pepducins that activated the cells independent of the receptor, and the mechanism was shown to involve direct activation of the downstream G_s-protein. When the effects of this G_s-activating pepducin were subsequently studied in neutrophils, the earlier-described functional effects induced by G_s-activation were not induced by these pepducins in neutrophils, suggesting differences related to the cell type in which the G_s-protein is expressed [64, 78]. Another receptor-independent effect of pepducins is a direct bactericidal activity (**Paper I**). Based on similarities in the physico-chemical properties between pepducins and a group of naturally occurring antibiotic lipopeptides, we hypothesized that pepducins also kill bacteria. We found that pepducins exert direct killing of both Gram-positive and Gram-negative bacteria, as well as clinical isolates of pathogenic bacterial species (**Paper I**). In an era increasing microbial resistance to classical antibiotics, there is a need for new antimicrobial drugs. Since the first approval of a lipopeptide as an antimicrobial drug back in 2003, this group of molecules has received much attention and represents one of the fastest growing areas of research in antimicrobial drug discovery [79-81].

INNATE IMMUNITY AND THE ROLE OF NEUTROPHILS

While our immune system is vitally important in protection against invading microorganisms, it also contributes to tissue injury and disease, as well as to the resolution of inflammation and damage repair. The inflammatory reaction is a process that is designed to kill, clean, heal, and repair. The immediate local reaction is swelling, redness, pain, heat, and possibly dysfunction of the inflamed tissues. These local reactions depend on an increased blood flow, relaxation effects on blood vessels, the release of pro-inflammatory mediators, the extravasation of fluids from the circulation into the infected/inflamed tissue, and the influx of pro-inflammatory cells, which are predominantly neutrophil granulocytes, the primary cells in the first line of host defense [82]. Dysregulation of the inflammatory response may lead to chronic inflammation, as well as auto-inflammatory disorders. Innate immune reactivity is very rapid and is constituted by three fundamental steps: i) the recognition of 'danger' molecules from pathogens or damaged tissue cells; ii) the ability to kill microbial pathogens and clean up cell debris; and iii) the ability to minimize host tissue-destructive activities thereby maintaining self-tolerance.

The innate immune apparatus is composed of cellular and humoral components, and these two parts are linked to recognition and/or effector functions that interact within a complex network. The humoral parts consist of many different soluble molecules that are present in extracellular compartments, including liver-produced acute-phase proteins, such as LBP (the lipopolysaccharide-binding protein), SAA (serum amyloid A), the C-reactive protein, and complement components [83, 84]. The major cellular apparatus in inflammatory reactions is the professional phagocyte of myeloid origin. Phagocytes differentiate and mature in the bone marrow, and when recruited to the bloodstream their commission is to seek and sense invading microbes in the tissue and thereafter engulf (phagocytose) and kill these invaders. The neutrophil granulocyte is one of the specialized killer cells, the so-called 'professional phagocytes', being endowed with a broad array of weapons and being of prime importance in innate immunity and inflammation [85-87].

The neutrophil granulocyte

Neutrophils are produced in the bone marrow; in a human adult, $1–2×10^{11}$ neutrophils are produced every day [88]. The neutrophil differentiation/maturation process takes approximately 14 days [89]. Mature neutrophils recruited from the bone marrow to the bloodstream will circulate in a naive/resting state, waiting to be recruited in response to danger signals emanating from a microbial infection or tissue injury. In humans, neutrophils are the most common white blood cell type, and they account for 50%–70% of all leukocytes in the peripheral blood. Together with eosinophils and basophils, they comprise the subgroup of polymorphonuclear leukocytes (PMN; named after the appearance of their multi-lobulated nuclei). These cells are also known as granulocytes, a name that reflects the high number of granules in their cytoplasm. In neutrophils, these granules (small membrane-enclosed organelles) act as storage organelles, containing numerous antimicrobial compounds, proteolytic enzymes, and membrane-localized receptors. Neutrophils have at least four different types of granules/vesicles, described in more detail below [90-93].

Two pools of neutrophils are found in peripheral human blood. One is the circulating pool (around 50% of the cells), and the other pool comprises neutrophils that are loosely attached to the vascular endothelium (known as the 'marginating pool´) [94]. In response to a local infection/inflammation, blood neutrophils are recruited to and accumulate at the affected site [94]. This recruitment is a dynamic process that involves several neutrophil functions, all of which are of vital importance for a successfully operating immune system. Using an aseptic inflammation skin chamber model, *in vivo* studies have revealed that during the recruitment process substantial amounts of various inflammatory mediators and neutrophil granule constituents are produced/released [95]. These factors are involved in the killing of microbes and in the resolution of the inflammatory reaction. The recruited neutrophils are also functionally adapted to the conditions at the inflammatory site. Compared to peripheral blood neutrophils, the tissue-recruited neutrophils produce higher levels of superoxide upon stimulation with certain chemoattractants that are generated by the electron-transporting NADPH-oxidase within these cells [96]. Functional analysis, as well as analysis of cell surface-exposed granule markers reveal that the granule mobilization that occurs during tissue recruitment of neutrophils is accompanied by the exposure of new receptors for specific chemoattractants, with these receptors being potentially mobilized from storage pools through the fusion of granule membranes with the plasma membrane [95-102]. Other chemoattractant receptors are downregulated/desensitized, possibly through a hierarchal receptor cross-talk mechanism that is of importance for the recruitment process. In order to be able to exit rapidly from the bloodstream and transmigrate through the endothelium and the extravascular tissue,

neutrophils are equipped with receptors that recognize PAMPs (pathogen-associated molecular patterns, from microbes) or DAMPs (danger-associated molecular patterns, from damaged tissues). Many PAMPs and DAMPs recognize receptors that belong to the GPCR superfamily [88, 103].

FUNCTIONS OF NEUTROPHILS EXPRESSING GPCRS

Neutrophils express a number of G-protein-coupled chemoattractant receptors, including those that recognize the platelet-activating factor (PAFR), leukotriene B4 (BLT1/2), and complement fragment 5a (C5aR) [104]. Of the 18 human chemokine GPCRs that have been identified, neutrophils are known to express CXCR4, CXCR1, and CXCR2 [105]. Most neutrophil GPCRs are coupled to pertussis toxin-sensitive G-proteins of the $G\alpha_i$ subgroup, and activation of the chemoattractant/chemokine receptors in neutrophils induces not only cellular directional migration, but also the release of reactive oxygen species (ROS) generated by the phagocyte NADPH-oxidase [106]. This oxidase is a multicomponent enzyme made up of a membrane-bound heterodimeric b-type cytochrome ($p22^{phox}$ and $gp91^{phox}$/Nox2), the soluble cytosolic components of $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, and the small GTPase Rac. Upon chemoattractant stimulation, the soluble components translocate to the b cytochrome, thereby forming an active enzyme that transfers electrons from NADPH in the cytosol across the membrane to reduce the oxygen to superoxide anions (O_2^-) (Figure 6) [107-110].

In addition to their abilities to activate the NADPH-oxidase, GPCR agonists may trigger a secretion process that leads to the mobilization of receptors and adhesion molecules from the intracellular storage granules in neutrophils. As mentioned above, neutrophils contain at least four different types of granules/vesicles, which are formed at different stages of neutrophil maturation in the bone marrow [90]. The first granules to be formed are the azurophilic (or primary) granules, followed by the specific (or secondary) granules. These granules contain numerous antimicrobial and potentially tissue-destructive components and they fuse primarily with phagocytic vacuoles. They are rather difficult to mobilize through fusion with the plasma membrane. The gelatinase (or tertiary) granules, which are formed at a later time-point in the maturation process, are easily mobilized, and their content of receptors is moved to the cell surface. The most easily mobilized and receptor-rich secretory vesicles are the last to be formed, from the plasma membrane through an endocytic process [91, 92]. Receptors that are mobilized to the cell surface from granules/vesicles include

the recently identified and partially characterized pattern recognition receptor FFA2R (a short-chain free fatty acid receptor, also called GPR43), which recognizes products derived from gut bacteria during the fermentation of dietary fibers, and the formyl peptide receptors (FPRs) that recognize formyl peptide, a hallmark of bacterial protein synthesis (described in more detail below) [111, 112].

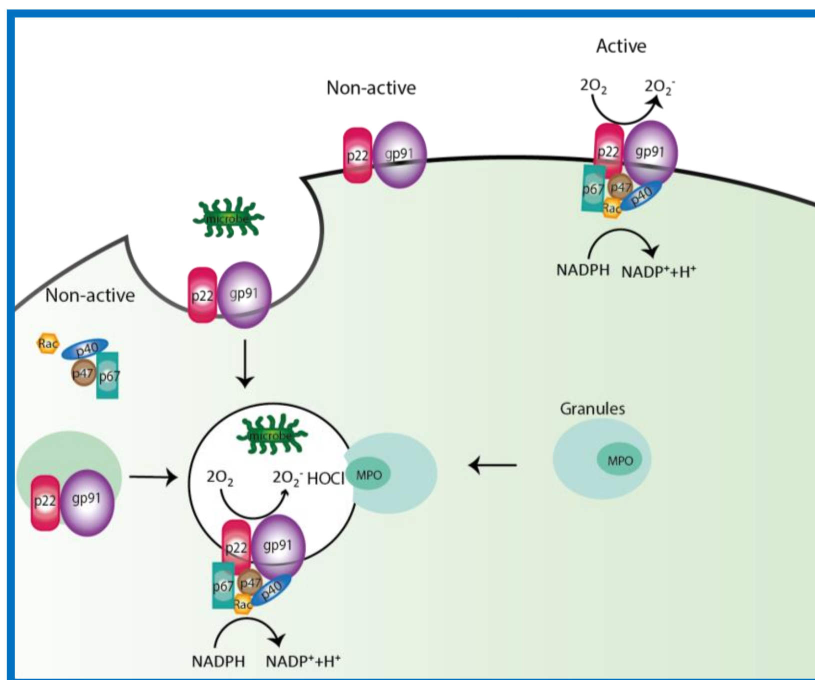

Figure 6. The neutrophil NADPH-oxidase. The NADPH-oxidase comprises a membrane-localized b-type cytochrome (also referred to as the heterodimer of p22phox and gp91phox) and the cytosolic components p40phox, p47phox, and p67phox, as well as the cofactor Rac. Upon activation, cytosolic components translocate to the b-type cytochrome-containing membranes to form a functional NADPH-oxidase, which is capable of producing reactive oxygen species (ROS). ROS production can occur either on the plasma membrane, resulting in the release of extracellular ROS, or on the phagosomal (or granule) membrane during phagocytosis. The b-type cytochrome in the phagosome originates from the plasma membrane and from the fusion of specific granules. Another type of granule (azurophilic) that contains myeloperoxidase (MPO) is also recruited and participates together with the formed hydrogen peroxide to generate hypochlorous acid ($HOCl^-$).

Formyl peptide receptors

In the mid-1970's, Shiffmann *et al.* showed that synthetic peptides that contained an N–terminal formylated methionine (fMet) could act as chemoattractants for macrophages and neutrophils [113]. They postulated that peptides that contained N-fMet should be produced by prokaryotes and possibly constitute the chemotactic activity observed for the supernatant fluids obtained from bacterial cultures. Indeed, highly potent chemotactic fMet-containing peptides have since then been isolated from culture filtrates of a number of bacteria, including *E. coli* [114], *S. aureus* [115-118], *M. avium* [119], and *L. monocytogenes* [117, 120, 121]. It is important to note that not only bacteria, but also mitochondria initiate protein synthesis with an N-formylated methionine, which means that damaged mitochondria also release danger signals in the form of formyl peptides that possess chemotactic activity for neutrophils [120, 122, 123].

The work of Shiffmann *et al* was soon followed by other studies that identified the formylated tripeptide fMLF (in the older literature, this is known as fMLP) as the most potent agonist of many different tested peptides. In 1990, the human receptor (originally FPR, now FPR1) for this peptide was cloned by screening a cDNA expression library that was constructed with mRNA species from differentiated HL-60 cells [124-126]. Shortly thereafter, using low-stringency DNA hybridization screening (under conditions of reduced temperature and/or increased salt concentration), with the cDNA of human FPR1 as the probe, two additional FPR-like receptors were cloned and named FPRL1 (now FPR2) and FPRL2 (now FPR3), and the genes for all three are clustered together on chromosome 19 q13.3 in the human genome [127-130]. Polymorphisms of the *FPR1* gene have been described in patients who are suffering from localized juvenile periodontitis, resulting in defects in G_i-protein coupling and reduced cell function [131, 132]. In addition, the FPR1 allele that contains an amino acid substitution in the C-terminal tail (abolishing its function) was associated with poor survival outcomes following chemotherapeutic treatment in patients who were suffering from breast and colorectal cancer [133, 134]. No polymorphisms in the coding regions of *FPR2* or *FPR3* have been described.

Although FPRs are mainly expressed by cells of myeloid origin, indicating a key role in innate immune reactions, they are also found in many other organs and tissues, including epithelial cells, liver hepatocytes, and Kuppfer cells, smooth muscle, and endothelial cells, as well as in neurons of the motor, sensory, and cerebellar systems [135]. The wide expression pattern of FPRs in non-immune cells suggests that they are also participating in other activities. Since this thesis deals mainly with neutrophils that express only FPR1 and FPR2, these two FPRs will be the focus hereafter.

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FPR1_HUMAN      METNSSLPTNISGGTPAVSAGYLFLDIITYLVFAVTFVLGVLGNGLVIWVAGFRMTHTVT     60
FPR2_HUMAN      METNFSTPLNEYEEVSYESAGYTVLRILPLVVLGVTFVLGVLGNGLVIWVAGFRMTRTVT     60
FPR3_HUMAN      METNFSIPLNETEEVLPEPAGHTVLWIFSLLVHGVTFVFGVLGNGLVIWVAGFRMTRTVN     60
                **** * * *    .    **: .* *:  :* .****:*****************:**.

FPR1_HUMAN      TISYLNLAVADFCFTSTLPFFMVRKAMGGHWPFGWFLCKFVFTIVDINLFGSVFLIALIA     120
FPR2_HUMAN      TICYLNLALADFSFTATLPFLIVSMAMGEKWPFGWFLCKLIHIVVDINLFGSVFLIGFIA     120
FPR3_HUMAN      TICYLNLALADFSFSAILPFRMVSVAMREKWPFGSFLCKLVHVMIDINLFVSVYLITIIA     120
                **.*****:***.*:: *** :*  **  :**** ****::. ::***** **:** :**

FPR1_HUMAN      LDRCVCVLHPVWTQNHRTVSLAKKVIIGPWVMALLLTLPVIIRVTTVPGKTGTVACTFNF     180
FPR2_HUMAN      LDRCICVLHPVWAQNHRTVSLAMKVIVGPWILALVLTLPVFLFLTTVTIPNGDTYCTFNF     180
FPR3_HUMAN      LDRCICVLHPAWAQNHRTMSLAKRVMTGLWIFTIVLTLPNFIFWTTISTTNGDTYCIFNF     180
                ****:*****.*:*****:*** :*: * *:::::**** ::  **:   .* . * ***

FPR1_HUMAN      SPWTNDPKERINVAVAMLTVRGIIRFIIGFSAPMSIVAVSYGLIATKIHKQGLIKSSRPL     240
FPR2_HUMAN      ASWGGTPEERLKVAITMLTARGIIRFVIGFSLPMSIVAICYGLIAAKIHKKGMIKSSRPL     240
FPR3_HUMAN      AFWGDTAVERLNVFITMAKVFLILHFIIGFSVPMSIITVCYGIIAAKIHRNHMIKSSRPL     240
                : *     **::* ::* ..  *::*:**** ****:::.**:**:***:: :*******

FPR1_HUMAN      RVLSFVAAAFFLCWSPYQVVALIATVRIREL-LQGMYKEIGIAVDVTSALAFFNSCLNPM     299
FPR2_HUMAN      RVLTAVVASFFICWFPFQLVALLGTVWLKEMLFYGKYKIIDILVNPTSSLAFFNSCLNPM     300
FPR3_HUMAN      RVFAAVVASFFICWFPYELIGILMAVWLKEMLLNGKYKIILVLINPTSSLAFFNSCLNPI     300
                **:: *.*:**:** *::::.:: :* ::*: : * ** * : :: **:**********:

FPR1_HUMAN      LYVFMGQDFRERLIHALPASLERALTED--STQTSDTATNSTLPSAEVELQAK     350
FPR2_HUMAN      LYVFVGQDFRERLIHSLPTSLERALSED--SAPTNDTAANSASPPAETELQAM     351
FPR3_HUMAN      LYVFMGRNFQERLIRSLPTSLERALTEVPDSAQTSNTDTTSASPPEETELQAM     353
                ****:*::*:****::**:******:*   *: *.:* :.*: *  *.****
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Figure 7. Sequence alignments of the human FPR1, FPR2, and FPR3 proteins. The UniProt alignments of FPR1 (UniProt ID: P21462), FPR2 (P25090) and FPR3 (P25089) are shown. Highlighted in pink are the trans-membrane domains of the proteins. Under the sequences, the colons (:) indicate conservation. of residues between proteins with strongly similar properties, the asterisks (*) indicate fully conserved residues, and the periods (.) indicate conservation of residues between proteins with weakly similar properties.

FPR signaling and regulation in neutrophils

Human neutrophils express FPR1 and FPR2, whereas monocytes express all three members of the FPR family [136]. At the primary sequence level, FPR2 shares 69% identity with FPR1 and a higher level of homology (83%) with FPR3 (Figure 7) [127, 130]. The two neutrophil FPRs share the highest levels of sequence similarities in their cytoplasmic signaling domains, which suggests that they transduce very similar signals through similar pathways and indeed, they trigger almost indistinguishable

cellular responses in neutrophils (Figure 7). The signaling pathways located downstream of FPR1 have been extensively studied, and details about the signal transduction pathways that participate in the induction of discrete neutrophil functions can be found in several recent reviews [137-139]. Agonist binding to FPRs leads primarily to signaling through the $G\alpha_i\beta\gamma$-regulated signaling route, and once activated, the dissociated $G\alpha_i$-protein subunits activate multiple downstream second messengers, including various phospholipases and protein kinases [140]. Based on the results obtained using a simple and straightforward system to measure β-arrestin binding, both FPR1 and FPR2 trigger translocation of β-arrestin, although the roles of this binding in signaling and functional responses have not been clearly defined. It is important to mention that unlike other GPCRs that rely on β-arrestin as the structural entity that is responsible for termination of G-protein signaling, desensitization of the FPRs relies in large part on binding to the actin cytoskeleton [141, 142]. That desensitized FPRs can be reactivated to produce superoxide by the addition of cytoskeleton-disrupting agents, such as cytochalasin B and latruculin A, strongly supports the idea that the cytoskeleton plays an important role. In addition, recent research has revealed that FPR reactivation can be induced by a novel receptor cross-talk mechanism, as illustrated by the reactivation of desensitized FPRs induced by ATP and PAF upon binding to their respective receptor [143]. This cross-talk signal generates a biased FPR response, as the reactivated receptor triggers assembly/activation of the NADPH-oxidase but no transient rise in the level of intracellular calcium [143].

Conventional FPR agonists

FPR1 was originally identified as a high-affinity receptor for formyl peptides. However, it has subsequently been discovered that one of the most prominent features of the FPRs is their ability to recognize many and diverse ligands, ranging from the formylated peptides, through non-formylated microbial/synthetic peptides and small molecules, to allosteric modulators, which include peptidomimetics and lipopeptides (see next section and **Papers I–IV**). Compared to FPR1, FPR2 displays a much more diverse ligand profile, and this receptor recognizes a broad range of molecules, including the GP-41 envelope protein of the human immunodeficiency virus type 1 (HIV-1), a peptide derived from glycoprotein G of herpes simplex virus type 2, Hp2-20 from *Helicobacter pylori*, and the synthetic peptides WKYMVM/m. The reader is directed to other recent reviews for a full description of FPR -specific/-selective ligands [140, 144-147]. FPR2 was for a long time regarded as an orphan, even though fMLF was known to be a low-affinity agonist. Soon after proper deorphanization, this receptor was shown to recognize a number of non-formylated agonists. However, formylated, phenol-soluble modulin (PSM) peptides, which are secreted by

methicillin-resistant *Staphylococcus aureus* (CA-MRSA), were recently identified as selective and potent agonists of FPR2 [121]. In addition, several mitochondrion-derived formyl peptides, such as mitocryptide, are preferentially recognized by FPR2 but not by FPR1 [113, 120, 121, 148].

Table 2. Overview of select FPR agonists, showing their origins and receptor specificities.

Ligand(s)	Origin	Receptor preference	Reference
		N-formylated	
fMLF	*E. coli*	FPR1 >> FPR2	[124, 149]
fMIFL	*S. aureus*	FPR1 >> FPR2	[115, 150]
fMIVIL	*L. monocytogenes*	FPR1 >> FPR2	[117, 120]
PSMα2, PSMα3	*CA-MRSA*	FPR2 >> FPR1	[121]
Hp2-20	*H. pylori*	FPR2	[151],
		Host-derived	
LL37	Cathelicidin	FPR2	[152, 153]
Annexin I	Endogenous human protein	FPR1, 2	[154-156]
Aβ (1-42)	Amyloid precursor	FPR2	[157]
		Peptide library	
WKYMVM	Synthetic peptide	FPR2 > FPR3	[158]
WKYMVm	Synthetic peptide	FPR2 > FPR1	[158-160]
MMK-1	Synthetic peptide	FPR2	[161, 162]
		Small molecules	
Comp 43	High-throughput screening	FPR1 > FPR2	[163, 164]
		Peptoidomimetic	
F2M2	Synthetic peptide	FPR2	[165]

When one compares the receptor preferences for formyl peptides of the two neutrophil FPRs, it appears that size is of importance, in that longer peptides prefer FPR2 whereas shorter peptides (<10 amino acids) prefer FPR1, and the in-between peptide lengths are equally potent for FPR1 and FPR2 [120]. Moreover, host-derived molecules have been suggested to act as FPR2 ligands, most notably the acute-phase protein SAA [166-168]. However, most (if not all) studies of the SAA-FPR2 complex have been performed with a recombinant protein that is a hybrid of two human SAA isoforms (SAA1 and SAA2) that do not exist *in vivo* [169]. It is debatable whether the idea of acute-phase SAA being a cytokine-like protein with pro-inflammatory properties really reflects the true biological activity of the endogenous SAA. Another host-derived molecule that interacts with the FPRs belongs to the annexin family of calcium-regulated, phospholipid-binding proteins that are involved in the regulation of

innate and adaptive immunity, although the receptor(s) involved in the different pathways have not been properly defined [148, 170-172].

As the FPRs have important regulatory functions in inflammation and in the pathogenesis of various diseases, targeting FPRs with receptor-specific ligands (agonists, antagonists, and allosteric modulators) has great therapeutic potential for treating diseases in which the inflammatory reaction is uncontrolled. Indeed, many FPR-selective peptide/protein ligands, as well as stable and selective small-molecule ligands have been identified over the last decades using high-throughput screening. It should be noted that it is of importance to determine the precise receptor specificity of "screening hits", as illustrated by the case of the potent agonist compound 43, which although it was originally identified in a screening process with FPR2-expressing cells, has been shown to interact preferentially with FPR1 [164]. The small molecules that are described as FPR agonists activate the preferred receptor also when expressed in naive human neutrophils, and the induced activities resemble those of pro-inflammatory peptides.

Lipid inhibitors of innate immune cell function have recently been shown to be of physiological relevance for resolving inflammation, and it has been claimed that FPR2 is one of the receptors shared by mediators of the lipoxin and resolvin groups of lipid metabolites. When LXA_4 (lipoxin A_4) analogues from two commercial sources were used neither induced any translocation of β-arrestin, as measured in an enzyme fragment complementation assay [173]. Based on these results, it was concluded that no signal is generated from FPR2 by LXA_4 in neutrophils, and that the LXA_4 effects on other cells are most likely mediated through an as yet unidentified receptor that is different from FPR2 [174]. In agreement with this conclusion, others have also failed to observe any FPR2-related effect of LXA_4 [175].

Conventional and allosteric FPR antagonists

A recent search for new FPR antagonists, using a ligand-based virtual screening technique, identified 30 FPR antagonistic compounds, including the potent Quin-C7, belonging to different chemical families [176]. The same research group identified WKYMVM from a peptide library and subsequently, they discovered the FPR2 antagonist WRWWWW (WRW_4) [177]. With respect to FPR1 antagonists, the cyclic undecapeptide cyclosporine H (CysH) produced by fungi is the most potent and selective. The mode of action is reduction of the basal activity of FPR1, which means that CysH is an inverse agonist [178, 179]. Replacing the formyl group of fMLF with a tertiary butyloxycarbonyl group (Boc-MLF, also known as Boc1) or replacement of the MLF sequence with FLFLF to yield Boc-FLFLF (also known as Boc2) generates FPR1 antagonists [149]. Boc1 and Boc2, when used at higher concentrations, partially inhibit FPR2 also [180].

A rhodamine-conjugated, gelsolin-derived peptide (PBP_{10}) has been identified as a potent inhibitor of FPR2. While it blocks FPR2-mediated responses without affecting FPR1 signaling, the inhibitory effect is not entirely FPR2-specific, since some non-FPR2-mediated signaling is also inhibited [181-183]. The rhodamine group is required for the PBP_{10} peptide to pass through the plasma membrane and for the FPR2-specific inhibitory function of the peptide [181, 183, 184]. A core PBP peptide (RhoB-QRLFQVG) for FPR2 inhibition has been identified, and this shorter peptide partly inhibits also FPR1 [181], which suggests that a structure of importance for inhibition is present also in FPR1, although this is obviously not accessible for the longer peptide. It has been suggested that PBP_{10} modulates FPR2 from the cytosolic side, although it is difficult to prove conclusively that it interacts with its specific receptor from the inside of the plasma membrane. This is also the case for the FPR2 pepducins (see below). The physicochemical properties (charge and hydrophobicity) that permit these membrane-permeable molecules to enter the cytoplasm are required for proper functionality, although that does not mean that they modulate receptor function from the cytosolic side of the membrane. The precise site of action of PBP_{10} remains unresolved. It is worth noting, however, that PBP_{10} inhibits the cellular response induced by allosteric FPR2-activating pepducins (see below).

Table 3. List of select FPR antagonists, showing their origins and receptor specificities.

Ligands	Source	Receptor	Literature
		Conventional ligands	
CysH	*T. inflatum*	FPR1	[185]
CHIPS	*S. aureus*	FPR1	[186]
FLIPr	*S. aureus*	FPR2 >> FPR1	[187]
PBP_{10}	Binding domain of gelsolin	FPR2 >> FPR3	[181]
		Peptide library	
WRW_4	Synthetic peptide	FPR2 >> FPR3	[177]
Boc1	Synthetic peptide	FPR1 >> FPR2	[149]
Boc2	Synthetic peptide	FPR1 >> FPR2	[149]
		Peptidomimetic	
Cmp. 1	Synthetic peptidomimetic	FPR2	[188]

FPR2-derived pepducins activate FPR2

Given that they have the capacity to permeate cell membranes and allosterically modulate GPCR function, pepducins should also be able to interact with neutrophil GPCRs, thereby providing unique tools for the regulation of innate immune-related activities. Accordingly, neutrophil-activating pepducins were recently described as having in common, peptides with amino acid sequences identical to the whole or parts of the third intracellular loop of FPR2 linked to a fatty acid [75]. Interestingly, the most potent peptide is not the one that contains the entire loop (16 amino acids) but the $F2Pal_{10}$ peptide, which contains 10 amino acids (**Paper I**). These FPR2-activating pepducins are highly FPR2-selective, as it has been shown that they are inactive in FPR1-transfected cells and that their activities in neutrophils are insensitive to the FPR1-specific antagonist CysH [75]. The pepducin concept proposes that receptor selectivity is manifested through the sequence identity between the pepducin and the intracellular loop of the targeted receptor, although it is difficult to understand how these two sequences act together to modulate receptor intracellular signaling. Studies using a chimeric FPR1-FPR2 receptor in which the third intracellular loop of FPR2 (from which the FPR2-activating pepducin is derived) was replaced with that of the FPR1 (pepducin-insensitive) showed that the chimeric receptor still recognizes the pepducin, suggesting that there is no direct linkage between the amino acid sequence in the activating pepducin and that in the third intracellular loop of the activated receptor [75]. $F2Pal_{10}$ triggers a neutrophil activation pattern that is very similar to that induced by conventional FPR2 agonists, despite the fact that pepducins and conventional agonists initiate signaling through different mechanisms, with "in-

side-in" signaling seen for pepducins and "outside-in" signaling observed for extracellular agonists. It is known that FPR2 cross-talks with other GPCRs, such as PAFR and $P2Y_2R$, through a unique but not yet understood signaling pathway that leads to FPR2 reactivation [143]. Both conventional and allosteric FPR2 modulators can induce this cross-talk, although the $F2Pal_{10}$ pepducin is more strongly biased toward this signaling than the conventional FPR2 agonist [189]. Thus, there may be some signaling differences between conventional and allosteric agonists.

The process termed "insertion and inversion", which entails the pepducin incorporating its lipid tail into the phospholipid bilayer, thereby enabling the peptide part of the construct to flip over the membrane and become exposed on the cytosolic side of the membrane, is suggested as the basic mechanism through which pepducins mediate their activities (Figure 5). As a consequence of this process, the lipid part is a prerequisite for activity, and this is also true for the FPR2 pepducins, as no activity was obtained with non-lipidated peptides [54]. It is also clear that the FPR2 pepducins are highly selective for the receptor, possessing sequence identity with the pepducin peptide, which is in agreement with the concept. However, some of the reported results raise questions regarding the precise mechanism of action. For example, the FPR2 pepducin inhibits the binding of a conventional peptide agonist that is selective for FPR2, and this inhibition occurs also at temperatures (e.g., 4°C) at which it must be very difficult for the pepducins to pass through the plasma membrane (**Paper II**). We should perhaps not assume that all pepducins act on the intracellular region of the specifically targeted GPCR and induce their effects through so-called allosteric modulation of intracellular receptor domains that couple to G-proteins and other signaling/regulating proteins. Instead, we should consider the possibility that there are different, and possibly unique, modes of action related to each individual pepducin/receptor pair.

Bacterial killing and immunomodulation

It is well established that the allosteric modulatory activity of pepducins relies on both the lipid anchor and the peptide sequence, and this is also true for the bacterial killing activities of known antimicrobial lipopeptides. Thus, immunomodulatory pepducins, being lipopeptides, may also have direct killing effects on bacteria [153, 190]. Accordingly, the direct antimicrobial activities of FPR2 pepducins were assayed, and the structural/physicochemical features of pepducins that link receptor activation and bacterial killing were exploited. The physicochemical properties of the lipopeptides are of importance both for their abilities to activate neutrophils and for killing bacteria, although there are no direct linkages between the two functions (**Paper I**). Although FPR2 pepducins have off-target effects that are independent of FPR2, this

functional dualism of pepducins could be explored as a novel class of antibacterial drugs with immunomodulatory properties. These results raise the possibility that other antimicrobial lipopeptides have immunomodulatory effects– an idea that warrants investigation.

Peptidomimetics, which are partly composed of unnatural residues, are proteolytically stable against endogenous protease degradation and may be designed to mimic immunomodulatory host defense peptides [188], as well as to share structural similarities with lipopeptides, such as the pepducins. Accordingly, the recently discovered FPR2-selective modulators belong to the class of lipidated α-peptide/β-peptoid hybrids. By screening a small array of a peptidomimetic library, using GPCR-dependent neutrophil production of superoxide as a read-out, both activating and inhibiting peptidomimetics that target specifically FPR2 were recently identified [165, 188]. The most promising compound, Pam-(Lys-βNSpe)$_6$-NH_2, was shown to inhibit FPR2 agonist-induced neutrophil granule mobilization and the release of ROS. The potency of Pam-(Lys-βNSpe)$_6$-NH_2 was comparable to that of PBP_{10}, which is the most potent FPR2-selective inhibitor known, and combining these two agents gave an additive effect [188]. The rhodamine group in the core PBP_{10} peptide cannot be exchanged for palmitic acid [181], suggesting that the mechanisms of action might differ between the lipopeptoid and PBP_{10}. This notion is supported by the finding that PBP_{10} has no effect on the mouse receptor, whereas the lipopeptoid inhibits the function of this receptor (see below and **Paper III**). The immunomodulatory effects of structural analogs of Pam-(Lys-βNSpe)$_6$-NH_2 emphasize the importance of both the lipid and peptidomimetic parts [188]. In addition, we have identified Lau-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH_2 as a potent FPR2 activator [165]. Further analyses of a number of structural variants have revealed that the *N*-acyl 2-aminooctanoic acid residue and the peptidomimetic backbone of the molecule are both required for the agonistic activity. This novel class of FPR2-modulating peptidomimetic ligands may serve as valuable tools for further delineation of the ligand recognition and signaling mediated by FPR2, as well as for exploring the therapeutic potential of targeting FPR2 in disease.

Receptor hijacking: FPR2-interacting pepducins with amino acid sequences derived from other GPCRs

Pepducins can activate receptors, as shown for the FPR2 third intracellular loop pepducins that are highly specific for this receptor, and despite their extensive sequence similarity with FPR1 (69% amino acid identity), there is no cross-activation of this receptor. Pepducins can, however, have the opposite effect and inhibit the

function of the targeted receptor, and this is true for the pepducin derived from the third intracellular loop of FPR1-inhibited neutrophils, although the target in this case is not FPR1. The 16-amino acid pepducin derived from the third intracellular loop of FPR1 potently inhibited neutrophil functions, and unexpectedly, the identity of the targeted receptor was found to be FPR2. Thus, FPR1 pepducin hijacks the closely related FPR2 (**Paper II**). This type of cross-reactivity has been described earlier for other closely related receptors, such as the PAR and the β-adrenergic receptors [191]. The concept of inherent receptor selectivity for a given pepducin is obviously not always valid. In agreement with results obtained with FPR2-activating pepducins, FPR1 also competes for binding with FPR2-specific agonists (**Paper II**), once again suggesting that there is no single mechanism through which pepducins selectively activate/inhibit GPCRs. One possible explanation for the results obtained from the competitive binding experiments is that FPR2-specific pepducins, irrespective of origin, are also recognized by the cell surface-exposed agonist-binding domain of FPR2. Regardless of the precise mechanism of action, it is clear the neither of the two pepducin parts work on their own. As there is only a very small difference (actually, one amino acid) between the activating and inhibiting pepducins, it is reasonable to hypothesize that this is the basis for the hijacking phenomenon. Results obtained with pepducins that have peptide sequences identical to the intracellular loops of CXCR4 (the CXCL12 receptor) and $P2Y_2R$ (the ATP receptor) are discrepant with this hypothesis. The receptor-desensitization profiles and the inhibitory effects of receptor-selective agonists/inhibitors indicate that these pepducins also hijack FPR2 [78, 189]. It is also worth noting that the $P2Y_2R$ pepducin converts its natural agonist to an activator of the neutrophil NADPH-oxidase. The third intracellular loop pepducin both activates and desensitizes FPR2, and when it comes to direct activation, the pepducin is a partial agonist [143]. However, the reactivation of deactivated receptors is much more pronounced, and in this system, the pepducin should be classified as a full agonist. These results raise general questions not only about the precise mechanism by which pepducins specifically activate GPCRs, but also about ligand recognition of lipopeptides by FPR2. It is clear from earlier studies on the structure-function relationships of pepducins, as well as other peptides, that their activities as FPR agonists are not dictated solely by the amino acids that access the presumed ligand-binding pockets of the receptors [75, 150] (**Paper II**). The physicochemical properties of the regions of agonistic peptides that do not have direct access to the binding site are just as important for the interaction with FPR2, as are the amino acids that putatively fit into the binding pocket [150, 181]. This suggests that endogenous or microbe-derived, lipid-substituted peptides represent an additional molecular pattern that is recognized by FPR2. However, it is important to point out that there are stringent structural requirements for recognition. This is illustrated by the facts that a number of pepducins are not recognized by FPR2 and that the amino

acid sequence is of importance for recognition, as are the positions of the charged amino acids in the peptide chain, rather than the net charge, as well as the length of the peptide (**Paper I)** [189].

MODULATION OF FPR FUNCTIONS IN MICE NEUTROPHILS

The innate immune system differs substantially between mouse and man. In the mouse, neutrophils constitute only around 10%–25% of the circulating leukocytes, whereas 50%–70% of the leukocytes in humans are neutrophils [192, 193]. Another prominent difference between these species is the appearance of the family of FPRs, which has undergone extensive expansion across species [145]. The receptors differ substantially in terms of their ligand recognition profiles, making it very challenging to translate experimental data obtained in mouse models to the human setting. However, to understand the basic biochemical mechanisms underlying cell function and to develop new strategies for therapeutic intervention and preclinical testing, mouse models of human disease are necessary.

Fprs in mice neutrophils

Functional FPR1 orthologs have been identified in several animal species, such as primates, rabbits, horses, rats, guinea pigs, and mice [194-199]. With respect to the mouse, there are at least eight Fpr family members (Fpr1 and Fpr-rs 1–7) encoded in the mouse genome, clustered on chromosome 17 in a region of conserved synteny with human chromosome 19, where the three *FPR* genes are located [200, 201]. All of the mice Fprs, with the exceptions of Fpr-rs5, which most probably is a pseudogene, are expressed as functional receptors [200]. Fpr1, Fpr2, and Fpr-rs1 are expressed by mice leukocytes, and Fpr1 is the FPR1 mice ortholog with 72% sequence identity, whereas Fpr2 is suggested to be the FPR2 ortholog with 76% sequence identity [145]. The remaining Fpr members are expressed mainly by the vomeronasal organ (an olfactory structure in the nasal septum that detects pheromones and other social cues) and skeletal muscle cells [202, 203].

Studies using animals that are genetically knocked out for Fpr1 or/and Fpr2 reveal that these receptor-deficient animals are viable, fertile, and display normal anatomy and physiology. Both $Fpr1^{-/-}$ and $Fpr2^{-/-}$ mice have dysregulated immune responses

under stress conditions, such as bacterial infections and sterile inflammation [123, 204-208]. Animals that were treated with the Fpr agonist WKYMVm were shown to be protected against disease in a severe sepsis mouse model [209], suggesting that the receptor(s) are important modulators of inflammation.

Table 4. Sequence identity (percentage) between the human and mice FPRs[1].

Size[2]	Name	FPR1	FPR2	FPR3	Fpr 1	Fpr-rs1	Fpr 2	Fpr-rs3	Fpr-rs4	Fpr-rs6	Frp-rs7
350	FPR1	100	69	58	72	60	64	56	52	51	50
351	FPR2		100	72	64	74	76	65	62	59	58
353	FPR3			100	52	61	63	54	52	50	51
364	Fpr1				100	56	60	53	50	50	50
347	Fpr-rs1					100	81	66	62	58	60
351	Fpr2						100	66	64	59	60
343	Fpr-rs3							100	78	74	73
323	Fpr-rs4								100	70	70
339	Fpr-rs6									100	94
338	Fpr-rs7										100

[1]Data listed were obtained from UniProt: FPR1 (UniProt ID: P21462); FPR2 (P25090); FPR3 (P25089); Fpr1 (P33766); Fpr-rs1 (O08790); Fpr2 (O88536); Fpr-rs3 (O88537); Fpr-rs4 (A4FUQ5); Fpr-rs6 (Q3SXG2); and Fpr-rs7 (Q71MR7)
[2]Number of amino acids

Ligand recognition differences between the receptors in mouse and man

Earlier work clearly demonstrated that FPRs and their mice counterparts differ substantially with respect to their ligand-binding profiles, as illustrated by the fact that the most potent and selective FPR1 and FPR2 antagonists, i.e., CysH and PBP_{10}, respectively, have no effects on the mouse receptors (**Paper III**). Moreover, the prototype and potent FPR1 agonist fMLF is a very poor agonist for the Fprs. Similarly, the potent FPR2 agonists WKYMVM and MMK-1 have low (WKYMVM) and no (MMK-1) activities in terms of activating mice neutrophils (**Paper III** and **Table 5**). Formyl peptides derived from *L. monocytogenes* (with the sequence fMIVIL) and *S. aureus* (with the sequence fMIFL) have been found to be much more potent than fMLF in stimulating mice neutrophils [117], and this phenomenon has been confirmed with both wild-type and $Fpr2^{-/-}$ cells (**Paper III**). Fpr1 has been identified as

the preferred receptor for a number of fMet-containing peptides that are also recognized by the H2-M3 complex, which presents N-formylated peptides to cytotoxic T cells [210]. In this study, we show no direct correlation between the activities induced by the different peptides in human and mice neutrophils, respectively, although we found that some of the peptides were more potent activators of Fpr1 than of the human receptor. Moreover, the structural requirements differed between the H2-M3 and FPR/Fpr, which suggests that these two recognition systems have followed different evolutionary paths.

The *S. aureus*-derived, phenol-soluble modulin PSMα2, which was earlier shown to be a potent FPR2 agonist, proved to be a potent activator of neutrophils that originated from normal wild-type animals, whereas no activity was induced in $Fpr2^{-/-}$ cells, clearly identifying this peptide as an Fpr2-selective agonist (**Paper III**). Some dual FPR1/FPR2 agonists act as dual agonists also in the mouse, being recognized by both Fpr1 and Fpr2 [174, 211].

Until very recently, there were very few molecular tools available in the form of well-characterized Fpr-specific antagonists. The earlier-mentioned Boc1 and Boc2 peptides have been suggested to inhibit primarily Fpr2, although Boc1 has no inhibitory effect and Boc2 is primarily an Fpr1 antagonist. The FPR2-selective peptide inhibitor WRW_4 is active also against Fpr2, albeit with a potency that is lower than that of the newly identified and described peptidomimetic inhibitor (**Paper III**). Taken together, the observed similarities and difference between FPRs and Fprs in terms of ligand recognition profiles highlight how important it will be in future research studies to choose appropriate ligands when designing animal experiments.

Table 5. Effects of selected human FPR agonists on human and mice neutrophils[1].

Agonist	Activity in human neutrophils[2]		Activity in mouse neutrophils[2]	
	FPR1	FPR2	Fpr1	Fpr2
fMIFL	+++	-	+++	-
fMLF	+++	-	+	-
fMIFL-PSMα2_{5-16}	+++	+++	++	+++
Comp43	++	+	++	+
WKYMVm	++	+++	+++	+++
WKYMVM	-	+++	-	+
PSMα2	-	+++	-	+++
PSM α2_{1-16}	-	++	-	++
MMK-1	-	++	-	-

[1] The figure is adapted from Paper III.
[2] +++ = very potent; ++ = potent; + = weak; – = inactive.

Table 6. Effects of selected human FPR antagonists on human and mice neutrophils.

Antagonist	Activity in human neutrophils[2]		Activity in mouse neutrophils[2]	
	FPR1	FPR2	Fpr1	Fpr2
CysH	+++	-	-	-
PBP_{10}	-	+++	-	-
WRW_4	-	++	-	++
Boc1	+	-	+	-
Boc2	++	-	++	-
Lau-(Lys-βNSpe)$_6$-NH_2	-	++	-	++
Pam-(Lys-βNSpe)$_6$-NH_2	-	+++	++	+++
Ac-(Lys-βNSpe)$_6$-NH_2	-	-	-	-

[1] The listed activities are taken from the results presented in Paper III.
[2] +++ = very potent; ++ = potent; + = weak; – = inactive.

Allosteric modulators of Fpr function

In similarity to FPR2, Fpr2 recognizes both pepducins and proteolytically stable peptidomimetics, as illustrated by the finding that the recently described α-peptide/β-peptoid (F2M2) stimulates superoxide production when it interacts with both mice and human neutrophils, and the targeted receptors are FPR2/Fpr2 [165]. When examining the FPR2-activating pepducins in relation to mice neutrophils, it became clear that mice neutrophils are also activated, and that basically this is achieved through the targeting of Fpr2 (**Paper IV**). It is worth mentioning that in contrast to its inhibitory effect on FPR2, the FPR1 pepducin ($F1Pal_{16}$) positively modulates Fpr2 and activates mice neutrophils. Future studies should aim at elucidating the modulation mechanism that underlies the activation effect of $F1Pal_{16}$ on Fpr2 and the inhibitory effect of $F1Pal_{16}$ on FPR2. Pepducins that have inhibitory effects on Fpr2 have also been identified, with the most potent one having a peptide sequence that is identical to the third intracellular loop of Fpr2. In similarity to the FPR1-derived pepducin $F1Pal_{16}$, the corresponding Fpr1-derived pepducin is an agonist for mice neutrophils but acts as an inhibitor for human neutrophils, and the effect is mediated through the hijacking of Fpr2 and FPR2, respectively, without affecting the closely related FPR1/Fpr1 (**Paper IV**). The identification of these Fpr-activating and -inhibiting pepducins provides not only valuable tools to study in greater detail FPR/Fpr allosteric modulation, but it also raises questions regarding the pepducin concept with

respect to the mechanism used to achieve receptor specificity. In addition, the presented data suggest that there may be an as yet unidentified molecular pattern for FPR2/Fpr2 recognition.

FUTURE PERSPECTIVES

GPCRs are the largest and most important group of cell-surface receptors that participate in the regulation of almost all aspects of cellular functioning. Recent research on GPCRs has led to a number of novel concepts with respect to receptor regulation, including biased signaling, allosteric modulation (both positive and negative), different levels of receptor cross-talk, as well as functional selectivity. The pepducin principle is an illustrative example of the selective allosteric modulation concept, in which a unique type of molecule acts from the cytosolic side of the receptor-expressing membrane and affects the signaling part of the targeted GPCR. Many GPCR-modulating pepducins have been successfully generated, and it is clear that allosteric modulators have potential advantages over orthosteric agonists/antagonist as therapeutic agents. In line with this, some pepducins have already entered into clinical trials. Nonetheless, the precise mechanism of action of pepducins is currently unclear, and this is particularly evident when it comes to the FPR-interacting pepducins, as illustrated by the fact that some of the results presented in this thesis are discrepant with the current concept of how pepducins regulate GPCRs. Future studies should aim to elucidate the general mechanisms (if any) of action (positive as well as negative) of the pepducin group of allosteric modulators.

There is strong evidence that FPRs are involved in several aspects of tissue homeostasis, host defense reactivity, and regulation of immune reactions and inflammation, which serves to highlight these receptors as promising targets for the future design of anti-infective and anti-inflammatory agents. However, more knowledge is needed regarding the basic pharmacological characterization and fine-tuning of FPR activities before it will be possible to define the full therapeutic potential. In this thesis, the identification of cross-species ligand recognition provides useful tools to investigate the physiological function of FPR2, as well as the therapeutic potential of targeting Fpr2 in a mouse model of human disease. While the molecular pattern recognized by FPR1 is well-established, no such pattern has yet been defined for FPR2. The studies presented in this thesis reveal that both lipopeptides and lipopeptidomimetics specifically bind to FPR2, suggesting a common feature that could be the basis for recognition by FPR2. Nevertheless, future research should aim to understand the basis of

FPR2/Fpr2 as a pattern recognition receptor, as well as to identify such FPR2-binding peptides *in vivo*.

Finally, knowledge obtained from the studies using FPRs as a model GPCR should facilitate our understanding of receptor modulation in general, and more importantly, will be of importance in terms of our abilities to develop FPR2-based immunomodulatory therapeutics in the future.

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