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Pepducins and Other Lipidated Peptides as Mechanistic Probes and Therapeutics

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Abstract

Lipopeptides based on the intracellular loops of cell-surface receptors, known as “Pepducins,” represent a promising new class of compounds used for the study of membrane proteins and as potential therapeutics in a variety of diseases. Detailed knowledge of the three-dimensional structure of G-protein-coupled receptors (GPCRs) and delineation of the mechanisms of pepducin activation and biased G-protein signaling has facilitated the development of even more potent pepducin allosteric modulators.

Keywords

Pepducins; Lipopeptides; GPCRs; PAR1; PAR2; PAR4; CXCR1; CXCR2; CXCR4

1 Introduction

G-protein-coupled receptors (GPCRs) are integral membrane proteins that are involved in a broad range of normal and pathological processes. GPCRs generally share high sequence conservation within their seven-transmembrane-domain (TM) core but have high diversity in their cytoplasmic loops (i1, i2, and i3) and C-terminal i4 domain. These intracellular surfaces play a critical role in the binding, selectivity, and activation of specific G proteins and non-G protein effectors [1, 2]. Pepducins are a new technology that selectively targets the intracellular receptor–effector interface [3]. Pepducins are derived from the intracellular i1–i4 domains of their cognate GPCR and gain the ability to readily penetrate cell membranes by the attachment of a lipid group such as a palmitate or steroid [2, 4, 5]. The rapid and efficient cell membrane flipping and membrane-tethering of pepducins [5–9] makes them well-suited for interrogation of the role of intracellular regions of receptors in G-protein coupling, cell signaling, and as potential therapeutics. The imbalance of GPCR signaling contributes to many diseases, and over 30 % of the U.S. Food and Drug Administration-approved therapeutics target GPCRs [10, 11]. Since 2007, the determination of new GPCR structures and the detection of previously unseen conformational states [11–14] have spurred new research and development efforts to identify drugs with fewer side effects and more favorable pharmacological properties. However, most drug discovery efforts have focused on the design and discovery of ligands that bind at the orthosteric site located near the extracellular surface of the GPCR. Pepducins or other lipidated peptides target intracellular regions or transmembrane domains [15] of GPCRs in an allosteric manner and are now being investigated as a potential treatment strategy for cardiovascular diseases [16–19], cancer [20–24], inflammation and sepsis [25–27], asthma [28], and bone

marrow transplant [29]. In this chapter, we summarize the current status of pepducins as mechanistic probes and as novel therapeutics (*see* Table 1).

2 Pepducins as Mechanistic Probes of G-Protein-Coupled and Other Cell-Surface Receptors

In the simplified orthosteric activation mechanism of a GPCR, an agonist typically binds to the orthosteric binding pocket localized inside the receptor transmembrane bundle near the extracellular surface. This induces a conformational change in the receptor including a large outward movement of the lower part of TM6 and rotation of TM7 with disruption of the DRY-ionic lock between TM3 and TM6. In this agonist-stabilized on-state, the intracellular portion of the receptor will present a large hydrophobic cavity for engagement of the Galpha protein C-terminal helix and exchange of GDP for GTP [12, 30, 31].

By comparison, pepducin agonists must first translocate across the plasma membrane in order to activate the receptor at an allosteric site(s) on the intracellular surface of the receptor–G protein interface [2, 5]. Attachment of a sufficiently hydrophobic lipid tether, typically a myristate, palmitate or lithocholic moiety to the peptide, confers the ability to partition to the outside leaflet of the lipid bilayer and then rapidly flip across the membrane to the inner leaflet, where it remains tethered [5–9]. Pepducins activate receptor–G protein signaling in the presence of their cognate receptor and are usually highly selective for receptor type [2, 4, 5]. For instance, the PAR1 i3-loop pepducin, P1pal-19, loses all ability to stimulate Gq-PLC-β-induced inositol phosphate production in the absence of PAR1 [4]. The i1-pepducin ATI-2766 bound to both the full-length native CXCR4 as well to mutants with truncations of the receptor N-terminus, which harbors an essential extracellular chemokine agonist docking site [8]. The CXCR4 i1 pepducin did not interact with the closely related chemokine receptor CCR5. The pepducin, ICL3–9, based on the β2AR receptor i3-loop promoted receptor interactions with Gs and increased Gs signaling, consistent with previous findings that pepducin activity requires the presence of its cognate receptor [28]. ICL3–9 induced an increase in the BRET signal between β2AR and Gs, in striking contrast to the decreased BRET signal observed with the orthosteric agonist isoproterenol. This result clearly demonstrated that the β2AR-Gs active conformation induced by the pepducin is different from that stabilized by isoproterenol. However, a more N-terminally based i3-loop pepducin, ICL3–8, induced receptor-independent Gs signaling [28]. The mechanism of this Gs direct activator pepducin remains unclear, however, this N-terminal region of the i3 loop of the β2AR is a critical interaction site with the Gs α5-β4 loop [12].

The i3 loop region is likewise critical for fine tuning G-protein-specific responses at the molecular level. For example, the i3 loop sequences of the formyl peptide receptors FPR1 and FPR2 differ by only two amino acids and differ in their response to orthotopic ligands [32]. A pepducin with the FPR2-specific K231 replaced by the FPR1-specific Q231 lost all activity to FPR2. However, the FPR2 pepducin F2Pal-10 triggered a response in cells expressing wild-type or FPR2 chimeras containing the i3 loop of FPR1, but not to wild-type FPR1 [32]. These data are consistent with the notion that i3 loop pepducins are not simply

replacing the intracellular loops but utilize the specific receptor–G protein interactions encoded in the native receptor loops [4].

Pepducin agonists can also favor receptor–G protein engagement of one class of G proteins over others. The PAR1 i3 loop agonist pepducin, P1pal-13, preferentially activates PAR1 G12/13-Rho signaling in quiescent endothelial cells, rather than Gi pathways [33]. However, when co-expressed with PAR2, P1pal-13 was able to transactivate PAR2-Gi signaling as a PAR1–PAR2 heterodimer, and lost the ability to activate PAR1-G12/13 signaling [33]. The CXCR4 pepducin ATI-2431 corresponding to the i1 intracellular loop of CXCR4 [29] selectively triggered coupling to Gαi, not Gα12/13 with a negative bias toward the β-arrestins [34]. This negative bias toward β-arrestin was due to the inability to strongly stimulate GPCR-kinase (GRK) phosphorylation of the receptor. Likewise, the Gs-biased pepducin agonist of the β2 adrenergic receptor, ICL3–9, did not induce interaction with GRKs and β-arrestins [28]. Similar to the case with i3 agonist pepducins, an i3 antagonist pepducin, P2pal-18S, was strongly biased toward blocking PAR2-Gq and PAR-Gi signaling pathways including MAPK activation, but had no effect on triggering PAR2-ligand-activated endocytosis [27]. As PAR2 endocytosis is dependent on β-arrestin engagement, it was not surprising that P2pal-18S was unable to inhibit β-arrestin interactions with PAR2 [35].

Recently, an array of 389 pepducins and pepducin-like 12mer lipopeptides were screened for agonist activity against the apelin APJ receptor [15]. Their approach was to synthesize lipopeptides each containing 12 contiguous amino acids of the entire amino acid sequence of the APJ receptor from the N-terminus to the C-terminus with 1 amino acid residue increments. Lipopeptides derived from the C-terminal portion of TM2 were found to be robust agonists of APJ activity with EC50 values as low as 34 nM. Subsequent alanine point mutations resulted in a APJ pepducin-like compound (Palm-VTLPLWATYTAR) with 2.7 nM agonist activity [15]. Although the mechanism of activation of the APJ receptor by this pepducin-like compound is not understood, this TM2 segment is known to comprise part of the binding site for many hydrated orthosteric ligands within the transmembrane core of GPCRs [36]. Shield’s group earlier employed a more sophisticated bioinformatics/high-throughput screening technique to identify pepducin and lipopeptide modulators of platelet function targeted to signaling-rich juxtamembrane regions of GPCRs and other transmembrane receptors [37]. Palmitoylated peptides derived from the insulin-like growth factor-1 (IGF-1) receptor juxtamembrane region were identified to be specific inhibitors of IGF-1-mediated Akt activation and breast cancer growth [37].

In addition to GPCRs, the pepducin approach has been successfully used as a mechanistic probe of other membrane proteins including ion channels such as M (Kv7) potassium channels and transient receptor potential vanilloid channels (TRPV). Cell-penetrating lipidated peptides were found to be selective antagonists of the TRPV1 channel by targeting the intracellular C-terminal TRP domain that was predicted to interact with the highly conserved receptor internal gate [38]. G proteins were also directly targeted by synthesizing palmitoylated peptides corresponding to the C terminus of Gq/11 with the intent to inhibit the muscarinic acetylcholine receptor (mAChR) [39]. Selectivity was demonstrated between Gq/11- and Go-targeted palmitoylated peptides. Du’s group [40] used a myristoylated C-

terminal peptide fragment of G13 [41] to inhibit signaling from the platelet receptor αIIbβ3 to associated G13.

Given the large number of GPCRs and non-GPCRs and membrane-associated proteins, it is likely that the pepducin approach will continue to serve as a unique mechanistic probe of these critical juxtamembrane and transmembrane regions in the context of cell signaling and pharmacology.

3 Pepducin-Based Therapeutics

PAR1—The serine protease thrombin is one of the most potent activators of platelet aggregation, endothelial cell activation, and other responses in many cell types [42]. There are three known thrombin receptors, PAR1, PAR3 and PAR4, which share the same proteolytic mechanism of activation. Human platelets express PAR1 and PAR4 on their surface. PAR1 has recently emerged as a promising new target for therapeutic intervention in patients with thrombotic diseases and acute coronary syndromes [43]. PZ-128 (P1pal-7) is a first-in-class cell-penetrating pepducin inhibitor of PAR1 that targets the receptor–G-protein interface on the inside cell including platelets [16–18]. PZ-128 (P1pal-7) is a cell-penetrating lipopeptide derived from the juxtamembrane region of the i3 loop/N-terminus of TM6 of PAR1. This region has been shown to be essential for coupling of PAR1 with associated G proteins [4]. The NMR structure of PZ-128 revealed a well-defined α-helix extending from the palmitate lipid, which is highly similar with the structure of the corresponding region of PAR1 (residues 307–313) in the off-state with an RMSD of 1.4 Å [17].

PZ-128 is a highly efficacious inhibitor of PAR1-dependent platelet aggregation [17]. PZ-128 also inhibited collagen-MMP1-induced platelet aggregation of PAR1, blocked G12/13-Rho and p38 MAPK activation, and significantly reduced the propagation of platelet–platelet thrombi in human whole blood, under arterial flow conditions [18]. The onset of action of PZ-128 occurred within 15 min after intravenous administration and suppressed PAR1 aggregation and arterial thrombosis in guinea pigs and baboons and strongly synergized with the oral P2Y12 inhibitor clopidogrel [16–18]. Importantly, PZ128 had no effect on bleeding or coagulation parameters in primates or in blood from patients undergoing percutaneous coronary intervention (PCI) [17]. PZ-128 has successfully completed a Phase I clinical trial in 31 subjects with coronary artery disease risk factors with assessments for safety, tolerability, pharmacokinetics, and pharmacodynamics (anti-platelet efficacy). A large Phase II study in patients undergoing PCI is planned to be initiated in 2015.

In addition to its well-recognized roles in platelet and vascular biology, PAR1 has been shown to be involved in the invasive and metastasis process of cancer [44]. The i3 loop pepducin PZ-128 and i1 loop inhibitor P1pal-i1 PAR1 showed significant inhibition of cell migration in both primary and established lung cancer cell lines similar to silencing of PAR1 expression with short hairpin RNA (shRNA). Unlike the i1 loop P1pal-i1, PZ-128 was an effective inhibitor of PAR1-mediated ERK activation and tumor growth. Comparable in

efficacy with Bevacizumab, PZ-128 monotherapy provided significant 75 % inhibition of lung tumor growth in nude mice [45].

Matrix metalloprotease-1 (MMP-1) robustly activates the PAR1-Akt survival pathway in breast carcinoma cells [23, 44]. Blockade of Matrix metalloprotease-1 (MMP-1)/PAR1 signaling by PZ-128 or MMP-1 inhibitor significantly promote apoptosis in breast tumor xenografts and inhibit metastasis to the lungs by up to 88 % [23]. Dual therapy with PZ-128 and taxotere inhibited the growth of MDA-MB-231 xenografts by 95 % [23]. Together, these findings indicate that blockade of MMP1-PAR1 signaling may provide a benefit beyond treatment with Taxotere alone in advanced, metastatic breast cancer [23, 44]. PZ-128 significantly reduced the peritoneal dissemination of OVCAR4 ovarian cancer cell line through inhibition of MMP1-PAR1-induced paracrine communication between ovarian carcinoma cells and endothelial cells [46]. PZ-128 almost completely blocked angiogenesis of peritoneal ovarian [46] and breast cancer [44] suggesting that the pepducin approach may prove useful as an anticancer strategy.

PAR4—The binding and subsequent activation of PAR1 and PAR4 by thrombin are mechanistically different [47, 48] and PAR4 may be an additional therapeutic target to prevent platelet-dependent thrombosis [3, 5, 49–52]. PAR1 and PAR4 also form a heterodimer on human platelets [16] which presents the opportunity to design pepducins that inhibit both receptors at the dimer interface or interaction site(s). PAR1-mediated platelet aggregation is transient and reversible unless strengthened by additional signaling inputs from P2Y 12 -ADP receptors or from the PAR4 receptor [47, 52, 53]. To tease out mechanistic differences between PAR1 and PAR4 thrombin receptors, the i1 loop pepducin P4pal-i1 and the i3 loop P4pal-10 were designed [16]. P4pal-i1 specifically inhibited the PAR4 but not the PAR1 signal during platelet aggregation. Dual PAR1/PAR4 inhibition with the addition of P4pal-i1 to small-molecule PAR1 antagonist RWJ-56110 effectively suppressed aggregation of human platelets to even high concentrations of thrombin [16]. The i3 loop pepducin P4pal-10 was found to be a dual PAR1/PAR4 [5] inhibitory pepducin. P4pal-10 inhibited 85 % of human platelet aggregation in response to 20 nM thrombin conditions where normally both PAR1 and PAR4 would be fully activated by thrombin [5]. P4pal-10 at a dose of 0.37 mg/kg afforded 50 % less platelet deposition in Dacron vascular graft segments in a femoral arterio-venous shunt-bearing baboon [16]. Inhibition of PAR1 with RWJ-56110 conferred partial protection against arterial thrombosis. Likewise, inhibition of PAR4 with P4pal-i1 gave partial blockade of arterial occlusion. However, combination inhibition of PAR1 and PAR4 gave significant protection against occlusive thrombus formation [16]. These data indicate that inhibiting PAR1 and PAR4 or the PAR1/PAR4 complex might offer alternative routes to suppressing pathophysiological activation of platelets during PCI and stenting.

PAR2—Protease-activated receptor-2 is a receptor for trypsin-like proteases such as tryptase and factors VIIa/Xa, and plays a key role in a number of acute and chronic inflammatory and fibrotic diseases of the skin, lungs, liver and gastrointestinal tract, joints, and vascular systems. As PAR2 exhibits significant constitutive activity, many inhibitors based on the extracellular ligand have partial agonist activity, potentially by stabilizing the latent on-state [27]. The constitutive activity of PAR2 could be ablated or enhanced by mutation of critical

i3 loop pharmacophores in the intact receptor. Incorporation of these mutation led us to the discovery of PAR2 pepducin antagonists that lost residual agonist activity [27]. The P2pal-18S i3 loop pepducin, with a mutation of a critical Arg for Ser residue, was found to be a full antagonist of PAR2-dependent calcium signaling (Gq) and neutrophil (Gi) chemotaxis. P2pal-18S significantly attenuated mast cell tryptase-dependent neutrophil migration and paw edema in mice in models of acute skin inflammation [27]. P2pal-18S was also found to protect pancreatic acinar cells against bile acid-induced injury/death but did not affect bile acid-induced intracellular zymogen activation [54]. P2pal-18S significantly reduced the severity of experimental biliary pancreatitis induced by retrograde intraductal bile acid infusion, which mimics injury induced by endoscopic retrograde cholangiopancreatography [54].

3.1 Chemokine Receptors

Chemokines comprise a large family of small secreted proteins that regulate nearly all aspects of innate and acquired immunity. In particular, CXCR1 and CXCR2 are important chemokine receptors for IL-8 responsible for the activation of neutrophils, endothelium, epithelium, macrophages and other cells, and can contribute to adverse outcomes in systemic inflammatory response syndrome/sepsis. Opposing the action of CXCR1 and CXCR2, CXCR4 directs the removal of senescent neutrophils and retains immature leukocytes in the bone marrow [26, 55]. Pepducins derived from either the i1 (x1/2LCA-i1) or i3 (x1/2pal-i3) intracellular loops of CXCR1 and CXCR2 prevent the IL-8 response of both receptors and reverse the lethal sequelae of sepsis, including disseminated intravascular coagulation (DIC) and multi-organ failure in mice [26]. Conversely, pepducin antagonists selective for CXCR4 i1 (x4pal-i1) and i3 (x4pal-i3) blocked SDF-1-dependent neutrophil chemotaxis and caused a massive leukocytosis that did not affect survival of mice in sepsis models [26].

The CXCR4 i1-loop pepducin agonist ATI-2341 induced CXCR4-G protein-dependent signaling, receptor internalization, and chemotaxis in CXCR4-expressing cells [29]. ATI-2341 also induced dose-dependent peritoneal recruitment of polymorpho-nuclear neutrophils (PMNs) when administered i.p. to mice. When administered systemically by i.v. bolus, ATI-2341 acted as a functional antagonist and dose-dependently mediated release of PMNs from the bone marrow of both mice and cynomolgus monkeys. ATI-2341 also mediated release of granulocyte/macrophage progenitor cells from the bone marrow. ATI-2341 may hold promise as a new therapeutic approach for the recruitment of hematopoietic stem and progenitor cells (HSPCs) before autologous bone marrow transplantation [29].

Inflammation, angiogenesis, and tumorigenesis are intimately linked [56]. CXCR2 is a key driver of tumor development and angiogenesis. CXCR2 deficiency profoundly suppressed inflammation-driven tumorigenesis in skin and intestine as well as spontaneous adenocarcinoma formation in a model of invasive intestinal adenocarcinoma [56]. The x1/2pal-i3 pepducin reduced CXCR2-driven tumorigenesis in *ApcMin*/+ mice [56]. CXCR1/2 pepducins also blocked IL-8 angiogenesis in ovarian cancer and be beneficial in the setting of advanced ovarian disease where other treatment options are limited [24]. CXCR4 pepducin x4pal-i1 completely abrogated CXCL12-mediated cell migration of

lymphocytic leukemias and lymphomas [57]. Combination treatment with CXCR4 pepducins and the CD20-targeted antibody rituximab significantly increased the apoptotic effect of rituximab which can be impacted by stromal-cell interactions. Furthermore, treatment of mice bearing disseminated lymphoma xenografts with pepducins alone or in combination with rituximab significantly increased survival [57] representing a potential new treatment strategy for lymphoid malignancies.

3.2 Sphingosine-1-Phosphate Receptor

Sphingosine-1-phosphate (S1P) is a bioactive lipid that plays key functions in the immune, inflammatory, and cardiovascular systems. S1P exerts its action through the interaction with a family of five GPCRs, named $S1P_{1–5}$. Among them, $S1P_3$ has been implicated in the pathological processes of a number of diseases, including sepsis and cancer. KRX-725 is a pepducin agonist derived from the i2 intracellular loop, which mimics the effects of S1P by specifically activating $S1P_3$ [58]. KRX-725 derivatives with deletions of two amino acids from both N- and C-termini have the ability to inhibit both KRX-725-induced vasorelaxation and fibroblast proliferation [58].

3.3 Formyl Peptide Receptors

The formyl peptide receptors (FPR) are Gi-coupled receptors involved in chemotaxis and inflammation. These receptors were originally identified by their ability to bind *N*-formyl peptides such as *N*-formylmethionine produced by the degradation of bacterial pathogens. Human neutrophils express FPR1 and FPR2 that are important regulators of inflammation as well as innate defense reactions [59, 60]. FPR-based pepducins have been shown to possess potent immune regulatory functions. F2Pal-16, containing 16 amino acids of the i3 loop of FPR2, induced superoxide production in human neutrophils which was sensitive to FPR2 antagonists. A C-terminal six-amino acid deletion of Fpal-16 generated a more potent neutrophil activator, F2pal-10 [61]. Neutrophils desensitized with the FPR2-specific pepducin F2pal-10 displayed increased cellular responses to stimulation with the platelet-activating factor (PAF) or ATP by receptor cross-talk signals generated through PAF receptor and ATP receptor $P2Y_2R$ [61]. The pepducin F2pal-12 selectively inhibited FPR2 agonists MMK-1 and serum amyloid A-stimulated neutrophil chemotaxis and human umbilical vein endothelial cell (HUVECs) migration and tube formation [62]. Recent work has documented that FPR2 pepducins possess potent antibacterial activity against both gram-negative *E. coli* and gram-positive *S. aureus* in inhibition zone assays [61]. Both palmitoylated and myristoylated FPR2 pepducins were effective in killing clinical isolates for *Pseudomonas aeruginosa* from patients with cystic fibrosis and *Enterococcus faecalis* from septic patients. The mechanism of action required both the fatty acid and peptide portions acting together, which may facilitate specific interactions and perturbations of the bacterial membrane components [61]. The dual function of FPR2 pepducins warrants further exploration as a novel class of antibacterial agents with immunomodulatory properties.

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Table 1

Pepducin applications and outcomes in diverse human diseases

Pepducin	Sequence	Target	Outcome	Ref.
PZ128 (P1pal-7)	pal-KKSRALF	PAR1	Inhibits platelet aggregation in humans and arterial thrombosis in primate models, suppresses tumor growth, metastasis and angiogenesis in mice	[16–18, 23, 44–46]
P1pal-12	pal-RCLSSSAVANRS	PAR1	Inhibits platelet aggregation, decreases thrombin-induced relaxation in rat aortic rings and limits pulmonary fibrosis	[18, 63, 64]
P1pal-12S	pal-RSLSSSAVANRS	PAR1	Temporally modulates sepsis survival, vascular leakage, and DIC	[33]
P1pal-13	pal-AVANRSKKSRALF	PAR1	Temporally modulates sepsis survival, vascular leakage, and DIC	[33]
P2pal-18S	pal-RSSAMDENSEKKRKSAIK	PAR2	Reduces acute inflammation and edema in skin inflammation models and reduces the severity of experimental biliary pancreatitis	[27, 54]
P4pal-10	pal-SGRRYGHALR	PAR4	Inhibits platelet aggregation and platelet deposition in guinea pig and baboon thrombosis models	[5]
P4pal-i1	pal-ATGAPRLPST	PAR4	Inhibits platelet aggregation and prolongs the occlusion time in guinea pig arterial thrombosis	[16]
x1/2pal-i3	pal-RTLFKAHMGQKHR	CXCR1 CXCR2	Inhibits neutrophil migration toward interleukin-8, decreases transmigration of neutrophils into the peritoneal cavity, reverses the lethal sequelae of sepsis and improves survival in mice	[26]
x4pal-i3	pal-HSKGHQKRKALK	CXCR4	Completely inhibits the response of neutrophils toward SDF-1α	[26]
x4pal-i1	pal-MGYQKKLRSMTD	CXCR4	Completely inhibits the response of neutrophils toward SDF-1α, mobilizes leukocytes from the bone marrow	[26]
ATI-2341	pal-MGYQKKLRSMTDKYRL	CXCR4	Mediates release of granulocyte/macrophage progenitor cells from the bone marrow	[29, 34]
KRX-7	myristoyl-GMRPYDANKR	S1P3	Induces vasorelaxation and fibroblast proliferation	[58]
Compd 16	myristoyl-GRPYDAN	S1P3	Blocks KRX-725 activity	[58]
F2pal-16	pal-KIHKKGMIKSSRPLRV	FRP2	Activates neutrophils to produce superoxide	[65]
F2Pal-10	pal-KIHKKGMIKS	FRP2	Activates neutrophils to produce superoxide	[65]
F2pal-12	pal-KIHKKGMIKSSR	FRP2	Inhibits neutrophil chemotaxis and superoxide production	[61]