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The proteolytically stable peptidomimetic Pam-(Lys-βNSpe)$_6$-NH$_2$ selectively inhibits human neutrophil activation via formyl peptide receptor 2

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ABSTRACT

Immunomodulatory host defense peptides (HDPs) are considered to be lead compounds for novel anti-sepsis and anti-inflammatory agents. However, development of drugs based on HDPs has been hampered by problems with toxicity and low bioavailability due to *in vivo* proteolysis. Here, a subclass of proteolytically stable HDP mimics consisting of lipidated α-peptide/β-peptoid oligomers was investigated for their effect on neutrophil function. The most promising compound, Pam-(Lys-βNSpe)$_6$-NH$_2$, was shown to inhibit formyl peptide receptor 2 (FPR2) agonist-induced neutrophil granule mobilization and release of reactive oxygen species. The potency of Pam-(Lys-βNSpe)$_6$-NH$_2$ was comparable to that of PBP10, the most potent FPR2-selective inhibitor known. The immunomodulatory effects of structural analogs of Pam-(Lys-βNSpe)$_6$-NH$_2$ emphasized the importance of both the lipid and peptidomimetic parts. By using imaging flow cytometry in primary neutrophils and FPR-transfected cell lines, we found that a fluorescently labeled analog of Pam-(Lys-βNSpe)$_6$-NH$_2$ interacted selectively with FPR2. Furthermore, the interaction between Pam-(Lys-βNSpe)$_6$-NH$_2$ and FPR2 was found to prevent binding of the FPR2-specific activating peptide agonist Cy5-WKYMWM, while the binding of an FPR1-selective agonist was not inhibited. To our knowledge, Pam-(Lys-βNSpe)$_6$-NH$_2$ is the first HDP mimic found to inhibit activation of human neutrophils via direct interaction with FPR2. Hence, we consider Pam-(Lys-βNSpe)$_6$-NH$_2$ to be a convenient tool in the further dissection of the role of FPR2 in inflammation and homeostasis as well as for investigation of the importance of neutrophil stimulation in anti-infective therapy involving HDPs.

Abbreviations: 2-Aoc, 2-aminooctanoic acid; Ac, acetyl; AMP, antimicrobial peptide; βNphe, *N*-phenyl-β-alanine; βNSpe, *N*-(*S*)-1-phenylethyl-β-alanine; C5a, complement fragment 5a; C5aR, receptor for C5a; $[Ca^{2+}]_i$, concentration of free intracellular calcium; CF, carboxyfluorescein; CL, chemiluminescense; Cmp. 43, compound 43 (FPR1 ligand); CsH, cyclosporine H; CXCR, CXC chemokine receptor; DAD, diode array detector; DMF, dimethylformamide; ESI-MS, electrospray ionization mass spectrometry; DIPEA, diisopropylethylamine; FCS, fetal calf serum; F2Pal10, FPR2-derived pepducin; FPR, formyl peptide receptor; GPCR, G-protein coupled receptor; hArg, homoarginine; HDP, host defense peptide; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; HR-MS, high-resolution mass spectrometry; IDR, innate defense regulator; KRG, Krebs-Ringer phosphate buffer with glucose; Lau, lauryl; Lys, lysine; Lys(Pam), N^{ε}-palmitoyl-lysine; mAb, monoclonal antibody; Me, methyl; NLys, α-peptoid lysine; Oct, octanoyl; OSu, *N*-hydroxysuccinimidyl; PAF, platelet-activating factor; PAFR, receptor for PAF; Pam, palmitoyl; PBP10, rhodamine-B labeled phosphatidylinositol 4,5-bisphosphate-binding peptide derived from gelsolin; PE, phycoerythrin; PFA, paraformaldehyde; Ph, phenyl; PLC, phospholipase C; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; PRR, pattern recognition receptor; PSM, phenol-soluble modulin; PyBOP, (benzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate; ROS, reactive oxygen species; RPMI, RPMI-1640 culture medium; SOD, superoxide dismutase; Ste, steroyl; TFA, trifluoroacetic acid.

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1. Introduction

The innate immune response constitutes the front-line defense against infection [1]. It consists of a complex network of cells and inducible soluble factors that interact to recognize and combat incoming pathogens in a series of immediate and relatively unspecific and generalized reactions. Microbial molecular components are recognized via pattern recognition receptors (PRRs) on host immune cells resulting in the release of pro-inflammatory and antibacterial factors such as cytokines, chemokines, lipid mediators, and reactive oxygen species (ROS) [2]. Local inflammation plays an important role in this response by orchestrating the recruitment of various immune cells to the infection focus often resulting in clearance of the infection. However, excessive, unbalanced, or prolonged inflammation can be detrimental to the host. In the case of sepsis, the massive release of pro-inflammatory factors into the circulation causes tissue damage possibly leading to organ dysfunction and ultimately death [3–5]. Novel anti-infective therapies are urgently needed due to the fact that excessive and non-compliant use of antibiotics has selected for multidrug-resistant bacterial strains. Consequently, infectious diseases are once again becoming a severe health threat as we are rapidly approaching what has been termed "the post-antibiotic era" [6–8]. As the innate immune response is involved in the initial protection against invasive microorganisms as well as in the pathogenesis of infectious and inflammatory diseases, immunomodulation has been proposed as an attractive novel non-antibiotic therapeutic approach [9,10]. Natural host defense peptides (HDPs) possess many of the properties essential for anti-infective agents as a number of these peptides exhibit both direct microbicidal activity and potent immunomodulatory functions via interaction with various immune-competent cells such as neutrophils [10–12]. Being among the first cells to be recruited to the site of infection, neutrophils are important early effector cells of the innate immune system. Moreover, dysregulation of neutrophil function has been linked to both aseptic and septic inflammatory and autoimmune diseases, highlighting the importance of this cell type in maintaining a balanced inflammatory response [13–18]. Recruitment and activation of neutrophils occur through integration of signals from cell-surface G-protein coupled receptors (GPCRs) recognizing host factors such as chemotactic proteins and peptides, e.g. chemokines via CXCR1/2, and complement anaphylatoxins via C5aR, as well as pathogen-derived N-formylated peptides via formyl peptide receptors (FPRs) [2,19]. Human neutrophils express two closely related FPRs, namely FPR1 and FPR2 [20]. Activation of neutrophils through FPRs induces a variety of pro-inflammatory and antibacterial effector mechanisms including production of ROS and release of antimicrobial peptides (AMPs) and hydrolytic enzymes from intracellular granules [20]. Furthermore, FPRs regulate the inflammatory reactions in neutrophils by modulating signaling through many other receptors in a process termed receptor cross-talk [21–24]. The role of FPRs in regulation of inflammation is highlighted by their suggested involvement in both systemic [25] and local [26–28] inflammatory responses. Thus, recently various groups have suggested FPRs as therapeutic targets in inflammatory and infectious diseases [29,30] and several selective FPR agonists and inhibitors have been discovered: the cyclic undecapeptide cyclosporine H (CsH) is the most potent selective FPR1 inhibitor [31,32], and rhodamine B-labeled PIP_2-binding peptide of gelsolin (PBP10) is the most potent selective FPR2 inhibitor known to date [33,34]. Also several HDPs have been shown to interact with FPRs, thereby modulating the responses of human neutrophils [35–40], e.g. human cathelicidin LL-37, a chemoattractant that activates neutrophils through FPR2 [36,37,39,40]. Furthermore, a synthetic derivative of bactenecin (a bovine HDP), referred to as innate defense regulator peptide 1 (IDR-1), has been shown to induce neutrophil migration and activation, thereby augmenting neutrophil-mediated killing of bacteria via FPR1 [41]. Development of anti-infective drugs based on HDPs has been hampered by problems with toxicity and poor bioavailability due to *in vivo* proteolytic degradation [42]. To circumvent these problems, we and others have developed synthetic HDP mimics with improved characteristics such as increased protease resistance [43–45]. Stable HDP mimics based on a design with alternating α-amino acids and peptoid residues (see Fig. 1A) have been found to exhibit antimicrobial activity against planktonic bacteria and biofilm and to possess antiplasmodial as well as immunomodulatory activities [43,46–49]. The aim of the present study was to investigate the effects of lipidated peptidomimetics, belonging to the subclass of α-peptide/β-peptoid hybrids, on the inflammatory responses of human neutrophils. The most promising compound, Pam-(Lys-βNSpe)$_6$-NH_2 (Cmp. **1**, Fig. 1B), displayed receptor-selective inhibition of cellular responses, such as production of ROS and degranulation induced in neutrophils by FPR2-specific agonists, with a potency comparable to that of the most potent known FPR2 inhibitor PBP10. Based on these results, Pam-(Lys-βNSpe)$_6$-NH_2 is considered to be a promising anti-inflammatory drug lead that may prove useful for the treatment of inflammation-driven disease, including sepsis. Furthermore, Pam-(Lys-βNSpe)$_6$-NH_2 may be a useful tool in the further dissection of the role of FPR2 in inflammation and homeostasis.

2. Materials and methods

2.1. Chemicals, reagents, and peptides

Solvents, Rink amide resin, α-amino acid building blocks, and coupling reagents were obtained from IrisBiotech (Marktredwitz, Germany), while octanoic acid, Lau-OSu and Pam-OSu, stearic acid, and 5(6)-carboxyfluorescein (CF) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany); Fmoc-Lys(Pam)-OH was acquired from Bachem (Bubendorf, Switzerland). Horseradish peroxidase (HRP) and phorbol myristate acetate (PMA) were from Sigma-Aldrich (St. Louis, MO, USA). PBP10 peptide (RhB-QRLFQVKGRR) and the FPR2-derived pepducin F2Pal10 (palmitoyl (Pam)-KIHKKGMIKS) were obtained from Caslo Laboratory (Lyngby, Denmark). The receptor antagonist WRWWWW (WRW4) was from Genscript Corporation (Scotch Plains, NJ, USA), and cyclosporin H was kindly provided by Novartis Pharma (Basel, Switzerland). The hexapeptides WKYMWM/m were purchased from AltaBioscience (University of Birmingham, Birmingham, U.K.), and the phenol-soluble modulin (PSMα2, MGIIAGIIKFIKGLIEKFTGK) was obtained in its α-N-formylated form from American Peptide Company (Sunnyvale, CA, USA). The formylated tripeptide fMLF and C5a were purchased from Sigma-Aldrich (St. Louis, MO) and PAF was from Avanti Polar Lipids Inc. (Alabama, USA). The FITC-fNLFNYK and the Cy5-WKYMWM peptides were from Phoenix Pharmaceutical (Burlingame, CA). All peptides were dissolved in dimethyl sulfoxide to a concentration of 10 mM and stored at −80 °C until use. Further dilutions were made in Krebs-Ringer phosphate buffer that was supplemented with glucose (10 mM), Ca^{2+} (1 mM), and Mg^{2+} (1.5 mM) (KRG; pH 7.3). RPMI 1640, fetal calf serum (FCS), penicillin and streptomycin, and G418 were from PAA Laboratories GmbH, Austria.

2.2. General procedure for purification and compound characterization

Analytical HPLC was performed on a Shimadzu HPLC system with diode array detector (DAD) consisting of an SCL-10A VP controller, an SIL-10AD VP auto injector, an LC-10AT VP Pump, an