A non-peptide receptor inhibitor with selectivity for one of the neutrophil formyl peptide receptors, FPR 1

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ABSTRACT

The neutrophil formyl peptide receptors (FPR1 and FPR2) are members of the G-protein coupled receptor family. The signals generated by occupied FPRs are both pro-inflammatory and anti-inflammatory. Accordingly, these receptors have become a therapeutic target for the development of novel drugs that may be used to reduce injuries in inflammatory diseases including asthma, rheumatoid arthritis, Alzheimer's disease and cardiovascular diseases. To support the basis for a future pharmacological characterization, we have identified a small molecular non-peptide inhibitor with selectivity for FPR1. We used the FPR1 and FPR2 specific ligands fMLF and WKYMVM, respectively, and an earlier described ratio technique, to determine inhibitory activity combined with selectivity. We show that the compound 3,5-dichloro-N-(2-chloro-5-methyl-phenyl)-2-hydroxy-benzamide (BVT173187) fulfills the criteria for an FPR1 inhibitor selective for FPR1 over FPR2, and it inhibits the same functional repertoire in neutrophils as earlier described peptide antagonists. Accordingly, the new inhibitor reduced neutrophil activation with FPR1 agonists, leading to mobilization of adhesion molecules (CR3) and the generation of superoxide anion from the neutrophil NADPH-oxidase. The effects of a number of structural analogs were determined but these were either without activity or less active/specific than BVT173187. The potency of the new inhibitor for reduction of FPR1 activity was the same as that of the earlier described FPR1 antagonist cyclosporine H, but signaling through the C5aR and CXCR (recognizing IL8) was also affected by BVT173187.

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1. Introduction

Professional phagocytes including neutrophil granulocytes and monocytes/macrophages play important roles in host defense against invading microbes, and they are also key regulators in the fine-tuning and resolution of inflammatory reactions. The functional repertoires of these cells are triggered and regulated through binding of different cytokines and chemoattractants, and these are in most instances recognized by specific receptors exposed on the cell surface of resting or primed cells [1,2]. One family of receptors, the G-protein coupled seven transmembrane receptors (GPCRs), is abundant on phagocytes. The basic functions and regulatory roles of one of these, the pattern recognition chemoattractant receptor, FPR1, which binds formylated peptides, have been extensively studied. This receptor is a member of the chemoattractant receptor subfamily and recognizes N-formylated peptides of microbial or mitochondrial origin [3–6]. Since such agonists of mitochondrial origin act as "danger signals", it has been proposed that a primary function of FPR1 is to promote trafficking of phagocytic myeloid cells to infected or damaged tissues [7]. The FPR1 expressing cells that leave the blood stream and enter the tissues, thus, exert antibacterial effector functions and clear cell debris. Accordingly the prototype agonist formylmethionyl-leucyl-phenylalanine (fMLF) is a high-affinity FPR1 agonist produced by bacteria and it triggers a variety of biologic activities in neutrophils, including granule secretion, and superoxide release, the latter generated through an activation of an electron transporting NADPH-oxidase [8,9]. Both neutrophils and monocytes/macrophages have the ability to recognize and respond to a number of additional molecules that bind FPRs and serve as "danger signals", and the list of such more or less well characterized agonists has steadily grown [6]. Many of the defined agonists are proteins or peptides of different length, but through the use of small molecule screening approaches, non-peptide agonists for the FPR sub-family have been identified during the last couple of years [10–12]. The importance of FPR1 and the closely related FPR2 is obvious as they both have, in

different animal models, been linked to chronic inflammation of systemic and auto-immune diseases, and through this type of research a few inhibitors/antagonists have been identified and characterized [13,14]. Small molecule FPR1 and FPR2 selective antagonists belonging to different chemical series were also recently described [15,16]. The expectation is, that once identified, such molecules may form the basis for development of clinically useful anti-inflammatory drugs. The most potent and receptor specific FPR1 antagonist described so far, is the fungal peptide metabolite cyclosporin H [13,17]. We have earlier described a number of FPR1 specific receptor agonists, identified through a screen with a small molecular library [10]. This screen was also accomplished in antagonist mode and one inhibitory compound was identified.

The aim of this study was to identify new inhibitors of FPRs in the small molecule library mentioned above; the hit and selected analogues of that were further analyzed in order to determine potency and receptor preference. A newly described method designed to identify agonists/antagonists for the FPR family of receptors [18] was used to determine the ability of the new inhibitors, to affect neutrophil generation of superoxide anions from the electron transporting NADPH-oxidase. In the present study, the compound 3,5-dichloro-N-(2-chloro-5-methyl-phenyl)-2-hydroxy-benzamide (BVT173187) was found to possess functional characteristics that are similar to those of earlier described FPR1 antagonists. The new inhibitor reduced the rise in intracellular calcium, granule mobilization, and production of superoxide anion triggered by FPR1 agonists, and it was found to be as potent as cyclosporin H in inhibiting FPR1 over FPR2. The new inhibitor reduced, however, also the activity induced by C5a and IL8, neutrophil chemoattractants that bind to two others GPCRs. We suggest that the identified small non-peptide compound (or rather future variants of it) could serve as good tools for enhanced understanding of the structure-function relationships of different GPCRs and for in vitro/in vivo therapeutic studies in which a stable inhibitor is required.

2. Material and methods

2.1. Materials

The chemical library as well as the assay used in the initial screen has been described earlier [10]. The purity and identity of the used compounds were verified using HPLC and mass spectroscopy. The compounds were dissolved in DMSO at a concentration of 10 mM and stored at $-80\,^\circ$C. Isoluminol, fMLF, catalase, H_2O_2 as well as C5a were obtained from Sigma Chemical Co. (St. Louis, MO, USA). IL-8 was from R&D systems (Minneapolis, MN, USA). The hexapeptide Trp-Lys-Tyr-Met-Val-Met-NH2 (WKYMVM) was synthesized and purified by HPLC by Alta Bioscience (University of Birmingham, Birmingham, UK). The FPR2 selective PSMα2 peptide (fMGIIAGIIKVIKSLIEQFTGK) [19] in its N-formylated form was synthesized by American Peptide Company (Sunnyvale, CA, USA). The horse radish peroxidase (HRP) was from Roche Diagnostics (Bromma, Sweden). Dextran and Ficoll-Paque were from Pharmacia (Uppsala, Sweden). The receptor antagonist Trp-Arg-Trp-Trp-Trp-Trp-CONH$_2$ (WRW$_4$) was from GenScript Corp. (Piscataway, NJ, USA) and cyclosporine H was kindly provided by Novartis Pharma (Basel, Switzerland). The peptides/receptor antagonists were dissolved in dimethyl sulfoxide to 10^{-2} M and stored at $-80\,^\circ$C until use. Further dilutions were made in Krebs–Ringer phosphate buffer containing glucose (10 mM), Ca^{2+} (1 mM), and Mg^{2+} (1.5 mM) (KRG; pH 7.3). The specific gelsolin-derived inhibitory peptide PBP10 (gelsolin residues 160–169 with the amino acid sequence QRLFQVKGRR), prepared by solid phase peptide synthesis and coupled to

rhodamine as described [20] was synthesized by K.J. Ross-Petersen (Holte, Denmark). The peptides/receptor antagonists were dissolved in dimethyl sulfoxide to 10^{-2} M and stored at $-80\,^\circ$C until use. Further dilutions were made in KRG.

MPO was obtained from Calbiochem (Darmstadt, Germany). RPMI 1640, FCS, PEST and G418 were from PAA Laboratories GmbH (Pasching, Austria).

2.2. Isolation of human neutrophils

Neutrophil granulocytes were isolated from buffy coats obtained from healthy adults [21]. After dextran sedimentation at $1 \times g$, hypotonic lyses of the remaining erythrocytes, and centrifugation in a Ficoll-Paque gradient, the neutrophils were washed twice and resuspended (1×10^7/ml) in KRG; pH 7.3. The cells were stored on melting ice and used within 120 min of preparation.

2.3. Expression of formyl peptide receptors in HL-60 cells

The procedures used to obtain stable expression of FPR1 and FPR2 in undifferentiated HL60 cells have been previously described [22]. To prevent possible auto-differentiation due to the accumulation of differentiation factors in the culture medium, cells were passed twice a week before they reached a density of 2×10^6 cells/ml. At each passage, an aliquot of the cell culture was centrifuged, the supernatant was discarded and the cell pellet was resuspended in fresh medium RPMI 1640 containing FCS (10%), PEST (1%), and G418 (1 mg/ml).

2.4. Measurement of superoxide anion production

The production of superoxide anion by the neutrophil NADPH-oxidase was measured by isoluminol-amplified chemiluminescense in a six-channel Biolumat LB 9505 (Berthold Co., Wildblad, Germany) and details about the technique and the precise oxygen metabolite measured has been described in detail earlier [23,24]. In short 2×10^5/ml neutrophils were mixed (in a total volume of 900 µl) with horse radish peroxidase (HRP, 4 U), and isoluminol (6×10^{-5} M) in KRG, pre-incubated at 37 °C after which the stimulus (100 µl) was added. The light emission was recorded continuously. The specific receptor inhibitors were included in the CL mixture for 5 min at 37 °C before stimulation.

2.5. Determination of changes in cytosolic calcium

Neutrophils at a density of 2×10^7 cells/ml in KRG without Ca^{2+} containing 0.1% BSA were loaded with 2 µM Fura 2-AM (Molecular Probes, Eugene, OR) for 30 min, at RT. Cells were then diluted to twice the original volume with RPMI 1640 culture medium without phenol red (PAA Laboratories GmbH, Austria) and centrifuged followed by washing once in KRG (with 1.0 mM Ca^{2+} from here on), and resuspended in KRG at a density of 2×10^7 cells/ml. Calcium measurements were carried out with a PerkinElmer fluorescence spectrophotometer (LC50).

The changes in cytosolic calcium levels were determined through measurement of the fluorescence, emitted at 510 nm, during excitation at 340 and 380 nm.

2.6. Cell surface receptor exposure by FACS analysis

To determine the effect of antagonists/inhibitors on cell surface CR3 exposure, neutrophils (2×10^6 cells/ml) were incubated in the absence or presence of an inhibitor at 37 °C for 5 min, and the cells were then activated with a receptor agonist. The exposure of CR3 on the cell surface was determined through the binding of a PE-conjugated antibody against CR3. The amount of specifically bound