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Stable formyl peptide receptor agonists that activate the neutrophil NADPH-oxidase identified through screening of a compound library

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ABSTRACT

The neutrophil formyl peptide receptors (FPR1 and FPR2) are G-protein coupled receptors that can induce pro-inflammatory as well as anti-inflammatory activities when activated. Accordingly, these receptors may become therapeutic targets for the development of novel drugs to be used for reducing the inflammation induced injuries in asthma, rheumatoid arthritis, Alzheimer's disease, cardiovascular diseases and traumatic shock. We screened a library of more then 50 K small compounds for an ability of the compounds to induce a transient rise in intracellular Ca^{2+} in cells transfected to express FPR2 (earlier called FPRL1 or the lipoxin A_4 receptor). Ten agonist hits were selected for further analysis representing different chemical series and five new together with five earlier described molecules were further profiled. Compounds 1–10 gave rise to a calcium response in the FPR2 transfectants with EC_{50} values ranging from 4×10^{-9} M to 2×10^{-7} M. All 10 compounds activated human neutrophils to release superoxide, and based on the potency of their activity, the three most potent activators of the neutrophil NADPH-oxidase were further characterized. These three agonists were largely resistant to inactivation by neutrophil produced reactive oxygen species and shown to trigger the same functional repertoire in neutrophils as earlier described peptide agonists. Accordingly they induced chemotaxis, granule mobilization and secretion of superoxide. Interestingly, the oxidase activity was largely inhibited by cyclosporine H, an FPR1 selective antagonist, but not by PBP10, an FPR2 selective inhibitor, suggesting that FPR1 is the preferred receptor in neutrophils for all three agonists.

1. Introduction

Professional phagocytes such as neutrophil granulocytes play important roles in host defence against pathogenic microbes, but they are also key regulators in the fine-tuning of inflammatory reactions. The functional repertoires of these cells are triggered and regulated through binding of different inflammatory mediators that are recognized by specific cell surface receptors expressed on resting or primed cells [1,2]. Receptors that belong to the "G-protein coupled, seven transmembrane family of receptors (GPCRs)" are well represented on neutrophils, and the function of one of these, the formyl peptide receptor (FPR1) has been extensively studied. This receptor is a member of the chemoattractant receptor subfamily that recognizes N-formylated peptides [3–5]. As such peptides are derived from bacterial or mitochondrial proteins, it has been proposed that a primary FPR1 function is to promote trafficking of phagocytic myeloid cells to sites of infection and tissue damage, where they exert antibacterial effector functions and clear cell debris. Accordingly the prototype agonist formylmethionyl-leucyl-phenylalanine (fMLF) is a bacterial produced high-affinity FPR1 agonist that triggers a variety of biological activities in neutrophils, including chemotaxis, granule secretion, cytokine production, and superoxide release, the latter generated through an activation of an electron transporting NADPH-oxidase [6]. The reactive oxygen species (ROS) derived from the activated NADPH-oxidase are essential for a successful clearance of bacteria during infection as illustrated by the fact that patients lacking a functional oxidase suffer repeatedly from severe bacterial infections [7]. In addition to FPR1, neutrophils express also a closely related receptor, FPR2, that belongs to the same receptor family [4,5]. This receptor was initially orphan, but over the last 15 years, a wide range of agonists have been identified. All peptide agonists described but two, possess the same pro-

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inflammatory activities as the FPR1 agonists. The two exceptions are (i) peptides cleaved off from the N-terminus of the calcium regulated protein annexin I [8,9] and (ii) a 21 amino acids long synthetic peptide identified through a library screen [10]. Activation of FPR2 with these two peptide agonists triggers a unique signalling pathway that inhibits neutrophil function and induces anti-inflammatory activities in different model systems. The same functional link – FPR2 and induction of anti-inflammatory activities – has been established for the eicosanoid LXA_4 [11], but our earlier published data suggest that this lipid mediator uses a receptor distinct from FPR2 [12,13]. The eicosanoid is easily oxidized and by that biologically inactivated [11–13], and this is true also for many of the peptide agonists [14].

Through the use of small molecule libraries screening, a series of non-peptide agonists for the FPRs have been identified during the last couple of years. When investigated, the functional repertoire triggered by small non-peptide agonists such as the Quinazolinonoe (Quin-C1) derivatives that selectively bind FPR2 [15], AG14 that selectively binds FPR1 [16], and pyrazolone that binds both receptors [17], resembles that of the pro-inflammatory peptides and not that of the anti-inflammatory agonists. Using a chemolibrary screening approach it was recently shown that agonists of FPR1/FPR2 include compounds with wide chemical diversity [18]. Owing to the fact that the function of FPR2 during recent years has been linked not only to innate immune functions but also to chronic inflammation in systemic inflammatory and auto-immune diseases, clinically oriented research focusing on FPR2 has been carried out.

The aim of this study was to identify novel small molecule FPR2 agonists by screening a small molecule library, and of particular interest was to determine the ability of the new agonists, to activate the neutrophil NADPH-oxidase. This could possibly be used to determine if a compound should be regarded as pro-inflammatory or have the potential of being anti-inflammatory. After screening more than 50,000 compounds, 5 structurally diverse FPR2 agonists were selected (from the more than 500 hits that were confirmed as agonists from the screen) and further investigated together with five "hits" identical to earlier described FPR2 agonists. All the agonists were found to activate the neutrophils to produce superoxide. The three most potent agonists, one earlier described pyrazolone compound [19] and two new compound, were further characterized and found to be insensitive to inactivation by oxidants and possess all the functional characteristics of earlier described pro-inflammatory peptide agonists. Upon binding to human neutrophils, they induce granule mobilization, directional cellular migration and production of superoxide anion. Our data also show that these novel agonists preferentially use FPR1, and in contrast to many of the earlier described peptide agonists, they are resistant to oxidation by neutrophil-derived oxygen radicals. We suggest that these small non-peptide compounds could serve as good tools for enhanced understanding of the structure–function relationships of FPRs and for *in vitro*/*in vivo* therapeutic studies in which long lived agonist are required.

2. Materials and methods

2.1. Materials

Part of the chemical library was selected on the basis of diversity from Biovitrum and iNovica compound collections which consists of 40,000 compounds. The other part (an additional 10,000 compounds) was based on reference compounds collected from the literature ([19], http://www.wipo.int/pctdb/en/wo.jsp?wo=2005047899), and used as input to computational techniques allowing us to detect compounds with similar properties. In order to do this a pharmacophore was built in Phase [20] and it consisted of a central acceptor/donor pair flanked on one side of an acceptor and a hydrophobic group and on the other side by one aromatic group and one hydrophobic group (see Supplementary Fig. 1). A Tuplet hypothesis was then constructed using triplets [21], and used in parallel with the Phase pharmacophore to find matching compounds. The search results were used to select targeted libraries that showed a large proportion of hits in either of the searches. In addition discrete compounds that were found using either method were also selected for the screening, and the additional 10,000 compounds were selected in this way. The purity and identity of the used compounds were verified using NMR spectroscopy, HPLC, and mass spectroscopy. The compounds were dissolved in DMSO at a concentration of 10 mM and stored at −80 °C. Isoluminol, TNF-α, fMLF, 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, United Kingdom). Horse radish peroxidase (HRP) was purchased from Boehringer-Mannheim (Mannheim, Germany). 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 °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 peptide QRLFQVKGRR (gelsolin residues 160–169), prepared by solid phase peptide synthesis and coupled to rhodamine as described [22].

2.2. Screening assay with FPR2 transfectants – determination of changes in cytosolic calcium

CHO-K1 cells stably expressing the human FPR2 and Galpha16 were plated in HAMSF12 with glutamax, 10% dialyzed FBS 250 mg/l zeocin and 400 mg/l geneticin (GIBCO/Invitrogen AB, Stockholm, Sweden) at a density of 10,000 cells/well and incubated at 37 °C and 5% CO_2 over night. After two washes with assay buffer, HBSS with 20 mM HEPES (GIBCO/Invitrogen AB, Stockholm, Sweden) cells were loaded with 3.6 μM Fluo4 AM (Molecular Probes, Eugene, OR, USA) diluted in assay buffer, including 2.5 mM probenecid (Sigma Chemical Co., St. Louis, MO, USA) for 60 min, at 37 °C. Prior to measurement cells were washed and incubated in assay buffer for another 5 min. Compounds dissolved in DMSO at a concentration of 10 mM were diluted to 0.08 mM in assay buffer. In FLIPR 2 (Molecular Devices Inc., Sunnyvall, CA, USA) cells were stimulated with compounds at a final concentration of 20 μM and changes of cytosolic calcium were measured. Excitation wavelength was 488 nm and a 510–570 nm filter was used for emission measurements. Maximum emission data from each compound stimulation was collected and percentage of response elicited by 2 nM WKYMVm-peptide was calculated. Compounds that induced a calcium response over 60% of the response elicited by 2 nM WKYMVm-peptide were defined as hits. All hits were counter screened in CHO-K1 cells transfected with G-alpha16 to exclude unspecific calcium agonists.

Selected hits were confirmed in calcium release analysis in FPR2 and Galpha16 transfected cells described above in a dose dependent manner from 20 μM to 3.4 pM in 11 steps.

The ability to activate FPR1 transfectants was determined in a calcium release assay basically as described above. CHO-K1 cells stably expressing the human FPR1 and Galpha16 were plated as

described above (but with 800 mg/l of geneticin) at a density of 10,000 cells/well and incubated at 37 °C and 5% CO_2 over night. Cells were labelled with Fluo4 and the change in intracellular calcium ions was determined as described above.

2.3. Isolation of human neutrophils

Neutrophil granulocytes were isolated from buffy coats obtained from healthy adults [23]. After dextran sedimentation at $1 \times g$, hypotonic lysis of the remaining erythrocytes, and centrifugation in a Ficoll-Paque gradient, the neutrophils were washed twice and resuspended (1×10^7/ml) in KRG. The cells were stored on melting ice and used within 120 min of preparation.

2.4. Neutrophil NADPH-oxidase activity

Neutrophil superoxide anion production was determined using an isoluminol-enhanced chemiluminescence (CL) system [24,25]. The CL activity was measured in a 6-channel Biolumat LB 9505 (Berthold Co., Wildbad, Germany) using disposable 4-ml polypropylene tubes with a 1-ml reaction mixture. Tubes containing isoluminol (2×10^{-5} M), horseradish peroxidase (HRP, 4 U), and neutrophils (2×10^5/ml) were allowed to equilibrate for 5 min at 37 °C, after which 0.1 ml of stimuli was added and the light emission was recorded continuously. All experiments (with resting as well as primed cells) were performed with neutrophils in suspension.

By a direct comparison of the superoxide dismutase (SOD) inhibitable reduction of cytochrome C and SOD inhibitable CL, 7.2×10^7 counts were found to correspond to a production of 1 nmol of superoxide (a millimolar extinction coefficient for cytochrome C of 21.1 was used; details about the CL technique is given in [25,26]).

2.5. Chemotaxis assay

Neutrophil migration was determined using a 96-well microplate chemotaxis chambers with hydrophobic filters of pore size 3 μm (ChemoTx; Neuro Probe Inc., Gaithersburg, MD, USA) according to the instructions given by the manufacturer. In short, compounds and the positive control, N-formyl-Met-Leu-Phe, fMLF (10^{-8} M) were added to wells in the lower chamber. All dilutions were made in KRG buffer supplemented with 0.3% BSA. Cell suspensions (30 μl) neutrophils (10^6/ml) were placed on top of the filters and allowed to migrate for 90 min at 37 °C. Cell migration was examined under microscope. For quantification, the content of myeloperoxidase was assessed in the lysates of transmigrated cells by adding a peroxidase substrate, o-phenylenediamine.

2.6. Cell surface receptor exposure by FACS analysis

To determine the effect of compounds on cell surface CR3 exposure, cells were incubated with various concentrations of neutrophil activators at 37 °C for 15 min. After which the cells were labelled with a PE-conjugated CR3 antibody on ice for 30 min. The amount of specifically bound antibody after washing off excess of unbound antibody was determined by flow cytometry.

The release of myeloperoxidase (MPO), an azurophil granule marker of importance for inactivation of chemoattractants [14], was determined as described earlier [27]

3. Results

3.1. Identification of novel non-peptide molecules that interact with FPR2

We used a small molecule screening set approach to identify novel agonists for formyl peptide receptor 2 (FPR2). Transfected CHO cells stably expressing FPR2 were loaded with Fluo4 AM and then stimulated with 50,000 compounds in 384 format FLIPR and analyzed for increase in the concentration of cytosolic calcium. The screen was performed at 20 μM to identify only agonists.

Out of the 50 K compounds screened, more then 500 was confirmed as agonist hits and five compounds representing different chemical series were selected for further profiling. In addition, five earlier described FPR2 agonists (compounds 1, 2, 3, 4 and 7 in Fig. 1) were also profiled. All 10 compounds were found to trigger an intracellular Ca^{2+} response in FPR2 expressing cells. The EC_{50} values for the different compounds were determined and were found to vary from 4×10^{-9} to 2×10^{-7} M (Fig. 1). Based on the activity, they could be divided into three groups. The EC_{50} value for compounds 8 and 9 were higher (in the 10^{-7} M range) than the corresponding values for compounds 1, 2, 3, 6 and 7 (in the 10^{-8} M range), whereas the most potent compounds (the compounds 4, 5, and 10) were active in the nM concentration range (Fig. 1).

The potential pro-inflammatory activity of FPR2 binding compounds derived from the small molecule library was investigated using human neutrophils as model cells and the NADPH-oxidase mediated superoxide production as readout system.

3.1.1. The non-peptide FPR2 agonists activate neutrophils to produce superoxide anions

The abilities of the 10 compounds identified as FPR2 agonists to induce a respiratory burst activity in resting and TNF-α primed neutrophils were determined. All compounds induced a neutophil respiratory burst and with respect to the potency they grouped in the same way as described above. The compounds 8 and 9 induced a respiratory burst only in TNF-α primed cells and in concentrations higher that 1 μM, whereas the compounds 1, 2, 3, 6 and 7 triggered also resting cells to produce superoxide anions (data not shown). The three compounds (4, 5 and 10) that had the highest activity in the calcium assay, were the most potent also in triggering a respiratory burst, and a robust superoxide production was induced in resting cells (Fig. 2). The response induced in TNF-α primed cells were strongly increased with all compounds including the three most potent ones (data not shown). We decided to characterize the activities induced in neutrophils by the three most active compounds 4, 5 and, 10, in more detail, but it should be noticed that although the magnitude of superoxide production differed between the compounds they all showed the same kinetics. This is shown for the three most potent compounds in Fig. 2. The time course was characterized by a very short lag phase followed by a rapid increase of superoxide release and a peak of activity was reached after around 1 min. This is a response that is typical for most chemoattractants that bind to a neutrophil GPCR and activate the oxidase, and one such, fMLF response, is shown for comparison (Fig. 2 inset).

The magnitudes of the responses induced by compounds 4 and 5 at a concentration of 1 μM were in the same level as that induced by WKYMVM (10^{-7} M) and fMLF (10^{-7} M), two very potent agonists for FPR2 and FPR1, respectively (shown for fMLF; Fig. 2 inset). The EC_{50} values for activation of the neutrophil oxidase were in the range 0.1–0.2 μM for both compounds 4 and 5. Even though the EC_{50} value for activation of the oxidase with compound 10 was in the same range as the other two, the maximal amount of radicals produced was lower (Fig. 2).

3.2. The compounds 4, 5, and 10 are not inactivated by reactive oxygen species generated by neutrophils

Neutrophil derived reactive oxygen species are highly reactive not only with bacterial proteins during bacterial clearance process, they could also oxidize and inactive small molecules very rapidly.

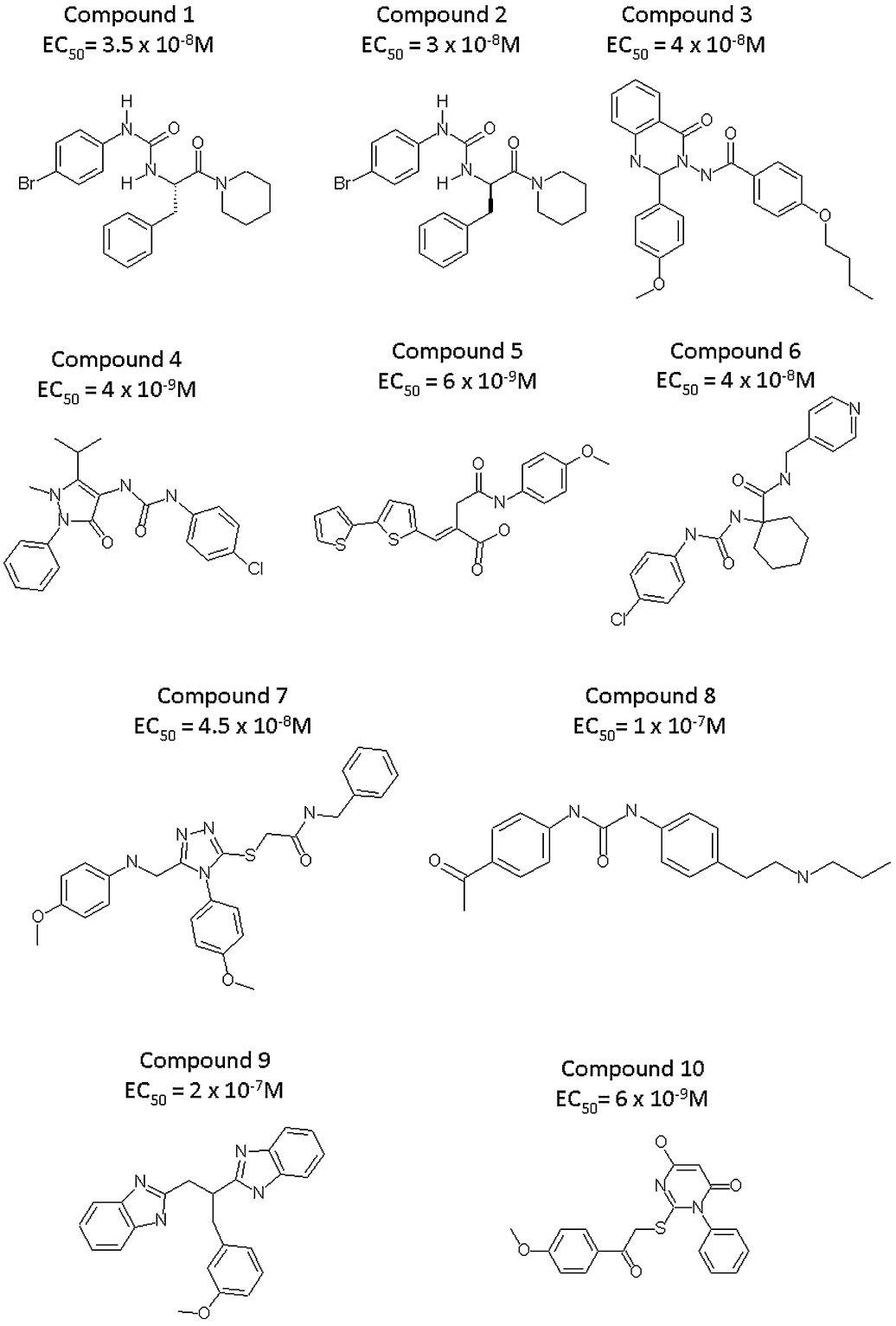

Fig. 1. Chemical structures of compounds 1 through 10. The chemical structure for the 10 compounds identified in the screening assay as FPR2 agonists. The EC_{50} values for the compounds, determined through their abilities to induce a Ca^{2+} response in cells stably expressing FPR2, are also given.

Accordingly, we recently showed that the FPR agonists fMLF and WKYMVM are rapidly inactivated by ROS producing neutrophils in a process that is dependent on the azurophil granule protein MPO [14,28]. To determine the sensitivity of the new compounds for neutrophil derived oxygen radicals, we added the compounds 4, 5, or 10 at high concentrations (10 μM) to neutrophils. After an incubation period of 10 min, the cells were removed by centrifugation and the agonists remaining in the supernatant after interaction with the neutrophils were used to trigger a new/fresh sample of cells to generate ROS. A high concentration (10 μM) of the agonists was chosen for inactivation, making it possible to use a small volume (0.1 ml) of each supernatant, and add that to a new/fresh sample of cells. Inactivation of a compound would then result in a reduced cellular response induced by a cell

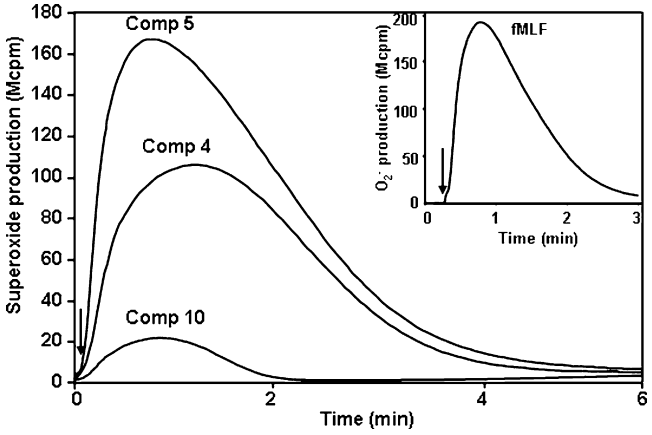

Fig. 2. Activation of the neutrophil NADPH-oxidase by the compounds 4, 5, and 10. Neutrophils were pre-incubated at 37 °C for 5 min and then challenged with the agonists (1 μM final concentration). The extracellular release of superoxide anion was monitored. The time point for addition of the agonist is indicated by an arrow and the amount of superoxide is expressed in arbitrary units. For comparison the response induced by the prototype agonist fMLF (10^{-7} M final concentration) is shown in the inset. Abscissa; time of study (min); ordinate; superoxide production given as light emission and expressed in cpmx10^{-6}.

free supernatant (with 10 μM from the beginning), compared to the corresponding control pre-incubated without any cells. The activities induced by the three compounds were, however, basically unchanged after interaction with the neutrophils (Fig. 3; Table 1) whereas the triggering effect of fMLF in a comparable system was reduced by more than 75% and a complete inactivation of WKYMVM (Fig. 3D; Table 1) was obtained.

We know from earlier studies that the inactivation is dependent on the granule enzyme MPO [14], but no MPO secretion was induced by any of the compounds used (data not shown), suggesting that the basal level MPO is sufficient. Moreover, the neutrophil activity induced by a cell free supernatant, originating from a mixture of cells, WKYMVM (an FPR2 agonist) and compound 5 (primarily an FPR1 agonist; see below) was totally inhibited by cyclosporine H (an FPR1 antagonist), showing that when one sensitive and one stable agonist are added to the cells simultaneously, only one of them "survives".

Table 1
Neutrophil-mediated inactivation of fMLF, WKYMVM, compound 4, compound 5 and compound 13. The agonists were added to neutrophils, and after an incubation period of 10 min, the cells were removed by centrifugation and the agonists remaining in the supernatants were used to trigger a new/fresh sample of cells to generate ROS and the activities were compared to a control sample of the respective compound. The ratios were calculated (using the peak values of the response) between samples incubated with cells and the controls, and a value of 1 is expected for compounds that are resistant to inactivation whereas those that are fully inactivated will have a ratio value close to 0.

Agonist incubated with neutrophils	Ratio (mean ± SD, *n*)
The FPR1 agonist fMLF (10^{-6})	0.19 ± 0.25, *n* = 8
The FRP2 agonist WKYMVM (10^{-6} M)	0.07 ± 0.02, *n* = 4
Compound 4 (10^{-5} M)	1.01 ± 0.10, *n* = 4
Compound 5 (10^{-5} M)	1.02 ± 0.09, *n* = 4
Compound 13 (10^{-5})	0.76 ± 0.09, *n* = 4

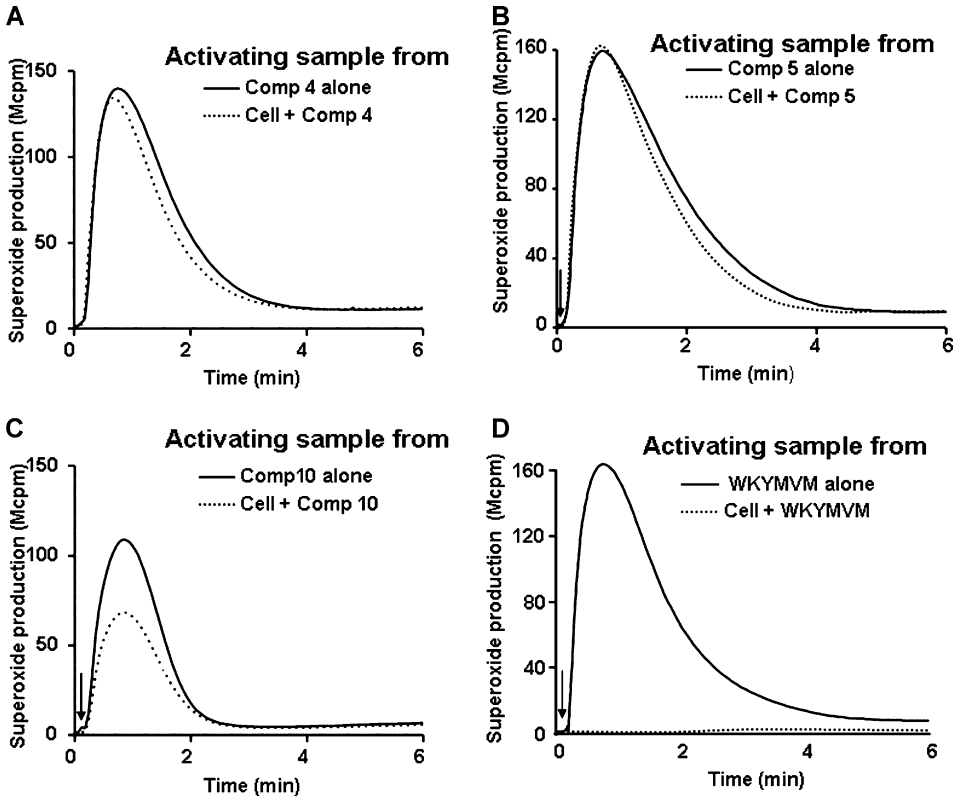

Fig. 3. Compound 4, 5 and 10 are not inactivated by the neutrophils. Neutrophils (10^7/ml) were incubated at 37 °C for 10 min with compound 4, 5 or 10 (10 μM final concentration; A–C) or WKYMVM (1 μM final concentration; D). The cells were then removed by centrifugation and the supernatants were used to trigger freshly prepared cell samples. The cell-free supernatant containing compound 4, 5 or 10 induced a respiratory burst (broken line in the upper panel) comparable to the control sample (solid line), whereas no activity was induced by the cell-free supernatant containing WKYMVM from the beginning (broken line in the lower panel). The time point for addition of the supernatant or control agonist is indicated by arrows and the amount of superoxide is expressed in arbitrary units. Abscissa; time of study (min); ordinate; superoxide production given as light emission and expressed in cpmx10^{-6}.

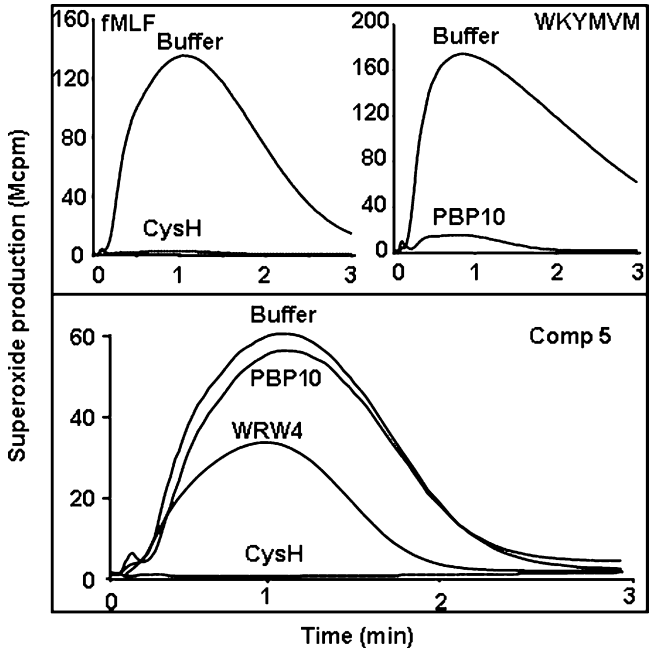

Fig. 4. Effects of the PBP 10 (an FPR2 selective inhibitor) and cyclosporine H (an FPR1 selective antagonist) on the NADPH-oxidase activity induced by compound 5. Neutrophils were pre-incubated at 37 °C for 5 min with or without the inhibitors. The concentration of cyclosporine chosen (5 μM final concentration) inhibited the fMLF induced activity by more than 90% (upper left) and that of PBP10 (1 μM) inhibited the WKYMVM induced activity to the same degree (upper right). Neutrophils were challenged with the FPR1 specific agonist fMLF (10^{-7} M final concentration; upper left), the FPR2 specific agonist WKYMVM (10^{-7} M; upper right) or compound 5 (1 μM final concentration; lower panel) ± cyclosporine H or WRW_4 (5 μM final concentration), respectively. The extracellular release of superoxide anion was monitored. The time point for addition of the agonist is indicated by an arrow and the amount of superoxide is expressed in arbitrary units. Abscissa; time of study (min); ordinate; superoxide production given as light emission and expressed in cpmx10^{-6}.

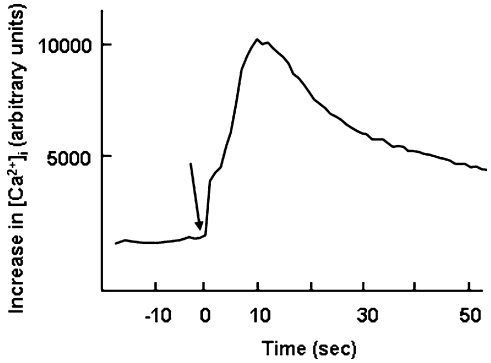

Fig. 5. A rise in the concentration of intracellular Ca^{2+} induced by compound 5 in stable transfectants expressing FPR1. FPR1 transfected cells loaded with the calcium sensitive dye Fluo4 were incubated at 37 °C for 5 min. The cells were then stimulated with compound 5 (10 nM final concentration) added at the time point indicated by the arrow. The changes in cytosolic Ca^{2+} levels were determined through measurement of the fluorescence emitted at 510–570 nm, during excitation at 488 nm. The result is presented in fluorescence intensities, and a representative experiment is shown.

3.3. Formyl peptide receptor1 (FPR1) is the preferred receptor for compounds 4, 5 and, 10

Neutrophils express in addition to FPR2 also the closely related receptor FPR1 and these receptors have the ability to recognize a large range of structurally unrelated agonists that are either specific or overlapping. An agonist that can bind and activate both receptors sometimes has a preference for either of the receptors [29], meaning that one of the receptors is not in use unless the preferred receptor is blocked by a specific antagonist or inhibitor. In order to determine which of the receptors that is used by compounds 4,5 and, 10, we used a couple of earlier characterized receptor antagonists and a receptor specific inhibitor. The receptor specific inhibitors used were cyclosporine H [30,31] and the PBP10 [31–33], antagonists/inhibitors for FPR1 and FPR2, respectively (Fig. 4). The FPR2 specific inhibitor PBP10, is a peptide derived from one of the PIP_2 binding domains present in the cytoskeletal protein gelsolin. The FPR1 specific antagonist cyclosporine H completely abolished the neutrophil response both to compounds 4 and 5 as well as to compound 10 (Table 2 and shown for compound 5 in Fig. 4). No inhibitory effect was obtained with the FPR2 specific inhibitor PBP10, but when the FPR1 antagonist was replaced by the specific FPR2 antagonist WRWWWW a partial inhibition was observed. Taken together, these data suggest that all three compounds (4,5 and 10) activate neutrophils preferentially through FPR1 rather than through FPR2.

Using transfected CHO cells stably expressing FPR1, we could show that the three compounds (4, 5 and 10) trigger an intracellular Ca^{2+} response also in these cells (Table 2 and shown for compound 5 in Fig. 5), confirming the results obtained with normal neutrophils.

3.4. Receptor desensitization and hierarchy

To further elucidate the receptors involved in the cellular responses induced by three novel compounds used, we performed receptor desensitization experiments using agonist with known receptor specificity. It is well established that neutrophils challenged with one GPCR agonist are non-responsive to a second dose of the same agonist, a process known as homologous desensitization. Accordingly, compounds 4, 5 and 10 induced a

Table 2
Basic characteristics of compounds 4 and 10 with respect to neutrophil functional responses.

Basic character/function investigated	Results obtained with	
	Compound 4	Compound 10
Sensitivity to inactivation by ROS	Completely resistant	Largely resistant
Effect of the FPR2 inhibitor PBP10	Not inhibited	Not inhibited
Effect of the FRP2 antagonist WRWWWW	Partially inhibited	Partially inhibited
Effect of the FPR1 antagonist cyclosporine H	Completely inhibited	Completely inhibited
Desensitization of the IL-8 response	Inhibited/desensitized	Inhibited/desensitized
Desensitization of the fMLF response	Partially inhibited	Partially inhibited
Desensitization of the WKYMVM response	Partially inhibited	Partially inhibited
Response following activation with IL-8	Not inhibited	Not inhibited
Response following activation with fMLF	Completely inhibited	Completely inhibited
Response following activation with WKYMVM	Partially inhibited	Partially inhibited
Calcium response in FPR1-expressing cells	Yes	Yes

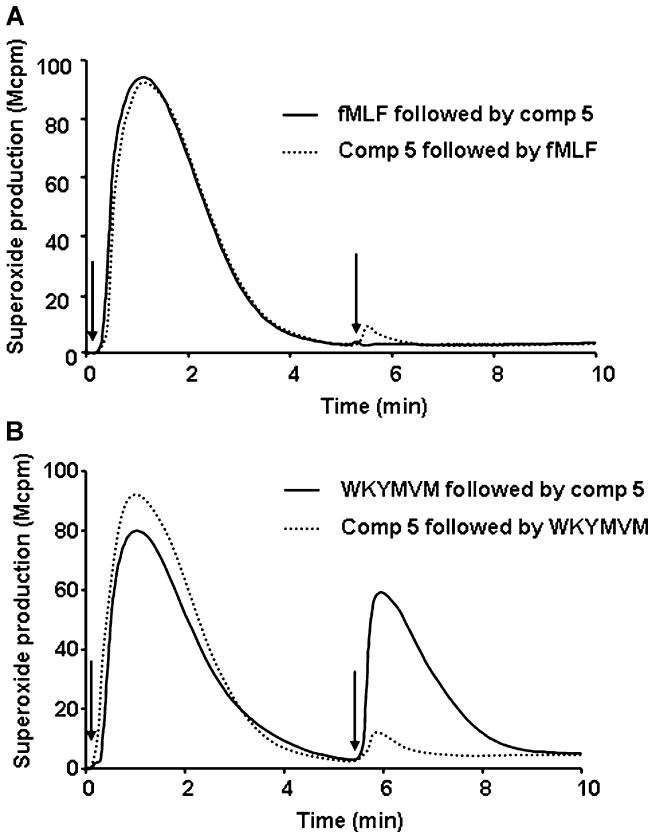

Fig. 6. Receptor desensitization induced by compound 5, fMLF and WKYMVM. Neutrophils were pre-incubated at 37 °C for 5 min and then challenged with the FPR1 specific agonist fMLF or as indicated by the first arrow (A; solid line). The same cell population was then triggered by compound 5 (second arrow in A, solid line). The agonist order was also reversed; the cells were first challenged with compound 5 (dotted line) and then with fMLF (dotted line). The experiment is also shown (B) in which fMLF is replaced by WKYMVM. The time points for addition of the agonists are indicated by arrows and the amount of superoxide is expressed in arbitrary units. Abscissa; time of study (min); ordinate; superoxide production given as light emission and expressed in cpmx10^{-6}.

homologues desensitization in neutrophils (data not shown). Cells that were desensitized with the FPR1 agonist fMLF were desensitized not only to this agonist, but also to the compounds 4, 5 and 10 and the desensitized state was there when the order for agonist addition was reversed, i.e., cells triggered with any of the three compounds were desensitized to fMLF (Table 2 and shown for compound 5 in Fig. 6A). Neutrophils challenged with any of the three compounds were also desensitized to the FPR2 specific agonist WKYMVM, but when the order was reversed – first WKYMVM and then one of the compounds – the cells were only partially desensitized to the three compounds (Fig. 6B; Table 2). This suggests that all three compounds prefer FPR1 over FPR2.

Neutrophils may also be desensitized heterologously, meaning that an occupied receptor not only desensitize itself but also an adjacent receptor with another agonist specificity. This type of desensitization also disclose a receptor hierarchy, illustrated by the fact that an occupation of FPR1 leads to a desensitization of this receptor as well as CXCR (the IL-8 receptor), whereas occupation of CXCR does not desensitize FPR1 [34,35]. To further explore hierarchy, we determined the desensitization patterns with compounds 4, 5 and, 10, three compounds and the intermediate receptor agonist CXCR as well as the end type receptor C5aR. We show that neutrophils challenged with any of the three compounds were non-responsive to a second stimulation with IL-8 whereas cells first triggered with IL-8 were fully responsive to compounds 4, 5 and 10 (Table 2 and shown for compound 5 in Fig. 7A) suggesting a receptor hierarchy that is in line with the known desensitization model in which the FPRs are superior to CXCR. For comparision, the desensitization pattern obtained when fMLF replaces compounds 4, 5 and, 10, and showing that fMLF could desensitize neutrophils to IL-8 but not vice versa, is also shown (Fig. 7C).

Neutrophils activated with either of the three compounds were non-responsive also to C5a whereas no such desensitization is obtained when the order of agonist addition is reversed (Table 2 and shown for compound 5 in Fig. 7B). This type of desensitization pattern is obtained also when fMLF replaces compounds 4, 5 and, 10, i.e., fMLF could desensitize neutrophils to C5a, but not vice versa (data not shown). The precise molecular mechanism underlying this cross/heterologous-desensitization is not yet known.

3.5. The compounds 4, 5, and, 10 are neutrophil chemoattractants

Having identified three novel neutrophil FPR1 agonists and shown that they have the ability to activate the neutrophil NADPH-oxidase to generate superoxide anions, we next investigated whether they could induce other cellular functions that are induced by most peptide agonists that are recognized by these receptors. Using a trans-well chamber assay system we could show that cells migrated to the lower chamber where the novel compounds were present suggesting that these substances are chemotactic (Fig. 8). The positive control peptides fMLF and WKYMVM (at 10 nM concentration) triggered migration under similar conditions (shown for fMLF in Fig. 8). Around 3% of the cells were recovered in the lower compartment when this contained buffer alone, and this value thus represent spontaneous cellular migration (Fig. 8). The potency by which compound 5 induced cellular migration was similar to that of the positive control fMLF, but the concentration required to induce this migration was much higher (1 μM compared to 10 nM). The two other compounds (4 and 10) were less potent.

3.6. The compounds 4, 5, and 10 induce granule mobilization

Neutrophil activation by chemoattractants is associated with an induction of granule secretion, and such a process leads to a mobilization to cell surface of new receptors. To investigate the ability of these compounds in triggering granule mobilization, we examined the degree of surface exposure of CR3, a marker protein localized in easily mobilizable granules in neutrophils (i.e., secretory vesicles, gelatinase granules, and to some extent specific granules), upon stimulation with various concentrations of the compounds 4, 5, and 10. The chemoattractants fMLF and WKYMVM were used as controls and they both induced a pronounced increased in CR3 exposure, in a concentration dependent manner (Fig. 9 inset). Similarly, pre-incubation of cells with either compound resulted in a large increase of CR3 exposure and this increase was also found to be concentration dependent (Fig. 9). Compound 4 was found to be the most potent whereas compound 5, the most potent in triggering the production of ROS, was the least potent in mobilizing CR3 (Fig. 9).

4. Discussion

Agonists as well as antagonists for FPR2 have been suggested to be of therapeutic value [36,37], and in addition, if they in one way or another selectively stimulate/inhibit neutrophil functions, they may be used also as research tools [5]. In order to find agonists with this potency, we screened a library of small compounds and several ligands for FPR2 with varying EC_{50} values were identified. The identification of small-molecule ligands represent an attractive

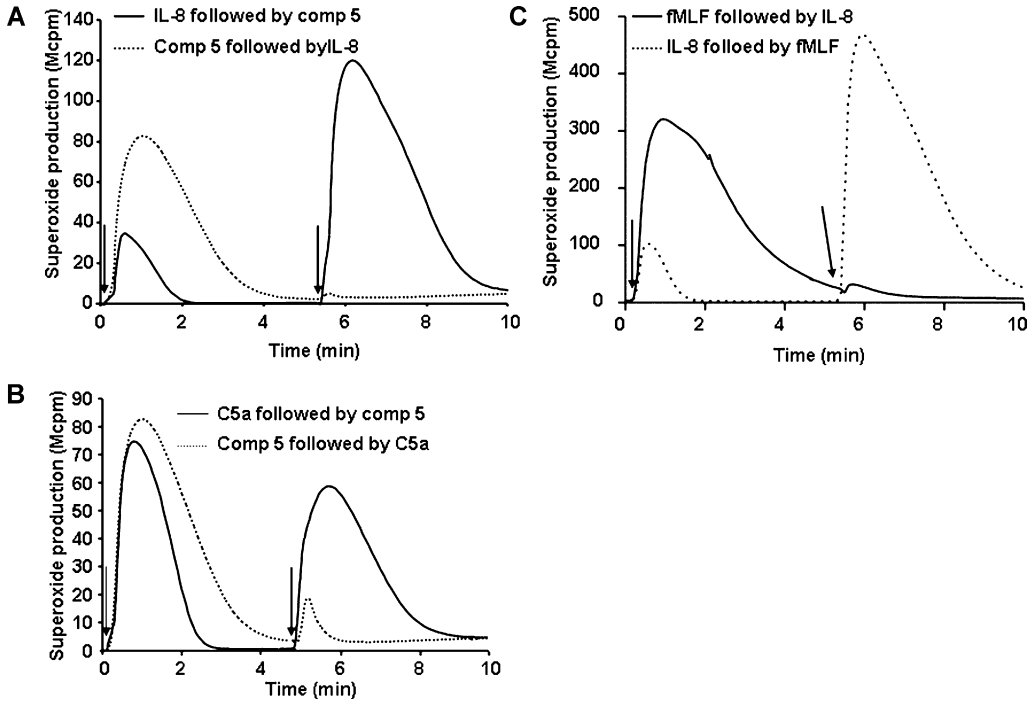

Fig. 7. Receptor desensitization of C5aR and CXCR induced by compound 5. Neutrophils were pre-incubated at 37 °C for 5 min and then challenged with compound 5 (1 μM; in A and B first arrow; broken lines). The same cell population was then triggered with IL8 (second arrow in A) or C5a (second arrow in B). The agonist order was also reversed; the cells were first challenged with IL8 (solid line in A) or C5a (solid line in B) and then with compound 5 (1 μM final concentration; second arrow in A and B). The time points for addition of the agonists are indicated by arrows and the amount of superoxide is expressed in arbitrary units. For comparison the response in neutrophils first challenged with fMLF (10^{-7} M; in C first arrow; solid line) and then triggered with IL8 (second arrow) is also shown. The agonist order was also reversed; the cells were first challenged with IL8 (broken line in C) and then with fMLF (10^{-7} M final concentration; second arrow in C). Abscissa; time of study (min); ordinate; superoxide production given as light emission and expressed in cpmx10^{-6}.

approach to analyze structure and function of FPRs, and in line with this several research articles using the same approach have been published during the last couple of years [16,38–40]. Of the compounds that we used and found to activate human neutrophils to produce/release superoxide anions, 5 (4 unique and one enantiomer) have been described earlier. Compounds 1 and 2 in this study (Fig. 1) are enantiomers of an earlier described FPR2 agonist (Acadia C7; http://www.wipo.int/pctdb/en/wo.jsp?wo=2005047899), that has been shown to dose dependently prevent hyperalgesia induced by carrageenan in a rat model. It should be noticed that large differences have been

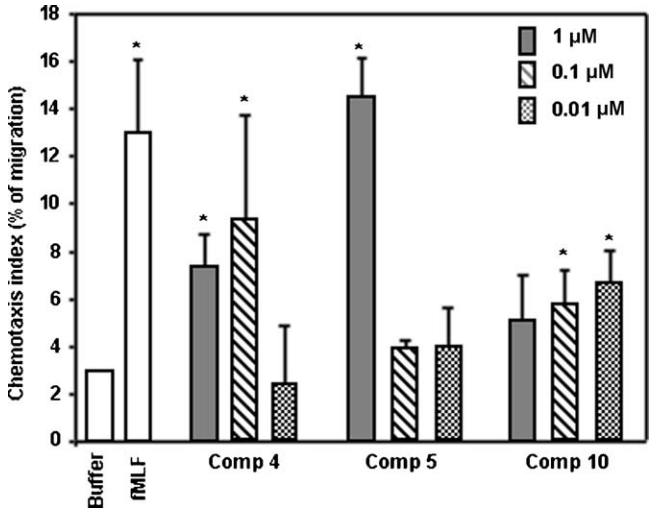

Fig. 8. Chemotaxis induced by compounds 4,5, and 10 expressed as percent of added cells recovered in the lower compartment of the migration chambers. Neutrophils were added on top of the filter that separated the upper compartment containing cells but no agonist, from the lower compartment containing the attractant. Three different concentrations of the compounds were used. The migration chambers were incubated at 37 °C for 90 min and the fraction cells (percent of those added) in the lower compartments were determined. Data are expressed as mean migration± SEM, of triplicate samples obtained from two experiments. Mann-Whitney U test was used to analyze statistical significance and p values <0.05 were considered significant.

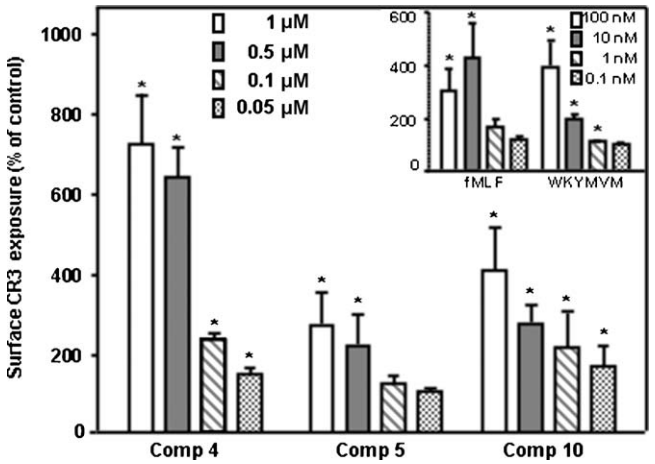

Fig. 9. Complement receptor 3 (CR3) mobilization by compounds 4, 5, and 10. Neutrophils were incubated for 15 min at 37 °C in the presence of compound 4,5 or 10 in concentrations ranging from 0.05 μM to 1 μM. The cells were then fixed through addition of ice-cold paraformyldehyde and stained with phycoeryterin-labelled anti-CR3 (clone CBRM1/5, ebioscience) for 30 min on ice followed by washing and resuspending the cells in FACS buffer (PBS with 0.1 mM EDTA). The amount of surface exposed CR3 was analyzed by flow cytometry and expressed as percentage of the mean fluorescence intensity (MFI) obtained with control cells incubated under the same conditions but without stimulation. For comparison, surface CR3 exposure upon stimulation with various concentrations of fMLF and WKYMVM is shown in the inset. Data are expressed as Mean ± SEM (n = 3). Mann-Whitney U test was used to analyze statistical significance and p values <0.05 were considered significant.

described for receptor agonists and their enantiomers [41], but in our hands compounds 1 and 2 were equally potent, but not among the most potent neutrophil activators. Compound 3 used in this study is a substituted quinazinolione (QuinC1), that has been described to be chemotactic and induce degranulation in neutrophils [42], but be unable to stimulate superoxide production [15]. We show using a more sensitive technique to detect ROS formation that compound 3 activates the NADPH-oxidase in resting as well as primed cells, and we could from experiments with receptor antagonists confirm that FPR2 is the preferred receptor (data not shown). It might well be that ligands may behave differently in functional assays because of their intrinsic ability to induce different receptor conformational changes, but we have not looked into this issue since compound 3 was not among the most potent neutrophil activators and thus not further investigated. Compound 4 is a pyrazolone originally described as an FPR2 agonist that dose dependently inhibit chemotaxis of human neutrophils in response to fMLF and IL8 [19]. This is in accordance with earlier studies suggesting that FPR2 is a down-regulating receptor when interacting with the lipoxygenase-derived eicosanoid lipoxin A_4 or peptides derived from the N-terminus of the calcium regulated protein annexin I [11,43]. We found that compound 4 (Fig. 1) activates neutrophils preferentially through FPR1 (see below) which could explain the down-regulating effects on fMLF and IL8 induced chemotaxis, and we also found this compound to be one of the three most potent agonists in our ROS production assay, and based on the potency of activity, the three most potent activators were further characterized.

Many FPR agonists can trigger their own inactivation when interacting with neutrophils (reported here for fMLF and WKYMVM) and the activity of the MPO-hydrogen peroxide system is the basis for the loss of biological activity [14,44]. Agents sensitive to this type of inactivation is not restricted to chemotactic factors [45,46], but the three most potent agonists in our ROS production assay (4, 5 and, 10), were all stable in relation to the radicals generated.

Cyclosporine H is a well established antagonist that abrogates FPR1-dependent responses but not those related to FPR2 [31]. The NADPH-oxidase response induced by the three most potent neutrophil activators, compounds 4, 5, and 10 were all inhibited by cyclosporine H, suggesting that they activate the neutrophil oxidase through FPR1 rather than FPR2. Although it is clear that the three compounds can bind and activate both FPR1 and FPR2, it is obvious that in neutrophils FPR1 is the preferred receptor. This conclusion is based on the following: cross-desensitization was obtained with cells desensitized with fMLF, i.e., the fMLF response was downregulated by the three compounds; the receptor specific inhibitor PBP10 (an FPR2 inhibitor) was without effects whereas the receptor specific antagonist cyclosporine H inhibited the neutrophil response to the three compounds. The fact that the FPR2 antagonist WRW_4 partly inhibited the activity by compounds 4, 5 an 10 is most probably due to a cross reactivity of the antagonist with FPR1, when the agonist used is present in low concentration or has a lower affinity for the receptor than fMLF. PBP10 is a more specific FPR2 inhibitor, and the fact that this was without effect suggests that FPR1 is the preferred receptor for all three compounds.

We show that all compounds trigger an activation of the neutrophil NADPH-oxidase in neutrophils primed with TNF-α. These cells are primed, not only in response to the new compounds, but to various inflammatory mediators, chemoattractants, and other ligands [47–50]. The primed response may be a regulatory mechanism, granting a cellular response (such as release of toxic oxygen radicals) only at sites where it is functional and necessary, e.g., in inflamed or infected areas. Although the molecular mechanism(s) responsible for induction of the primed state is unclear, the exposure of new receptors is an attractive model as the molecular basis for an augmented response. We know from an earlier study that intracellular organelles are mobilized to the cell surface during TNF-α priming [13], resulting in an increased exposure of various receptors including CR3, FPR1, and FPR2. The molecular background to the primed response to the new peptides may, thus, be the result of an increased exposure of these receptors.

Our study demonstrates several important functional features of the three compounds characterized: (i) they are neutrophil chemoattractants; (ii) they mobilize intracellular stores containing the integrin receptor for complement factor iC3b; (iii) they are potent activators of the NADPH-oxidase, especially in primed neutrophils; (iv) they trigger neutrophils through FPR1 and (v) they are not inactivated by the reactive oxygen species generated. Availability of potent, cheep, easy to handle, and stable activators of FPRs will undoubtedly be of great help for screening of antagonists for these receptors. The described non-peptide ligands for FPR1 and 2 may also serve as the nucleus for further structural modifications leading to the discovery of more potent and efficacious agonists. This type of work has been performed already, using the pyrazolone originally described as an FPR2 agonist, and an even more potent FPR2 agonist has been described together with a 100 fold less active enatiomer [41]. The selective activation property of Quin-C1 (our compound 3) has also been suggested as an interesting starting structure for future development of small non-peptide molecules that may be clinically useful antagonists for FPR2. The two compounds not earlier described, compounds 5 and 10, and found to be agonist for both FPR2 and FPR1, may also be used as starting structures for the development of clinically useful compounds that should be valuable tools also in future studies to reveal structural functional relationships for FPR ligands and their receptors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.11.005.

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