Published in final edited form as:

Biochem Pharmacol. 2017 October 15; 142: 120–132. doi:10.1016/j.bcp.2017.07.004.

4-Aroyl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-ones as *N*-Formyl Peptide Receptor 1 (FPR1) Antagonists

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Abstract

Formyl peptide receptors (FPRs) are expressed on a variety of leukocytes and play important roles in inflammation. Thus, FPR antagonists may represent novel therapeutics for modulating innate immunity and treating inflammatory diseases. Previously, 1*H*-pyrrol-2(5*H*)-ones were reported to be potent and competitive FPR1 antagonists. In the present studies, 42 additional 1*H*-pyrrol-2(5*H*)-one analogs were evaluated for FPR1 antagonist activity. We identified a number of novel competitive FPR1 antagonists that inhibited *N*-formylmethionyl-leucyl-phenylalanine (fMLF)-induced intracellular Ca²⁺ mobilization in FPR1-transfected HL60 cells and effectively competed with WKYMVm-FITC for binding to FPR1 in FPR1-transfected RBL cells. The most active pyrroles inhibited human neutrophil Ca²⁺ flux, chemotaxis, and adhesion to human epithelial cells, with the most potent being compounds **14** (4-benzoyl-1-hexyl-3-hydroxy-5-(4-hydroxy-3-methoxyphenyl)-2,5-dihydro-1*H*-pyrrol-2-one) and **17** (4-benzoyl-5-(2,5-dimethoxyphenyl)-3-hydroxy-1-(2-methoxyethyl)-2,5-dihydro-1*H*-pyrrol-2-one). In addition, these FPR1 antagonists inhibited fMLF-induced phosphorylation of extracellular signal-regulated kinases (ERK1/2) in FPR1-RBL cells, differentiated HL-60 cells, and human neutrophils. Most of the antagonists were specific for FPR1 and did not inhibit WKYMVm/WKYMVm-induced intracellular Ca²⁺ mobilization in FPR2-HL60 cells, FPR3-HL60 cells, or interleukin 8-induced Ca²⁺ flux in human neutrophils. Moreover, molecular modeling showed that the active pyrroles had a significantly higher degree of similarity with the FPR1 antagonist pharmacophore template

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Conflict of Interest Disclosure

The authors declare no conflicts of interest.

as compared to inactive analogs. Thus, the 4-aryl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-one scaffold represents an important backbone for the development of novel FPR1 antagonists and could provide important clues for understanding the molecular structural requirements of FPR1 antagonists.

Graphical abstract

Keywords

antagonist; formyl peptide receptor; 1*H*-pyrrol-2(5*H*)-one; neutrophil; molecular modeling

1. Introduction

Formyl peptide receptors (FPRs) are G protein-coupled receptors (GPCR) that play an important role in leukocyte activation and chemotaxis [1]. In humans, there are three FPR isoforms: FPR1, FPR2, and FPR3 [1]. FPR1 is expressed on a variety of cell types, including neutrophils, macrophages, natural killer (NK) cells, immature dendritic cells, astrocytes, microglial cells, hepatocytes, and bone marrow-derived mesenchymal stem cells [1–6]. FPR1 was originally identified as a receptor for *N*-formyl peptides, which are produced by bacteria but can also be released from damaged mitochondria during tissue injury [2, 7, 8]. In phagocytes, FPR1 activation induces cell migration, the release of reactive oxygen species (ROS), and phagocytosis [1, 4]. In addition to its role in phagocyte activation, FPR1 seems to have physiological roles in other cell types. For example, *N*-formylmethionyl-leucyl-phenylalanine (fMLF) induces osteoblast differentiation and upregulates expression of osteogenic markers [9]. Likewise, fMLF suppresses adipocyte differentiation in human mesenchymal stem cells [9]. Annexin A1 peptides can also activate FPR1 similarly to *N*-formyl peptides and induce inflammatory responses [10]. Recently, it was found that FPR1 binds scolopendrasins, which are anti-microbial peptides from *Scolopendra subspinipes mutilans* [11, 12]. Cytokine-like proteins FAM19A4 (family with sequence similarity 19 member A4) and FAM3D (family with sequence similarity 3 member D) were also reported as a novel FPR1 ligands [13, 14], and FAM19A4 was shown to stimulate chemotactic migration, phagocytosis, and release of ROS in macrophages via FPR1 [13].

FPR1 has been reported to contribute to the pathogenesis of several diseases. For example, FPR1 expression is associated with tumor progression and survival in gastric cancer [15], and FPR1 mediates the tumorigenicity of human hepatocellular carcinoma cells [16]. High expression of FPR1 in neuroblastoma primary tumors corresponds with high-risk disease and poor patient survival [17]. Likewise, interaction of endogenous annexin A1 with FPR1

leads to transactivation of the epithelial growth factor receptor (EGFR), which promotes invasion and growth of glioma cells [18]. Gliadin, the immunogenic component within gluten and a trigger of celiac disease, induces neutrophil migration via engagement of FPR1 [19]. The efficacy of FPR1 blockade in hepatic ischemia-reperfusion injury was reported recently [20]. In addition, an aurantiamide analog HCH6–1 demonstrated protective effects on lipopolysaccharide-induced acute lung injury by blocking FPR1 in mice [21, 22]. Thus, bioactive ligands acting as FPR1 antagonists might serve as useful therapeutics in host defense in order to reduce detrimental effects associated with inflammation and cancer [23].

Currently, the most potent FPR1-specific antagonists described are the fungal cyclic peptides, cyclosporine A and H [24]. However, *in vivo* studies of cyclosporines should be interpreted carefully, because their main therapeutic effects appear to involve signaling pathways unrelated to FPR1 [25–27]. However, growing evidence supporting the anti-inflammatory and tissue-protective effects of FPR1 antagonists led to the screening of natural products and commercial libraries for novel small-molecule FPR1 antagonists. As result of these screening efforts and/or structure–activity relationship (SAR)-directed design and synthesis, a number of synthetic non-peptide FPR1 antagonists with a wide range of chemical diversity have been identified (reviewed in [28, 29]). In addition, a variety of natural molecules have been shown to be FPR1 antagonists [30]. Among the most potent and specific small-molecule FPR1 antagonists are compounds with a 4*H*-chromen-4-one scaffold [31]. Several FPR1 antagonists with a 4-aryl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-one scaffold were previously reported [32, 33] (Figure 1), but their activities in primary cells, SAR analysis of related compounds, as well as molecular modeling have not been described. It should be noted that compounds having the same scaffold were also reported as small molecule blockers of the interaction between S100A10 and annexin A2 [34]. Because both annexin A2 and FPR1 are involved in pathogenesis of tumor growth and invasion of neuroblastoma, glioma, and hepatocellular carcinoma [35–37], development of FPR1 antagonists based on the 4-aryl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-one scaffold could lead to promising dual functional agents for the treatment of these diseases.

In the present study, we evaluated forty two 1*H*-pyrrol-2(5*H*)-ones for their ability to antagonize FPR-dependent signaling in neutrophils and FPR-transfected cells and identified novel and relatively potent FPR1 antagonists. Most of these antagonists were specific for FPR1 and did not inhibit FPR2-, FPR3-, or chemokine (C-X-C motif) receptor (CXCR) 1/2-dependent responses. SAR analysis of these compounds revealed the importance of a small hydrophobic group at position R₄ of the 4-aryl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-one scaffold. In addition, molecular modeling showed a high degree of similarity for low-energy conformations of these antagonists with the pharmacophore model of FPR1 antagonists.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO), *MLF*, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and Histopaque 1077 were from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium and penicillin–streptomycin solution were from Mediatech (Herdon, VA,

USA). DMEM/F12 was from Lonza (Walkersville, MD, USA). Fetal bovine serum (FBS) was from Atlas Biologicals (Fort Collins, CO, USA). Peptides Trp-Lys-Tyr-Met-Val-L-Met-NH₂ (WKYMVm) and Trp-Lys-Tyr-Met-Val-D-Met-NH₂ (WKYMVM) were from Calbiochem (San Diego, CA, USA) and Tocris Bioscience (Ellisville, MO, USA), respectively. 2',7'-Bis-(carboxyethyl)-5(6)-carboxyfluorescein pentaacetoxymethyl ester (BCECF-AM) was from Biotium (Fremont, CA, USA). Human interleukin-8 (IL-8) was from Peprotech Inc (Rocky Hill, NJ, USA). Hanks' balanced salt solution (HBSS), Fluo-4 AM, and G418 were from Life Technologies (Grand Island, NY, USA). HBSS containing 1.3 mM CaCl₂ and 1.0 mM MgSO₄ is designated as HBSS⁺; HBSS without Ca²⁺ and Mg²⁺ is designated as HBSS⁻. Selected 1*H*-pyrrol-2(5*H*)-ones were purchased from ChemDiv (San Diego, CA, USA), ChemBridge (San Diego, CA, USA), Princeton BioMolecular Research (Monmouth Junction, NJ, USA), Vitas-M Laboratory (Moscow, Russia), and InterBioScreen (Moscow, Russia). Fluorescein isothiocyanate (FITC) was conjugated to the lysine residue of WKYMVm to produce a fluorescent ligand (WKYMVm-FITC) that binds to both FPR1 and FPR2 (custom synthesis by Bachem, Torrance, CA, USA).

2.2. Cell culture

Human promyelocytic leukemia HL60 cells stably transfected with FPR1 (FPR1-HL60 cells) or FPR2 (FPR2-HL60 cells) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 100 µg/ml streptomycin, 100 U/ml penicillin, and G418 (1 mg/mL), as described previously [38]. Rat basophilic leukemia (RBL-2H3) cells transfected with human FPR1 (FPR1-RBL) were cultured in DMEM supplemented with 20% (v/v) FBS, 10 mM HEPES, 100 µg/ml streptomycin, 100 U/ml penicillin, and G418 (250 µg/ml), as described previously. Although stable cell lines are cultured under G418 selection pressure, G418 may affect some assays, so it was removed in the last round of culture before assays were performed. Wild-type HL60 and RBL-2H3 cells were cultured under the same conditions, but without G418. For differentiation of HL60 into neutrophil-like cells, DMSO was added to a final concentration of 1.2%, and the cells were cultured for 6 days. Differentiation was monitored by a gain of the responsiveness of cells to Δ MLF by measuring Δ MLF-induced superoxide generation (data not shown). Human T84 colonic adenocarcinoma cells were cultured in DMEM/F12 1:1 media supplemented with 10% (v/v) FBS, 15 mM HEPES, 100 µg/ml streptomycin, and 100 U/ml penicillin.

2.3. Isolation of human neutrophils

Blood was collected from healthy donors in accordance with a protocol approved by the Institutional Review Board at Montana State University. Neutrophils were purified from the blood using dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of red blood cells, as previously described [39]. Isolated neutrophils were washed twice and resuspended in HBSS⁻. Neutrophil preparations were routinely >95 % pure, as determined by light microscopy, and > 98 % viable, as determined by trypan blue exclusion.

2.4. Ca²⁺ mobilization assay

Changes in intracellular Ca²⁺ were measured with a FlexStation II scanning fluorometer (Molecular Devices, Sunnyvale, CA, USA), as described previously [38]. The cells,

suspended in HBSS⁻ containing 10 mM HEPES, were loaded with Fluo-4 AM dye (1.25 µg/mL final concentration) and incubated for 30 min in the dark at 37 °C. After dye loading, the cells were washed with HBSS⁻ containing 10 mM HEPES, resuspended in HBSS⁺ containing 10 mM HEPES, and aliquoted into the wells of a flat-bottom, half-area-well black microtiter plates (2 × 10⁵ cells/well). For evaluation of direct agonist activity, the compound of interest was added from a source plate containing dilutions of test compounds in HBSS⁺, and changes in fluorescence were monitored (λ_{ex} = 485 nm, λ_{em} = 538 nm) every 5 s for 240 s at room temperature after automated addition of compounds.

Antagonist activity and selectivity were evaluated after a 30 min pretreatment with test compounds at room temperature, followed by addition of peptide/chemokine agonist (5 nM Δ MLF, 5 nM WKYMVM, 10 nM WKYMVM, or 25 nM IL-8). In some experiments, a range of Δ MLF concentrations was used. Maximum change in fluorescence during the first 3 min, expressed in arbitrary units over baseline, was used to determine a response. Responses for FPR1 antagonists were normalized to the response induced by 5 nM Δ MLF for FPR1-HL60 cells and neutrophils, which were assigned a value of 100%. Curve fitting (5–6 points) and calculation of median effective inhibitory concentrations (IC₅₀) were performed by nonlinear regression analysis of the dose–response curves generated using Prism 7 (GraphPad Software, Inc., San Diego, CA, USA). Efficacy is expressed as % inhibition by an antagonist of the response induced by 5 nM Δ MLF at the maximal applied concentration of an antagonist (~ 50 µM).

2.5. Chemotaxis assay

Human neutrophils were suspended in HBSS⁺ containing 2% (v/v) heat-inactivated FBS (2 × 10⁶ cells/ml), and chemotaxis was analyzed in 96-well ChemoTx chemotaxis chambers (Neuroprobe, Gaithersburg, MD, USA), as described previously [31]. In brief, neutrophils were preincubated with the indicated concentrations of the tested compounds or DMSO for 30 min at room temperature and added to the upper wells of the ChemoTx chemotaxis chambers. The lower wells were loaded with 30 µl of HBSS⁺ containing 2% (v/v) heat-inactivated FBS with the indicated concentrations of test compounds plus 1 nM Δ MLF, DMSO plus 1 nM Δ MLF (positive control), or DMSO alone (negative control). Neutrophils were allowed to migrate through the 5.0-µm pore polycarbonate membrane filter for 60 min at 37 °C and 5% CO₂. The number of migrated cells was determined by measuring ATP in lysates of transmigrated cells using a luminescence-based assay (CellTiter-Glo; Promega, Madison, WI, USA) and a Fluoroscan Ascent FL microplate reader. Luminescence measurements were converted to absolute cell numbers by comparison of the values with standard curves obtained with known numbers of neutrophils. Curve fitting (at least eight to nine points) and calculation of median effective concentration values (IC₅₀) were performed by nonlinear regression analysis of the dose–response curves generated using Prism software.

2.6. Competition binding assay

Dose–response assays were performed to measure test compound competition with the high-affinity fluorescent ligand WKYMVM-FITC for binding to human FPR1 in RBL transfected cells, as described previously [33]. Briefly, FPR1-RBL cells were preincubated with

different concentrations of test compound for 30 min at 4°C, followed by addition of 0.5 nM WKYMVm-FITC. After incubation for an additional 30 min at 4°C, the samples were immediately analyzed using flow cytometry (LSRII, BD Biosciences, San Jose, CA, USA) without washing. The assay response range was defined by replicate control samples containing 1 μ M of unlabeled α MLF (positive control) or buffer (negative control). In an individual dose–response experiment, each compound was tested in duplicate, resulting in 9 data points. The ligand competition curves were fitted by Prism software using nonlinear least-squares regression in a sigmoidal dose–response model to determine the concentration of added test compound that inhibited fluorescent ligand binding by 50% (i.e., IC₅₀). In equilibrium binding experiments with the labeled ligand, the K_d values for FPR1 in RBL-FPR1 cells were found to be ~0.5 and 0.4 nM, respectively. K_i values were calculated from IC₅₀, as reported previously [33].

2.6. Neutrophil adhesion assay

Neutrophil adhesion to confluent T84 epithelial cells was evaluated as described previously [40] with modifications. Briefly, T84 epithelial monolayers were seeded at a density of 2×10^5 T84 cells/well in 96-well plates and grown overnight. Human neutrophils were labeled for 30 min at 37 °C with BCECF-AM (5 μ M final concentration) and washed three times with HBSS⁻. The BCECF-labeled neutrophils were then added to the epithelial monolayers (10⁵ cells/monolayer), followed by the addition of test compounds at the indicated concentrations or 1% DMSO (vehicle control). After a 10-min pre-incubation, 10 nM α MLF was added, the plates were centrifuged at 150 g for 4 min to uniformly settle the neutrophils and synchronize the process, and adhesion was allowed for 10 min at 37 °C. The monolayers were gently washed three times with HBSS⁻, and fluorescence intensity (excitation, 485 nm; emission, 530 nm) was measured on a Fluoroscanner Ascent FL microplate reader. Adherent cell numbers were determined from standard curves generated by serial dilutions of known neutrophil numbers.

2.7. ERK phosphorylation assay

Phosphorylation of p44/42 mitogen-activated protein kinases (ERK1/2) was determined based on activation-associated phosphorylation. Both neutrophil-like HL60 cells and FPR1-transfected RBL cells were used. Cells cultured in six-well plates were serum starved for 4 h before stimulation. Samples were treated with the indicated concentrations of the compounds under investigation or 0.5% DMSO (vehicle) for 10 min and then stimulated with α MLF (20 nM) for 5 min. The reactions were terminated by adding 150 μ l of ice-cold SDS-PAGE loading buffer. Samples were analyzed by SDS-PAGE and Western blotting using rabbit anti-ERK1/2 (9102) and rabbit anti-ERK1/2 phosphorylated at Thr202 and Tyr204 (9101) (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA). Horseradish peroxidase-conjugated AffiniPure goat anti-rabbit IgG (1:3000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was used as secondary antibody. The immunoblots were visualized using a SuperSignal West Pico Chemiluminescence kit (ThermoFisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. The levels of phosphorylated ERK1/2 were determined by densitometry using NIH ImageJ software (NIH, Bethesda, MD) and normalized against total ERK1/2 on the same blot (n=3). The results are expressed as relative phosphorylation of ERK1/2. For determination of

ERK1/2 phosphorylation in human neutrophils, the cells were incubated for 10 min with the selected compounds or negative control (1% DMSO) at 37 °C, followed by addition of 10 nM AMLF. The cells were lysed by adding lysis buffer (R&D Systems), and the levels of phosphorylated ERK1/2 were measured in the cell lysates using an ELISA kit for human phospho-ERK1 (Thr202/Tyr204)/ERK2 (Thr185/Tyr187) (R&D Systems). The concentrations of phospho-ERK1/2 in the cell lysates were determined using a SuperSignal West Pico Chemiluminescence kit.

2.8. Assessment of compound cytotoxicity

Cytotoxicity was analyzed with a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. Briefly, HL60 cells (wild-type) were cultured at a density of 1×10^5 cells/well with different concentrations of compound under investigation for 18 h at 37 °C and 5% CO₂. Following treatment, the cells were allowed to equilibrate to room temperature for 30 min, substrate was added, and the samples were analyzed with a Fluoroscan Ascent FL microplate reader.

2.9. Molecular modeling

For pharmacophore modeling, we used a ligand-based approach for molecular modeling known as field point methodology, as described previously [31]. The structures of compounds were preoptimized by the semiempirical PM3 method using HyperChem 8.0 software and saved in Tripos MOL2 format. The structures were then imported into the FieldAlign program (FieldAlign Version 2.0.1; Cresset Biomolecular Discovery Ltd., Hertfordshire, UK). We used *S*-enantiomers of compounds for modeling, as preliminary studies demonstrated that FPR1 antagonists **1**, **2**, **15**, and **17** in their *S*-configurations exhibited a noticeably higher degree of similarity to the pharmacophore template than the corresponding *R*-enantiomers. The conformation hunter algorithm incorporated in FieldAlign was used to generate representative sets of conformations corresponding to local minima of energy calculated within the extended electron distribution force field [41, 42]. Up to 200 conformations with specific field point patterns were obtained for each molecule. For the generation of field point patterns, probe atoms having positive, negative, and zero charge were placed in the vicinity of a given conformation, and the energy of their interaction with the molecular field was calculated using the extended electron distribution parameter set. Positions of energy extrema for positive probes give “negative” field points, whereas energy extrema for negative and neutral probe atoms correspond to “positive” and steric field points, respectively. Hydrophobic field points were also generated with neutral probes capable of penetrating into the molecular core and reaching extrema in the centers of hydrophobic regions (e.g., benzene rings). The size of a field point depends on magnitude of an extremum. The field points are colored according to the following convention: blue, electron-rich (negative field, i.e. obtained with a positive probe); red, electron-deficient (positive field, i.e. obtained with a negative probe); yellow, van der Waals attractive (steric); and orange, hydrophobic. A detailed description of the field point calculation procedure has been published elsewhere [43].

A pharmacophore template based on known active FPR1 antagonists was also imported into the FieldAlign program. The template was created previously as a superimposition of the

bioactive conformations of three potent FPR1 antagonists: 7-acetoxy-6-ethyl-2-methyl-3-(1-methylbenzimidazol-2-yl)-4-oxo-chromene, (*S*)-*N*-(1-(benzimidazol-2-yl)-3-(methylthio)propyl)-5-ethoxy-3-methylbenzofuran-2-carboxamide, and 6-benzyl-3-(2-chlorophenyl)-5-methyl-2-(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-7(1*H*)-one [31]. The molecules under investigation were then superimposed onto the template, taking into account mutual correspondence of field points. Superimpositions with the best similarity scores were collected. Statistical analysis of the similarity scores was performed with the use of ANOVA methodology as implemented in STATISTICA 8.0 software.

The FPR1 homology model was created using the crystal structure of bovine rhodopsin, as reported previously [44] and imported into Molegro Virtual Docking (MVD) software (MVD 2010.4.2, MolegroApS) together with the structures of modeled compounds, and docking computations were performed using a spherical search space, as reported previously [45].

3. Results

3.1. SAR analysis of pyrrole-based FPR1 antagonists

Based on structures of three published FPR1 antagonists **1–3** with a 4-benzoyl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-one scaffold [32, 33] (Figure 1), 42 additional analogs were selected and evaluated for FPR1 antagonist activity in FPR1-HL60 cells by monitoring effects on MLF-induced Ca²⁺ mobilization. As result of this secondary screening, 18 novel FPR1 antagonists (45.2%) were identified with the most potent compounds being **4** and **17** (Table 1), supporting the significance of this scaffold for FPR1 antagonist activity.

Analysis of derivatives with an unsubstituted R₁ showed that replacement of the terminal methyl group at R₄ with a methoxy group (compare **4** and **17**) did not change FPR1 antagonist activity. However, more bulky substitution with morpholine (**41**) decreased activity, and subsequent replacement of the terminal methyl group with dimethylamino, dimethylaminomethyl, or diethylamino groups (compounds **29**, **33**, and **39**, respectively) resulted in a complete loss of FPR1 antagonist activity. Similarly, in the series of derivatives with a methyl group at R₁, compounds containing normal or branched alkyl groups (**2** and **15**) or a methoxy group (**18**) at R₄ were highly active FPR1 antagonists. Derivatives with nitrogen-containing R₄ groups, such as *N*-morpholino (**42**) or dimethylamino alkyl groups (**30** and **34**), were completely inactive. If the alkoxy chain at R₄ was elongated to three carbons, activity was increased 2- fold (compare **20** and **27**). Although compound **41** containing a morpholine group at R₄ was active, additional introduction of a methyl group at position R₁ or methoxy group at R₂ was associated with complete loss of activity (compounds **42** and **43**, respectively). All other compounds with 3-(*N*-morpholino)-1-propyl (**40** and **44**), 2-(dimethylamino)ethyl (**28**, **31**, **32**) and 3-(dimethylamino)-1-propyl (**35** and **36**) groups were also inactive. Thus, although a long aliphatic chain at R₄ (up to 6 carbon atoms; e.g. compare compounds **14** and **15**) can be beneficial, nitrogen-containing substituents at this position are generally detrimental, perhaps, due to higher basicity of these groups.

No clear SAR emerged from modification of other positions of the molecules. However, moving CH₃ group from R₂ to R₃ did not affect activity (compare **20** and **21**). Introduction of a methoxy group led to complete loss of activity (compare **41** and **43**). Note that all three reference compounds (**1–3**) that were previously discovered have methoxy groups at R₅ and R₇, suggesting the importance of these groups for FPR1 antagonist activity. Although we did not explore deeply substitutions at these positions, elimination of one methoxy group at R₇ decreased activity 2-fold (compare **20** and **24**). Similarly, elimination of the methoxy group at R₇ or moving this group to R₆ had a detrimental effect as well (compare compound pairs **18/19** and **16/17**). However, elimination of the methoxy group at position R₆ did not affect activity (compare **22** and **26**). Similarly, replacing the methyl group at R₆ with ethyl groups had no effect on FPR1 antagonist activity (compounds **23** and **25**, respectively).

3.2. Competition binding of pyrrole-based FPR1 antagonists

All active compounds were analyzed for their ability to compete with WKYMVm-FITC for binding to FPR1, as described previously [31], and values of the calculated binding constants (K_i) are presented in Table 1. As an example, a representative dose–response curve of competitive inhibition of WKYMVm-FITC binding by compound **4** in FPR1-RBL cells is shown in Figure 2. Compounds **4**, **15**, **17**, **18**, and **27** had the highest binding affinities ($K_i < 2.0 \mu\text{M}$) among all 1*H*-pyrrol-2(5*H*)-ones evaluated, which are comparable to the K_i of previously reported 1*H*-pyrrol-2(5*H*)-one FPR1 antagonists **1** and **2** [32, 33].

3.3. Selectivity of pyrrole-based FPR1 antagonists

Eight of the most potent FPR1 antagonists with $\text{IC}_{50} < 10 \mu\text{M}$ were evaluated for their ability to activate and inhibit Ca²⁺ mobilization in FPR2 and FPR3-transfected HL60 cells, as well as in primary human neutrophils stimulated by the FPR2-specific agonist WKYMVM or the CXCR1/2 agonist IL-8. Importantly, all of these competitive antagonists did not directly activate Ca²⁺ flux in any of the FPR-transfected cell lines or neutrophils, demonstrating that they are indeed competitive receptor antagonists and not just desensitizing the cells.

To evaluate FPR selectivity of the antagonists, FPR2-HL60 and FPR3-HL60 cells were pretreated with the selected compounds for 30 min, followed by stimulation with WKYMVm (FPR2-HL60) or WKYMVM (FPR3-HL60 cells). All but one of the selected FPR1 antagonists did not inhibit FPR2- or FPR3-dependent Ca²⁺ flux, demonstrating specificity for FPR1. Likewise, these compounds inhibited the FPR1-dependent neutrophil response induced by ΔMLF but did not inhibit FPR2-dependent neutrophil responses induced by WKYMVM (Table 2). As examples, representative dose–response curves showing the effects of compounds **14** and **17**, which were the most potent antagonists of ΔMLF -induced Ca²⁺ mobilization in FPR1-HL60 cells ($\text{IC}_{50} = 3.0$ and $1.7 \mu\text{M}$, respectively, see Table 1), on Ca²⁺ flux in ΔMLF and WKYMVM-stimulated neutrophils are shown in Figure 3A. The one exception was compound **15**, which inhibited Ca²⁺ flux in FPR2-HL60 cells and IL-8-stimulated human neutrophils (Table 2). The reason for the broad inhibitory activity of this compound is not clear; however, its lack of specificity indicates it would not be useful to target FPR1.

Activation of neutrophils by α MLF induces various neutrophil function responses, including chemotaxis and adhesion [46]. Thus, to further investigate the effects of the FPR1 antagonists on these human neutrophil functions. We first evaluated the effects of these compounds on neutrophil chemotaxis. All compounds dose-dependently inhibited α MLF-induced neutrophil migration, with IC_{50} values of 2.2 to 36.3 μ M (Table 2). As an example, representative dose–response curves for the inhibition of α MLF-induced neutrophil chemotaxis by compounds **14** and **17** are shown in Figure 3B. We next evaluated the ability of selected FPR1 antagonists to inhibit neutrophil adhesion to epithelial cells. As with previous studies [47], α MLF stimulated neutrophil adhesion in a concentration–dependent fashion with a EC_{50} value of $\sim$ 7.5 nM. Consistent with the chemotaxis results, α MLF-induced neutrophil adhesion was significantly inhibited by most of the selected pyrrole-based FPR1 antagonists, with IC_{50} values of 1.1 to 25.8 μ M (Table 2), and representative dose–response curves for the inhibition of α MLF-induced neutrophil adhesion by compounds **14** and **17** are shown in Figure 4. Note, however, that the compounds with the lowest binding activity (compounds **6** and **16**) were inactive in this assay. Interestingly, compound **15** actually enhanced α MLF-induced neutrophil adhesion to epithelial cells. Likewise, this compound was the only pyrrole that inhibited Ca^{2+} mobilization activated via FPR2 and CXCR1/2 (Table 2). Together, these results support our conclusion that compound **15** is not specific for FPR1.

All FPR1 antagonists that blocked agonist-induced Ca^{2+} flux, with the exception of **15**, also inhibited chemotaxis and/or adhesion in a dose-dependent manner. Note, however, that the levels of inhibition (i.e., IC_{50} values) of our antagonists in the Ca^{2+} flux assay did not always correlate with the respective IC_{50} values measured in the chemotaxis and adhesion assays. For example, plotting of the IC_{50} values of Ca^{2+} flux inhibition versus chemotaxis inhibition resulted in a correlation coefficient of 0.72. Interestingly, the correlation between these activities for chromone-based FPR1 antagonists was also in this range ($r=0.73$, $n=13$) [31]. The reason for the low correlation between these activities for the FPR1 antagonists is currently not understood. One likely contributor to lack of correlation between Ca^{2+} flux and downstream function responses in studying receptor pharmacology is the times involved in these assays, where Ca^{2+} flux response occurs very rapidly after agonist addition (<1 min) while the functional adhesion and chemotaxis assays involve much longer times of incubation with the agonist (10 and 60 min, respectively). Charlton and Vauquelin [48] proposed that the K_d value of an antagonist is influenced by its dissociation rate, the agonist's intrinsic efficacy, and the cellular amplification of the signal and that these various influences become more extreme when comparing Ca^{2+} assays to functional assays requiring much longer times [48]. In fact, this can result in assay-related antagonist potency inversions, which have been seen for other receptors [48] and may explain the low correlation of EC_{50} values observed for some of our compounds in the different assays (e.g., see compound **6**). In any case, further kinetic studies on this issue are clearly necessary.

Activation by α MLF leads to phosphorylation of ERK1/2 in human neutrophils and FPR1-transfected cells [49]. Thus, we evaluated whether the selected FPR1 antagonists inhibited phosphorylation of ERK1/2 in DMSO-differentiated, neutrophil-like HL60 cells and primary human neutrophils. Pretreatment with the potent FPR1 antagonists **14** and **17** effectively

inhibited α MLF-induced phosphorylation of ERK1/2 (Figure 5A and Figure 6). Since differentiated HL60 and neutrophils express both FPR1 and FPR2, we next examined FPR1-transfected RBL (FPR1-RBL) cells. Consistent with our results in differentiated HL60 cells and neutrophils, pretreatment with FPR1 antagonists **14** and **17** inhibited α MLF-induced ERK1/2 phosphorylation in FPR1-HL60 cells with similar potency (Figure 5B). Importantly, untransfected RBL cells did not respond to α MLF, and no ERK1/2 phosphorylation was observed (data not shown).

To ensure that the results were not influenced by possible compound toxicity, cytotoxicity of the most potent FPR1 antagonists was evaluated at various concentrations up to 50 μ M in wild-type HL60 cells. None of the active FPR1 antagonists affected cell viability at the highest tested concentrations (data not shown), thereby verifying that these compounds were not cytotoxic, at least during the 18-h incubation period.

3.4. Molecular modeling of FPR1 antagonists

For molecular modeling, we developed a pharmacophore model of FPR1 antagonists, which was based on the structure of previously reported potent FPR1 antagonists with diverse chemical scaffolds [31]. As designated previously [31], this pharmacophore template has three hydrophobic sites (*Protrusion*, *Area I*, and *Area II*), one compact grouping of H-bond donors or positively charged centers (*Area III*), and one region of H-bond acceptors or negatively charged centers (*Area IV*) (Figure 7A). Indeed, overlay of a recently reported FPR1 antagonist, HCH6-1 [(*R*)-methyl 2-((*S*)-2-benzamido-3-(1*H*-indol-3-yl)propanamido)-3-phenylpropanoate] [21, 22], on the template shows a high level of similarity. Importantly, the *Protrusion* area is occupied by the indole fragment and a large “blue” field point is located in *Area III* (Figure 7A), verifying that these two structural features are important for FPR1 antagonist activity and confirming that this pharmacophore template is applicable to FPR1 antagonists of different chemical classes.

A visual inspection of the molecule overlays on the FPR1 template revealed that pyrrole-based FPR1 antagonists occupy three hydrophobic pockets (*Area I*, *Area II*, and the *Protrusion*) with their R_4 , phenyl, and aroyl (benzoyl/pyridine) moieties in different combinations (Table 3). As an example, Figure 7B shows that the potent FPR1 antagonist **14** superimposes quite well with the template. Thus, we explored optimal alignments of the most potent antagonists and several inactive pyrroles onto the pharmacophore template of FPR1 antagonists using the FieldAlign program. The alignments with higher similarity scores were analyzed in terms of molecular moiety coincidence with key regions in the pharmacophore model described above. The values of similarity scores obtained for the active and inactive pyrroles are presented in Table 3. According to Field Point methodology [42], the template reflects the main geometrical and electronic features of a receptor binding site. Hence active and inactive compounds should differ in their level of similarity, and a statistical analysis of similarity scores using ANOVA was performed in order to estimate the difference between the antagonists and inactive compounds in terms of the score values. Notably, a statistically significant difference ($P < 0.05$) between groups of active and inactive compounds with respect to their similarity scores was found.

The similarity score is an integral quantity. However, structural peculiarities of the alignments can be of greater importance for molecular recognition. Analysis of data in Table 3 shows that most active FPR1 antagonists possess two common features. First, the hydrophobic pocket called the *Protrusion* is occupied by a moiety of the overlaid molecule. Second, an antagonist molecule in its superimposed conformation produces blue field point(s) in *area III* where the analogous field points of the template are located. In other words, an antagonist interacts with some positively charged center in the receptor, e.g. forms a H-bond in the FPR1 binding site. These two simple features give correct classifications for 13 active FPR1 antagonists (81% of all the active compounds investigated). Likewise, 12 of the 16 inactive compounds were also classified correctly, as they lacked at least one of these features. However, for the alignments of molecules **28–30**, the R₄ group in the *Protrusion* area extended outside of the template boundaries (see an example for the compound **30** in Figure 7C). Thus, an unfavorable orientation of the R₄ substituent results in inactivity of these three compounds despite fulfillment of the other two above-mentioned features. Thus, our pharmacophore model [31] can be used to explain differences between 1*H*-pyrrol-2(5*H*)-one-based FPR1 antagonists and their inactive analogs.

In order to map the important features of the template onto the FPR1 binding site, we performed a docking study for three pyrrole-based FPR1 antagonists (**14**, **15**, and **17**) using MVD software. An homology model of the FPR1 ligand-binding region described previously [45] was taken as a target protein for the docking computations. The FPR1 binding site regions are designated according to our previous publication [45], where *cavity B* is restricted by Val160, Leu198, Arg201, Gly202, and Arg205; the *bottom D* of the binding site is associated with Ala261, Ala264, and Val283; and *cavity E* is located near Trp91, Trp95, Cys98, and Lys99 (Figure 8). Consistent with our pharmacophore modelling above, poses of the three compounds overlaid with each other within the ligand binding site, and the molecular moieties of compounds **14**, **15**, and **17** overlapped in the same manner as with the pharmacophore template. For example, substituted phenyl rings directly linked to pyrrole heterocycles are mutually overlaid like within the template where they fall into *area I*. Likewise, the R₄ groups of molecules **14** and **15** and the benzoyl moiety of compound **17** all occupy *area II* of the template. Similar correspondence between the pharmacophore model and docking results also existed for moieties that fell in the *Protrusion* region of the template. These observations confirm applicability of the pharmacophore template to predict correct binding modes of pyrrole derivatives to FPR1.

4. Discussion

FPR1 is a key regulator of the inflammatory environment and may represent unique target for therapeutic drug design. For example, conventional FPR1 agonist *f*MLF is involved in the pathogenesis of multiple inflammatory diseases, such as pouchitis, colitis, ulcerative colitis, Crohn's disease and juvenile periodontitis (reviewed in [1]). Since FPR1 represents a potentially important pharmacological target, significant attention has been focused on the identification of ligands that interact with this receptor and/or interfere with FPR1-dependent pathways. Although many small-molecule compounds have been reported as FPR1 agonists (reviewed in [28, 30]), less attention has focused on synthetic FPR1 antagonists. Previously, several 1*H*-pyrrol-2(5*H*)-one derivatives were identified as small-

molecule competitive FPR1 antagonists [32, 33]. In the current work, we report further characterization and development of related analogs with FPR1 antagonist activity in functional tests using transfected cells and primary neutrophils. Screening of a library of forty two additional 1*H*-pyrrol-2(5*H*)-ones, which are structural derivatives of previously reported competitive FPR1 antagonists **1–3** [32, 33], resulted in the discovery of novel and potent pyrrole-based FPR1 antagonists. We also showed that these compounds can compete with FITC-labeled WKYMVm for binding with FPR1 in FPR1-RBL transfected cells.

Several of the pyrrole-based FPR1 antagonists identified here specifically blocked α MLF-induced responses mediated via FPR1 in FPR1-HL60 cells and human neutrophils, but not responses mediated via FPR2 or FPR3. These compounds also did not inhibit IL-8-induced Ca^{2+} mobilization in human neutrophils. Thus, 4-aryl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-one may represent a unique chemical scaffold for development of specific FPR1 antagonists.

The selected pyrrole derivatives (Table 2) were also inactive as direct agonists of either FPR1 and FPR2 and did not activate Ca^{2+} flux in human neutrophils. Although there are several examples in which compounds derived from similar chemical scaffolds have opposite functional activities (agonist vs. antagonist) at FPR1 and FPR2 (e.g., see [50]), no agonists of human FPR1 with a 1*H*-pyrrol-2(5*H*)-one scaffold have been reported. Thus, similar to 4*H*-chromen-4-one [31], the 1*H*-pyrrol-2(5*H*)-one may represent another chemical scaffold for development of specific FPR1 antagonists that do not have undesirable off-target effects.

Endogenous α MLF and its formylated analogs are released from bacteria and/or mitochondria in dead host cells and could exert a chemotactic function by promoting neutrophil Ca^{2+} mobilization and mitogen-activated protein kinase (MAPK) activation [51]. Here we found that in addition to mobilizing Ca^{2+} from intracellular stores, α MLF activates the MAPKs ERK1/2 [49]. We evaluated this response to test the relative potency of selected pyrrole-based FPR1 antagonists. Compounds **14**, **15**, and **17**, which exhibited high potency in the Ca^{2+} flux assay, also inhibited α MLF-induced ERK phosphorylation. Moreover, eight pyrrole-based FPR1 antagonists decreased α MLF-driven neutrophil chemotaxis and five of these compounds inhibited neutrophil adhesion to epithelial cells *in vitro* (Table 2). Neutrophils play an essential role in proper resolution of inflammation, and when these processes are not tightly regulated, neutrophils can trigger positive feedback amplification loops that promote neutrophil chemotaxis, adherence to endothelial cells, and activation, leading to significant tissue damage and evolution toward chronic disease [52]. Recently, Honda et al. [53] demonstrated that FPR1 blockade by cyclosporine H attenuated hepatic ischemia-reperfusion injury by inhibition of neutrophil chemotaxis. Thus, the pyrrole-based FPR1 antagonists reported here and their analogs may offer pharmacological means to treat acute and chronic inflammation by reducing FPR1-dependent neutrophil chemotaxis and adherence to epithelial cells. However, these FPR1 antagonists are active in the micromolar range and further work will be necessary to increase a potency of these inhibitors by scaffold optimization and evaluation of their effectiveness in inflammatory models *in vivo*.

SAR analysis and computer-aided design can be beneficial in the identification of new molecules based on the features of known structures. Comprehensive SAR analysis of all 45 pyrrole analogs suggests that the nature of the group at position R₄ has a major impact on FPR1 antagonist activity. Subsequent molecular modeling also indicated that the presence of a hydrophobic moiety in the *Protrusion* region of the FPR1-antagonist pharmacophore template [31] and interaction of a ligand with a positively charged group or H-bond donors in the FPR1 binding site were critical for antagonist activity. Our molecular docking results for 1*H*-pyrrol-2(5*H*)-ones agreed with the spatial arrangement of the field point pharmacophore template, which was developed based on the structures of previously reported potent FPR1 antagonists with diverse chemical scaffolds [31]. Notably, the docking poses and overlays on the pharmacophore template for chromone-based FPR1 antagonists investigated previously [31] are very similar to the present results. However, some substituted chromones possessed molecules longer in size than the pyrrole derivatives studied here, and substituents in these chromone-based FPR1 antagonists can also occupy *channel C* of the FPR1 binding site [31]. This detail of the binding mode can be considered less important, taking into account that pyrrole-based FPR1 antagonists occupy mainly *bottom D*, *cavity B*, and *cavity E* (see Figure 6B). Importantly, the degree of similarity of the pyrrole FPR1 antagonists to the pharmacophore model is significantly higher as compared to the inactive 1*H*-pyrrol-2(5*H*)-ones.

3-Hydroxy-1*H*-pyrrol-2(5*H*)-one could serve as a functional building block for the construction of promising molecules with diverse bioactivities. For example, 5-aryl-4-benzoyl-3-hydroxy-1-(2-arylethyl)-2*H*-pyrrol-2-ones have been identified as competitive and specific vasopressin-2 receptor (V2R) antagonists [54]. Certain 4-aryl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-one derivatives showed specificity for targeting trypanothione synthetase in *Leishmania infantum* [55]. Previously, a series of 4-aryl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-ones were reported as inhibitors of the annexin A2–S100A10 protein interaction [34]. In the present study, we also evaluated seven relatively potent inhibitors of the annexin A2–S100A10 protein interaction (compounds **7–13**) [34], but these compounds did not exhibit any FPR1 antagonist activity. However, further studies using pyrrole-based FPR1 antagonists compounds will be necessary to evaluate their inhibitory effect on annexin A2–S100A10 protein interaction using published [34] methodology.

In conclusion, we have identified a number of specific, competitive FPR1 antagonists with 1*H*-pyrrol-2(5*H*)-one scaffold. Several of these pyrrole derivatives could represent important leads for therapeutic development focused on FPR1 function and attenuation of neutrophil-mediated inflammatory diseases. These compounds can also serve as scaffolds for the development of additional potent and selective FPR1 antagonists. Furthermore, characterization of this class of antagonists and analysis of additional derivatives should provide important clues for understanding the molecular structural requirements of FPR1 antagonists.

Acknowledgments

This research was supported in part by National Institutes of Health IDeA Program COBRE Grant GM110732; the Ministry of Education and Science of the Russian Federation (project No. 4.8192.2017/8.9); the Science and Technology Development Fund of Macau (FDCT 072/2015/A2); USDA National Institute of Food and Agriculture

Hatch project 1009546; Montana University System Research Initiative: 51040-MUSRI2015-03; and the Montana State University Agricultural Experiment Station. Molecular modeling was supported by the Tomsk Polytechnic University Competitiveness Enhancement Program grant.

Abbreviations

BCECF	2',7'-Bis-(carboxyethyl)-5(6)-carboxyfluorescein
CXCR	chemokine (C-X-C motif) receptor
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
ERK1/2	p44/42 mitogen-activated protein kinases 1 and 2
FBS	fetal bovine serum
fMLF	N-formyl-methionyl-leucyl-phenylalanine
FPR	formyl peptide receptor
FITC	fluorescein isothiocyanate
IL-8	interleukin 8
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HBSS⁻	Hanks' balanced salt solution without Ca ²⁺ and Mg ²⁺
HBSS⁺	Hanks' balanced salt solution containing 10 mM HEPES and Ca ²⁺ and Mg ²⁺
WKYMVm	Trp-Lys-Tyr-Met-Val-D-Met
SAR	structure–activity relationship
WKYMVm	Trp-Lys-Tyr-Met-Val-L-Met-NH ₂
WKYMVM	Trp-Lys-Tyr-Met-Val-D-Met-NH ₂

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Figure 1. Previously reported FPR1 antagonists **1–3** with a 4-benzoyl-3-hydroxy-5-phenyl-1 *H*-pyrrol-2(5*H*)-one scaffold.

Figure 2.

Pyrrole-based FPR1 antagonists compete with WKYMVm-FITC for binding to FPR1. FPR1-RBL cells were incubated with the indicated concentrations of compound **4**, followed by addition of WKYMVm-FITC, and bound FITC was analyzed by flow cytometry, as described under *Materials and Methods*. The data are from one experiment with a single replicate for each concentration and are representative of three independent experiments.

Figure 3.

Inhibition of Ca^{2+} mobilization by pyrrole-based FPR1 antagonists. **Panel A:** Human neutrophils were preincubated with the indicated concentrations of compounds **14** and **17** for 30 min at 25 °C and then stimulated with 5 nM Δ MLF (○, compound **14**; □, compound **17**) or 10 nM WKYMVM (●, compound **14**; ■, compound **17**). The responses induced by peptide agonist alone were assigned a value of 100%. **Panel B:** Human neutrophils were preincubated with the indicated concentrations of the compounds **14** (○) and **17** (□) or DMSO for 30 min at room temperature, and chemotaxis toward 1 nM Δ MLF was measured, as described under *Materials and Methods*. The results are expressed as the number of

migrated cells per well. In the panels **A** and **B**, the data are from one experiment with a single replicate for each concentration and are representative of three independent experiments.

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Figure 4.

Inhibition of neutrophil adhesion to epithelial cells by pyrrole-based FPR1 antagonists. T84 epithelial cells were preincubated with BCECF-labeled neutrophils and the indicated concentrations of the compounds **14** (○) and **17** (■) or DMSO for 10 min at room temperature, and neutrophil adhesion induced by 10 nM α MLF was measured, as described under *Materials and Methods*. The results are expressed as the number of adherent neutrophils per well. For reference, the control level of α MLF-activated neutrophil adhesion to the epithelial monolayer (pretreated with 1% DMSO) was $\sim 3.3 \times 10^4$ neutrophils/well.

The data are from one experiment with triplicate samples for each concentration and are representative of three independent experiments.

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Figure 5.

Effect of the pyrrole-based FPR1 antagonists on ERK1/2 phosphorylation. Serum-starved HL60 cells differentiated into neutrophil-like cells (**Panel A**) and FPR1-RBL cells (**Panel B**) were incubated for 10 min with various concentrations of the indicated antagonists followed by treatment for 5 min with fMLF, and cell lysates were analyzed by Western blotting for phospho-ERK1/2 (upper panel) and total ERK1/2 (lower panel). Control: cells treated with vehicle (DMSO at same concentration as in samples). The images in panels A and B were cut from single blots and joined (see stitch lines). The relative levels of ERK1/2 phosphorylation determined by densitometry (Mean $\pm$ SEM, based on 3 independent

experiments) are shown below the immunoblots. *, $p < 0.05$ and **, $p < 0.01$, compared with untreated samples (0 μM) in each group.

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Figure 6.

Effect of potent FPR1 antagonists on ERK1/2 phosphorylation in human neutrophils. Human neutrophils were incubated for 10 min with the indicated concentration of FPR1 antagonists followed by treatment for 5 min with 10 nM *f*MLF, and the cell lysates were analyzed by ELISA for phospho-ERK1/2. Negative control samples were treated with 1% DMSO. The data are from one experiment with triplicate samples for each concentration and are representative of three independent experiments. **, $p < 0.01$ compared with untreated samples (0 μM) in each group.

Figure 7.

Superimposition of active and inactive pyrroles on a multi-molecule pharmacophore template for FPR1 antagonists. **Panel A.** FPR1 antagonist HCH6-1 (grey bold backbone, spherical field points) superimposed onto the FPR1 antagonist pharmacophore template. **Panel B.** Pyrrole-based FPR1 antagonist **14** (grey bold backbone, spherical field points) superimposed onto the FPR1 antagonist pharmacophore template. **Panel C.** Inactive pyrrole **30** superimposed onto the FPR1 antagonist pharmacophore template. Arrow indicates a dimethylamino ethyl group of the molecule in the area of the *Protrusion*, but which extends outside of the template boundaries. In the panels **A-C**, field points are colored as follows:

blue = located near electron-rich groups (produced by a positive probe atom); red = located near electron-deficient groups (produced by a negative probe atom); yellow = van der Waals attractive (steric); and orange = hydrophobic. Molecules of the template are shown with thin backbones and icosahedral field points.

Figure 8.

Docking of pyrrole-based FPR1 antagonists in the FPR1 binding site. **Panel A.** Docking poses of pyrroles **14** (magenta), **15** (light-blue), and **17** (yellow). Hydrogen atoms are not shown. Residues within 10 Å from the center of the spherical search space are visible. **Panel B.** Docking poses of pyrroles **14** (magenta) and **17** (yellow). Arrows indicate: curved **cavity B** located behind the blue-colored ledge; **bottom D** of the binding site; and large **cavity E**, located between **channel C** and larger blue colored ledge. See [45] for further details.

Surface coloring was made according to electrostatic properties, whereby negatively and positively charged areas are shown in red and blue, respectively.

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Table 1FPR1 antagonist activity of 1*H*-pyrrol-2-(5*H*)-ones

Compd.	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	Ca ²⁺ flux IC ₅₀ (μM)	Binding K _i (μM)
1	H	H	H	(CH ₂) ₄ CH ₃	OCH ₃	H	OCH ₃	-	0.55
2	CH ₃	H	H	CH ₂ CH(CH ₃) ₂	OCH ₃	H	OCH ₃	-	4.0
3	F	H	H	(CH ₂) ₅ COOH	OCH ₃	H	OCH ₃	-	12.0
4	H	H	H	(CH ₂) ₂ CH ₃	OCH ₃	H	OCH ₃	1.5	1.0
5	OCH ₃	H	H	(CH ₂) ₃ CH ₃	H	CH ₃	H	3.8	9.2
6	CH ₃	H	H	CH ₂ CHCH ₃ OH	OCH ₃	H	H	8.3	10.4
7	CH ₃	H	H	CH ₂ CHCH ₃ OH	H	iPr	H	N.A.	N.D.
8	CH ₃	H	H	CH ₂ CHCH ₃ OH	H	H	H	N.A.	N.D.
9	CH ₃	H	H	CH ₂ CHCH ₃ OH	H	CH ₃	H	N.A.	N.D.
10	CH ₃	H	H	CH ₂ CHCH ₃ OH	H	CH ₂ CH ₃	H	N.A.	N.D.
11	CH ₃	H	H	CH ₂ CH ₂ OCH ₃	H	tBu	H	N.A.	N.D.
12	CH ₃	H	H	CH ₂ CH ₂ OCH ₃	H	N(CH ₃) ₂	H	N.A.	N.D.
13	CH ₃	H	H	CH ₂ CH ₂ OCH ₃	H	Cl	H	N.A.	N.D.
14	H	H	H	(CH ₂) ₅ CH ₃	H	OH	OCH ₃	3.0	3.0
15	CH ₃	H	H	(CH ₂) ₅ CH ₃	OCH ₃	OCH ₃	H	3.7	1.9
16	H	H	H	(CH ₂) ₂ OCH ₃	OCH ₃	H	H	4.6	7.8
17	H	H	H	(CH ₂) ₂ OCH ₃	OCH ₃	H	OCH ₃	1.7	1.4
18	CH ₃	H	H	(CH ₂) ₂ OCH ₃	OCH ₃	H	OCH ₃	5.3	1.6
19	CH ₃	H	H	CH ₂) ₂ OCH ₃	OCH ₃	OCH ₃	H	10.5	4.1

	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	Ca ²⁺ -flux IC ₅₀ (μM)	Binding K _i (μM)
1-37, 39-44									
38									
45									
42				(CH ₂) ₃ N-Morph	OCH ₃	H	OCH ₃	N.A.	N.A.
				(CH ₂) ₃ N-Morph	OCH ₃	H	OCH ₃	N.A.	N.A.
				(CH ₂) ₃ N-Morph	OCH ₃	H	H	N.A.	N.A.
				(CH ₂) ₃ N-Morph	OCH ₃	H	H	N.A.	N.D.

Antagonist activity in the Ca^{2+} flux assay was evaluated after 30 min pretreatment of FPRL1-HL60 cells with test compounds, followed by addition of 5 nM ΔMLF (EC50 for ΔMLF in FPRL1-HL60 cells was 3 nM). Effect of the compounds in the competition binding assay was evaluated after 30 min pretreatment of FPRL1-RBL cells with test compounds, followed by addition of 0.5 nM WKYMVm-FITC. N.A.: No activity or binding was observed at the highest tested concentration (50 μM) in the Ca^{2+} flux or competition binding assays, respectively. N.D.: competition binding was not performed. Ph Phenyl group; Ar: Aroyl group; Morph: morpholine group.

Table 2

Antagonist activity and selectivity of pyrrole-based FPR1 antagonists

Compd.	Ca ²⁺ flux					Chemotaxis	Adhesion
	Transfected cells		PMN (+ fMLF)	PMN (+ WKYMVM)	PMN (+ IL-8)		
		FPR2-HL60	FPR3-HL60	IC ₅₀ (μM)			
4	N.A.	N.A.	10.7 ± 2.5	N.A.	N.A.	6.3 ± 2.2	2.9 ± 1.1
5	N.A.	N.A.	26.7 ± 4.6	N.A.	N.A.	21.3 ± 7.6	25.8 ± 3.2
6	N.A.	N.A.	12.4 ± 3.2	N.A.	N.A.	36.3 ± 4.0	N.A.
14	N.A.	N.A.	4.8 ± 0.5	N.A.	N.A.	4.2 ± 1.3	1.6 ± 0.6
15	6.5 ± 0.9	N.A.	3.8 ± 1.8	1.7 ± 0.1	6.2 ± 2.1	12.8 ± 4.8	—*
16	N.A.	N.A.	30.3 ± 6.7	N.A.	N.A.	16.9 ± 4.3	N.A.
17	N.A.	N.A.	6.7 ± 0.2	N.A.	N.A.	2.2 ± 1.2	1.1 ± 0.2
18	N.A.	N.A.	5.2 ± 1.4	N.A.	N.A.	12.4 ± 4.2	13.3 ± 2.7

Antagonist activity was evaluated as inhibition of Ca²⁺ mobilization induced by 5 nM WKYMVM in FPR2-HL60 cells (EC₅₀ for WKYMVM in FPR2-HL60 cells was 0.5 nM), 10 nM WKYMVM in FPR3-HL60 cells (EC₅₀ for WKYMVM in FPR1-HL60 cells was 5 nM), 5 nM /MLF in human neutrophils (PMN; EC₅₀ for /MLF in PMNs was 5 nM), or 25 nM IL-8 (EC₅₀ for IL-8 in PMNs was 2.5 nM). Inhibition of PMN chemotaxis was evaluated in the presence of 1 nM /MLF (EC₅₀ for /MLF-induced PMN chemotaxis was 0.5 nM). Inhibition of PMN adhesion to epithelial cells was evaluated in the presence of 10 nM /MLF (EC₅₀ for /MLF-induced adhesion was 7.5 nM). N.A.: No activity was observed at the highest concentration tested (50 μM)

* Compound **15** enhanced /MLF-induced neutrophil adhesion. Chemical names for the selected compounds are: compound **4** (4-benzoyl-5-(2,5-dimethoxyphenyl)-3-hydroxy-1-propyl-2,5-dihydro-1H-pyrrol-2-one); compound **5** (1-butyl-3-hydroxy-4-(4-methoxybenzoyl)-5-(4-methylphenyl)-2,5-dihydro-1H-pyrrol-2-one); compound **6** (3-hydroxy-1-(2-methoxypropyl)-5-(2-methoxyphenyl)-4-(4-methylbenzoyl)-2,5-dihydro-1H-pyrrol-2-one); compound **14** (4-benzoyl-1-hexyl-3-hydroxy-5-(4-hydroxy-3-methoxyphenyl)-2,5-dihydro-1H-pyrrol-2-one); compound **15** (5-(2,4-dimethoxyphenyl)-1-hexyl-3-hydroxy-4-(4-methylbenzoyl)-2,5-dihydro-1H-pyrrol-2-one); compound **16** (4-benzoyl-3-hydroxy-1-(2-methoxyethyl)-5-(2-methoxyphenyl)-2,5-dihydro-1H-pyrrol-2-one); compound **17** (4-benzoyl-5-(2,5-dimethoxyphenyl)-3-hydroxy-1-(2-methoxyethyl)-2,5-dihydro-1H-pyrrol-2-one); compound **18** (5-(2,5-dimethoxyphenyl)-3-hydroxy-1-(2-methoxyethyl)-4-(4-methylbenzoyl)-2,5-dihydro-1H-pyrrol-2-one).

Table 3
Superimposition of selected 1*H*-pyrrol-2(5*H*)-ones onto the template of FPR1 antagonists using the FieldAlign program

Compd.	Activity class	Molecular fragment superimposed onto:			Matching field points in:		Similarity score
		Area I	Area II	Protrusion	Area III	Area IV	
1	Active	Ph	R ₄	Ar	No	No	0.514
2	Active	Ph	Ar	R ₄	Yes	No	0.533
4	Active	Ph	Ar	R ₄	Yes	No	0.509
5	Active	Ph	Ar	R ₄	Yes	Yes	0.572
R-6	Active	Ar	R ₄	Ph	Yes	Yes	0.527
S-6	Active	Ph	Ar	R ₄	Yes	Yes	0.540
14	Active	Ph	R ₄	Ar	Yes	No	0.526
15	Active	Ph	R ₄	Ar	No	No	0.498
16	Active	R ₄	Ar	Ph	Yes	No	0.508
17	Active	Ph	Ar	R ₄	Yes	No	0.502
18	Active	Ph	Ar	R ₄	Yes	No	0.512
19	Active	Ph	Ar	R ₄	Yes	No	0.525
23	Active	R ₄	Ar	Ph	Yes	No	0.524
24	Active	R ₄	Ar	Ph	Yes	No	0.521
26	Active	R ₄	Ar	Ph	Yes	No	0.505
27	Active	R ₄	Ar	-	Yes	Yes	0.499
28	N.A.	Ph	Ar	R ₄	Yes	No	0.504
29	N.A.	Ph	Ar	R ₄	Yes	No	0.518
30	N.A.	Ph	Ar	R ₄	Yes	No	0.525
31	N.A.	R ₄	Ar	Ph	No	No	0.513
32	N.A.	R ₄	Ar	Ph	No	No	0.504
33	N.A.	Ph	R ₄	Ar	No	No	0.516
34	N.A.	R ₄	Ar	-	Yes	Yes	0.501

Compd.	Activity class	Molecular fragment superimposed onto:			Matching field points in:	Similarity score
		Area I	Area II	Protrusion		
35	N.A.	R ₄	Ar	Ph	Yes	0.517
36	N.A.	R ₄	Ar	Ph	No	0.502
37	N.A.	Ph	R ₄	Ar	No	0.515
38	N.A.	Ph	R ₄	Ar	No	0.496
39	N.A.	Ph	R ₄	Ar	No	0.504
40	N.A.	R ₄	Ar	-	Yes	0.496
42	N.A.	Ph	R ₄	Ar	No	0.517
44	N.A.	Ph	R ₄	Ar	No	0.492
45	N.A.	Ph	R ⁴	Ar	No	0.499

Ph Phenyl group; Ar: Aroyl group; R₄: radical R₄ (see Table 1).

“Yes” and “No”: area was occupied or not, respectively by a molecular fragment.

N.A.: No activity was observed at the highest concentration tested in the Ca²⁺ flux assay (50 μM) (see Table 2).

R- or S-configuration refers to an additional stereocenter in R₄ group of molecule 6.