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Antagonism of Human Formyl Peptide Receptor 1 (FPR1) by Chromones and Related Isoflavones

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Abstract

Formyl peptide receptors (FPRs) are G protein-coupled receptors (GPCRs) expressed on a variety of cell types. Because FPRs play an important role in the regulation of inflammatory reactions implicated in disease pathogenesis, FPR antagonists may represent novel therapeutics for modulating innate immunity. Previously, 4*H*-chromones were reported to be potent and competitive FPR1 antagonists. In the present studies, 96 additional chromone analogs, including related synthetic and natural isoflavones were evaluated for FPR1 antagonist activity. We identified a number of novel competitive FPR1 antagonists that inhibited *f*MLF-induced intracellular Ca²⁺ mobilization in FPR1-HL60 cells and effectively competed with WKYMVm-FITC for binding to FPR1 in FPR1-HL60 and FPR1-RBL cells. Compound **10** (6-hexyl-2-methyl-3-(1-methyl-1*H*-benzimidazol-2-yl)-4-oxo-4*H*-chromen-7-yl acetate) was found to be the most potent FPR1-specific antagonist, with binding affinity K_i~100 nM. These chromones inhibited Ca²⁺ flux and chemotaxis in human neutrophils with nanomolar-micromolar IC₅₀ values. In addition, the most potent novel FPR1 antagonists inhibited *f*MLF-induced phosphorylation of extracellular signal-regulated kinases (ERK1/2) in FPR1-RBL cells. These antagonists were specific for FPR1 and did not inhibit WKYMVm/WKYMVm-induced intracellular Ca²⁺ mobilization in FPR2-HL60 cells, FPR3-HL60 cells, RBL cells transfected with murine Fpr1, or interleukin 8-induced Ca²⁺ flux in human neutrophils and RBL cells transfected with CXC chemokine receptor 1 (CXCR1). Moreover, pharmacophore modeling showed that the active chromones had a significantly higher degree of similarity with the pharmacophore template as compared to inactive analogs. Thus, the chromone/isoflavone scaffold represents a relevant backbone for development of novel FPR1 antagonists.

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Keywords

antagonist; formyl peptide receptor; Ca²⁺ mobilization; isoflavone; neutrophil; molecular modeling

1. Introduction

Formyl peptide receptors (FPRs) are G protein-coupled receptors (GPCR) that play important roles as sensors of pathogen- and host-derived products and recruit leukocytes to sites of infection where these cells exert microbicidal effector functions and clear cellular debris [1, 2]. In humans, there are three FPR isoforms: FPR1, FPR2, and FPR3 [3]. These receptors are expressed on a variety of cell types, including macrophages, neutrophils, T lymphocytes, epithelial cells, dendritic cells, fibroblasts, and astrocytes (reviewed in [3, 4]). Being expressed in the majority of white blood cells, FPRs play an important role in the regulation of inflammatory reactions and cellular dysfunction and, thereby, represent an attractive family of pharmacological targets for therapeutic development [4–6].

FPR1 exhibits high affinity for formyl peptides, which are produced by bacteria and can also be released from damaged mitochondria during tissue injury [7, 8]. In addition, FPR1 has recently been reported to contribute to disease pathogenesis. For example, interaction of endogenous annexin A1 with FPR1 leads to transactivation of the receptor for epithelial growth factor (EGFR), which promotes invasion and growth of glioma cells [2]. Likewise, Cheng et al. [9] reported that FPR1 expression is associated with tumor progression and survival in gastric cancer. 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.

The receptor-specific and most potent FPR1 antagonists described so far are the fungal hydrophobic cyclic peptides, cyclosporines A and H [10]. Although cyclosporine H blocked *N*-formyl-Met-Leu-Phe (fMLF)-induced analgesia [11] and attenuated the acute inflammatory response evoked by cigarette smoke [12], *in vivo* studies of cyclosporines should be interpreted carefully because their main therapeutic effects appear to involve signaling pathways unrelated to FPR1 [13–16]. Other known peptide FPR antagonists are Boc-1 (Boc-MLF) and Boc-2 (Boc-FLFLFL), and there are several reports of *in vivo* application of Boc-2 [17–20]. Recently, analogs of Boc-2 were reported as FPR1 antagonists [21, 22]. Several non-steroidal anti-inflammatory drugs (NSAIDs), including diclofenac, piroxicam, sulfinpyrazone, and tenoxicam have been reported as low activity FPR1 antagonists [23–25]. However, because NSAIDs exhibit a variety of pharmacological properties, these drugs are not suitable for *in vivo* studies designed to probe the physiological roles of FPR1.

Growing evidence supporting the anti-inflammatory and tissue-protective effects of FPR antagonists led to the screening of commercial libraries for novel small-molecule FPR antagonists. As result of these screening efforts and/or structure–activity relationship (SAR)-directed design and synthesis, a number of synthetic non-peptide FPR1/FPR2 antagonists with a wide range of chemical diversity have been identified ([26–33]). Structures of the

most potent small-molecule FPR1 antagonists are shown in Figure 1. Among these competitive FPR1 antagonists are some compounds with a 4*H*-chromen-4-one scaffold (Figure 1, compounds **1-4**) [26, 27]. However, activities of these chromones in primary cells, SAR analysis of related chromones and isoflavones, as well as molecular modeling have not been described.

In the present study, we evaluated 96 4*H*-chromen-4-ones, including synthetic and naturally occurring isoflavones, for their ability to antagonize FPR-dependent signaling in neutrophils and FPR-transfected cells and identified novel and potent FPR1-specific antagonists. These antagonists were specific for FPR1 and did not inhibit FPR2-, FPR3-, or CXCR1-dependent responses. SAR analysis of these compounds revealed the importance of a small hydrophobic group at position 2 and the type of substituent at position 7 of the 4*H*-chromen-4-one scaffold. In addition, molecular modeling showed a high degree of similarity for low-energy conformations of these antagonists to the pharmacophore model for FPR1 antagonists. Overall, the isoflavone scaffold represents an appropriate backbone to develop novel FPR1 antagonists.

2. Materials and Methods

2.1. Materials

Phorbol-12-myristate-13-acetate (PMA), dimethyl sulfoxide (DMSO), fMLF, HEPES, Percoll, and Histopaque 1077 were from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium and penicillin-streptomycin solution were from Mediatech (Herdon, VA). Fetal bovine serum (FBS) was from Atlas Biologicals (Fort Collins, CO). Peptides WKYMVm and WKYVM were from Calbiochem (San Diego, CA) and Tocris Bioscience (Ellisville, MO), respectively. Human interleukin-8 (IL-8) was from Peprotech Inc (Rocky Hill, NJ). Hanks' balanced salt solution (HBSS), Fluo-4 AM, and G418 were from Life Technologies (Grand Island, NY). HBSS containing 1.3 mM CaCl₂ and 1.0 mM MgSO₄ is designated as HBSS⁺; HBSS without ions Ca²⁺ and Mg²⁺ is designated as HBSS⁻. Selected 4*H*-chromen-4-ones were purchased from ChemDiv (San Diego, CA), ChemBridge (San Diego, CA), Princeton BioMolecular Research (Monmouth Junction, NJ), Indofine (Hillsborough, NJ), Otava (Toronto, Canada), Vitas-M Laboratory (Moscow, Russia), and InterBioScreen (Moscow, Russia). Fluorescein isothiocyanate (FITC) was conjugated to the lysine residue of the WKYMVm peptide to produce a fluorescent ligand (WKYMVm-FITC) that binds to both FPR1 and FPR2 (custom synthesis by Bachem, Torrance, CA).

2.2. Cell Culture

Human promyelocytic leukemia HL-60 cells stably transfected with FPR1 (FPR1-HL60 cells) or FPR2 (FPR2-HL60 cells) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 100 µg/ml streptomycin, 100 U/ml penicillin, and G418 (1 mg/mL), as described previously [34]. Rat basophilic leukemia (RBL-2H3) cells transfected with human FPR1 (FPR1-RBL), human CXCR1, or mouse Fpr1 (Fpr1-RBL) were cultured in DMEM supplemented with 20% (v/v) heat-inactivated 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. Wild-type HL-60 and RBL-2H3 cells were cultured under the same conditions, but without G418.

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 described previously [35]. 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. Isolation of Bone Marrow Leukocytes and Murine Neutrophils

All animal use was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Montana State University. Bone marrow leukocytes were flushed from tibias and femurs of BALB/c mice with HBSS, filtered through a 70- μ m nylon cell strainer to remove cell clumps and bone particles, and resuspended in HBSS⁻ at 1×10^6 cells/ml. Bone marrow neutrophils were isolated from the bone marrow leukocyte preparations as described previously [36]. Briefly, bone marrow leukocytes were resuspended in 3 ml of 45% Percoll solution and layered on top of a Percoll gradient consisting of 2 ml each of 50, 55, 62, and 81% Percoll solutions in a conical 15-ml polypropylene tube. The gradient was centrifuged at 1600g for 30 min at 10°C, and the cell band located between the 62 and 81% Percoll layers was collected. The cells were washed, layered on top of 3 ml of Histopaque 1119, and centrifuged at 1600g for 30 min at 10°C to remove contaminating red blood cells. The purified neutrophils were collected, washed, and resuspended in HBSS⁻.

2.5. Ca²⁺ Mobilization Assay

Changes in intracellular Ca²⁺ were measured with a FlexStation II scanning fluorometer (Molecular Devices, Sunnyvale, CA) in human neutrophils, HL-60 cells, and RBL cells, as described previously [34]. The cells, suspended in HBSS⁻ containing 10 mM HEPES, were loaded with Fluo-4 AM dye (Invitrogen) (1.25 μ g/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 aliquotted into the wells of flat-bottom, half-area-well black microtiter plates (2×10^5 cells/well). For evaluation of direct agonist activity, the compounds of interest were added from a source plate containing dilutions of test compounds in HBSS⁺, and changes in fluorescence were monitored ($\lambda_{\text{ex}} = 485$ nm, $\lambda_{\text{em}} = 538$ nm) every 5 s for 240 s at room temperature after automated addition of compounds.

Antagonist activity and selectivity were evaluated after 5–30 min pretreatment with test compounds at room temperature, followed by addition of peptide/chemokine agonist (5 nM fMLF, 5 nM WKYMVm, 10 nM WKYMVM, or 25 nM IL-8). In some experiments, a range of fMLF 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 β 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 (IC_{50}) were performed by nonlinear regression analysis of the dose-response curves generated using Prism 6 (GraphPad Software, Inc., San Diego, CA). Efficacy is expressed as % inhibition by an antagonist of the response induced by 5 nM β MLF at the maximal applied concentration of an antagonist ($\sim 50 \mu M$).

2.6. Chemotaxis Assay

Human neutrophils were suspended in HBSS⁺ containing 2% (v/v) heat-inactivated FBS (2×10^6 cells/ml), and chemotaxis was analyzed in 96-well ChemoTx chemotaxis chambers (Neuroprobe, Gaithersburg, MD), as described previously [35] with modifications. 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 μ l of HBSS⁺ containing 2% (v/v) heat-inactivated FBS with the indicated concentrations of test compounds plus 1 nM β MLF, DMSO plus 1 nM β MLF (positive control), or DMSO alone (negative control). Neutrophils were allowed to migrate through the 5.0- μ m 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), and 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 (IC_{50}) were performed by nonlinear regression analysis of the dose-response curves generated using Prism software.

2.7. 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 HL-60 and RBL transfected cells, as described previously [27] with modifications. Briefly, FPR1-HL60 or FPR1-RBL cells were preincubated with different concentrations of test compound for 30 min at 4°C. WKYMVm-FITC (0.5 nM) was added, and after incubation for an additional 30 min at 4°C, the samples were immediately analyzed using flow cytometry (LSRII; BD Biosciences, San Jose, CA) without washing. The assay response range was defined by replicate control samples containing 1 μ M of unlabeled β 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., IC_{50}). In equilibrium binding experiments with the labeled ligand, the K_d values for FPR1 in HL60-FPR1 and RBL- FPR1 cells were found to be ~ 0.5 and 0.4 nM, respectively. K_i values were calculated from IC_{50} , as reported previously [27].

2.7. ERK Phosphorylation Assay

Phosphorylation of p44/42 mitogen-activated protein kinases (ERK1/2) was determined based on activation-associated phosphorylation. Cells cultured in six-well plates were serum starved for 4 hr before stimulation. Samples were treated with the indicated compounds at 50 μ M or 0.5% DMSO (vehicle) for 10 min and then stimulated with fMLF (20 nM) for various time periods (0 – 15 min) or 200 ng/ml of PMA. 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). Horseradish peroxidase-conjugated AffiniPure goat anti-rabbit IgG (1:3000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as secondary antibody. The immunoblots were visualized using a SuperSignal West Pico Chemiluminescence kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. The results of Western blotting were quantified using ImageJ software from the National Institutes of Health.

2.8. Compound Cytotoxicity

Cytotoxicity was analyzed with a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI), according to the manufacturer's protocol. Briefly, HL-60 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

Four FPR1 antagonists (compounds **1**, **5-7**) with different chemical scaffolds and relatively high FPR1 antagonist activity were chosen as reference compounds for pharmacophore modeling (structures shown in Figure 1). We used a ligand-based approach for molecular modeling known as field point methodology [37, 38], as described previously [39]. The structures of the compounds were pre-optimized by the semi-empirical PM3 method using HyperChem 8.0 software. The Polak-Ribiere conjugate gradient method was applied for the minimization with a RMS gradient less than $0.01 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-1}$ as a convergence condition. The structures in Tripos MOL2 format were then imported into the FieldTemplater program (FieldTemplater Version 2.0.1; Cresset Biomolecular Discovery Ltd., Hertfordshire, UK). The conformation hunter algorithm was used to generate representative sets of conformations corresponding to local minima of energy calculated within the extended electron distribution force field [37, 40]. This algorithm incorporated in the FieldTemplater and FieldAlign software allowed us to obtain up to 200 independent conformations that were used in further calculation of field points surrounding each conformation of each molecule. To decrease the number of rotatable bonds during the conformation search, the “force amides trans” option was enabled in the program. 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); red, electron-deficient (positive); yellow, van der Waals attractive (steric); and orange, hydrophobic. A detailed description of the field point calculation procedure has been published elsewhere [41]. A clique-matching algorithm with further simplex optimization was applied to obtain the conformations of four active molecules giving good mutual overlays in terms of geometric and field similarity. However, it was possible to obtain a satisfactory superimposition of three FPR1 antagonists (**1**, **5**, and **7**). The best overlay was taken as a template representative of a FPR1 antagonist bioactive conformation.

Structures of additional FPR1 antagonists and inactive analogs were pre-optimized by the PM3 method using the approach described above, converted into Tripos MOL2 format, and imported into FieldAlign program (FieldAlign Version 2.0.1; Cresset Biomolecular Discovery Ltd., Hertfordshire, UK) together with the template. The conformation hunter algorithm was used to generate up to 200 conformations for each ligand, which 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 [42]. Structure of compound **10** was pre-optimized by the semi-empirical PM3 method using HyperChem software, saved in Tripos MOL2 format, imported into the Molegro Virtual Docker (MVD) program (MVD 2010.4.2, MolegroApS), and docked as reported previously [43].

3. Results

3.1. SAR Analysis of 4*H*-Chromen-4-one FPR1 Antagonists

Based on structures of four published FPR1 antagonists **1-4** with a 4*H*-chromen-4-one scaffold [26, 27] (Figure 1), 96 additional chromen-4-one analogs and related isoflavones were selected and evaluated for FPR1 antagonist activity in FPR1-HL60 cells by monitoring effects on *f*MLF-induced Ca²⁺ mobilization. These analogs included 25 derivatives of compound **1** (benzimidazoles **8-32**, series **A**), 22 derivatives of compounds **2** and **3** (furoyl and thienoyloxy derivatives **33-54**, series **B**), nine 7-benzoyloxy-substituted isoflavones (**55-63**, series **C**), 26 derivatives of compound **4** (2-trifluoromethyl-2'-methoxy isoflavones **64-89**, series **D**), and 14 additional natural and synthetic isoflavones (**90-103**, series **E**). Structures of these analogs and reference compounds **1-4** are presented in Table 1.

In previous studies of potential FPR1 antagonists where inhibition of *f*MLF-induced Ca²⁺ mobilization was evaluated, cells were pretreated for 5 to 30 min with putative antagonists

prior to *f*MLF addition [26, 27]. Here, we found that pretreatment of FPR1-HL60 cells with novel chromone analogs for 5 min at room temperature blocked *f*MLF-induced Ca^{2+} flux up to 90% of control level (see example in Figure 2A). However, the response was completely blocked only after 30-min pretreatment (Figure 2B). Thus, we used a 30-min pretreatment period for further screening of chromone analogs and dose-response analysis. As an example, a representative dose-response curve for inhibition of *f*MLF-induced Ca^{2+} mobilization in FPR1-HL60 cells by compound **10** is shown in Figure 2C. Antagonist activity (expressed as IC_{50}) of all chromone analogs tested from scaffolds **A–E** is presented in Table 1.

All benzimidazole derivatives from series **A** that inhibited *f*MLF-induced Ca^{2+} flux in FPR1-HL60 cells (compounds **1**, **10**, **15**, **27**, and **28**) contained a CH_3 group at position 2 of the chromone scaffold (R_3) (Table 1). This methyl moiety was essential for antagonist activity, as elimination of this group led to inactive compounds (compare active **1** and inactive **9**). Substitution at position 7 of the chromone scaffold (R_2) also had effects on activity, but a wide range of modifications was tolerated. Although substitution of OCOCH_3 in reference compound **1** with *tert*-butyl-carboxyl or hydroxyl groups led to inactive compounds **11** and **17**, compound **28** with a bulky *ortho*-chloro-benzoyloxy ($\text{OCO-C}_6\text{H}_4\text{Cl-}o$) substituent at R_2 retained FPR1 antagonist activity. Other active FPR1 antagonists from series **A** contained hydroxyl (**15**) or furan-2-carboxylate (**27**) groups at this position.

All active FPR1 antagonists in series **B** (compounds **2**, **3**, **36**, **38**, and **45–48**) contained CH_3 or CF_3 groups at position 2 of the chromone heterocycle (R_3), supporting the importance of a small hydrophobic group at this position for antagonist activity, which was noted above for series **A** compounds (Table 1). Indeed, elimination of the CF_3 group in compound **36** or substitution of CF_3 in reference compound **2** with an ethyl-carboxylate group resulted in inactive compounds **37** and **54**, respectively. Although replacement of CF_3 by CH_3 resulted in decreased activity for some analogs (compare **2** and **45** or **38** and **48**), this same replacement converted inactive compound **34** into active **47**. Replacement of the furan ring by thiophene in the aryloxy group led to variable effects on activity, depending on the presence of substituents at other positions in a given molecule. Most active derivatives within this series contained Cl (compounds **3** and **36**) or OCH_3 (compounds **2**, **35**, **45**, and **46**) in the *ortho* position of the benzene ring (R_4). However, the presence of a substituent at this position was not absolutely essential for antagonist activity, as compounds **38** and **47** were also active.

From series **C**, only compound **55** had FPR1 antagonist activity (Table 1). Addition of substituents in the benzoyloxy phenyl ring led to complete loss of activity among the 2-trifluoromethyl derivatives (**56–62**). Elimination of the CF_3 group in compound **55** resulted in inactive compound **63**, again supporting the importance of this group at position 2 of the chromone scaffold for FPR1 antagonist activity. Based on analysis of benzoyloxy-containing compounds **28–31** (**A** series) and **55–63** (**C** series), the presence of a benzoyloxy group at R_2 is not an essential feature for antagonist activity, probably because of the bulkiness and/or high hydrophobicity of this substituent. Hence, we did not consider further compounds with a benzoyloxy moiety at R_2 in subsequent screening of 4*H*-chromen-4-ones.

Keeping substituents CF₃ and OCH₃ at positions R₃ and R₄, respectively, since these groups led to the best FPR1 antagonist activity, we next evaluated a variety of alkoxy, acyloxy, and other oxygen-containing groups at position 7 of the chromone scaffold (R₂) (Table 1, series **D**). Five novel FPR1 antagonists (**64**, **68**, and **73-75**) were identified in the series of 2-trifluoromethyl-2'-methoxy isoflavones, with the most potent being compound **68**. It is interesting to note that replacement of cyclopropyl in compound **68** with isopropyl decreased antagonist activity by ~20-fold (reference compound **4**), while substitution of the cyclopropyl group with *tert*-butyl resulted in inactive compound **69**. A possible reason for this decrease is the smaller effective size of the cyclopropyl group compared to the isopropyl and *tert*-butyl substituents. Other compounds with antagonist activity were molecules bearing hydroxyl (**64**), OCOOCH₂CH₃ (**73**), OCH₂COOH (**74**), and OCH₂CONH₂ (**75**) groups. On the other hand, longer R₂ chains in compounds **76-89** led to loss of activity, probably due to steric hindrance within the binding pocket.

Four novel FPR1 antagonists, including the naturally occurring isoflavone biochanin A (compound **94**), were identified among the 14 natural and synthetic isoflavones tested (series **E**). Note, however, that the closely related isoflavone genistein (compound **93**) was inactive. Removal of a hydroxyl group at position 5 of the isoflavone scaffold (R₁₁) resulted in loss of antagonist activity (compare compounds **95** and **97**), suggesting this OH group may increase tolerance for a modification at position 2 (R₃), because removing CF₃ in compound **95** led to decreased but not complete loss of antagonist activity (compound **94**), which was a feature of the compounds in series **A-C**. On the other hand, introduction of a hydroxyl group at position 5 of compound **98** resulted in inactive compound **99**. Thus, the effect of this hydroxyl substitution may depend on the presence of substituents at other positions of a molecule. Overall, no clear SAR emerged from other modifications in this series, and additional evaluation of synthetic and natural occurring isoflavones, especially 5-hydroxy isoflavones, will be necessary.

All 100 chromone derivatives were evaluated for FPR-independent activation in non-transfected HL-60 cells, and only compounds **94** and **95** directly activated Ca²⁺ mobilization in wild-type cells (data not shown), indicating their apparent FPR antagonist activity could be due to receptor cross-desensitization or some other nonspecific effect on the cell. Thus, these compounds were not included in further studies of specific antagonists.

3.2. Competition Binding of Selected FPR1 Antagonists

Compounds that inhibited fMLF-induced Ca²⁺ mobilization in FPR1-HL60 cells with IC₅₀ <5 μM and efficacy >75% were analyzed for their ability to compete with WKYMVm-FITC for binding to FPR1 in FPR1-HL60 and FPR1-RBL cells, as described previously [44]. Values of IC₅₀ for the competitive binding assay and calculated inhibition constants (K_i) are presented in Table 2. As an example, a representative dose-response curve of competitive inhibition of WKYMVm-FITC binding by compound **10** in FPR1-RBL transfected cells is shown in Figure 3A. Compound **10** had the highest binding affinity (K_i = 110 and 90 nM in FPR1-HL60 and FPR1-RBL cells, respectively) among all chromones evaluated, including previously reported chromone FPR1 antagonists **1-4** [27, 45]. Notably, compound **10** had a 5.7-fold higher binding affinity and ~4.5-fold greater antagonist activity than compound **1**,

which was the most potent, selective small-molecule FPR1 antagonist previously reported. Overall, the binding affinity (K_i) of the active compounds in FPR1-HL60 cells exhibited a good linear correlation ($r=0.92$, $n=11$) with potency (IC_{50}) of the ligands in *f*MLF-stimulated FPR1-HL60 cells, with one exception (compound **3**) (Figure 3B).

In contrast to the other compounds tested, compound **94** did not displace WKYMVm-FITC (data not shown), again supporting the conclusion that inhibition of *f*MLF-induced Ca^{2+} mobilization in FPR1-HL60 cells by this isoflavone involves an FPR1-independent mechanism.

3.3. Selectivity of FPR1 Antagonists

The selected FPR1 antagonists (Table 2) were evaluated for their ability to activate and inhibit Ca^{2+} mobilization in FPR1-, FPR2, or FPR3-transfected HL60 cells, Fpr1-transfected RBL cells, and CXCR1-transfected RBL cells, as well as in primary human and murine neutrophils. Importantly, all but one of these competitive antagonists did not directly activate Ca^{2+} flux in any of the cell lines or primary cells, supporting the conclusion that they are competitive receptor antagonists. The one exception was compound **2**, which did not have an agonist effect in FPR-transfected cells or human neutrophils but did have agonist activity in murine neutrophils. The reason for this activity is currently not understood; however, we have observed differing response patterns to some agonists and/or antagonists in human versus murine neutrophils, respectively (data not shown).

To evaluate receptor selectivity of the antagonists, FPR2-HL60, FPR3-HL60, Fpr1-RBL, and CXCR1-RBL cells were pretreated with the selected compounds for 30 min, followed by stimulation with WKYMVm (FPR2-HL60 or Fpr1-RBL cells), WKYMVm (FPR3-HL60 cells), or IL-8 (CXCR1-RBL cells). None of the selected FPR1 antagonists inhibited FPR2-, FPR3-, Fpr1-, or CXCR1-dependent Ca^{2+} flux (Table 2), demonstrating specificity for FPR1. Likewise, these compounds also did not inhibit human FPR2-dependent or murine Fpr1-dependent neutrophil responses induced by WKYMVm (Table 2 and Figure 2C) and WKYMVm (Table 2), respectively. Moreover, the selected FPR1 antagonists did not inhibit Ca^{2+} flux induced in human neutrophils by IL-8 (data not shown). Thus, these antagonists are FPR1-specific and do not target human FPR2, human FPR3, mouse Fpr1, or other chemokine GPCRs, such as human CXCR1. On the other hand, all selected antagonists inhibited *f*MLF-induced FPR1 activation in human neutrophils, further supporting their specificity for FPR1 (Table 2). As an example, a representative dose-response curve for the inhibition of *f*MLF-induced Ca^{2+} flux in human neutrophils by compound **10** is shown in Figure 4A. Since the IC_{50} value is relative and depends on agonist concentration, we also evaluated inhibition of *f*MLF-induced Ca^{2+} flux in human neutrophils by compound **10** at different *f*MLF concentrations and found that IC_{50} values for the antagonist were ~0.032, 0.12, and ~2.0 μ M, at *f*MLF concentrations of 1, 5, and 25 nM, respectively (Figure 4A). Furthermore, activity of these FPR1 antagonists in *f*MLF-stimulated human neutrophils generally had a good linear correlation ($r=0.77$, $n=12$) with IC_{50} values in *f*MLF-stimulated FPR1-HL60 cells, with only two exceptions (compounds **38** and **47**) (Figure 4B). Although naturally occurring isoflavones including genistein, daidzein, afrormosin, formononetin, and 7-hydroxyisoflavone were inactive in FPR1-HL60 cells, these compounds were also tested

in human neutrophils. None of these compounds inhibited β MLF-induced Ca^{2+} mobilization in these primary cells at the tested concentrations ($50 \mu\text{M}$) (data not shown).

To further investigate the effects of competitive FPR1 antagonists on human neutrophil function, we also evaluated the ability of these compounds to inhibit chemotaxis. All 14 compounds dose-dependently inhibited β MLF-induced neutrophil migration, with IC_{50} values of 24 nM to $4.5 \mu\text{M}$ (Table 2). As an example, a representative dose–response curve for the inhibition of β MLF-induced neutrophil chemotaxis by compound **10** is shown in Figure 4C.

β MLF stimulation results in phosphorylation of ERK1/2 in human neutrophils and FPR1-transfected cells [46, 47]. Thus, we evaluated whether selected FPR1 antagonists altered phosphorylation of ERK1/2 in FPR1-RBL cells. Stimulation of FPR1-RBL cells with β MLF resulted in rapid phosphorylation of ERK1/2 (Figure 5A–C). Pretreatment with two of the most potent FPR1 antagonists (**10** and **36**) effectively inhibited β MLF-induced phosphorylation of ERK1/2 (Figure 5A,B). This effect was specific for β MLF activation, as compounds **10** and **36** did not significantly alter PMA-induced phosphorylation of ERK in untransfected RBL cells (Figure 5D). Importantly, untransfected RBL cells did not respond to β MLF with ERK1/2 phosphorylation (Figure 5D). Consistent with the relative potency of FPR1 antagonists (Table 1), low activity antagonists **15** and **38** only weakly inhibited ERK1/2 phosphorylation, while non-active compound **35** also had no effect on ERK1/2 phosphorylation induced by β MLF (Figure 5).

To ensure that the results were not influenced by possible compound toxicity, cytotoxicity of the most potent FPR1 antagonists was evaluated at various concentrations ($25 \mu\text{M}$) 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-hr incubation period.

3.4. Molecular Modeling of FPR1 Antagonists

For development of the ligand-binding site template for FPR1 antagonists, four potent small-molecule FPR1 antagonists with different chemical scaffolds were selected from the literature (reviewed in [33]). These molecules were identified previously using high-throughput screening and/or lead optimization and included compounds **1** and **7** [27], **5** [28], and **6** [30]. For all independent conformations generated for these compounds, a search for optimal superimpositions was performed. We found that only three of these compounds (**1**, **5**, and **7**) could be overlaid simultaneously with good correspondence of the molecular fields, and the superimposition with the highest similarity score calculated by FieldTemplater was chosen for further investigation as a pharmacophore model/template. This pharmacophore template has three hydrophobic sites (*protrusion* and two *areas I* and *II*), one compact grouping (*area III*) of H-bond donors or positively charged groups in a proposed receptor, and one region of H-bond acceptors (*area IV*) or negatively charged centers (Figure 6A). The template is slightly curved in shape with the *protrusion* where CH_3 and CF_3 groups of compounds **1** and **7** are located together with the methylthioethyl substituent of molecule **5**. On this *protrusion*, four hydrophobic (orange) pharmacophore

points are positioned according to the nature of these substituents. Another compact grouping of hydrophobic points lies in *area I*, where fused nitrogen-containing heterocycles of compounds **1** and **5** are located. Molecule **7** has an *ortho*-chlorophenyl substituent in this region. A more diffuse group of hydrophobic field points (*area II*) corresponds to the benzofuran moiety of compound **5**, substituted benzene ring of the chromone moiety in molecule **1**, and benzyl group of compound **7**, which are superimposed within the template. Additionally, several negative (blue) and positive (red) points lay very near heteroatoms and H-bond donors/acceptors (*areas III* and *IV*, respectively). A few blue field points are also surrounding *area II*, because it contains electronegative oxygen atoms.

A visual inspection of the molecule overlays on the 3-molecule FPR1 template revealed that chromone-derived FPR1 antagonists have very similar alignments on the template, with the R₃ group (CH₃ or CF₃) lying within the *protrusion*. Substituent R₂ coincides with *area II*, while a moiety at position 3 of the chromone heterocycle (i.e., benzimidazole or substituted phenyl ring) falls into *area I*. As an example, the most potent FPR1 antagonist (compound **10**) superimposed onto the 3-molecule template is shown in Figure 6A.

Since the geometry and location of the FPR1-binding site were not considered explicitly in the field point methodology described above, a homology model of FPR1 was applied. Several subpockets of the FPR1 binding site, including two channels (**A** and **C**), two cavities (**B** and **E**), and the bottom (**D**) were recently described for this model [43], and the same designation of the subpockets (**A-D**) is used here for comparison (Figure 6B). To determine if these subpockets could accommodate the FPR1 antagonist pharmacophore, receptor-docking poses of compound **10** were determined. A pose with minimal energy score of $-114.7 \text{ kcal mol}^{-1}$ had a geometry close to its best-fit configuration in the FPR1 antagonist pharmacophore template. A slight difference was found between conformations of the flexible hexyl group (R₂) in the docking pose and field point superimposition. According to our docking study, the pharmacophore *area I* with hydrophobic *area III* are located near the bottom **D** of the FPR1 binding site, the *protrusion* is fitted in cavity **B**, and *area II* is located in cavity **E** with the long hexyl chain positioned in channel **C** (Figure 6B). FPR1 residues corresponding to these areas of the binding pocket were enumerated previously [43].

We next explored optimal alignments of all active and most inactive 4*H*-chromen-4-ones onto the template with the use of FieldAlign program. The alignments with higher similarity scores were analyzed in terms of molecular moieties coincidence with key regions in the pharmacophore model described above. Software options of the calculations are described in *Materials and Methods*. The values of similarity scores obtained for active and inactive 4*H*-chromen-4-ones with different chemical scaffolds are presented in Table 1. According to Field Point methodology [37], 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. The data were analyzed for the total set of compounds and separately for compounds with common scaffolds, with the exception of compounds with scaffold **C**, because this set had only one active analog (compound **55**). Although there was no a linear correlation between the similarity scores and inverse ($1/IC_{50}$)

values of FPR1 antagonist activity ($r = 0.23$), the ANOVA gave a statistically significant difference ($p < 0.001$) between groups of active and inactive compounds with respect to their similarity scores (Figure 7A). Within individual chemical scaffolds classes evaluated, the similarity scores also differed significantly between FPR1 antagonists and inactive analogs (Figure 7B).

Absence of a correlation between the similarity score and antagonist activity can be partially explained by variations in a binding mode to FPR1. For example, inactive compound **63** has a reverse mode of superimposition onto the template, as compared with its active trifluoromethyl analogue **55**, although their level of similarity is equal (Table 1C). Compound **63** does not occupy the protrusion, while antagonist **55** has a CF_3 group (R_3) in this area, which is similar to other active compounds. Clearly, a region corresponding to the *protrusion* is crucial for antagonist activity. Another important feature influencing activity was the presence of bulky substituents that encountered a wall of the proposed binding site. For example, inactive compound **60** has a large *o*-methoxy group outside of the template boundaries, although the *protrusion* of the FPR1 pharmacophore template is occupied by the desired CF_3 group. Besides the global measure of similarity to the template, some local similarity can also be important for emergence of FPR1 antagonist activity. “Local” representation of chemical structures can be encoded by other descriptors, such as frontal polygons [48] and/or atom pairs [49]. Further studies using more sophisticated SAR analysis and comprehensive molecular docking will be necessary to address these questions in the future.

4. Discussion

4*H*-Chromones are an important class of synthetic and natural compounds that exhibit a broad range of biological activities. Their closely related derivatives are isoflavones, a large family of secondary plant metabolites with several variants of a heterocyclic ring substitution pattern. These compounds, along with their synthetic analogues, possess a wide variety of biological activities, including antiinflammatory, antiproliferative, antiosteoporotic, antihyperglycemic, antifungal, antiviral, antiparasitic, antioxidant, and cardiovascular effects [50–55]. Although reactive oxidant scavenging activity of flavonoids/isoflavones may contribute to modulation of phagocyte functional activity [56, 57], these compounds also interact with other cellular targets, including receptors, enzymes, and other macromolecules (for review [58–60]). Such properties include actions upon peroxisome proliferator-activated receptor isoforms, estrogen receptors, tyrosine kinases, DNA topoisomerase II, and NF- κ B activation [61–63]. There are a few reports about regulation of GPCRs by isoflavones, although most describe effects on estrogen receptor 1 (GPER1/GPR30) [64, 65].

Previously, several 4*H*-chromen-4-one derivatives were identified as small-molecule competitive FPR1 antagonists [26, 27]. In the current work, we report further characterization and development of related analogs with improved potency and FPR1 antagonist activity in functional tests using transfected cells and primary neutrophils. Screening of a library of 96 4*H*-chromen-4-ones, which are structural derivatives of four known chromone-derived competitive FPR1 antagonists **1–4**, resulted in the discovery of

novel potent FPR1 antagonists. We also showed that these compounds can compete with FITC-labeled peptide WKYMVm for binding with FPR1 in both FPR1-HL-60 and FPR1-RBL transfected cells. Compound **10** was the most potent FPR1 antagonist and exhibits the highest binding affinity among all known chromone analogs, including previously reported compounds **1-4**.

A review of the current literature regarding small-molecule FPR1 antagonists [66] showed that pyrazole-4-carboxamides are even more active at inhibition of *f*MLF-induced Ca^{2+} flux in human neutrophils, with the most potent compound **6** (see the structure in Figure 1), but competition binding at FPR1 for these compounds was not evaluated [29]. In addition, all independent conformations generated for molecule **6** in our modeling experiments did not exhibit good correspondence with the molecular fields of the other three antagonists with different chemical scaffolds (**1**, **5**, and **7**) that were used for building the FPR1 pharmacophore template. Thus, inhibition of *f*MLF-induced Ca^{2+} flux in human neutrophils by pyrazole-4-carboxamides may involve FPR1-independent mechanisms.

Compound **10** and several additional FPR1 antagonists identified here specifically blocked *f*MLF-induced responses mediated via FPR1 in FPR1-HL60 cells and human neutrophils, but not responses mediated via FPR2 or FPR3 (in human neutrophils and transfected HL60 cells) or Fpr1 (murine neutrophils and transfected RBL cells). These compounds also did not inhibit IL-8-induced Ca^{2+} mobilization in RBL cells transfected with CXCR1 and human neutrophils. The selected chromone/isoflavone derivatives (Table 2) were also inactive as direct agonists of FPR2 and Fpr1 and did not activate Ca^{2+} flux in murine and 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 (for example, see [31, 67]), no agonists of human FPR1 with a 4*H*-chromen-4-one scaffold have been reported. Thus, 4*H*-chromen-4-one may represent a unique chemical scaffold for development of specific FPR1 antagonists that do not have undesirable off-target effects.

In addition to mobilizing calcium from intracellular stores, *f*MLF activates the mitogen-activated protein kinases ERK1/2 [46, 47]. We evaluated this response to test the relative potency of selected FPR1 antagonists. Compounds **10** and **36**, which exhibited high potency in the Ca^{2+} flux assay, also inhibited *f*MLF-induced ERK phosphorylation. In comparison, antagonists with low potency in the Ca^{2+} flux assay, such as **15** and **38**, or the inactive compound **35** had much less or no effect on ERK1/2 phosphorylation. These results verify the Ca^{2+} flux data and together established a dose-effect relationship for these FPR1 antagonists.

Comprehensive SAR analysis of all 100 analogs was performed mainly at positions 2 and 7 of the chromone scaffold due to the apparent importance of these regions of the molecules. Our analysis suggests the importance of a small hydrophobic group (CH_3/CF_3) at position 2 of the heterocycle (R_3 functionality) for FPR1 antagonist activity. Substitution at position 7 of the chromone scaffold also had effects on antagonist activity, but a wider range of modifications was tolerated. Although several substituents at positions 6 and 2' of the

chromone and isoflavone scaffolds were evaluated, modification of these regions will be necessary for further optimization of chromone-based FPR1 antagonists.

Our molecular modeling showed a noticeably higher degree of similarity of the active molecules to the pharmacophore model, which was developed based on the structure of previously reported potent FPR1 antagonists with diverse chemical scaffolds, including chromone **1**, methionine-derived benzimidazole **5**, and pyrazolo[1,5-a]pyrimidin-7(1*H*)-one **7**. This reflects the main hypothesis of molecular recognition using a field point approach [38]: molecules of different chemical groups have the same biological action if they have similar geometrical arrangement of key pharmacophore features, including hydrophobic, electronegative, or electropositive features. Indeed, a significant statistical difference between groups of FPR1 antagonists with different sub-scaffolds and their inactive derivatives with respect to their similarity scores compared to the 3-molecule FPR1 antagonist pharmacophore was observed. The docking study of the most potent FPR1 antagonist **10** showed that the subpockets of FPR1 binding site could also fully accommodate the FPR1 antagonist pharmacophore. Further studies are clearly required to define the nature of molecular interactions between the chromone/isoflavone based ligands and FPR1 binding site.

Although biochanin A (compound **94**) inhibited *f*MLF-induced Ca^{2+} flux in FPR1-HL60 cells and human neutrophils, this natural occurring isoflavone did not displace WKYMVM-FITC from FPR1-HL60 cells. In addition, biochanin A and its synthetic 2-trifluoromethyl derivative **95** had direct agonist activity in cell lines and primary neutrophils. Thus, compounds **94** and **95** may activate Ca^{2+} flux via FPR-independent pathways, which leads to cross-desensitization of FPR1 and/or cross-talk with the receptor. Indeed, several other synthetic and natural flavonoids (wogonin, apigenin, genistein, kazinol B, and ugonin U) have been reported to activate intracellular Ca^{2+} flux in various cell lines [68, 69] or neutrophils [70, 71]. Clearly, additional research is required to define the mechanisms of biochanin A action on neutrophils.

In the present studies, we found that genistein did not antagonize *f*MLF-induced Ca^{2+} mobilization in FPR1-HL60 cells and human neutrophils. Thus, the previously reported inhibitory effect of genistein on *f*MLF-stimulated degranulation, aggregation, and reactive oxygen species (ROS) production in human neutrophils [47, 72, 73] may be related to pathways downstream of Ca^{2+} -mobilization, such as tyrosine kinase-dependent signaling [46, 47] and ROS scavenger activity [74]. Indeed, the broad modulatory activity of genistein on neutrophil activity suggests a non-receptor mode of action. For example, genistein inhibited neutrophil adhesion/adherence activated by agonists of protease-activated receptor 2 (PAR2) [75] or TNF [76]. Moreover, pretreatment of neutrophils with genistein potentiated *f*MLF-induced cell transmigration [77]. Another isoflavone, afrormosin, inhibited *f*MLF-stimulated neutrophil degranulation at very high concentrations only ($\text{IC}_{50} \sim 67 \mu\text{M}$) [78]. Thus, our current studies suggest that FPR1-independent molecular mechanisms could play a role in the previously reported inhibition of *f*MLF-induced neutrophil responses by genistein and afrormosin.

Although isoflavones are abundant in the members of the *Fabaceae* (i.e., *Leguminosae*) [79], these compounds were also isolated from species belong to other families. Dozens of novel isoflavonoids are discovered every year (for review, see [79]) and further broad screening of natural isoflavones for FPR antagonist activity and anti-inflammatory activity should now be considered. Furthermore, previously reported inhibitory effects of 4*H*-chromen-4-ones and related flavonoids (e.g., [80]) on fMLF-induced neutrophil responses should be reevaluated in light of our current findings because their biological effects may involve FPR1 antagonist activity.

In conclusion, we have identified a number of specific, competitive FPR1 antagonists with isoflavone backbones. Several of these 4*H*-chromen-4-one 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 to understanding the molecular structural requirements of FPR1 antagonists.

Supplementary Material

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Figure 1.
Structures of previously reported small-molecule FPR1 antagonists.

Figure 2. Inhibition fMLF-induced Ca^{2+} mobilization in FPR1-HL60 but not FPR2-HL60 cells by compound 10

Panel A. FPR1-HL60 cells were preincubated for 5 min (at 25 °C) with 1 μM of compound **10** or DMSO (vehicle control) and treated with 5 nM fMLF or DMSO (vehicle control). Kinetics of Ca^{2+} mobilization were monitored for 5 minutes. **Panel B.** FPR1-HL60 cells were preincubated for the indicated times (at 25 °C) with 1 μM of compound **10** or DMSO (vehicle control) and treated with 5 nM fMLF or DMSO (vehicle control). Inhibition of fMLF-induced response was calculated as indicated under *Materials and Methods* (mean $\pm$ S.D.; n=3). *Significance difference from 100% inhibition ($p < 0.05$). **Panel C.** FPR1-HL60 cells (●) and FPR2-HL60 cells (□) were preincubated with the indicated concentrations of compound **10** for 30 min and 25 °C, and the cells were stimulated with 5 nM of fMLF or WKYMVM, respectively. The response induced by peptide agonist alone was assigned a value of 100%, and % of control responses are shown. In each panel, values are samples from one experiment that is representative of three independent experiments.

Figure 3. Selected antagonists compete with WKYMVm-FITC for binding to FPR1
Panel A. FPR1-RBL cells were incubated with the indicated concentrations of compound **10**, followed by addition of WKYMVm-FITC, and bound FITC was analyzed by flow cytometry, as described under Materials and Methods. Values are samples from one experiment that is representative of three independent experiments. **Panel B.** Plot of IC_{50} values for inhibition of f MLF-induced Ca^{2+} mobilization in FPR1-HL60 cells versus K_i values reflecting binding affinity to the receptor. IC_{50} and K_i values were selected from Tables 1 and 2, respectively.

Figure 4. Inhibition of Ca^{2+} mobilization by selected FPR1 antagonists

Panel A. Human neutrophils (hPMN) were preincubated with the indicated concentrations of compound **10** for 30 min at 25 °C and then stimulated with 1 nM (●), 5 nM (○), or 25 nM (■) of fMLF or 10 nM WKYMVM (△). The response induced by peptide agonist alone was assigned a value of 100%. **Panel B.** Plot of IC_{50} values for inhibition of fMLF-induced Ca^{2+} mobilization in FPR1-HL60 cells versus IC_{50} values for inhibition of fMLF-induced Ca^{2+} mobilization in the human neutrophils. IC_{50} values for inhibition of Ca^{2+} flux in FPR1-HL60 cells and human neutrophils were selected from Tables 1 and 2, respectively. Compound numbers are indicated for each symbol. **Panel C.** Human neutrophils were preincubated with the indicated concentrations of the compound **10** or DMSO for 30 min at room temperature, and chemotaxis toward 1 nM fMLF was measured as described under

Materials and Methods. The results are expressed as the number of migrated cells per well (mean $\pm$ S.D.; n=3). In the panels **A** and **C**, values are samples from one experiment that is representative of three independent experiments.

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Figure 5.

Effect of the FPR1 antagonists on ERK1/2 phosphorylation. FPR1-RBL cells (Panels **A**, **B**, and **C**) or un-transfected RBL-2H3 cells (**Panel D**) were serum-starved for 4 h and then stimulated with the indicated compounds at 50 μ M for 10 min. The samples were then stimulated with 20 nM fMLF for different time points. Phosphorylated ERK1/2 (top panel) as well as total ERK1/2 (middle panel) were determined by Western blotting, as described. Relative ERK1/2 phosphorylation level was quantified using ImageJ software (bottom panel). In **Panel D**, RBL-2H3 cells were pretreated with compounds **10** and **36**, as indicated, followed by stimulation with 200 ng/ml PMA for 0, 5, 15 min or 20 nM fMLF for 5 min. The data shown are representative of two independent experiments.

Figure 6.

Multi-molecule template for FPR1 antagonists and superimposition of the compound **10** on the template and docking pose of compound **10** into homology model of FPR1 binding site.

Panel A. Compound **10** (magenta backbone, spherical field points) superimposed onto 3-molecular template (grey backbones, icosahedral field points), developed from compounds **1** (light blue), **5** (pink), and **7** (violet). Field points are colored as follows: blue = electron-rich (negative); red = electron-deficient (positive); yellow = van der Waals attractive (steric); and orange = hydrophobic. **Panel B.** Model of antagonist docking to FPR1. Geometry of the

hydrophobic field surface of the compound **10** (left side) matches to the binding site geometry of FPR1 (right side). FPR1 antagonist can approach the FPR1 binding site, which includes two channels (**A** and **C**), two cavities (**B** and **E**), and the bottom (**D**). These key features of the FPR1 binding site are designated in accordance to our previous publication [43] and indicated with light-blue arrows. Matching key ligand areas to key areas of the FPR1 binding site is indicated by green arrows under the **Panel B**. Surface coloring in the FPR1 was made according to electrostatic properties, whereby negatively and positively charged areas are shown in red and blue, respectively. It should be noted, that red (negatively charged) surface areas of the receptor correspond to red field points obtained with negative probe atom and blue (positively charged) surface areas of the receptor correspond to blue field points obtained with positive probe atom.

Figure 7.

Similarity scores of FPR1 antagonists and inactive analogs in whole set of *4H*-chromen-4-ones (**Panel A**) and in sub-sets of the analogs with related chemical scaffolds (**Panel B**). Selected molecules with different chemical scaffolds (see Table 1) were superimposed onto 3-molecular template for FPR1 antagonists and similarity score between aligned molecule and the template was calculated using the MVD program. Sub-set of the chemical scaffold **C** was excluded, because the set contains only one active compound. Position of biochanin A (compound **94**) with minimal similarity score with the FPR1 template among active compounds is indicated on the **Panel A**. Statistical analysis of the similarity scores was performed with the use of ANOVA.

Table 1

FPR1 Antagonist activity of 4*H*-chromen-4-ones

A. 3-(Benzimidazol-2-yl)-4 <i>H</i> -chromen-4-ones (1, 8-32) ^a

Compd	R ₁	R ₂	R ₃	IC ₅₀ (μM) and efficacy (%) in FPR1-HL60	Sim. ^b	
1	CH ₂ CH ₃	OCOCH ₃	CH ₃	1.4 ± 0.24 (100)	0.676	
8	H	OCOCH ₃	H	N.A.	0.549	
9	CH ₂ CH ₃	OCOCH ₃	H	N.A.	0.562	
10	(CH ₂) ₅ CH ₃	OCOCH ₃	CH ₃	0.31 ± 0.13 (100)	0.609	
11	CH ₂ CH ₃	OCO- <i>t</i> Bu	CH ₃	N.A.	0.552	
12	H	OCO- <i>t</i> Bu	CH ₃	N.A.	0.613	
13	CH ₂ CH ₃	OCO- <i>t</i> Bu	(CH ₂) ₂ COOH	N.A.	0.562	
14	H	OCO- <i>t</i> Bu	<i>i</i> Pr	N.A.	0.538	
15	H	OH	CH ₃	24.7 ± 3.2 (80)	0.666	
16	CH ₂ CH ₃	OH	H	N.A.	0.569	
17	CH ₂ CH ₃	OH	CH ₃	N.A.	0.566	
18	CH ₂ CH ₃	OH	<i>i</i> Pr	N.A.	0.541	
19	CH ₂ CH ₃	OH	CF ₃	N.A.	0.551	
20	H	OH	CH ₂ CH ₃	N.A.	0.563	
21	H	OH	<i>i</i> Pr	N.A.	0.543	

A. 3-(Benzimidazol-2-yl)-4H-chromen-4-ones (1, 8-32) ^a						
Compd	R ₁	R ₂	R ₃	IC ₅₀ (μM) and efficacy (%) in FPR1-HL60	Sim. ^b	
22	H	OH	(CH ₂) ₂ COOH	N.A.	0.569	
23	CH ₂ CH ₃	OH	(CH ₂) ₂ COOH	N.A.	0.600	
24	CH ₂ CH ₃	OH	COOCH ₂ CH ₃	N.A.	0.560	
25	CH ₂ CH ₃	OH	CH ₂ OCH ₂ COOH	N.A.	0.591	
26	CH ₂ CH ₃	OCO-2-Furyl	H	N.A.	0.606	
27	H	OCO-2-Furyl	CH ₃	3.1 ± 1.1 (100)	0.620	
28	CH ₂ CH ₃	OCO-C ₆ H ₄ Cl- <i>o</i>	CH ₃	5.2 ± 1.8 (90)	0.634	
29	CH ₂ CH ₃	OCO-Ph	H	N.A.	0.538	
30	CH ₂ CH ₃	OCO-C ₆ H ₄ OMe- <i>p</i>	H	N.A.	0.564	
31	H	OCH ₂ -C ₆ H ₄ NO ₂ - <i>p</i>	CH ₃	N.A.	0.547	
32	CH ₂ CH ₃	OSO ₂ CH ₃	H	N.A.	0.554

Compd	X	R ₃	R ₄	R ₅	R ₆	IC ₅₀ (μM) and efficacy (%) in FPR1-HL60	Sim.
2	O	CF ₃	OCH ₃	H	H	0.44 ± 0.13 (100)	0.576
3	S	CF ₃	Cl	H	H	0.42 ± 0.11 (100)	0.567
33	S	CF ₃	OCH ₃	H	H	N.A.	0.566
34	O	CF ₃	H	H	H	N.A.	0.579
35	S	H	OCH ₃	H	H	N.A.	0.558
36	O	CF ₃	Cl	H	H	0.38 ± 0.17 (100)	0.559
37	O	H	Cl	H	H	N.A.	0.563
38	S	CF ₃	H	H	H	3.9 ± 1.3 (90)	0.574
39	O	CF ₃	H	H	Cl	N.A.	0.558
40	S	CF ₃	H	H	OCH ₃	N.A.	0.556
41	S	CH ₃	H	H	OCH ₃	N.A.	0.539
42	S	H	H	H	H	N.A.	0.572
43	S	CH ₃	H	H	Cl	N.A.	0.541
44	S	CH ₃	H	OCH ₃	OCH ₃	N.A.	0.559
45	O	CH ₃	OCH ₃	H	H	1.9 ± 0.24 (100)	0.580
46	S	CH ₃	OCH ₃	H	H	2.6 ± 0.41 (100)	0.571
47	O	CH ₃	H	H	H	2.9 ± 0.32 (100)	0.581
48	S	CH ₃	H	H	H	7.2 ± 1.8 (95)	0.577
49	O	CH ₃	H	H	Br	N.A.	0.549
50	O	H	H	H	OCH ₃	N.A.	0.566
51	O	H	H	H	Cl	N.A.	0.565

B. 7-(2-Furoyloxy)- and 7-(2-thienoyloxy)-substituted isoflavones (2,3,33-54)

Compd	X	R ₃	R ₄	R ₅	R ₆	IC ₅₀ (μM) and efficacy (%) in FPR1-HL60	Sim.
52	O	H	H	H	Br	N.A.	0.551
53	O	COOCH ₂ CH ₃	H	H	OCH ₃	N.A.	0.542
54	O	COOCH ₂ CH ₃	OCH ₃	H	H	N.A.	0.536

C. 7-Benzoyloxy-substituted isoflavones (55-63)

Compd	R ₃	R ₄	R ₇	R ₈	R ₉	IC ₅₀ (μM) and efficacy (%) in FPR1-HL60	Sim.
55	CF ₃	OCH ₃	H	H	H	1.3 ± 0.19 (90)	0.562
56	CF ₃	OCH ₃	H	H	Cl	N.A.	0.548
57	CF ₃	OCH ₃	Cl	H	H	N.A.	0.551
58	CF ₃	OCH ₃	H	H	OCH ₃	N.A.	0.537
59	CF ₃	OCH ₃	H	OCH ₃	H	N.A.	0.549
60	CF ₃	OCH ₃	OCH ₃	H	H	N.A.	0.559
61	CF ₃	Cl	Br	H	H	N.A.	0.555
62	CF ₃	Cl	NO ₂	H	H	N.A.	0.549

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C. 7-Benzoyloxy-substituted isoflavones (55-63)

Compd	R ₃	R ₄	R ₇	R ₈	R ₉	IC ₅₀ (μM) and efficacy (%) in FPR1-HL60	Sim.
63	H	OCH ₃	H	H	H	N.A.	0.562

D. 2-Trifluoromethyl-2'-methoxy isoflavones (4,64-89)

Compd	R ₂	IC ₅₀ (μM) and efficacy (%)in FPR1-HL60	Sim.
4	OCO- <i>i</i> Pr	1.4 ± 0.3 (100)	0.591
64	OH	3.9 ± 1.2 (70)	0.600
65	OCH ₃	N.A.	0.530
66	OCH ₂ CH ₃	N.A.	0.549
67	OCH ₂ CN	N.A.	0.552
68	OCO- <i>cyclo</i> Pr	0.8 ± 0.3 (100)	0.590
69	OCO- <i>t</i> Bu	N.A.	0.564
70	OCOCH-Et ₂	N.A.	0.540
71	OCOCH ₂ - <i>t</i> Bu	N.A.	0.561
72	OCO- <i>cyclo</i> C ₆ H ₁₁	N.A.	0.577

D. 2-Trifluoromethyl-2'-methoxy isoflavones (4,64-89)				
Compd	R ₂	IC ₅₀ (μM) and efficacy (%)in FPRI-HL60	Sim.	
73	OCOOCH ₂ CH ₃	2.2 ± 0.6 (80)	0.596	
74	OCH ₂ COOH	6.1 ± 1.7 (100)	0.592	
75	OCH ₂ CONH ₂	15.6 ± 3.3 (65)	0.595	
76	OCH ₂ COOCH ₃	N.A.	0.562	
77	OCH ₂ COOCH ₂ CH ₃	N.A.	0.580	
78	OCH ₂ COO(CH ₂) ₂ CH ₃	N.A.	_a	
79	OCH ₂ COOCH(CH ₃) ₂	N.A.	-	
80	OCH ₂ COOCH ₂ CH(CH ₃) ₂	N.A.	-	
81	OCH ₂ COO(CH ₂) ₃ CH ₃	N.A.	-	
82	OCH(CH ₃)COOCH ₃	N.A.	-	
83	OCH ₂ C≡C(CH ₃) ₂	N.A.	-	
84	OCH ₂ (CH ₃)(=CH ₂)	N.A.	-	
85	OCH ₂ COO(CH ₂) ₄ CH ₃	N.A.	-	
86	OCH(CH ₃)COOCH(CH ₃) ₂	N.A.	-	
87	OCH(CH ₃)COO(CH ₂) ₃ CH ₃	N.A.	-	
88	OCH(CH ₃)COOCH ₂ CH(CH ₃) ₂	N.A.	-	
89	OCH(CH ₂ CH ₃)COOCH ₂ CH ₃	N.A.	-	

E. Miscellaneous natural and synthetic isoflavones (90-103)
90-101					103				
Compd	R ₂	R ₃	R ₄	R ₁₀	R ₁₁	IC ₅₀ (μM) and efficacy (%) in FPR1-HL60	Sim.		
90 ^a	OH	H	H	H	H	N.A.	0.530		
91	OH	H	H	OH	H	N.A.	0.569		
92	OH	H	H	OCH ₃	H	N.A.	0.561		
93	OH	H	H	OH	OH	N.A.	0.566		
94	OH	H	H	OCH ₃	OH	10.3 ± 1.7 (80)	0.551		
95	OH	CF ₃	H	OCH ₃	OH	2.6 ± 0.8 (100)	0.591		
96	OH	CH ₃	H	OCH ₃	H	N.A.	0.558		
97	OH	CF ₃	H	OCH ₃	H	N.A.	0.554		
98	OH	CH ₃	OCH ₃	H	H	23.1 ± 1.8 (50)	0.608		
99	OH	CH ₃	OCH ₃	H	OH	N.A.	0.584		
100	OCOCH ₃	CH ₃	OCH ₃	H	H	6.9 ± 2.7 (100)	0.596		
101	OCH ₂ COOCH ₂ CH ₃	CH ₃	OCH ₃	H	H	N.A.	0.588		
102	OCH ₂ COOH	CH ₃	H	OCH ₃	H	N.A.	0.553		
103						N.A.	0.593		

^a Substituents at similar positions of the molecules in series A–E have the same numbers in the enumeration scheme chosen in this paper.

^b Similarity (Sim.) between aligned molecule and the template was calculated using the FieldAlign program.

N.A.: No activity was observed at the highest tested concentration (50 μM).

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^a Not calculated.

N.A.: No activity was observed at the highest tested concentration (50 μM).

^a Common names of the natural isoflavones are: **90**, 7-hydroxy isoflavone; **91**, daidzein; **92**, formononetin; **93**, genistein; **94**, biochanin A; **103**, afformosin.
N.A.: No activity was observed at the highest tested concentration (50 μM).

Table 2

Antagonist activity and binding affinity of selected 4*H*-chromen-4-ones^a

Compd	Competition Binding		Calcium Flux				Chemotaxis	
	FPR1-RBL	FPR1-HL60	FPR2-HL60	FPR3-HL60	Fpr1-RBL	mPMN	hPMN	hPMN
	K _i (μM)		IC ₅₀ (μM)					
1	0.85	0.65	N.A.	N.A.	N.A.	N.A.	1.3	0.027
2	2.5	1.2	N.A.	N.A.	N.A.	N.A.	0.48	0.18
3	2.9	6.1	N.A.	N.A.	N.A.	N.A.	0.15	0.08
4	2.6	1.6	N.A.	N.A.	N.A.	N.A.	1.1	0.20
10	0.09	0.11	N.A.	N.A.	N.A.	N.A.	0.12	0.024
27	3.5	5.7	N.A.	N.A.	N.A.	N.A.	5.1	0.41
36	6.1	0.79	N.A.	N.A.	N.A.	N.A.	0.65	0.05
38	3.8	6.5	N.A.	N.A.	N.A.	N.A.	0.69	0.64
45	3.6	1.7	N.A.	N.A.	N.A.	N.A.	3.5	0.43
46	4.0	4.1	N.A.	N.A.	N.A.	N.A.	2.6	0.32
47	2.0	4.0	N.A.	N.A.	N.A.	N.A.	20.2	0.88
55	(45%) ^b	(30%)	N.A.	N.A.	N.A.	N.A.	4.5	0.12
68	(45%)	(30%)	N.A.	N.A.	N.A.	N.A.	0.9	0.36
73	5.6	5.1	N.A.	N.A.	N.A.	N.A.	1.7	1.0

^a Antagonist activity was evaluated as inhibition of Ca²⁺ mobilization induced by 5 nM WKYMVM in FPR2-HL60 cells, 10 nM WKYMVM in FPR3-HL60 cells, 5 nM WKYMVM in Fpr1-RBL cells and murine neutrophils (mPMN), or 5 nM fMLF in human neutrophils (hPMN). Inhibition of chemotactic activity in human neutrophils was evaluated in the presence of 1 nM fMLF.

^b Binding did not exceed 50% inhibition at the highest tested concentration (50 μM), so K_i values for these compounds were not calculated. Chemical names for the selected compounds are: **1**, 6-ethyl-2-methyl-3-(1-methyl-1*H*-benzimidazol-2-yl)-4-oxo-4*H*-chromen-7-yl acetate; **2**, 3-(2-methoxyphenyl)-4-oxo-2-(trifluoromethyl)-4*H*-chromen-7-yl 2-furoate; **3**, 3-(2-chlorophenyl)-4-oxo-2-(trifluoromethyl)-4*H*-chromen-7-yl thiophene-2-carboxylate; **4**, 3-(2-methoxyphenyl)-4-oxo-2-(trifluoromethyl)-4*H*-chromen-7-yl 2-methylpropanoate; **10**, 6-hexyl-2-methyl-3-(1-methyl-1*H*-benzimidazol-2-yl)-4-oxo-4*H*-chromen-7-yl acetate; **27**, 2-methyl-3-(1-methyl-1*H*-benzimidazol-2-yl)-4-oxo-2-(trifluoromethyl)-4*H*-chromen-7-yl furan-2-carboxylate; **36**, 3-(2-chlorophenyl)-4-oxo-2-(trifluoromethyl)-4*H*-chromen-7-yl furan-2-carboxylate; **38**, 4-oxo-3-phenyl-2-(trifluoromethyl)-4*H*-chromen-7-yl thiophene-2-carboxylate; **45**, 3-(2-methoxyphenyl)-4-oxo-4*H*-chromen-7-yl furan-2-carboxylate; **46**, 3-(2-methoxyphenyl)-2-methyl-4-oxo-4*H*-chromen-7-yl thiophene-2-carboxylate; **47**, 2-methyl-4-oxo-3-phenyl-4*H*-chromen-7-yl furan-2-carboxylate; **55**, 3-(2-methoxyphenyl)-4-oxo-2-(trifluoromethyl)-4*H*-chromen-7-yl benzoate; **68**, 3-(2-methoxyphenyl)-4-oxo-2-(trifluoromethyl)-4*H*-chromen-7-yl cyclopropanecarboxylate; **73**, ethyl 3-(2-methoxyphenyl)-4-oxo-2-(trifluoromethyl)-4*H*-chromen-7-yl carbonate.

N.A.: No activity was observed at the highest tested concentration (50 μM).