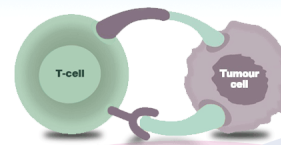


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Identification of Peptides That Antagonize Formyl Peptide Receptor-Like 1-Mediated Signaling¹

Yoe-Sik Bae,^{2*} Ha Young Lee,* Eun Jin Jo,* Jung Im Kim,* Hyun-Kyu Kang,* Richard D. Ye,[†] Jong-Young Kwak,* and Sung Ho Ryu[‡]

Formyl peptide receptor-like 1 (FPRL1) is an important classical chemoattractant receptor that is expressed in phagocytic cells in the peripheral blood and brain. Recently, various novel agonists have been identified from several origins, such as host-derived molecules. Activation of FPRL1 is closely related to inflammatory responses in the host defense mechanism and neurodegenerative disorders. In the present study we identified several novel peptides by screening hexapeptide libraries that inhibit the binding of one of FPRL1's agonists (Trp-Lys-Tyr-Met-Val-D-Met-CONH₂ (WKYMVm)) to its specific receptor, FPRL1, in RBL-2H3 cells. Among the novel peptides, Trp-Arg-Trp-Trp-Trp-Trp-CONH₂ (WRWWWW (WRW⁴)) showed the most potent activity in terms of inhibiting WKYMVm binding to FPRL1. We also found that WRW⁴ inhibited the activation of FPRL1 by WKYMVm, resulting in the complete inhibition of the intracellular calcium increase, extracellular signal-regulated kinase activation, and chemotactic migration of cells toward WKYMVm. For the receptor specificity of WRW⁴ to the FPR family, we observed that WRW⁴ specifically inhibit the increase in intracellular calcium by the FPRL1 agonists MMK-1, amyloid β 42 (A β 42) peptide, and F peptide, but not by the FPR agonist, fMLF. To investigate the effect of WRW⁴ on endogenous FPRL1 ligand-induced cellular responses, we examined its effect on A β 42 peptide in human neutrophils. A β 42 peptide-induced superoxide generation and chemotactic migration of neutrophils were inhibited by WRW⁴, which also completely inhibited the internalization of A β 42 peptide in human macrophages. WRW⁴ is the first specific FPRL1 antagonist and is expected to be useful in the study of FPRL1 signaling and in the development of drugs against FPRL1-related diseases. *The Journal of Immunology*, 2004, 173: 607–614.

Formyl peptide receptor-like 1 (FPRL1),³ which was initially cloned as a formyl peptide receptor (FPR) homologue by low stringency hybridization with the human FPR cDNA probe (1), is one of the classic chemoattractant receptors, i.e., a G protein-coupled seven-transmembrane receptor. Previous reports suggested the role of FPRL1 in the regulation of various cellular responses in several cell types: Ca²⁺-dependent Cl[−] secretion by human airway epithelial cells (2); the involvement in host defense in the brain by astrocytoma cell lines (3); the contribution to innate and adaptive immunities by recruiting neutrophils, monocytes, and T cells to sites of microbial invasion (4); and the induction of hyperadhesiveness in HUVECs to increase in the binding of neutrophils (5).

FPRL1 has important roles in the regulation of immune responses against pathogen infection by modulating the activities of phagocytes (6, 7). In particular, FPRL1 has been reported to mediate phagocyte chemotaxis (8, 9) and to cause superoxide generation and exocytosis in human neutrophils. FPRL1 also has been reported to attenuate HIV-1 infection by desensitizing important chemokine receptors (CCR5 and CXCR4) that act as coreceptors of HIV infection (10). FPRL1, which was found to be highly expressed in mononuclear phagocytes that infiltrated the brain tissues of Alzheimer's disease patients, also plays a role in the neuronal system. FPRL1 has important implications in several disease states, such as amyloidosis, neurodegenerative disease, and prion disease (11–13).

Recently, several FPRL1 agonists have been identified. They include host-derived agonists, such as LL-37 and a mitochondrial peptide fragment. MYFINILTL (4, 14). One of the important lipid mediators, lipoxin A4 (LXA4), also has been reported to bind to FPRL1 (14). Some peptides (T21/DP107, F peptide, and V3 peptide) derived from HIV-1 envelope proteins have been demonstrated to bind FPRL1 (15–17). Trp-Lys-Tyr-Met-Val-D-Met-CONH₂ (WKYMVm), a potent leukocyte-stimulating synthetic peptide (18, 19), was also reported to be a potent peptide ligand for FPRL1 (20).

Although the recruitment of phagocytes into sites of tissue damage and their activation are required for the immune responses, excess recruitment of phagocytes into the infection site causes several adverse effects, for example, tissue damage and inflammatory disease (21). In terms of FPRL1, although the receptor has been believed to play a critical role in the innate immune responses and in some neurodegenerative diseases, FPRL1-specific antagonists have not been reported. To reveal the role of FPRL1 in physiological and pathological conditions, specific FPRL1 antagonists would be highly desirable.

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³ Abbreviations used in this paper: FPRL1, formyl peptide receptor-like 1; FPR, formyl peptide receptor; LXA4, lipoxin A4; WKYMVm, Trp-Lys-Tyr-Met-Val-D-Met-CONH₂; fura-2/AM, fura-2 penta-acetoxymethyl ester; A β 42, amyloid β 42; ERK, extracellular signal-regulated kinase; PS-SPCL, positional scanning synthetic peptide combinatorial library; [Ca²⁺]_i, intracellular calcium concentration; HSA, human serum albumin; WRW⁴, Trp-Arg-Trp-Trp-Trp-Trp-CONH₂; RHW⁴, Arg-His-Trp-Trp-Trp-Trp-CONH₂; DRW⁴, Asp-Arg-Trp-Trp-Trp-Trp-CONH₂; RRW⁴, Arg-Arg-Trp-Trp-Trp-Trp-CONH₂; LFMYHP, Leu-Phe-Met-Tyr-His-Phe-CONH₂; WKYMVM, Tyr-Met-Val-Met-CONH₂.

Among the known ligands for FPRL1, WKYMVm, which has potent phagocytic activity with only six amino acids, has several merits with respect to FPRL1 antagonist screening. In this study we identified novel FPRL1 antagonist peptides by screening hexapeptide libraries. We also found that the novel peptides could block FPRL1-induced cellular signaling and cellular responses. We expect that these novel peptides will prove useful in the study of FPRL1-mediated physiological responses.

Materials and Methods

Materials

F-moc amino acids were obtained from Millipore (Bedford, MA). Rapid-amide resin was obtained from DuPont (Boston, MA). PBM separation medium (Histopaque-1077) and cytochrome *c* were purchased from Sigma-Aldrich (St. Louis, MO). Fura-2 penta-acetoxymethyl ester (fura-2/AM) was obtained from Molecular Probes (Eugene, OR). RPMI 1640 was obtained from Invitrogen (Carlsbad, CA), and dialyzed FBS and supplemented bovine serum were purchased from HyClone Laboratories (Logan, UT). Radioiodinated WKYMVm (125 I-labeled), which was prepared by conjugation with monoiodinated 125 I-labeled Bolton and Hunter reagent using a method developed by Nycomed Amersham as described in the product information sheet of the producer (Amersham Pharmacia Biotech), was a gift from Amersham Pharmacia Biotech (Little Chalfont, U.K.). Biotinylated WRWWWW was prepared as described previously (22). Amyloid β 42 (A β 42) was purchased from Bachem Bioscience (King of Prussia, PA). LXA4 was obtained from Biomol (Plymouth Meeting, PA). Anti-phospho-extracellular signal-regulated kinase (ERK) Abs and anti-ERK Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-A β 42 Ab was obtained from Zymed Laboratories (South San Francisco, CA).

Positional scanning synthetic peptide combinatorial libraries

The hexapeptide libraries were prepared by the Peptide Library Support Facility of Pohang University of Science and Technology, as described previously (18, 19). Briefly, peptides were synthesized on a Rapidamide support resin and assembled by following the standard F-moc/*t*-butyl strategy on an acid-labile linker. Peptide compositions were confirmed by amino acid analysis, as previously described (18, 19). Finally, 114 peptide pools (Cys was excluded in the construction of the libraries) were individually dissolved in water at a final concentration of 27 nM/peptide sequence in each peptide pool. Peptides were synthesized using a previously described solid phase method (18).

Cell culture

FPRL1-expressing RBL-2H3 cells and vector-transfected RBL-2H3 cells were maintained as previously described (23). Human neutrophils and PBMCs were isolated from healthy donors, as previously described (24). PBMCs were differentiated to macrophages by culturing the cells on four-well chamber slides (Nalge Nunc International, Rochester, NY) in RPMI 1640 medium containing 0.1% BSA, 0.01 M HEPES (pH 7.4), and 20 ng/ml monocyte CSF (PeproTech, Rocky Hill, NJ).

Screening of peptide libraries and ligand binding analysis

For the initial screening of the positional scanning synthetic peptide combinatorial libraries (PS-SPCLs), we measured the effect of each peptide pool on the binding of 125 I-labeled WKYMVm to its specific receptor, FPRL1 in RBL-2H3 cells. Ligand binding analysis was performed as previously described (25). Briefly, FPRL1-expressing RBL-2H3 cells were seeded at 1×10^5 cells/well into a 24-well plate and cultured overnight. After blocking the cells with blocking buffer (33 mM HEPES (pH 7.5) and 0.1% BSA in RPMI 1640) for 2 h, 50 pM labeled WKYMVm was added to the cells in binding buffer (PBS containing 0.1% BSA) in the absence or the presence of unlabeled peptides (final concentration, 0.5 nM/peptide sequence for the initial screening), then incubated for 3 h at 4°C with continuous shaking. The samples were then washed five times with ice-cold binding buffer, and 200 μ l of lysis buffer (20 mM Tris (pH 7.5) and 1% Triton X-100) was added to each well. After 20 min at room temperature, the lysates were collected and counted using a gamma counter (25).

Flow cytometric analysis

To determine whether WRWWWW binds to FPRL1, FPRL1-expressing RBL-2H3 cells were labeled with 10 μ M biotin-WRWWWW for 30 min in PBS containing 0.02% sodium azide. The unbound biotin-WRWWWW

was washed extensively with PBS containing 0.2% BSA and 0.02% sodium azide. Then the cells were incubated with 5 μ g/ml streptavidin-FITC at 4°C for 40 min in the dark. Subsequently, cells were washed twice in washing solution (PBS plus BSA and azide) and fixed with 0.2% paraformaldehyde. Fixed cells were analyzed in single laser for FITC with FACSCalibur (BD Biosciences, San Jose, CA).

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was determined by Grynkiewicz's method using fura-2/AM (24). For DNP-human serum albumin (DNP-HSA) stimulation, RBL-2H3 cells were sensitized with 1 μ g/ml mouse DNP-specific IgE overnight before fura-2 loading (26).

Stimulation of cells with peptides for Western blot analysis

FPRL1-expressing RBL-2H3 cells or isolated human neutrophils (2×10^6) were stimulated with the indicated concentrations of peptides for predetermined times. After stimulation, the cells were washed with serum-free RPMI 1640 and lysed in lysis buffer (20 mM HEPES (pH 7.2), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4 , 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 1 mM PMSF). Detergent-insoluble materials were pelleted by centrifugation ($12,000 \times g$, 15 min, 4°C), and the soluble supernatant fraction was removed and either stored at -80°C or used immediately. Protein concentrations in the lysates were determined using the Bradford protein assay reagent.

Electrophoresis and Western blot analysis

Proteins were separated in 8% SDS-polyacrylamide gel, and the proteins were blotted onto a nitrocellulose membrane, which was then blocked by incubating with Tris-buffered saline/0.05% Tween 20 containing 5% non-fat dry milk. Subsequently, membranes were incubated with specific Abs and washed with TBS. Ag-Ab complexes were visualized after incubating the membrane with 1/5000 diluted goat anti-rabbit IgG or goat anti-mouse IgG Ab coupled to HRP and were detected by ECL.

Chemotaxis assay

Chemotaxis assays were performed using multiwell chambers (NeuroProbe, Gaithersburg, MD), as described previously (24, 27). Migrated cells in five randomly chosen high power fields ($\times 400$) were then counted.

Measurement of superoxide generation

Superoxide anion generation was determined by measuring cytochrome *c* reduction using a microtiter 96-well plate ELISA reader (EL312e; Bio-Tek Instruments, Winooski, VT) as previously described (24). Superoxide generation was determined for the change in light absorption at 550 nm over 5 min at 1-min intervals.

Fluorescence confocal microscopy

Confocal microscopic analysis using anti-A β 42 Ab was performed as described previously (28). Briefly, human macrophages grown on four-well chamber slides were treated with 10 μ M A β 42 peptide in the absence or the presence of 10 μ M WRWWWW for different periods at 37°C. The cells were then fixed in 4% paraformaldehyde for 10 min at room temperature, washed with PBS, and incubated with 5% goat serum (Sigma-Aldrich) in PBS containing 0.05% Tween 20 for 1 h to block nonspecific binding and permeabilization. Samples were incubated with anti-A β 42 Ab for 1 h at room temperature, and slides were washed three times with PBS and incubated with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich; 1/500 dilution in TBS containing 3% BSA) for 30 min. Mounted samples were observed under a laser scanning confocal fluorescence microscope (Zeiss, Oberkochen, Germany).

Results

Identification of peptides that inhibit the binding of WKYMVm to FPRL1

In this study a total of 114 peptide pools (~47 million peptides) from hexapeptide PS-SPCLs were screened to identify peptides that inhibit the binding of WKYMVm to its cell surface receptor. Fig. 1 shows the results of the initial screening. We observed that amino acids at different positions induced different levels of inhibition of WKYMVm binding to FPRL1. The results shown in Fig. 1 are representative of four independent experiments. The results were very reproducible, because most of the SE values were

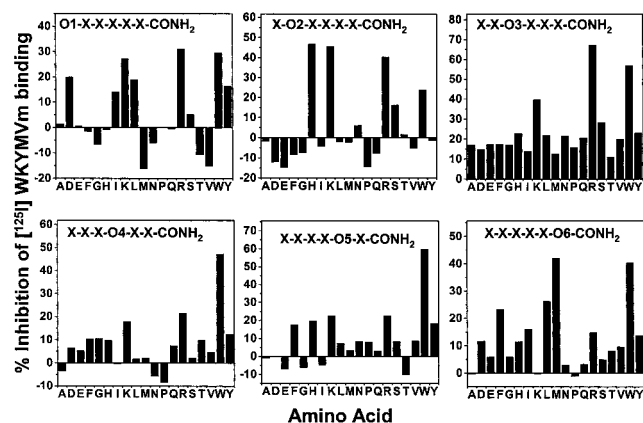


FIGURE 1. The initial screening of the PS-SPCLs for peptides that inhibit the binding of ^{125}I -labeled WKYMVm (50 pM) in FPRL1-expressing RBL-2H3 cells. FPRL1-expressing RBL-2H3 cells (1×10^5 cells/200 μl) were used for the binding assay. The ligand binding assay was monitored. The results shown are representative of four independent experiments.

<15% of each mean value. The most active peptides and position were as follows: Lys (K), Arg(R), or Trp(W) in the first position; His (H), Lys(K), or Arg(R) in the second position; Arg(R) or Trp (W) in the third position; Trp (W) in the fourth position; Trp (W) in the fifth position; and Met (M) or Trp (W) in the sixth position of hexapeptides.

Based on the results of the first screening of the peptide libraries, we generated, by reiterative synthesis, four peptide pools, each containing $3 \times 3 \times 1 \times 1 \times 1 \times 1 = 9$ individual hexapeptides. Each peptide mixture contained (D/R/W)(H/K/R)RWWM, (D/R/W)(H/K/R)RWWW, (D/R/W)(H/K/R)WWWM, or (D/R/W)(H/K/R)WWWW. The effect of each peptide mixture on the binding of labeled WKYMVm to FPRL1 was examined. A peptide mixture with the sequence (D/R/W)(H/K/R)WWWW showed the greatest activity in terms of competing with WKYMVm binding to FPRL1 (data not shown). The peptide mixture was purified into four fractions by reverse phase HPLC using a C_{18} column (Vydac, Hesperia, CA; 218TP1022, 22×250 mm). The effectiveness of the peptides at inhibiting WKYMVm binding to FPRL1 in RBL-2H3 cells was evaluated using the method described for the initial screening. Peptides within the fraction with the greatest activity were identified by mass spectrometry. After confirming the effects of the synthesized single peptides on the inhibition of labeled WKYMVm, we finally identified Trp-Arg-Trp-Trp-Trp-CONH₂ (WRWWWW (WRW⁴)), Arg-His-Trp-Trp-Trp-CONH₂ (RHW WWW (RHW⁴)), Asp-Arg-Trp-Trp-Trp-CONH₂ (DRWWWW (DRW⁴)), and Arg-Arg-Trp-Trp-Trp-CONH₂ (RRWWWW (RRW⁴)) as being the most active peptide in terms of inhibiting the binding of WKYMVm to FPRL1 in RBL-2H3 cells. The inhibitory effects of the four hexapeptides were tested at several concentrations (Fig. 2). The peptide WRW⁴ was found to most effectively inhibit ^{125}I -labeled WKYMVm binding. Three other peptides, RHW⁴, DRW⁴, and RRW⁴, also effectively inhibited the binding of labeled WKYMVm (Fig. 2). As a negative control experiment, we confirmed that the random sequence of the hexapeptide (Leu-Phe-Met-Tyr-His-Pro-CONH₂ (LFMYHP)) caused no inhibitory effect at up to 10 μM (Fig. 2). The IC₅₀ values for the inhibition of 50 pM ^{125}I -labeled WKYMVm binding to FPRL1 were 0.23 μM for WRW⁴, 3.2 μM for RHW⁴, 2.4 μM for RRW⁴, and 3.4 μM for DRW⁴.

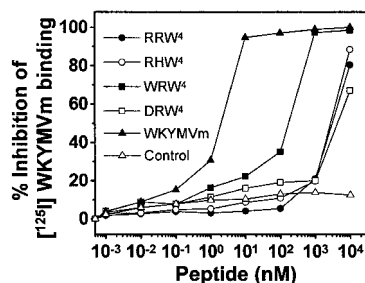


FIGURE 2. Effects of several candidate peptides based on the PS-SPCL screening results with regard to the inhibition of WKYMVm binding in FPRL1-expressing RBL-2H3 cells. FPRL1-expressing RBL-2H3 cells (1×10^5 cells/200 μl) were used for binding assay, and various concentrations of each unlabeled peptide (RRW⁴, RHW⁴, WRW⁴, and DRW⁴) were pretreated before the addition of ^{125}I -labeled WKYMVm (50 pM). Specifically bound ^{125}I -labeled WKYMVm was measured. The results shown are representative of four independent experiments.

The novel peptide, WRW⁴, specifically binds to FPRL1

To confirm that WRW⁴ binds to FPRL1, we synthesized a peptide probe, biotin-WRW⁴. At first we tested whether the peptide inhibited the binding of ^{125}I -labeled WKYMVm to FPRL1. The peptide probe biotin-WRW⁴ was also found to effectively inhibit ^{125}I -labeled WKYMVm binding (data not shown). Then, we performed a more rigorous experiment using flow cytometric analysis for the specific receptor identification in FPRL1-expressing RBL-2H3 cells. For flow cytometric analysis, vector- or FPRL1-expressing RBL-2H3 cells were labeled with 10 μM of the peptide probe, biotin-WRW⁴, and subsequently with streptavidin-FITC (5 $\mu\text{g}/\text{ml}$). Although FPRL1-expressing RBL-2H3 cells are bound by peptide and thereby stained by FITC (Fig. 3A), vector-expressing RBL-2H3 cells are not bound with the peptide (Fig. 3B). Furthermore, staining of FPRL1-expressing RBL-2H3 cells with biotin-WRW⁴ was almost completely inhibited by the addition of 30 μM unlabeled WKYMVm (Fig. 3A). The results indicate that WRW⁴ specifically binds to FPRL1.

The novel peptide, WRW⁴, inhibited FPRL1-mediated $[\text{Ca}^{2+}]_i$ increase

The stimulation of FPRL1 by WKYMVm elicited a $[\text{Ca}^{2+}]_i$ increase in FPRL1-expressing RBL-2H3 cells (Fig. 4A). To investigate whether the novel peptides inhibited WKYMVm-induced $[\text{Ca}^{2+}]_i$ increase, we stimulated fura-2-loaded FPRL1-expressing RBL-2H3 cells with various concentrations of WRW⁴, and subsequently stimulated cells with an effective concentration of WKYMVm. As shown in Fig. 4A, 10 μM WRW⁴ alone did not change the intracellular calcium concentration. However, 10 μM WRW⁴ pretreatment before stimulation with 10 nM WKYMVm completely inhibited the $[\text{Ca}^{2+}]_i$ increase induced by WKYMVm in FPRL1-expressing RBL-2H3 cells (Fig. 4A). To confirm specific inhibition by WRW⁴ on FPRL1-induced signaling, we examined the effect of WRW⁴ on another extracellular signal-induced $[\text{Ca}^{2+}]_i$ increase. Fc ϵ RI cross-linking has been reported to induce a $[\text{Ca}^{2+}]_i$ increase in RBL-2H3 cells (26). Stimulation of FPRL1-expressing RBL-2H3 cells (sensitized with 1 $\mu\text{g}/\text{ml}$ mouse DNP-specific IgE) with 1 $\mu\text{g}/\text{ml}$ DNP-HSA caused a dramatic increase in $[\text{Ca}^{2+}]_i$ (Fig. 4A). However, pretreatment with 10 μM WRW⁴ before DNP-HSA stimulation did not significantly change the DNP-HSA-induced $[\text{Ca}^{2+}]_i$ increase in RBL-2H3 cells (Fig. 4A). These results indicate that the WRW⁴-induced inhibition of $[\text{Ca}^{2+}]_i$ increase is a FPRL1-specific event. On examining the concentration-dependency of the WRW⁴-induced inhibition of

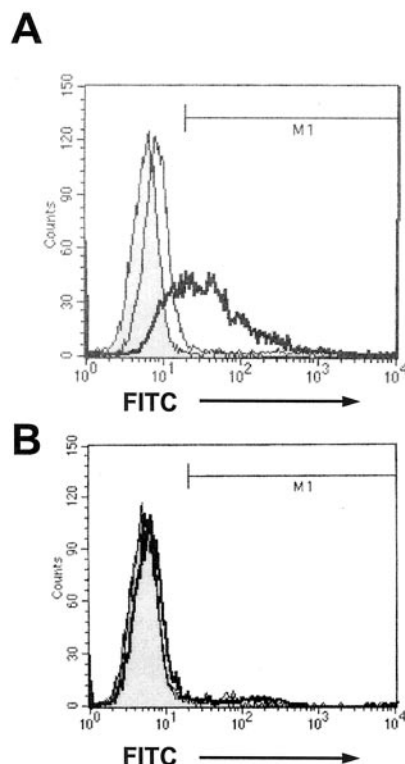


FIGURE 3. Flow cytometric analysis of FPRL1- or vector-expressing RBL-2H3 cells with biotin-WRW⁴. Cultured FPRL1-expressing (A) or vector-expressing (B) RBL-2H3 cells (1×10^7 /ml) were labeled with 10 μ M biotin-WRW⁴ in the absence (bold solid line) or the presence (solid line) of 30 μ M WKYMVm. The samples were further incubated with streptavidin-FITC, fixed with 0.2% paraformaldehyde, and analyzed in FACSCalibur (BD Biosciences). The shaded area indicates unstained cells.

[Ca²⁺]_i increase by WKYMVm, we observed that WRW⁴ inhibited the WKYMVm-mediated process in a concentration-dependent manner, showing maximal inhibition at 10 μ M (Fig. 4B); 1 μ M WRW⁴ inhibited WKYMVm-induced [Ca²⁺]_i increase by ~55% (Fig. 4B). These results strongly suggest that WRW⁴ is a specific antagonist of FPRL1.

WRW⁴ inhibits FPRL1-mediated ERK phosphorylation

To support our idea that WRW⁴ peptide antagonizes FPRL1 signaling, we examined its effect on WKYMVm-induced ERK phosphorylation. Thus, we stimulated FPRL1-expressing RBL-2H3 cells with various concentrations of WRW⁴ and measured the ERK phosphorylation level by Western blot with an anti-phospho-ERK antibody. As shown in Fig. 5A, WRW⁴ alone did not affect ERK phosphorylation in the cells. However, when we stimulated the cells with 10 nM WKYMVm for 2 min, we observed a dramatic increase in the ERK phosphorylation level (Fig. 5A). Moreover, pretreatment with WRW⁴ inhibited WKYMVm-elicited ERK phosphorylation in a concentration-dependent manner (Fig. 5A), and pretreatment with an inactive control peptide, LFMYHP, did not inhibit ERK phosphorylation event induced by WKYMVm (Fig. 5A). In addition, we confirmed that same amounts of proteins were used for this experiment by Western blot with anti-ERK Ab (Fig. 5A). To demonstrate that WRW⁴ does not inhibit ERK phosphorylation induced by non-FPRL1 agonist, we performed a control experiment using DNP-HSA. Preincubation of FPRL1-expressing RBL-2H3 cells with 10 μ M before the stimulation with DNP-HSA did not affect DNP-HSA-induced ERK phosphorylation. Fig. 5B shows the quantitative inhibitory effect of WRW⁴ in terms of ERK phosphorylation by WKYMVm or DNP-

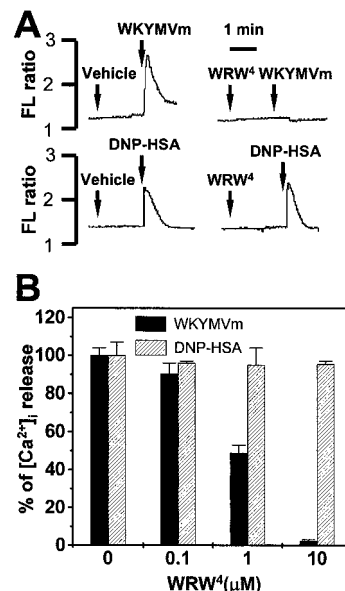


FIGURE 4. Effect of WRW⁴ on the WKYMVm-induced [Ca²⁺]_i increase in FPRL1-expressing RBL-2H3 cells. Cells were stimulated with vehicle or WRW⁴ (10 μ M) and then with WKYMVm (10 nM) or the Ag DNP-HSA (1 μ g/ml). The changes in 340/380 nm were monitored. The results are representative of three independent experiments (A). Cells were stimulated with various concentrations of WRW⁴ before adding 10 nM WKYMVm or 1 μ g/ml DNP-HSA. The results shown are the mean \pm SE of four independent experiments (B).

HSA. These results indicate that WRW⁴ specifically blocked ERK phosphorylation downstream of FPRL1 by WKYMVm.

WRW⁴ inhibits FPRL1-mediated cellular chemotaxis

FPRL1 is a classical chemoattractant receptor, and a previous report demonstrated that WKYMVm induces cellular chemotaxis via FPRL1 (20), which is one of the more important physiological roles of FPRL1. Thus, we investigated the effect of the novel FPRL1 antagonist, WRW⁴, on WKYMVm-induced chemotaxis by examining the effect of WRW⁴ alone on the chemotaxis of FPRL1-expressing RBL-2H3 cells at various peptide concentrations. As shown in Fig. 6A, WKYMVm caused cellular chemotaxis in FPRL1-expressing RBL-2H3 cells in a concentration-dependent manner. However, WRW⁴ alone showed no effect on cellular chemotaxis at concentrations of 1 nM to 10 μ M in FPRL1-expressing RBL-2H3 cells (Fig. 6A). We then checked the effect of WRW⁴ on WKYMVm-induced cellular chemotaxis in FPRL1-expressing RBL-2H3 cells and found that the addition of several concentrations of WRW⁴ before chemotaxis assay using 10 nM WKYMVm caused a concentration-dependent inhibition of WKYMVm-induced chemotaxis of the cells (Fig. 6B). WRW⁴ (1 μ M) blocked ~60% of WKYMVm-induced chemotaxis, and 10 μ M WRW⁴ almost completely blocked this WKYMVm-induced process (Fig. 6B). The addition of 10 μ M of an inactive control peptide, LFMYHP, did not affect WKYMVm-induced chemotaxis (Fig. 6B). These results demonstrate that WRW⁴ blocks WKYMVm-induced chemotaxis.

WRW⁴ specifically inhibits FPRL1-induced cell signaling in human neutrophils

The effect of WRW⁴ on endogenous ligand-induced FPRL1 signaling was investigated in human neutrophils. A β 42 peptide has previously been reported to act as an endogenous ligand for FPRL1 (12). We found that treatment of human neutrophils with

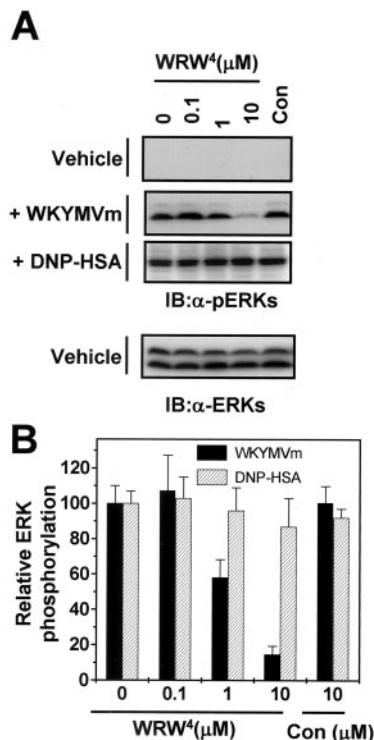


FIGURE 5. Effect of WRW⁴ on WKYMVm-stimulated ERK phosphorylation in FPRL1-expressing RBL-2H3 cells. FPRL1-expressing RBL-2H3 cells were treated with various concentrations of WRW⁴ for 5 min, then stimulated with vehicle, 10 nM WKYMVm, or 1 μg/ml DNP-HSA for 5 min (A). Phosphorylated ERK was determined by immunoblot analysis with anti-phospho-ERK Ab (A). ERK phosphorylation was quantified by densitometry. Results are presented as the mean ± SE of at least six independent experiments (B).

40 μM Aβ42 peptide dramatically changes $[Ca^{2+}]_i$ (Fig. 7A), but WRW⁴ alone did not affect this $[Ca^{2+}]_i$ increase in human neutrophils (Fig. 7A). However, when the neutrophils were pretreated with 10 μM WRW⁴ before stimulation with 40 μM Aβ42 peptide, this Aβ42 peptide-induced $[Ca^{2+}]_i$ increase was completely inhibited (Fig. 7A). To determine the specificity of WRW⁴ on FPRL1 signaling, we examined the effect of WRW⁴ on fMLF-stimulated $[Ca^{2+}]_i$ increase in human neutrophils. Stimulation with 1 μM fMLF caused a transient $[Ca^{2+}]_i$ increase, but the preincubation of human neutrophils with 10 μM WRW⁴ had no effect on the $[Ca^{2+}]_i$ increase by fMLF (Fig. 7A). We also tested the effect of WRW⁴ on other FPRL1-specific agonists (MMK-1 and F peptide)-induced $[Ca^{2+}]_i$ increase. As shown in Fig. 7B, when the cells were pretreated with 10 μM WRW⁴ before stimulation with 1 μM MMK-1 and 30 μM F peptide, the increase in $[Ca^{2+}]_i$ caused by the two FPRL1 agonists was completely inhibited. Furthermore, the $[Ca^{2+}]_i$ increase induced by 1.4 μM LXA4 was also inhibited by 10 μM WRW⁴ (Fig. 7B). The result indicates that WRW⁴ inhibits all tested FPRL1 ligand-induced $[Ca^{2+}]_i$ increases in human neutrophils. However, stimulation with 500 μM ATP caused a transient $[Ca^{2+}]_i$ increase that was not inhibited by preincubation of human neutrophils with 10 μM WRW⁴ (Fig. 7B). These results indicate that WRW⁴ specifically inhibits the FPRL1-induced, but not the FPR-induced, signaling event in human neutrophils.

WRW⁴ inhibits Aβ42 peptide-induced superoxide generation and chemotactic migration of neutrophils

Superoxide generation is one of the important functions of phagocytic leukocytes such as neutrophils (29). In this study we found

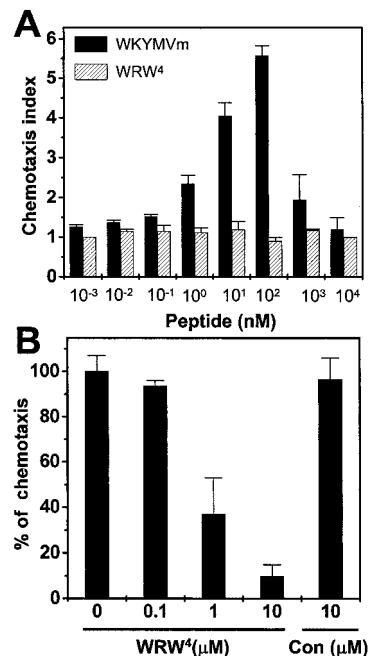


FIGURE 6. Effect of WRW⁴ on WKYMVm-induced cellular chemotaxis in FPRL1-expressing RBL-2H3 cells. Cultured FPRL1-expressing RBL-2H3 cells (1×10^6 cells/ml RPMI 1640) were added to the upper wells of a 96-well chemotaxis chamber for 4 h at 37°C. The numbers of migrated cells were determined by counting in a high power field ($\times 400$). Various concentrations of WRW⁴ or WKYMVm were used in the assays (A). Several concentrations of WRW⁴ or 10 μM LFMYPH were added before the chemotaxis assay using 10 nM WKYMVm (B). Results are presented as the mean ± SE of three independent experiments, each performed in duplicate.

that Aβ42 increased superoxide generation in human neutrophils. Moreover, this Aβ42 peptide-induced superoxide-generating activity was concentration dependent and showed a maximal effect at a peptide concentration of 40 μM (Fig. 8A). The addition of WRW⁴ to human neutrophils at up to 100 μM did not affect superoxide generation (Fig. 8A). However, when neutrophils were preincubated with various concentrations of WRW⁴, Aβ42 peptide-induced superoxide generation was inhibited in a concentration-dependent manner (Fig. 8B), e.g., 10 μM WRW⁴ almost completely inhibited superoxide production by Aβ42 peptide (Fig. 8B). As a control experiment we examined the effect of inactive control peptide (LFMYHP) on Aβ42 peptide-induced superoxide generation in human neutrophils. Preincubation with several concentrations of LFMYPH before Aβ42 peptide stimulation did not affect Aβ42 peptide-stimulated superoxide generation in human neutrophils (Fig. 8B).

It has been reported that Aβ42 peptide induces chemotactic migration in human neutrophils via activation of FPRL1 (30). Thus, we examined the effect of Aβ42 on neutrophil chemotaxis and found that Aβ42 peptide induces neutrophil chemotaxis in a concentration-dependent manner (Fig. 8C). Aβ42 (40 μM) induced a 10-fold increase in the number of migrated cells (Fig. 8C). WRW⁴ alone did not affect neutrophil chemotaxis between 1 and 100 μM (Fig. 8C). Thus, to investigate the effect of WRW⁴ on Aβ42 peptide-induced neutrophil chemotaxis, we pretreated human neutrophils with several concentrations of WRW⁴ before the chemotaxis assay with 40 μM Aβ42. Preincubation of neutrophils with WRW⁴ was found to inhibit Aβ42 peptide-induced neutrophil chemotaxis in a concentration-dependent manner (Fig. 8D), and 10 μM

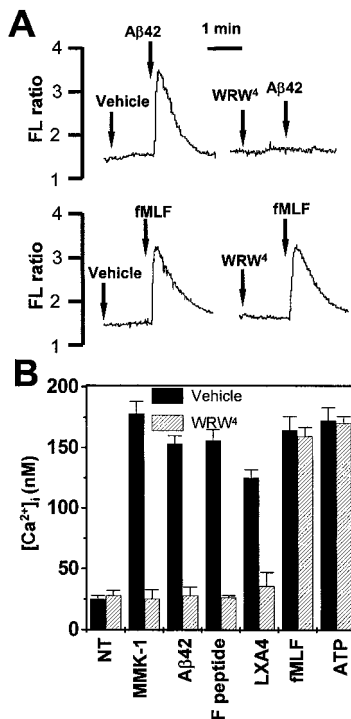


FIGURE 7. Specific inhibition of the FPRL-1-induced $[Ca^{2+}]_i$ increase by WRW⁴ in human neutrophils. Fura-2-loaded human neutrophils were treated with vehicle or WRW⁴ (10 μ M), then stimulated with Aβ42 (40 μ M) or fMLF (1 μ M). Changes in 340/380 nm were monitored. The results shown are representative of three independent experiments (A). Neutrophils were stimulated with vehicle or WRW⁴ (10 μ M), then stimulated with MMK-1 (1 μ M), Aβ42 (40 μ M), F peptide (30 μ M), LXA4 (1.4 μ M), fMLF (1 μ M), or ATP (500 μ M) (B). Changes in 340/380 nm were monitored, and the calibrated fluorescence ratio was converted to $[Ca^{2+}]_i$. Results are presented as the mean \pm SE of three independent experiments, each performed in duplicate (B).

WRW⁴ almost completely inhibited the neutrophil chemotaxis induced by Aβ42 peptide (Fig. 8D). Moreover, the control peptide, LFMYHP, did not affect Aβ42 peptide-induced neutrophil chemotaxis (Fig. 8D). These results demonstrate that the selective FPRL1 antagonist, WRW⁴, inhibits two important Aβ42 peptide-induced cellular responses, namely, superoxide generation and chemotactic migration in human neutrophils.

WRW⁴ inhibits internalization of Aβ42 peptide in human macrophages

Aβ42 peptide has been reported to internalize via FPRL1 in human macrophages (28). Because WRW⁴ was found to inhibit the intracellular signaling induced by Aβ42 peptide in human neutrophils, we examined the effect of WRW⁴ on the internalization of Aβ42. When 10 μ M Aβ42 peptide was incubated in human macrophages, Aβ42 internalization was induced in a time-dependent manner (Fig. 9). Aβ42 peptide internalization began at 5 min, showing maximal internalization at 30 min after incubation (Fig. 9). When human macrophages were pretreated with 10 μ M WRW⁴ before Aβ42 peptide incubation, we observed that Aβ42 peptide internalization was completely inhibited by WRW⁴ (Fig. 9). This result indicates that the preoccupation of FPRL1 by WRW⁴ inhibits Aβ42 peptide internalization via FPRL1 in human macrophages.

Discussion

In this study by screening peptide libraries we identified several hexapeptides that antagonize FPRL1 signaling. W-rich peptides,

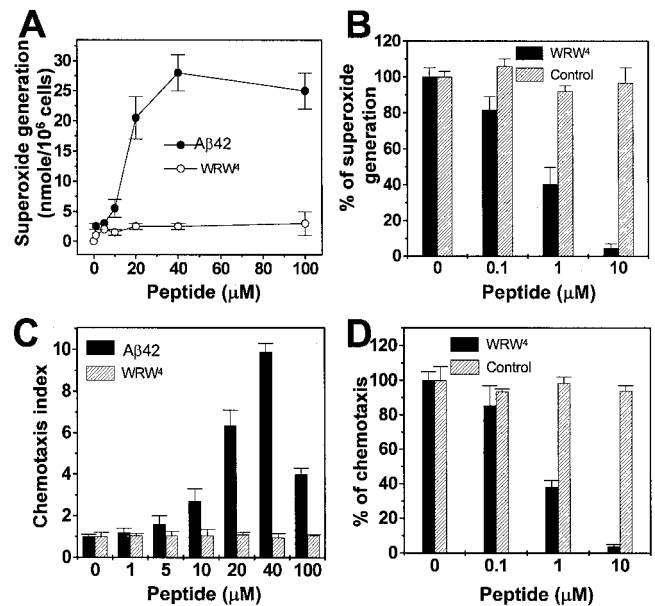


FIGURE 8. Effect of WRW⁴ on Aβ42-induced superoxide generation and chemotaxis in human neutrophils. Human neutrophils (1×10^6 cells/100 μ l) were stimulated with various concentrations of WRW⁴ or Aβ42 (A). Cells were preincubated with several concentrations of WRW⁴ or with 10 μ M control peptide (LFMYHP) for 1 min before adding 40 μ M Aβ42 peptide (B). Cytochrome *c* reduction was monitored. The results shown are representative of four independent experiments (A and B). Chemotaxis assays were performed with various concentrations of WRW⁴ or Aβ42 (C). Several concentrations of WRW⁴ or 10 μ M control peptide (LFMYHP) were pretreated before the chemotaxis assay using 40 μ M Aβ42 (D). The data are presented as the mean \pm SE of three independent experiments, each performed in duplicate (C and D).

such as WRW⁴, RHW⁴, RRW⁴, and DRW⁴, were found to interact directly with FPRL1 and to inhibit the binding of WKYMVM to its cell surface receptor in a concentration-dependent manner. Moreover, WRW⁴ exerted an antagonistic effect on WKYMVM-induced FPRL1 signaling and blocked not only chemotactic migration, but also the superoxide generated by Aβ42 peptide in human neutrophils.

In the process of immunomodulating activity of chemoattractants, it is crucial to induce phagocytic cell accumulation into infected tissues (6, 31). Although the excessive recruitment of phagocytic cells causes adverse effects, such as an inflammatory response, few studies have reported the possible negative regulation of chemoattractant-induced responses. The direct negative regulation of chemoattractant signaling can be induced by interrupting agonist-receptor binding. This study shows that a number of W-rich peptides blocked WKYMVM binding to FPRL1 (Fig. 2). In the experiment using the most efficient peptide, WRW⁴, we demonstrated that biotin-WRW⁴ binds to FPRL1-expressing RBL-2H3 cells, but not to vector-expressing RBL-2H3 cells (Fig. 3). Taken together the results show that WRW⁴ may bind to FPRL1 and inhibit the binding of WKYMVM to FPRL1. In terms of the peptide, WRW⁴, we found that it blocked all the tested cellular activities induced by WKYMVM investigated in FPRL1-expressing RBL-2H3 cells. Specifically, WRW⁴ blocked the WKYMVM-induced $[Ca^{2+}]_i$ increase, ERK activation, and chemotactic migration (Figs. 4–6). Taken together, these findings show that WRW⁴ blocks WKYMVM-initiated FPRL1 signaling by blocking the binding of WKYMVM to the receptor. As FPRL1 is an important

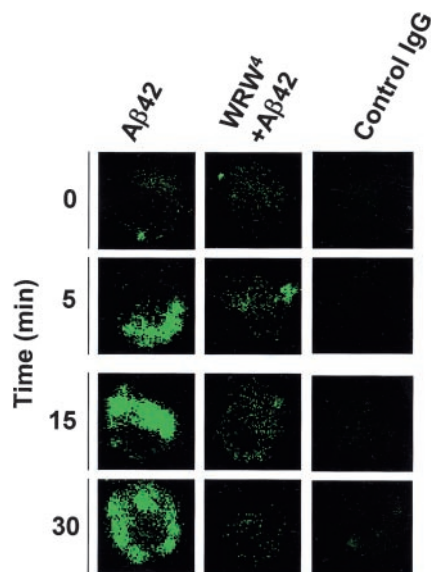


FIGURE 9. Effect of WRW⁴ on the internalization of A β 42 peptide in human macrophages. Human macrophages were cultured on chamber slides and incubated with 10 μ M A β 42 at 37°C for various times in the absence or the presence of 10 μ M WRW⁴. The cells were then rinsed, permeabilized, and stained with anti-A β 42 Ab or control Ab. The samples were further incubated with FITC-conjugated goat anti-mouse IgG. A β 42 staining was examined under a confocal microscope.

chemoattractant receptor that is involved in the host defense mechanism against pathogen infections, we believe that the W-rich peptides will be useful agents for the development of anti-inflammatory drugs.

Due to the crucial role of FPR family receptors in inflammatory responses, many research groups have tried to identify receptor antagonists for the receptor family. To date, a few antagonists for FPR have been reported (32–34). Two FPR antagonists (*t*-butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe-OH and *i*-butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe-OH) have been developed by replacement of the formyl group of fMLF with *t*-butyloxycarbonyl or *i*-butyloxycarbonyl (32, 33). A cyclic undecapeptide, cyclosporine H has been developed as a potent and selective FPR antagonist (34). Cyclosporine H has been reported to inhibit FPR-mediated Ca²⁺ mobilization, chemotaxis, and release of proinflammatory mediators (34–36). Even though some FPR-specific antagonists have been developed and investigated for their putative role as therapeutic agents for modulators of inflammatory responses, FPRL1-specific antagonists have not been reported until now. In this study we developed several synthetic hexapeptides that act as FPRL1 antagonists. We also demonstrated that one of the potent FPRL1 antagonists, WRW⁴, specifically inhibited the [Ca²⁺]_i increase induced by all the tested FPRL1 agonists (MMK-1, A β 42 peptide, and F peptide), but not by fMLF (Fig. 7).

Concerning the efficiency of the novel FPRL1 antagonist, WRW⁴ inhibited some FPRL1 agonist-induced signaling within 1–10 μ M (Figs. 4–8). Some chemokine receptor antagonists have been reported. One example is the CCR1 antagonist CP-481,715. This antagonist inhibits the binding of 50 pM [¹²⁵I]-labeled CCL3 in a concentration-dependent manner, showing the complete inhibition at ~10 μ M (37). Another CXCR4 antagonist, KRH-1636, inhibits the effective concentration of stromal cell-derived factor 1 (1 μ g/ml)-induced Ca²⁺ mobilization at 10 μ M (38). As WRW⁴ peptide inhibits binding of the homologous ligand by 2-log, 10-fold higher concentrations, this seems to be a weak competitor. Keeping in mind that WRW⁴ peptide is the first FPRL1 antagonist,

it will be used for the development of more improved FPRL1-specific antagonists.

In Fig. 7B, we showed that the increase in [Ca²⁺]_i induced by LXA4 was also inhibited by WRW⁴, even though LXA4 has been reported to be a potent anti-inflammatory mediator. Concerning the effect of WRW⁴ on the FPRL1 ligand peptides or LXA4-induced signaling, WRW⁴ inhibited the increase in [Ca²⁺]_i induced by FPRL1 ligand peptides (WKYMVm, F peptide, A β 42, and MMK-1) and LXA4. Previously, Chiang et al. (14) demonstrated that two different ligands of FPRL1 (LXA4 and MMK-1) could compete with each other. Taken together, it appears that WRW⁴ may compete for the binding of FPRL1 ligand peptides and LXA4 on FPRL1.

Previous reports have shown that inflammation is critically involved in the pathogenesis of Alzheimer's disease (39). Moreover, A β 42 peptide is one of the enzymatic cleavage fragments of amyloid precursor protein (40) and has been reported to play a significant role in the proinflammatory responses of systemic amyloidosis, such as Alzheimer's disease (39, 40). Recently, A β 42 peptide was found to bind to FPRL1 and to modulate the generation of reactive oxygen species and cellular chemotactic migration in human neutrophils via FPRL1 (30). Fig. 8 demonstrates that preincubation of human neutrophils with WRW⁴ before A β 42 peptide treatment caused complete inhibition of A β 42 peptide-induced neutrophil chemotaxis and reactive oxygen species generation. As A β 42 peptide is critically involved in Alzheimer's disease, WRW⁴ could be used to antagonistically block the action of A β 42 peptide.

In terms of Alzheimer's disease, A β 42 peptide is known to play a central role in mediating neurotoxicity and in the formation of senile plaques (39, 40). It has been reported that mononuclear phagocytes in the brain express FPRL1, and that FPRL1 gene expression is elevated in CD11b-positive mononuclear phagocytes that infiltrate senile plaques in the brain tissues of Alzheimer's disease patients (12). A β 42 peptide also has been reported to increase neurodestructive reactive oxygen species and reactive nitrogen and TNF- α in brain microglia cells and peripheral blood mononuclear phagocytes (41). These molecules are elevated in Alzheimer's disease (39–41). More recently, Yazawa et al. (28) reported that A β 42 peptide is internalized via FPRL1 and forms fibrillar aggregates in macrophages. In the present study we found that WRW⁴ is an FPRL1-specific antagonist that blocks the internalization of A β 42 peptide in human macrophages (Fig. 9). Our results suggest that WRW⁴ provides a developmental basis for a putative drug capable of blocking the internalization of A β 42 peptide and fibrillar formation.

In addition to A β 42 peptide, several other ligands derived from the HIV-1 envelope domains or host-derived agonists have been reported to bind to FPRL1 (14–17). Further studies are needed to evaluate the effects of WRW⁴ on these and other FPRL1 agonist-related cellular responses and diseases. In summary, we believe that WRW⁴, which is the first identified FPRL1 antagonist, can be developed as a useful molecule for the study of FPRL1 signaling and as a candidate drug for the treatment of several diseases in which FPRL1 is known to play a role.

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