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A Novel Fluorescent Cross-Reactive Formylpeptide Receptor/ Formylpeptide Receptor-Like 1 Hexapeptide Ligand[1]

J. Jacob Strouse[2], **Susan M. Young**[2], **Hugh D. Mitchell**[3], **Richard D. Ye**[5], **Eric R. Prossnitz**[3], **Larry A. Sklar**[2,4], and **Bruce S. Edwards**[2,4,*]

[2]Cytometry, Cancer Research and Treatment Center, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

[3]Department of Cell Biology and Physiology, Cancer Research and Treatment Center, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

[4]Department of Pathology, Cancer Research and Treatment Center, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

[5]Department of Pharmacology, University of Illinois at Chicago College of Medicine, Chicago, Illinois

Abstract

Background—Formylpeptide receptors are implicated in a variety of immunological and inflammatory response cascades. Further understanding FPR-family ligand interactions could play an integral role in biological and therapeutic discovery. Fluorescent reporter ligands for the family are desirable experimental tools for increased understanding of ligand/receptor interactions.

Methods—The ligand binding affinity and fluorescent reporting activity of the peptide WK(FL)YMVm was explored though use of the high throughput HyperCyt® flow cytometric platform. Relative binding affinities of several known FPR and FPRL1 peptide ligands were compared in a duplex assay format.

Results—The fluorescent W-peptide ligand, WK(FL)YMVm, proved to be a high affinity, cross-reactive reporter ligand for the FPR/FPRL1 duplex assay. Ligand specificity was demonstrated for each receptor with known, selective peptide ligands. The binding site specificity of the reporter ligand was further verified by a fluorescent confocal microscopy internalization experiment.

Conclusions—The fluorescent peptide ligand WK(FL)YMVm bound with high affinity to both FPR and FPRL1. The differential affinities of known peptide ligands were observed with the use of this fluorescent probe in HTS flow cytometry.

Key terms

formylpeptide receptor; FPR; formylpeptide receptor-like 1; FPRL1; fluorescent ligand; cross-reactive; W-peptide; WKYMVm; flow cytometry; GPCR

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[*]Contact information: Bruce S. Edwards, PhD, Cytometry and Department of Pathology, CRTC, UNM HSC, MS08-4630, Albuquerque, NM, 87131, bedwards@salud.unm.edu; (505) 272-6206; Fax (505) 272-6695.

Introduction

The G protein coupled formylpeptide receptor (FPR) is one of the first discovered members of the chemoattractant receptor superfamily (1,2). FPR is expressed in several cell types including neutrophils, monocytes, hepatocytes, immature dendritic cells, astrocytes, microglial cells, and the tunica media of coronary arteries (3-6). Two other FPR variants have been described; formylpeptide receptor-like 1 (FPRL1) and formylpeptide receptor-like 2 (FPRL2) (7). Also a 7-transmembrane G protein-coupled receptor (GPCR), FPRL1 shares 69% primary sequence identity with FPR (8). FPRL2 encodes a receptor that has 56% and 83% amino acid sequence identity to FPR and FPRL1, respectively. FPRL1 is expressed in an even greater variety of cell types than FPR including phagocytic leukocytes, hepatocytes, epithelial cells, T lymphocytes, neuroblastoma cells, astrocytoma cells, and microvascular endothelial cells (1,8-11). In addition, a recent study has documented expression of both FPR and FPRL1 on normal human lung and skin fibroblasts (12). The diverse tissue expression of these receptors suggests the possibility of as yet unappreciated complexity in the innate immune response and perhaps other unidentified functions for the receptor family. Exploring this diversity could be facilitated by molecular tools targeted at elucidation of ligand-receptor interactions. Based on known peptide ligands, our group sought a fluorescent reporter probe for use in flow cytometric analysis of FPR and FPRL1.

The most commonly studied class of FPR activators are protein/peptide based ligands. N-formylated peptides such as the *E. coli* derived N-formyl-Met-Leu-Phe (fMLF) are high affinity FPR ligands that elicit a variety of biologic activities in myeloid cells and it has been proposed that a primary FPR function is to promote trafficking of phagocytic myeloid cells to sites of infection and tissue damage where they exert anti-bacterial effector functions and clear cell debris. While FPR and its ligands have been studied in great depth (8,13), a growing awareness of the biological importance of FPRL1 makes it increasingly the subject of new, and joint FPR-family investigations. Many potent formylpeptide FPR agonists only weakly activate FPRL1 (14). N-formylated hexapeptides derived from the N-terminus of mitochondrial NADH dehydrogenase subunits 4 and 6 and cytochrome C oxidase subunit 1 prove to be an exception and are agonists of both FPR and FPRL1 (14). Non-N-formyl phagocyte chemotactic activation was shown with peptides of varied N-terminal substitution including free amino, acetyl, ureido, and carbamate functional groups (15-17). Despite the limited activation by N-formyl peptides, FPRL1 can be considered exceptionally promiscuous, responding to ligands of multiple origins spanning a wide range of structural diversity (1,18). A number of host-derived FPRL1 agonists have been identified that are associated with pathophysiological settings. These include amyloidogenic proteins, serum amyloid A (19), the 42 amino acid form of β amyloid, Aβ42 (20), and a prion protein fragment, PrP1206-26 (21), which are involved in chronic inflammation-associated systemic amyloidosis (22), Alzheimer's disease (23), and prion diseases (21), respectively. Since infiltration of activated mononuclear phagocytes is a common feature, cells responding to FPRL1 ligands may contribute to the inflammatory pathology observed in the diseased tissues (24). Other FPRL1 agonists include an enzymatic cleavage fragment of the neutrophil granule derived cathelicidin (25), and HIV-1 envelope protein domains which are also capable of binding FPR (26).

The present study was motivated by the need for a fluorescent probe with which to efficiently screen libraries of small molecules to identify selective FPR and FPRL1 ligands. The goal was to produce a high affinity probe that could simultaneously report binding interactions of test compounds with both receptors in a single assay volume. Baek et al. previously reported a class of peptides with an amino-terminal W residue, known as W-peptides, that were selective high affinity ligands for FPRL1 (27). In one series of W-peptides, it was shown that various substitutions could be made at the second amino acid

residue without significantly affecting the FPRL1 binding interaction (28). We exploited the second residue binding ambiguity in this high affinity FPRL1 ligand class to generate a fluorescent, cross-reactive, high affinity FPR/FPRL1 probe. Incorporation of a amide conjugated fluoresceinyl-lysine residue at that position resulted in a fluorescent peptide probe, H_2N-Trp-(Fluoresceinyl-Lys)-Tyr-Met-Val-(D-Met)-amide (WK(FL)YMVm), that not only retained high affinity for FPRL1 but also acquired a substantially increased affinity for FPR.

Materials and Methods

The W-peptide series (WK(FL)YMVm, WKYMVm, WKRMVm, and WKGMVm) were synthesized by New England Peptide, Inc. (Gardner, MA) and supplied at > 95% purity by HPLC with identity verified by MALDI-TOF MS. All peptide dilutions were done in DMSO prior to the final in well additions where the DMSO concentration was no more than 1%. Chemical reagents, including the formyl peptides N-formyl-Met-Leu (fML), fMLF, and N-formyl-Met-Leu-Phe-Phe (fMLFF), were obtained from Sigma (St. Louis, MO) unless otherwise specified. Flow cytometric analysis was done on a CyAn flow cytometer (Beckman-Coulter, Fullerton, CA). Fluorescence was excited at 488 nm and detected with 530/40 and 680/30 optical band pass filters for WK(FL)YMVm and FuraRed™, respectively. The resulting time-resolved data files were analyzed with IDLeQuery software to determine compound activity in each well. The HyperCyt® high throughput flow cytometry platform was used to sequentially sample cells from 384-well microplates (2 μL/sample) for flow cytometer presentation at a rate of 40 samples/min (29,30). The HyperCyt® platform and associated analysis software are commercially available from IntelliCyt™ (Albuquerque, NM). Ligand competition curves were fitted by Prism® software (GraphPad Software, Inc., San Diego, CA) using nonlinear least-squares regression in a sigmoidal dose response model with variable slope, also known as the four parameter logistic equation.

Rat Basophilic Leukemia (RBL-2H3) cells expressing human FPRL1 (RBL/FPRL1) were grown as adherent cell cultures in TCM supplemented with 2.5 μg/mL Amphotericin B (CellGro, Mediatech Inc., Manassas, VA). U937 cells expressing human FPR were grown as 100 mL suspensions in TCM. Unless otherwise indicated, U937 cells were used that expressed a mutant FPR with glycine and alanine substituted for serine and threonine residues in the C-terminal tail (ΔST) which do not internalize the receptor when stimulated with fMLF (31). Cultures were grown at 37°C in a 5% CO_2 atmosphere, and passaged every 3 days. RBL/FPRL1 cells were detached with 0.25% Trypsin-EDTA (37° C, 2-5 min.), suspended in TCM, centrifuged 10 min. at 450×g and resuspended at 4×10^6 / mL in PDB. U937/FPR cells were centrifuged 10 min. at 450×g, resuspended in PDB at 5×10^5 cells/mL and color-coded by incubation 15 min. at 37°C with Fura Red™, AM (InVitrogen, Carlsbad, CA) at a final concentration of 6 μM. After two subsequent centrifugation washes in PDB to remove unincorporated dye, the cell pellet was resuspended by addition of the RBL/FPRL1 cell suspension to achieve a final U937/FPR cell concentration of 4×10^6 / mL, equal to that of the RBL/FPRL1 cells. The cell mixture was stored on ice until used in the assay.

Duplex Flow Cytometric Analysis

The experiment was performed in duplex format in which ΔST-U937 cells expressing FPR were tested together with RBL-2H3 cells expressing FPRL1. The FPR-expressing cells were stained red to allow them to be distinguished from the FPRL1-expressing cells during flow cytometric analysis. Assays were performed in polystyrene 384-well plates with small volume wells (#784101, Greiner, Monroe, NC). The assay optimized order of addition was done in the following sequence: 1) test compounds and control reagents, 5 μL/well; 2) a combination of FPR- and FPRL1-expressing cell lines; 3) fluorescent WK(FL)YMVm

peptide (after 30 min., 4°C incubation, 5 μL/well). After an additional 45 min. at 4 °C incubation, plates were analyzed by flow cytometry. The assay response range was defined by replicate control wells containing unlabeled receptor-binding peptide (positive control) or buffer (negative control). The formyl peptide fMLFF was used as the FPR-blocking peptide and unlabeled WKYMVm as the FPRL1-blocking peptide. Assay wells were directly analyzed on the flow cytometer without wash steps. Supplemental material is available demonstrating the gating strategies used for these analyses. A recent publication describing the use of the fluorescent ligand described here in a screening campaign further illustrates these duplex-assay analysis methods (32). FPR/FPRL1 expression ranged from 100,000 to 200,000 receptors per cell as determined by comparison to standard curves generated with Fluorescein Reference Standard Microbeads (Bangs Laboratories, Fishers, IN). This corresponded to total FPR/FPRL1 concentrations of 0.6 to 1.2 nM. Potential quenching effects from both conjugation of fluorescein and receptor binding of the peptide were addressed by comparison to the previously characterized peptide, N-formyl -Met-Leu-Phe-Lys-fluorescein (fMLFK(FL)) (33). At saturation the fluorescence intensity of WK(FL)YMVm was 66% of that observed for fMLFK(FL) indicating that receptor number estimates were comparable within the indicated range.

Fluorescent Cross-Reactive Reporter Ligand Binding Affinity

The K_d of WK(FL)YMVm was determined in both FPR and FPRL1 expressing cell lines. To account for fluorescence from non-specific binding, the blocking peptides fMLFF and WKYMVm were used to saturate receptors prior to addition of WK(FL)YMVm. Each titration series of the fluorescent probe was done in duplicate and in total there were sixteen points per concentration over two separate days of data collection. The inhibitory peptide (5 μL) was added to the wells first, with final concentrations of 250 nM of fMLFF for FPR and 67 nM of WKYMVm for FPRL1, followed by 5 μL of cells. The plates were then incubated at 4°C for 30 min. followed by addition of 5 μL of the fluorescent WK(FL)YMVm ligand dilution series. The concentration of fluorescent ligand ranged in wells from 0.1 to 66.67 nM over a nine point span. The plates were incubated overnight (18 hrs.) at 4°C to allow receptor-ligand binding interactions to attain approximate equilibrium, then analyzed on the flow cytometer.

Comparative Formylpeptide Ligand Binding

The fluorescent WK(FL)YMVm peptide was then used as the reporter ligand for exploring relative binding affinities of known FPR and FPRL1 peptide ligands using the duplex protocol outlined above. Opposite to the K_d determination experimentation, the fluorescent ligand WK(FL)YMVm was held at a constant concentration (5 nM) and the peptide ligands were subjected to serial dilution. The nine point concentration range of the peptide ligands spanned from 1.0 nM to 6.7 μM. Incubation times and order of addition for compounds and cells were identical to the previous protocol.

Fluorescent Ligand Internalization

Stable FPRL1-transfected RBL cells were transiently transfected with RFP-tagged arrestin-3 using the Nucleofector transfection system with Solution L, Program T-020 (Amaxa Inc., Gaithersburg, MD). These dually transfected RBL cells were then plated on coverslips and allowed to recover overnight. The coverslips were incubated for 10 minutes at 37°C with 5 nM WK(FL)YMVm in growth medium (10% fetal bovine serum in RPMI 1640), washed, immediately fixed, and subsequently mounted. Images were acquired using a Zeiss laser scanning confocal fluorescence microscope (Thornwood, NY).

Results

WK(FL)YMVm Cross-Reactive Binding Affinity

Prior to incorporation of fluoresceinated lysine, the W-peptide affinity for FPRL1 was approximately a hundred fold higher than for FPR (28). In equilibrium binding experiments with the W-peptide fluorescein conjugate, the K_d for FPR and FPRL1 were found to be 1.21 ± 0.36 nM and 1.82 ± 0.78 nM, respectively (Figure 1). To quantify non-specific binding of WK(FL)YMVm to cells, a solution of unlabeled high-affinity peptide ligands was added to the staining reaction. The concentration of inhibitory peptides used in the reported K_d determination experiment were optimized for use in the duplex system. The K_d values for each receptor were also determined independently in each cell line at 1 μM inhibitory peptide concentrations, more that 100 times the K_d of each peptide for its respective receptor. The values found were within the error range of those reported for the optimized duplex conditions (data not shown).

Receptor Binding Specificity

To evaluate the ligand affinity reporting capability of the probe at both receptors binding affinities of several unlabeled FPR and FPRL1 selective peptides were measured in competitive binding assays using the new fluorescent probe. The ligand dependent specificity was demonstrated by comparison of high affinity FPR binding formylpeptide ligands and FPRL1 selective WKYMVm (Figure 2 A, U937/FPR cells & B, RBL/FPRL1 cells). The formyl peptide ligands, fML, fMLF, and fMLFF, bound with moderate to high affinity to FPR in U937/FPR cells (EC_{50} values ranged from 110 nM for fML to 1 nM for fMLFF) but exhibited no detectable ligand activity for the FPRL1 expressing RBL cells (Table 1). Binding affinity measurements of WKYMVm for the two receptors yielded EC_{50} values of 43.8 nM and 1.8 nM for FPR and FPRL1, respectively. The single residue substitution peptides WKGMVm and WKRMVm showed exclusive binding for FPRL1 with EC_{50} values of 176 nM and 705 nM respectively (Figure 2 C, U937 cells & D, RBL cells). Bae et al. previously observed Ca^{2+} flux responses in FPR and FPRL1 expressing cell lines for a series of W-peptides including WKYMVm (EC_{50} = 47 nM and 0.6 nM respectively for FPR and FPRL1), WKGMVm (EC_{50} = 21 nM), and WKRMVm (EC_{50} = 2 nM) (28). Despite the apparent quantitative correlation of data across the two experiments for WKYMVm, the comparison of ligand binding affinity and the efficiency with which the ligand induces calcium flux does not necessarily have a direct association. Rather, it is important to note the qualitative relationship of the two data sets, particularly the cross-activity of WKYMVm for both receptors and the FPRL1 specificity seen for the residue analogs WKGMVm and WKRMVm.

FPRL1/WK(FL)YMVm Internalization

Arrestins are adaptor proteins that uncouple phosphorylated GPCRs from G proteins and regulate receptor internalization (34). FPR and FPRL1 agonists induce internalization of the receptors. Although FPR internalization is reportedly not dependent on the presence of arrestins (35), Huet et al. have demonstrated that upon agonist stimulation FPRL1 remains co-localized with arrestin during endocytosis (36). As a means of further validating FPRL1 binding specificity of the WK(FL)YMVm ligand, arrestin colocalization experiments were performed. Figure 3 shows the observed internalization data for WK(FL)YMVm in RBL cells. Slides A and B show both wild type and FPRL1 expressing RBL cell lines treated with 5 nM WK(FL)YMVm at 37°C to demonstrate receptor expression and internalization in the transfected cell line. Internalized ligand was not apparent in the wild type cells but is readily seen in cells expressing FPRL1. Internalized ligand was shown to colocalize extensively with arrestin-3, as demonstrated in Figure 3C-E.

Discussion

Baek et al. originally identified hexapeptide ligands with the consensus sequence XKYX(P/V)M, where X is one of 19 amino acids (cysteine excluded), that stimulate the formation of inositol phosphates (InoPs) in B lymphocyte cell types through the action of phospholipase C via a pertussis toxin sensitive G protein coupled cell-surface receptor (27). Since their discovery, this W-peptide family of ligands has been widely studied and modified to explore formylpeptide receptor family pharmacology. Specifically, the peptide WKYMVM was shown to initiate InoP generation in U266 (human B myeloma), U937 (human histiocytic lymphoma), and HL60 (human promyelocytic lymphoma) cell lines at 1 μM. Increased affinity was later seen after substitution of the L-Met^6 with D-Met (WKYMVm) (37), with a hundred fold better affinity seen for FPRL1 over FPR (28). Expanding on the structure affinity relationship of the W-peptide series, Bae et al. demonstrated that the lysine residue of WKYMVm was less crucial to binding than any other position (28). Wan et al. also noted that the last two residues could be removed with little change in affinity and that the 4-peptide sequence with the highest affinity included a norleucine replacement of the lysine (WNleYM) (38). This primary amine free alkyl chain showed similar binding to its lysine counterpart, as well as the original hexapeptide (EC_{50}: WKYMVm = 3.31 nM, WKYM = 43.6 nM, WNleYM =5.34 nM). The authors proposed that the binding pocket consisted of at least two aromatic interactions (tryptophan and tyrosine) as well as a hydrogen bonding region (methionine) and a hydrophobic interaction at the norleucine.

The high affinity binding and the potential to modify the second residue in the W-peptide family was taken advantage of to generate a fluorescent reporter ligand for two FPR family receptors. Modification of the FPRL1 ligand, WKYMVm, with a fluoresceinated lysine (WK(FL)YMVm) afforded an FPR/FPRL1 cross-reactive, high affinity reporter ligand. Addition of the large fluorescein carboxamide yielded a ligand with increased, affinity for both receptors. The ligand was also shown to internalize and co-localize with arrestin-3 by confocal microscopy in RBL cells, further demonstrating its formylpeptide receptor family specific binding.

To our knowledge there is no other cross-reactive fluorescent reporter ligand for both FPR and FPRL1. We have previously reported the use of fMLFK-FITC as a fluorescent reporter for FPR with a K_d of 3 nM, but this ligand does not bind FPRL1 (39). A radioactive iodinated WKYMVm ligand has been described as a binding affinity reporter for both FPR and FPRL1 (40). Although this probe can be used at subnanomoler concentrations, the need for physical sample processing (centrifugation through a 10% sucrose-PBS cushion) and the signal output (γ-ray emissions) are not conducive to HTS-methods. Replacing the natural amino acid residue with the bulky, relatively hydrophobic fluorescein group could have forced a more sterically favorable conformation change which in turn affected binding affinity. The fact that a fluorescein is involved in providing increased binding affinity suggests that there is potentially a large hydrophobic region involved in the interaction as suggested by Wan et al. (38).

The new fluorescent probe was then used in HTS flow cytometric analysis to demonstrate specific ligand interactions with both FPR and FPRL1 expressing cell lines in a single well. Known high affinity FPR formylpeptide ligands were shown to bind with specificity to FPR and the parent WKYMVm ligand was shown to be selective for FPRL1. Thus, the fluorescent WK(FL)YMVm reporter ligand can be used in a duplex format assay to explore chemical libraries for selective ligands for FPR and FPRL1 in a high content, high throughput manner. This fluorescent probe has been successfully used in the duplex competitive binding assay to screen more than 27,000 compounds from the NIH Small

Molecule Repository and other sources to identify a number of novel and selective small molecule antagonists for FPR and FPRL1 (32).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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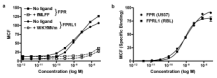

Figure 1.

Comparison of the binding affinity of WK(FL)YMVm on FPR and FPRL1. A: Raw median channel fluorescence values of bound receptor with and without the presence of nonfluorescent ligand (dashed and solid line, respectively). Receptor saturation was achieved in FPR (squares) with fMLFF and for FPRL1 (circles) with WKYMVm. B: Compensation for the nonspecific binding afforded *K*d values of 1.21 ± 0.36 nM and 1.82 ± 0.78 nM, respectively, for FPR (solid circles) and FPRL1 (solid squares).

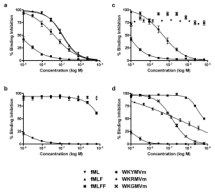

Figure 2.

Direct comparison of unlabeled FPR and FPRL1 peptide ligands through a competitive binding assay with the fluorescent WK(FL)YMVm probe. A: This plot shows the direct comparison of the FPR selective formylpeptide ligands (fML, fMLF, fMLFF) with the FPRL1 selective WKYMVm in FPR expressing U937 cells. The high-affinity fMLFF stands out with a 1.0 ± 0.6 nM EC_{50} while WKYMVm is only 43.8 ± 4.3 nM. The other ligands, fML and fMLF, had EC_{50} values in the 100 nM range. B: In FPRL1 expressing RBL cells the WKYMVm ligand shows high affinity (EC_{50} = 1.8 ± 0.3 nM), whereas the formyl peptides are all effectively nonbinding. C: The W-peptides WKRMVm and WKGMVm show no binding in FPR expressing U937 cells. D: Moderate binding of the W-peptides is demonstrated in FPRL1 expressing RBL cells at EC_{50} values of 704.8 ± 384.1 nM and 175.6 ± 40.6 nM for WKRMVm and WKGMVm, respectively.

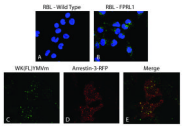

Figure 3.

Internalization of fluorescent ligand bound FPRL1 and colocalization with arrestin-3 in RBL cells. The cells were all exposed to 5 nm WK(FL)YMVm for 10 min at 37°C before being washed and fixed. A,B: Internalization of the fluorescent WK(FL)YMVm ligand (green) is demonstrated in wild-type RBL cells (A) and FPRL1 expressing cells (B). Nuclei are stained with DAPI (blue). C_E: Colocalization of fluorescent ligand with arrestin-3. RBL cells transiently transfected with RFP-arrestin-3 (D) were allowed to internalize ligand as before (C); merged image in E.

Table 1

Comparison of peptide ligand binding reported through the fluorescent probe WK(FL)YMVm. EC_{50} Values for Peptide Ligands

Peptide	U937 FPR EC_{50} (nM)	RBL FPRL1 EC_{50} (nM)
fML	110.0 ± 17.6	-
fMLF	79.0 ± 26.9	-
fMLFF	1.0 ± 0.6	-
WKYMVm	43.8 ± 4.3	1.8 ± 0.3
WKRMVm	-	704.8 ± 384.1
WKGMVm	-	175.6 ± 40.6