A Novel Nonpeptide Ligand for Formyl Peptide Receptor-Like 1

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ABSTRACT

Formyl peptide receptor-like 1 (FPRL1) is a G protein-coupled receptor that binds natural and synthetic peptides as well as lipoxin A4 and mediates important biological functions. To facilitate its pharmacological characterization, we screened a compound library and identified a substituted quinazolinone (Quin-C1, 4-butoxy-N-[2-(4-methoxy-phenyl)-4-oxo-1,4-dihydro-2H-quinazolin-3-yl]-benzamide) as a ligand for FPRL1. Quin-C1 induces chemotaxis and secretion of bactericidal functions including degranulation and production of superoxide (Prossnitz and Ye, 1997; Mills et al., 1999; Le et al., 2002). Intriguingly, the bactericidal activities triggered by these chemotactic peptides contribute to tissue damage when neutrophils are activated in certain pathological conditions such as ischemia reperfusion. Therefore, an understanding of the pharmacological basis of FPR binding and signaling has the potential to enhance anti-infective activity as well as to reduce unwanted neutrophil activation and the resulting tissue damage.

The human FPR gene family consists of 3 members: fpr1, first cloned in 1990 (Boulay et al., 1990), encodes a high-affinity receptor for fMLF (FPR); fpr1, initially identified by several groups based on sequence homology to fpr1, encodes a receptor (formyl peptide receptor-like 1, or FPRL1) that share 69% identity at the amino acid level to human FPR (Bao et al., 1992; Murphy et al., 1992; Perez et al., 1992; Ye et al., 1992); the fpr2 gene encodes a receptor (FPRL1) that interacts with MAP, mitogen-activated protein; GPCR, G protein-coupled receptor.

Phagocytic leukocytes play important roles in host defense against invading microorganisms. In response to pathogenic challenges, phagocytes such as neutrophils migrate to the site of infection, where they engulf and destroy bacteria (Snyderman and Uehling, 1992). The tripeptide N-formyl-Met-Leu-Phe (fMLF) is a prototype of formylated chemotactic peptides for neutrophils owing to its ability to activate the G protein-coupled formyl peptide receptor (FPR). At subnanomolar to nanomolar concentrations, this binding event translates into directional movement of neutrophils. At higher concentrations (~100 nM), the same peptide also stimulates bactericidal functions including degranulation and production of superoxide (Prossnitz and Ye, 1997; Mills et al., 1999; Le et al., 2002). Intriguingly, the bactericidal activities triggered by these chemotactic peptides contribute to tissue damage when neutrophils are activated in certain pathological conditions such as ischemia reperfusion. Therefore, an understanding of the pharmacological basis of FPR binding and signaling has the potential to enhance anti-infective activity as well as to reduce unwanted neutrophil activation and the resulting tissue damage.

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shares 56% amino acid sequence identity with human FPR (Bao et al., 1992). Although FPR has been extensively characterized pharmacologically and physiologically because the 1980s, studies of FPRL1 and FPRL2 began only after molecular cloning of the respective genes. Initially known as a low-affinity receptor for fMLF (Ye et al., 1992; Quehenberger et al., 1993), FPRL1 now has more than 10 ligands and is the most promiscuous in this group of receptors with respect to agonist selectivity (Prossnitz and Ye, 1997; Le et al., 2002). Lipoxin A4, an autacoid, is a high-affinity ligand for FPRL1 (Fiore et al., 1994). The acute-phase protein serum amyloid A and the β-amylod peptide Aβ1–42 bind and activate FPRL1 (Su et al., 1999; Le et al., 2001). Of interest is the ability of serum amyloid A to stimulate cytokine gene expression (He et al., 2003) and of Aβ1–42 to induce superoxide production and to use FPRL1 for its internalization into microglia (Tiffany et al., 2001; Yazawa et al., 2001). Other natural ligands for FPRL1 include the prion peptide PrP1, peptides derived from the HIV-1 gp41 protein (Le et al., 2002), a fibrinolytic peptide from urokinase-type plasminogen activator receptor (Resnati et al., 2002), and the antiapoptotic peptide humanin (Ying et al., 2004). In addition, potent synthetic peptides such as WKYMVM (Baek et al., 1996; Le et al., 1999) and MMK1 (Klein et al., 1998) have been identified and used widely in in vitro and ex vivo studies of FPRL1.

There have been ongoing efforts in several laboratories to study the ligand and FPRL1 interaction in part because of the potential for FPRL1 to become a therapeutic target. Such studies will be greatly facilitated by synthetic, nonpeptide compounds that are not available currently. Given that peptides are difficult to make and administer as therapeutic agents, small molecular weight chemical compounds would be a better choice for clinical use. In an attempt to identify novel nonpeptide compounds for FPRL1, we designed a screening protocol based on FPRL1-mediated reporter gene expression and calcium signaling. We report here the identification and characterization of a substituted quinazolinone as an FPRL1-selective ligand that exhibits partial agonistic activity for FPRL1-mediated cell activation.

Materials and Methods

Materials. W-peptide (WKYMVM) and MMK1 (LESIFRSLLFRVM) were synthesized at Macromolecular Resources (Fort Collins, CO). The chemotactic peptide FMLF was purchased from Sigma (St. Louis, MO). 252I-WKYVMV (Bolton-Hunter labeled) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). The cells were incubated with 4 μM Plus-3AM in HBSS/BSA at 37°C for 1 h and examined for calcium mobilization in response to the compounds using a fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA). Some cells were pretreated with 100 ng/ml pertussis toxin for 18 h. After a brief wash, cells were aliquoted into 5 × 105 cells per assay. More detailed calcium mobilization assays were conducted in a spectrofluorometer (Photon Technology International, Lawrenceville, NJ) with excitation wavelength at 488 nm and emission wavelength at 525 nm.

Synthesis of 2-Substituted Quinazolinones. The 2-substituted quinazolinones were obtained through the cyclization of antrinilhydrazine and aldehyde under acidic conditions. The synthesis route (Mayer et al., 1997) permits the introduction of a wide range of substituents into the 2-position of this ring system using different benzaldehydes. The structure of the original compound, based upon which Quin-C1 is synthesized, is shown in Fig. 1A.

Chemotaxis. Agonist-induced migration of cells was assessed in a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MA), as reported previously (He et al., 2000). In brief, different concentrations of the agonists WKYMVM (0.01 nM–10 μM) and Quin-C1 (1 nM–1 mM) were placed in the lower compartment (30 μl). Purified human neutrophils (35 μl of a 2 × 106 cells/ml suspension) were seeded in the upper compartment, which was separated from the lower compartment by a polycarbonate filter with pore size of 5 μm. For checkerboard analysis, agonists were also added to the upper wells and cell migration in the absence of chemotactic gradient (chemokinesis) was determined for calculation of chemotaxis index. After incubation at 37°C for 1 h, the filter was removed, fixed, and stained with Diff-Quick staining solutions (IMEB Inc., San Marcos, CA). The scanned filter image was quantified for cell density using the ImageQuant software (Amersham Biosciences). After subtraction of migrating cells because of chemokinesis, data were presented as chemotaxis index that represents the ratio of the density of the area where cells migrated toward agonists over the density of the area where cells migrated toward medium.

Degranulation. For release of β-hexosaminidase, RBL-FPRL1 and RBL-FPR cells were cultured in a 24-well plate for 48 h. Before assay, cells were washed briefly and preincubated with 10 μM cytochalasin B in HBSS containing 20 mM HEPES, pH 7.4, and 0.2% BSA (HBSS-HB) for 15 min on ice followed by 15 min at 37°C. After
a brief wash, cells were stimulated for 10 min with indicated amounts of agonists or vehicle (buffer containing the same amount of organic solvent as in agonist preparation) at 37°C. The degranulation reaction was terminated by rapid incubation in sample buffer with ice. The amount of secreted β-hexosaminidase was quantified by incubating 20 μl of supernatant with 10 μl of 1 mM \( p \)-nitrophenyl-N-acetyl-β-D-glucosaminide in 0.1 M sodium citrate buffer, pH 4.5, at 37°C for 1 h in a 96-well plate. The reaction was terminated by adding 200 μl of 0.1M Na2CO3 and 0.1M NaHCO3, pH 10, and absorbance at 405 nm was determined in a SpectroMax 340 plate reader (Molecular Devices). Total cellular β-hexosaminidase was determined with cell lysate in 0.1% Triton X-100. Data are collected from several experiments and presented as percentage of total β-hexosaminidase released.

For β-glucuronidase release, purified human neutrophils were preincubated with 10 μM cytochalasin B in HBSS-HB for 15 min on ice followed by 15 min at 37°C. The cells were then stimulated for 10 min with indicated amounts of agonists at 37°C. The reaction was terminated by rapid incubation in sample buffer with ice, and the samples were immediately centrifuged to separate supernatant from cell pellet. The amount of released β-glucuronidase was quantified by incubating 20 μl of supernatant with 20 μl of 10 mM 4-methylumbelliferyl β-D-glucuronide hydrate in 0.1 M sodium acetate, pH 4.0, and 0.1% Triton X-100 at 37°C for 15 min. The reaction was terminated by adding 300 μl of Stop Solution, pH 10.4, containing 50 mM glycine with 5 mM EDTA. Fluorescence was measured immediately in a spectrofluorometer with excitation wavelength at 365 nm and emission wavelengths at 460 nm. Total cellular β-glucuronidase was determined with cell lysate in 0.1% Triton X-100. Data are presented as percentage of total β-glucuronidase released.

**Detection of Superoxide Production.** Production of superoxide was determined based on superoxide dismutase-inhibitable reduction of cytochrome c. In brief, neutrophils (1 × 10⁶ cells per sample) were resuspended in 0.25 ml of PBS plus 0.5 mM MgCl₂, 0.9 mM CaCl₂, and 7.5 mM dextrose) containing 75 μM cytochrome c. The neutrophils were stimulated with fMLF, WKYMVm, MMK1, or the compounds to be tested. Samples containing superoxide dismutase (250 U) were run in parallel. Superoxide production was determined in a SpectroMax 340 microplate reader set at 37°C, and quantified by means of an extinction coefficient of 21.1 mM⁻¹cm⁻¹ for cytochrome c. The maximum rate of superoxide generation over a 3-min interval was calculated with the use of the SoftMax software (Molecular Devices).

**Ligand Binding Assay.** RBL-FPRL1 cells (1.2 × 10⁵) were seeded in 24-well cell culture plates 2 days before experiment. Confluent cells were washed twice with blocking buffer (RPMI 1640 supplemented with 33 mM HEPES, pH 7.5, and 0.1% BSA), and incubated in blocking buffer at 37°C for 2 h. After removing the blocking buffer, the compounds to be tested were added at different concentrations together with 0.5 nM 125I-WKYMVm (specific activity, 2200 Ci/mmol; PerkinElmer Life and Analytical Sciences), in a final volume of 125 μl in binding buffer (PBS with 10% BSA). In a parallel experiment, unlabelled WKYMVm was added at different concentrations. After incubation on ice for 3 h, the buffer with unbound radioligand was removed, and the cells were washed five times with binding buffer and lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, and 1% Triton X-100). Samples were collected, and the retained radioactivity was determined in a MINAXI γ 5650 Auto-γ counter (PerkinElmer Life and Analytical Sciences).

**FPRL1 Internalization.** An FPRL1-EGFP construct was prepared by ligation of an FPRL1 cDNA to the N terminus of EGFP (BD Biosciences Clontech, Palo Alto, CA). An RBL-2H3 cell line expressing the FPRL1-EGFP was generated by stable transfection using a procedure described above. The cells responded normally to WKYMVm in calcium mobilization assay (data not shown). For internalization assay, the RBL-FPRL1-EGFP cells were grown on glass coverslip for 16 h in DMEM supplemented with 20% FBS. The cells were washed with HBSS-HB and stimulated with the testing compounds at 37°C for 30 min. Internalization was determined by adding fixation buffer (3% paraformaldehyde in PBS) followed by incubation at room temperature for 15 min. The cells were then washed twice with PBS, once with H₂O, and mounted with VectaShield mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA). Fluorescent images were taken with a Nikon TE300 inverted epifluorescence microscope (Nikon, Tokyo, Japan).

**Phosphorylation of Mitogen-Activated Protein Kinases.** Activation of the p44/p42 MAP kinases (ERK1/2) was determined based on activation-associated phosphorylation (Payne et al., 1991). Cells were cultured in 12-well plates and serum-starved overnight before fMLF (100 nM) stimulation in HBSS/BSA. Some samples were pre-treated with 100 ng/ml pertussis toxin during serum starvation. The cells were then washed three times with PBS, once with H₂O, and stimulated with fixation buffer containing 10% FBS, 25 mM HEPES, pH 7.5, 0.1% Triton X-100. Cells were incubated at room temperature for 15 min. The cells were then washed twice with PBS, once with H₂O, and mounted with VectaShield mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA). Fluorescent images were taken with a Nikon TE300 inverted epifluorescence microscope (Nikon, Tokyo, Japan).

**Results**

**Identification and Chemical Synthesis of Substituted Quinazolinone As Ligand for FPRL1.** In previous studies, we reported that transfected cells expressing FPR or FPRL1 could respond to their respective agonists with NF-κB activation (Browning et al., 1997; Yang et al., 2001). Based on these findings, a stable cell line of HeLa that expresses FPRL1 and a NF-κB-driven firefly luciferase reporter was...
generated. Using this cell line, a synthetic compound library was screened for ligands that induce FPRL1-mediated expression of the luciferase reporter. Of the 15,760 synthetic compounds and 400 natural compounds screened, 56 (Table 1) were found to induce the NF-κB luciferase reporter activity, ranging from 6- to 10-fold over the unstimulated cells. These compounds were subsequently tested in a HeLa cell line that expresses the NF-κB luciferase reporter but not FPRL1, and 53 were found to stimulate NF-κB activation in the absence of FPRL1. The three compounds that induce the luciferase reporter only in the presence of FPRL1 were selected for further analysis and structural modification.

Quinazolinone derivatives were synthesized based on one of the 2 benzoheterocyclic derivatives, the core structure of which is shown in Fig. 1A. One of the heterocyclic quinazolinone derivatives displayed enhanced ability in the NF-κB luciferase reporter assay and was analyzed further in calcium mobilization assay (see below) as well as neutrophil functional assays. The structure of this compound (Quinazolinone-C1, abbreviated as Quin-C1; 4-butoxyphenyl)-4-oxo-1,4-dihydro-2H-quinazolin-3-yl-benzamide) is shown in Fig. 1B.

**Stimulation of Neutrophil Chemotaxis by Quin-C1.** Quin-C1 was examined in neutrophil chemotaxis assay. When applied to the bottom wells in a microchemotaxis chamber, Quin-C1 stimulated directional migration of human neutrophils with a typical bell-shaped dose curve (Fig. 2). Maximal chemotaxis was induced by 100 nM to 1 μM Quin-C1, and chemotaxis of neutrophils declined gradually when Quin-C1 concentrations were above 10 μM. In comparison, WKYMVm (W-peptide), one of the well characterized agonists for FPRL1, is more efficacious in stimulating neutrophil chemotaxis than Quin-C1.

**Quin-C1 Induces Release of β-Glucuronidase.** Blood neutrophils prepared from healthy donors were stimulated with Quin-C1 at various concentrations, and the release of β-glucuronidase was determined as described under Materials and Methods. As shown in Fig. 3, Quin-C1 induced degranulation in a dose-dependent manner. For this experiment, WKYMVm, fMLF and the FPRL1-selective agonist MMK1 were also examined for their abilities to induced degranulation. Both WKYMVm and fMLF exhibited higher potency and efficacy than Quin-C1. MMK1 displayed efficacy similar to that of Quin-C1. No further increase in degranulation was observed with MMK1 at concentrations higher than 1 μM or Quin-C1 beyond 100 μM (data not shown). MMK1, however, is more potent than Quin-C1 in stimulating β-glucuronidase release (Fig. 3).

**Quin-C1 Is Unable to Stimulate a Potent Superoxide Production in Neutrophils.** Freshly prepared human neutrophils were stimulated with Quin-C1, and the result was compared with those obtained with WKYMVm and MMK1. A shown in Fig. 4, Quin-C1 at concentrations of up to 100 μM failed to induce significant amount of O2- . The O2- produced by neutrophils from other donors (n = 3) did not exceed 1.7 nmol/min/10^7 cells with the maximal concentration of Quin-C1 used in this study (100 μM). Therefore, Quin-C1 is a partial agonist that selectively stimulates some but not all neutrophil bactericidal activities.

**Quin-C1 Selectively Activates FPRL1 over FPR.** The above results show that human neutrophils respond well to WKYMVm and fMLF in all functional assays. Both agonists activate FPR, and WKYMVm is a more potent agonist for FPRL1 than FPR (Le et al., 1999). Neutrophils are less responsive to MMK1, which selectively binds and activates FPRL1 (Klein et al., 1998). Quin-C1 is the least potent among the four ligands tested, and its efficacy equals that of MMK1 in β-glucuronidase release assay. Because neutrophils express both FPRL1 and FPR, a receptor that shares 69% of sequence identity at the amino acid level, it was necessary to determine whether Quin-C1 is a selective agonist for FPRL1 or a dual agonist for both receptors. We used RBL-2H3 cells transfected to express either FPRL1 (RBL-FPRL1) or FPR (RBL-FPR) for this study. The cells were loaded with the Ca2+-sensitive fluorescent probe Fluo3/AM, and stimulated either with WKYMVm, Quin-C1 or MMK1. Although the untransfected RBL cells did not respond to any one of these ligands (data not shown), we observed a marked increase in intracellular Ca2+ level in the RBL-FPRL1 cells. At 100 μM, Quin-C1 induced Ca2+ mobilization with magnitude similar to that induced by 100 nM WKYMVm (Fig. 5, A and B). Dose-response curve was subsequently obtained with the three ligands. As shown in Fig. 5C, the EC50 for Quin-C1 is 1.41 × 10^-6 M compared with the EC50 values for MMK1 (5.67 × 10^-8 M) and WKYMVm (4.45 × 10^-9 M). However, Quin-C1 is more efficacious than MMK1 in stimulating calcium mobilization through FPRL1.

In contrast to the above results, the RBL-FPR cells responded well to WKYMVm (Fig. 6A) but poorly to Quin-C1, which induced minimal Ca2+ mobilization (Fig. 6B). MMK1, an FPRL1-specific agonist, did not induce Ca2+ mobilization in RBL-FPR cells (Fig. 6C).

To confirm that Quin-C1 activates cellular functions through FPRL1, we examined its ability to stimulate the release of β-hexasominidase from RBL-FPRL1 cells and compared the result with that from the RBL-FPR cells. Quin-C1,
WKYMVm, and MMK1 all induced β-hexosaminidase release from RBL-FPRL1 cells, with EC₅₀ values of 1.88 × 10⁻⁶ M, 2.29 × 10⁻⁸ M, and 7.17 × 10⁻⁸ M, respectively (Fig. 7A). As expected, fMLF failed to induce β-hexosaminidase release in RBL-FPRL1 cells at concentrations up to 1 μM. In the FPR-expressing RBL cells, however, Quin-C1 and MMK1 were unable to stimulate degranulation whereas equally potent agonistic activities were observed for fMLF and WKYMVm in this cell line (Fig. 7B).

**Quin-C1 Stimulates Internalization of FPRL1.** G protein-coupled receptors rapidly internalize upon ligand binding (von Zastrow, 2003). Based on this property, we examined Quin-C1 for its ability to bind to FPRL1 and stimulate its internalization. An FPRL1-EGFP chimeric receptor was prepared by fusing the enhanced green fluorescent protein (EGFP) to the carboxyl terminus of FPRL1 and expressing in RBL-2H3 cells. As shown in Fig. 8, Quin-C1 effectively stimulated FPRL1 internalization in 30 min when used at concentrations above 1 μM. In comparison, WKYMVm, a more potent agonist, induced FPRL1 internalization at 100 nM.

**Partial Competition with WKYMVm for Binding to FPRL1.** Competitive binding assay was conducted with ¹²⁵I-labeled WKYMVm (0.5 nM), a commercially available radioligand for FPRL1. As shown in Fig. 9, Quin-C1 inhibited binding of ¹²⁵I-WKYMVm at concentrations above 1 μM. When used at a higher concentration (1 mM), Quin-C1 was able to reduce ¹²⁵I-WKYMVm binding by 36.1 ± 2.5%. In comparison, the unlabeled WKYMVm competed more effectively for binding ¹²⁵I-WKYMVm, with a half-maximal dose of 15 nM.

**Quin-C1 Stimulates ERK Phosphorylation.** Activation of mitogen-activated protein kinases results in their phosphorylation. Various agonists including chemoattractants can stimulate MAP kinase activation (Torres et al., 1993). We examined whether Quin-C1 was able to induce phosphorylation of the extracellular signal-regulated protein kinases, ERK1 (p44) and ERK2 (p42). As shown in Fig. 10, Quin-C1 stimulated ERK1/2 phosphorylation in RBL-FPRL1 cells in a time-dependent manner, with peak phosphorylation at 2 to 5 min. This pattern of phosphorylation is similar to that of MMK1.

**Quin-C1–Stimulated Transmembrane Signaling Involves Gi Proteins.** To characterize the proximal signaling pathway initiated by Quin-C1, we treated RBL-FPRL1 cells with pertussis toxin that catalyzes ADP ribosylation of the Gi proteins and disrupts their interaction with receptors (Bokoch and Gilman, 1984; Okajima et al., 1985). Pertussis toxin markedly reduced Quin-C1-induced Ca²⁺ mobilization.

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**Fig. 2.** Chemotaxis of neutrophils. Neutrophils from different donors (n = 3) were stimulated with WKYMVm or Quin-C1 at different concentrations. The chemotaxis assays were conducted in a 48-well microchemotaxis chamber as detailed under Materials and Methods. Checkerboard analysis was conducted by placing agonist in the upper chamber as well as the same agonist in the bottom chamber, and the cell counts resulting from random cell movement was subtracted from calculation of chemotaxis index (see Materials and Methods). Data shown (mean ± S.E.M.) is from one representative experiment of a total of three, with similar results.

**Fig. 3.** Release of β-glucuronidase in neutrophils stimulated with different agonists. Human neutrophils were stimulated with different concentrations of fMLF, WKYMVm (W-pep), MMK1, and Quin-C1. The results show dose-dependent secretion of β-glucuronidase from the stimulated cells as mean ± S.E.M., based on triplicate measurements from three independent experiments, using neutrophils from different donors.

**Fig. 4.** Differential activation of neutrophil NADPH oxidase by fMLF, WKYMVm, MMK1, and Quin-C1. The ability of the above agonists to stimulate neutrophil O₂⁻ production was compared using freshly prepared neutrophils from three different donors. Based on results from cytochrome c reduction assays, the SOD-inhibitable release of O₂⁻ varies among the agonists with WKYMVm being most potent and Quin-C1 the least potent and efficacious. Data shown are from one set of experiments from a total of three, and are displayed as mean ± S.D. from duplicate samples. Similar results were obtained from two other sets of experiments using neutrophils from different donors.
In a parallel experiment, pertussis toxin also reduced Ca\(^{2+}\) mobilization initiated by WKYMVm (Fig. 11, A and B). The inhibition, however, was incomplete. Because calcium mobilization is a highly sensitive assay and can be induced when a small fraction of the receptor is occupied, we examined the effect of pertussis toxin on degranulation, a less sensitive assay that requires a higher percentage of the receptors to be bound with agonist. As shown in Fig. 11C, a nearly complete blockade of β-hexosaminidase release was observed in cells treated with pertussis toxin.

**Discussion**

We report here the identification and characterization of a substituted quinazolinone as a novel nonpeptide ligand for FPRL1. This study takes advantage of FPRL1-mediated activation of NF-κB and the resulting expression of a NF-κB-directed luciferase reporter. The sensitivity and high-throughput capability of the assay make it suitable for the identification of agonists that induce the expression of the luciferase reporter. With modifications, this approach is expected to be useful in screening of antagonists that inhibit

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**Fig. 5.** Induction of Ca\(^{2+}\) mobilization in RBL-FPRL1 cells. RBL-FPRL1 cells were stimulated with WKYMVm (W-pep), MMK1, or Quin-C1 at different concentrations. Real-time changes in intracellular Ca\(^{2+}\) concentrations were recorded in Fluo3-labeled cells and shown as relative fluorescence intensity (counts per second or CPS). The Ca\(^{2+}\) mobilization trace from cells stimulated with WKYMVm (100 nM; A) is compared with that from cells stimulated with Quin-1 (100 μM; B). C, dose-response curve derived from RBL-FPRL1 stimulated with WKYMVm, MMK1, Quin-C1, and fMLF. Shown in the figure are means ± S.E.M. based on data collected from four independent experiments.

**Fig. 6.** Ca\(^{2+}\) mobilization in RBL-FPR cells. Experimental condition were the same as in Fig. 5 above, except that the RBL-FPR cells were stimulated with the following agonists. A, WKYMVm; B, Quin-C1; and C, MMK1. One set of representative traces, selected from a total of four independent experiments, is shown.
the activity induced by a known agonist, such as WKYMVm. Although antagonists for FPRL1 have obvious therapeutic value, agonists that selectively stimulate neutrophil functions may be used as research tools and therapeutics. As a first step toward this goal, we chose to screen for FPRL1 agonists and prove the concept that small, nonpeptide ligands for FPRL1 can be identified using the reporter-based approach.

Quin-C1 exhibits agonistic activities in neutrophil chemotaxis and degranulation assays. In addition, Quin-C1 stimulates calcium mobilization and phosphorylation of the MAP kinases ERK1 and ERK2. In several comparative studies, we have shown that Quin-C1 is approximately 1000-fold less potent than WKYMVm, one of the most potent and efficacious agonists for FPRL1. Other FPRL1 agonists such as Aβ1–42 and serum amyloid A are less potent, and their EC50 values in most functional assays are in the micromolar range. Compared with MMK1, a synthetic peptide and surrogate ligand for FPRL1 (Klein et al., 1998), Quin-C1 is equally efficacious in degranulation assay and slightly more efficacious in calcium mobilization assay. Both Quin-C1 and MMK1 can induce activation of ERK 1 and 2 with similar kinetics. In comparison, the WKYMVm-induced ERK phosphorylation peaks earlier. In chemotaxis assay, Quin-C1 is similar to MMK1 in efficacy (data not shown). These results indicate that Quin-C1 is a partial agonist for FPRL1.

In contrast to the above results, Quin-C1 is unable to stimulate superoxide production even when used at a concentration of 100 μM. One possibility for this discrepancy is that superoxide production requires higher concentrations of chemotactic peptides, whereas chemotaxis and calcium mobilization can be induced when a smaller percentage of receptors are occupied by agonist. For example, fMLF induces chemotaxis at lower nanomolar concentrations, and stimulates superoxide generation at 100 nM or above. Although the low potency of Quin-C1 limits the range of concentrations that can be tested in these functional assays, it is notable that Quin-C1 induces only marginal superoxide production at 1 μM (Fig. 4) but substantial amount of β-hexosaminidase release at the same concentration (Fig. 7). Its potency in these two functional assays is not comparable with that of MMK1, which induces significantly more superoxide production at 1 μM (Fig. 7). This brings us to the second possibility, that ligands may behave differently in functional assays because of their intrinsic ability to induce different receptor

Fig. 7. FPRL1-selective release of β-hexosaminidase by Quin-C1. RBL-FPRL1 (A) and RBL-FPR (B) cells were stimulated with fMLF, WKYMVm, MMK1, and Quin-C1, each used at several different concentrations. After 10 min, the released β-hexosaminidase was determined. The results (mean ± S.E.M.) are expressed as percentage release of total cell-associated β-hexosaminidase activity and are based on three independent experiments with triplicate measurements.

Fig. 8. Internalization of FPRL1. RBL-2H3 cells expressing an FPRL1-EGFP construct were treated with buffer, vehicle (0.5% DMSO), WKYMVm (W-pep), and Quin-C1 at three different concentrations as indicated. After 30 min, the cells were fixed, stained with 4,6-diamidino-2-phenylindole for nuclei, and viewed under an epifluorescence microscope. Representative images from three different experiments are shown.
conformational changes. This notion is substantiated by published data showing that lipixin A₄ and several newly identified peptide agonists for FPRL1 activate selected functions of neutrophils, with no clear correlation to their binding affinity and potency in other functional assays (Bae et al., 2003). In addition, the amyloid peptide Aβ₁₋₄₂ is a low-affinity ligand for FPRL1 and requires micromolar concentrations for Ca²⁺ mobilization (Le et al., 2001). But it is able to induce superoxide production in microglial cells (Tiffany et al., 2001). Although the complex behavior of Quin-C1 and other FPRL1 ligands may not be fully explained at this time, the ligand-specific receptor conformation change is a property that may be explored for designing novel ligands capable of stimulating selected functions, thus reducing tissue injury caused by release of reactive oxygen species.

Because FPRL1 shares considerable sequence homology with human FPR, we determined whether Quin-C1 also affects cellular functions in transfected RBL cells expressing human FPR. Our results demonstrate a very high selectivity of this compound for FPRL1, with only a minor increase in calcium mobilization in RBL-FPR cell when Quin-1 was used at the concentration of 100 μM. The selectivity of Quin-C1 for FPRL1 is similar to that of MMK1, whereas several other peptide agonists, including WKYMVm, can activate both FPR and FPRL1. This result demonstrates the potential utility of Quin-C1 as an FPRL1-specific ligand. Because an exhaustive survey of GPCRs has not been conducted, we cannot rule out the possibility that Quin-C1 activates other GPCRs. However, based on the observation that Quin-C1 induces Ca²⁺ mobilization in RBL-FPRL1 cells but not in the untransfected RBL cells, it is safe to conclude that this compound does not activate the endogenous GPCRs in RBL cells under the experimental conditions.

Quin-C1 is quite different in structure from WKYMVm and other FPRL1 agonists. Its identification as an FPRL1 ligand provides further evidence for the diversity of the ligands that can interact with this receptor. A systematic study of the structure-activity relationship will be necessary to understand how FPRL1 binds such a diverse array of ligands. In addition to structural modifications of the ligand, mutagenesis study of FPRL1 will also help to identify the domains and residues that constitute the binding pockets for different ligands. Given the very limited information available for FPRL1 structure, it is not possible to accurately determine the Quin-C1 binding site on the receptor at present. The incomplete competitive binding assay suggests that Quin-C1 interacts with FPRL1 on a site that is different from but probably adjacent to the WKYMVm binding site, such that partial competition can be achieved. It also reflects the technical limitation of using Quin-C1 at concentrations above 1 mM. Although it is difficult to calculate the exact $K_D$ based on the partial competition binding data, we estimate that Quin-C1 interacts with FPRL1 with dissociation constant in the micromolar range. This relatively low binding affinity may limit the use of Quin-C1 as a therapeutic agent. However, this nonpeptide ligand for FPRL1 may serve as the

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**Fig. 9.** Competitive binding with ¹²⁵I-labeled WKYMVm. RBL-FPRL1 cells were plated in 24-well plates and incubated on ice for 3 h with 0.5 nM ¹²⁵I-WKYMVm in the absence or presence of unlabeled WKYMVm or Quin-C1 at the indicated concentrations. Data shown are triplicate samples with mean ± S.E.M. from two experiments.

**Fig. 10.** Stimulation of ERK phosphorylation. RBL-2H3 cells were serum-starved overnight and stimulated with one of the three ligands as indicated. At different time points, samples were collected and cell lysates were prepared. The total as well as phosphorylated ERK1 (p44) and ERK2 (p42) were determined by Western blotting. A set of blots, representative of three different experiments, is shown in this figure.
nucleus for further structural modifications leading to the discovery of more potent and efficacious agonists. Based on published data, it is possible to develop quinazolinone derivatives with higher binding affinities for cell surface receptors including GPCRs (Oshit et al., 1986; Chern et al., 1993; Padia et al., 1998). The selective activation property of Quin-C1 will be of particular interest for future development of small molecular weight, nonpeptide compounds devoid of cell-stimulating functions associated with tissue injury. It is also hopeful that structural modification of Quin-C1 will result in clinically useful antagonists for FPRL1.

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**References**


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