

A novel nonpeptide ligand for formyl peptide receptor-like 1

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Running title: A novel nonpeptide ligand for FPRL1

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Number of text pages: 30

Number of tables: 1

Number of figures: 11

Number of references: 35

Number of words in

Abstract: 241

Introduction: 605

Discussion: 1051

Non-standard abbreviations: FPR, formyl peptide receptor; FPRL1, formyl peptide receptor-like 1; Quin-C1, quinazolinone-C1 (4-butoxy-N-[2-(4-methoxyphenyl)-4-oxo-1,4-dihydro-2H-quinazolin-3-yl]-benzamide); fMLF, N-formyl-Met-Leu-Phe; NF- κ B, nuclear factor kappa B; ERK, extracellular signal regulated kinase.

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 β -glucuronidase in peripheral blood neutrophils with a potency of approximately 1/1,000 of that of the peptide agonist WKYMVm. In studies using transfected rat basophilic leukemia (RBL) cell lines expressing either FPR or FPRL1, Quin-C1 induced enzyme release from RBL-FPRL1 but not RBL-FPR cells. Likewise, Quin-C1 selectively stimulates calcium mobilization in RBL-FPRL1 cells, a response that was markedly inhibited by pertussis toxin. Quin-C1 also stimulates phosphorylation of extracellular signal-regulated protein kinases 1 and 2, and induces internalization of an FPRL1 fused to green fluorescent protein. In degranulation assays, both the FPRL1-selective peptide agonist MMK1 and Quin-C1 exhibited lower efficacy and potency than WKYMVm, with EC₅₀ values of 7.17×10^{-8} M and 1.88×10^{-6} M, respectively, as compared to the EC₅₀ value for WKYMVm (2.29×10^{-8} M). However, Quin-C1 did not induce neutrophil superoxide generation at up to 100 μ M. Based on these results, we conclude that Quin-C1 is a novel nonpeptide ligand that binds to FPRL1 and selectively stimulates FPRL1-mediated functions. Quin-C1 is a prototype of substituted quinazolinones based on which further structural modifications may be made to improve its efficacy and potency for FPRL1.

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 Uhing, 1992). The tripeptide N-formyl-Met-Leu-Phe (fMLF) is a prototype of formylated chemotactic peptides for neutrophils, owing to its ability to bind and 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 (Le et al., 2002; Mills et al., 1999; Prossnitz and Ye, 1997). 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); *Fpr11*, 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 *fpr12* gene encodes a receptor (FPRL2) that share 56% amino acid sequence identity to human FPR (Bao et al., 1992). While FPR has been extensively characterized pharmacologically and physiologically since 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 (Quehenberger et al., 1993; Ye et al., 1992),

FPRL1 now has more than 10 ligands and is the most promiscuous in this group of receptors with respect to agonist selectivity (Le et al., 2002; Prossnitz and Ye, 1997). 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 -amyloid peptide A β ₁₋₄₂ bind and activate FPRL1 (Le et al., 2001; Su et al., 1999). Of interest is the ability of serum amyloid A to stimulate cytokine gene expression (He et al., 2003), and of A β ₁₋₄₂ 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 PrP^{Sc}, 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 anti-apoptotic 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 (LESIFRSLFRVM) were synthesized at Macromolecular Resources, Fort Collins, CO. The chemotactic peptide fMLF was purchased from Sigma (St. Louis, MO). [¹²⁵I]-WKYMVm (Bolton-Hunter labeled) was obtained from Perkin Elmer (Boston, MA). Fluo3/AM, used in the calcium mobilization assay, was purchased from Molecular Probes (Eugene, OR). Pertussis toxin was obtained from List Laboratories (Campbell, CA). The anti-ERK 1/2 and anti-phospho-ERK antibodies were from Cell Signaling Technologies (Beverly, MA). Other reagents were obtained from Sigma.

Cell Culture. The rat basophilic leukemia cell line RBL-2H3 was originally obtained from Dr. John Apgar (Scripps Research Institute, La Jolla, CA), and maintained in DMEM supplemented with 20% FBS. The cells were transfected with an expression vector containing the human FPRL1 or FPR cDNA as described previously (He et al., 2000). Stable transfectants were selected against G418 for 3-4 weeks, and 30-50 individual transfectants were pooled for functional analysis. HeLa cells were transfected with a pNFκB-Luc reporter plasmid containing 5 copies of NF-κB binding sequence (Stratagene, La Jolla, CA), with or without co-transfection with the human FPRL1 expression vector. After G418 selection, the cell line was maintained in DMEM supplemented with 10% FBS.

Isolation of human neutrophils. Peripheral blood was drawn from healthy donors using a protocol approved by the Institutional Review Board at The University of Illinois at Chicago. Neutrophils were purified using Percoll gradient (Amersham Pharmacia, Piscataway, NJ) according to the methods of Ulmer and Flad (Ulmer and Flad, 1979). Cell viability was 98% or higher, and ~97% of the isolated cells were neutrophils. The cells were kept in RPMI 1640 medium and used on the same day of isolation.

Whole Cell-based compound screening. HeLa cells expressing FPRL1/NFκB-Luc were seeded in 96-well plates at a density of 1.5×10^4 cells per well. After cells became adherent, they were serum-starved in DMEM without phenol red for 16 h prior to library screening. For each well, samples containing 10 individual compounds (approximately 0.5 μg/ml per compound) were applied. After a 5-hour incubation period, the expressed luciferase activity was determined in a Wallac 1420 multilabel counter (VICTOR², Perkin Elmer) using the Steady-Glo Luciferase Assay solutions from Promega (Madison, WI).

A total of 15760 synthetic compounds and 400 natural compounds were screened. Library compounds were designed as the XY matrix to facilitate positive identification. Fifty-six lead compounds, that produce 6-10 fold induction in luciferase activity, were tested in HeLa cells expressing pNFκB-Luc but not FPRL1. Three compounds that do not induce the luciferase reporter activity in the absence of FPRL1 were further analyzed in calcium mobilization assay using the RBL-FPRL1 cell line.

The Calcium Mobilization Assay. RBL-FPRL1 cells were harvested with enzyme-free cell dissociation buffer (Invitrogen, Carlsbad, CA). The cells were incubated with 4 μM Fluo-3/AM 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 5×10^5 cells per assay. More detailed calcium mobilization assays were conducted in a PTI 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 anthranilhydrazine 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 benzaldehyde. The structure of the original compound, based on which Quin-C1 is synthesized, is shown in Figure 1A.

Chemotaxis. Agonist induced migration of cells was assessed in a 48-well micro-chemotaxis chamber (Neuro Probe, Cabin John, MA), as previously reported (He et al., 2000). Briefly, different concentrations of the agonists WKYMVm (0.01 nM - 10 μ M) and Quin-C1 (1 nM - 1 mM) were placed in the lower compartment (30 μ l). Purified human neutrophils (35 μ l of a 2×10^6 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 μ m. 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 (Molecular Devices). After subtraction of migrating cells due to chemokinesis, data were presented as chemotaxis index that represents the ratio of the density of the area where cells migrated towards agonists over the density of the area where cells migrated towards 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 pre-incubated with 10 μ M cytochalasin B in HBSS containing 20 mM HEPES (pH 7.4) and 0.2% of BSA (HBSS-HB) for 15 min on ice followed by 15 min at 37°C. Following 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 a rapid reduction in sample temperature with ice. The amount of secreted β -hexosaminidase was quantified by incubating 20 μ l of supernatant with 10 μ l of 1 mM *p*-nitrophenyl-*N*-acetyl- β -*D*-glucosamide 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 μ l 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 percent of total β -hexosaminidase released.

For β -glucuronidase release, purified human neutrophils were pre-incubated with 10 μ M 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 a rapid reduction in sample temperature with ice, and the samples were immediately centrifugated to separate supernatant from cell pellet. The amount of released β -glucuronidase was quantified by incubating 20 μ l of supernatant with 20 μ l 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 μ l of Stop Solution (pH 10.4) containing 50 mM glycine with 5 mM EDTA. Fluorescence was measured immediately in a PTI 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 percent of total β -glucuronidase released.

Detection of superoxide production. Production of superoxide was determined based on superoxide dismutase-inhibitable reduction of cytochrome c. Briefly, neutrophils (1 x 10⁶ cells per sample) were resuspended in 0.25 ml of PBSG (PBS plus 0.5 mM MgCl₂, 0.9 mM CaCl₂,

and 7.5 mM dextrose) containing 75 μ M 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 $\text{mM}^{-1}\text{cm}^{-1}$ for cytochrome c. The maximum rate of superoxide generation over a 3-minute interval was calculated with the use of the SoftMax software (Molecular Devices).

Ligand Binding Assay. RBL-FPRL1 cells (1.2×10^5) 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 of ^{125}I -WKYMVm (Perkin Elmer; specific activity 2,200 Ci/mmol), in a final volume of 125 μ l in binding buffer (PBS with 10% BSA). In a parallel experiment, unlabeled WKYMVm was added at different concentrations. After incubation on ice for 3 h, the buffer with unbound radioligand were removed, and the cells were washed 5 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-Gamma counter (Packard Instrument Co, Downers Grove, IL).

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 terminated 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 DAPI (Vector Laboratories, Inc. Burlingame, CA). Fluorescent images were taken with a Nikon TE300 inverted epifluorescence microscope.

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 prior to fMLF (100 nM) stimulation in HBSS/BSA. Some samples were pretreated with 100 ng/ml pertussis toxin during serum starvation. The reaction was terminated by adding 300 μ l of ice-cold SDS-PAGE loading buffer [15% (v/v) glycerol, 125 mM Tris-Cl, pH 6.8, 5 mM EDTA, 2% (w/v) SDS, 0.1% bromophenol blue and 1% β -mercaptoethanol]. Samples were transferred to microcentrifuge tubes and sonicated two times for 5 sec each to disperse DNA contents. After boiling, samples were analyzed by SDS-PAGE and Western blot using anti-ERK1/2 and anti-phospho-ERK1/2 antibodies at 1:1,000 dilution. HRP-conjugated anti-rabbit antibody (1:3,000) was used as secondary antibody. The resulting immunocomplex was visualized by SuperSignal West Pico Chemiluminescence kit (Pierce) according to manufacture's instruction.

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- κ B 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- κ B-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 15760 synthetic compounds and 400 natural compounds screened, 56 (Table 1) were found to induce the NF- κ B 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- κ B luciferase reporter but not FPRL1, and 53 were found to stimulate NF- κ B activation in the absence of FPRL1. The 3 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 Figure 1A. One of the heterocyclic quinazolinone derivatives displayed enhanced ability in the NF- κ B 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-butoxy-N-[2-(4-methoxy-phenyl)-4-oxo-1,4-dihydro-2H-quinazolin-3-yl]-benzamide) is shown in Figure 1B.

Stimulation of neutrophil chemotaxis by Quin-C1. Quin-C1 was examined in neutrophil chemotaxis assay. When applied to the bottom wells in a micro-chemotaxis chamber, Quin-C1 stimulated directional migration of human neutrophils with a typical bell-shaped dose curve (Figure 2). Maximal chemotaxis was induced by 100 nM to 1 μ M of Quin-C1, and chemotaxis of neutrophils declined gradually when Quin-C1 concentrations were above 10 μ M. 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 in *Materials and Methods*. As shown in Figure 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 similar efficacy to Quin-C1. No further increase in degranulation was observed with MMK1 at concentrations higher than 1 μ M or Quin-C1 beyond 100 μ M (data not shown). MMK1, however, is more potent than Quin-C1 in stimulating β -glucuronidase release (Figure 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. As shown in Figure 4, Quin-C1 at concentrations of up to 100 μ M failed to induce significant amount of O_2^- . The O_2^- produced by neutrophils from other donors (n=3) did not exceed 1.7 nmol/min/ 10^7 cells with maximal concentration of Quin-C1 used in this study (100 μ M). 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 4 ligands tested, and its efficacy equals that of MMK1 in β -glucuronidase release assay. Since 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 Ca^{2+} -sensitive fluorescent probe Fluo3/AM, and stimulated either with WKYMVm, Quin-C1 or MMK1. While the untransfected RBL cells did not respond to any one of these ligands (data not shown), we observed a marked increase in intracellular Ca^{2+} level in the RBL-FPRL1 cells. At 100 μM , Quin-C1 induced Ca^{2+} mobilization with magnitude similar to that induced by 100 nM of WKYMVm (Figure 5A, 5B). Dose-response curve was subsequently obtained with the 3 ligands. As shown in Figure 5C, the EC_{50} for Quin-C1 is 1.41×10^{-6} M as compared to the EC_{50} 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 (Figure 6A) but poorly to Quin-C1, which induced minimal Ca^{2+} mobilization (Figure 6B). MMK1, an FPRL1-specific agonist, did not induce Ca^{2+} mobilization in RBL-FPR cells (Figure 6C).

To confirm that Quin-C1 activates cellular functions through FPRL1, we examined its ability to stimulate the release of β -hexosaminidase 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 EC_{50} values of 1.88×10^{-6} M, 2.29×10^{-8} M and 7.17×10^{-8} M, respectively (Figure 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 (Figure 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 expressed in RBL-2H3 cells. As shown in Figure 8, Quin-C1 effectively stimulated FPRL1 internalization in 30 minutes when used at concentrations above 1 μ M. 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 125 I-labeled WKYMVm (0.5 nM), a commercially available radioligand for FPRL1. As shown in Figure 9, Quin-C1 inhibited binding of 125 I-WKYMVm at concentrations above 1 μ M. When used at a higher concentration (1 mM), Quin-C1 was able to reduce 125 I-WKYMVm binding by 36.1 ± 2.5 %. In comparison, the unlabeled WKYMVm competed more effectively for binding 125 I-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 is able to induce phosphorylation of the extracellular signal-regulated protein kinases, ERK1 (p44) and ERK2 (p42). As shown in Figure 10, Quin-C1 stimulated ERK1/2 phosphorylation in RBL-FPRL1 cells in a time-dependent manner, with peak phosphorylation at 2-5 minutes. 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 Ca^{2+} mobilization. In a parallel experiment, pertussis toxin also reduced Ca^{2+} mobilization initiated by WKYMVm (Figure 11A, B). The inhibition, however, was incomplete. Since 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 Figure 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- κ B and the resulting expression of a NF- κ B-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 the activity induced by a known agonist, such as WKYMVm. While 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 towards 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 1,000-fold less potent than WKYMVm, one of the most potent and efficacious agonists for FPRL1. Other FPRL1 agonists such as A β ₁₋₄₂ and serum amyloid A are less potent and their EC₅₀ values in most functional assays are in the micromolar range. When compared to 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 μ M. 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 100 μ M (Figure 4) but substantial amount of β -hexosaminidase release at the same concentration (Figure 7). Its potency in these two functional assays is not comparable to that of MMK1, which induces significantly more superoxide production at 1 μ M (Figure 7). This brings to the second

possibility that ligands may behave differently in functional assays due to their intrinsic ability to induce different receptor 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 A β ₁₋₄₂ 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). While 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 due to release of reactive oxygen species.

Since 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 μ M. 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. Since 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 interact 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 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 (Chern et al., 1993; Oshita et al., 1986; 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.

ACKNOWLEDGEMENTS:

The authors would like to thank Dr. Rong He for helpful discussions.

REFERENCES:

- Bae YS, Park JC, He R, Ye RD, Kwak JY, Suh PG and Ho Ryu S (2003) Differential signaling of formyl peptide receptor-like 1 by Trp-Lys-Tyr-Met-Val-Met-CONH₂ or lipoxin A₄ in human neutrophils. *Mol Pharmacol* **64**:721-30.
- Baek SH, Seo JK, Chae CB, Suh PG and Ryu SH (1996) Identification of the peptides that stimulate the phosphoinositide hydrolysis in lymphocyte cell lines from peptide libraries. *J Biol Chem* **271**:8170-5.
- Bao L, Gerard NP, Eddy RL, Shows TB and Gerard C (1992) Mapping genes for the human C5a receptor (C5AR), human FMLP receptor (FPR), and two FMLP receptor homologue orphan receptors (FPRH1, FPRH2) to chromosome 19. *Genomics* **13**:437-40.
- Bokoch GM and Gilman AG (1984) Inhibition of receptor mediated release of arachidonic acid by pertussis toxin. *Cell* **39**:301-8.
- Boulay F, Tardif M, Brouchon L and Vignais P (1990) The human N-formylpeptide receptor: characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. *Biochemistry* **29**:11123-33.
- Browning DD, Pan Z, Prossnitz ER and Ye RD (1997) Cell type and developmental stage-specific activation of NF- κ B by fMet-Leu-Phe in myeloid cells. *J. Biol. Chem.* **272**:7995-8001.
- Chern JW, Tao PL, Yen MH, Lu GY, Shiau CY, Lai YJ, Chien SL and Chan CH (1993) Studies on quinazolines. 5. 2,3-dihydroimidazo[1,2-c]quinazoline derivatives: a novel class of potent and selective alpha 1-adrenoceptor antagonists and antihypertensive agents. *J Med Chem* **36**:2196-207.

- Fiore S, Maddox JF, Perez HD and Serhan CN (1994) Identification of a human cDNA encoding a functional high affinity lipoxin A₄ receptor. *J Exp Med* **180**:253-60.
- He R, Sang H and Ye RD (2003) Serum amyloid A induces IL-8 secretion through a G protein-coupled receptor, FPRL1/LXA₄R. *Blood* **101**:1572-81.
- He R, Tan L, Browning DD, Wang JM and Ye RD (2000) The synthetic peptide trp-lys-tyr-met-val-D-Met is a potent chemotactic agonist for mouse formyl peptide receptor. *J Immunol* **165**:4598-605.
- Klein C, Paul JJ, Sauve K, Schmidt MM, Arcangeli L, Ransom J, Trueheart J, Manfredi JP, Broach JR and Murphy AJ (1998) Identification of surrogate agonists for the human FPRL-1 receptor by autocrine selection in yeast. *Nat Biotechnol* **16**:1334-7.
- Le Y, Gong W, Li B, Dunlop NM, Shen W, Su SB, Ye RD, Murphy PM and Wang JM (1999) Utilization of two seven-transmembrane, G-protein coupled receptors FPRL1 and FPR by the synthetic hexapeptide WKYVMm for human phagocyte activation. *J. Immunol.* **163**:6777-84.
- Le Y, Gong W, Tiffany HL, Tumanov A, Nedospasov S, Shen W, Dunlop NM, Gao JL, Murphy PM, Oppenheim JJ and Wang JM (2001) Amyloid (beta)₄₂ activates a G-protein-coupled chemoattractant receptor, FPR-like-1. *J Neurosci* **21**:RC123.
- Le Y, Murphy PM and Wang JM (2002) Formyl-peptide receptors revisited. *Trends Immunol* **23**:541-48.
- Mayer JP, Lewis GS, Curtis MJ and Zhang J (1997) Solid phase synthesis of quinazolinone. *Tetrahedron Lett* **38**:8445-8.

- Mills JS, Miettinen HM and Jesaitis AJ (1999) The N-formyl peptide receptor: Structure, signaling and disease, in *Molecular Biology of Inflammation* (Serhan CN and Ward PA eds) pp 215-45, Humana Press, Totowa, NJ.
- Murphy PM, Ozcelik T, Kenney RT, Tiffany HL, McDermott D and Francke U (1992) A structural homologue of the N-formyl peptide receptor: characterization and chromosome mapping of a peptide chemoattractant receptor family. *J Biol Chem* **267**:7637-43.
- Okajima F, Katada T and Ui M (1985) Coupling of the guanine nucleotide regulatory protein to chemotactic peptide receptors in neutrophil membranes and its uncoupling by islet-activating protein, pertussis toxin. *J Biol Chem* **260**:6761-8.
- Oshita M, Morikawa K, Aratani T, Kato H and Ito Y (1986) Pharmacological studies of 2-(3-(3-(1-piperidinylmethyl)-phenoxy)propylamino)-4 (3H)-quinazolinone (NO-794), a new histamine H₂-receptor antagonist. *Jpn J Pharmacol* **42**:229-35.
- Padia JK, Field M, Hinton J, Meecham K, Pablo J, Pinnock R, Roth BD, Singh L, Suman-Chauhan N, Trivedi BK and Webdale L (1998) Novel nonpeptide CCK-B antagonists: design and development of quinazolinone derivatives as potent, selective, and orally active CCK-B antagonists. *J Med Chem* **41**:1042-9.
- Payne DM, Rossomando AJ, Martino P, Erickson AK, Her JH, Shabanowitz J, Hunt DF, Weber MJ and Sturgill TW (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J* **10**:885-92.
- Perez HD, Holmes R, Kelly E, McClary J and Andrews WH (1992) Cloning of a cDNA encoding a receptor related to the formyl peptide receptor of human neutrophils. *Gene* **118**:303-4.

- Prossnitz ER and Ye RD (1997) The N-formyl peptide receptor: A model for the study of chemoattractant receptor structure and function. *Pharmacol Ther* **74**:73-102.
- Quehenberger O, Prossnitz ER, Cavanagh SL, Cochrane CG and Ye RD (1993) Multiple domains of the N-formyl peptide receptor are required for high-affinity ligand binding. Construction and analysis of chimeric N-formyl peptide receptors. *J Biol Chem* **268**:18167-75.
- Resnati M, Pallavicini I, Wang JM, Oppenheim J, Serhan CN, Romano M and Blasi F (2002) The fibrinolytic receptor for urokinase activates the G protein-coupled chemotactic receptor FPRL1/LXA₄R. *Proc Natl Acad Sci U S A* **99**:1359-64.
- Snyderman R and Uhing RJ (1992) Phagocytic cells: Stimulus-response coupling mechanisms, in *Inflammation: Basic Principles and Clinical Correlates* (Gallin JI, Goldstein IM and Snyderman R eds) pp 421-439, Raven Press, New York.
- Su SB, Gong W, Gao JL, Shen W, Murphy PM, Oppenheim JJ and Wang JM (1999) A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. *J Exp Med* **189**:395-402.
- Tiffany HL, Lavigne MC, Cui YH, Wang JM, Leto TL, Gao JL and Murphy PM (2001) Amyloid-beta induces chemotaxis and oxidant stress by acting at formylpeptide receptor 2, a G protein-coupled receptor expressed in phagocytes and brain. *J Biol Chem* **276**:23645-52.
- Torres M, Hall FL and O'Neill K (1993) Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein-kinases. *J Immunol* **150**:1563-78.

- Ulmer AJ and Flad HD (1979) Discontinuous density gradient separation of human mononuclear leucocytes using Percoll as gradient medium. *J. Immunol. Methods* **30**:1-10.
- von Zastrow M (2003) Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway. *Life Sci* **74**:217-24.
- Yang M, Sang H, Rahman A, Wu D, Malik AB and Ye RD (2001) Galpha(16) couples chemoattractant receptors to NF-kappa B activation. *J Immunol* **166**:6885-92.
- Yazawa H, Yu ZX, Takeda, Le Y, Gong W, Ferrans VJ, Oppenheim JJ, Li CC and Wang JM (2001) Beta amyloid peptide (Abeta42) is internalized via the G-protein-coupled receptor FPRL1 and forms fibrillar aggregates in macrophages. *FASEB J* **15**:2454-62.
- Ye RD, Cavanagh SL, Quehenberger O, Prossnitz ER and Cochrane CG (1992) Isolation of a cDNA that encodes a novel granulocyte N-formyl peptide receptor. *Biochem Biophys Res Commun* **184**:582-9.
- Ying G, Iribarren P, Zhou Y, Gong W, Zhang N, Yu ZX, Le Y, Cui Y and Wang JM (2004) Humanin, a newly identified neuroprotective factor, uses the G protein-coupled formylpeptide receptor-like-1 as a functional receptor. *J Immunol* **172**:7078-85.

FOOTNOTES:

This study was supported in part by NIH grant AI33503 (to RDY), grants from the Ministry of Science and Technology of China (2002AA2Z343A; to MWW) and the Chinese Academy of Sciences (KSCX1-SW-11-2; to MWW).

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M. N. and X. C. made equal contribution to this work.

LEGENDS FOR FIGURES:

Figure 1. Chemical structure of the core compound and Quin-C1. A, the core structure based on which Quin-C1 is developed. B, the structure of Quin-C1 (4-butoxy-N-[2-(4-methoxyphenyl)-4-oxo-1,4-dihydro-2H-quinazolin-3-yl]-benzamide), in which $R_1 = -OCH_3$, and $R_2 = H$.

Figure 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 micro-chemotaxis chamber as detailed in *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 \pm SEM) is from one representative experiment of a total of three, with similar results.

Figure 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 \pm SEM, based on triplicate measurements from 3 independent experiments, using neutrophils from different donors.

Figure 4. Differential activation of neutrophil NADPH oxidase by fMLF, WKYMVm, MMK1 and Quin-C1. The ability of the above agonists to stimulate neutrophil O_2^- production was compared using freshly prepared neutrophils from 3 different donors. Based on results from

cytochrome c reduction assays, the SOD-inhibitable release of O_2^- 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 3, and are displayed as mean \pm SD from duplicate samples. Similar results were obtained from two other sets of experiments using neutrophils from different donors.

Figure 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, panel A) is compared to that from cells stimulated with Quin-1 (100 μ M, panel B). C, dose-response curve derived from RBL-FPRL1 stimulated with WKYMVm, MMK1, Quin-C1 and fMLF. Shown in the figure are means \pm SEM based on data collected from 4 independent experiments.

Figure 6. Ca^{2+} mobilization in RBL-FPR cells. Experimental condition were the same as in Figure 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 4 independent experiments, is shown.

Figure 7. FPRL1-selective release of β -hexosaminidase by Quin-C1. RBL- FPRL1 (panel A) and RBL-FPR (panel 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 \pm SEM) are expressed as % release of total cell-associated β -hexosaminidase activity, and are based on 3 independent experiments with triplicate measurements.

Figure 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 3 different concentrations as indicated. After 30 minutes, the cells were fixed, stained with DAPI for nuclei, and viewed under an epifluorescence microscope. Representative images from 3 different experiments are shown.

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

Figure 10. Stimulation of ERK phosphorylation. RBL-2H3 cells were serum-starved overnight and stimulated with one of the 3 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 3 different experiments, is shown in this figure.

Figure 11. Effect of pertussis toxin on Quin-C1 induced Ca^{2+} mobilization and degranulation. RBL- FPRL1 cells were treated for 16 hours with pertussis toxin (PTX, 100

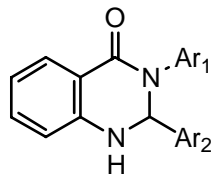
ng/ml) or vehicle (culture medium), before stimulation with either WKYMVm (W-pep, panel A) or Quin-C1 (panel B) for measurement of Ca^{2+} mobilization. Representative traces from one of the 4 experiments are shown. C, RBL-FPRL1 cells were treated with PTX as described above, and then stimulated with the agonists at the indicated concentrations. Release of β -hexosaminidase was determined. Data shown are means \pm SEM from 2 independent experiments, each with triplicate measurements.

Table 1. Chemical properties of the initial “hits” identified by primary screening.

Chemical Structure	Number of “hits”	Comments
Amine	5	
Aromatic carboxylic acid and ester	5	
Aromatic amide compound	12	
Urea	3	
Sulfonamide	3	
Five or six-membered N-containing heterocyclic derivatives	12	Including pyrimidine, thiazolinone, imidazolone, dihydropyridine, <i>etc.</i>
Benzoheterocyclic derivatives	11	Including quinazolinone, indole, benzoimidazole, quinoline, benzothiazole, <i>etc.</i>
Others	5	
Total	56	

Figure 1

A



B

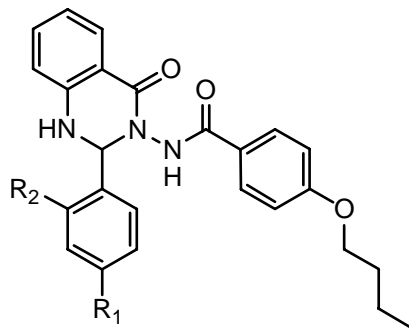


Figure 2

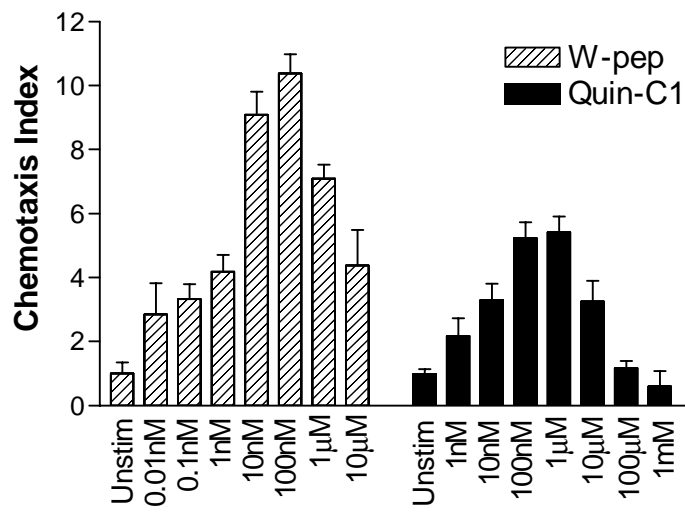


Figure 3

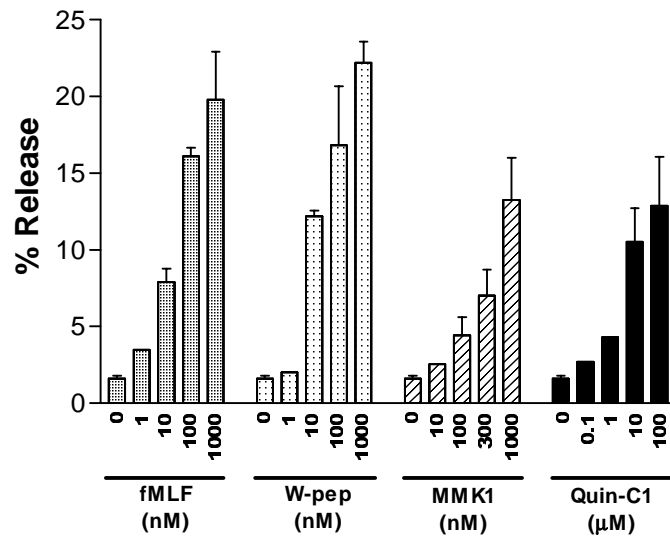


Figure 4

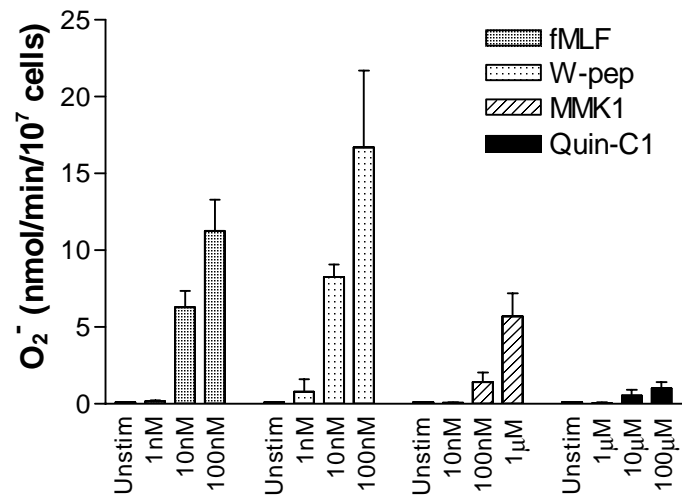


Figure 5

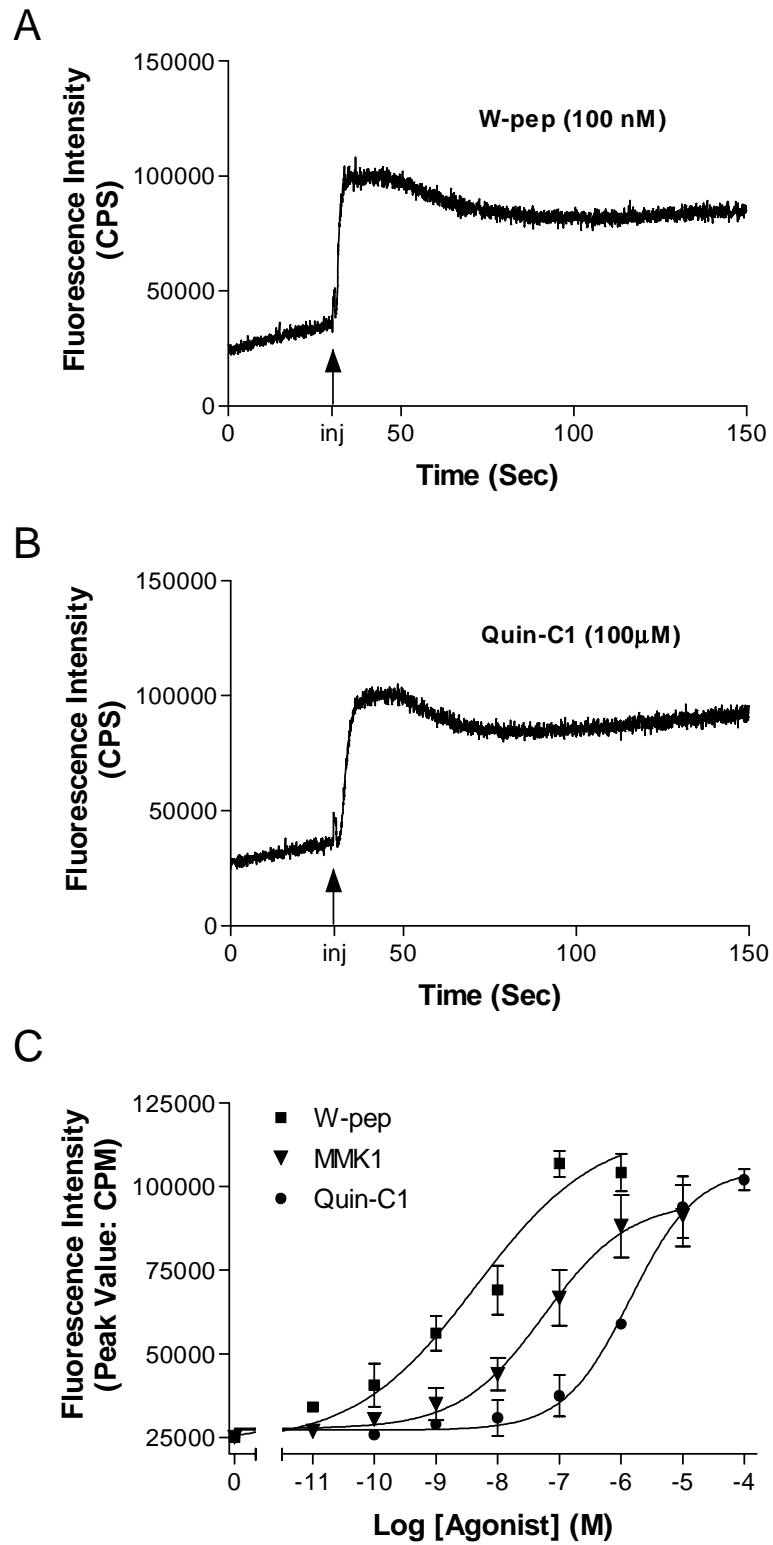


Figure 6

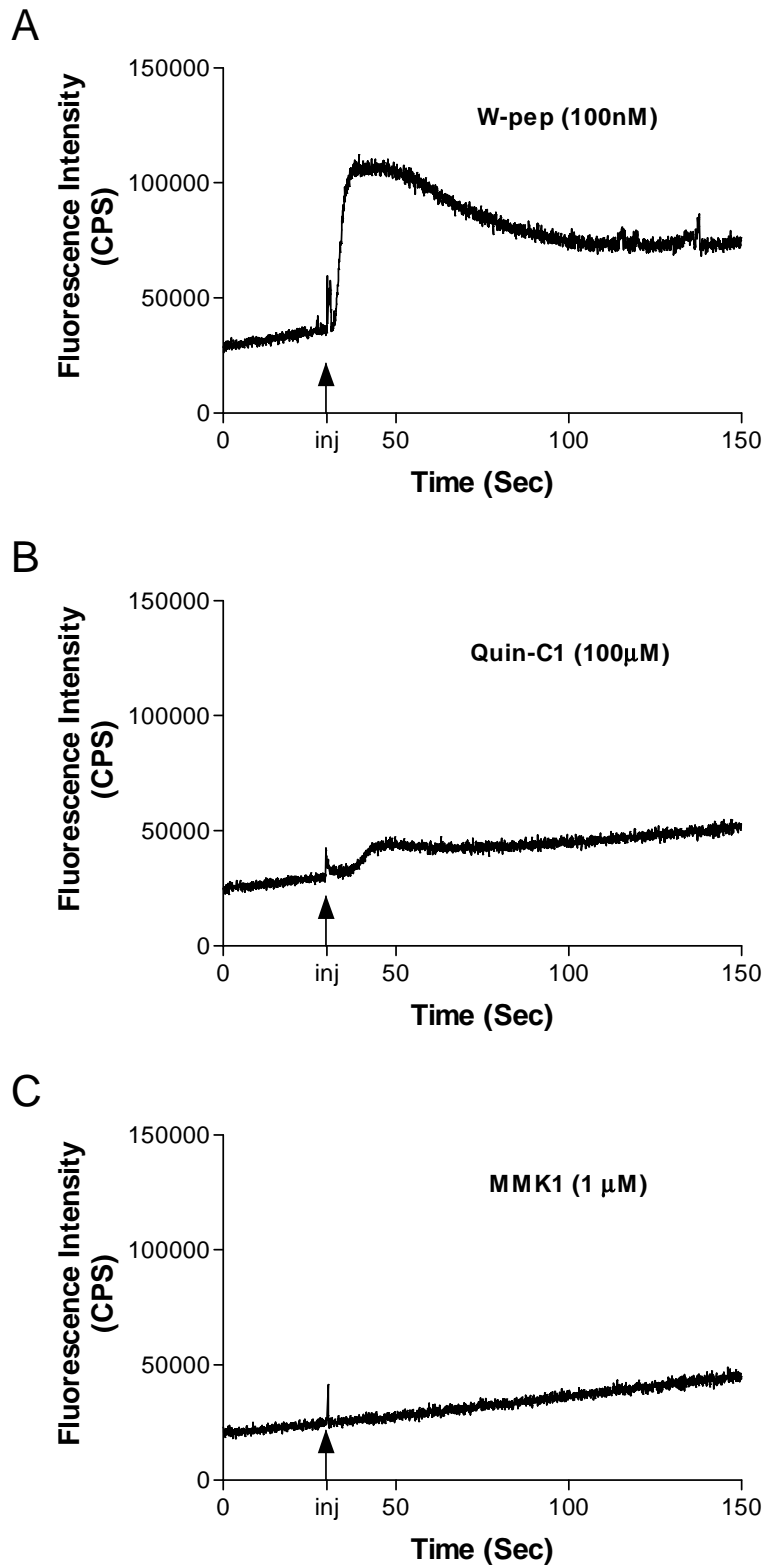


Figure 7

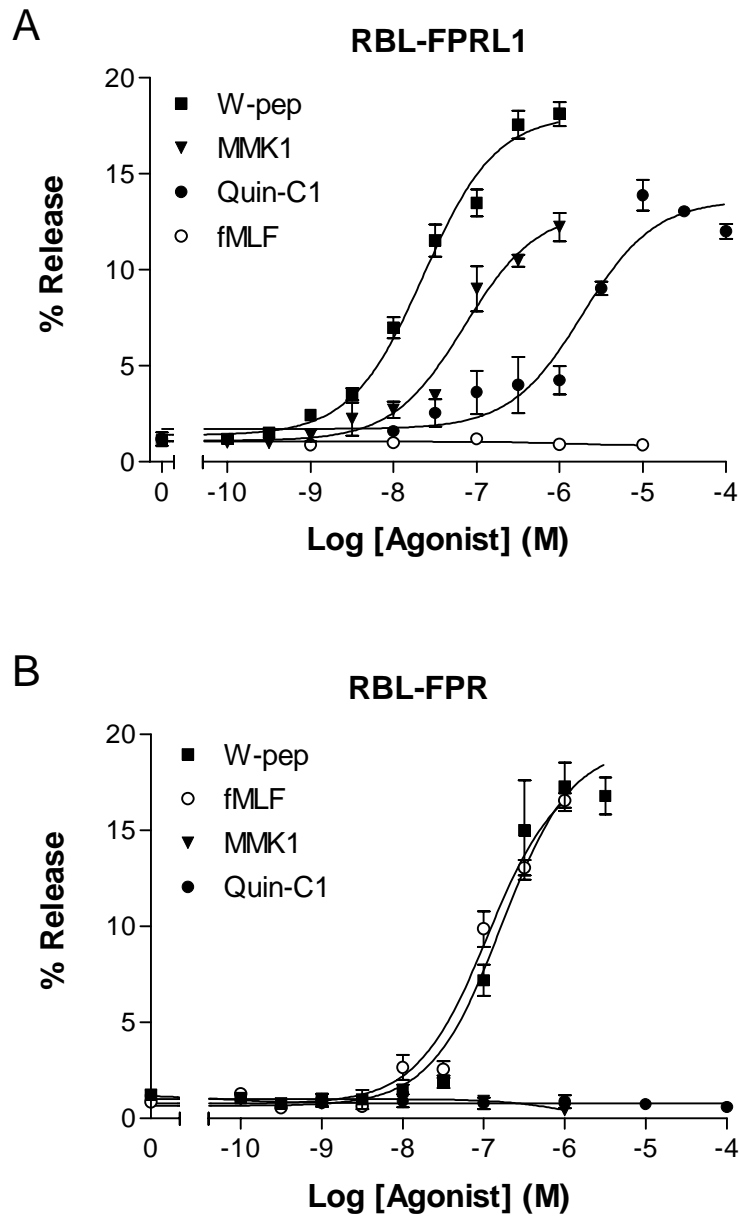


Figure 8

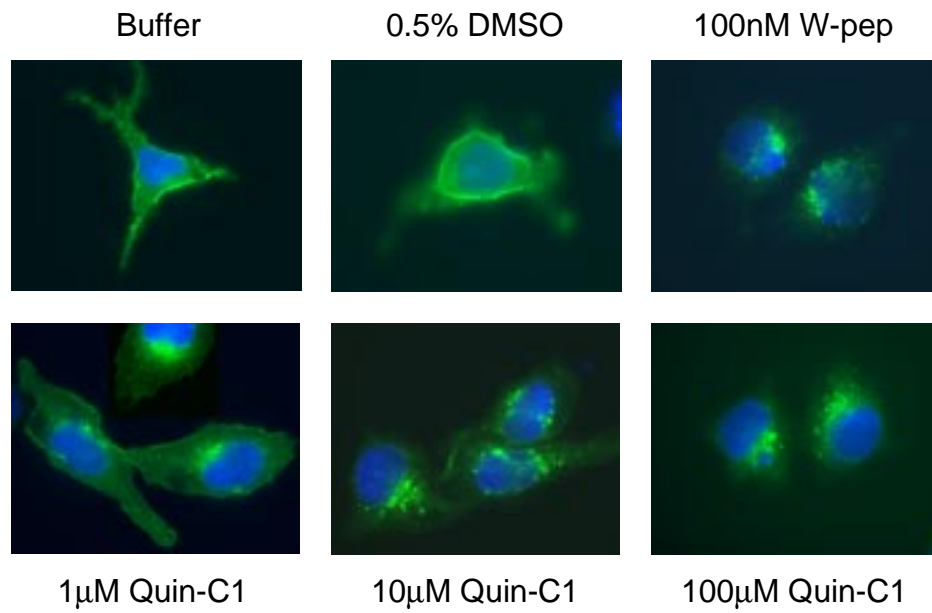


Figure 9

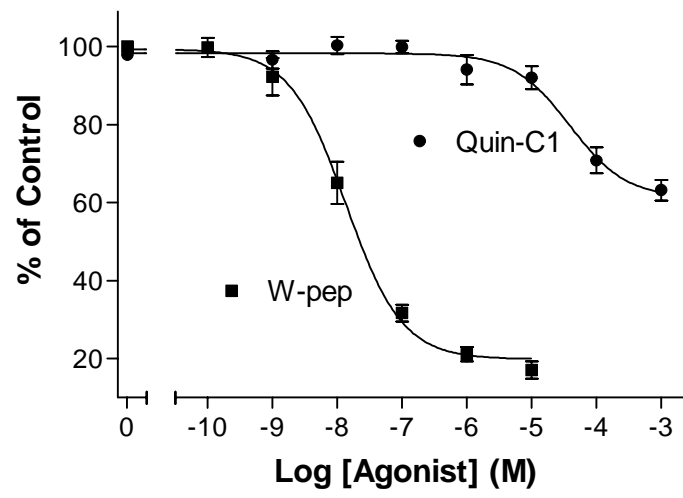


Figure 10

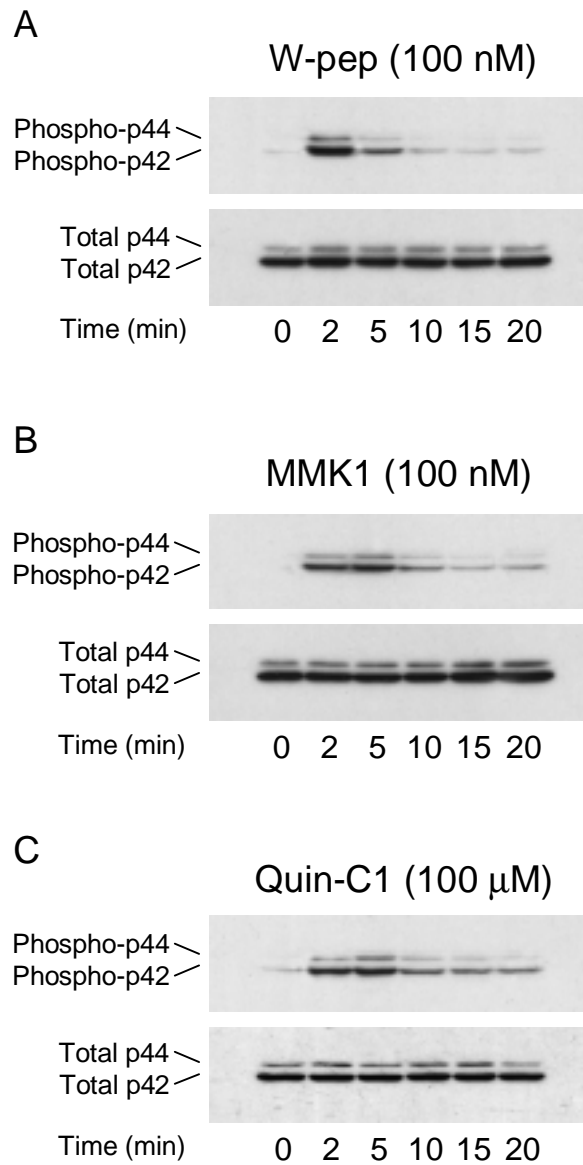


Figure 11

