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Discovery of Trp-Nle-Tyr-Met as a novel agonist for human formyl peptide receptor-like 1

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ABSTRACT

Formyl peptide receptor-like 1 (FPRL1) is a structural homologue of FPR, which binds chemotactic peptides as small as three amino acids (e.g., fMet-Leu-Phe, fMLF) and activates potent bactericidal functions in neutrophils. In comparison, FPRL1 ligands include peptides of 6-104 amino acids, such as Trp-Lys-Tyr-Met-Val-[D]Met (WKYMVm) and other synthetic peptides. To determine the core peptide sequence required for FPRL1 activation, we prepared various analogues based on WKYMVm and evaluated their bioactivities in an FPRL1-transfected cell line. Although substitution of D-Met^6 resulted in loss of activity, removal of Val^5 together with D-Met^6 produced a peptide that retained most of the bioactivities of the parent peptide. The resulting peptide, WKYM, represents a core structure for an FPRL1 ligand. Further substitution of Lys^2 with Nle slightly improved the potency of the tetrapeptide, which selectively activates FPRL1 over FPR. Based on these structure–activity relationship studies, we propose a model in which the modified tetrapeptide Trp-Nle-Tyr-Met (WNleYM) binds to FPRL1 through aromatic interactions involving the side chains of Trp^1 and Tyr^3, hydrophobic interaction of Nle^2, and the thio-based hydrogen bonding of Met^4, with the respective residues in FPRL1 which have not been identified. The identification of the core sequence of a potent peptide agonist provides a structural basis for future design of peptidomimetics as potential therapeutic agents for FPRL1-related disorders.

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Abbreviations: Nle, L-norleucine; met, D-methine; 1-Nap, L-3-(1-naphthyl)-alanine; 3-Thi, L-3-(3-thiophenyl)-alanine; Cha, L-3-(cyclohexyl)-alanine; K (Ac) or Lys (Ac), L-ε-N-acetyl-lysine; Y (4-Me) or Tyr (4-Me), L-(4-methyl)-tyrosine; asn, D-asparagine; Phe (4-F), L-(4-fluoro)-phenylalanine; f, formyl; Ac, acetyl; FPR, formyl peptide receptor; FPRL1, formyl peptide receptor-like 1; WKYMVm, Trp-Lys-Tyr-Met-Val-[D]Met; fMLF, *N*-formyl-Met-Leu-Phe; Quin-C1, 4-butoxy-N-[2-(4-methoxy-phenyl)-4-oxo-1,4-dihydro-2H-quinazolin-3-yl]-benzamide; DMEM, Dulbecco's Modified Eagle's Medium; BSA, bovine serum albumin; $A\beta_{(1–42)}$, amyloid β-peptide (1–42); HIV, human immunodeficiency virus; LC–MS, liquid-phase chromatography mass spectrometry; NF-κB, nuclear factor κB; ACN, acetonitrile

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1. Introduction

The human formyl peptide receptor gene family consists of three members: formyl peptide receptor (hFPR), formyl peptide receptor-like 1 (hFPRL1) and formyl peptide receptor-like 2 (hFPRL2) [1,2]. These receptors are G-protein coupled, seven-transmembrane cell surface proteins that can bind natural and synthetic peptides of various sequences and mediate leukocyte chemotaxis and other important biological functions. The prototype receptor, hFPR, was first cloned in 1990 by Boulay et al. and binds the chemoattractant fMLF (N-formyl-Met-Leu-Phe) with high affinity [3]. The binding of fMLF by hFPR induces a cascade of G protein-mediated signaling events leading to phagocyte adhesion, chemotaxis, release of superoxide anions, enhanced phagocytosis and bacterial killing [4].

Human FPRL1 and FPRL2 were initially cloned as homologues of hFPR, with 69% and 56% identity at the amino acid level to hFPR, respectively [4–6]. Although the function of hFPRL2 is still unclear, hFPRL1 has been found to play important roles in inflammation and immunity. Initially, the tripeptide fMLF was found as a low-affinity ligand for hFPRL1 [5,6]. More recently, a number of natural and synthetic ligands have been shown to interact with FPRL1 [1,2]. Lipoxin A4 is a high-affinity ligand for hFPRL1 [7]. Other FPRL1 ligands include the β-amyloid peptide $A\beta_{(1–42)}$, the acute-phase protein serum amyloid A, the antiapoptotic peptide humanin, the prion peptide PrP1 and peptides derived from the HIV-1 gp41 and gp120 protein [8–14]. Among the synthetic peptides, MMK-1 and other modified peptides were found to be the ligands for hFPRL1 [15]. In addition, a synthetic peptide, WKYMVm, obtained from a random peptide library, has been identified as a potent agonist for hFPRL1 and is widely used in *in vitro* and *ex vivo* studies on hFPRL1 (Fig. 1) [16–19].

As a member of the hFPR family, hFPRL1 is also capable of binding bacterial chemotactic formyl peptides and plays a role in host defense against pathogen infection. In particular, hFPRL1 has been reported to mediate phagocyte chemotaxis, and the activation of hFPRL1 was found to cause superoxide generation and exocytosis in human neutrophils. In terms of the modulation of HIV-1 infection, hFPRL1 has been reported to attenuate HIV-1 infection through innate immunity and its transition to adaptive immunity, or through desensitizing important chemokine receptors, CCR5 and CXCR4 that act as co-receptors of HIV-1 infection [20]. hFPRL1 plays a role not only in the immune system, but also in the neuronal system. hFPRL1 has potential implications in several disease states, such as amyloidosis and neurodegenerative disease. hFPRL1 was also found to be highly expressed in mononuclear phagocytes that infiltrate the brain tissues of Alzheimer's disease patients and believed to play a role in the inflammatory aspects of prion disease [12,13]. To reveal the role of hFPRL1 in physiological and pathological conditions, specific hFPRL1 agonists and antagonists are highly desirable.

Recently, a substituted quinazolinone compound (Quin-C1) has been identified in our laboratories as a novel nonpeptidic ligand for hFPRL1, with an EC_{50} value of 1.88×10^{-6} M [21]. However, Quin-C1 does not induce neutrophil superoxide generation at up to 100 μM and is a partial agonist for hFPRL1. Lately, a pyrazolone compound was discovered to be a potent hFPRL1 agonist by high-throughput screen of a compound library, but the pharmacophore of the analogues and the binding mode between the compound and hFPRL1 is still uncharacterized [22]. To further elucidate the interaction between hFPRL1 and its ligands and ultimately obtain peptidomimetic ligands, we generated various analogues of WKYMVm and evaluated their bioactivities. Herein, we report the identification of novel peptide agonists for FPR and FPRL1 based on a structure–activity relationship (SAR) study of WKYMVm.

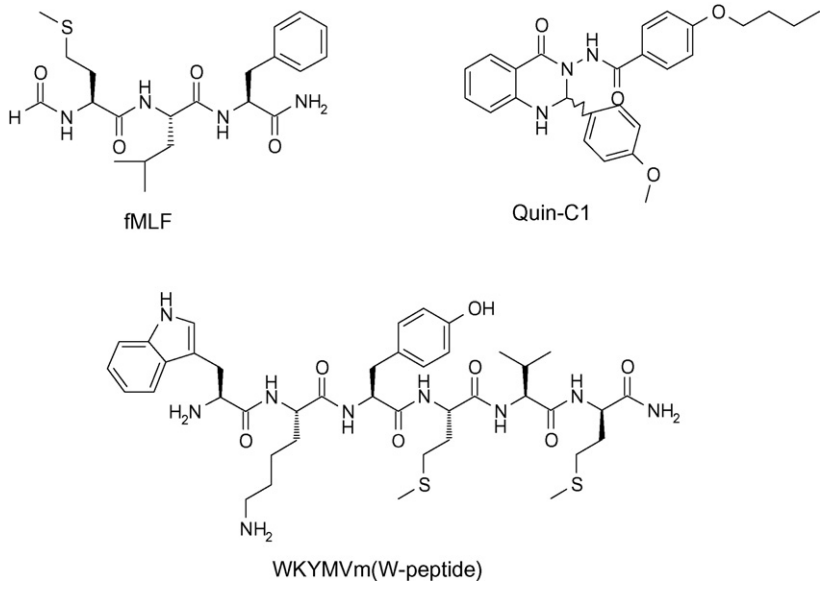

Fig. 1 – Chemical structures of fMLF, Quin-C1 and WKYMVm.

2. Materials and methods

2.1. Materials

All amino acids, Rink Amide-AM resin (0.3–0.8 mmol/g, 100–200 mesh, 1% DVB), *N*-methylpyrrolidin-2-one (NMP), *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), 1-hydroxy-7-azabenzotriazole (HOAt), 1-hydroxybenzotriazole (HOBt); benzotriazole-1-yl-oxy-trispyrrolinophosphonium hexafluorophosphate (PyBOP); pyridine; *N*,*N*-diisopropylcarbodiimide (DIC), 4-dimethylaminopyridine (DMAP) were purchased from GL Biochem (Shanghai, China); DIPEA, trifluoroacetic acid (TFA) were obtained from Sigma–Aldrich Chimie (St. Quentin Fallavier, France); formic acid, acetic anhydride, triethylsilane (TES), were from Acros-Organics (Geel West Zone 2, Belgium). LC–MS was carried out on Thermo Finnigan LCQ DCA XP and purity was confirmed on Gilson high-performance liquid chromatography (HPLC) (306 pump, UV/Vis-156 Detector, 215 liquid handle). [^{125}I]-WKYMVm (Bolton-Hunter labeled) and FlashBlue^{TM} GPCR Scintillating Beads were obtained from PerkinElmer (Boston, MA). Steady-Glo^{TM} Luciferase Assay Solutions were obtained from Promega (Madison, WI). Fluo-4/AM was the product of Molecular Probes (Eugene, OR). DMEM culture medium and trypsin were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone Co. (St. Louis, MO). All other reagents were of analytical grade and used without further purification.

2.2. Peptide synthesis and purification

All peptide analogues were synthesized (0.05 mmol scale) on a Rink Amide-AM resin using the standard Fmoc strategy. All Fmoc-amino acids (0.2 mmol, 4 eq.) were coupled via *in situ* activation with DIC/HOAt (0.25 mmol:0.25 mmol, 5 eq.), and DMAP (0.025 mmol, 0.5 eq.) in DMF. Reactive side chains were protected as follows: Trp and Lys, *t*-butyloxycarbonyl (Boc); Tyr, *t*-butyl (^{t}Bu). Acetylation of the peptide was performed on the resin by addition of a mixture of acetic anhydride/pyridine (0.2 mmol:0.2 mmol, 4 eq.) in DMF for 16 h at room temperature. Formylation of the peptide was performed on the resin by addition of a mixture of formic acid/PyBOP/HOBt/DIPEA (0.2 mmol:0.2 mmol:0.2 mmol:0.4 mmol, 4 eq. or 8 eq.) for 30 min at 0 °C and for 24 h at room temperature. The reaction was monitored by Kaiser test. After completion of the reaction, the peptides were deprotected and cleaved from the resin by addition of 5 ml of the mixture TFA:DCM:TES:water (50:40:5:5, v/v/v/v) and incubated for 90 min at room temperature. After the resin was filtered and the filtrate was concentrated under vacuum, crude peptides were precipitated by addition of diethyl ether. The crude peptides which showed >90% purity by HPLC were used without any further purifications, and the peptides with <90% purity were purified by semipreparative-HPLC (Thermo Finnigan LCQ DECA XP, YMC ODS, 3 μm, 2.0 mm × 50 mm).

2.3. Cell culture

The human cervical carcinoma cell line HeLa was transfected with pNF-κB-Luc reporter plasmid that contains five copies of NF-κB binding sequence (Stratagene, La Jolla, CA) and a human FPRL1 cDNA in pSFFV.neo vector as reported [21]. The transfected cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Rat basophilic leukemia cell line RBL-2H3 stably transfected with a human FPRL1 cDNA (RBL-FPRL1) or FPR cDNA (RBL-FPR) were described previously and maintained in DMEM supplemented with 20% FBS [23].

2.4. Reporter gene assay

HeLa cells expressing FPRL1-NF-κB-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 before screening assay. Different concentrations of compounds were added to the cells for 5 h, the expressed luciferase activity was determined in an EnVision 2101 Multilabel Reader (PerkinElmer) using the Steady-Glo^{TM} Luciferase Assay solutions.

2.5. Ligand binding assay

Ligand binding assay was performed as previously described [24]. RBL-FPRL1 (~10^9) were harvested and washed twice with PBS. Cell membrane was prepared with BioNeb Cell Disruption System (Glas-Col, Terre Haute, IN). Various concentrations of compounds were incubated together with RBL-FPRL1 cell membrane preparation and 0.16 nmol/l [^{125}I]WKYMVm (PerkinElmer; catalog number NEX386025UC; K_d = 0.32 nM), FlashBlue^{TM} GPCR beads (100 μg/well) to give a final volume of 0.1 ml. The plates were incubated at 4 °C for 12 h and centrifuged for 3 min at 2500 × *g* before counting on a MicroBeta scintillation counter (PerkinElmer).

2.6. Calcium mobilization assay

Calcium mobilization assay was performed as previously described [24]. Briefly, RBL-FPRL1 or RBL-FPR cells were detached and collected by centrifugation, loaded with 5 μM fluo-4/AM (Molecular Probes, Eugene, OR) in Hank's balanced salt solution (HBSS) supplemented with 2.5 mmol/l probenecid for 45 min, and then washed twice with HBSS. Cell suspensions were adjusted to a density of 5×10^6 cells/ml and seeded onto 96-well plates (100 μl per well). Cells were reattached by centrifugation and then analyzed for calcium mobilization using FlexStation^{TM} (Molecular Devices, Sunnyvale, CA) with excitation wavelength at 485 nm and emission wavelength at 525 nm.

2.7. Chemotaxis

Agonist-induced migration of cells was assessed in a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MA), as reported previously [24]. Different concentrations of WKYMVm or WNleYM were placed in the lower chamber, RBL-FPRL1 cells (50 μl at 1×10^6 cells/ml) were loaded in the upper chamber, which was separated from the lower chamber by a polycarbonate filter (pore size 8 μm). After incubation at 37 °C for 4 h, the filter was removed, fixed and stained with Diff-Quick staining solutions (IMEB Inc., San Marcos, CA).

Chemotaxis was quantified by counting migrated cells in five randomly chosen high power fields (400×). 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.

2.8. Degranulation

The amount of β-hexosaminidase released from RBL-FPRL1 cells was measured as reported previously [23]. In brief, cells (2×10^5/well) were cultured overnight in 24-well tissue culture plates. They were washed twice with HBSS 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 concentrations of WKYMVm or WNleYM before being terminated by placing the samples on ice. The amount of β-hexosaminidase secreted into the medium was determined by incubating 20 μl of supernatant or cell lysate with 10 μl of 5 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide in sodium citrate

Table 1 – The sequences and analytical data for WKYMVm analogues

Entry	Peptide sequence	HPLC purity (%)[a]		LC–MS[b]	
		Gradient A	Gradient B	Calculated	Observed
0	WKYMVm	100	100	855	856
1	AKYMVm	98.7	99.1	740	741
2	WAYMVm	100	100	798	799
3	WKAMVm	100	99.8	763	764
4	WKYAVm	100	99.3	795	796
5	WKYMAm	100	98.7	827	828
6	WKYMVA	99.7	98.4	795	796
7	WKYMVa	100	100	795	796
8	(1-Nap) KYMVm	100	100	866	867
9	HisKYMVm	100	99.5	806	807
10	PheKYMVm	100	98.9	816	817
11	ChaKYMVm	100	100	822	823
12	(3-Thi) KYMVm	100	99.6	822	823
13	WK (Ac) YMVm	96.9	95.2	897	898
14	WRYMVm	100	100	883	884
15	WNleYMVm	100	96.4	840	841
16	WKY (4-Me) MVm	91.8	90.7	869	870
17	WKPheMVm	100	100	839	840
18	WKHisMVm	100	100	829	830
19	WKPhe (4-F) MVm	97.3	95.6	857	858
20	WKChaMVm	99.5	98.8	845	846
21	WK (3-Thi) MVm	98.9	96.2	845	846
22	WKYNleVm	90.6	92.3	837	838
23	WKYMVM	94.0	93.7	855	856
24	WKYMVasn	92.0	90.2	838	839
25	f-WKYMVm	92.3	91.9	883	884
26	Ac-WKYMVm	96.2	94.0	897	898
27	f- (1-Nap) KYMVm	91.5	90.6	894	895
28	Ac-(1-Nap) KYMVm	97.8	95.8	908	909
29	WKYMV	93.8	92.4	724	725
30	WKYM	92.6	92.1	625	626
31	WKY	90.3	91.0	494	495
32	KYMVm	93.8	93.4	669	670
33	YMVm	96.4	94.9	541	542
34	MVm	93.8	92.5	378	379
35	(1-Nap) KYM	94.0	92.0	636	637
36	(3-Thi) KYM	100.0	99.7	592	593
37	f-WKYM	92.4	90.1	653	654
38	Ac-WKYM	90.8	90.5	667	668
39	f- (1-Nap) KYM	92.2	92.5	664	665
40	Ac- (1-Nap) KYM	93.7	91.8	678	679
41	WK (Ac) YM	97.4	94.0	667	668.4
42	WNleYM	94.6	93.1	610	611
43	WKY (4-Me) M	92.5	90.4	639	640
44	WKYNle	94.6	91.0	607	608

[a] Purity was performed on Gilson high-performance liquid chromatography (HPLC) (Column YMC-ODS, 4.6 mm × 50 mm, 5 μm; 306 pump, UV/Vis-156 Detector, 215 liquid handle); Mobile phase: 0.1% TFA in ACN-H_2O; Flow rate: 2.5 ml/min; Gradient A: 4–95% ACN in 4.1 min, plateau at 95% ACN for 2 min; Gradient B: 4–50% ACN in 6.6 min, plateau at 50% ACN for 1 min.

[b] LC–MS was carried out on Thermo Finnigan LCQ DCA XP, ESI$[M+H]^+$, *m/z* from 105 to 1500.

buffer (0.1 M, pH 4.5), at 37 °C for 1 h. At the end of the incubation, 200 μl of 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$, pH 10, were added. Absorbance at 405 nm was determined in a VERSAmax Tunable Microplate 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.

2.9. Data analysis

Dose responses and concentration responses were analyzed by using the Prism software (GraphPad Software, San Diego, CA) to fit four-parameter sigmoid functions.

3. Results

3.1. Peptide synthesis and characterization

We designed and synthesized five series of WKYMVm analogues by methods of alanine scanning, natural or unnatural amino acid replacement, or acylation of N-terminus and amino acid deletion. Synthesis of the peptide analogues was performed on Rink Amide-AM resin by the standard Fmoc strategy, which is commonly used in solid-phase peptide synthesis [25]. Acylation of the N-terminus was carried out with formic acid and acetic anhydride, respectively. All the peptides were analyzed by HPLC and LC–MS (Table 1).

3.2. Structure–activity relationship of WKYMVm analogues

Analogues of WKYMVm were evaluated for their activities using a screening protocol based on hFPRL1-mediated reporter gene expression and using a radioligand binding assay with RBL-FPRL1 cells [21]. To determine the amino acid side chains of WKYMVm involved in ligand–receptor interaction, systematical replacement of each amino acid in WKYMVm by alanine was performed (Table 2). This approach was commonly used in the literature. The activities of the resulting peptides were evaluated by hFPRL1-mediated reporter gene expression and in radioligand-binding assays. As can be seen, replacement of Val^5 by Ala retained the activity of hFPRL1-mediated cell activation with an EC_{50} of 9.04 nM. Similarly, alanine replacement of Trp^1 or Met^4 did not significantly affect the activity of the peptide (EC_{50} for peptide **1** was 19.98 and 40.79 nM for peptide **4**). However, replacement of Lys^2, Tyr^3 or D-Met^6 by Ala resulted in a dramatic decrease in activity: peptide **2** displayed 97-, peptide **3** displayed 37- and peptide **6** displayed 490-fold lower activity in EC_{50} values relative to WKYMVm, respectively. Moreover, replacement of D-Met^6 by D-Ala resulted in an EC_{50} of 1152.35 nM, 348-fold less potent than that of the WKYMVm.

In contrast, substitution of Trp^1, Tyr^3, Met^4 or Val^5 by Ala did not markedly impact the peptide on the interaction with the receptor. However, substitution of Lys^2 by Ala led to a 71-fold decrease in ligand-binding affinity. Moreover, substitution of D-Met^6 by Ala or D-Ala significantly reduced the binding activity of hFPRL1, by 917- and 593-fold, respectively.

To further characterize the structure–activity relationship of WKYMVm for receptor activation and ligand binding based on the above results, we made a general substitution of each amino acid in WKYMVm with other natural or unnatural amino acids. The results shown in Table 3 exhibited another interesting scenario of the structure–activity relationship for analogues of WKYMVm. Substitution of Trp^1 with Nap resulted in a four-fold increase in EC_{50} values for peptide **8**, but substitution with His resulted in a 22-fold decrease for

Table 2 – Replacement of each amino acid in WKYMVm by alanine

Entry	Peptide sequence	Reporter assay (EC_{50}, nM)[a]	Ligand binding assay (IC_{50}, nM)[b]
0	WKYMVm	3.31 ± 1.27	0.5 ± 0.1
1	AKYMVm	19.98 ± 1.95	5.4 ± 4.0
2	WAYMVm	322.50 ± 14.71	35.8 ± 11.2
3	WKAMVm	121.9 ± 12.02	6.9 ± 2.4
4	WKYAVm	40.79 ± 2.14	7.6 ± 2.8
5	WKYMAm	9.04 ± 4.20	2.3 ± 2.1
6	WKYMVA	1651.33 ± 423.46	458.9 ± 222.2
7	WKYMVa	1152.35 ± 317.70	296.9 ± 199.5

[a] The peptides' agonistic activities were assessed in HeLa cells stably transfected with a human FPRL1 cDNA and a pNF-κB-Luc reporter plasmid [23].
[b] Peptides were evaluated for their binding activities to FPRL1 in competition with 0.16 nM of [^{125}I]WKYMVm [24]. Data were mean ± S.D. of two to three independent experiments.

Table 3 – Replacement of each amino acid in WKYMVm by other amino acids

Entry	Peptide sequence	Reporter assay (EC_{50}, nM)[a]	Ligand binding assay (IC_{50}, nM)[b]
0	WKYMVm	3.31 ± 1.27	0.5 ± 0.1
8	(1-Nap) KYMVm	0.80 ± 0.01	0.7 ± 0.2
9	HisKYMVm	85.61 ± 2.69	7.6 ± 2.3
10	PheKYMVm	4.75 ± 1.58	2.1 ± 2.0
11	ChaKYMVm	4.50 ± 0.75	1.7 ± 1.7
12	(3-Thi) KYMVm	1.81 ± 0.68	1.2 ± 1.4
13	WK (Ac) YMVm	3.28 ± 0.69	4.7 ± 0.7
14	WRYMVm	1.45 ± 0.12	1.2 ± 1.2
15	WNleYMVm	89.71 ± 62.92	3.7 ± 0.4
16	WKY (4-Me) MVm	2.32 ± 0.87	1.0 ± 0.1
17	WKPheMVm	20.5 ± 3.91	0.9 ± 0.2
18	WKHisMVm	18.30 ± 5.10	5.5 ± 3.7
19	WKPhe (4-F) MVm	3.53 ± 0.91	2.4 ± 0.5
20	WKChaMVm	7.32 ± 1.30	1.2 ± 0.6
21	WK (3-Thi) MVm	8.91 ± 1.06	1.9 ± 0.8
22	WKYNleVm	3.13 ± 0.25	0.4 ± 0.1
23	WKYMVM	>10000	>10000
24	WKYMVasn	1204 ± 93.34	2085.5 ± 1208.4

[a] The peptides' agonistic activities were assessed in HeLa cells stably transfected with a human FPRL1 cDNA and a pNF-κB-Luc reporter plasmid [23].
[b] Peptides were evaluated for their binding activities to FPRL1 in competition with 0.16 nM of [^{125}I]WKYMVm [24]. Data were mean ± S.D. of two to three independent experiments.

peptide **9**. On the other hand, the EC_{50} values were retained at the same level when Trp^1 was substituted with Phe (Peptide **10**; Pep **10**), Cha (Pep **11**) and Thi (Pep **12**). Similarly, the values of EC_{50} were minimally affected by replacement of Lys^2 with Lys (Ac) (Pep **13**) or Arg (Pep **14**), however, the activity was reduced by 27-fold when the Lys^2 was substituted with Nle. Accordant to our expected results, substitution of Tyr^3 with Tyr (4-Me) (Pep **16**), Phe (Pep **17**), Phe (4-F) (Pep **19**), Cha (Pep **20**) or Thi (Pep **21**) caused a small change in the activity of receptor activation, with EC_{50} values ranging from 2.3 to 20.5 nM. In contrast, the polar aromatic histidine residue, which is likely charged at physiological pH, appeared to be required for full activity. Clearly, the activity of the receptor was retained at a high level of 3.13 nM when the Met^4 was replaced by Nle (Pep **22**), whereas peptides **23** and **24** almost lost their activities when the D-Met^6 was replaced by L-Met or D-Asn. In the same way, replacement of Trp^1, Lys^2, Tyr^3 and Met^4 by other amino acids including aromatic, aliphatic and neutral amino acid residues had little influence on binding affinity. In contrast, replacement of D-Met^6 with D-Asn (Pep **24**) or its L-isomer (Pep **23**) caused a loss of its binding affinity.

Based on the finding that aromatic aliphatic replacement of the amino acid at Trp^1 position can induce the increase of activity, we made acylation (formylation or acetylation) of N-terminus of peptide **0** and **8** to improve their lipophilicity. The results of the modified peptides shown in Table 4 confirmed our hypothesis. The EC_{50} values of peptide **26** and **28** exhibited a six- or three-fold decrease compared with the corresponding **0** and **8**, while retaining their binding affinity with the receptor. Results from both the reporter and binding assays demonstrated that acylation of the N-terminus of the peptides can promote the activities of the receptor and ligand-binding affinity, but is not essential.

According to the above results, we would like to elucidate the roles of N-terminal and C-terminal amino acids in WKYMVm for receptor activation and ligand-binding affinity. Toward this goal, a stepwise truncation of the amino acid from N-terminal or C-terminal was performed. As can be taken from Table 5, peptide **29** and peptide **32**, which had the C-terminal residue D-Met^6 and N-terminal residue Trp^1 deleted, respectively, lost almost all activities. Interestingly, peptide **30** and peptide **33**, which had the next amino acid from the C- and N-terminus deleted, kept moderate activities as compared with WKYMVm. Peptide **30** demonstrated a moderate activity with an EC_{50} of 43 nM and an IC_{50} of 7.4 nM, while peptide **33** exhibited about 35- and 60-fold decrease in EC_{50} and IC_{50} values. However, the peptides with three amino acids deleted from the C- and N-termini again showed even lower activities. These results suggest that the N- and C-terminal residues play a crucial role in receptor activation and ligand binding. Based on the data we can predict that the sequence WKYM is the core sequence in WKYMVm representing a part of the physiological ligand essential for its biological activity.

To explore the importance of amino acids in the core sequence and to improve the activities of WKYM for hFPRL1, analogues of the core peptide WKYM were designed and synthesized. The results shown in Table 6 indicate that replacement of Trp^1 with Nap (Pep **35**) and Thi (Pep **36**) could cause a small decrease in activity compared with peptide **30**

Table 4 – Acylation of the N-terminus of peptide 0 and 8

Entry	Peptide sequence	Reporter assay (EC_{50}, nM)[a]	Ligand binding assay (IC_{50}, nM)[b]
0	WKYMVm	3.31 ± 1.27	0.5 ± 0.1
8	(1-Nap) KYMVm	0.80 ± 0.01	0.7 ± 0.2
25	f-WKYMVm	0.57 ± 0.23	0.5 ± 0.4
26	Ac-WKYMVm	0.49 ± 0.17	1.1 ± 0.8
27	f- (1-Nap) KYMVm	0.28 ± 0.06	0.6 ± 0.2
28	Ac- (1-Nap) KYMVm	0.22 ± 0.04	0.5 ± 0.3

[a] The peptides' agonistic activities were assessed in HeLa cells stably transfected with a human FPRL1 cDNA and a pNF-κB-Luc reporter plasmid [23].
[b] Peptides were evaluated for their binding activities to FPRL1 in competition with 0.16 nM of [^{125}I]WKYMVm [24]. Data were mean ± S.D. of two to three independent experiments.

Table 5 – Amino acid deletion of WKYMVm

Entry	Peptide sequence	Reporter assay (EC_{50}, nM)[a]	Ligand binding assay (IC_{50}, nM)[b]
0	WKYMVm	3.31 ± 1.27	0.5 ± 0.1
29	WKYMV	>10000	>50000
30	WKYM	43.60 ± 0.29	7.4 ± 3.9
31	WKY	669.50 ± 112.41	146.2 ± 56.9
32	KYMVm	4652.67 ± 1768.09	2179.5 ± 1017.5
33	YMVm	117.56 ± 2.39	30.8 ± 27.5
34	MVm	>10000	5732.0 ± 2081.7

[a] The peptides' agonistic activities were assessed in HeLa cells stably transfected with a human FPRL1 cDNA and a pNF-κB-Luc reporter plasmid [23].
[b] Peptides were evaluated for their binding activities to FPRL1 in competition with 0.16 nM of [^{125}I]WKYMVm [24]. Data were mean ± S.D. of two to three independent experiments.

Table 6 – Replacement of each amino acid in WKYM by other amino acids

Entry	Peptide sequence	Reporter assay (EC_{50}, nM)[a]	Ligand binding assay (IC_{50}, nM)[b]
0	WKYMVm	3.31 ± 1.27	0.5 ± 0.1
30	WKYM	43.60 ± 0.29	7.4 ± 3.9
35	(1-Nap) KYM	199.30 ± 51.62	44.4 ± 16.7
36	(3-Thi) KYM	369.63 ± 152.60	618.8 ± 375.5
37	f-WKYM	36.60 ± 10.60	15.2 ± 2.0
38	Ac-WKYM	661.13 ± 158.06	252.5 ± 41.9
39	f- (1-Nap) KYM	382.85 ± 17.89	637.9 ± 347.3
40	Ac- (1-Nap) KYM	2705.50 ± 309.01	1169.0 ± 182.4
41	WK (Ac) YM	2215.50 ± 1035.91	939.6 ± 383.9
42	WNleYM	5.35 ± 0.30	5.6 ± 2.2
43	WKY (4-Me) M	368.85 ± 121.69	26.9 ± 10.0
44	WKYNle	2658.57 ± 1182.99	1308.9 ± 874.2

[a] The peptides' agonistic activities were assessed in HeLa cells stably transfected with a human FPRL1 cDNA and a pNF-κB-Luc reporter plasmid [23].
[b] Peptides were evaluated for their binding activities to FPRL1 in competition with 0.16 nM of [^{125}I]WKYMVm [24]. Data were mean ± S.D. of two to three independent experiments.

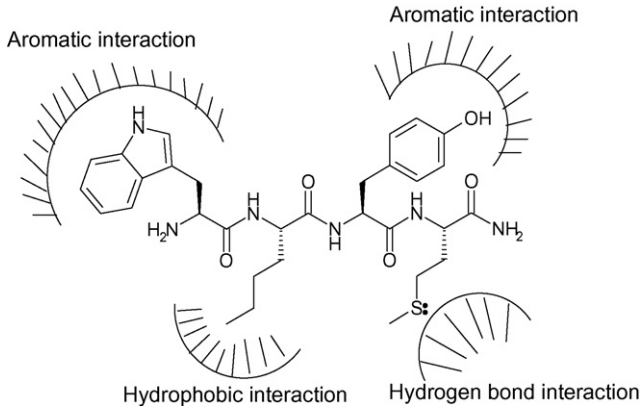

Fig. 2 – Proposed interaction mode between the tetrapeptide WNleYM with the receptor.

(with EC_{50} values from 43.6 nM to 199.3 and 369.6 nM, respectively), while formylated peptides retained the activity (Peps **30**, **35**, **37** and **39**) and acetylated peptides had about 15-fold decrease in activity. Replacement of Tyr^3 with Tyr (4-Me) (Pep **43**) caused less than 10-fold decrease in receptor activation and ligand-binding affinity. On the other hand, replacement of Lys^2 and Met^4 with Lys (Ac) (Pep **41**) and Nle (Pep **44**) led to more than 50-fold decreases. Interestingly, when Lys^2 was substituted with Nle, peptide **42** demonstrated a distinct improvement in receptor activation. These results indicate that the novel peptide, WNleYM, is more potent and efficacious than the core sequence, WKYM.

Based upon the above structure–activity relationship studies, a possible interaction mode between the core peptide and the receptor was proposed. It was probable that two aromatic groups of Trp^1 and Tyr^3, together with an alkyl group of Nle^2 may be responsible for binding to the receptor under hydrophobic interaction (Fig. 2). Additionally, an amino acid residue with a sulfur atom on a large side chain (Met^4) may affect the interaction through a thio-based hydrogen bond with the receptor.

3.3. Stimulation of chemotaxis by WNleYM

The newly discovered peptide WNleYM was further examined with chemotaxis assay in RBL-FPRL1 cells. When applied to the bottom wells in a microchemotaxis chamber, WNleYM stimulated directional migration of RBL-FPRL1 with a typical bell-shaped dose curve (Fig. 3). Maximal chemotaxis was induced by 1–10 nM WNleYM, and chemotaxis of cells declined gradually when its concentrations were above 10 nM. In comparison, WKYMVm, one of the well-characterized agonists for FPRL1, is more efficacious in stimulating chemotaxis in RBL-FPRL1 cells than WNleYM.

3.4. WNleYM induces release of β-hexosaminidase

While tested in the degranulation assay in RBL-FPRL1 cells, as shown in Fig. 4, WNleYM induced the release of β-hexosaminidase in a similar dose-dependent manner as WKYMVm. Its EC_{50} was 69.0 nM, which is about 10-fold less potent than the value (6.5 nM) of WKYMVm. No further increase in degranulation was observed with WKYMVm at concentrations above 100 nM or WNleYM beyond 1 μM.

3.5. WNleYM selectively activates FPRL1 over FPR

Because neutrophils express both FPRL1 and FPR, two receptors that share 69% of sequence identity at the amino acid level, it is necessary to determine whether the novel peptide, WNleYM, is a selective agonist for FPRL1 or a dual agonist for both receptors. We conducted Ca^{2+} mobilization with RBL-2H3 cells transfected to express either FPRL1 or FPR

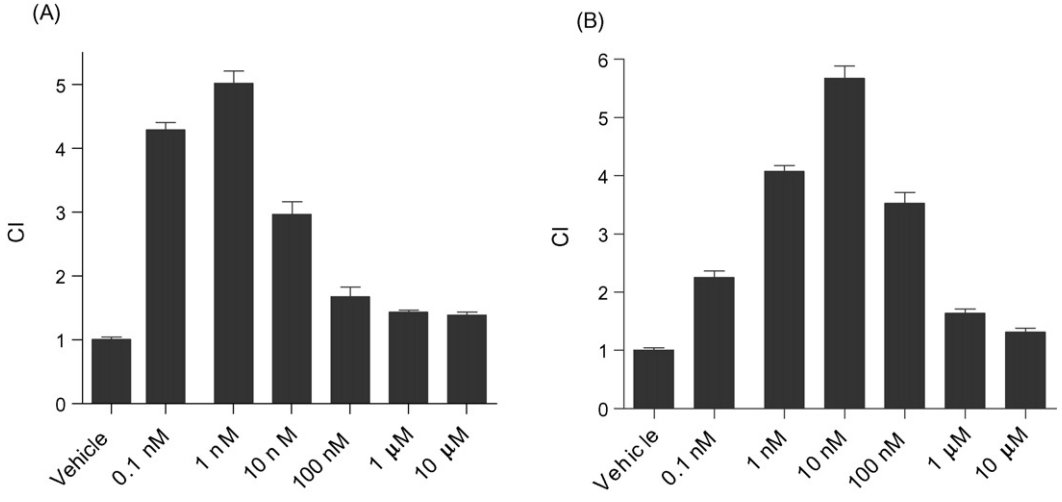

Fig. 3 – Effects of WKYMVm and WNleYM stimulated chemotaxis in RBL-FPRL1 cells. Different concentrations of WKYMVm (A) and WNleYM (B) were placed in the lower compartment of a 48-well chemotaxis chamber and cells were seeded in the upper compartment. Chemotaxis assay was conducted at 37 °C for 4 h and the number of migrated cells was determined by counting in a high power field (×400). Data was presented as relative chemotaxis index (CI). The results are presented as means ± S.E.M. of three independent experiments.

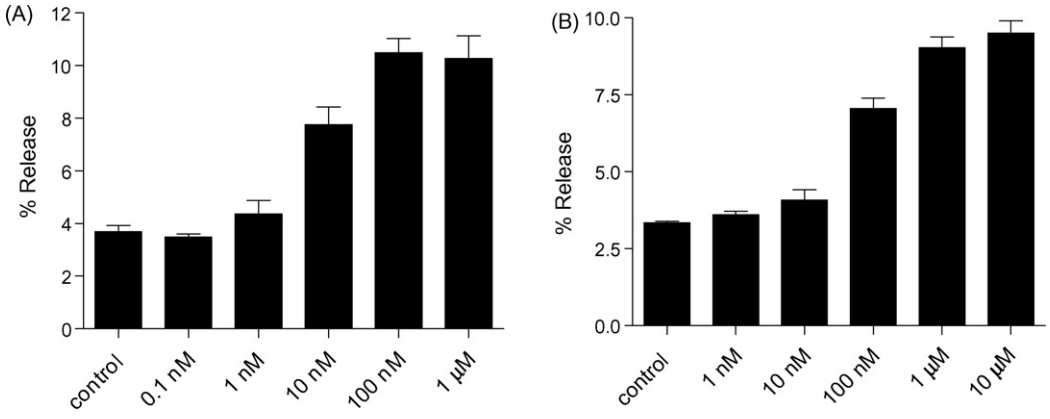

Fig. 4 – Effects of WNleYM stimulated β-hexosaminidase release in RBL-FPRL1 cells. Cells were treated with WKYMVm (A) and WNleYM (B) at various concentrations for 10 min. The released hexosaminidase was measured as described in Section 2. The results are presented as means ± S.E.M. of three independent experiments.

for this study. The cells were loaded with the Ca^{2+}-sensitive fluorescent probe fluo-4/AM and stimulated with WKYMVm, fMLF, WNleYM and selected analogues of WKYMVm. As shown in Table 7, WNleYM selectively stimulated Ca^{2+} mobilization in RBL-FPRL1 cells while having a much lower potency (104-fold difference) for RBL-FPR. The EC_{50} value of WKYMVm for RBL-FPRL1 is 0.91 nM compared with 56.4 nM for RBL-FPR. Of the 7 WKYMVm analogues examined, Nap-KYMVm (Pep **8**) displayed the highest selectivity for FPRL1 (234-fold over FPR) while Ac-WKYMVm (Pep **26**) had the lowest selectivity (17-fold over FPR). Interestingly, the core peptide (Pep **30**) could not stimulate Ca^{2+} mobilization in RBL-FPR cells even at the concentration of 10 μM. To verify the responsiveness of the cell lines, fMLF was used for stimulation of both RBL-FPR and RBL-FPRL1 cells. As expected, fMLF is highly selective for FPR (1412-fold difference compared to FPRL1), and the EC_{50} values are consistent with those reported by other groups. Based on these results, we conclude that WNleYM selectively activates FPRL1 over FPR, but at high concentrations may also activate FPR.

Table 7 – Effect of WKYMVm derivatives on calcium mobilization in RBL-FPRL1 and RBL-FPR cells

Entry	Peptide sequence	Calcium mobilization (EC_{50}, nM)[a]	Calcium mobilization (EC_{50}, nM)[b]
0	WKYMVm	0.91 ± 0.46	56.6 ± 17.8
8	NapKYMVm	0.37 ± 0.01	86.7 ± 15.4
16	WKY (4-Me) MVm	0.36 ± 0.03	24.5 ± 1.7
25	f-WKYMVm	0.80 ± 0.46	35.6 ± 15.8
26	Ac-WKYMVm	1.30 ± 1.21	22.5 ± 2.7
28	Ac- (1-Nap) KYMVm	0.49 ± 0.01	41.4 ± 7.2
30	WKYM	8.66 ± 4.63	NA
42	WNleYM	3.18 ± 3.04	330.1 ± 57.5
	fMLF	8193 ± 1.76	5.8 ± 3.0

[a] Calcium responses were examined in RBL-FPRL1 cells [24].
[b] Calcium responses were examined in RBL-FPR cells. NA means not active. Each value represents mean ± S.D. of at least three independent experiments.

4. Discussion

Interaction of peptide ligands with G protein-coupled receptors is of considerable interest to pharmacologists, as a better understanding of the structure–activity relationship can provide guidance for the development of peptidomimetic agents with potential therapeutic values. The formyl peptide receptor family contains three structurally similar receptors: FPR, FPRL1 and FPRL2. Of these receptors, FPR and FPRL1 (69% amino acid sequence identity) are known to bind formylated peptides with different affinities. The interaction between fMLF with FPR is of high affinity with K_d value at single-digit nanomolar range, but the interaction between fMLF and FPRL1 is of low affinity, with K_d value 100-fold higher. FPRL1 is known for its broad binding characteristics, and its ligands include large peptides (serum amyloid A, $A\beta_{(1-42)}$, LL-37), lipid (lipoxin A4) and peptide fragments from HIV-1 [1,2]. In addition, FPRL1 interacts with short peptides such as MMK-1 and WKYMVm. The fact that FPRL1 can bind a large number of ligands that share little sequence homology indicates the presence of various structural determinants capable of forming binding sites for these ligands.

Baek et al. first reported the identification of WKYMVm as a potent agonist for neutrophil activation [16]. Le et al. subsequently identified FPRL1 and FPR as functional receptors for WKYMVm [18]. The D-Met at position 6 apparently is important in determining the binding preference to FPR *versus* FPRL1 [26]. Further characterization of the peptide led to the production of substitution at position 2 (Lys to Arg, and Lys to Gly) that possess selective activities for FPR-mediated functions [27]. Since the original screening was conducted using a hexapeptide library that also identified other hexapeptides as FPRL1 and FPR ligands [16], the minimal size of the core peptide for FPRL1 is unclear. The current study addresses this issue with an experimental scheme that allows us to first evaluate the importance of each residue in WKYMVm through alanine scanning mutagenesis. This approach confirmed that D-Met at position 6 is critical to the bioactivity of the peptide, and led to a new finding that the Val at position 5 is less important for the activity of the peptide. The potency of the

peptides resulting from alanine substitutions is in the order Val^5–Ala > Trp^1–Ala > Met^4–Ala > Tyr^3–Ala > Lys^2–Ala > D-Met^6–Ala. This result is critical to subsequent reduction in peptide size that produced a tetrapeptide representing the core sequence for FPRL1 activation.

Although D-Met^6 indeed is important for the bioactivity of WKYMVm, our experimental data indicate that removal of both Val^5 and D-Met^6 produced a peptide with higher potency than one with removal of D-Met^6 only. This finding suggests that, in the absence of D-Met^6, Val^5 may interfere with receptor binding of the peptide produced from the deletion experiment. We have subsequently identified that the core peptide, WKYM, may be further improved through Nle substitution of Lys^2, which introduced a neutral amino acid in place of a positively charged residue. Based on these findings, we have proposed a model of interaction between WNleYM and FPRL1, in which maximal binding is facilitated by the aromatic interactions involving Trp^1 and Tyr^3, and the hydrophobic interaction involving Nle^2. In addition, hydrogen bonding between the sulfur atom and an unidentified residue in the receptor also plays a role in optimal ligand–receptor interaction.

Data of reporter gene assay and ligand binding assay in RBL-FPRL1 cells indicate the receptor activation and binding affinity, respectively. We noted a considerable difference in the concentrations required for the expression of the reporter gene and the IC_{50} value in binding assays. For most peptides tested, the EC_{50} value for reporter expression is higher than the IC_{50} value in the binding assay. This difference most likely results from the requirement of higher receptor occupancy for complex responses such as reporter expression. In a few cases, the IC_{50} values in binding assay were larger than the EC_{50} values for the reporter assay (e.g., Pep **24** in Table 3; Peps **26**, **27**, **28** in Table 4). Since indirect binding assay was used in this study, it is possible that the peptide (WKYMVasn) is less efficient in competing with the radiolabeled ligand (^{125}I-WKYMVm) than its agonistic activity in the reporter assay. Therefore, caution should be taken in the interpretation of the ratio between the binding assay and the reporter assay, as indirect binding assay adds another layer of complexity. With some peptides, acylation of the tetrapeptide results in more decline in binding affinity than receptor activation. The positive charged residue in Lys^2 seems to play more important roles in receptor activation than in ligand binding. The different results between ligand binding assay and reporter assay may indicate that hexapeptide ligands and tetrapeptide ligands interact with different binding domains in FPRL1.

Similar with the parent peptide **0**, its six-amino acid analogues display higher potency in RBL-FPRL1 than in RBL-FPR cells in receptor activations. The truncated analogue, Pep **30**, is not active in RBL-FPR cells. However, Pep **42** with neutral residue replacement of the positive charged residue shows selectivity similar to that of WKYMVm. This indicates that modification of the core peptide may be helpful to alter potency and selectivity for formyl peptide receptors.

Structural studies of peptide ligand interaction with its receptor, particularly small peptides such as fMLF and WKYMVm with the formyl peptide receptors, have met with difficulties of the number of conformations that small peptides may resume in solution. Although the changes in peptide structures make it more difficult to use chimeric receptors for the localization of ligand binding sites as compared to studies of ligands with more rigid structures [28], it remains possible to use the FPR/FPRL1 chimeric receptors for binding studies due to the selectivity of the WKYMVm analogues for FPRL1 (Table 7) [29]. The fact that all the analogues display higher selectivity for FPRL1 than FPR indicates that these analogues preferentially use binding sites on FPRL1. When bound to the receptor, peptides provide far more advantage than chemicals of rigid structure in maximizing the activation of the receptor and its biological functions. With this consideration, it is of potential significance to determine the minimal requirements for a peptide to activate a receptor, therefore providing critically important information in designing peptidomimetic reagents for therapeutic use. The size of WKYM and WNleYM approaches to that of fMLF, suggesting a common feature shared by FPR and FPRL1 in binding small peptides.

Acknowledgements

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