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Potent hFPRL1 (ALXR) agonists as potential anti-inflammatory agents

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Abstract—We report the discovery of potent agonists for the human formyl-peptide-like 1 receptor (hFPRL1). These compounds did not act at a closely related receptor denoted human formyl peptide receptor (hFPR) up to 10 μM concentration. Recent studies have indicated that agonizing this receptor may promote resolution of inflammation. In an exploratory study, a novel hFPRL1 agonist showed efficacy in a mouse ear inflammation model following oral administration.

Inappropriate inflammatory processes play a central role in many diseases such as asthma and rheumatoid arthritis. Many anti-inflammatory therapeutic agents have been developed; however, most have some limitations.[1] Most of the currently used anti-inflammatory drugs interfere with the action of pro-inflammatory mediators, whereas less is understood about the biochemical processes that resolve inflammation. Principally, selective activation of such a pathway might lead to an alternative treatment for inflammation.[1] For instance, Lipoxin A_4 (LXA_4) is an endogenously occurring metabolite of arachidonic acid, which is formed in response to inflammation.[2] LXA_4 and related structures have been shown to promote resolution of inflammatory processes, and it has been postulated that their anti-inflammatory effect is related to selective agonism of the G-protein-coupled receptor hFPRL1 (also known as ALXR or LXA_4R).[3–8] Recent studies suggest that this receptor might also be involved in neuro-inflammatory diseases.[9] It is possible that an application of exogenous Lipoxin A_4 (or close analogs)[10–12] for the treatment of inflammation is hampered by the inherent physicochemical properties of the poly-olefinic natural product. Therefore, we set out to investigate whether a small-molecule agonist for hFPRL1 would have potential as an anti-inflammatory therapeutic agent. First non-peptidic hFPRL1 agonists have recently been described.[13,14]

Using a cell-based assay in a high-throughput screen of our compound library,[15] we identified the pyrazolone **24** as a potent hFPRL1 agonist (Table 1). In absence of structural information about the receptor, our next goal was to establish a pharmacophore model by preparing and testing analogs of **24**. The synthesis of these compounds is shown in Schemes 1 and 2. Condensation of phenylhydrazine and various β-keto-esters (**1**–**3**),[16] followed by N-methylation of the cyclic intermediates, led to the corresponding pyrazolones on multi-gram scales. Subsequently, these pyrazolones were selectively nitrosylated or nitrated at C(4), and the crude products were reduced by hydrogenation to provide the respective amines. Without further purification, the amines were treated with a variety of aryl isocyanates in CH_2Cl_2. Collection of the precipitated products by filtration, followed by washing with CH_2Cl_2 or Et_2O, provided the final ureas **24**–**48**. The *N*-ethyl derivative **49** was prepared by analogy to this reaction sequence using ethyl tosylate for the N-alkylation. The acetamide **14** and

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Table 1. Influence of substituents on hFPRL1-mediated Ca^{2+} mobilization (generic structure shown in Scheme 1, EC_{50} values in [μM])

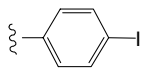
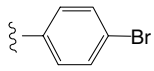
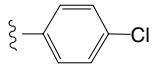
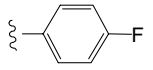
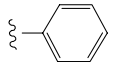
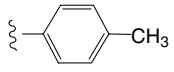
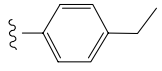
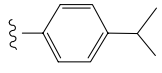
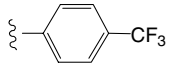
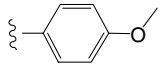
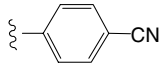
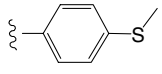
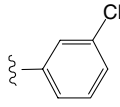
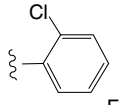
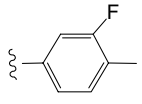
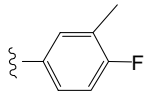
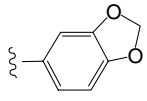
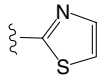
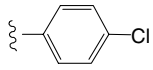
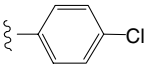
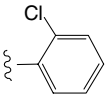
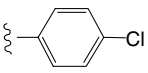
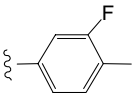
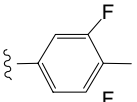
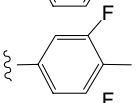
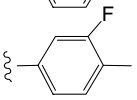
Compound	R^1	R^2	R^3	hFPRL1[a] EC_{50} [μM]
24		CH_3	CH_3	0.03 ± 0.01
25		CH_3	CH_3	0.09 ± 0.03
26		CH_3	CH_3	0.49 ± 0.09
27		CH_3	CH_3	4.85 ± 1.16
28		CH_3	CH_3	>10
29		CH_3	CH_3	1.26 ± 0.53
30		CH_3	CH_3	0.77 ± 0.30
31		CH_3	CH_3	12.3 ± 6.37
32		CH_3	CH_3	0.10 ± 0.05
33		CH_3	CH_3	2.73 ± 1.74
34		CH_3	CH_3	3.70 ± 0.80
35		CH_3	CH_3	0.17 ± 0.03
36		CH_3	CH_3	>10
37		CH_3	CH_3	>10
38		CH_3	CH_3	0.98 ± 0.50
39		CH_3	CH_3	>10
40		CH_3	CH_3	5.88 ± 2.48
41		CH_3	CH_3	>10
42		CH_2CH_3	CH_3	0.14 ± 0.03

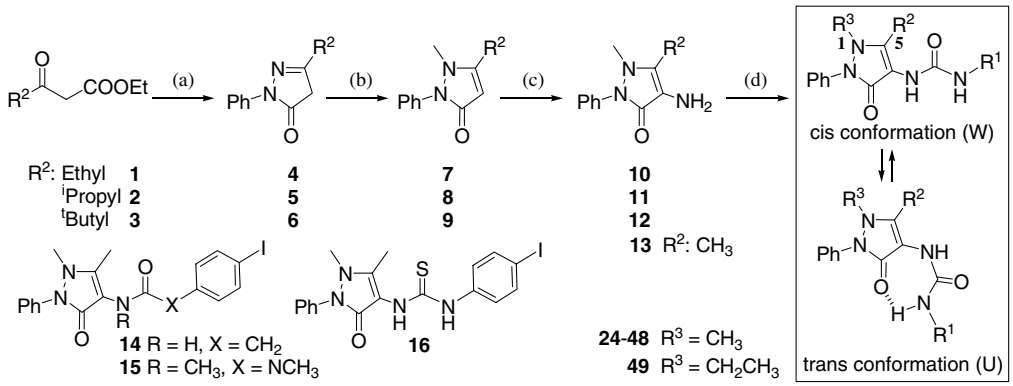
Table 1 (*continued*)

Compound	R^1	R^2	R^3	hFPRL1[a] EC_{50} [μM]
43			CH_3	0.044 ± 0.005
44			CH_3	>10
45			CH_3	0.023 ± 0.006
46		CH_2CH_3	CH_3	0.31 ± 0.12
47			CH_3	0.081 ± 0.023
48			CH_3	0.083 ± 0.015
49			CH_2CH_3	0.10 ± 0.02

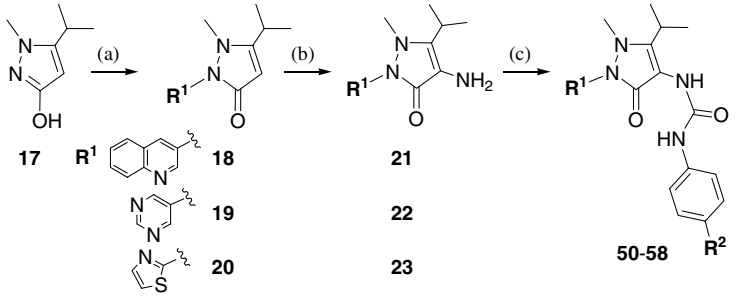
[a] EC_{50} values ($n \geqslant 4$) have been determined by Ca^{2+} flux in CHO recombinant cells co-expressing hFPRL1, $G_{\alpha 15}$ protein, and aequorin.

the thiourea **16** were synthesized from the commercially available aniline **13** applying standard amide and thiourea formation conditions, whereas the N,N-dimethylated urea **15** was obtained from urea **24** (MeI, NaH, DMF).[17] Compounds with purities below 95% (HPLC) were purified by crystallization or trituration. Coupling of the known hydroxypyrazole **17**[18] with various aryl bromides under Buchwald-type amidation conditions gave the pyrazolones **18**–**20** in modest yields, as well as O-alkylated pyrazoles as major side products.[19] The intermediates **18**–**20** were converted to the final ureas **50**–**58** as described above. All compounds that were tested for biological activity showed purities in excess of 95% as evidenced by HPLC and ^1H NMR spectroscopy.[20]

As the 4-iodo-substituent in molecule **24** may constitute a potential liability (e.g., homolytic C–I bond cleavage), we started to investigate alternative groups at position 4 of the phenyl group (Table 1). Replacement of this iodide with other halides resulted in decreasing hFPRL1 potency, correlating well with the size of the halo-substituent. In fact, the unsubstituted phenyl urea **28** was completely inactive up to at least 10 μM. The 4-ethyl compound **30** was slightly more active than the methyl derivative **29**. In contrast, the sterically more demanding isopropyl compound **31** lost significant potency (12.3 μM). A more detailed survey of substituent changes pending off the phenyl-urea moiety demonstrated that the steric and electronic nature of the para-substituent on the phenyl group has incisive effects on hFPRL1 activity. For instance, the 4-CF_3–phenyl compound **32**



Scheme 1. Synthesis of **24**–**49**. Reagents and conditions: (a) $PhNHNH_2$ (1 equiv), 50% aq AcOH, 115 °C, 3 h, **4** (53%), **5** (>95%), **6** (38%); (b) CH_3OTos (2 equiv), 160 °C, 2 h, **7** (66%), **8** (23%), **9** (53%); (c) $NaNO_2$, AcOH, aq HCl, 5 °C, 1 h or 70% aq HNO_3 (5 equiv), CF_3COOH, 50 °C, 30 min, then H_2, Pd (black), AcOEt, MeOH, crude; (d) CH_2Cl_2, R-NCO (1.1 equiv), ca. 1 h, 24 °C, 40–80%.



Scheme 2. Synthesis of **50**–**58**. Reagents and conditions: (a) R^1Br, CuI (0.05 equiv), 1,10-phenanthroline (0.11 equiv), Cs_2CO_3 (1.4 equiv), dioxane (for **20**, DMF), 110 °C (sealed tube), 48 h, **18** (44%), **19** (28%); (b) 70% aq HNO_3 (5 equiv), CF_3COOH, 50 °C, 30 min, then H_2, Pd (black), AcOEt, MeOH, **21** (34%), **22** (30%), **23** (5%, from **17**, 3 steps); (c) CH_2Cl_2, R-NCO (1.1 equiv), ca. 1 h, 24 °C, 50–90%.

was about as active as the one bearing the SMe group **35** or bromide **25** (100, 170, and 90 nM, respectively), whereas the methoxy analog **33** showed potency only in the micromolar range. Likewise, nitrile **34** was only moderately active (3.7 μM). Alternating the position of the substituent resulted in dramatic loss of potency, as exemplified by the meta- and ortho-substituted chlorides **36** and **37**, respectively. Various disubstituted ring systems were investigated and compounds **38**–**40** stand as representative examples: as above, subtle modifications in the substitution pattern at the phenyl moiety were found to alter hFPRL1 activity dramatically; for example, the 4-methyl-3-fluoro-compound **38** showed low micromolar potency, while its isomer **39** was not active below 10 μM. Replacing the phenyl group by a thiazole ring (**41**) resulted in reduced activity as well, suggesting that heterocyclic moieties at this position might not be well tolerated.

Generally, ureas are well known to adopt a cis or trans conformation or to exist as a mixture of both conformers.[21] For molecules like **24**, it is conceivable that an intramolecular hydrogen bond stabilizes the trans or U-shaped conformation (illustrated in Scheme 1). Such a conformation is not expected to be highly populated for the acetamide **14**, since the H-bond donor has been removed. As the acetamide **14** did not show hFPRL1 activity (<10 μM), we reasoned that the bioactive conformation of the ureas described herein might be U-shaped. Quantum mechanic calculations suggest that the urea **24** prefers a U-shaped conformation by ~1.4 kcal.[22] Interestingly, for thioureas such as **16**, the U-shaped conformation is predicted to be more stable than the W-conformer by about 4.6 kcal. Compound **16** demonstrated roughly equal potency as compared to the urea analog **24** (26 nM vs 30 nM). Similarly to the acetamide **14**, the N,N-dimethylated derivative **15** did not show increased Ca^{2+} mobilization below 10 μM concentration. Next, we modified the substituent at C(5) of the pyrazolone ureas and synthesized several ethyl, isopropyl, and tertiary butyl derivatives (**42**–**48**). We found that the potency of compounds with bulkier C(5)-substituents was consistently enhanced [e.g., for **26** (Me) 490 nM; for **42** (Et) 140 nM; for **43** (^{i}Prop) 44 nM; for **45** (^{t}Butyl) 23 nM]. The observation that increased steric demand of the group at C(5) gives rise to enhanced activity may be a result of a shift in the conformational equilibrium between the U- and W-shaped conformation and/or additional van der Waals interactions.

As above, the ortho-chloro substituted compound **44**, an isomer of the potent pyrazolone **43**, did not show activity up to 10 μM. Replacing the methyl group at

N(1) with the larger ethyl substituent had no major impact on potency (see **49**).

We also studied the influence of the N(2)-substituent of the pyrazolone core on hFPRL1 activity (Table 2) and found that modifications within this portion of the molecule are relatively well tolerated. For example, the *N*(2)-pyrimidines (**50**, **53**, and **56**) and quinolines (**51**, **54**, and **57**) were roughly equally or more potent than the corresponding *N*(2)-phenyl derivatives, while thiazoles (**52**, **55**, and **58**) showed somewhat reduced activity.

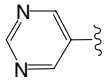
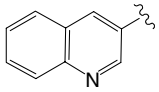
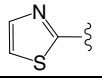
Notably, the compounds described above did not show significant activity (up to 10 μM) for the human formyl peptide receptor (hFPR, counter-screen),[23] which is the closest homolog of hFPRL1.

Two pyrazolones with good hFPRL1-mediated Ca^{2+} flux activity (**24**, **43**) were tested in a functional cell-based assay side by side with two inactive analogs (**15**, **44**): polymorphonuclear human neutrophils (PMN) were preincubated for 45 min with compound or vehicle. Subsequently, their migration against a gradient of chemo-attractant (either IL-8 or *N*-formyl-methyl-leucyl-phenylalanine = fMLP) was determined by fluorescence according to a standardized protocol by Martin and coworkers (Table 3).[24] In essence, the agonists **24** and **43** showed a dose-dependent inhibition of PMN migration, irrespective of the nature of the stimulant, while their inactive analogs **15** and **44** had no impact. The concentrations required for inhibition of PMN migration are somewhat higher than the respective EC_{50} values determined for Ca^{2+} mobilization, which may be due to high protein binding of the compounds and/or different levels of receptor expression in neutrophils versus transfected CHO cells. Interestingly, 16-phenoxy-Lipoxin,[10] a well-documented stable analog of Lipoxin A_4 (prepared according to Phillips et al.),[12] did not demonstrate blockage of PMN migration under these conditions.

As part of an exploratory profiling, the active and inactive iodo-pyrazolones **24** (30 nM) and **15** (>10 μM) were studied in a mouse ear inflammation model (topical).[25,26] For this, mice (10 animals per group) were treated topically with test compound 15 min prior to inducing an ear edema with a combination of prostaglandin E_2 and leukotriene B_4 (5 μg each). Ear thickness was measured 3 h post induction and compared to the thickness prior to the inflammatory insult. As illustrated in Figure 1, the active pyrazolone **24** caused reduction of ear edema, whereas the inactive analog **15** did not show a significant effect. Under these conditions (applied topically), 16-phenoxy-Lipoxin demonstrated considerable reduction of ear swelling (at 6.2 μg/ear: 22%, at 12.5 μg/ear: 33%, at 25 μg/ear, 69% reduction of swelling).

The poor solubility characteristics of the iodides **15** and **24** did not allow formulation necessary for advanced in vivo studies. Therefore, we investigated the pharmacokinetic behavior and efficacy of the more soluble pyrazolone **43**. Following iv administration to rats (0.5 mg/kg), the compound showed relatively low

Table 2. hFPRL1 activity of compounds **50**–**58**[a]

R^1	R^2		
	Cl	SMe	CF_3
	**50** 0.25 ± 0.07	**53** 0.043 ± 0.005	**56** 0.15 ± 0.02
	**51** 0.080 ± 0.013	**54** 0.030 ± 0.003	**57** 0.044 ± 0.006
	**52** 0.54 ± 0.46	**55** 5.13 ± 1.75	**58** 1.47 ± 0.68

EC_{50} values in [μM] (generic structure shown in Scheme 2).
[a] EC_{50} values ($n \geqslant 4$) have been determined by Ca^{2+} flux in CHO recombinant cells co-expressing hFPRL1, $G_{\alpha 15}$ protein, and aequorin.

Table 3. Inhibition of neutrophil (PMN) migration by pyrazolones (IC_{50} in [μM])

Compound	IC_{50} [μM]		hFPRL1[b] EC_{50} [μM]
	fMLP[a]	IL-8	
24	~2[c]	~2[c]	0.03 ± 0.01
15	>10	>10	>10
43	0.64 ± 0.20	0.24 ± 0.05	0.044 ± 0.005
44	>10	>10	>10

[a] Mean IC_{50} values determined using PMNs from at least three donors. IL-8 or fMLP were used as chemo-attractants (each 10 nM). Final [DMSO] = 0.1%.
[b] hFPRL1 activity for comparison.
[c] Approximate IC_{50} as determined by mean value of two donors. PMNs of one donor did not show activity below 10 μM.

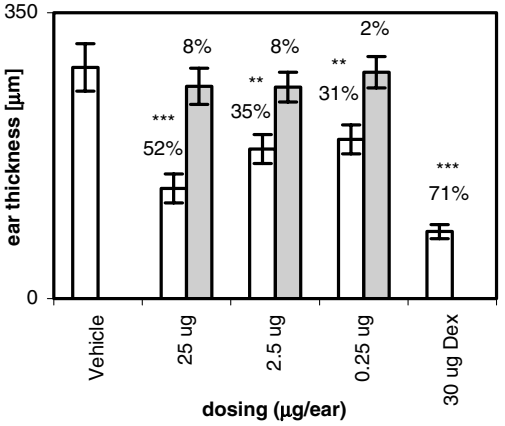


Figure 1. Reduction of ear swelling in mice: topical administration of agonist **24** and inactive analog **15**. (*X*-axis: vehicle alone, topical administration of 25, 2.5, and 0.25 µg/ear of **24** (white blocks), **15** (grey blocks), and 30 µg of dexamethasone (Dex). *Y*-axis: ear thickness [µm], 3 h post induction. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.005$.)

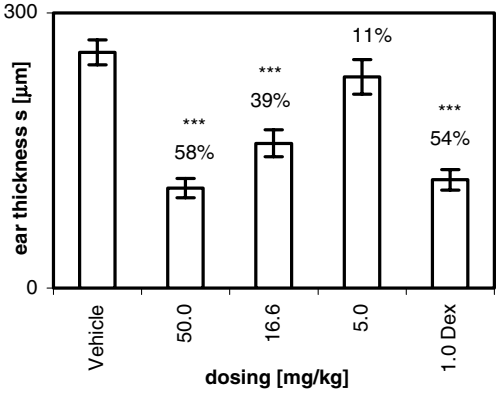


Figure 2. Reduction of ear swelling in mice: oral administration of agonist **43**. (*X*-axis: vehicle alone, **43** (po, 50, 16.6, 5 mg/kg), dexamethasone (Dex) at 1 mg/kg (iv). *Y*-axis: ear thickness [µm], 3 h post induction. Statistical significance: $^{***}P < 0.005$.)

clearance (0.126 L/h/kg) and a half-life of 2.78 h. Significant levels of **43** were detected in the systemic circulation such that the bioavailability following po administration (2 mg/kg) was 91%. Encouraged by these data, we tested **43** in the mouse ear inflammation model described above following oral administration (Fig. 2):[25] mice (10 animals per group) were treated orally with compound **43** 60 min prior to induction of edema and the ear thickness was analyzed 3 h post insult. At the 50 mg/kg dose, a reduction in edema by 58% was observed, which is similar to that of the positive control (dexamethasone, 1 mg/kg iv).[27] At this dose, the plasma concentration of **43** at the end of the experiment (free fraction, corrected for plasma protein binding) was 185 ± 42 nM, that is, about four times higher than the EC_{50} value for Ca^{2+} mobilization and in the range of the IC_{50} value determined for IL-8 induced PMN migration.[28] At the lower doses (16.6 and 5 mg/kg, respectively), which resulted in only partial or no efficacy, the endpoint concentration of free **43** was below the EC_{50} value (Ca^{2+} flux).

Notably, compound **43** did not show significant cross-interaction with a series of unrelated protein targets including the mitogen-activated P38α kinase, the chemokine receptor CXCR2, the histamine receptor 1, and the vanilloid receptor 1.

In summary, a high-throughput screen and subsequent medicinal chemistry effort resulted in potent and novel hFPRL1 agonists. Two active compounds (**24**, **43**) inhibited fMLP and IL-8 induced human neutrophil migration, whereas closely related structures with no hFPRL1-mediated Ca^{2+} mobilization activity (**15**, **44**) did not show inhibition. Under these conditions, 16-phenoxy-Lipoxin did not regulate neutrophil migration. In a mouse model of acute inflammation (topical application), compound **24** and 16-phenoxy-Lipoxin were efficacious. Again, an inactive analog **15** did not have an effect. The observation that a potent hFPRL1 agonist showed inhibitory activity in a functional cell-based assay and in vivo (topical), while its inactive derivative did not have an impact, indicates that the observed anti-inflammatory effect is target-mediated. The differential effects of the pyrazolones and 16-phenoxy-Lipoxin on PMN migration may suggest that the signal transduction events triggered by these different classes of agonists differ from each other. Such differences between synthetic and endogenous agonists have been precedented.[29] Studies to further address the mode of action of these molecules are ongoing.

Importantly, a prototypic hFPRL1 agonist and PMN migration inhibitor (**43**) with improved properties and very good oral bioavailability showed a dose-dependent inhibition of ear swelling in mice following oral administration.

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27. Dexamethasone (positive control) was administered as an iv bolus. For the analysis, the 3-h time point was found to provide an optimal window as the ear swelling slowly declines afterwards (data not shown).
28. Compound **43** is 97.9% bound to mouse plasma as determined by equilibrium dialysis. Analysis of blood samples taken at the endpoint of the study revealed that at the highest dose (50 mg/kg), the concentration of free agent **43** was 185 ± 42 nM (corrected for plasma protein binding). Lower concentrations of unbound **43** were found in blood samples from mice treated at lower dose ([**43**] corrected for plasma protein binding: 17 ± 7.0 nM at 16.6 mg/kg, 14 ± 3.2 nM at 5 mg/kg).
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