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Structure—Function Characteristics and Signaling Properties of Lipidated Peptidomimetic FPR2 Agonists: Peptoid Stereochemistry and Residues in the Vicinity of the Headgroup Affect Function

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ABSTRACT: Formyl peptide receptor 2 (FPR2) plays important roles in inflammation. In the present study, 20 analogues of the FPR2-selective lipidated α-peptide/β-peptoid agonist Lau-[((S)-Aoc)-Lys-βNnPhe]-NH₂ were generated, which allowed two novel subclasses of more potent FPR2 agonists to be distinguished. Critical factors influencing FPR2 recognition comprise the presence of β-peptoid phenylaniline-like residues (i.e., βNnPhe, βNspe, or βNrpe) in the peptidomimetic tail, configuration of the 2-aminooctanoic acid (Aoc) in the headgroup, and the length of the N-terminal fatty acid. Intriguingly, a single βNrpe residue in the vicinity of the N-terminus (i.e., Lau-[(S)-Aoc]-Lys-βNrpe-[Lys-βNnPhe]-NH₂) proved to increase the agonist potency, whereas the βNspe-containing analogue was a weak FPR2-selective antagonist. Another subclass displaying potent agonism comprised analogues possessing two α-amino acids vicinal to the headgroup. The optimized FPR2-activating lipidated peptidomimetics exhibited biased signaling: PLC-PIP2-Ca²⁺ signaling was activated, but without recruitment of β-arrrestin or induction of chemotaxis. These FPR2-interacting compounds are considered to be useful tools in future studies of receptor–ligand interactions.

INTRODUCTION

Recruitment of neutrophils to inflammatory sites is directed by host- or pathogen-derived danger molecules recognized by G-protein-coupled receptors (GPCRs), for example, receptors for platelet-activating factor (i.e., PAFR), adenosine triphosphate (i.e., P2Y2R), and formyl peptide receptors FPR1 and FPR2. The third FPR subclass (i.e., FPR3) is not expressed by human neutrophils, and its biological functions are not yet known.† FPRs are high-affinity receptors for formyl peptides, being danger molecules of bacterial and mitochondrial origin, albeit these receptors also recognize a large number of non-formylated peptides and small-molecule ligands, representing different chemical classes.‡−⁵ FPRs are important not only for neutrophil recruitment and initiation of inflammation but also for resolution of such processes. Thus, aberrant FPR expression is associated with a wide range of immunity-mediated diseases, for example, atherosclerosis, cancer, neurodegeneration, and sepsis,⁶,⁷ and thus, FPRs constitute attractive therapeutic targets. Consequently, an increasing number of FPR ligands with therapeutic potential have been identified and characterized in recent years.³,⁴

A major complication in therapeutic use of peptide-based ligands is their intrinsic susceptibility to in vivo degradation by peptidases, conferring low bioavailability. Hence, peptidomimetics (partly composed of non-natural residues) that resist enzymatic degradation constitute favorable alternatives. Stable α-peptide/β-peptoid hybrid oligomers displaying an alternating design with cationic α-amino acids and aromatic hydrophobic β-peptoid residues (Figure 1) have been found to mimic the biological activities of peptides, such as microbial killing, reduction of biofilm formation, and immunomodulation. This class of peptidomimetics has proved to constitute a promising source of lead compounds for the development of potential pharmaceuticals within these therapeutic areas.⁷−⁹

FPRs belong to the superfamily of GPCRs that mediate fundamental cellular responses upon agonist binding, with subsequent G-protein-dependent signal transduction.⁶ Both pro- and anti-inflammatory responses are mediated by FPRs,
and this dual functionality appears to be in line with the “biased signaling” concept, often termed “functional selectivity.” Classically, activation of a GPCR signaling pathway initiates the dissociation of a heterotrimeric G-protein, triggering production of multiple downstream secondary messengers, for example, phospholipases and protein kinases. The resulting signaling cascade is terminated by recruitment of β-arrestin to the GPCR, a coupling that sterically blocks further G-protein recruitment/signaling. Recent research has highlighted that β-arrestins, besides this involvement in termination, also possess a G-protein-independent signaling capacity. The concept of biased signaling states that a certain ligand may selectively activate one (or a few) of several signaling pathways possible for a given receptor: that is, an activated receptor may induce signals both downstream from the G-protein and downstream from β-arrestin, or signaling may only occur through one of these pathways. In line with this, we have recently shown that an FPR2-selective agonist, F2Pal10, belonging to the pepducin-type lipopeptides, in fact is a biased FPR2 agonist that activates PLC-PIP2-Ca2+ signaling and assembly of the superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the absence of β-arrestin recruitment. We have earlier identified both neutrophil-inhibiting and -activating lipopeptidomimetics (e.g., 1 and 2, respectively; Figure 2), for which it was shown that FPR2 is the preferred receptor. The presence of a hydrophobic N-terminal headgroup as well as the β-peptoid units in the tail moiety were essential for the biological activities of these lipopeptidomimetics. Nevertheless, several structural features of these ligands remain to be investigated in order to delineate the more precise requirements for the optimal interaction with FPRs, in particular, the factors determining whether an agonistic or inhibitory receptor-selective outcome results from the interaction.

A major purpose of the present study was identification of structural determinants in lipopeptide/β-peptoid hybrid peptidomimetics that are of importance for the FPR2-dependent activation of neutrophils by agonists resembling compound 2. This included comparison of the FPR2 interaction of 2 with that of 3 known and 20 novel analogues (i.e., 3–25). The present structure–function optimization of compound 2, a previously described cross-species FPR2 agonist, resulted in identification of two subclasses of more potent FPR2 agonists. The full agonistic activity was found to rely not only on the presence of a fatty acid of appropriate length and the stereochemistry of the adjacent 2-amino-octanoic acid (Aoc) residue, but also on the nature of the two vicinal residues as well as the presence/absence of α-chirality in the peptoid residues of the tail region. Moreover, our data disclose a propensity for biased signaling of FPR2 agonists belonging to this class of lipated peptidomimetics that trigger the PLC-PIP2-Ca2+ signaling pathway without recruitment of β-arrestin or induction of chemotaxis.

### RESULTS AND DISCUSSION

#### Introduction of α-Chiral β-Peptoid Units Influences the Type of Activity and Potency

Through screening of a focused library of lipated α-peptide/β-peptoid hybrids, Lau-[[(S)-Aoc]-Lys-βNspe]2-NH2 (2, Figure 2) was previously identified as the first FPR2-selective peptidomimetic agonist, and the FPR2 preference was confirmed by using FPR2 overexpressing cells. Compound 2 is a partial FPR2 agonist that induces release of superoxide with a slightly lower potency and efficacy in comparison to that of the prototypical peptide FPR2 agonist WKYMVM. Incorporation of shorter or longer N-terminal fatty acids (to give 3 or 4, respectively; Table 2) resulted in reduced or unaffected potency as compared to that of 2, while FPR2 activation proved to be dependent on an (S)-configured N-terminal Aoc residue. These findings prompted us to investigate the influence of chirality in other parts of the molecule, and therefore, several analogues were designed as depicted in Figure 2.

Introduction of chirality in the β-peptoid unit by incorporation of βNspe (to give 5) conferred increased efficacy in triggering superoxide release because 5 induced a higher maximum response, but similar efficacy when compared to the response induced by WKYMVM, and is therefore considered to be a full agonist for the receptor (Figure 3A, and Supporting Information, Figure S1). Furthermore, analysis of the dose–response curves showed that compound 5 is ∼3-fold more potent than 2, with EC50 values of 58 nM (95% CI: 51–66) and 167 nM (95% CI: 142–197), respectively (Figure 3B). The response induced by compound 5 was mediated through FPR2 as inferred by the inhibition profiles, obtained when applying well-characterized selective FPR1 and FPR2 antagonists (see the list of prototypical ligands in Table 1). Thus, the activity of 5 was completely abolished by preincubation with two FPR2-selective antagonists, i.e., PBP10 and the earlier identified peptidomimetic inhibitor 1 (Figure 4A,B). In contrast, the FPR1-selective antagonist cyclosporine H (CysH) did not influence the response induced by 5. Furthermore, the FPR2 selectivity of 5 was confirmed by receptor desensitization experiments, showing that neutrophils activated by the FPR2 agonist WKYMVM were desensitized to a subsequent dose of 5 and of WKYMVM. In contrast, neutrophils stimulated by fMLF were fully responsive to a subsequent dose of 5 but not to fMLF (Figure 4C,D).

The influence of peptoid stereochemistry on activity was further investigated by examining analogue 8 in which the α-chiral βNpe peptoid residues were replaced by βNspe units,
displaying the opposite side-chain stereochemistry (see Figure 1). Interestingly, 8 lacked the ability to confer activation of neutrophil NADPH oxidase (Figure 5A). Generally, the level of superoxide released by neutrophils depends on the cellular state examined, being either naïve (i.e., low-responding) or primed (i.e., high-responding). The activity of 8 was examined on cells in the primed state, achieved by incubating naïve cells with tumor necrosis factor-α (TNF-α) for 20 min at 37 °C. No
superoxide release was inducible by 8 from such primed cells (Figure 5A), thus further confirming the lack of agonistic effect of 8.

Our experience gained from the work on FPR2-interacting lipopeptides belonging to the pepducin family suggests that subtle structural modification, for example, a single exchange of an amino acid, may result in a switch of FPR2 ligands from agonist to antagonist.25,26 On this basis, the inhibitory effect of 8 on FPR2 was examined, showing that 8 dose-dependently, but incompletely, inhibited the response generated by the FPR2-selective agonist WKYMVM with an IC50 value of 0.29 μM (CI: 0.22–0.37 μM; Figure 5B and inset herein). The ability of peptidomimetic 8 to produce a transient increase in the concentration of cytosolic Ca2+, a very early downstream signaling event following FPR activation, was also investigated. Expectedly, 8 proved unable to trigger a transient Ca2+ response by itself. The receptor preference of 8 for FPR2 over FPR1, measured as its inhibitory effect in the NADPH oxidase assay, was confirmed because 8 also blocked the WKYMVM-induced Ca2+ transient (Figure 5C). By contrast, the transient increase in the intracellular calcium level, induced by the FPR1-selective agonist fMLF, was not influenced by the presence of 8 (Figure 5C inset).

**Influence of Fatty Acid Length and Stereochemistry of the Aoc Residue in the Headgroup.** Interestingly, while the size of the N-terminal fatty acid moiety was of minor importance for the parent compound 2, replacement of the N-terminal lauric acid in 8 with the shorter decanoic acid (to give 6) decreased potency, while a two-carbon increased length of the fatty acid (to give 7) led to a ~2-fold increased potency (Table 2). In addition, the structural requirements for agonism/antagonism of analogues of 8, displaying different lengths of N-terminal fatty acids (i.e., 9 and 10), were examined, but neither 9 nor 10 induced any release of superoxide in neutrophils, demonstrating their lack of agonistic effects, while they retained a weak FPR2-selective antagonistic profile like the parent 8 (Table 2). The highest antagonist
from at least three independent experiments (i.e., $n \geq 3$) is shown. (B) Neutrophils (10^5 cells/mL) were preincubated (5 min at 37 °C) with or without compound 8 (1 μM), and were then stimulated with WKYMVM (100 nM). Subsequently, the release of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10^6 counts/min; Mcpm). A representative data set from at least three independent experiments (i.e., $n \geq 3$) is shown. (C) The intracellular calcium transient was measured in neutrophils labeled with Fura-2. Cells were preincubated (10 min at 37 °C) with or without compound 8 (1 μM), and were then stimulated with WKYMVM (10 nM), after which the concentration of free intracellular calcium was measured by the Fura-2 fluorescence. Inset: Cells were preincubated (10 min at 37 °C) with or without compound 8 (1 μM), and were then stimulated with fMLF (10 nM). Abscissa: time of study (s); ordinate: fluorescence (arbitrary units). A representative data set from at least three independent experiments (i.e., $n \geq 3$) is shown.

Concentration tested was 1 μM, at which inflection of the inhibition curve was not observed. By contrast, our earlier published closely related but strongly antagonistic peptidomimetics (e.g., 1) had IC_{50} values in the range 50–100 nM. Consequently, the antagonistic analogues, identified in the present work, were considered to display only weakly antagonistic properties. Hence, on the basis of these findings, the stereochemistry of the peptoid units appears to be a most critical determinant of the type of FPR2 interaction displayed by this class of peptidomimetic ligands, but notably FPR2 selectivity is retained for all these peptidomimetic analogues (i.e., 2−10).

The precise function of the N-terminal fatty acid moiety in the FPR2 interaction is not clear, but many lipidated molecules are modulators of FPR2 function, and these include the FPR2-modulating pepducins. For pepducins, suggested to mediate their modulating action on the intracellular signaling domains of the targeted receptor, the fatty acid is considered to enable peptide translocation across the lipid bilayer.^{7,25} This concept may well apply to other GPCR-derived pepducins that interact with their respective receptors, but the mode of action of FPR2-activating/inhibiting pepducins appears to be unique because recent results infer that they interact with extracellular receptor parts. This issue has been discussed in detail in earlier publications.^{6,16,25,29} This pattern recognition model is supported by the finding that lipidated peptidomimetics also preferentially display affinity for FPR2 on the cell surface.^{17,18}

Nevertheless, when only an N-terminal fatty acid is present on a peptidomimetic tail or the headgroup (N-acylated Aoc) is conjugated to an α-peptide/β-peptoid tail sequence possessing inappropriate chirality of the hydrophobic β-peptoid side chains, the resulting compounds lack agonistic effects on neutrophils, showing that interactions of the peptidomimetic tail sequence also contribute to FPR2 agonism.^{17–19}

In line with our previous results for compound 2, the stereochemistry of the Aoc residue was also of utmost importance for both type and degree of activity. Thus, compounds 11 and 12, both displaying an (R)-Aoc residue instead of (S)-Aoc present in 5 (Table 2), were almost devoid of neutrophil-activating effect. On the other hand, for antagonistic analogues, the stereochemistry of the Aoc residue did not substantially affect the activity (i.e., 13 vs 8, Table 2).

Collectively, these findings suggest that the functional groups displayed by the peptidomimetic tail and the headgroup residues all interact with sites within FPR2, which, depending on the degree of the fit, will result in activation or inhibition. The latter most likely arise from a slight deviation from the optimal orientation of one or more crucial functional groups in an antagonist ligand that, despite lack of ability to induce the conformational change associated with activation, blocks binding of agonists or locks the receptor in an inactive conformation.

Substitutions Vicinal to the N-Terminal Hydrophobic Headgroup Alter Activity and Potency. The quite specific

Figure 5. Peptoid backbone stereochemistry is critical for agonist/antagonist activity. (A) Naïve neutrophils (10^5 cells/mL) or neutrophils pretreated with TNF-α (for 20 min at 37 °C) were preincubated (5 min at 37 °C), and were then stimulated with compound 8 (1 μM). Subsequently, the release of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10^6 counts/min; Mcpm). A representative data set from at least three independent experiments (i.e., $n \geq 3$) is shown. (B) Neutrophils (10^5 cells/mL) were preincubated (5 min at 37 °C) with or without compound 8 (1 μM), and were then stimulated with WKYMVM (100 nM). Subsequently, the release of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10^6 counts/min; Mcpm). A representative data set from at least three independent experiments (i.e., $n \geq 3$) is shown. The inset shows an inhibitory dose−response curve for compound 8. Data are presented as the normalized peak response (mean ± SD; $n = 3$), together with the fitted curve and the calculated IC_{50} value (95% CI). (C) The intracellular calcium transient was measured in neutrophils labeled with Fura-2. Cells were preincubated (10 min at 37 °C) with or without compound 8 (1 μM), and were then stimulated with WKYMVM (10 nM), after which the concentration of free intracellular calcium was measured by the Fura-2 fluorescence. Inset: Cells were preincubated (10 min at 37 °C) with or without compound 8 (1 μM), and were then stimulated with fMLF (10 nM). Abscissa: time of study (s); ordinate: fluorescence (arbitrary units). A representative data set from at least three independent experiments (i.e., $n \geq 3$) is shown.
requirements for both the fatty acid and Aoc residues for FPR2 activation infer that the entire N-terminal headgroup is indeed involved in crucial ligand–receptor interactions initiating signaling. In accordance with this, even small changes to the Lau–(S)-Aoc headgroup, characteristic of potent peptidomimetic agonists, give rise to analogues with weakened agonistic or even antagonistic properties.17 In the present study, an analogue of the FPR2 agonist WKYMVM (Figure 6B), but in contrast to WKYMVM, the neutrophil-activating effect of compound 14 was not affected by myeloperoxidase (MPO)-derived reactive oxygen species (Figure 6C). Peptidomimetic 14 thus shows better oxidative stability than the conventional peptide agonist.

These data indeed infer that interactions with several functional groups in the N-terminal part of the peptidomimetic tail region are involved in mediating FPR2 activation. In support of this hypothesis, introduction of a single βNpe in this position (to give 15) proved sufficient to abolish its capability for FPR2 activation. Nonetheless, the ability of 15 to inhibit the WKYMVM response was lowered (Table 2), suggesting that other groups contribute more to the propensity for inhibition.

Thus, the data obtained so far indicate the importance of functional groups within the N-terminal headgroup and in the residues adjacent to this (i.e., Lau–[(S)-Aoc]-Lys-X with X = βNpe conferring highest potency) in the activation of FPR2 by such peptidomimetics. In order to investigate the nature of these interactions further, we prepared a series of variants of 2, in which the lysine and/or the βNpe residues in the extended headgroup were replaced by α-amino acids with different but related functional groups. Replacement of the first βNpe in 2 with the corresponding α-amino acid Phe (resulting in 16) had no significant effect on the relative potencies [EC\textsubscript{50} of 167 nM (95% CI: 142–197) and 140 nM (95% CI: 117–163) for 2 and 16, respectively]. In contrast, similar replacement with Tyr (to give 17) further increased the potency [to an EC\textsubscript{50} of 49 nM (95% CI: 45–53)], while substitution with Trp (to give 18) led only to a slightly reduced potency [EC\textsubscript{50} of 208 nM (95% CI: 134–321); Table 2]. These data suggest that the hydrophobic functional group in the side chain of the residue in this position is involved in the receptor interaction.

**Table 2. Compounds Tested for FPR2 Interaction by Using the NADPH Oxidase Activation Assay**

<table>
<thead>
<tr>
<th>no.</th>
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<th>EC\textsubscript{50} nM (CI)</th>
<th>comment</th>
<th>ref</th>
</tr>
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<tbody>
<tr>
<td>Variation of Fatty Acid and Peptoid Chirality</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>2</td>
<td>Lau–[(S)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>167 (142–197)</td>
<td>partial agonist\textsuperscript{a,b}</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Dec–[(S)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>&gt;200</td>
<td>partial agonist\textsuperscript{a,b}</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Myr–[(S)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>176 (153–202)</td>
<td>partial agonist\textsuperscript{a}</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>Lau–[(S)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>58 (51–66)</td>
<td>full agonist</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>Dec–[(S)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>177 (138–226)</td>
<td>partial agonist</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>Myr–[(S)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>35 (25–45)</td>
<td>full agonist</td>
<td>17</td>
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</table>

**Variation of Aoc Stereochemistry**

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<td>11</td>
<td>Lau–[(R)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>too weak\textsuperscript{c}</td>
<td>agonist</td>
<td>17</td>
</tr>
<tr>
<td>12</td>
<td>Lau–[(R)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>too weak\textsuperscript{c}</td>
<td>agonist</td>
<td>17</td>
</tr>
<tr>
<td>13</td>
<td>Lau–[(R)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>weak antagonist\textsuperscript{d}</td>
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**Variation of Peptoid Residue Adjacent to Headgroup**

<table>
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<th>EC\textsubscript{50} nM (CI)</th>
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<tr>
<td>14</td>
<td>Lau–[(S)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>29 (22–37)</td>
<td>full agonist</td>
<td>17</td>
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<tr>
<td>15</td>
<td>Lau–[(S)-Aoc]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>weak antagonist\textsuperscript{d}</td>
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**Insertion of Hydrophobic Amino Acid (Instead of β-Peptoid) Adjacent to Headgroup**

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<tr>
<td>16</td>
<td>Lau–[(S)-Aoc]-Lys-Phe-βNpe]-NH\textsubscript{2}</td>
<td>140</td>
<td>full agonist</td>
<td></td>
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<tr>
<td>17</td>
<td>Lau–[(S)-Aoc]-Lys-Tyr-βNpe]-NH\textsubscript{2}</td>
<td>49 (45–53)</td>
<td>full agonist</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Lau–[(S)-Aoc]-Lys-Trp-βNpe]-NH\textsubscript{2}</td>
<td>208 (134–321)</td>
<td>partial agonist\textsuperscript{a}</td>
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**Replacement of Lys Adjacent to Headgroup**

<table>
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<td>19</td>
<td>Lau–[(S)-Aoc]-Dab-βNpe]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>weak antagonist\textsuperscript{d}</td>
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<tr>
<td>20</td>
<td>Lau–[(S)-Aoc]-Dab-βNpe]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>weak antagonist\textsuperscript{d}</td>
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<tr>
<td>21</td>
<td>Lau–[(S)-Aoc]-D-Lys]-βNpe]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>weak antagonist\textsuperscript{d}</td>
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</tr>
<tr>
<td>22</td>
<td>Lau–[(S)-Aoc]-βNlys-Lys-βNpe]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>weak antagonist\textsuperscript{d}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Lau–[(S)-Aoc]-Nle-Phe-βNpe]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>87 (76–105)</td>
<td>full agonist</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Lau–[(S)-Aoc]-Arg-Phe-βNpe]-Lys-βNpe]-NH\textsubscript{2}</td>
<td>45 (42–49)</td>
<td>full agonist</td>
<td></td>
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Reversed Backbone

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<th>no.</th>
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<th>EC\textsubscript{50} nM (CI)</th>
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<tr>
<td>23</td>
<td>Lau–[(S)-Aoc]-βNlys-Lys-Phe]-βNpe]-NH\textsubscript{2}</td>
<td>weak antagonist\textsuperscript{d}</td>
<td></td>
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</table>

*Defined as significant lower max response (in O\textsubscript{2}⁡ production) than that induced by WKYMVM (Supporting Information, Figure S1). EC\textsubscript{50} values are provided in ref 17 and in Supporting Information, Figure S1. “Determined by the inhibition of the O\textsubscript{2}⁡ production induced by WKYMVM. “Inhibitory activity but no IC\textsubscript{50} could be determined because inflection of the dose–response curve was not achieved at the highest concentration tested (1 μM). “Activation of superoxide release was observed, but the inflection point of the dose–response curve was not reached at the maximum concentration tested (1 μM), and thus no EC\textsubscript{50} value could be determined.

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promoting activation, albeit some polarity appears to be preferable in this position. Furthermore, replacement of the N-terminal lysine in 2 or 16 with (S)-2,3-diaminobutanoic acid (Dab), having a two-carbon shorter side chain (to give 19 or 20), abrogated all FPR activation when the adjacent hydrophobic residue was Phe or βNPhe; however, the resulting analogues exhibited weak antagonistic properties. Likewise, replacement of this Lys with D-Lys or βNLys, conferring altered or absence of chirality (in the resulting 21 or 22), respectively, also led to a loss of agonistic activity. However, in line with previous minor modifications of 2, these molecules possessed inhibitor/antagonistic properties, implying that the cationic Lys adjacent to Aoc is also directly involved in receptor interactions. Moreover, analogue 23 displaying a completely reversed design with alternating cationic β-peptoid residues (i.e., βNLys units) and hydrophobic Phe residues proved to possess only weak FPR2-antagonistic properties. In compound 24, the first Lys was replaced by the noncharged Nle, which increased its potency almost 2-fold as compared to that of 16. Interestingly, the receptor selectivity of 24 was reduced, as it activated both FPR1 and FPR2. This was evident from the observation that in order to achieve full inhibition of the response induced by 24, the antagonists CysH (FPR1-selective) and PBP \textsubscript{10} (FPR2-selective) had to be employed in combination (Supporting Information, Figure S2). Analogue 25, displaying a Lys→Arg substitution (and thus a slightly elongated cationic side chain), possessed a ~3-fold increased potency as compared to that of 16, while retaining FPR2 selectivity (Table 2).

These structure–activity relationships indicate that chirality as well as length and charge of the side chain in the residue adjacent to Aoc influences the ability of the analogue to activate FPRs. Noticeably, charge determines whether the analogue exhibits full selectivity for FPR2 over FPR1. These findings correspond well with recent results obtained in a combined mutagenesis/computer modeling study, showing that the primary difference between FPR1 and FPR2, regarding their respective orthosteric binding sites for formyl peptides, relates to their electrostatic potential: the binding site in FPR2 is negatively charged because of the presence of an Asp in position 281, whereas FPR1 contains a neutral Gly in the same position.\textsuperscript{30}

Pronounced FPR2 Reactivation via Cross-Talk with the Receptors for PAF and ATP in Peptidomimetic-Desensitized Neutrophils. During infection/inflammation, microbes or damaged host cells release several danger

Figure 6. (A) Compound 14 is a highly potent FPR2-activating peptidomimetic. Neutrophils (10\textsuperscript{5} cells/mL) were preincubated (5 min at 37 °C) with the FPR2-selective inhibitor PBP \textsubscript{10} (1 μM) or buffer before stimulation with compound 14 (100 nM), and then the production of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10\textsuperscript{6} counts/min; Mcppm). A representative data set out of three independent experiments (n = 3) is shown. Inset: Dose-dependent superoxide release induced by compound 14; data are presented as normalized peak response (mean ± SD; n = 3), and the fitted curve as well as the EC\textsubscript{50} value and 95% confidence interval are calculated. (B) Neutrophils (10\textsuperscript{5} cells/mL) were preincubated for 5 min at 37 °C, and were then stimulated with compound 14 (1 μM) or WKYMVM (100 nM), and then release of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10\textsuperscript{6} × counts/min; Mcppm). A representative data set out of three independent experiments (n = 3) is shown. Inset: Comparison of the responses induced by compound 14 and WKYMVM is presented as the ratio between the peak values obtained with compound 14 and WKYMVM, respectively (mean ± SD; n = 3). (C) Compound 14 is not sensitive to oxidation by the MPO–H\textsubscript{2}O\textsubscript{2} system. The peptide WKYMVM (1 μM) and compound 14 (1 μM) were incubated with MPO (1 μg/mL) and H\textsubscript{2}O\textsubscript{2} (10 μM). The remaining activity of the agonists (WKYMVM and compound 14 after 100× dilution in the oxidase-measuring system) was determined by their ability to activate the neutrophil NADPH oxidase. Neutrophils (10\textsuperscript{5} cells/mL) were activated with untreated and MPO-H\textsubscript{2}O\textsubscript{2}-treated compound 14 (100 nM final concentration) or WKYMVM (100 nM final concentration), and then the peaks of the responses were determined. The remaining activation potencies are presented as the percentage relative to the responses induced by the untreated agonists (controls) and treated agonists (mean ± SD; n = 3). Statistical analysis was performed by using paired Student’s t-test, * *p < 0.01.

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molecules/chemoattractants, including formyl peptides that are sensed by neutrophils through surface-expressed GPCRs. These receptors cross-talk through different mechanisms and together they regulate neutrophil directional migration and other effector responses. Furthermore, it was found that upon desensitization FPR2 may be reactivated by receptor cross-talk.

Figure 7. Neutrophils desensitized with compound 14 can be reactivated by PAF to a greater extent than neutrophils desensitized with WKYMVM. (A) Neutrophils (10⁵ cells/mL) were first desensitized with compound 14 (250 nM) and subsequently stimulated with PAF (100 nM; as indicated by the arrow: solid line). The PAF responses induced in naive cells (dotted line) are shown for comparison. A representative data set out of three independent experiments (n = 3) is shown. Inset: Neutrophils (10⁵ cells/mL) were first desensitized with compound 14 (250 nM; solid line) or WKYMVM (100 nM; dashed line) and subsequently stimulated with PAF (100 nM; as indicated by the arrow), and then release of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10⁶ × counts/min; Mcpm). A representative data set out of three independent experiments (n = 3) is shown.

Figure 8. Compound 14 dose-dependently induces an intracellular calcium transient but no β-arrestin recruitment. (A) Intracellular calcium transient was measured in neutrophils loaded with Fura-2. Cells were preincubated (10 min at 37 °C) and then stimulated with compound 14 in different concentrations (50, 25, and 10 nM) or (B) with WKYMVM (10, 5, and 1 nM), after which the concentration of free intracellular calcium was measured by the Fura-2 fluorescence. Abscissa: time of study (s); ordinate: fluorescence (arbitrary units). Representative data sets out of three independent experiments (n = 3) are shown in A and B. (C/D) FPR2-induced β-arrestin translocation was measured in PathHunter CHO cells from DiscoverX that coexpressed prolinked FPR1/FPR2 and β-galactosidase. Binding of β-arrestin was quantified by fluorescence through enzyme complementation. The prototypic FPR1 agonist fMLF (100 and 10 nM) induced β-arrestin translocation in FPR1-overexpressing cells, whereas the FPR2 agonist WKYMVM (100 and 25 nM) induced β-arrestin translocation in FPR2-overexpressing cells. In contrast, peptidomimetic 14 (at 250 and 100 nM) failed to induce any β-arrestin translocation in both cell lines. Data are expressed as mean ± SD from three independent experiments (i.e., n = 3). The statistical significance was calculated with one-way ANOVA using Dunnett’s multiple comparison test against the buffer control. **p < 0.001.
induced by ATP or PAF upon binding to their respective receptors. The molecular origin of this cross-talk reactivation is not clear, but we have shown that the reactivation signaling pathway bypasses the PLC-PIP2-Ca2+ signal generated downstream of the receptors.33 To determine the ability of PAF and ATP to reactivate peptidomimetic-desensitized FPR2 (for more details about the receptor cross-talk reactivation process, see refs 32−36), neutrophils desensitized with 14 were subjected to reactivation through addition of PAF (an agonist for PAFR) or ATP (an agonist for P2Y2R). The PAF-induced response was primed in comparison to the response induced in naive cells (Figure 7A). In agreement with a cross-talk mechanism for reactivation of FPR2, the PAF-induced response proved sensitive to the FPR2-selective antagonist PBPI0 when this was added prior to stimulation with PAF (Figure 7A). A very similar reactivation pattern was observed when PAF was replaced with ATP that activates the neutrophil P2Y2R (Figure 7A inset). More importantly, when the cross-talk reactivation in neutrophils desensitized with 14 was compared to that observed for cells desensitized with the conventional peptide FPR2 agonist WKYMVM, the response was substantially higher in cells desensitized with 14 (Figure 7B). A higher concentration of WKYMVM proved not to compensate for the increased potency of 14 in cross-talk reactivation. We have shown earlier that PAF/ATP is also capable of potent reactivation of neutrophils desensitized with FPR2-selective lipopeptides belonging to the pepducin family of GPCR-activating agonists,34,35 suggesting similarities between the signaling induced by FPR2-selective pepducins and lipated peptidomimetics.

**FPR2-Selective Peptidomimetics Trigger the Calcium Signaling Pathway without Recruitment of β-Arrestin.**

Signaling induced by the FPR2-selective neutrophil-activating pepducin (F2Pal10) was recently shown to be biased toward activation of the PLC-PPI2-Ca2+ pathway, while β-arrestin was not recruited.16 The β-arrestin signaling pathway cannot be quantitatively determined in neutrophils, and therefore, the PathHunter enzyme fragment (EA) complementation technology in FPR-overexpressing CHO cells was used to compare the signaling properties of 14 with those of the conventional FPR2-selective agonist WKYMVM.

At concentrations of 25 and 50 nM, compound 14 evoked a transient increase in intracellular Ca2+ level in neutrophils (Figure 8A), whereas WKYMVM at concentrations of 5 and 10 nM induced a similar concentration-dependent increased Ca2+ level (Figure 8B). Expectedly, fMLF (at 10 nM) only triggered β-arrestin recruitment in FPR1-overexpressing cells because of its FPR1 selectivity (Figure 8C,D), while neither WKYMVM nor compound 14 affected these cells (Figure 8C). From the β-arrestin translocation experiments with FPR2-
overexpressing cells, WKYMVM (at 25 nM) proved capable of β-arrrestin recruitment, suggesting that FPR2 is properly expressed, and that the system to recruit β-arrrestin upon stimulation is intact in these cells (Figure 8D). In contrast, compound 14 failed to induce β-arrrestin recruitment even at concentrations that induced maximal superoxide release; however, this was not due to adverse effects on the cells caused by the peptidomimetics because the cells treated with 14 proved to be viable (see Supporting Information, Figure S3).

Overall these findings demonstrate that both WKYMVM (a conventional FPR2 agonist) and the FPR2-activating peptidomimetics trigger a G-protein-dependent Ca²⁺ response, whereas only binding of WKYMVM to FPR2 results in β-arrrestin recruitment. Thus, the peptidomimetic agonists (e.g., 2, 5, and 14) exhibited biased FPR2 signaling (see Supporting Information, Figure S1), and thus, these compounds share this signaling property with FPR2-activating pepducins such as F2Pal10.16 Interestingly, biased FPR agonism has been proposed as a new approach in the therapy for myocardial injury.17

Notably, despite the fact that β-arrrestin was not recruited by FPR2 when triggered by these peptidomimetic agonists, the activated receptor is rapidly desensitized after activation (Figure 4). This is in line with reports showing that FPR desensitization is achieved primarily through a cytoskeleton-dependent process in which polymerized actin replaces β-arrrestin, thereby segregating the agonist-bound receptor from the signaling G-protein.16,38,59 Even though the precise signals involved in receptor cross-talk remain unknown, we show that neutrophils desensitized with the lipidated peptidomimetic 14 can be reactivated to produce substantial amounts of superoxide anions when PAFR or P2Y₂R is triggered by PAF or ATP, respectively.

**Peptidomimetic 14 Is Not a Chemoattractant and Does Not Compete in Binding with the FPR2-Selective Agonist WKYMVM.** To further elucidate key features regarding the mechanism for neutrophil activation/desensitization/reactivation induced by 14, binding experiments were performed by using a fluorescently labeled peptide agonist for FPR2. As expected, nonlabeled WKYMVM in excess (i.e., the ratio between nonlabeled and labeled peptide was 100:1) reduced Cy5-WKYVMVM binding significantly (Figure 9A), whereas no significant reduction of binding was obtained with 14 in concentrations up to 1 μM (i.e., nonlabeled 14 to Cy5-WKYVMVM in the ratio 1000:1; Figure 9A). These findings corroborate previously reported data, inferring that binding of the fluorophore-labeled FPR2-selective Cy5-WKYVMVM ligand cannot be competitively displaced by compound 2.17 We have previously identified F2Pal10 as a biased FPR2 agonist, which lacks the capacity to recruit β-arrrestin and is devoid of chemotactic properties.16 To investigate whether compound 14 possessed the same activation profile, the ability of compound 14 to trigger a chemotactic response in neutrophils was determined. In contrast to WKYMVM (a positive control), compound 14 did not induce any chemotactic migration (Figure 9B,C).

Clearly, FPR2 has a broad ligand recognition profile, as it displays high affinity for the microbial/mitochondrial formyl peptide molecular pattern, but, on the other hand, it is promiscuous as it accommodates binding of several other types of ligands that may activate the receptor or inhibit receptor function. Although the precise binding pocket for the well-characterized FPR2 ligands has not yet been clearly defined, it is now apparent that biased agonists may stabilize receptors in conformations different from that triggered by conventional agonists. Hence, these different subactive conformations appear to trigger distinct sets of signaling modes (e.g., G-protein-dependent and/or β-arrrestin-dependent), leading to selective induction of certain cellular responses.10,15,40 The present data indicate that WKYMVM and peptidomimetic ligands (such as 14) may utilize different binding sites and that the peptidomimetic ligands may act as allosteric agonists. Furthermore, this difference in binding-site occupancy may contribute to the characteristic FPR2-mediated signaling properties exerted by each of these ligand types.

Indeed, it would be interesting to compare the in vivo effects of biased and nonbiased agonists in models of infectious and/ or inflammatory diseases to delineate the contribution of these different FPR-dependent signaling pathways and cellular responses to induction and resolution of inflammation, and thus the therapeutic potential of targeting these pathways. However, for such a comparison to be accurate, biased and nonbiased agonists should preferably belong to the same compound type to ensure similar biodistribution and stability. Thus, future development of non-biased FPR2-selective agonists belonging to the present class of peptidomimetics would allow for a delineation of the molecular requirements for induction of β-arrrestin recruitment and chemotaxis, which both are highly relevant signaling properties.

**CONCLUSIONS**

Regarding FPR2 activation and inhibition by lipidated α-peptide/β-peptoid hybrids, the present study provides structural insights into the critical role of the two residues vicinal to the N-terminal hydrophobic headgroup as well as the effects of presence/absence of α-chirality in the peptid residues of the tail region. These peptidomimetics constitute a lipided subclass of α-peptide/β-peptoid hybrids, which earlier were found to resist proteolytic degradation by generalist enzymes such as pronase and specific enzymes such as trypsin and chymotrypsin.37,59 Also, these FPR2-selective agonists were found to exert biased signaling and mediate functional selectivity in human neutrophils. Nevertheless, further studies are required to elucidate the exact role of β-arrrestin in signaling, causing a reactivation of desensitized FPRs. In summary, these proteolytically stable peptidomimetics with unique signaling properties may constitute excellent tools for elucidating the mechanistic details in FPR2-mediated signaling as well as for disclosing its immunoregulatory function in vivo. In addition, future studies should also increase our understanding of the impact of these novel FPR2 ligands on other cells that endogenously express FPR2.

**EXPERIMENTAL SECTION**

**Materials.** Solvents, Fmoc-protected amino acid building blocks, Rink amide resin, and coupling reagents were purchased from IrisBiotech (Marktredwitz, Germany), while Lau-OSu, decanoic acid, and myristic acid were from Sigma-Aldrich Chemie (Steinheim, Germany). Dextran and Ficoll-Paque were obtained from GE-Healthcare Bio-Science (Uppsala, Sweden). Horseradish peroxidase (HRP) was obtained from Boehringer Mannheim (Germany). The hexapeptide WKYMVM was obtained from AltaBioscience.
(University of Birmingham, Birmingham, UK), and the PIP₂ binding peptide PBP₁₀ was obtained from Caslo Laboratory (Lyngby, Denmark). Isolinum, MLF, bovine serum albumin (BSA), TNF-α, and ATP/γS were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Fura-2 was from Molecular Probes, while CyS-WKYWMV was from Phenomenex Pharmaceutical (Burlingame, CA). Cyclosporin H (CysH) was kindly provided as a gift from Novartis Pharma (Basel, Switzerland), while PAF was obtained from Avanti Polar Lipids (Birmingham, Alabama, USA). Reagents were ninefold diluted with NaCl, 5 mM KCl, 1.7 mM KH₂PO₄, 8.3 mM NaH₂PO₄, and 10 mM glucose) supplemented with Ca²⁺ (1 mM) and Mg²⁺ (1.5 mM).

**General Methods for Compound Characterization.** The identity of compounds was determined by high-resolution mass spectrometry, while purity was measured by ultrahigh-performance liquid chromatography (UHPLC) to be at least 97% (detection at \( \lambda = 220 \) nm). Analytical UHPLC was performed on a Shimadzu Prominance UHPLC system by using a Phenomenex Luna C18(2) HTS column (100 × 2.1 mm; particle size: 5 µm) on a Shimadzu system composed of a CBM-20A Prominence diode array communicatio nb u sm o d u l e , t w o L C - 2 0 A P . Subsequently, all dilutions of reagents were made with 95% MeCN + 0.1% TFA. Peptidomimetic tail region, the N-terminus was functionalized via coupling of Fmoc-Aoc-OH (5 equiv) for 1 h. After the last Fmoc removal, acylation was performed with decanoic acid or myristic acid with PyBOP (5 equiv) as the coupling reagent or via coupling with Lau-OSu (5 equiv, 16 h; 5 equiv DIPEA). Finally, the peptidomimetics were cleaved from the resin by using TFA—water—95:5 (5 mL, 1 h at room temperature). Following evaporation in vacuo, the resulting residues were purified by preparative HPLC. Upon lyophilization, the target compounds were stored at \(-20^\circ C\) until use.

**Peptidomimetic 5.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.29 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2161.4873; found, 2161.4882; ΔM = 0.4 ppm.

**Peptidomimetic 6.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 5.71 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2133.4560; found, 2133.4564; ΔM = 0.1 ppm.

**Peptidomimetic 7.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.94 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2189.5195; found, 2189.5167; ΔM = 1.2 ppm.

**Peptidomimetic 8.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.46 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2161.4873; found, 2161.4860; ΔM = 0.6 ppm.

**Peptidomimetic 9.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 5.86 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2133.4560; found, 2133.4564; ΔM = 0.1 ppm.

**Peptidomimetic 10.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 7.07 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2189.5182; found, 2189.5183; ΔM = 0.1 ppm.

**Peptidomimetic 11.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.31 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2161.4973; found, 2161.4913; ΔM = 1.8 ppm.

**Peptidomimetic 12.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.45 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2161.4873; found, 2161.4913; ΔM = 1.8 ppm.

**Peptidomimetic 13.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.45 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2161.4873; found, 2161.4913; ΔM = 1.8 ppm.

**Peptidomimetic 14.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.13 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2091.4091; found, 2091.4091; ΔM = 0.0 ppm.

**Peptidomimetic 15.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.21 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2091.4091; found, 2091.4091; ΔM = 0.0 ppm.

**Peptidomimetic 16.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.13 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2163.3778; found, 2163.3763; ΔM = 0.6 ppm.

**Peptidomimetic 17.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 5.79 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2079.3727; found, 2079.3722; ΔM = 0.2 ppm.

**Peptidomimetic 18.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.15 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2102.3887; found, 2102.3873; ΔM = 0.6 ppm.

**Peptidomimetic 19.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.23 \) min. MALDI-TOF: calcd for \([M + H]^+\), 2049.3621; found, 2049.3603; ΔM = 0.8 ppm.

**Peptidomimetic 20.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. \( t \_R = 6.27 \) min. MALDI-TOF:
calcld for [M + H]⁺, 2035.3465; found, 2035.3471; ΔM = 0.2 ppm.

**Peptidomimetic 21.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 5.96 min. MALDI–TOF: calcld for [M + H]⁺, 2063.3778; found, 2063.3779; ΔM = 0.1 ppm.

**Peptidomimetic 22.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.05 min. MALDI–TOF: calcld for [M + H]⁺, 2077.3934; found, 2077.3919; ΔM = 0.7 ppm.

**Peptidomimetic 23.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.02 min. MALDI–TOF: calcld for [M + H]⁺, 2077.3934; found, 2077.3940; ΔM = 0.2 ppm.

**Peptidomimetic 24.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 7.44 min. MALDI–TOF: calcld for [M + H]⁺, 2048.3669; found, 2048.3690; ΔM = 1.0 ppm.

**Peptidomimetic 25.** Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.25 min. MALDI–TOF: calcld for [M + H]⁺, 2091.3839; found, 2091.3843; ΔM = 0.1 ppm.

**Ethics Statement.** In the present study, conducted at the Sahlgrenska Academy, Gothenburg, Sweden, we used buffy coat preparations that were obtained from the blood bank at Sahlgrenska University Hospital, Gothenburg, Sweden. According to Swedish legislation section code 4 § 3p SFS 2003:460 (Lag om etikprövning av forskning som avser människor), no ethical approval was required because the buffy coats were provided anonymously and thus could not be traced back to a specific donor.

**Isolation of Human Neutrophils.** Peripheral blood neutrophils were isolated from human buffy coats from healthy blood donors by using a dextran sedimentation and Ficoll/Paque gradient centrifugation: the cell-rich plasma/buffy coat was mixed with physiological saline containing dextran (2% w/v; MW 500 000), and then sedimentation of erythrocytes was allowed to take place for 30 min at ambient temperature. The remaining erythrocytes in the Ficoll/Paque solution (density of 1.077 g/mL) were mixed with physiological saline containing dextran (2% w/v) and then centrifuged at 200 g for 1 min at room temperature. The supernatant was then centrifuged at 960 g for 10 min. The cell-rich plasma/buffy coat preparations that were obtained from the blood bank at Sahlgrenska University Hospital, Gothenburg, Sweden, we used buffy coats that were obtained from the blood bank at Sahlgrenska University Hospital, Gothenburg, Sweden. According to Swedish legislation section code 4 § 3p SFS 2003:460 (Lag om etikprövning av forskning som avser människor), no ethical approval was required because the buffy coats were provided anonymously and thus could not be traced back to a specific donor.

**Neutrophil NADPH Oxidase Activity.** NADPH oxidase activity was measured in an isoluminol-enhanced chemiluminescence (CL) assay: neutrophils were added to a 4 mL polypropylene tube containing isoluminol (2 × 10⁻³ M) and HRP (2U) in KRG (final volume 900 µL). The tubes were subjected to equilibration in the Biolumat for 5 min at 37 °C, after which the stimulating agent (100 µL) was added, and then light emission was recorded continuously. Receptor reactivation and cross-talk were achieved by initial stimulation of cells by exposure to a receptor-specific agonist, and when the response returned to baseline (i.e., the desensitized state), the cells were reactivated by receiving a second stimulation to induce release of superoxide. In these reactivation experiments, the antagonists were added to the CL assay mixture 1 min prior to the second stimulation. The CL response was measured with a six-channel Biolumat LB 950S (Berthold Co., Germany).

**β-Arrestin Recruitment Assay.** The capability of FPR2 agonists to promote recruitment of β-arrestin was evaluated in PathHunter eXpress CHO-K1 FPR2-overexpressing cells from DiscoverX (Fremont, USA). The CHO-K1 cells coexpress β-arrestin (isoform 2) tagged with an EA of β-galactosidase and FPR2 tagged with the complementary part (PK) of the β-galactosidase enzyme. Thus, activation of FPR2-PK induces β-arrestin-EA recruitment, forcing complementation of the two β-galactosidase EAs, leading to the assembly of a functional enzyme capable of hydrolyzing the substrate, thereby generating a CL signal. The CHO-K1 cells were seeded into 96-well plates at a density of 10 000 cells/well, which were then generated for 24 h at 37 °C in 5% CO₂. The overnight-cultured cells were activated with agonists and allowed to recruit β-arrestin for 90 min at 37 °C, after which a CL-based detection solution was added (DiscoverX), and then enzyme activity was determined. Light generation, given as relative CL units, was measured with a Multilabel Microtiter Plate Reader (Clariostar, BMG Labtech, Germany).

**Calcium Mobilization.** Freshly isolated neutrophils in KRG containing 0.1% BSA (5 × 10⁷ cells/mL) were exposed to 5 µM FURA 2-AM for 30 min in the dark at room temperature according to the supplier’s protocol (Thermo-Fisher, USA). The cell suspensions were then diluted 1:2 with RPMI 1640 culture medium without phenol red (PAA Laboratories GmbH, Pasching, Austria), and the resulting suspensions were then centrifuged. Next, the cells were washed once with KRG and were resuspended in KRG to yield a density of 2 × 10⁷ cells/mL. The FURA 2-labeled neutrophils were added to a polystyrene cuvette that was equilibrated in the fluorometer for 10 min at 37 °C, upon which the stimulatory agent (100 µL) was added, and then fluorescence was recorded continuously. These measurements were performed with a PerkinElmer fluorescence spectrophotometer (LC50) having excitation wavelengths set to 340 and 380 nm, an emission wavelength set to 509 nm, and slit widths of 5 and 10 nm, respectively. The transient increase in the intracellular Ca²⁺ level is presented as the ratio between the fluorescence intensities detected (i.e., 340 nm: 380 nm).

**Evaluation of Ligand–Receptor Binding by Flow Cytometry.** Neutrophils suspended in ice-cold KRG (1 × 10⁶ cells/mL) were preincubated with unlabeled ligands for 10 min while being kept on ice. A fluorophore-labeled FPR2-selective agonist (Cy5-WKYMWM; 1 nM final concentration) was added, and then incubation on ice was continued for an additional 60 min. Binding of the fluorescent FPR2 agonist to the neutrophils was then analyzed by flow cytometry by using the settings for the Cy5 fluorophore (Ex 488 nm/Em 670 nm). For each sample, 10 000 cells were collected by using an Accuri C6 flow cytometer (Becton Dickinson Sparks, MD, USA). Neutrophil samples incubated with Cy5-WKYMWM, either alone or in combination with WKYMWM (100 nM final concentration), were included as controls to determine total binding (Cy5-WKYMWM alone) and nonspecific binding (labeled and unlabeled WKYMWM together), respectively.

**Stability of Receptor Ligands to MPO–H₂O₂-Mediated Oxidation.** In order to investigate the sensitivity of the FPR2 agonists WKYMWM and peptidomimetic 14 to MPO–H₂O₂-mediated oxidation, the agonists (1 µM concentration) were incubated with MPO (1 µg/mL; 5 min 37 °C) prior to addition of H₂O₂ (final concentration, 10 µM). The samples were incubated for an additional 10 min at room temperature. Agonists incubated under the same conditions, but without
MPO and H₂O₂ were used as controls. The remaining activity of the agonists (WKYMVM and 14) was determined by their ability to induce neutrophil NADPH oxidase activity as described above.

**Neutrophil Chemotaxis.** Neutrophil chemotaxis was determined in a filter assay system (ChemoTx, Neuro probe, UK) used essentially as recommended by the manufacturer. In short, neutrophils suspended in KRG supplemented with BSA (0.3% v/w) were added on top of the filter (pore size: 3 μm), and then cell migration was allowed to proceed for 90 min in a cell culture incubator (at 37 °C under 5% CO₂) in response to different agonist receptors present in the lower compartment separated from the cells by the filter. The number of neutrophils recovered in the lower chamber was determined through the measurement of the MPO activity. The FPR agonists fMLF (10 nM) and WKYMVM (50 nM) were used as positive controls, while buffer was used as a negative control (i.e., spontaneous migration).

**Data Analysis.** Data analysis was carried out with GraphPad Prism 7.0 (Graphpad Software, San Diego, CA, USA). Curve fitting was performed by nonlinear regression using the sigmoidal dose–response equation (variable-slope). One-way ANOVA and Dunnett’s multiple or paired Student’s t-test were employed for statistical analysis, and the method used is given in the figure legends; *p < 0.05, **p < 0.01, ***p < 0.001. Each independent experiment was performed with neutrophils isolated from different individual blood donors or cell lines obtained after different passages.

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**ABBREVIATIONS**

βNLys, N-aminobuty1-β-alanine; βNphe, N-phenylmethyl-β-
alanine; βNpe, N-(R)-1-phenethyl-β-alanine; βNepe, N-(S)-
1-phenethyl-β-alanine; Aoc, 2-aminoacetoic acid; BSA,
bovine serum albumin; CHO, chinese hamster ovary; CL,
chemiluminescence; CsYs, cyanine-5; CysH, cyclosporine H;
Dab, (S)-2,3-diaminobutanol acid; Dec, decanoyl; DCM,
dichloromethane; DlPEA, diisopropylethylamine; DMF,
N,N-dimethylforamide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene;
FPR, formyl peptide receptor; Fmoc, fluoroen-9-ylmethylxycarbonyl; Fura-2 AM, Fura-2 acetoxyethyl ester; GPCR,
G-protein-coupled receptor; HRP, horseradish peroxidase; IP₃,
inositol trisphosphate; KRG, Krebs-Ringer phosphate bu
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**ASSOCIATED CONTENT**

1. Supporting Information
   The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00098.

   - Eₘₐₓ values for agonistic peptidomimetics; dual FPR selectivity of peptidomimetic 24; no off-target effects, shown with peptidomimetic 14; and characterization of peptidomimetics (PDF)
   - Molecular formula strings (CSV)

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**Notes**
The authors declare no competing financial interest.

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