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Unexpected Enhancement in Biological Activity of a GPCR Ligand Induced by an Oligoethylene Glycol Substituent

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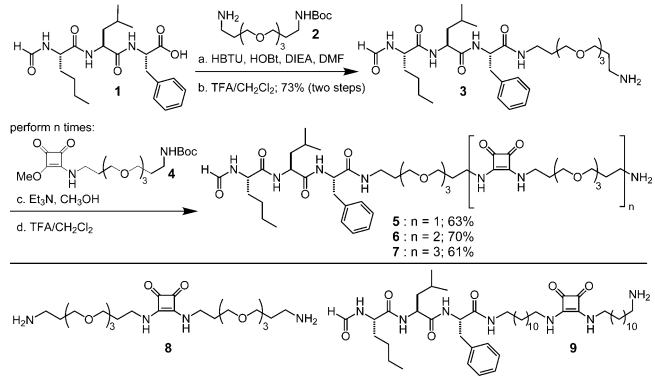
Polyethylene glycol (PEG) is often used to enhance key properties of biologically active molecules.[1,2] PEG substituents can augment the efficacy of protein and peptide therapeutic agents by protecting them from proteolytic degradation[3] or decreasing their rate of clearance from plasma.[2] The widespread use of PEG for these purposes stems from its low toxicity, excellent aqueous solubility, and low antigenicity. These properties appear to be shared by ethylene glycol oligomers. Indeed, oligoethylene glycol groups have been employed to tether biological recognition elements (e.g., to form dimers[4] or higher-order oligomers[5]). Because their persistence length can be estimated,[6] oligoethylene glycol moieties are attractive linkers in the construction of potent multivalent ligands.[4,5] Moreover, oligoethylene glycol moieties on surfaces or in biomaterials resist nonspecific protein binding.[7] Thus, the widespread use of oligoethylene substituents also stems from their presumed inertness. Here we present results demonstrating that an oligoethylene glycol substituent can enhance the potency of a ligand for a transmembrane G-protein-coupled receptor (GPCR).

Our studies originated from our interest in chemotactic signaling. A key initiator of neutrophil chemotaxis is the formyl peptide receptor (FPR). FPR belongs to the largest and most diverse family of integral membrane signaling receptors, the GPCR family.[8] FPR, which is present at high levels on the surface of neutrophils and monocytes, mediates chemotactic responses to N-formylated peptides, including the canonical chemoattractant *N*-formyl-methionine-leucine-phenylalanine (fMLF). Formylated peptides are produced from sources that include the mitochondrial proteins of ruptured host cells and the proteins of invading pathogens.[9] The molecular details of FPR−ligand complexes have not been elucidated to date; however, modeling of the seven transmembrane α-helices[10] suggests that the FPR binding site can accommodate four to five amino acids.[11] Structure−activity relationship data indicate that formyl peptide derivatives with C-terminal substituents can retain the activity of the parent compound.[12,4a] Because we were interested in generating formyl peptide probes of chemotactic signaling, we tested the consequences of adding C-terminal linker substituents.

Precedent suggested that a tether based on oligoethylene glycol would have little effect on signaling. To test this assumption, we appended a series of ethylene glycol oligomers to the C-terminus of a formyl peptide. The FPR ligand we employed, *N*-formyl-norleucine-leucine-phenylalanine (fNleLF), is a chemoattractant.[13] Though less potent than fMLF, its chemical stability is superior. Specifically, the methionine residue in fMLF can undergo oxidation, thereby complicating the synthesis and handling of its derivatives. In contrast, fNleLF-based compounds are stable. To assemble the target compounds, oligoethylene glycol building blocks **2** and **4** were synthesized.[5a] These precursors could be conjugated to the peptidic chemoattractant to yield a series of C-terminal-modified fNleLF derivatives (Scheme 1).

Scheme 1. Route to Formyl Peptides Designed To Bind to FPR



We used squarate-derived building block **4** and free peptide **1** to assemble a series of derivatives possessing C-terminal substituents with six (**5**), nine (**6**), or twelve (**7**) ethylene glycol units. The resulting compounds were evaluated for their abilities to activate signaling in FPR-transfected U937 cells, a monocytic cell line.[14] Like neutrophils, these cells can respond to even a shallow gradient of chemoattractant.[15] To assay chemotactic responses, we employed a simplified multiwell Boyden chamber assay, and the number of migrating cells was determined by using a cell proliferation assay.[16,17] All of the fNleLF derivatives promote cell migration and therefore serve as attractants. Their differential effects on chemotaxis, however, were surprising. Specifically, the more hydrophilic ethylene glycol unit might be expected to decrease the ability of fNleLF to bind to its transmembrane receptor and thereby mitigate attractant activity. Unexpectedly, these substituents had a dramatic positive effect on chemotaxis (Figure 1A). Compound **5,** with six ethylene glycol units, is a more powerful attractant than the free *N*-formyl peptide. Compound **6** with nine ethylene glycol units is even more potent. Indeed, compared with the nonderivatized formyl peptide **1**, compound **6** is >20-fold more active. The trend, however, did not continue beyond nine units. Compound **7**, which possesses an oligoethylene glycol substituent of 12 units, is less active than compound **6**. These results suggest that the oligoethylene glycol substituent is not inert but instead increases the chemotactic activity by an extent that depends upon its length.

To test whether the differences in the cell migration assay depended on FPR signaling, we evaluated the ability of the fNleLF derivatives to elevate a key indicator of chemotactic signaling, namely, intracellular calcium ion concentration ($[Ca^{2+}]_i$). As shown in Figure 1B, formyl peptide **1** induced an increase in $[Ca^{2+}]_i$, and oliogethylene glycol derivatives **5**−**7** likewise activated signaling. The most active fNleLF derivatives in this assay were also the most potent attractants. Specifically, compound **6**, a powerful chemoattractant, caused the greatest increase in $[Ca^{2+}]_i$; it was >10-fold more potent than formyl peptide **1**. These findings link the observed changes in chemotactic activity to increases in intracellular signaling.

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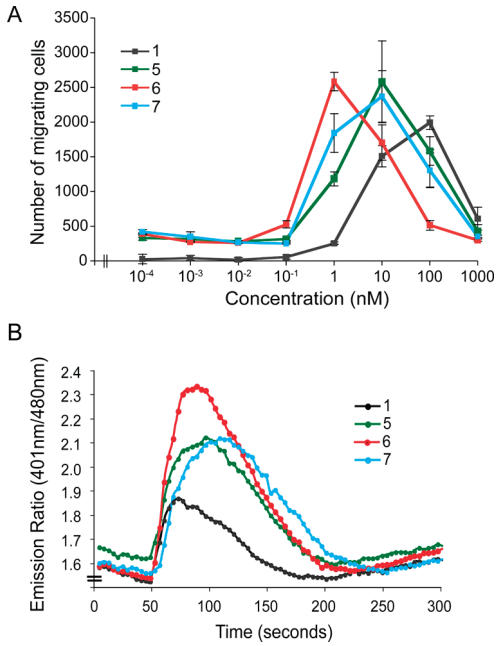

Figure 1. Effects of the formyl peptide derivatives. (A) Chemotactic responses of FPR-transfected U937 cells to formyl peptides. Data shown are from three separate experiments conducted in triplicate. The standard error is depicted. (B) Change in intracellular Ca^{2+} concentration induced by formyl peptides. Cells were loaded with the ratiometric dye Indo-1,[18] and emission ratios were measured using a Photon Technology International fluorimeter. The results shown are from a representative experiment using formyl peptides **1** and **5**−**7** (10 nM). Experiments also were performed at different peptide concentrations (see the SI).

We conducted several additional experiments to determine whether the responses observed were mediated through FPR. To test whether the oligoethylene glycol unit alone can elicit chemotaxis or FPR-mediated signaling, we synthesized compound **8** [see the Supporting Information (SI)]. This compound neither promoted chemotaxis nor signaling (see the SI). We also assessed whether the effects of **1** and **5**−**7** depended upon FPR by evaluating their ability to elicit chemotaxis of FPR-negative U937 cells. None of the compounds induced chemotactic activity in this cell line. These results indicate that the observed activity arises from formyl peptide recognition by FPR.

The observed enhancements in chemotactic activity might stem solely from C-terminal substitution and not from the nature of the substituent. To test for this possibility, we synthesized formyl peptide derivative **9** (see the SI), which possesses an alkyl linker with a length comparable to that of the ethylene glycol substituent in **5**. Though **5** is a more potent attractant than the unsubstituted **1**, alkyl derivative **9** had no enhanced activity in either assay. These data indicate that the unexpected increase in the activity of the formyl peptide derivatives **5**−**7** is due to the oligoethylene glycol substituent.

PEG substituents are known to exhibit a long-range protein repellent effect. At short range, however, the interaction between PEG and protein can become attractive and therefore facilitate binding.[19] Thus, oligoethylene glycol substituents might contribute to the binding affinity of the ligands to the receptor. To test for this possibility, we performed a competitive binding assay of oligoethylene glycol-substituented fNleLF derivatives using commercially available fNleLFNleYK-FITC. Our data indicate that oligoethylene glycol substitution could contribute to binding affinity, as the dissociation constants (K_d) of oligoethylene glycol substituents were found to be slightly lower (4−7-fold) than that of the nonderivatized fNleLF (see the SI). Still, the affinity differences for these formyl peptide derivatives are subtle, suggesting that other factors contribute to the differences in the cellular responses they elicit. For example, the oligoethylene glycol substituent may stabilize an active signaling conformation, increase the conformational flexibility of FPR, or alter its oligomerization state. Regarding the latter, several studies have shown that agonists can disrupt GPCR oligomerization.[20] An ethylene glycol group could serve in that capacity. It is also interesting to note that the compound with the largest oligoethylene glycol substituent, **7**, was less active than compound **6**. As the ethylene glycol substituent becomes more sterically demanding, it may impede binding.

In summary, ethylene glycol-substituted chemoattractants show enhanced activities. The magnitude of the increase depends upon the length of the oligoethylene glycol substituent. The physiological importance of GPCRs is underscored by the many drugs that target them. Our finding that an oligoethylene glycol unit can enhance the activity of a chemotactic agonist provides a blueprint for generating highly potent GPCR agonists.

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Supporting Information Available: Synthetic methods and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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