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Pharmacological profile of a bifunctional ligand of the formyl peptide receptor$_1$ fused to the myc epitope ☆

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ABSTRACT

In human peripheral blood neutrophils or in myeloid PLB-985 cells differentiated towards a neutrophil-like phenotype, the peptide N-formyl-L-norleucyl-L-leucyl-L-phenylalanyl-L-norleucyl-L-tyrosyl-L-leucyl-fluorescein isothiocyanate (f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC) binds to and activates formyl peptide receptor$_1$ (FPR$_1$) and is submitted to receptor-mediated endocytosis (microscopy, cytofluorometry). This peptide may be considered a C-terminally extended version of f-Met-Leu-Phe which carries a fluorescent cargo into cells. By analogy to other peptide hormones for which we have evaluated epitope-tagged agonists as carriers of antibody cargoes, we have designed and evaluated f-Nle-Leu-Phe-Nle-Tyr-Lys-myc, C-terminally extended with the 10-residue myc tag. This peptide is as potent as f-Met-Leu-Phe to compete for f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC uptake by PLB-985 cells, but did not mediate (10–1000 nM) the internalization of the fluorescent anti-myc monoclonal antibody 4A6 added to the extracellular fluid at ~7 nM (microscopy). The nonfluorescent version of the antibody (28 nM) acts as a pre-receptor antagonist of f-Nle-Leu-Phe-Nle-Tyr-Lys-myc, but not of f-Met-Leu-Phe (superoxide release assay in differentiated PLB-985 cells). A further prolonged analog, f-Nle-Leu-Phe-Nle-Tyr-Lys-(Asn-Gly)$_5$-myc, designed to decrease the possible steric hindrance between FPR$_1$ and the bound anti-myc antibody, has little affinity for the receptor, precluding a direct assessment of this issue. Thus, the relatively low-affinity anti-myc antibody used at a high concentration functionally behaves as a selective pre-receptor antagonist of the agonist f-Nle-Leu-Phe-Nle-Tyr-Lys-myc.

1. Introduction

The formyl peptide receptor$_1$ (FPR$_1$) is a G protein coupled receptor (GPCR) responsive to N-formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe), a possible model for proteins synthesized by the bacterial ribosome that could activate an innate form of immunity, notably the activation of neutrophil leukocytes [1]. The N-formyl-Met N-terminus is assembled in mitochondrial proteins/peptides as well, also synthesized by the prokaryotic molecular machinery, and there is evidence that FPR$_1$-mediated chemotaxis of neutrophils to sterile tissue injury sites may be driven by such a damage-associated molecular pattern [2]. The peptide N-formyl-L-norleucyl-L-leucyl-L-phenylalanyl-L-norleucyl-L-tyrosyl-L-leucyl-fluorescein isothiocyanate (f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC) binds to and selectively activates FPR$_1$ and is submitted to receptor- and arrestin-mediated endocytosis in neutrophils [1]. The fluorescent peptide supports a cytofluorometric competition assay based on human neutrophils to evaluate the binding of unlabeled peptides and drugs to FPR$_1$ [3]. The neutrophil FPR$_1$ mediates many functional responses in addition to chemotaxis, such as the respiratory burst, adhesion, and secretion of lysosomal enzymes.

We recently illustrated GPCR-mediated endocytosis of antibody cargoes by intact cells using myc-tagged agonist peptide hormones and an anti-myc monoclonal antibody, the 4A6 clone, with a view on possible applications in diagnosis and therapy (e.g., toxin-conjugated antibodies in oncology). This bifunctional agonist strategy applied particularly well to large recombinant myc-tagged peptides, CCL19 and parathyroid hormone, that formed complexes with the anti-myc antibody in the extracellular fluid; the complexes were internalized in endosomes by their cognate receptors, CCR7 and PTH$_1$R, respectively [4,5]. A similar strategy applied to the bradykinin B$_2$ receptor concerned myc-KGP-B-9972, a synthetic tagged analog agonist that carried some anti-myc antibody into receptor-expressing cells, but with a lesser efficacy [6]. This was due to either the relatively low, micromolar affinity of the agonist for the receptor or to its small size, leading to possible steric hindrance between the antibody and the receptor, two large molecules that must bind the bifunctional agonist at the same

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time. By analogy to other peptide hormones for which we have evaluated epitope-tagged agonists as carriers of antibody cargoes, we have designed and evaluated f-Nle-Leu-Phe-Nle-Tyr-Lys-myc, C-terminally extended with the 10-residue myc tag, as a putative bifunctional high affinity FPR_1 agonist. A further prolonged analog with a larger spacer between the N-terminal FPR_1 pharmacophore and the C-terminal epitope was also produced to address the steric hindrance issue. It was found that the relative affinities of the former bifunctional peptide for the monoclonal antibody and the FPR_1 are sufficient to explain its pharmacologic profile.

2. Materials and methods

2.1. Cells and stimulation

The institutional research ethics board approved the anonymous use of human citrated blood from healthy volunteers to obtain leukocytes. PMNLs (essentially neutrophils) were prepared according to Fernandes et al. [7], with some modifications. Briefly, after sedimentation of red blood cells in 2% dextran, PMNLs were aseptically purified by centrifugation on Ficoll-Paque cushions. Contaminating erythrocytes were removed by hypotonic lysis and PMNLs were resuspended (10^6/ml) in DMEM without serum (for microscopy) or supplemented with 10% FBS (for cytofluorometry). An undifferentiated myeloid cell line was used in most experiments, namely, the PLB 985 cell line [8], originally obtained from the German Collection of Microorganisms and Cell Cultures. These lines were cultured in RPMI 1640 medium containing 10% FBS, pyruvate, glutamine and antibiotics at 37 °C in a 5% CO_2 humidified atmosphere and chemical induction of differentiation was based on adding dimethylsulfoxide (DMSO, 1.3% *v*/*v*) for 5 to 7 days in the culture medium, a step known to upregulate the expression of the human FPR_1 in these cells [9].

2.2. Drugs

f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC was purchased from Molecular Probes, f-Met-Leu-Phe, Met-Leu-Phe, Boc-Phe-D-Leu-Phe-D-Leu-Phe, cytochrome c and phorbol 12-myristate 13-acetate (PMA), from Sigma-Aldrich. f-Nle-Leu-Phe-Nle-Tyr-Lys-myc and f-Nle-Leu-Phe-Nle-Tyr-Lys-$(Asn\text{-}Gly)_5$-myc, the myc sequence representing the 10-residue epitope (EQKLISEEDL), were custom-synthesized by Peptide 2.0, Inc. (Chantilly, VA) and provided as ≥98.6% pure peptides (HPLC) with confirmed identity (mass spectrometry). The $(Asn\text{-}Gly)_n$ linker has been successfully used as a spacer compatible with the extracellular fluid in recombinant protein constructions [10] and in the design of fluorescent bradykinin B_1 receptor ligands (X. Charest-Morin and F. Marceau, unpublished); it was used to increase the distance between the N-terminal FPR_1 pharmacophore and the C-terminal epitope. The anti-myc tag monoclonal antibody 4A6 was purchased from Millipore both in an unlabeled and an AlexaFluor-488 conjugated form.

2.3. Cell stimulation, microscopy and cytofluorometry

Epifluorescence microscopy [5] and cytofluorometry were used to ascertain the cellular uptake and subcellular distribution of formyl-Nle-Leu-Phe-Nle-Tyr-Lys, fluorescein isothiocyanate derivative (f-Nle-

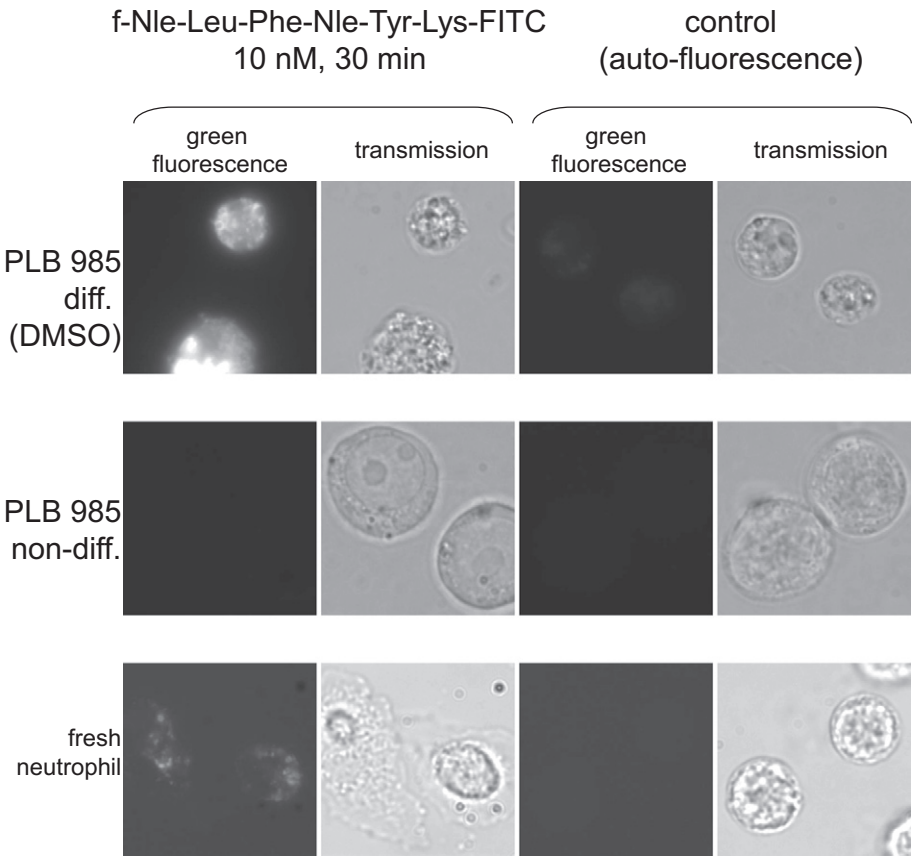

Fig. 1. Granular uptake of f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC (10 nM, 30 min) into cultured PLB 985 cells, differentiated or not, or intact purified PMNLs (green epifluorescence and microscopy, original magnification 1000 ×).