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.

Keywords: formylated peptide; formylated peptide receptor$_1$; receptor-mediated endocytosis; neutrophil.

1. Introduction

The formyl peptide receptor$_1$ (FPR_1) is a G protein coupled receptor (GPCR) responsive to N-formyl-methionyl-leucyl-phenylananine (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_1R, 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 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 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 and Boc-Phe-D-Leu-Phe-D-Leu-Phe, from Sigma-Aldrich. f-Nle-Leu-Phe-Nle-Tyr-Lys-myc and f-Nle-Leu-Phe-Nle-Tyr-Lys-$(Asn-Gly)_5$-myc, the myc sequence representing the 10-residue epitope (EQKLISEEDL), were custom-synthesized by Peptide 2.0, Inc. (Chantilly, VA) and provided as $\geq$ 98.6% pure peptides (HPLC) with confirmed identity (mass spectrometry). The $(Asn-Gly)_n$ 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-Leu-Phe-Nle-Tyr-Lys-FITC; Molecular Probes) in PMNLs or PLB 985 cells maintained in the culture media described above. The stimulation involved a saturating concentration of the fluorescent ligand (10 nM) and was performed at 37ºC for 30 min in order to label the endosomal cell compartment. Specific experiments involved the competition of this labeling by co-treatment with a relevant non-fluorescent ligand of the FPR_1 receptors. The cells were spun, resuspended in HBSS and the fluorescent probe uptake in cell populations assessed using the BD SORP LSR II cell analyzer (BD Biociences, Franklin Lakes, NJ; green fluorescence) and epifluorescence microscopy. The cytofluorometry results were analyzed using the BD FACS DIVA software. Other experiments involved incubating differentiated PLB-985 cells (37ºC, 30 min) maintained in their regular culture medium with the with f-Nle-Leu-Phe-Nle-Tyr-Lys-myc or other peptides or agents supplemented with the AlexaFluor-488 anti-myc monoclonal antibody, clone 4A6, to monitor the endocytosis of the fluorescent antibody cargo (epifluorescence microscopy).

2.4. Superoxide measurement

Differentiated PLB 985 cells support f-Met-Leu-Phe-induced the O_2^- release assay based on cytochrome c reduction that was applied as described [9].

2.5. Data analysis

Numerical values are means ± SEM. Superoxide release values were compared with ANOVA followed by Dunnett's test (comparison with a common control; InStat 3.05 computer program, GraphPad Software; San Diego, CA).

3. Results

f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC is a highly selective agonist of the FPR_1 receptor [1]. After 30 min of cell stimulation (10 nM) at 37°C, it strongly labeled many (but not all) differentiated PLB 985 cells in a granular manner that was consistent with extra-nuclear endocytic vesicles (Fig. 1, upper row for the microscopy, Fig. 2 for cytofluorometry). The differentiated PLB 985 cells were relatively small compared to the non-differentiated proliferating cells of the same line (Figs. 1, 2), but both types were non-adherent to plastic surfaces. The non-differentiated cells essentially did not internalize the fluorescent probe. Freshly isolated PMNLs took up f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC (10 nM, 30 min) in endosomal structures that were not very bright (Fig. 1), possibly related to fluorophore quenching at low pH, but that were present in the vast majority of cells (cytofluorometry, Fig. 2). Thus, the DMSO-differentiated PLB 985 cells represent a good model of the neutrophil FPR_1 receptors and were used as such in subsequent experiments.

To further validate the significance of the uptake of f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC by differentiated PLB 985 cells, non-fluorescent peptides were co-incubated with the fluorescent probe during the uptake period (Fig. 3). Non-fluorescent f-Met-Leu-Phe concentration-dependently displaced the fluorescent probe between 100 nM and 10 µM down to the cell autofluorescence level. The identity of the binding site with a functional receptor for f-Met-Leu-Phe is consistent with the lack of competition by non-formylated peptide, Met-Leu-Phe, and with the capacity of a known antagonist, Boc-Phe-D-Leu-Phe-D-Leu-Phe, to compete for some of the binding at 5-20 µM (Fig. 3). The myc-tagged peptide f-Nle-Leu-Phe-Nle-Tyr-Lys-myc also competed with the fluorescent probe with a potency comparable with that of f-Met-Leu-Phe (Fig.

3), but the prolonged design f-Nle-Leu-Phe-Nle-Tyr-Lys-$(Asn-Gly)_5$-myc, a 26-mer, was ~300-fold less potent, suggesting that the receptor discriminates against long peptides.

Differentiated PLB 985 cells, apt for the endocytosis of f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC, were further used to monitor the uptake of the fluorescent form of the anti-myc monoclonal antibody 4A6 (microscopy, Fig. 4). The used dilution of 4A6 corresponds to a nanomolar concentration. There was a small non-specific cellular uptake of the fluorescence just superior to cell autofluorescence (data not shown) and that was not prevented by co-treatment with a 100-fold excess of a mixture of irrelevant murine monoclonal IgGs, suggesting that the small uptake is not mediated by the Fc-γ receptors. f-Met-Leu-Phe, at highly active concentrations (10-1000 nM), failed to increase the cellular labeling when co-incubated with fluorescent 4A6. At 10-1000 nM, f-Nle-Leu-Phe-Nle-Tyr-Lys-myc or the prolonged myc-tagged peptide were also inactive in this respect (Fig. 4).

Both f-Met-Leu-Phe and f-Nle-Leu-Phe-Nle-Tyr-Lys-myc (10 nM) behaved as agonists in the superoxide release assay applied to differentiated PLB 985 cells (Fig. 5). While the addition of the antibody 4A6 to the intact cells had no direct effect on the assay and did not abate the effect of f-Met-Leu-Phe, it decreased the effect of f-Nle-Leu-Phe-Nle-Tyr-Lys-myc in a concentration-dependent manner (significantly at 28 nM, Fig. 5), supporting a selective pre-receptor antagonism of the tagged peptide.

4. Discussion

In view of the increasing availability of therapeutic monoclonal antibodies that carry cytotoxic cargoes for anti-cancer therapy [11] or other conjugates supporting diagnostic procedures, we studied the feasibility of forcing intact cells to internalize known monoclonal antibodies by exploiting agonist-induced endocytosis of the FPR_1 triggered by a novel bifunctional agonist. While a bifunctional FPR_1 ligand conjugated with an infrared fluorophore has been previously successfully designed and applied in live animals to detect inflammatory neutrophil accumulation [12], this specific agent had a large spacer (polyethylene $glycol_{76}$) between the receptor-binding peptide and the fluorophore. The present strategy has produced the relatively small 16-mer peptide f-Nle-Leu-Phe-Nle-Tyr-Lys-myc, modelled on the high affinity fluorescent probe agonist f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC, the fluorophore representing a comparatively small cargo compatible with receptor binding. However, microscopy failed to document the endocytosis of the antibodies in a situation where there is an approximate equimolar ratio of nanomolar concentrations of the ligand and the receptor (schematic representation: Fig. 6, left); in the presence of such a mixture, the agonist effect of the myc-tagged analog is not decreased (Fig. 5). This implies that the bifunctional ligand has a higher affinity for the FPR_1 than for the antibodies. However, increasing the antibody concentration revealed some pre-receptor antagonism (Figs. 5, 6, right), proving that the tagged peptide binds to the antibodies in a sizeable manner; this antagonism did not apply to f-Met-Leu-Phe, showing specificity. Increasing the concentration of the myc-tagged agonist was not favorable to record the endocytosis of bound fluorescent antibodies (Fig. 4) due to either the excess of free agonist in the reaction or to some steric hindrance between the agonist-bound antibody and the receptor. The prolonged peptide f-Nle-Leu-Phe-Nle-Tyr-Lys-$(Asn\text{-}Gly)_5$-myc, while presumably maintaining its affinity for the

antibodies, did not bind well enough to the FPR_1 (Fig. 3) to directly address the issue of the role of steric hindrance in the system.

The present experimental system illustrates a variant of the modular cargo approach applied to bifunctional peptide ligands: the discrepancy between the ligand affinity for the receptor and that for the antibody may determine unfavorable stoichiometric ratios of the ligand/antibody concentrations to reach cargo endocytosis.

Authorship

F.M. designed the experiments and drafted the manuscript. M.J.G.F. supplied cells and expertise in phagocyte biology. X.C.-M. and C.R. executed most experiments. All authors read and edited the manuscript.

Conflict of interest

The authors have no competing interests for this article.

Acknowledgments

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Figure legends

Figure 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 ×).

Figure 2. Cytofluorometric evaluation of f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC uptake by whole PLB 985 cells, differentiated or not, or intact purified PMNLs. The fluorescence median intensity, including that of cell autofluorescence without the ligand, is illustrated in the right hand side histograms (n = 2-4).

Figure 3. Competition of the uptake of f-Nle-Leu-Phe-Nle-Tyr-Lys-FITC (10 nM, 30 min, 37°C) to differentiated PLB 985 cells by non-fluorescent peptides (co-treatment, concentrations as indicated). Values are means ± s.e.m. of the number of replicates represented indicated between parentheses and are expressed as a percent of the median fluorescence in tubes with the fluorescent ligand but without competitor. In these experiments, the autofluorexence of cells amounted to 27.3 ± 3.3% (not subtracted).

Figure 4. Testing the uptake of the fluorescent form of the anti-myc 4A6 monoclonal antibody (diluted 1:1000, final concentration ~7 nM) incubated with FPR_1 agonists by differentiated PLB 985 cells. Controls include cells without agonist exposed to an excess of irrelevant mouse IgGs, to evaluate the non-speficic uptake of the 4A6 antibody. The agonists were non-tagged f-Met-Leu-Phe, f-Nle-Leu-Phe-Nle-Tyr-Lys-myc or f-Nle-Leu-Phe-Nle-Tyr-Lys-$(Asn-Gly)_5$-myc at the indicated concentrations. One representative cell per condition is shown (green epifluorescence,

1000×). The experiments were replicated at least twice using different batches of differentiated cells.

Figure 5. Selective antagonism of f-Nle-Leu-Phe-Nle-Tyr-Lys-myc by the 4A6 antibody functionally demonstrated in a superoxide release assay applied to differentiated PLB 985 cells. Values are means ± SE of the number of replicates indicated by n. ANOVA indicated that values were heterogeneous only in the group of values from cells stimulated with the myc-tagged peptide ($P < 0.01$). * $P < 0.01$ vs. the control without 4A6 (Dunnett's test).

Figure 6. Schematic representation of the pharmacologic effects of f-Nle-Leu-Phe-Nle-Tyr-Lys-myc interacting with the 4A6 anti-myc monoclonal antibodies. See Discussion for description.

Graphical Abstract

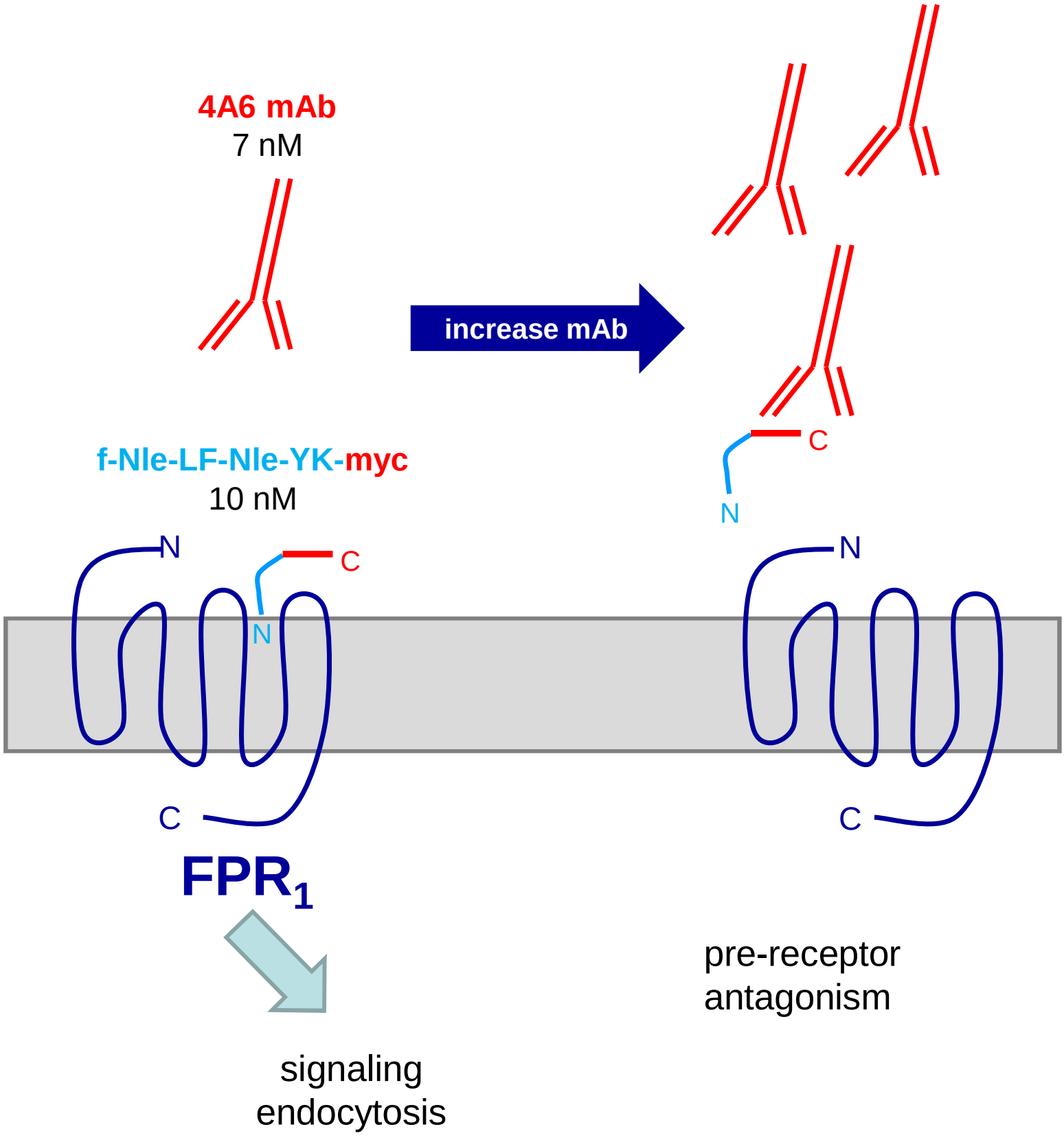


Figure 1

Fig. 1

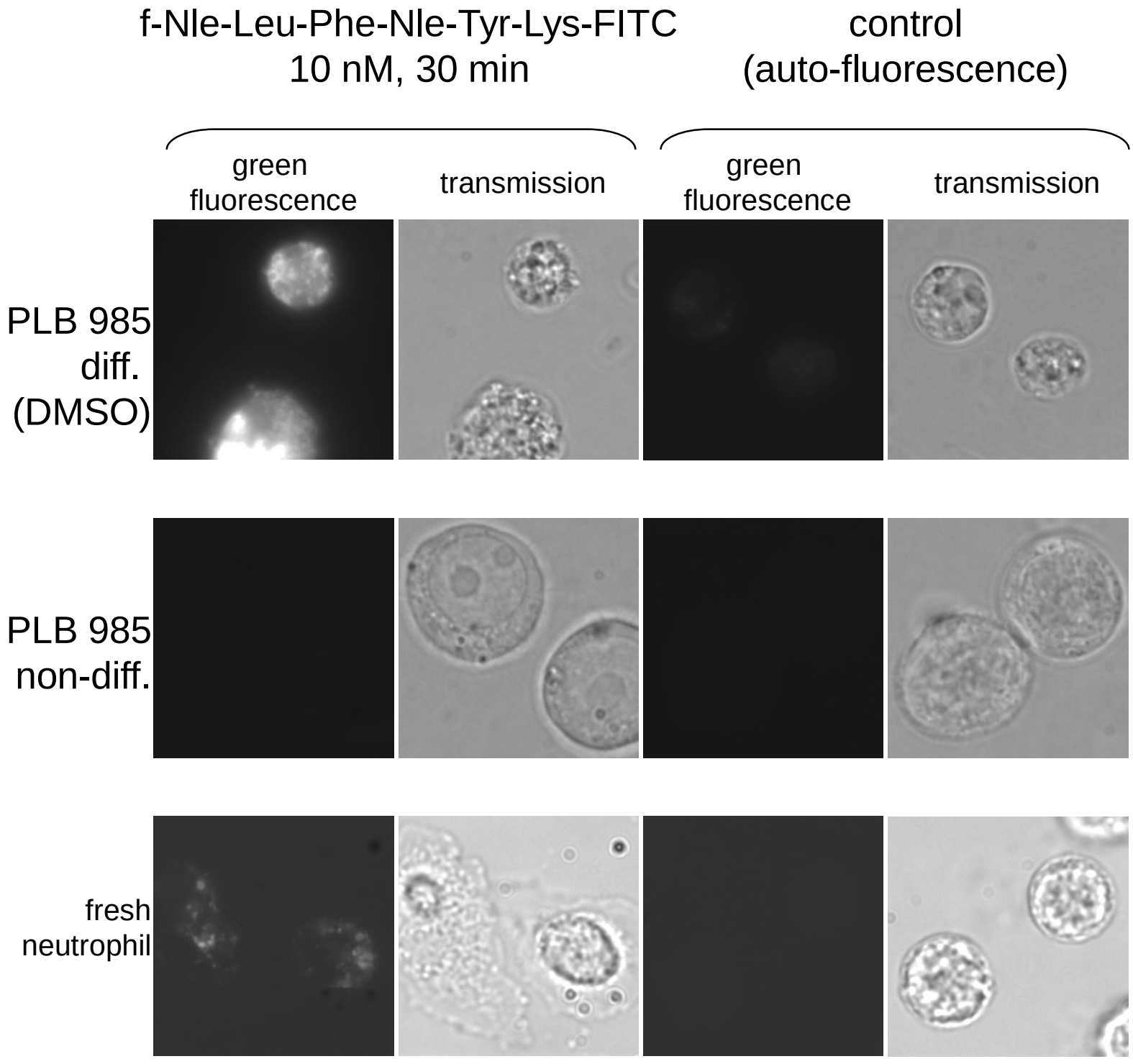
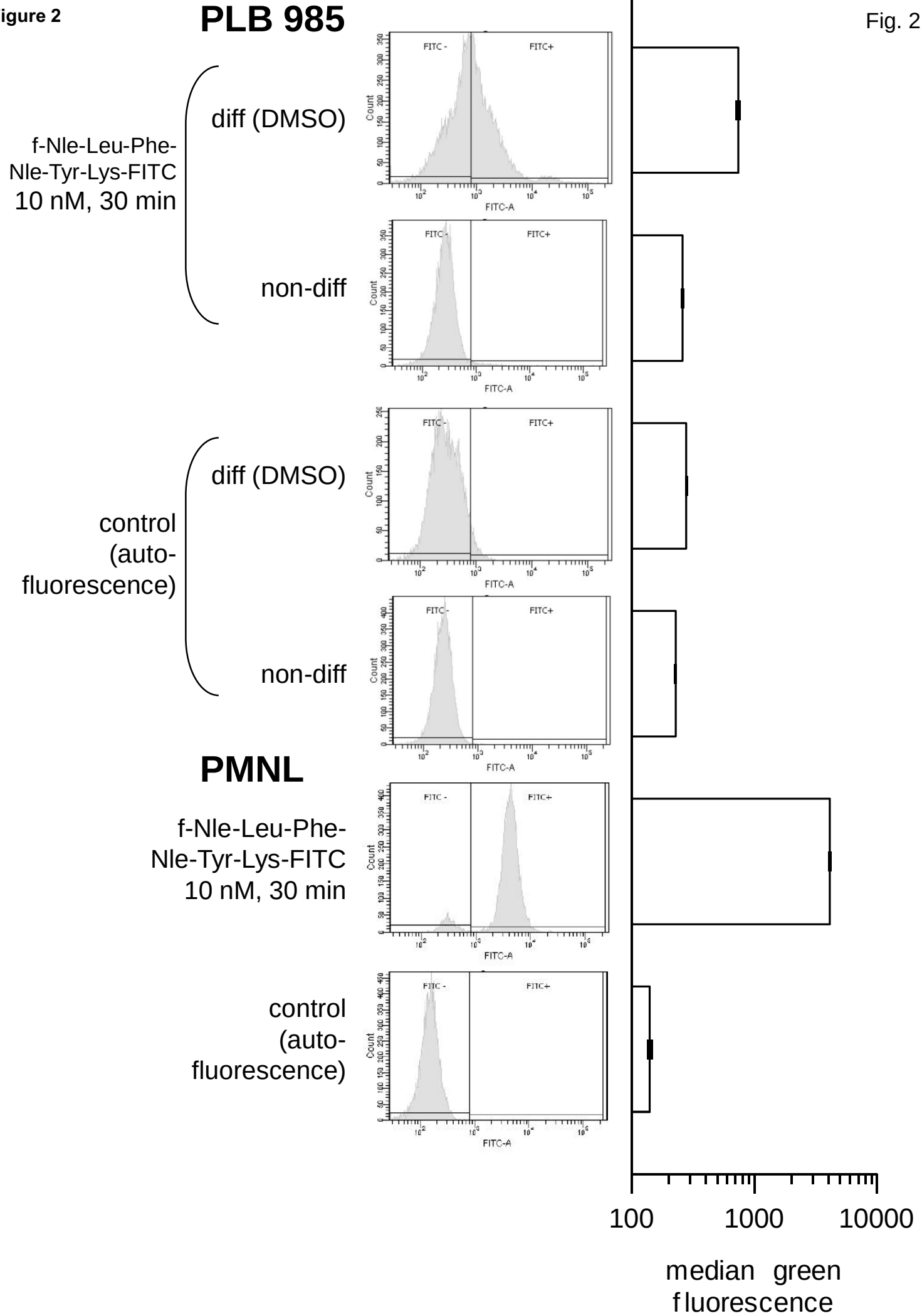
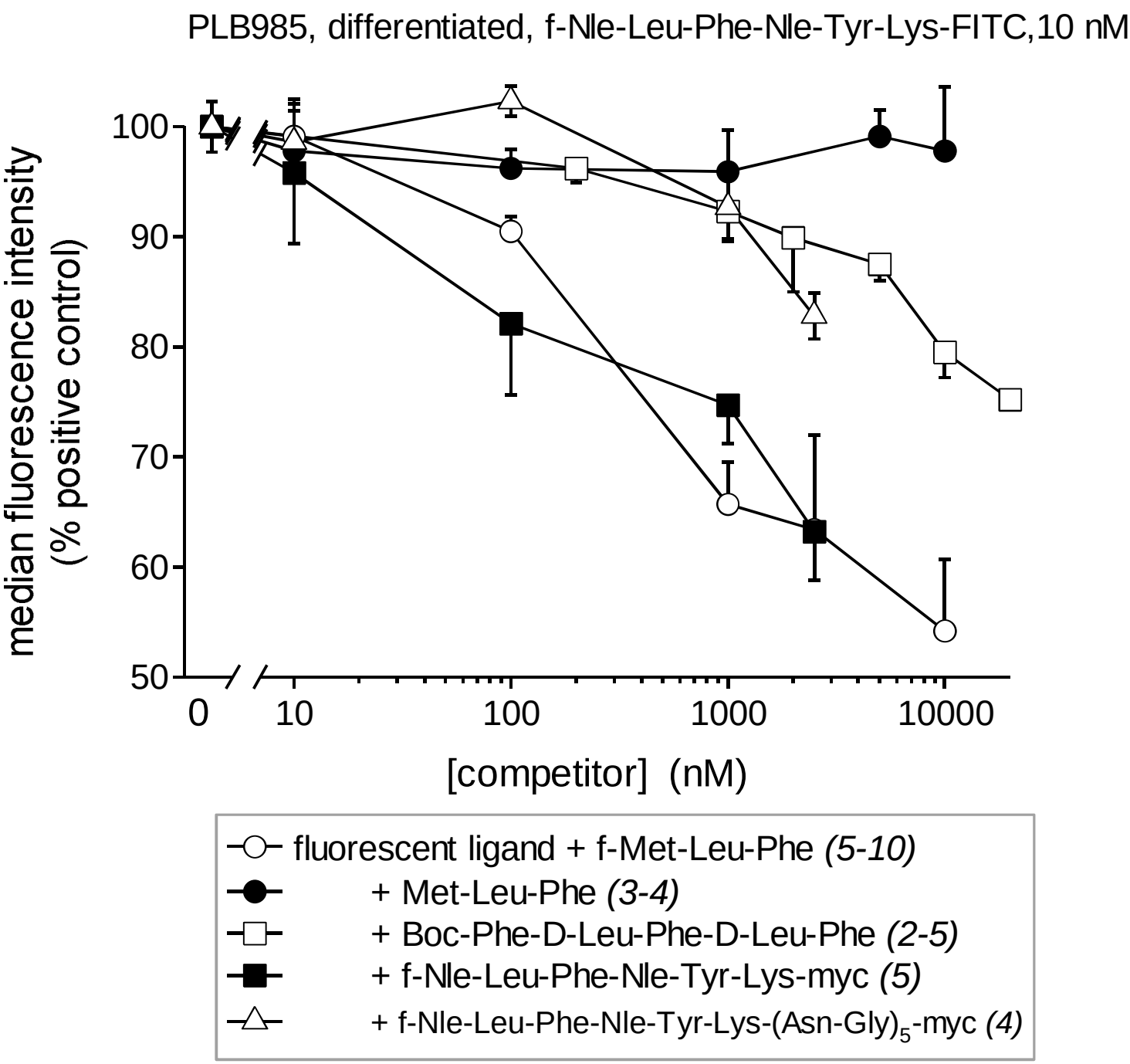


Figure 2

Fig. 2

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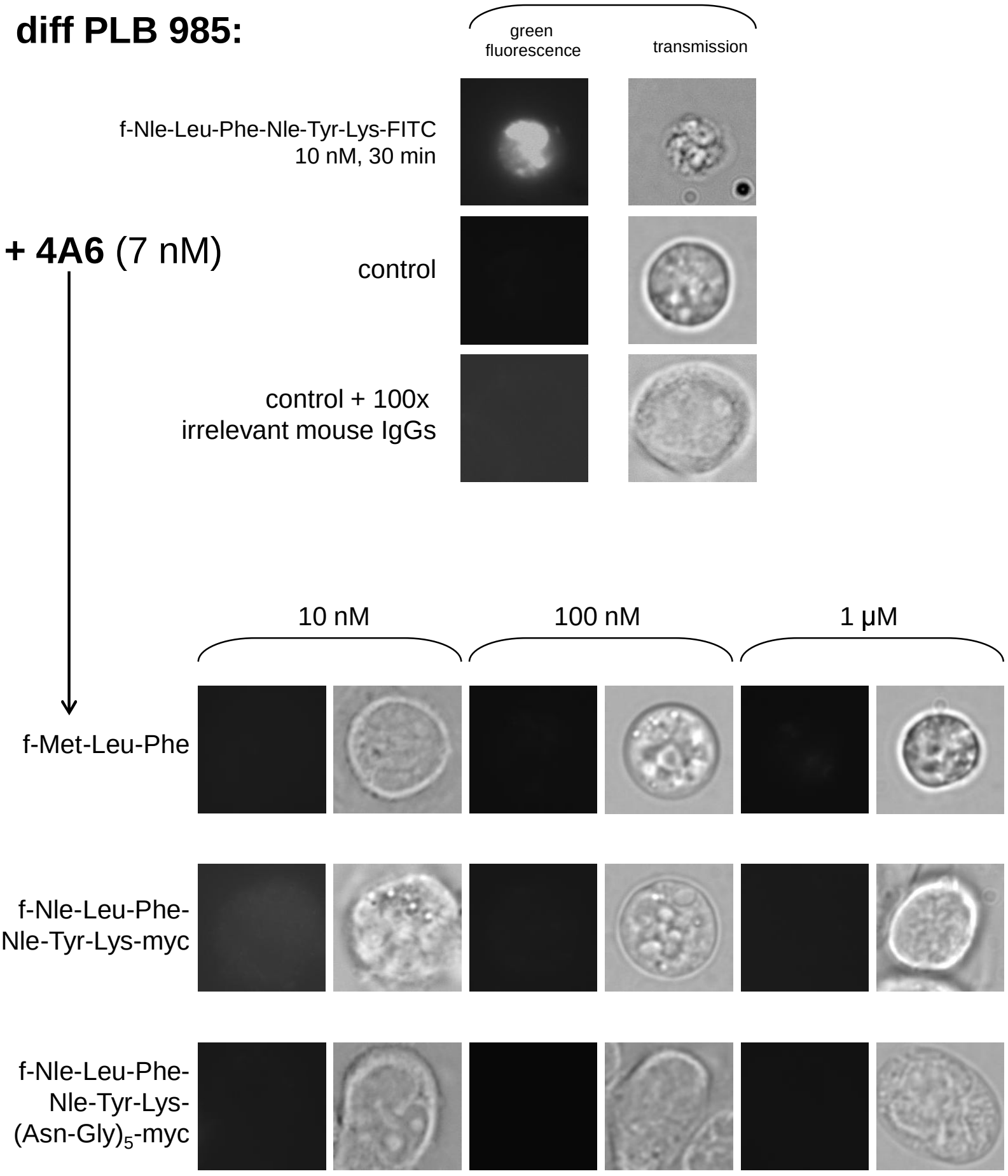
(% positive control)
100
90
80
70
60
50
0
10
100
1000
10000
[competitor] (nM)
fluorescent ligand + f-Met-Leu-Phe (5-10)
+ Met-Leu-Phe (3-4)
+ Boc-Phe-D-Leu-Phe-D-Leu-Phe (2-5)
+ f-Nle-Leu-Phe-Nle-Tyr-Lys-myc (5)
+ f-Nle-Leu-Phe-Nle-Tyr-Lys-(Asn-Gly)5-myc (4)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10 nM, 30 min
+ 4A6 (7 nM)
control
control + 100x
irrelevant mouse IgGs
10 nM
100 nM
1 µM
f-Met-Leu-Phe
f-Nle-Leu-Phe-
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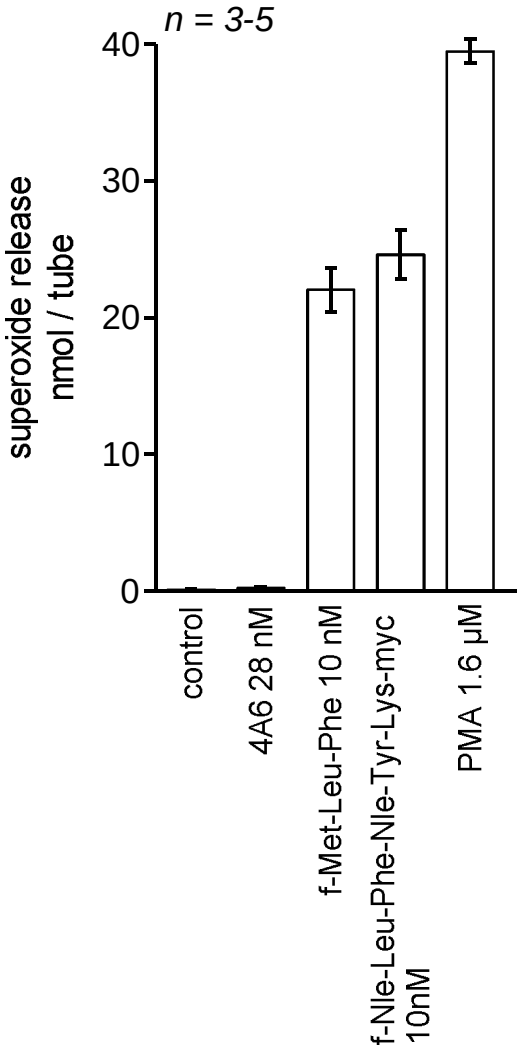
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superoxide release
nmol / tube
40
30
20
10
0
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PMA 1.6 µM

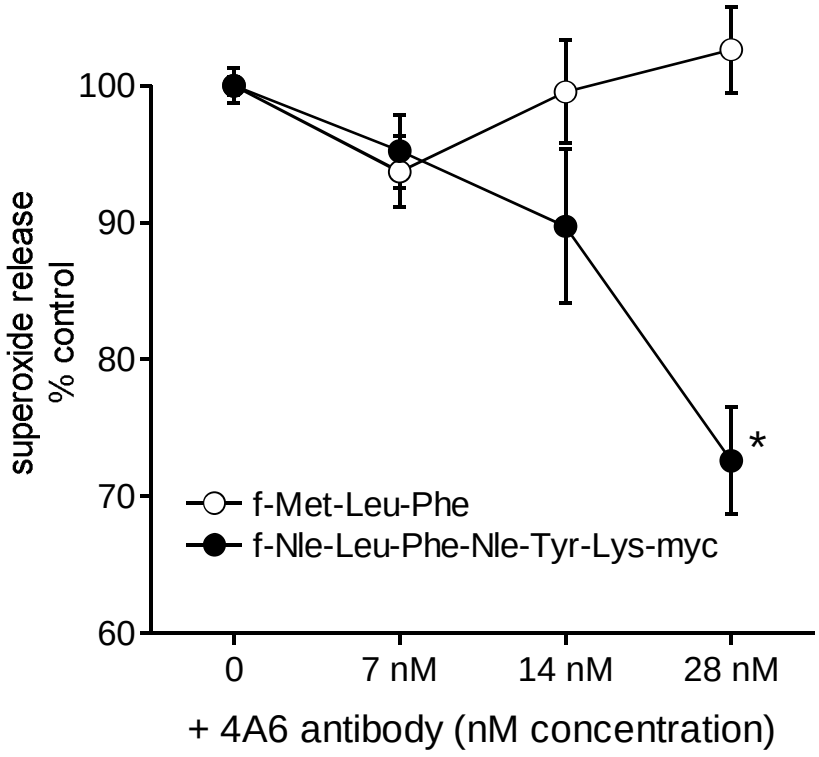
superoxide release
% control
100
90
80
70
60
*
f-Met-Leu-Phe
f-Nle-Leu-Phe-Nle-Tyr-Lys-myc
0
7 nM
14 nM
28 nM
+ 4A6 antibody (nM concentration)

Figure 6

Fig. 6
and graphical abstract