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Synthesis and Application of Fluorescein- and Biotin-Labeled Molecular Probes for the Chemokine Receptor CXCR4

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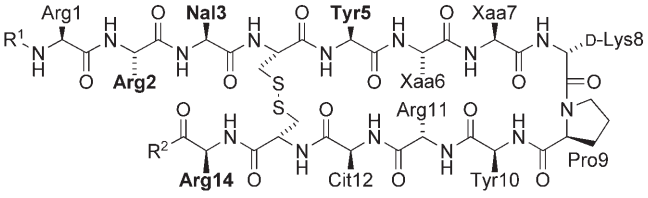
The design, synthesis, and bioevaluation of fluorescence- and biotin-labeled CXCR4 antagonists are described. The modification of D-Lys8 at an ε-amino group in the peptide antagonist Ac-TZ14011 derived from polyphemusin II had no significant influence on the potent binding of the peptide to the CXCR4 receptor. The application of the labeled peptides in flow cytometry and confocal microscopy studies demonstrated the selectivity of their binding to the CXCR4 receptor, but not to CXCR7, which was recently reported to be another receptor for stromal cell-derived factor 1 (SDF-1)/CXCL12.

Introduction

The CXC chemokine receptor 4 (CXCR4) is a G-protein-coupled cell-surface receptor that was identified previously as a coreceptor for infection by the T-cell-line-tropic (X4) human immunodeficiency virus type 1 (HIV-1).[1,2] Stromal cell-derived factor 1 (SDF-1)/CXCL12 is a homeostatic chemokine that regulates a number of physiological and pathologic processes through its interaction with and activation of CXCR4. SDF-1 secreted in bone-marrow stromal cells supports the retention of hematopoietic stem cells (HSCs), progenitor cells, and B-cell precursors in the hematopoietic microenvironment.[3] SDF-1 expression is implicated in the survival, growth, and development of CXCR4-expressing cells, including normal and malignant B lymphocytes, hematopoietic progenitors, and carcinoma cells.[3,4] It has also been demonstrated that concentration gradients of SDF-1 promote the homing of HSCs to bone marrow, the recruitment of progenitor cells to sites of ischemic tissue damage, and the metastasis of CXCR4-expressing neoplastic cells to target organs.[4,5]

Recently, CXCR7 (RDC1, CCX-CKR2) was reported to be another receptor for SDF-1.[6,7] CXCR7 promotes cell survival, growth, and adhesion in vitro and in vivo.[7,8] Furthermore, the expression pattern of CXCR7 is complementary to that of CXCR4 in the migrating primordium.[9,10] Therefore, the SDF-1–CXCR7 axis, like SDF-1–CXCR4, is relevant to the control processes of cell growth, migration, and recruitment. To investigate the distribution and localization of two binding partners of SDF-1, CXCR4 and CXCR7, both in vitro and in vivo, it would be useful to have access to selective and specific fluorescence- and otherwise-labeled ligands for these receptors.

To date, several CXCR4-receptor probes have been prepared and applied both in vitro[11–14] and in vivo.[15] Fluorescein-labeled SDF-1 was utilized to detect the CXCR4-dependent internalization of SDF-1 by stromal bone-marrow cells.[11] This labeled agonist was useful for evaluating the mechanism of receptor activation. We developed a potential radiopharmaceutical agent based on the polyphemusin II derived CXCR4 antagonist T140 (Scheme 1). Thus, [^{111}In]–diethylenetriaminepenta-



	R^1	R^2	Xaa6	Xaa7
T140	H	OH	Arg	Lys
Ac-TZ14011 (**1**)	Ac	NH_2	Cit	Arg

Scheme 1. Structure of the selective CXCR4 antagonists T140, which was used to design probe Ac-TZ14011 (**1**). Bold type indicates the pharmacophore residues.

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acetic acid (DTPA) labeled Ac-TZ14011 was designed for the in vivo imaging of CXCR4-expressing tumors.[15] Rhodamine-conjugated azamacrocycle antagonists were also developed; however, the small molecules were taken up into the cells by a potential active-transport process.[13]

On the basis of our previous research on peptide-based CXCR4 antagonists,[16] we conducted an extensive structure–activity-relationship analysis of labeled ligands with CXCR4 receptors expressed on the cell surface. Herein, we report the design of the labeled antagonists and their application in in vitro experiments, including flow cytometry. The selectivity of the ligand for CXCR4 versus CXCR7 was also investigated by confocal microscopy.

Results and Discussion

Peptide design and synthesis

Previous alanine-scanning experiments identified four indispensable pharmacophore residues of T140, which are located peripheral to the disulfide bridge.[17] On the other hand, modification of the N and C termini or β-turn region with several types of functional groups or peptidomimetics did not lead to a decrease in activity.[16] For example, arylacyl functional groups, such as fluorobenzoyl, at the N terminus of T140 analogues enhanced anti-HIV activity.[18] On the basis of these precedent structure–activity-relationship studies on T140 derivatives, we designed two types of potential labeled CXCR4 ligands (Tables 1 and 2). The functional groups for labeling were attached with an appropriate spacer by acylation to the α-amino group of the N-terminal Arg1 residue or on the ε-amino group of D-Lys8. To identify appropriate fluorophores that did not affect peptide binding affinity to CXCR4, carboxyfluorescein and Alexa Fluor 488, which have a similar fluorescence spectrum, were used for fluorescence labeling. The different functional groups on the fluorescent section of peptides could have an effect on the affinities of the peptides for CXCR4. The Alexa Fluor 488 dye, which contains an amino group, an imino group, and two sulfonate groups on a xanthene structure, exhibited greater photostability and pH insensitivity.

Table 1. Sequences and biological activity of labeled T140 analogues.

R-Arg-Arg-Nal-Cys-Tyr-Cit-Arg-**D-Xaa**-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2

Peptide	R	D-Xaa	IC_{50} [nM][a]
1	Ac	D-Lys	5.2 ± 0.1
2	Ac	D-Glu	6.7 ± 2.6
3	fluorescein	D-Lys	24 ± 0.3
4	fluorescein	D-Glu	199 ± 26
5	Alexa Fluor 488	D-Glu	5700 ± 769

[a] IC_{50} values for the peptides are based on the inhibition of [^{125}I]SDF-1 binding to CHO cells that were transfected with CXCR4.

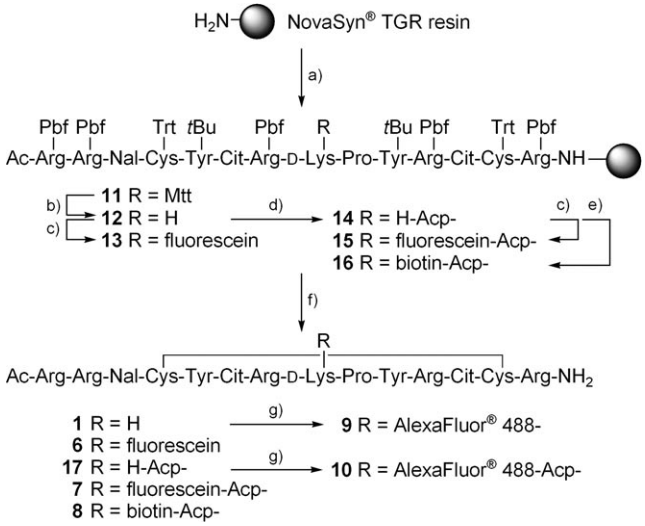
Table 2. Sequences and biological activity of Ac-TZ14011 analogues.

Ac-Arg-Arg-Nal-Cys-Tyr-Cit-Arg-D-**Lys**(R)-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2

Peptide	R	IC_{50} [nM][a]
1	H (amine)	5.2 ± 0.1
6	fluorescein	16 ± 0.8
7	fluorescein-Acp-	26 ± 2.4
8	biotin-Acp-	11 ± 0.1
9	Alexa Fluor 488	8.1 ± 3.5
10	Alexa Fluor 488-Acp-	267 ± 19

[a] IC_{50} values for the peptides are based on the inhibition of [^{125}I]SDF-1 binding to CHO cells that were transfected with CXCR4.

Peptide resins were constructed manually by standard Fmoc-based solid-phase peptide synthesis (SPPS) by using *N*,*N*′-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt). Fluorescein or acetyl modification at the N-terminal α-amino group of peptides **1**–**4** was carried out on the resin by using carboxyfluorescein/DIC/HOBt or Ac_2O/pyridine, respectively. For the preparation of peptides **6**–**8** and **10** with either a fluorescein label or a 6-aminocaproic acid (Acp) linker combined with a fluorescein, biotin, or Alexa Fluor 488 label on the ε-amino group of D-Lys8, a 4-methyltrityl (Mtt) group was used for side-chain protection (Scheme 2). After the removal of the Mtt group on peptide **11** with 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP), an Acp linker and/or labeling groups were attached to the peptide resin **12** by a standard protocol to afford the labeled protected peptide resins **13**–**16**. Treatment of the protected peptide resins with TFA/1,2-ethanedithiol (EDT)/H_2O (95:2.5:2.5) followed by air oxidation in aqueous solution yielded the expected peptides **1**–**4**, **6**–**8**, and **17**.



Scheme 2. Synthesis of D-Lys8-labeled CXCR4 antagonists: a) Fmoc-based peptide synthesis; b) CH_2Cl_2/HFIP/TFE/TES (65:20:10:5); c) carboxyfluorescein, DIC, HOBt; d) Fmoc-Acp-OH, DIC, HOBt, then 20% piperidine/DMF; e) biotin, DIC, HOBt; f) TFA/EDT/H_2O (95:2.5:2.5), then NH_4OH; g) Alexa Fluor 488–OSu, iPr_2NEt. DMF: *N*,*N*-dimethylformamide; Fmoc: 9-fluorenylmethoxycarbonyl; Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; TFE: 2,2,2-trifluoroethanol; TES: triethylsilane; Trt: triphenylmethyl (trityl).

Labeling with Alexa Fluor 488 was performed with the activated succinimidyl ester, which is commercially available. The

precursor peptides (e.g., **1**, **17**) were modified in DMF to provide the expected peptides **5**, **9**, and **10** with a single Alexa Fluor 488 dye moiety.[19]

Biological evaluation of fluorescein- and biotin-labeled peptides

The CXCR4-antagonistic activity of peptides **1**–**10** was evaluated with respect to the inhibition of [^{125}I]SDF-1 binding to CXCR4 Chinese hamster ovary (CHO) cell transfectants. The replacement of D-Lys8 in the parent peptide **1** with D-glutamic acid had no significant effect on the bioactivity of the peptide (IC_{50}(**1**) = 5.2 nM; IC_{50}(**2**) = 6.7 nM; Table 1); this is consistent with the results of previous Glu-scanning experiments of a related peptide.[20] This result suggested that the modification of the β turn $i+1$ position of the peptides with a functional group for labeling would be possible. Fluorescein modification of the N terminus of peptides **1** and **2** led to a slight and significant decrease in inhibitory activity (IC_{50}(**3**) = 24 nM; IC_{50}(**4**) = 199 nM), respectively. Although the substituted benzoyl and pyridinecarbonyl groups at the N terminus of the peptide improved its bioactivity,[18] an additional xanthene or carboxyl group might be unfavorable to ligand binding with CXCR4. The Alexa Fluor 488 labeled peptide **5** showed a significant decrease in inhibitory activity (IC_{50}(**5**) = 5.7 μM); this indicates that the N terminus is inappropriate for fluorescence labeling.

Modification of the ε-amino group of D-Lys8 in the parent peptide **1** was another promising approach to the creation of labeled CXCR4 antagonists (Table 2). The fluorescein-modified peptides **6** and **7** exhibited slightly decreased bioactivity but retained significant binding affinity for CXCR4 (IC_{50}(**6**) = 16 nM; IC_{50}(**7**) = 26 nM). The biotin-labeled peptide **8** containing an Acp spacer, which would be helpful for the simultaneous binding of **8** with CXCR4 and avidins, was also a potent inhibitor (IC_{50}(**8**) = 11 nM). Thus, the presence of a functional group at this position for labeling, with or without an Acp spacer, did not appear to influence the bioactivity of the peptide. We concluded that the D-Lys8 residue in the β-turn region might be unimportant for direct molecular recognition by CXCR4; consequently, this position was considered to be more appropriate for labeling. The Alexa Fluor 488 labeled peptide **9** without an Acp linker showed nearly equipotent inhibitory activity to that of the parent peptide **1** (IC_{50}(**9**) = 8.1 nM). In contrast, significantly lower bioactivity was observed for peptide **10**, which contains an Acp linker (IC_{50}(**10**) = 267 nM). This result implies that the modified xanthene structure of Alexa Fluor 488 might cause some unfavorable interactions with the receptor, contrary to our expectations. The two potent labeled peptides **6** and **9** were used for further experiments.

Application of fluorescence-labeled peptides to flow cytometry and confocal microscopy studies

The applicability of the fluorescence-labeled CXCR4 antagonists **6** and **9** to in vitro experiments was investigated (Figure 1A and B). CHO cells that expressed high levels of the CXCR4 receptor and CXCR4-negative control cells were incubated with peptide **6** or **9** (200 nM), and the resulting mixtures were analyzed by flow cytometry. The CXCR4-expressing cells bound the fluorescent ligand, but the cells that did not express CXCR4 were not stained. The binding of peptides **6** and **9** was inhibited by competition with the unlabeled specific CXCR4 antagonist T140 (200 nM). This result supports the specificity of the fluorescent ligands for CXCR4. With the fluorescent probe **6**, lymphocytes derived from mouse spleen were identified by light scatter gating and analyzed for binding of the fluorescent antagonists (Figure 1C). Peptide **6** bound to CXCR4-expressing lymphocytes, and the staining was inhibited competitively by the addition of unlabeled T140 (200 nM). Peptide **6** was also utilized for the detection of chemotactic cells in a transmigration assay with CXCL12 (Figure 1D). Whereas a low percentage of the cells in the top well of a chemotaxis chamber were positive, the cells which passed through 3-μm pores in response to the CXCL12 chemotactic gradient were all stained positively with peptide **6**.

The probing ability of **6** and **9** for CXCR4 was also verified by confocal microscopy studies on CXCR4-expressing HEK293 cells (Figure 2). The cell surface of CXCR4-positive cells was stained with peptides **6** and **9** in a dose-dependent manner (Figure 2A; see also the Supporting Information). This result is in contrast to a previous report that a rhodamine-labeled azamacrocycle localizes in the cytoplasm by nonspecific uptake.[13] Staining was not observed with CXCR4-negative control cells; this suggests that receptor recognition of these fluorescent peptides is specific to CXCR4 (Figure 2C). Furthermore, CXCR7-expressing HEK293 cells were not stained by the fluorescent peptides **6** and **9** (Figure 2B); this indicates that these T140 derivatives are selective inhibitors of the CXCR4 receptor.

Conclusions

In the current study the effects of labeling a peptide at different positions with various functionalities with a view to retaining indispensable interactions with the CXCR4 receptor was investigated. Fluorescein, biotin, and Alexa Fluor 488 moieties on the D-Lys8 ε-amino group of the parent peptide were appropriate labels. The resulting labeled peptides exhibited specific and high affinity for the CXCR4 receptor, but not for the CXCR7 receptor. The labeled peptides could be useful as selective molecular probes for the CXCR4 receptor in future in vitro and/or in vivo experiments.

Experimental Section

General procedure for peptide synthesis: Protected peptide resins were constructed manually by standard Fmoc-based SPPS on NovaSyn TGR resin (192 mg, 0.05 mmol) by using DIC (39 μL, 0.25 mmol) in combination with HOBt (38 mg, 0.25 mmol). The side chains Tyr, Glu, Lys, Cys, and Arg were protected with *t*Bu, *t*Bu ester, Boc, Trt, and Pbf groups, respectively. For the preparation of peptides **6**–**8** and **10**, the Mtt group was used to protect the D-Lys8 side chain. The N-terminal α-amino group was acetylated by treatment of the resin with Ac_2O (24 μL, 0.25 mmol) and pyridine (40 μL, 0.10 mmol). Biotin (61 mg, 0.25 mmol) or carboxyfluorescein (94 mg, 0.25 mmol) was coupled to the peptide by using DIC

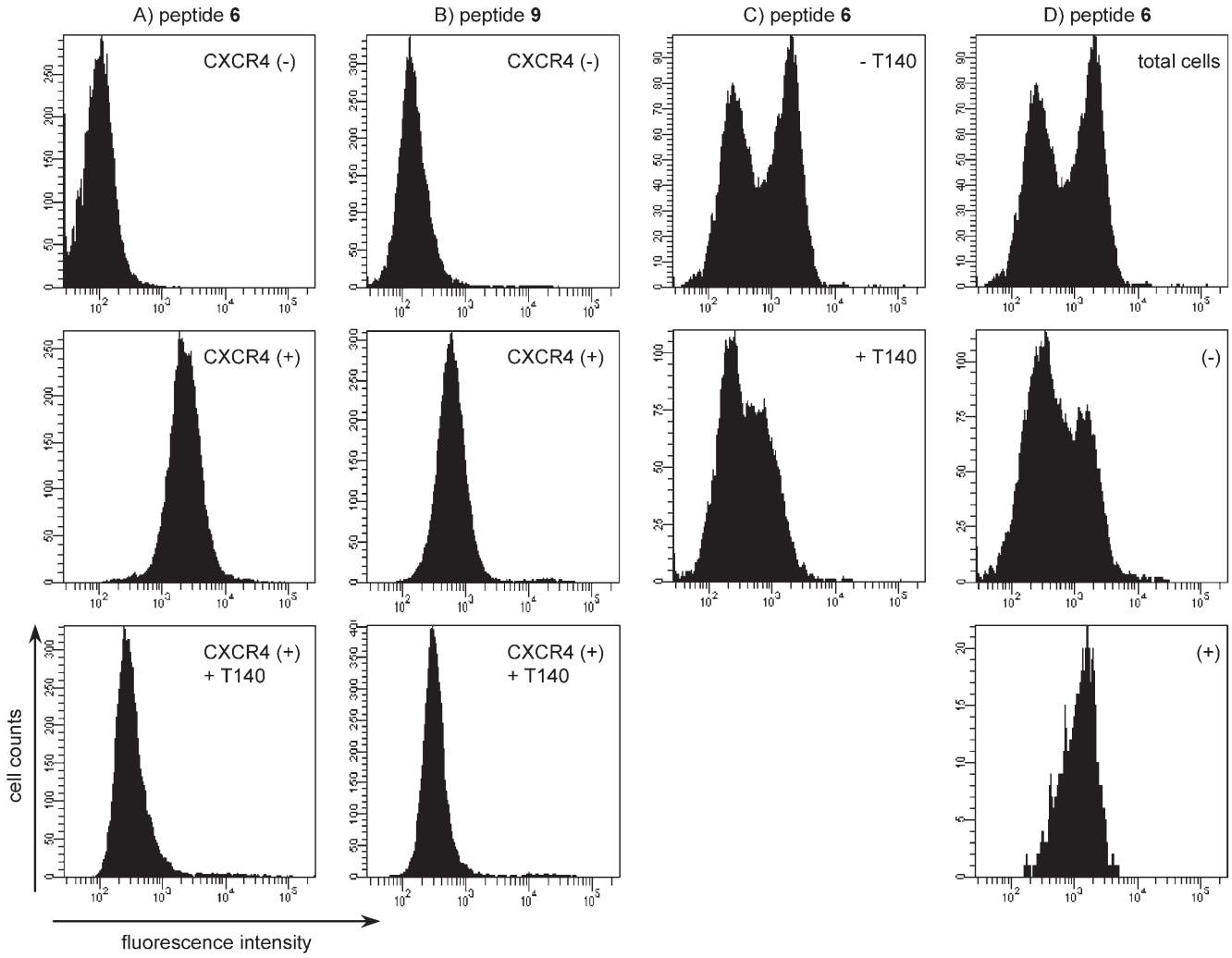


Figure 1. Application of fluorescent CXCR4 antagonists **6** and **9** to flow cytometry. CHO cells were incubated with labeled peptides (200 nM) A) **6**, and B) **9**. The top and middle panels show the results with cells that did not (−) and did express CXCR4 (+), respectively. Competitive binding was assessed with T140 (200 nM; lower panels). C) FACS (fluorescence-activated cell sorting) data for mouse spleen cells treated with peptide **6** (200 nM) in the presence (+) and absence (−) of T140 (200 nM). D) Chemotaxis experiment with mouse spleen cells (top panel). Cells from the total population that did not display chemotaxis are shown in the middle panel (−), and cells that migrated in response to a gradient of SDF-1 are shown in the lower panel (+).

(39 μL, 0.25 mmol) and HOBt (38 mg, 0.25 mmol) in DMF (2 mL). The resulting protected peptide resin (0.05 mmol) was treated with TFA/H_2O/EDT (95:2.5:2.5, 4 mL) for 2 h at room temperature. After removal of the resin by filtration, ice-cold dry Et_2O (100 mL) was added to the residue. The resulting powder was collected by centrifugation and then washed with ice-cold dry Et_2O (3×50 mL). The crude reduced peptide was dissolved in H_2O (100 mL), and the pH value was adjusted to 8.0 with NH_4OH. After oxidation by exposure to air for 1 day, the crude product in the solution was purified by preparative HPLC to afford the desired peptide as a white powder.

Removal of the Mtt protecting group: The resin (0.05 mmol) was treated with CH_2Cl_2/HFIP/TFE/TES (6.5:2:1:0.5, 10 mL) for 2 h at room temperature. It was then washed with the same mixture twice, treated with 10% iPr$_2$NEt in DMF, and used for the next coupling.

Conjugation of Alexa Fluor 488 succinimidyl ester with peptides: Lyophilized peptide (4.66 μmol) and iPr$_2$NEt (3.77 μL, 27.2 μmol) were added to a solution of Alexa Fluor 488 succinimidyl ester (2.50 mg, 3.88 μmol) in DMF (250 μL), and the resulting mixture was stirred in the dark for 12 h at room temperature. The crude mixture was then diluted with MeOH (100 μL) and purified by HPLC. Fractions containing Alexa Fluor 488 conjugates were collected and lyophilized to give **5** (3.3 mg, 27%), **9** (5.1 mg, 51% from **1**), or **10** (5.88 mg, 46% from **17**) as a red powder.

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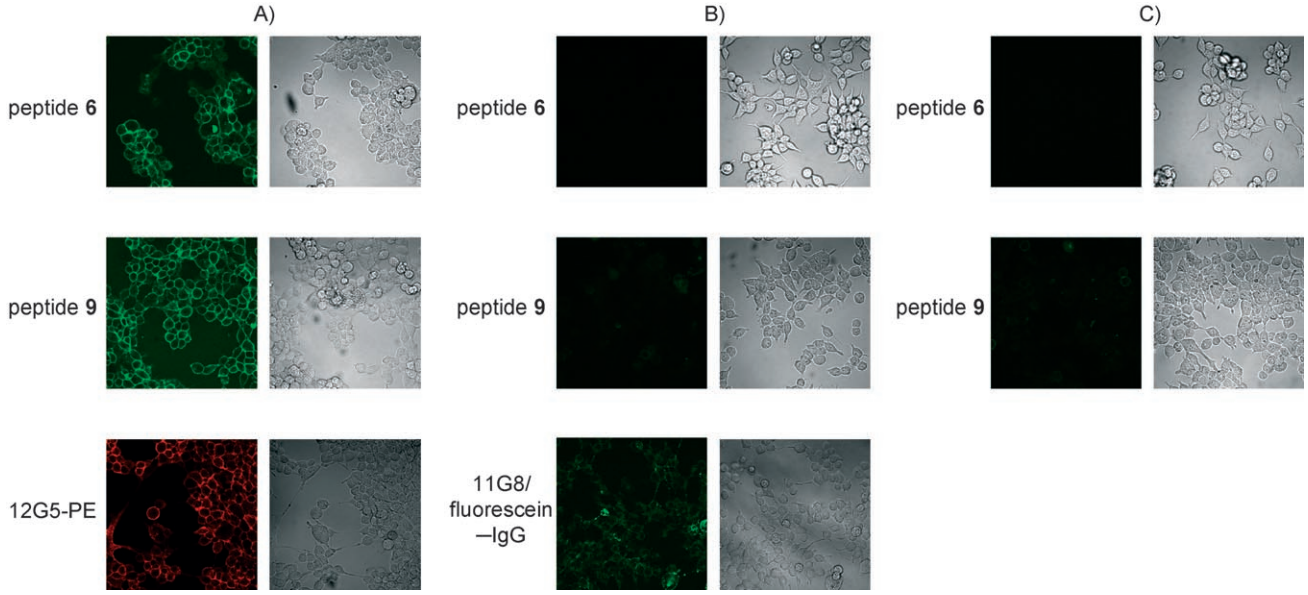

Figure 2. Confocal images of HEK293 cells stained with peptides **6** and **9** (100 nM): A) CXCR4-expressing cells, B) CXCR7-expressing cells, C) CXCR4-negative control cells. CXCR4- and CXCR7-receptor expression was verified by using the monoclonal antibodies 12G5 and 11G8.

Keywords: cell imaging · chemokine receptors · CXCR4 antagonists · fluorescent probes · peptides

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