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Modulation of nuclear internalization of Tat peptides by fluorescent dyes and receptor-avid peptides

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Abstract The nuclear internalization of biomolecules by Tat peptide provides a mechanism to deliver drugs to cells. However, translocation of molecular imaging probes to the nucleus may induce undesirable mutagenesis. To assess the feasibility of retaining its cell permeating effect without nuclear translocation, Tat-peptide was conjugated with a somatostatin receptor (STR)-avid ligand (Oct) and labeled with fluorescent dyes. The results show that Tat-Oct-5-FAM (fluorescein 5′-carboxylic acid) remained in the cytoplasm of STR-positive AR42J cells. Co-incubation of Tat-Oct-5-FAM with ATP induced nuclear translocation. These data suggest that both dye and Oct-STR endocytosis complex could modulate nuclear internalization of Tat peptides.

Keywords: Tat peptide; Nuclear internalization; Somatostatin receptor; Bombesin peptide analogue; Fluorescent probe

1. Introduction

Numerous studies have shown that the Tat protein basic domain residues 48–57 (GRKKRRQRRR) from human immunodeficiency virus (HIV-1) rapidly permeate plasma membranes and translocate into the nucleus [1–5]. This mechanism is currently used to deliver proteins and nucleic acids to cell nuclei through covalent linkages [2]. Similarly, conjugation of molecular probes such as fluorescein 5′-carboxylic acid (5-FAM) [4] and green fluorescent protein (GFP) [3,5] with Tat peptides provides an opportunity to image intracellular processes by optical methods.

Recent advances in laser technology and image reconstruction algorithms have re-ignited interest in the use of optical imaging as a powerful method to diagnose pathologic conditions as well as complement existing imaging modalities [6–10]. The use of exogenous optical molecular probes can enhance the rapid visualization of pathologic tissues. In previous studies, we demonstrated that conjugating a somatostatin receptor-avid peptide (octreotate, Oct) with fluorescent dyes improves the specificity of imaging cancers in rodents by contrast agent-mediated optical imaging [11]. Targeting somatostatin receptor subtype 2 (STR_2) is attractive because it is up-regulated in cancer cells and several clinical studies have successfully used STR-targeted radiopharmaceuticals to image various forms of tumors and pathologic conditions [12,13]. To increase the rate of tumor localization, we explored the effects of labeling a tandem Tat-Oct peptide conjugate with fluorescent dyes, which are useful for cellular and whole-body optical imaging studies. Unfortunately, Tat-peptides are known to translocate into cell nuclei, rendering this approach less attractive for imaging agents because of potential undesirable mutagenesis.

In this study, we prepared a pair of three groups of compounds and assessed their cellular uptake and localization in STR-positive and negative cell lines: (a) 5-FAM and Cypate2-Tat peptides, (b) Oct-5-FAM and Oct-Cypate2, and (c) Tat-Oct-5-FAM and Tat-Oct-Cypate2 conjugates. We also prepared bombesin (BN) receptor-avid peptide [14] to assess the effects of other secondary peptides on modulating the activity of Tat peptides. The results show that nearly all the Tat peptide conjugates internalized and localized rapidly in cellular nuclei but a careful choice of a dye and a secondary peptide can modulate the nuclear internalization of Tat peptides. Particularly, we found that Tat-Oct-Cypate2 promoted, but Tat-Oct-5-FAM inhibited, the nuclear internalization of the Tat peptide residue. Interestingly, addition of ATP to the culture medium can reestablish the internalization of the product in the cell nucleus. This finding is applicable to another receptor-avid bombesin peptide when conjugated to Tat peptide, suggesting the potential to use a variety of secondary peptides to alter the intracellular distribution of Tat peptides. Thus, the internalization of Tat peptides in the nucleus can be modulated by a combination of Oct and 5-FAM and the potential to reverse this process with ATP provides a strategy to selectively destroy target tissues by mutagenesis.

2. Materials and methods

2.1. Synthesis of probe Cypate2

A solution of triethyl orthoformate (0.6 g, 4.0 mmol) in acetonitrile (7.0 mL) was added dropwise into a stirred solution of 1,1,2-trimethyl[1H]-benz[e]indole-3-propanoic acid (0.72 g, 2.0 mmol) in pyridine (10.0 mL) under reflux for 2 h and concentrated. The resulting residue was triturated with dilute HCl (5%), filtered, washed with water, and dried to afford the desired product (0.61 g, 93% yield). The observed molecular mass in electrospray ionization mass spectrum (ESI-MS) corresponded to the calculated $[MH]^+$ value of 573.41.

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Abbreviations: STR_2, somatostatin receptor subtype 2; Oct, octreotate; BN, bombesin peptide analogue; 5-FAM, fluorescein 5′-carboxylic acid; NLS, nuclear localization signal; LRP, lipoprotein receptor-related protein; HSPGs, heparan sulfate proteoglycans

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2.2. Peptide synthesis and conjugation with 5-FAM or Cypate2

All amino acids were purchased from Novabiochem (San Diego, CA, USA). The peptides were prepared on solid support by standard fluorenylmethyloxy (Fmoc) chemistry as described previously [15]. The tandem Tat-octreotate peptide was prepared by a one-pot synthetic strategy on an automated peptide synthesizer (ACT 396). Starting with threonine on Wang resin the tandem Tat-Oct peptide moieties were prepared from the C-terminus of Oct to the N-terminus of Tat 48–57 (Fig. 1). The linker lysine between the OCT and Tat peptide sequences was protected with the orthogonal *N*-α-1-(4,4-dimethyl-2,6-dixoxcyclohex-1-ylidene)ethyl-*N*-ε-Fmoc group (Dde-Lys(Fmoc)-OH) to allow the automated synthesis of the tandem peptide moieties using Fmoc strategy. After peptide synthesis, the Dde group was removed with 2% hydrazine in DMF before dye conjugation. The carboxyl groups of Cypate2 or 5-FAM (Molecular Probes, Inc., Eugene, OR, USA) were pre-activated with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/*N*-hydroxybenzotriazole (HOBt) coupling reagent and coupled to the peptide on resin, as described previously [14]. A similar approach was used to prepare the analogous 5-FAM bombesin peptide analogues used in this study. Cleavage of the peptide–dye conjugate from the solid support and concomitant removal of the side-chain protecting groups with TFA afforded the desired products, which were purified by HPLC and characterized by ESI-MS and spectroscopic methods. Each of the molecular probes was characterized by absorption and fluorescence spectroscopy in 20% aqueous DMSO.

1 Cypate2-K(RRRQRRKKRG-Ac)-OH (Tat-Cypate2), ESI-MS: 693.8 $[M+3H^+]^{3+}$.
2 5-FAM-K(RRRQRRKKRG-Ac)-OH (Tat-5-FAM), ESI-MS: 942 $[M+H^+]^{2+}$.
3 Cypate2-K(Ac)-f-cyclo(C-Y-w-K-T-C)-T-OH (Oct-Cypate2), ESI-MS: 1604.5 $[M+H^+]$, 802.9 $[M+H^+]^{2+}$; f and w represent D-phenylalanine and D-tyrosine, respectively.
4 5-FAM-K(Ac)-f-cyclo(C-Y-w-K-T-C)-T-OH (Oct-5-FAM), ESI-MS: 1407 $[M+H^+]$.
5 Cypate2-K(RRRQRRKKRG-Ac)-f-cyclo(C-Y-w-K-T-C)-T-OH (Tat-Oct-Cypate2), ESI-MS: 1577.3 $[M+H^+]^{2+}$; 1051.9 $[M+3H^+]^{3+}$.
6 5-FAM-K(RRRQRRKKRG-Ac)-f-cyclo(C-Y-w-K-T-C)-T-OH (Tat-Oct-5-FAM), ESI-MS: 2957 $[M+H^+]$; 1478.4 $[M+2H^+]^{2+}$, 992.1 $[M+3H^+]^{3+}$
7 5-FAM-K(RRRQRRKKRG-Ac)-GSGGQWVAGHLM-NH_2 (Tat-BN-5-FAM), ESI-MS: 2957 $[M+H^+]$; 1478.4 $[M+2H^+]^{2+}$; 992.1 $[M+3H^+]^{3+}$.
8 5-Fam-Gly-Ser-Gly-Gly-Gln-Trp-Val-Ala-Gly-His-Leu-Met-NH2 (BN-5-FAM), ESI-MS: 1499 $[M+H^+]$; 749 $[M+2H^+]^{2+}$.

2.3. Cell culture and internalization studies

The STR-positive rat pancreatic carcinoma (AR42J) and the STR-negative human lung cancer (A549) cell lines were purchased from ATCC (Manassas, VA) and stored in 95% air/5% CO_2 37 °C. AR42J cells were cultured in Ham's F-12K medium supplemented with 20% FBS, 1.5 mg/ml $NaHCO_3$ and 2 mM L-glutamine. A549 cells were cultured in Ham's F-12K medium supplemented with 10% FBS, 1.5 mg/ml $NaHCO_3$ and 2 mM L-glutamine. Sub-culturing was performed in a solution of 0.25% trypsin/0.1% EDTA. STR-negative human embryonic kidney 293A (HEK 293) cells (Invitrogen, Carlsbad, CA) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS). The cells (1×10^5 cells/well) were grown on LabTek 8-chamber slides (Nunc Inc. Rochester, NY) overnight prior to the experiment. All compounds were dissolved in 20% aqueous DMSO and diluted in 0.01 M PBS (pH 7.4) (Sigma, St. Louis, MO) to a concentration of 10 μM. Each compound (10 μM) was added to the cells in chambers to obtain final concentration of 1 μM and incubated at 37 °C or 4 °C. The samples were analyzed at different time points. For blocking studies, cells were pre-incubated in 1 μM of the corresponding unlabeled peptides for 3–4 h at 37 °C before adding 1 μM of the molecular probes and incubated for another 15 h at 37 °C. After washing (4×) with 0.01 M PBS (pH 7.4), cells were fixed with 4% paraformaldehyde for 30 min and treated with 0.1% Triton-X for 2 min at room temperature. This step was omitted for the live cell study. Subsequently, the cells were stained for 30 min with 1:2000 ToPro3 (Invitrogen) for fixed cells at room temperature, and 2 h for live cells at 37 °C. Cells were mounted in 50% glycerol in 0.01 M PBS (pH 7.4), covered with coverslips and sealed with nail polish. Olympus confocal laser scanning microscope (FV1000) was used to analyze all samples.

2.4. Determination of molecular probe internalization by confocal microscopy

The internalization of Cypate2 labeled compounds was monitored by using 568 laser sources for excitation and monitored at 605–620 nm. The 5-FAM labeled compounds were excited at 488 nm and monitored at 510–550 nm. Internalization of the molecular probes in cells was observed at 0.5 h, 1 h, 2 h, 4 h, and 15 h incubation in fixed and live AR42J cells. The cells incubated with culture medium or Topro3 (nuclear dye) alone in 0.01 M PBS buffer were used as control. The imaging parameters were kept constant between control and sample groups. A 60× water-merged objective lens was used for microscopy. For optical sectioning by confocal microscopy, a few cells were used to avoid color overlap of the different cells in *X–Y* and *X–Z* images. *Z*-axis dimension of each image was 5–6 μm and the *X–Z* images are presented as short stacks of three optical sections reconstructed with Olympus Fluoview software (v1.4a). Quantitation was performed by counting the number of cells with nuclear internalization of the molecular probes (Table 1) and measurement of fluorescence intensity in the nucleus and cytoplasm at different time points. For the kinetics experiment, three lines were drawn in the nucleus of different cells in each image and the mean fluorescence intensity of the lines was measured by the Fluoview software. The fluorescence intensity was normalized to control (5-FAM or Cypate2) at different time points.

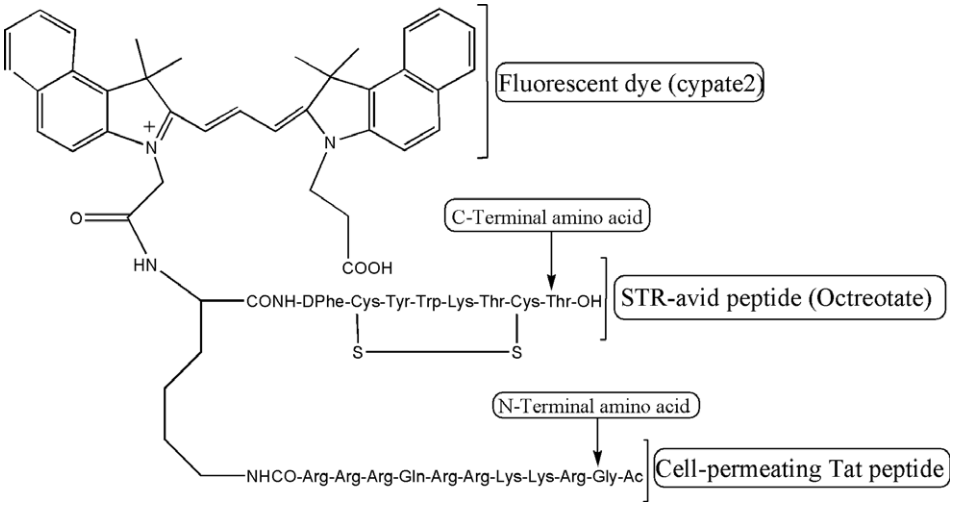

Fig. 1. Structure of Tat-Oct-Cypate2. The fluorescent dye was replaced with 5-FAM in Tat-Oct-5-FAM and octreotate was replaced with bombesin peptide analogue (BN7-14) in Tat-BN-Cypate2. See text for details.