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ORIGINAL PAPER

Synthesis and Characterization of Her2-NLP Peptide Conjugates Targeting Circulating Breast Cancer Cells: Cellular Uptake and Localization by Fluorescent Microscopic Imaging

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Abstract To synthesize a fluorescent Her2-NLP peptide conjugate consisting of Her2/neu targeting peptide and nuclear localization sequence peptide (NLP) and assess its cellular uptake and intracellular localization for radionuclide cancer therapy targeting Her2/neu-positive circulating breast cancer cells (CBCC). Fluorescent Cy5.5 Her2-NLP peptide conjugate was synthesized by coupling a bivalent peptide sequence, which consisted of a Her2-binding peptide (NH_2-GSGKCCYSL) and an NLP peptide (CGYGPKKKRKVGG) linked by a polyethylene glycol (PEG) chain with 6 repeating units, with an activated Cy5.5 ester. The conjugate was separated and purified by HPLC and then characterized by Maldi-MS. The intracellular localization of fluorescent Cy5.5 Her2-NLP peptide conjugate was assessed by fluorescent microscopic imaging using a confocal microscope after incubation of Cy5.5-Her2-NLP with Her2/neu positive breast cancer cells and Her2/neu negative control breast cancer cells, respectively. Fluorescent signals were detected in cytoplasm of Her2/neu positive breast cancer cells (SKBR-3 and BT474 cell lines), but not or little in cytoplasm of Her2/neu negative breast cancer cells (MDA-MB-231), after incubation of the breast cancer cells with Cy5.5-Her2-NLP conjugates in vitro. No fluorescent signals were detected within the nuclei of Her2/neu positive SKBR-3 and BT474 breast cancer cells, neither Her2/neu negative MDA-MB-231 cells, incubated with the Cy5.5-Her2-NLP peptide conjugates, suggesting poor nuclear localization of the Cy5.5-Her2-NLP conjugates localized within the cytoplasm after their cellular uptake and internalization by the Her2/neu positive breast cancer cells. Her2-binding peptide (KCCYSL) is a promising agent for radionuclide therapy of Her2/neu positive breast cancer using a β^- or α emitting radionuclide, but poor nuclear localization of the Her2-NLP peptide conjugates may limit its use for eradication of Her2/neu-positive CBCC using I-125 or other Auger electron emitting radionuclide.

Keywords Breast cancer · Her2/neu oncoprotein · Circulating tumor cells · Nuclear localization sequence peptide · Radionuclide cancer therapy

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Introduction

The spread of cancer cells from original site to a distant location or organ, a process known as metastasis, is the cause of most cancer deaths. One of the most important determinants of prognosis of cancer is the absence or presence of distant metastasis at the time of initial diagnosis. Circulating tumor cells (CTC) are malignant cancer cells present in the peripheral blood. Detection of CTC is associated with early relapse, metastasis and poor prognosis of the patients diagnosed with breast cancer [1, 2]. Her-2/neu oncoprotein is

overexpressed in 10 to 30 % of breast carcinoma and overexpression of Her-2/neu oncoprotein is associated with drug resistance and poor prognosis. Clinical evidences have shown that detection of circulating breast cancer cells (CBCC) overexpressing Her2/neu oncoprotein was associated with tumor recurrence, metastasis and poor clinical outcome in stage I to III breast cancer patients [3, 4]. Development of an effective therapy to eradicate Her2/neu positive CBCC is significant for prevention of metastasis and improving survival of the patients diagnosed with Her2/neu positive CBCC at early and advanced stage of the disease.

Auger-electron emitting radionuclides, such as I-125, may be used for eradication of CBCC because its short distance radiation (< 1 μm) is highly lethal to CBCC without harmful cross-fire radiation on normal cells. To be effective, an Auger-electron emitting radionuclide needs to be delivered into the nuclei of cancer cells. The NLP (CGYGPKKKRKVGG) is a peptide derived from SV40 T antigen which has been tested for nuclear delivery of Auger-electron emitting In-111 radionuclide for experimental radiotherapy of Her2/neu amplified breast cancer [5] or intranuclear localization of nanoparticles for cancer therapy [6]. This study aimed to synthesize a fluorescent Cy5.5 Her2-NLP peptide conjugate consisted of a Her2-binding peptide ligand [7] and an NLP peptide (CGYGPKKKRKVGG), and assess nuclear localization of Cy5.5 Her2-NLP peptide conjugates in the breast cancer cells for its potential use for targeted delivery of I-125 radionuclide for eradication of Her2/neu positive CBCC.

Materials and Methods

Chemical Reagents and Peptides

Commercially available starting materials were purchased from commercial vendors and used directly without further purification unless otherwise stated. Milli-Q water (18 MΩ-cm) was obtained from a Millipore Gradient Milli-Q water system (Billerica, MA). All aqueous solutions were prepared with Milli-Q water.

The Her2-binding peptide [GSG-K(dde)CCYSL($CONH_2$)], NLP peptide [CGYGPKKKRKVGG($CONH_2$)], and the Her2-NLP peptide [GSG-K(dde)CCYSL-(PEG)6-GYGPK(dde)K(dde)K(dde)RK(dde)VGG] were synthesized at Protein Chemistry Technology Center, UT Southwestern Medical Center. The Cyanine 5.5 NHS ester (Cy5.5-NHS) and Cyanine5.5 Maleimide (Cy5.5-mal) were purchase from Lumiprobe Corporation (Hallandale Beach, FL). All reactions were carried out under N_2 atmosphere in degassed dried solvents. Matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF/MS) were acquired on an Applied Biosystems Voyager-6115 mass spectrometer. High Performance Liquid Chromatography (HPLC) separation was performed on a Waters 600 Multisolvent Delivery System equipped with a Waters 2996 Photodiode Array detector. The mobile phase consisted of H_2O with 0.1 % TFA (solvent A) and acetonitrile with 0.1 % TFA (solvent B). The HPLC separation was performed on a semi-preparative XTerra RP18 Column (250× 10 mm) with a gradient of 0 % B to 100 % B in 50 min at a flow rate of 4.0 mL/min.

Synthesis of Cy5.5-Her2 Conjugate

The Her2 peptide [GSG-K(dde)CCYSL($CONH_2$)] (0.5 mg, 0.46 μmol) was mixed with the CY5.5-NHS(1.0 mg, 1.3 μmol) in 100 μL of anhydrous DMF, to which 10 μL of DIPEA was added. The mixture was stirred in dark at room temperature for 24 h under N_2. After removal of the solvent, the crude product was purified by semi-preparative reverse-phase HPLC. The collected fractions of multiple runs were combined and lyophilized to afford dde (N-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl)protected Cy5.5-Her2 conjugate as white powder. MALDI-TOF/MS: calcd for $[M+H]^+$:1644.8. Found: 1644.4. The dde-protected Cy5.5-Her2 conjugate (0.5 mg, 0.3 μmol) was dissolved in 100 μL of anhydrous DMF and treated with 2 % hydrazine hydrate in dichloromethane. The mixture was stirred at room temperature for 30 min. After evaporation of the solvent, the residue was purified by semi-preparative reverse-phase HPLC. The collected fractions of multiple runs were pooled and lyophilized to afford CY5.5-Her2 conjugate white solid in quantitative yield. MALDI-TOF/MS: calcd for $[M+H]^+$:1480.4. Found: 1480.3.

Synthesis of Cy5.5-NLP Conjugate

The NLP peptide [CGYGPKKKRKVGG($CONH_2$)] (0.6 mg, 0.44 μmol) was mixed with the Cy5.5-mal(1.0 mg, 1.3 μmol) in 100 μL of anhydrous DMF, to which 10 μL of DIPEA was added. The mixture was stirred in dark at room temperature for 24 h under N_2. After removal of the solvent, the crude product was purified by semi-preparative reverse-phase HPLC. The collected fractions of multiple runs were combined and lyophilized to afford Cy5.5-NLP conjugate white solid in quantitative yield. MALDI-TOF/MS: calcd for $[M+H]^+$:2082.0. Found: 2082.0.

Synthesis of Cy5.5-Her2-NLP Conjugate

Fluorescent Cy5.5 Her2-NLP peptide conjugate was synthesized by coupling a bivalent peptide sequence, which consisted of a Her2-binding peptide (NH_2-GSGKCCYSL) and an NLP peptide (CGYGPKKKRKVGG) linked by a polyethylene glycol (PEG) chain with 6 repeating units, with an activated Cy5.5 ester (Fig. 1). The Her2-NLP peptide conjugate [GSG-K(dde)CCYSL-$(PEG)_6$-GYGPK(dde)

K(dde)K(dde)RK(dde)VGG] (1.5 mg, 0.4 μmol) was mixed with the Cy5.5-NHS(1.0 mg, 1.3 μmol) in 100 μL of anhydrous DMF, to which 10 μL of DIPEA was added. The mixture was stirred in dark at room temperature for 24 h under N_2. After removal of the solvent, the crude product was purified by semi-preparative reverse-phase HPLC. The collected fractions of multiple runs were combined and lyophilized to afford dde-protected Cy5.5-Her2-NLP conjugate as white powder. MALDI-TOF/MS: calcd for $[M+H]^+$:3892.8. Found: 3892.6. The dde-protected Cy5.5-Her2-NLP conjugate (1.0 mg, 0.25 μmol) was dissolved in 100 μL of anhydrous DMF and treated with 2 % hydrazine hydrate in dichloromethane. The mixture was stirred at room temperature for 2 h. After evaporation of the solvent, the residue was purified by semi-preparative reverse-phase HPLC. The collected fractions of multiple runs were pooled and lyophilized to afford Cy5.5-Her2-NLP conjugate white solid in quantitative yield. MALDI-TOF/MS: calcd for $[M+H]^+$:3069.8. Found: 3069.9.

Intracellular Localization of Cy5.5-Labeled Peptide Conjugates

Human breast cancer cell lines (SKBR3, BT474, and MDA-MB-231) were purchased from American Type Culture Collection (ATCC). Her2/neu positive SKBR3 cells were cultured in McCoy's 5A medium supplemented with 10 % fetal bovine serum (FBS), and Her2/neu positive BT474 cells were cultured in Hybri-Care medium supplemented with 10 % FBS as described previously [8, 9]. Her2/neu negative MDA-MB-231 cells were cultured in Leibovitz's L-15 medium supplemented with 10 % FBS. Prior to incubation with peptide conjugates, the breast cancer cells were incubated with 2 % bovine serum albumin (BSA) solution in phosphate buffered saline (PBS) at 37 °C for 1 h placed in a cell culture incubator for blocking nonspecific binding. After removing PBS containing 2 % BSA, the cells were incubated with 30 μM Cy5.5-labeled peptide conjugates in cell culture medium containing 5 % FBS for 5 h at 37 °C in a cell culture incubator. Upon completion of incubation for a total of 5 h, cell culture medium containing Cy5.5-labeled peptide conjugates was removed and cells were treated with Hoechst 33342 (1:5000) for 10 min for nuclear staining. After washing the cells with PBS for 3 times, cellular uptake and intracellular localization of the Cy5.5-labeled peptide conjugates were assessed by fluorescent microscopic imaging using a Carl Zeiss LSM 510 META inverted laser scanning confocal microscope (Jena, Germany).

Results

Synthesis and Characterization of Cy5.5 Her2-NLP Peptide Conjugates

The fluorescent peptide conjugates (Cy5.5-Her2, Cy5.5-NLP, and Cy5.5-Her2-NLP) were successfully synthesized using well established conjugation chemistry (Fig. 1). The Her2 [GSG-K(dde)CCYSL($CONH_2$)] and Her2-NLP [GSG-K(dde)CCYSL-$(PEG)_6$-GYGPK(dde)K(dde)K(dde)RK(dde)VGG] peptides had NH_2 functional group present at N-terminal for acid-amine conjugation chemistry, while C-terminal was capped with amide group. The lysine groups in peptide Her2 and Her2-NLP were also protected with dde (N-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl) group to restrict the fluorescent probe (Cy5.5) conjugation at N-terminal amine. Conjugation of dde-protected Her2 and Her2-NLP peptide with 3 equivalents of Cy5.5-NHS in the presence of DIPEA provided the dde-protected conjugates in quantitative yields. Finally, the dde group was deprotected using 2 % hydrazine hydrate in dichloromethane to provide Cy5.5-Her2 and Cy5.5-Her2-NLP each in quantitative yield. The NLP peptide presented SH functional group for thiol-maleimide conjugation chemistry, while C-terminal was capped with amide group. Conjugation of NLP peptide with 3 equivalents of Cy5.5-mal in the presence of DIPEA provided final conjugate Cy5.5-NLP in quantitative yield. All of the peptide conjugates were characterized by their molecular ion peak by MALDI-mass spectrometry, and the purity of these conjugates was assured by observing a single peak in the reverse-phase HPLC.

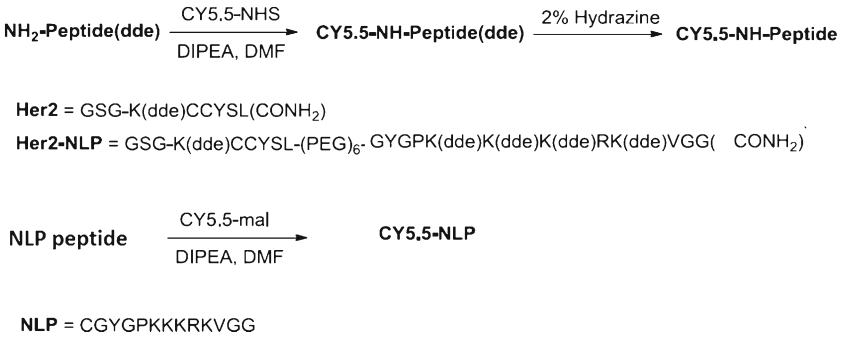

Fig. 1 Synthesis of fluorescent Cy5.5 Her2-NLP peptide conjugates

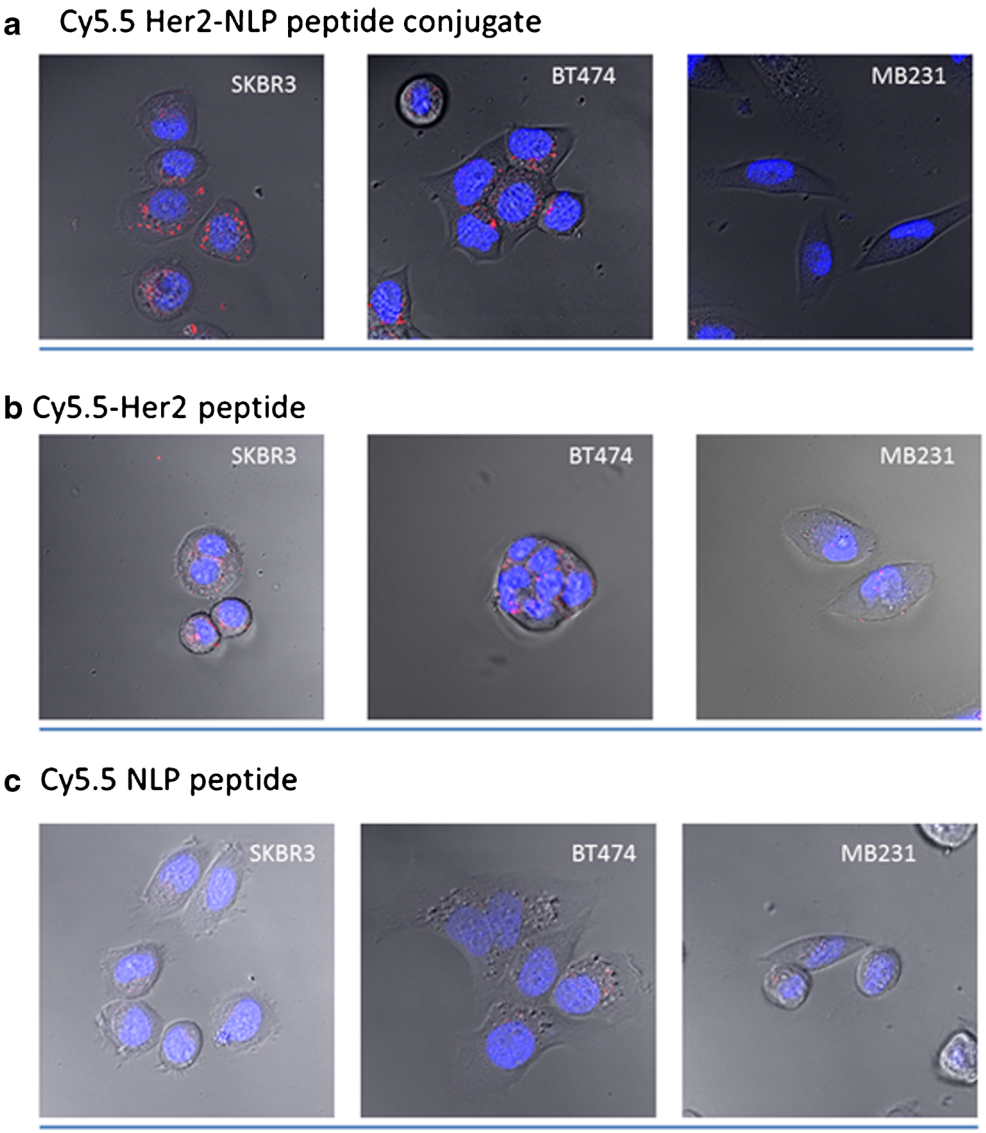

Fig. 2 Fluorescent confocal microscopic imaging of cellular uptake and intracellular localization of Cy5.5-Her2-NLP peptide conjugates in breast cancer cells. Fluorescent signals of Cy5.5-Her2-NLP peptide conjugates were visualized in the cytoplasm of Her2/neu positive breast cancer cells (SKBR3 and BT474), but not in the cytoplasm of Her2/neu negative breast cancer cells (MDA-MB-231). Furthermore, no fluorescent signals of Cy5.5-Her2-NLP peptide conjugates, Cy5.5-Her2 or Cy5.5-NLP peptides were detected in the nuclei of Her2/neu positive or Her2/neu negative breast cancer cells

Her2/neu-specific Cellular Uptake and Localization of Cy5.5-Her2-NLP Peptide Conjugates

Fluorescent signals were detected in cytoplasm of Her2/neu positive SKBR-3 and BT474 breast cancer cells incubated with the Cy5.5-Her2-NLP peptide conjugates, but minimal in cytoplasm of Her2/neu negative MDA-MB-231 breast cancer cells (Fig. 2). This demonstrated Her2/neu specific cellular uptake, and cytoplasm internalization of the Cy5.5-Her2-NLP peptide conjugates within the Her2/neu positive SKBR-3 and BT474 breast cancer cells. The quantity of the fluorescent signals detected in the cytoplasm of SKBR-3 cell was more than the quantity of fluorescent signals detected in the cytoplasm of BT474 breast cancer cells. However, no or little fluorescent signals were detected within the nuclei of Her2/neu positive SKBR-3 and BT474 breast cancer cells, neither within the nuclei of Her2/neu negative MDA-MB231 cells, despite intracellular localization of the Cy5.5-Her2-NLP peptide conjugates in the cytoplasm of the Her2/neu positive SKBR-3 and BT474 breast cancer cells (Fig. 2a). This indicated poor further nuclear localization or failure of trafficking from cytoplasm into the nuclei by the Cy5.5-Her2-NLP peptide conjugates localized within the cytoplasm of Her2/neu positive breast cancer cells. Similarly, fluorescent signals were detected in cytoplasm of Her2/neu positive SKBR-3 and BT474 breast cancer cells incubated with Cy5.5-Her2 peptide, but not in cytoplasm of Her2/neu negative MDA-MB-231 breast cancer cells (Fig. 2b). Little fluorescent signals were detected within the Her2/neu positive (SKBR-3 and BT474) or Her2/neu negative (MDA-MB-231) breast cancer cells incubated with Cy5.5-NLP peptide, indicating poor cellular uptake of Cy5.5-NLP peptide by breast cancer cells without Her2-specific targeting ligand (Fig. 2c).

Discussion

Targeted delivery of radionuclide is a promising therapeutic approach for prevention and early treatment of breast cancer metastasis by eradication of circulating breast cancer cells (CBCC) refractory to conventional systemic therapy. Using nuclear localization sequence peptide (NLP), a peptide conjugate consisting of a peptide ligand targeting Her2/neu oncoprotein and an NLP sequence may be used as a delivery vehicle for targeted delivery of I-125, an Auger-electron emitting therapeutic radionuclide, for radionuclide eradication of Her2/neu positive CBCC. Detection of fluorescent signals in the cytoplasm of Her2/neu positive breast cancer cells (SKBR-3 and BT-474 cells) incubated with the Cy5.5-Her2-NLP peptide conjugates, but not in the cytoplasm of Her2/neu negative breast cancer cells, demonstrated Her2/neu specific binding, cellular uptake, and cytoplasm localization of the Cy5.5-Her2-NLP peptide conjugates. This provided further evidence to support use of Her2-binding peptide (KCCYSL) as a ligand or delivery vehicle for targeted delivery of β^- or α emitting radionuclide for radionuclide cancer therapy.

No fluorescent signals were detected in the nuclei of the Her2/neu positive breast cancer cells incubated with Cy5.5-Her2-NLP peptide conjugates. This may be caused by: (1) dissociation of Cy5.5 dye from Her2-NLP peptide conjugates localized within cytoplasm of breast cancer cells despite nuclear localization of Her2-NLP peptide conjugate or (2) failure or poor nuclear localization of Cy5.5-Her2-NLP peptide conjugates localized within the cytoplasm of breast cancer cells due to loss of NLP's capability to facility nuclear localization after conjugation with Her2 peptide. This awaits further investigation by testing nuclear localization of fluorescent Her2-NLP peptide conjugates with Cy5.5 dye attached to different location of the conjugates. Nuclear localization efficacy of NLP peptide ligand itself is expected to be higher than a conjugate of NLP peptide loaded with other molecules, particularly those of large molecular weight. However, no significant fluorescent signals were detected in the nuclei of the breast cancer cells incubated with Cy5.5-NLP peptide, which might be due to poor cellular uptake of Cy5.5-NLP peptide lacking a high affinity targeting ligand, or dissociation of Cy5.5 from NLP peptide following cellular uptake of Cy5.5-NLP peptide. Additional studies are necessary to validate nuclear localization capability of NLP peptide by assessing nuclear localization of conjugates consisting of NLP ligand and various targeting ligands [10, 11]. Use of a cleavable conjugate with I-125 attached to NLP peptide sequence linked with a targeting ligand is an attractive approach to facilitate nuclear delivery of I-125 radionuclide [12]. Finally, it will be also interesting to explore use of modified NLP peptide with non-classical nuclear localizing sequence (NLS) conjugated with Her2 targeting ligand, or other delivery vehicles for eradication of circulating breast cancer cells using I-125 or other α emitting radionuclide [13, 14].

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Conflict of Interest The authors declare that they have no conflict of interest.

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