Bioorganic & Medicinal Chemistry 17 (2009) 5176–5181

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Toward preparation of antibody-based imaging probe libraries for dual-modality positron emission tomography and fluorescence imaging

Heng Xu [a], Peter K. Eck [b], Kwamena E. Baidoo [a], Peter L. Choyke [b], Martin W. Brechbiel [a,*]

[a] Radioimmune and Inorganic Chemistry Section, Radiation Oncology Branch, National Cancer Institute, National Institutes of Health, Building 10, Room 1B40, 10 Center Drive, Bethesda, MD 20892-1088, USA
[b] Molecular Imaging Program, Center for Cancer Research, National Cancer Institute, National Institutes of Health, 10 Center Drive, Bethesda, MD 20892-1088, USA

ARTICLE INFO

Article history:
Received 18 February 2009
Revised 15 May 2009
Accepted 20 May 2009
Available online 27 May 2009

Keywords:
Molecular imaging
PET
SPECT
Optical imaging
Monoclonal antibody
EGFR
HER2

ABSTRACT

Two novel imaging agents trastuzumab-Cy5.5-CHX-A″ **1** and cetuximab-Cy7-CHX-A″ **2**, bearing both a chelating moiety (CHX-A″) for sequestering metallic radionuclides (^{86}Y or ^{111}In) and the near infrared dye Cy5.5/Cy7, were prepared by a novel modular synthetic strategy as examples of dual-labeled, antibody-based imaging probe library. Fluorescent microscopy illustrated that **1** and **2** strongly bind to HER2-expressing cancer cells (e.g., NIH3T3–HER2^{+}, SKOV-3) and to EGFR-expressing cancer cells (e.g., A431), respectively, thereby demonstrating that the functionality of the targeting moiety is conserved. Hence, the described novel synthesis strategy can be applied to engineer other tumor-targeted monoclonal antibody based probes for multimodality imaging.

Published by Elsevier Ltd.

1. Introduction

Over the past decade, multimodality imaging has revolutionized medical imaging especially for cancer diagnostics.[1] Multimodality imaging usually incorporates two or more imaging modalities selected from such technologies as positron emission tomography (PET), single photon emission computed tomography (SPECT), computed tomography (CT), magnetic resonance imaging (MRI), or optical imaging (OI), all within the setting of a single examination. The combination of technologies collects information from different imaging modalities and thus allows for the strengths of each modality to be combined in a single imaging session to improve overall diagnostic accuracy. Development of imaging probes for multimodality imaging has thus received great attention.[2–5] However, preparation of these agents is frequently more challenging than that of single modality agents. Careful selection of nuclear, optical and MR tracers is required to avoid the chemical–physical conflicts or interference along with a more complex synthesis to integrate selected components into a single agent.[6]

The breakthrough technology in production of monoclonal antibodies (mAbs) by Kohler and Milstein in 1975 initiated extensive research into mAb based cancer imaging and therapies.[7] Monoclonal antibodies feature highly specific association with targeted antigens and have proven to be excellent targeting vectors to deliver a variety of 'payloads' such as radionuclides, toxins, imaging dyes, or enzymes to cancer cells.[8] Radiolabeled mAbs bearing either α- or β-emitting particles have been reported to have significant therapeutic efficacy of killing tumor cells,[9] and the FDA has recently approved clinical use of two anti-CD20 mAb regimens involving radionuclides for the treatment of NHL (^{90}Y ibritumomab and ^{131}I tositumomab).[10,11] Concurrently, mAbs labeled with suitable isotopes (e.g., ^{124}I, ^{18}F, ^{64}Cu, ^{86}Y for PET and ^{99m}Tc, ^{111}In for SPECT) have been used for PET or SPECT imaging to visualize specific biological processes at the cellular and molecular level.[12,13] Complementary, fluorescence-labeled mAbs have also been studied for tumor localization for more than a decade.[14] Coupling dyes with emissions suitable for imaging applications to mAbs directed against tumor associated antigen offers great sensitivity for detection of cancerous tissues. In particular, the commercial availability of near infrared (NIR) dyes promotes use of these dye-antibody conjugates as targeted NIR optical imaging probes for diagnostic imaging of tumors due to several unique advantages: high signal-background ratio; dynamic, real-time in vivo images; and potential imaging of a variety of molecular features.

While both radiolabeled and fluorescence labeled antibodies have made significant, independent advances in cancer imaging and/or therapy, we have been interested in exploring opportunities to integrate these two imaging strategies into a single antibody.

* Corresponding author. Fax: +1 301 402 1923.
E-mail address: martinwb@mail.nih.gov (M.W. Brechbiel).

0968-0896/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.bmc.2009.05.048

This fuses all the advantages and features of SPECT/PET with NIR optical imaging. Dual modality imaging, combining PET/SPECT with optical, will provide an enhanced level of detection sensitivity, and resolution of targets in tissues, as well as to potentially provide an intraoperative surgical aide that is unavailable by PET/SPECT alone. We recently reported a dual-modality trastuzumab based imaging probe, which bears both a chelating moiety (CHX-A″) for sequestering metallic radionuclides (^{86}Y or ^{111}In) and the near infrared dye Cy5.5 for dual modality PET (or SPECT) and fluorescence imaging, respectively.[6] In this paper, we further demonstrate our novel synthetic strategy to generally prepare dual technology antibody constructs using trastuzumab and cetuximab as examples (Fig. 1). The synthetic approach has the potential to allow the preparation of dual-labeled antibody-based imaging probe libraries. We have evaluated the binding capability of the modified antibodies to cancer cells by fluorescent microscopy. This in conjunction with our previously reported bioactivity of the radiolabeled trastuzumab analog demonstrate the dual modality nature of these probes.[6]

The monoclonal antibodies cetuximab and trastuzumab, which bind to the epidermal growth factor receptors EGFR and HER-2, respectively, were chosen because they are already established targeting vectors to deliver radioactive isotopes and fluorescent dyes separately.[15–26] EGFR and HER2 are expressed/over-expressed in a variety of cancer cell lines. The spatial distribution of the receptors has been demonstrated in pre-clinical molecular imaging of cancer. The squamous carcinoma cell line A431, with high EGFR expression, is widely used to study binding of the native EGFR substrate EGF and other targeting vectors like cetuximab or affibodies.[24] Many HER2 over-expressing cell lines have also been characterized such as the ovarian cancer cell line SKOV-3. In addition, the genetically engineered HER2 over-expressing murine fibroblast-like cell line NIH3T3–HER2$^+$ has been very useful as a model system in HER2 imaging.[25,26]

Our laboratory has a long-standing experience with radiolabeled antibodies for targeted radiation therapy. The creation of dual imaging probes would provide the means to monitor not only response to therapy in a broad sense, but also receptor behavior. The NIR dyes Cy5.5 and Cy7 were selected because they fluoresce at ~694 nm and ~776 nm in the NIR region, respectively, and are also far enough apart to be reasonably distinguishable in fluorescence based studies. While either trastuzumab-Cy5.5-CHX-A″ or cetuximab-Cy7-CHX-A″ could function as a targeted dual imaging probe, the combined use of these two antibodies provides unique opportunities to obtain fluorescent images while targeting and thus mapping different receptors on tumor cells.

2. Experimental

2.1. General methods

Proton NMR data were obtained using a Varian Gemini 300 MHz instrument and chemical shifts are reported in ppm on the δ scale relative to TMS, TSP, or residual solvent. Low and high resolution mass spectra (HRMS) were obtained on a Waters' LCT Premier time-of-flight mass spectrometer using electrospray ionization (ESI/TOF/MS) in positive ion mode operated at a resolution of 10,000. The electrospray capillary voltage was 3 kV and the sample cone voltage was 60 V. Desolvation temperature was 225 °C and the desolvation gas was nitrogen at 300 L/h. Accurate masses were obtained using the lock spray mode with Leu-Enkephalin as the external reference compound.

Trifunctional chelates **4** and **5** were purified by reversed-phase HPLC (RP-HPLC) using a Gilson system consisting of two Model 303 pumps, a Model 803 Manometric Module and a Model 811B solvent mixer monitored on line with a Knauer variable wavelength monitor for uv and an IN/US γ-RAM radioactivity detector and controlled by Gilson Unipoint System Software. RP-HPLC was performed on a Vydac 5 μm C_{18} reversed-phase 10 mm × 25 cm column equilibrated with 15 mM NH_4OAc (pH 7). A gradient of CH_3CN that increased from 0% at 0 min to 50% at 30 min was employed.

Size-exclusion HPLC (SE-HPLC) was performed using the same system in a single pump mode using a Tosohaas G3000SW, 10 μm, 7.8 mm × 30 cm column with phosphate buffered saline solution as the eluent (0.5 mL/min).

2.1.1. Materials

Solvents and reagents were used as purchased. Cy7 mono-NHS ester was purchased from GE Healthcare (Piscataway, NJ). Lysine derivative **3** and trastuzumab-Cy5.5-CHX-A″ were prepared as previously reported.[6] Cetuximab (Erbitux; Bristol-Myers Squibb, Princeton, NJ) was obtained from the Veterinary Resources Program Pharmacy at NIH. All experiments with moisture- and/or air-sensitive compounds were carried out under a dry Ar atmosphere. All H_2O was purified using a Hydro Ultrapure Water Purification system.

2.1.2. Cy7-Lys(SMCC)-CHX-A″ (5)

To a solution of amine **3** (3.0 mg, 3.34 μmol) in DMSO (3 mL) was added Cy7 mono-NHS ester (2.50 mg, 3.06 μmol) and Et_3N (10 μL). The reaction mixture was stirred at room temperature for 18 h and then diluted with Et_2O (40 mL). The precipitated product was purified by reverse-phase (RP) semi-preparative HPLC on a

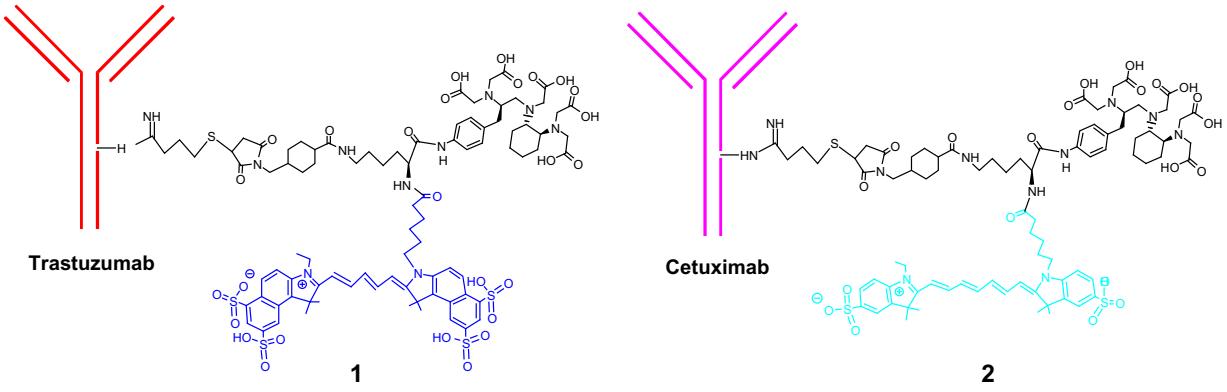

Figure 1. A schematic presentation of mAb conjugates trastuzumab-Cy5.5-CHX-A″ **1** and cetuximab-Cy7-CHX-A″ **2**.

C_{18} column using a 0–50% CH_3CN to 15 mM NH_4OAc gradient over 30 min followed by lyophilization of the solvent from the relevant fraction to yield trifunctional chelate **5** (1.50 mg, 31%). RP-HPLC: t_R = 21.5 min; ES-MS *m/z*: calcd for $C_{78}H_{100}N_9O_{21}S_2$ $[M-H]^-$, $[M-2H]^{2-}$: 1562.7, 780.9 found 1562.4, 780.7.

2.1.3. Conjugation of 5 to cetuximab (general procedure)

Cetuximab (~10 mg) was reacted with Traut's reagent[27] (Sigma Chemical Co., St. Louis, MO) at a 1:15 molar ratio for 1.5 h at room temperature in 1 mL of PBS plus 10 mM EDTA buffer. Excess Traut's reagent was removed by passage of the reaction solution through a PD-10 column eluted with PBS plus 10 mM EDTA buffer. The number of thiols introduced was determined using Ellman's reagent.[28] Just prior to protein conjugation, **5** was dissolved in the same buffer and then added drop wise to the mAb solution to achieve a molar reaction ratio of 10:1 (**5**: Cetuximab) and gently vortexed. The solution was then gently agitated in the dark at 25 °C for 1.5 h. Excess free unreacted SH groups were capped by the addition of iodoacetamide solution (2.0 mM). Finally, the reaction mixture was dialyzed into PBS buffer at 4 °C with 4 buffer changes (4 L total) over 48 h. Protein concentration was determined by Lowry assay and the number of dye (and chelate) molecules per antibody was calculated based on Cy 7 dye UV absorption at 747 nm.

2.1.4. Radiolabeling

Trastuzumab-Cy5.5-CHX-A″ and cetuximab-Cy7-CHX-A″ were each labeled with ^{111}In as follows. A solution of ^{111}InCl in HCl (0.05 M, 1–3 μL, 0.5–1 mCi) was added to either antibody conjugate (50 μg) contained in NH_4OAc buffer (100 μL, 0.15 M, pH 7), vortexed immediately and incubated at room temperature for 30 min. The reaction was quenched by the addition of EDTA solution (0.1 M, 4 μL). The ^{111}In labeled antibodies were purified by gel filtration chromatography using PD-10 columns (GE Healthcare) eluted with PBS. Radiolabeling yields as determined by the PD-10 separation ranged from 75% to 94%. The purity of the PD-10 purified product was ascertained by SE-HPLC as described above.

2.1.5. Radioimmunoassays

The immunoreactivity of the cetuximab-Cy7-CHX-A″ conjugate was evaluated in a competition radioimmunoassay. Fifty ng of EGFR (Sigma–Aldrich, St. Louis, MO) in 50 μL of PBS containing Mg^{2+} and Ca^{2+} was added to each well of a 96-well plate. Following an overnight incubation at 4 °C, wells were aspirated and 150 μL of PBS/BSA added to each well and allowed to sit for an additional hour at ambient temperature. The wells were aspirated and serial dilutions (0.01–500 ng in 50 μL BSA/PBS) of unmodified cetuximab or cetuximab-Cy7-CHX-A″ conjugate were added to the wells in triplicate (one set of wells received BSA/PBS without any competitor), along with ^{125}I-cetuximab (~50,000 cpm in 50 μL BSA/PBS). The wells were aspirated after 4 h incubation at 37 °C and washed three times with BSA/PBS. The bound radioactivity was removed with 100 μL 0.2 M NaOH, adsorbed to cotton filters, placed in 12 × 75 mm tubes and counted in a γ-scintillation counter (Wizard One, PerkinElmer, Shelton, CT). The percent inhibition was calculated using the buffer control and plotted. HuM195, a mAb that reacts with human CD33, served as a negative control.

2.1.6. Cell culture and treatment

A431, SKOV3, and NIH3T3 cells were obtained from ATCC (Manasass, VA). The NIH3T3 cells were transfected with HER2 genes (3T3/HER^{2+}), in order to over-express HER2 receptors.[22] All of the cell lines were grown at 37 °C in the presence of 5% CO_2 in DMEM medium supplemented with 1% L-glutamine, and 5% FBS. For the confocal fluorescence microscopy experiments cells were plated in Lab-Tek chambered slides (#178599 Nalge Nunc) at an appropriate dilution and allowed to attach overnight at 37 °C prior to treatment.

To demonstrate the binding of trastuzumab-Cy5.5-CHX-A″ (**1**) and cetuximab-Cy7-CHX-A″ (**2**) by fluorescent microscopy both monoclonal antibodies were used at a concentration of 10 μg/mL in culture medium. The individual cell lines seeded on chambered slides with 60–80% confluency were incubated for 2 h at 37 °C in the dark. After incubation the cell culture medium was removed and slides were washed three times with PBS (pH 7.4) and immediately fixed in 4% paraformaldehyde prepared in PBS (pH 7.4) for 5 min at room temperature. Fluorescent mounting medium (S3023, DakoCytomation) was added at the time the slide was covered with a cover glass to stabilize the fluorophores before and during confocal microscopy.

2.1.7. Confocal fluorescence imaging

For confocal images, cells were examined with a Zeiss LSM 510 confocal system (Carl Zeiss Inc., Thornwood, NY, USA) with an Axiovert 100 M inverted microscope operating with a 5 mW HeNe laser tuned to 633 nm. Fluorescence (Cy5.5 or Cy7) and DIC images were collected simultaneously using a 63× Plan-Apochromat 1.4 NA oil immersion objective and a long pass 650 nm emission filter. In the acquisitions for all channels the Zeiss AIM software version 3.2 sp2 (Carl Zeiss GmbH, Heidelberg, Germany) was used. All confocal images were acquired with a frame size of 512 by 512 with identical settings.

3. Results and discussion

Synthesis of lysine derivative **3**, bearing the radiometal chelate CHX-A″ and the thiol-reactive maleimide moiety attached to the α-COOH and ε-NH_2 of lysine functional groups, respectively, was recognized as a versatile intermediate that could serve as a nexus for the modular introduction of fluorescent dyes or other agents. NIR dyes Cy5.5 and Cy7 were then selected to demonstrate synthetic feasibility. Cy 5.5 derivative **4** and its antibody conjugate **1** were recently developed in our laboratory.[6] The synthetic approach to **4** was applied to the synthesis of the corresponding Cy 7 conjugates **5** and **2** using similar procedures with minor modifications. In brief, Cy 7 mono-NHS ester was reacted with the α-NH_2 of **5** in DMSO. The product was precipitated from diethyl ether and then purified by reverse-phase HPLC using a C18 column to isolate **5** in ~31% yield (Fig. 2). To conjugate **5** to cetuximab, the mAb was first thiolated with 15 equiv of Traut's agent using standard procedures.[27] In our hands, ~2–4 –SH groups per mAb were introduced as quantitated by Ellman's reagent.[28] Thiolated mAb was then reacted with 10 equiv of **5** at rt for 1.5 h to produce antibody conjugate **2**. Unreacted remaining thiols were capped with iodoacetamide to minimize cross-linking of the antibody conjugate products. Finally, the reaction mixture was dialyzed into phosphate-buffered saline (PBS; pH 7.2) at 4 °C with four buffer changes over 48 h. The number of Cy7 dye moieties per cetuximab was calculated to be ~2 based on UV absorption at 747 nm, which directly corresponds to the same number of CHX-A″ chelates on cetuximab according to the 1:1 ratio of Cy7 and CHX-A″ within **5**.

Both trastuzumab-Cy5.5-CHX-A″ and cetuximab-Cy7-CHX-A″ radiolabeled efficiently (~75–94%) with the SPECT radionuclide ^{111}In within 30 min at room temperature. HPLC profiles of both **1** and **2** reveal single symmetric peaks of near identical retention time (see Supplementary data).

Radioimmunoassay of the cetuximab-Cy7-CHXA″ conjugate showed that conjugation of **5** to cetuximab resulted in comparable binding to the EGFR receptor (Fig. 3). Fifty percent inhibition was achieved with 1.7 nmol cetuximab-Cy7-CHXA″ versus 1 nmol for the native cetuximab. The fluorescent labeling of HER2 expressing

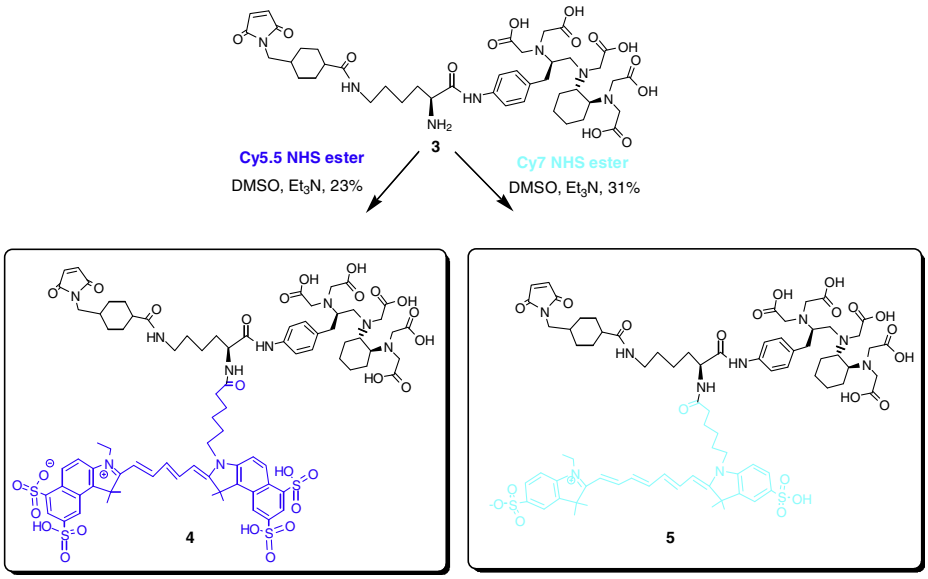

Figure 2. Synthesis of trifunctional chelate **4** and **5**.

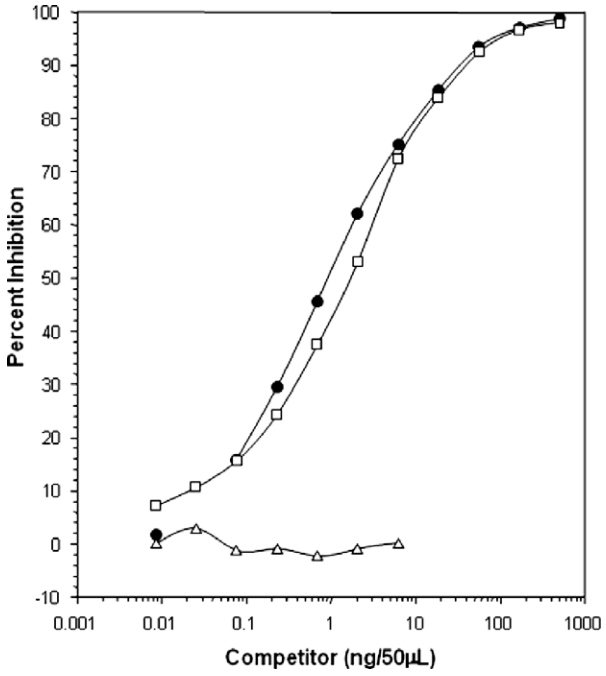

Figure 3. Analysis of cetuximab-Cy7-CHX-A″ immunoreactivity in a competition radioimmunoassay. The immunoreactivity of cetuximab-Cy7-CHX-A (□) for purified EGFR was compared to unmodified cetuximab (●). The anti-CD33 mAb, HuM195 (Δ) was used as a negative control.

cells as determined by confocal microscopy studies with trastuzumab-Cy5.5-CHX-A″ (**1**) is demonstrated in Figure 4, panels A and B. The images in panel A clearly show excellent binding capability of **1** to genetically engineered cells (NIH3T3–HER2^{+}) which over-express human HER2 receptors,[22] indicating that immunoreactivity of the trastuzumab component in **1** is well preserved after modification. Further, images in panel B illustrate that **1** strongly binds to intrinsically HER2 over-expressing human SKOV-3 cells as well. Panel C shows no or minimal binding when LS174T cells, known to marginally express HER2 receptors in vitro,[29] were incubated with **1**. In conclusion, trastuzumab-Cy5.5-CHX-A″ (**1**) binds HER2 expressing cells and fluorescently labels the cell membrane of both cells genetically engineered to express human HER2 as well as intrinsically HER2 over-expressing SKOV3 cells. This result is comparable to that reported for cell membrane binding of simple fluorescently labeled trastuzumab.[15–21] The images here confirm the role of HER2 as a cell surface–specific target and that the functionality of **1** in labeling HER2 over-expressing cancer cells was conserved.

The trifunctional agent, cetuximab-Cy7-CHX-A″ (**2**) was synthesized similarly to **1**. The monoclonal antibody cetuximab targets the human epidermal growth factor receptor EGFR.[23–26] In many cancers EGFR is over-expressed and up-regulated and therefore serves as another cell surface marker. The red cetuximab-Cy7-CHX-A″ (**2**) fluorescence specifically labels EGFR-expressing A431 cells as seen in panel D as compared to panel E where no cetuximab-Cy7-CHX-A″ was added (Fig. 4). The observed fluorescence was mainly membrane associated, but some initial internalization is also visible. In comparison, the cetuximab-Cy7-CHX-A″ labeled A431 cells presents the same labeling pattern as seen by others using confocal microscopy with only fluorescent labeled cetuximab.[23,24] These experiments confirm full functionality of the newly synthesized agent cetuximab-Cy7-CHX-A″ with regards to specific binding and labeling of living EGFR over-expressing cancer cells.

Overall, excellent specificity and functionality for the newly engineered and synthesized agents trastuzumab-Cy5.5-CHX-A″ and cetuximab-Cy7-CHX-A″ were demonstrated in regard to antigen recognition and binding in cell culture. The specific target binding of the monoclonal antibody in both constructs is conserved and they exhibit excellent fluorescence to characterize spatial resolution in confocal microscopy. Hence, the conjugated Cy-Dye-CHX-A″ component does not interfere with the specificity and binding properties of the antibody.

4. Conclusions

In summary, we demonstrate here a modular strategy exemplified by the preparation of trastuzumab-Cy5.5-CHX-A″ and cetuximab-Cy7-CHX-A″ as examples of a dual-modality antibody-based imaging probe library. The examples prepared here showed strong binding capabilities of the resulting conjugates to cancer cells by fluorescent microscopy. Through this strategy, other tumor

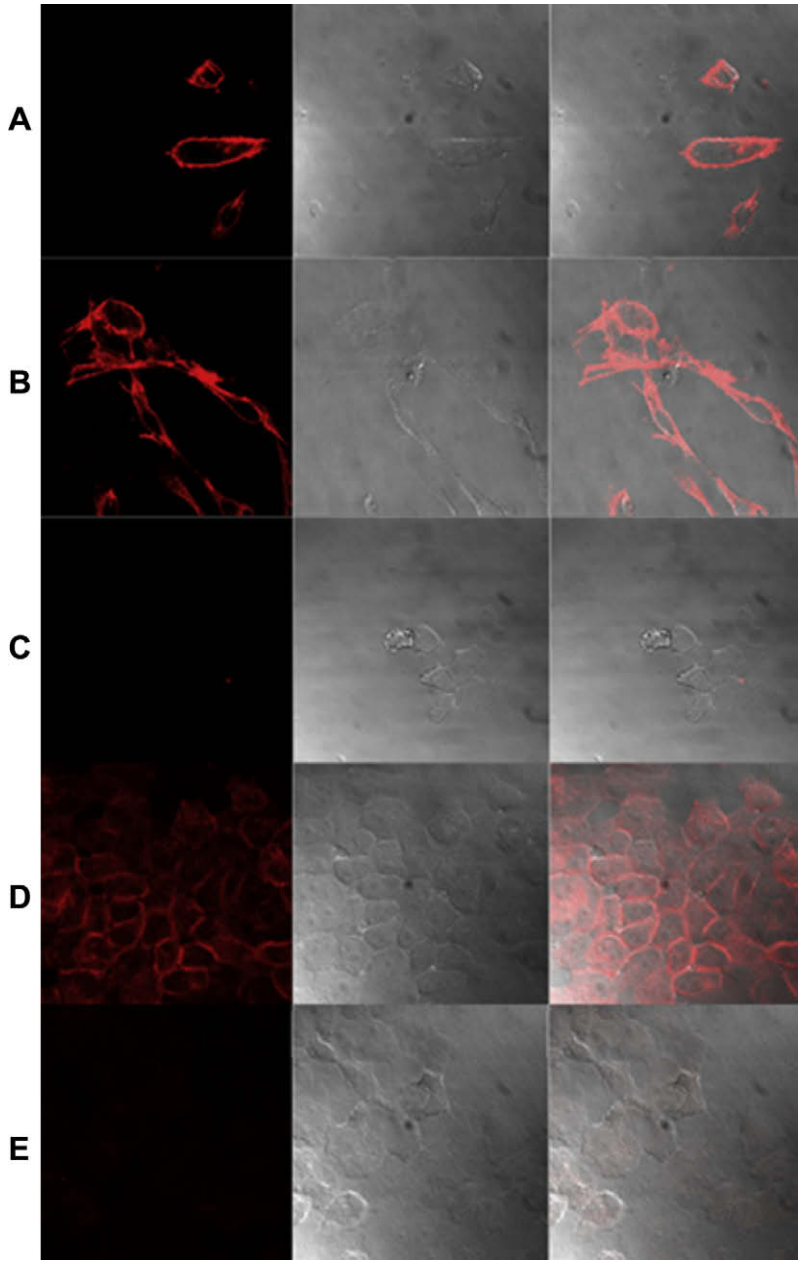

Figure 4. Confocal microscopy demonstrating specific binding of trastuzumab-Cy5.5-CHX-A″ and cetuximab-Cy7-CHX-A″ in cells: Confocal laser scanning microscopy images of cells exposed to trastuzumab-Cy5.5-CHX-A″ (panels A–C) and cetuximab-Cy7-CHX-A″ (panel D) for 1 h at 37 °C. The confocal microscopy images demonstrate bright probe fluorescence localized to the cell membrane for both probes. Genetically engineered HER2 over-expressing (NIH3T3–HER2$^+$) cells and intrinsically HER2 expressing SKOV3 cells show the trastuzumab-Cy5.5-CHX-A″ signal (panels A and B). LS174T cells marginally expressing the receptors of interests did not label (C). EGFR expressing A431 cells demonstrate binding of cetuximab-Cy7-CHX-A″ (D) compared with cells not incubated with cetuximab-Cy7-CHX-A″ (E).

targeting vectors (e.g., antibodies, affibodies, and peptides) can also be modified with both a radiolabeled chelating agent and near-infrared dye using efficient thiol-maleimide chemistry for multimodality imaging of cancers. Therefore, our synthetic approach opens an extensive range of possibilities to prepare tumor-targeted dual modality imaging probe libraries. Ongoing studies are evaluating these agents in both subcutaneous and intraperitoneal xenograft tumor targeting model systems.

Acknowledgment

This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

Supplementary data

Supplementary data (mass spectrum and RP-HPLC of **5** and SE-HPLCs of both **1** and **2** radiolabeled with ^{111}In) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.05.048.

References and notes

1. Moseley, M.; Donnan, G. *Stroke* **2004**, *35*, 2632.
2. Hüber, M. M.; Staubli, A. B.; Kustedjo, K.; Gray, M. H. B.; Shih, J.; Fraser, S. E.; Jacobs, R. E.; Meade, T. J. *Bioconjugate Chem.* **1998**, *9*, 242.
3. Dirksen, A.; Langereis, S.; Waal, B. F. M. d.; Genderen, M. H. P. v.; Meijier, E. W.; Lussanet, Q. G. d.; Hackeng, T. M. *Org. Lett.* **2004**, *6*, 4857.
4. Manning, H. C.; Goebel, T.; Marx, J. N.; Bornhop, D. J. *Org. Lett.* **2002**, *4*, 1075.

5. Talanov, V. S.; Regino, C. A. S.; Kobayashi, H.; Bernardo, M.; Choyke, P. L.; Brechbiel, M. W. *Nano Lett.* **2006**, *6*, 1459.
6. Xu, H.; Baidoo, K.; Gunn, A. J.; Boswell, C. A.; Milenic, D. E.; Choyke, P. L.; Brechbiel, M. W. *J. Med. Chem.* **2007**, *50*, 4759.
7. Kohler, G.; Milstein, C. *Nature* **1975**, *256*, 496.
8. Wu, A. M.; Senter, P. D. *Nat. Biotechnol.* **2005**, 1137.
9. Milenic, D. E.; Brady, E. D.; Brechbiel, M. W. *Nat. Rev. Drug Discov.* **2004**, *3*, 488.
10. Wahl, R. L. *J. Nucl. Med.* **2005**, *46*, 128s.
11. Borghaei, H.; Schilder, R. J. *Semin. Nucl. Med.* **2004**, *34*, 4.
12. *Cancer Imaging with Radiolabeled Antibodies*; Goldenberg, D. M., Ed.; Kluwer Academic: Norwell, MA, 1990.
13. van Dongen, G. A. M. S.; Visser, G. W. M.; Lub-de Hooge, M. N.; de Vries, E. G.; Perk, L. R. *The Oncologists* **2007**, *12*, 1397.
14. Ballou, B.; Fisher, G. W.; Hakala, T. R.; Farkas, D. L. *Biotechnol. Prog.* **1997**, *13*, 649.
15. Fazekas, Z.; Petrás, M.; Fábián, A.; Pályi-Krekk, Z.; Nagy, P.; Damjanovich, S.; Vereb, G.; Szöllosi, J. *Cytometry A* **2008**, *73*, 209.
16. Gee, M. S.; Upadhyay, R.; Bergquist, H.; Weissleder, R.; Josephson, L.; Mahmood, U. *Int. J. Cancer* **2007**, *121*, 2492.
17. Costantini, D. L.; Chan, C.; Cai, Z.; Vallis, K. A.; Reilly, R. M. *J. Nucl. Med.* **2007**, *48*, 1357.
18. Yang, T.; Choi, M. K.; Cui, F. D.; Kim, J. S.; Chung, S. J.; Shim, C. K.; Kim, D. D. *Control Release* **2007**, *120*, 169.
19. Tada, H.; Higuchi, H.; Wanatabe, T. M.; Ohuchi, N. *Cancer Res.* **2007**, *67*, 1138.
20. Belimezi, M. M.; Papanastassiou, D.; Merkouri, E.; Baxevanis, C. N.; Mamalaki, A. *Cancer Immunol Immunother.* **2006**, *55*, 1091.
21. Steffen, A. C.; Wikman, M.; Tolmachev, V.; Adams, G. P.; Nilsson, F. Y.; Ståhl, S.; Carlsson, J. *Cancer Biother. Radiopharm.* **2005**, *20*, 239.
22. Di Fiore, P. P.; Pierce, J. H.; Kraus, M. H.; Segatto, O.; King, C. R.; Aaronson, S. A. *Science* **1987**, *237*, 178.
23. Lin, Y.; Liu, Z.; Jiang, J.; Jiang, Z.; Ji, Y.; Sun, B. *Cell Mol. Immunol.* **2004**, *1*, 137.
24. Nordberg, E.; Friedman, M.; Göstring, L.; Adams, G. P.; Brismar, H.; Nilsson, F. Y.; Ståhl, S.; Glimelius, B.; Carlsson, J. *Nucl. Med. Biol.* **2007**, *34*, 609.
25. Koyama, Y.; Barrett, T.; Hama, Y.; Ravizzini, G.; Choyke, P. L.; Kobayashi, H. *Neoplasia* **2007**, *12*, 1021.
26. Barrett, T.; Koyama, Y.; Hama, Y.; Ravizzini, G.; Shin, I. S.; Jang, B. S.; Paik, C. H.; Urano, Y.; Choyke, P. L.; Kobayashi, H. *Clin. Cancer Res.* **2007**, *13*, 6639.
27. Traut, R. R.; Bolen, A.; Sun, T. T.; Hershey, J. W. B.; Sundberg, J.; Pierce, L. R. *Biochemistry* **1973**, *12*, 3266.
28. Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.
29. Milenic, D. E.; Garmestani, K.; Brady, E. D.; Albert, P. S.; Ma, D.; Abdulla, A.; Brechbiel, M. W. *Cancer Biother. Radiopharm.* **2005**, *20*, 557.