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A zwitterionic near-infrared dye linked TrkC targeting agent for imaging metastatic breast cancer†

Zhen Yang,^{‡a} Syed Muhammad Usama,^{‡b} Feng Li,^a
Kevin Burgess^{‡b} and Zheng Li^{‡a}

Much effort has been devoted to targeting agents for imaging and chemotherapy of tumors in cancer research, but there remain significant unmet needs in that area. We have reported a series of preclinical TrkC targeting agents for diagnoses and treatment of metastatic breast cancer; however, with respect to optical imaging, there are limitations to the agents already disclosed. In this work, a TrkC targeting fragment was equipped with a zwitterionic cyanine dye to give a near-infrared probe for *in vivo* imaging of metastatic breast tumors, with excitation and emission wavelengths of 760 and 780 nm, respectively, and facilitate the aqueous dissolution of the final design. To our delight, the newly developed probe maintained the specific targeting to TrkC⁺ 4T1 metastatic breast tumor cells as well as the TrkC⁺ metastatic breast tumor tissue. Upon injection into 4T1-tumor-bearing mice, the tumor was visualized *in vivo* and *ex vivo* through the fluorescence signal of the probe. These data seem to encourage further investigations toward developing a TrkC targeting agent for the diagnosis of metastatic breast tumors.

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Introduction

Development of imaging probes that selectively associate with metastatic tumor cells enhances useful diagnostic tools for the therapeutic assessment of cancer treatment.^{1,2} For breast cancer, there is an emerging demand for imaging probes that are capable for the recognition of *metastatic*, not just normal, breast tumor cells.^{3,4} Tropomyosin kinase receptor C (TrkC) has been identified as a characteristic regulator of breast cancer cell growth and metastasis and tends to be overexpressed in metastatic breast tumor cells.^{5,6} Consequently, it is a potential target for the active targeting of metastatic breast cancer.

Most often, monoclonal antibodies (mAbs) are used as targeting agents.^{7,8} Small-molecule alternatives, however, have advantages with respect to cost, reagent stabilities, immunogenic effects, circulation times in the course of patient imaging, and, most importantly, superior perme-

ation into solid tumors.^{1,9} Consequently, the focus of our research is on small molecules for active targeting of TrkC.

Targeting TrkC with small molecules has featured a bivalent targeting fragment (IY-IY, blue color throughout diagrams in this paper).^{10–12} In this work, that fragment, “IY-IY”, was covalently linked to a zwitterionic cyanine dye (purple color throughout) to give a near-infrared probe for TrkC targeted imaging. The polar, zwitterionic characteristics of the dye were anticipated to foster aqueous dissolution for *in vivo* administration and simultaneously diminish net-charges that might otherwise induce non-specific binding.¹³ Since the intrinsic structure of the IY-IY fragment was preserved, we hypothesized that the final assembly of the probe would maintain its characteristics of TrkC targeting.

Results and discussion

Probe design

Motivation for these studies came from positive results obtained from a study of the photodynamic therapy (PDT) agent A in mice impregnated with 4T1 mouse breast tumors.¹⁴ In that work, one single 10 mg kg^{−1} dose followed by illumination for 10 min caused near complete regression of the primary tumor over 28 days and inhibited metastatic spread relative to untreated control animals.

^a Center for Bioenergetics, Houston Methodist Research Institute, Houston, TX 77030, USA. E-mail: zli@houstonmethodist.org

^b Department of Chemistry, Texas A & M University, Box 30012, College Station, TX 77842, USA. E-mail: burgess@tamu.edu

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‡ Both authors contributed equally to this work.

The IY-IY targeting group has been made many times previously in these labs, but for the current study an improved procedure was developed to facilitate synthesis on a larger scale. Details of the modified route are given in the ESI,[†] and the concept is outlined in Scheme 1. The last step involved a coupling of the targeting fragment C with the activated cyanine part D.²⁵ The innovation in the modification was in the construction of C. Previously, IY-piperazine fragments had

Scheme 1 Retrosynthetic route to probe 1 illustrating an improved route to the IY-IY fragment.

been added to the triazine, but that step did not scale well. Instead, the current route involves an S_NAr reaction of a tyrosine-derived azide, then a click reaction to build up the IY-IY fragment in layers.

The following electronic spectral data summarize the key points from the full analysis shown in the ESI.† Probe 1 had an extinction coefficient of $140\,000\text{ L mol}^{-1}\text{ cm}^{-1}$ and a quantum yield of 5.6% (in 7.4 PBS buffer); these data are typical of the strong absorption from the cyanine fragment and indicate excellent overall brightness. Maximal absorption and emission were observed at 760 and 780 nm (in 7.4 PBS buffer), ideal for optical imaging.

Compound 2 is an isomer of 1 with the targeting amino acids inverted (YI-YI); this was a useful control in the cellular studies that are now described.

Probe behavior in live cell and tissue studies

Probe 1 was first tested for binding cells stably transfected with TrkC; the data obtained (Fig. 1a, horizontal row) show that the cells did, in fact, bind the probe, whereas the parent NIH3T3 cells that do not express this receptor, did not (Fig. 1b). Control experiments (Fig. S1–S3†), demonstrated that the control probe 2 did not stain TrkC⁺ or wild type (TrkC[−]) NIH3T3 cells, and neither did the parent cyanine dye without the targeting group. Confocal studies at a higher magnification revealed that 1 tends to localize in the lysosomes (Fig. 1c and d), just as natural neurotrophins do when they are imported *via* the TrkC receptor.²⁶ In the histology studies with commercial samples from breast cancer patients, the metastatic breast cancer tissue was stained but not the normal tissue (Fig. 1e and f).

In vivo imaging with probe 1

Mouse 4T1 breast tumor is a well-established animal model for the study of human metastatic breast cancer.²⁷ In this study, murine 4T1 tumor cells were subcutaneously injected into BALB/c nu/nu mice to create 8–10 mm diameter metastatic breast tumor xenografts. After intravenous injection of the imaging probe, NIR fluorescence images of the tumor-bearing mice were captured at various time points after injection of 1 (Fig. 2). The 4T1 tumors were clearly visualized through the fluorescence differences displayed from the tumor to muscle background as early as 15 min post injection of the targeting agent. Moreover, the agent 1 rapidly cleared from the body; fluorescence intensities of the tumor dropped from 15 min to 1 h, and further at 3 h, and almost to background at 24 h.

Fig. 1 a: Probe 1 binds to NIH3T3 TrkC⁺ cells, but not to NIH3T3 WT cells (b). Confocal experiments revealed that 1 colocalizes c: with the LysoTracker green, and d: not with the MitoTracker green. Histology studies of 1 with e: metastatic breast cancer tissue (invasive ductal carcinoma), and f: normal breast tissue (adjacent normal breast tissue). Throughout the blue channel, the nucleus is detected by nuclear staining with Nuc Blue.

Fig. 2 a: *In vivo* NIR fluorescence images of 4T1 tumor-bearing mice at 15 min, 1 h, 3 h, and 24 h post-injection of the targeting agent. White arrows indicate the tumors, and the circles mark the ROI study of the tumor for the quantification analysis in b. b: Quantification of fluorescence in the tumors. Data shown represent mean $\pm$ SD ($n = 3$ per group).

Fig. 3 a: Representative *ex vivo* fluorescence images of: 1 heart, 2 lungs, 3 liver, 4 kidney, 5 spleen, 6 gastrointestinal, 7 muscle, 8 tumor, 9 brain, and 10 spinal cord, collected from mice at 15 min, 30 min, 3 h, 6 h and 24 h post-injection of the targeting agent. White arrows mark the tumors. b: Quantification of fluorescence in the tumors. Data shown represent mean $\pm$ SD ($n = 3$ per group).

Compared to TrkC targeting agent B, the retention time of 1 was much shorter, presumably because of the more hydrophilic nature of the zwitterionic dye.

To further validate the tumor uptake of the targeting agent, the internal organs of the imaged mice were collected for *ex vivo* imaging. The relatively higher fluorescence intensities in tumors, compared to the muscle background, indicated the selective uptake (Fig. 3a). Trk receptors play important roles in the mammalian nervous system, and tend to exhibit high expression of the TrkC receptor;^{28,29} however, we did not observe uptake in the mice brain and spinal cord, and hence it appears that the size and charge of the probe suppress its penetration into the central nervous system. A kinetic curve of the tumor uptake extracted from the *ex vivo* imaging data corresponded to that from the *in vivo* imaging (Fig. 3b). The uptake of the targeting agent in the tumor reached its peak half an hour after the injection, then, clearance from the body dominated.

Probe 1 localized in the TrkC expressing tumors, as anticipated, providing a way to stain them with NIR fluorescence.

In retrospect, the observation that the probe was washed out of the system relatively rapidly can be attributed to the polarity of the cyanine dye. However, the observation of the rapid washout from tumors *in vivo* surprised us, for two reasons. First, the cell imaging data in this paper prove that probe 1 is selectively imported into the TrkC⁺ cells. Secondly, at least some members of the parent dye system E (particularly where R¹ and R² contain carboxylate or sulfonate functionalities) tend to localize in many types of solid tumors.^{2,30–32} Further experiments are in progress to try to understand these apparent inconsistencies.

Conclusions

In this work, an imaging probe that incorporated with the TrkC targeting agent and zwitterionic NIR dye was reported for imaging metastatic breast cancer. *In vitro* and *in vivo* imaging studies showed the specific association of the TrkC targeting probe with 4T1 tumors. This study is helpful for further development of a TrkC targeting agent for theranostics of metastatic breast cancer.

Materials and methods

Probe synthesis

All reactions were carried out under an inert atmosphere (nitrogen or argon where stated) with dry solvents under anhydrous conditions. Glassware for the anhydrous reactions was dried in an oven at 140 °C for a minimum of 6 h prior to use. Dry solvents were obtained by passing the previously degassed solvents through activated alumina columns. The yields refer to chromatographically and spectroscopically (¹H-NMR) homogeneous materials, unless otherwise stated. The reagents were purchased at high commercial quality (typically 97% or higher) and used without further purification, unless otherwise stated. Analytical thin layer chromatography (TLC) was carried out on Merck silica gel plates with a QF-254 indicator and visualized by UV. Flash column chromatography was performed using silica gel 60 (SiliCycle, 230–400 mesh). ¹H and ¹³C spectra were recorded on a 400 MHz spectrometer and were calibrated using a residual non-deuterated solvent as an internal reference. The detailed synthesis information is provided in the ESI†. The abbreviations or combinations in the ESI† thereof were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, ddd = doublet of doublet of doublets.

In vitro assays

NIH3T3-WT cells (from the American Type Culture Collection) were cultured on 75 cm² culture flasks in Dulbecco's Modified Eagle's Medium/nutrient mixture F-12 (DMEM/F12, Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS. NIH3T3-TrkC cells were obtained and cultured according to previous procedures.¹¹ The cells were cultured in a humidified incubator at 37 °C with 5% CO₂ and 95% air.

Fluorescence microscopy

Intracellular localization of the NIH3T3 cells was measured using an Olympus Fluoview FV1000. The images were taken using a 60×/1.20 water immersed objective. Lysosome staining LysoTracker Green DND 26 (ThermoFisher), mitochondria staining MitoTracker Green FM (ThermoFisher) and for the nucleus, Nuc Blue (ThermoFisher) was used. A 488 nm laser was used for the green channel dyes, a 205 nm laser was used for the nucleus and a 633 nm laser was used for 1 and 2. The rest of the fluorescence images were taken using an Evos FL Imaging System (ThermoFisher).

Intracellular localization

The NIH3T3 cells were incubated with 20 μM of compound 1 for 2 h at 37 °C. After 2 h, the cells were washed with PBS, and LysoTracker and MitoTracker were added for 40 min. The cells were washed again with PBS and the nucleus was stained using Nuc Blue.

Histology on patient's tissues

The slide of the human malignant melanoma tissue microarray (BR243w) was purchased from US Biomax, Inc.; the array includes 13 cases of malignant melanoma and 12 cases of adjacent normal cells. The slide was transferred to a xylene bath for 10 min and then rehydrated in two changes of fresh absolute ethanol for 7 min each. Excess liquid was shaken off and the slide was incubated in fresh 90% and 70% ethanol and then water for 7 min each. The slide was washed in two changes of PBS for 5 min each, then incubated with PBS containing 4% BSA for 30 min. The tissues were rinsed with PBS and incubated twice in PBS for 5 min each. The compound 1 solution in 4% PBS/BSA was added to the slide and incubated overnight at 4 °C. The slide was rinsed twice in PBS, then in water (10 min each). Then the slide containing 1 was mounted on permanent mounting media with DAPI (Vector) and incubated at 4 °C for 4 h. The tissues were imaged with an Evos FL Imaging System. Throughout, digital images were captured with a 20×/0.45 excitation at 730 nm for 1 and 405 nm for DAPI.

Animal model

Murine breast cancer 4T1 cells were obtained from the American Type Culture Collection and cultured as protocol suggested, *i.e.* in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO₂. Upon 80% confluence, the cells were collected and suspended in PBS, and then subcutaneously injected into 6 week old BALB/c nu/nu mice (Charles River) at 2 × 10⁶ cells per 100 μL per mouse. Then the mice were fed for two weeks to allow the tumor growth to reach 8–10 mm for the imaging study. The animal procedures were performed according to a protocol approved by the Animal Care and Use Committee of Houston Methodist Research Institute and in accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85–23 Rev. 1985).

In vivo and ex vivo fluorescence imaging

The fluorescence imaging was performed using an IVIS200 imaging system, and the ROI study was quantified using Living Imaging software (Xenogen, CA). Excitation and emission filters were set at 745 nm and 800 nm, respectively, for image acquisition. For injection, the synthesized agent was firstly prepared as a 1 mM stock solution in DMSO, and then diluted into 10 nmol per 100 μL in PBS for each mouse. Upon intravenous injection *via* the tail vein, the mice (3 per group) were anesthetized to accomplish the imaging acquisition. As to *ex vivo* imaging, the mice (3 per group) were euthanized as animal protocol described at various time points after injection of the targeting agent, and the internal organs (*i.e.* heart, lungs, liver, kidney, spleen, gastrointestinal, tumor, muscle, brain, and spinal cord) were collected for *ex vivo* image acquisition. Upon image acquisition, ROI analysis and

quantification of the fluorescence signals were performed using Living Imaging software.

Definitions

BODIPY 4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene
 NT3 Neurotrophin-3
 PET Positron emission tomography
 Trk Tropomyosin receptor kinases

Conflicts of interest

The authors declare no competing financial interests.

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