Novel Small Molecule Probes for Metastatic Melanoma

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S Supporting Information

ABSTRACT: Actively targeting probe **1b**, an unsymmetrical bivalent dipeptide mimic, selectively bound melanoma over healthy skin tissue in histological samples from patients and Sinclair swine. Modifications to **1b** gave agents **2–4** that contain a near-IR aza-BODIPY fluor. Contrary to our expectations, symmetrical probe **3** gave the highest melanoma-to-healthy skin selectivity in histochemistry and experiments with live cells; this was surprising because **2**, not **3**, is unsymmetrical like the original lead **1**. Optical imaging of **3** in a mouse melanoma model failed to show tumor accumulation *in vivo*, but the probe did selectively accumulate in the tumor (some in lung and less in the liver) as proven by analysis of the organs post mortem.

KEYWORDS: Targeting, cancer, melanoma, small molecule ligand

Molecular fragments that bind receptors selectively overexpressed on tumor cell surfaces can be useful for active targeting.¹ Active targeting is distinct from strategies designed to perturb specific biochemical pathways upregulated in cancer, e.g. involving kinases or proteases. Small molecules that are commonly used for active targeting include some vitamins (e.g., folic acid,^{2–5} biotin,⁶ and cobalamin^{7,8}), RGD peptidomimetics,^{9–13} a few carbonic anhydrase ligands,^{14–17} and mimics of the prostate specific antigen,^{18,19} but relatively little else. This is limiting because not all tumor types over-express the corresponding receptors at usable cell surface copy-numbers, and some of these ligands have suboptimal properties for targeting entities.²⁰

To facilitate discovery of novel ligands for targeting, we reported²¹ the method shown in Figure 1 to identify small molecules that bind unknown receptors selectively expressed on the surface of cancer cells. In that method, a set of monovalent dipeptide mimics were designed and prepared,^{22–25} with a bias toward two side-chain pharmacophores that correspond to the most common amino acids found at protein–protein interfaces (Trp, Arg, Tyr, Lys, Glu, then Ser, Asn, Leu).²⁶ Our hypothesis is that these small molecules mimic side-chain orientations on various dipeptides that have a relatively high tendency to bind other proteins. Amino acid side-chains are important because they tend to dominate the thermodynamics of protein–protein interactions.²⁶

These monovalent molecules were assembled into bivalent ones **1a** bearing a long hydrophobic chain substituent as shown above. Only n monovalent compounds are needed to make $n(n + 1)/2$ bivalent ones, so large libraries can be made from a small number of building blocks.²⁷ In a one-compound-per-well format, each bivalent molecule was allowed to associate with a liposome via capture of the hydrophobic side-chain into the surface bilayer. All the liposomes used in these experiments carried the plasmid encoding luciferase. Cells will fuse with liposomes at a certain rate, but this happens faster if the liposome-supported bivalent compounds bind a cell-surface receptor and mediate internalization. The degree of import after

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Figure 1. Liposome assay to facilitate identification of small molecule ligands that promote preferential recognition of cancer cells over healthy tissue.

a set time can be determined by lysing the cells and adding agents to induce bioluminescence.²¹ We now reveal one of the compounds found in this assay, **1a**, selectively bound to melanoma SK-MEL-28 cells and was internalized more than it was when interacting with HUVEC cells (normal cell control). The study reported here was designed to establish if (i) the targeting fragments in **1** bind other live melanoma cells; (ii) they bind fixed tissue slices; (iii) modifications are made that replace the triazine core with a near-IR dye influence their binding; and (iv) the new probes can be revealed by near-IR optical imaging *in vivo*.

A weakness of the liposome assay in Figure 1 is that the HUVECs cells used as the “healthy control” are not closely related to skin. Fortunately, we have access to the Sinclair swine model,^{28,29} which allowed us to take biopsy material from pigs with naturally occurring metastatic melanoma. This was done to obtain samples of melanoma tumor and healthy skin tissue adjacent to the melanoma region. That the tissue was obtained from the same animal is significant because comparison reveals the targeting effect for a particular individual (here the individual is a pig).

The first step in care of an individual suspected of having a melanoma tumor would be diagnosis. Figure 2 shows

Figure 2. Histochemistry with **1b**. (a) Healthy skin is not stained, but (b) melanoma tissue from the same Sinclair swine is. Scale bar is 10 μm .

healthy skin tissue from the pig was not stained by the fluorescent analogue **1b**, whereas the melanoma tissue stained strongly.

Melanoma cells derived from a pig biopsy were easy to culture compared to normal skin cells from the same pig, but we found *The Georgetown Method*^{30,31} could be used to obtain live cells from the normal skin biopsy. Thus, the normal skin cells were cocultured with 3T3 Swiss fibroblast cells in the presence of the ROCK kinase inhibitor Y27632 and stem-like cells were obtained after several passages.

Confocal microscopy was used to show how **1b** interacted with the melanoma and healthy skin cells. Figure 3 shows that **1b** was

Figure 3. (a) Melanoma cells from Sinclair swine. (b) Probe **1b** is internalized by these cells. (c) Internalization of a lysosome tracker into the melanoma cells. (d) Overlays with the fluorescence from **1b**. Scale bar is 20 μm .

internalized into the melanoma cells, and it colocalized with a lysosome tracker. No signal of **1b** was observed when this was exposed to the normal skin cells (Figure S1). This observation implies conjugates of **1** may have a tendency to accumulate in melanoma cells *in vivo*.

Experiments described above validate the melanoma targeting effects of the “warhead” fragments in **1**. This successful confirmation leads to a range of possibilities for subsequent research; the one featured in the remainder of this letter is formation of a theranostic that could be used for optical imaging of melanoma.

For *in vivo* optical imaging, the fluor in **1b**, which absorbs around 520 nm, is unsuitable because observation of non-superficial tissue requires agents that absorb at longer wavelengths (ideally, >700 nm).^{32,33} One possibility would be to replace the BODIPY dye in **1** with a similar, but near-IR-absorbing, fluor. This is particularly important when intrinsically dark melanoma tissue is involved; though dark, this tissue is permeable to light $\lambda_{\text{max}} > 700 \text{ nm}$.^{34,35} The conflicting parameters are that near-IR dyes tend to be large, but it is good practice to keep molecular size as small as possible to avoid adverse absorption, metabolism, and excretion effects. For this reason we

Scheme 1. Functionalization of Diamino aza-BODIPY A with the Targeting Groups in Compounds 1

chose to investigate replacement of *both* the 520 nm dye and the triazine splitter in **1**, with a near-IR dye that could also act as a scaffold to present the melanoma-targeting groups. Thus, Scheme 1 describes a synthesis of our new target, the “aza-BODIPY”^{36,37} system **2**. Fluorescence maxima of aza-BODIPY fluors tend to be longer than 700 nm; such wavelengths are suitable for excitation of fluors in deep-seated tissue imaging.

Scheme 1 begins with coupling one equivalent of an amino acid-derived azide^{23,38} with the diamine A.³⁹ Stereochemistry in the isoleucine side-chain is an intrinsic probe for epimerization in this coupling, and none was detectable by ^1H NMR. Copper-mediated Huisgen coupling gave **6**, which has one dipeptide mimic supported on the aza-BODIPY scaffold. Construction of the other dipeptide in **8** was achieved in the same way, i.e., via **7**. Finally, acid-mediated deprotection afforded the target system **2**; this compound is not water-soluble but can be dissolved in DMSO then diluted to 1% DMSO in PBS buffer.

Predictably, since the first coupling involving A is only controlled by stoichiometry, byproducts in the synthesis of **2** made it cost-effective to prepare the symmetrical probes **3** and **4** as interesting controls. In the event, they were far more interesting than we had anticipated.

Disappointingly, treatment of pig melanoma tissue with dye **2** showed only very weak staining (Figure 4a). However, to our surprise, the symmetrical control **3** gave a much stronger signal, whereas almost no staining was observed for the other symmetrical control **4** (Figure 4b,c, respectively). Figure 4d,e compares normal skin and melanoma stained with agents **2** and **3**, respectively; these data confirm that **3** gives a far better contrast between tumor and healthy tissue than our original target **2**. From this point on, **3** became our lead probe, and no further studies were performed on **2**. Overall, this is fortunate because **2** is significantly more time-consuming to make than the symmetrical analogue **3** (see Supporting Information for synthesis).

Figure 4. Metastatic melanoma tissue stained with agents: the unsymmetric probe 2 (a) and the symmetric probes 3 (b) and 4 (c). (d) Probe 2 gives weak staining and almost no contrast between normal and melanoma tissue from the Sinclair swine, but (e) agent 3 gives a distinct contrast.

Data from one particular Sinclair swine is not reliably indicative of a trend in human melanoma. Thus, a panel of commercially available human melanoma tissue samples was stained to estimate the fraction of cases that would bind agent 3 (Figure 5). Duplicate tissue samples from 22 metastatic melanoma patients were tested, and *every one of them stained with agent 3*. Conversely, no significant staining was observed when the two duplicate samples of normal skin available were treated with the same probe.

To further test if the featured ligands could be used *in vivo*, B16–F10 cells were chosen to induce a metastatic melanoma tumor in an orthotopic mouse model. In a preliminary *in vitro* experiment, the symmetric probe 3 internalized with greater selectivity for B16–F10 cells compared to normal endothelial cells (HUVECs; Figure 6). Moreover, 3 selectively bound to B16–F10, while agent 2 and 4 showed weak signal in the same cell line (Figure S2). These were surprising observations since we

Figure 5. (a) Human normal skin tissue did not stain with agent 3, but (b–d) human metastatic melanoma tissue did (illustrative data from tissue derived from three patients are shown).

Figure 6. (a) B16–F10 cells stain with agent 3, but (b) HUVECs cells did not.

had expected the unsymmetrical probe 2 to show the greatest selectivity. For example, the selectivity of the probes were observed in only melanoma cells, but not in breast cancer cells (Figure S3).

In vivo fluorescence imaging experiments were conducted using an IVIS imaging system. Unfortunately, none of the agents 2, 3, and 4 were sufficiently fluorescent to overcome the dark pigment of melanin in melanoma at any of the time points after intravenous (i.v.) injection of agents (Figure S4).

After 2 h postinjection (p.i.), tumors were excised from the mice and imaged. This phase of the study revealed agent 3 accumulated in B16–F10 tumor more than agents 2 and 4 (Figure 7a) implying that the targeting ligand on 3 is more specific to melanoma tumor. To confirm accumulation of 3 in the tumor, the tissues were sectioned and imaged by confocal microscopy. Figure 7b–e shows strong red fluorescence was clearly visualized in tumor injected with 3, but little to no fluorescence signal from the tumors with no injection or injected with 2 and 4.

Figure 7. (a) B16–F10 tumors excised from mice injected with agents 2, 3, and 4 compared to control tumor. (b–e) Confocal imaging of tumor slices stained with agents 3 (c), 2 (d), and 4 (e) compared with control (b). Blue color is nucleic acid stained with DAPI. Scale bar is 20 μm .

Experiments were then performed to reveal the distribution of agent 3 in tumors relative to other major organs. Thus, important organs from mouse injected with 3 were removed and *ex vivo* fluorescence imaging revealed that 3 accumulated mostly in the lungs, and some in liver after 2 h p.i. (Figure S5). We suspect the poor solubility of agent 3 in biological media that might have caused aggregation in lung, but cannot confirm that assertion.

In summary, we designed and synthesized the small molecule targeting agents for melanoma. In agents 2–4, an aza-BODIPY was used as a linker-spacer for the targeting groups and as a near-IR dye for optical imaging. Targeting agent 3 showed higher specificity to variety of melanoma tissues (pig, human, and mouse) than 2 and 4 based on histological data. In a mouse B16–F10 melanoma model, i.v. injection did not provide *in vivo* images even though differences between targeting and nontargeting compounds were evident in post mortem analyses. We suspect even though agent 3 presents preferential targeting of melanoma it has poor solubility characteristics that reduce the brilliance of its fluorescence *in vivo*.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00368.

Synthetic procedures for agents 2–4, *in vitro* and *in vivo* protocols (PDF)

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Notes

The authors declare no competing financial interest.

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