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# Small Molecule Ligands for Active Targeting of TrkC-Expressing Tumor Cells

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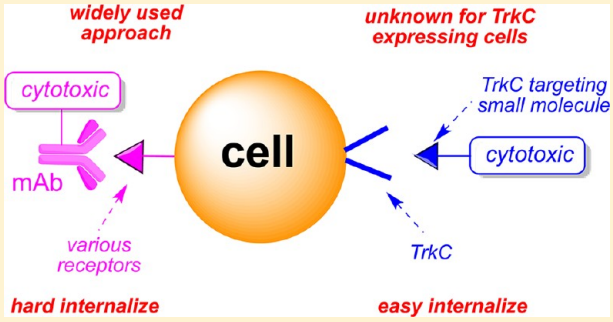
Supporting Information

**ABSTRACT:** A small molecule motif was used in "active targeting" to deliver cytotoxic substances into tumor cells that express the TrkC receptor. Underlying this study was the hypothesis that internalization of targeted conjugates into cells would be facile if mediated by receptor binding and receptor−ligand internalization. Initial experiments using 6-mercaptopurine gave encouraging data but demonstrated the importance of maintaining solubility and high cytotoxicity. Conjugates of the targeting agent with a cytotoxic rosamine (similar to a rhodamine) were more successful. Targeting of TrkC was observed, validated in a series of competition experiments featuring other TrkC ligands, and accumulation into lysosomes was observed, as expected for receptor-mediated internalization.




**KEYWORDS:** *TrkC, neurotrophin, targeting, cancer, small molecule ligand*

Current U.S.-approved small molecule pharmaceuticals for the treatment of cancer tend to have low therapeutic indices, that is, similarly toxic to cancerous and healthy tissue. Therapeutic indices of anticancer compounds can be increased by selective delivery; this is a form of "targeting". Unfortunately, the word "targeting" is ambiguous in biomedical research. For instance, compounds that inhibit enzymes overexpressed in tumor cells are sometimes described as "targeted", but they are not guided to the target via an extracellular chemical interaction.[1] Conversely, some physical or physiochemical properties (e.g., enhanced permeability and retention and pH effects) favor accumulation of nanoparticles or acid-sensitive compounds in tumors; for the purposes of this work, we refer to this as *passive targeting*. Passive targeting can be useful, but the particles can still accumulate in healthy tissue surrounded by either relatively impermeable vasculature or abnormally acidic interstitial fluid. *Active targeting* occurs when proteins or small molecule ligands form chemical interactions with macromolecules selectively expressed on the surface of the targeted cells.[2]

At least two possible factors can favor active targeting: (i) affinity of the ligand for that cell surface receptor and (ii) internalization of the ligand into the cells by the receptor. Actively targeted cytotoxicity occurs if one or both of these effects are in play. One cell surface receptor that could be targeted is TrkC. We hypothesized that this receptor is a good target because several tumor types overexpress TrkC, including neuroblastoma,[3] medulloblastoma,[4] and breast cancer.[5] Moreover, this receptor internalizes the natural (neurotrophin) ligands that bind to it, fulfilling criterion (ii).[6]

The research that is described in this letter was undertaken to demonstrate active targeting of TrkC-expressing tumor cells that could be affected using novel small molecule targeting ligands.

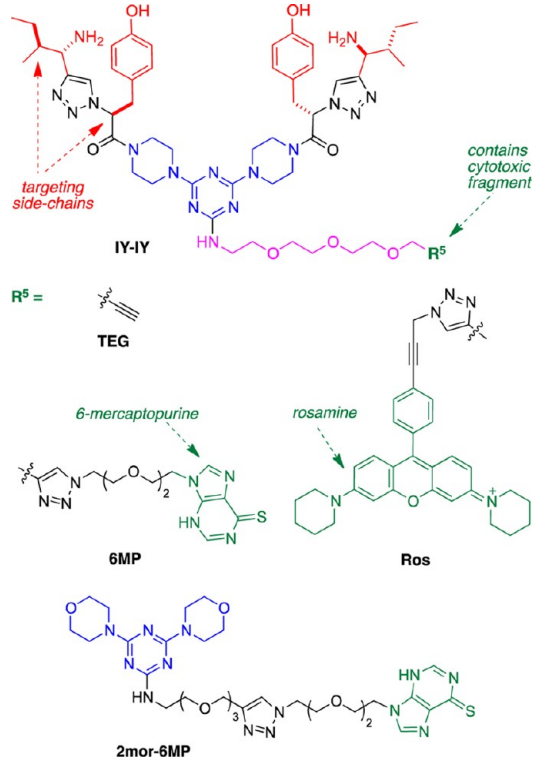



**Figure 1.** Targeting ligands used in this study and the nontargeting control compound **2mor-6MP**.

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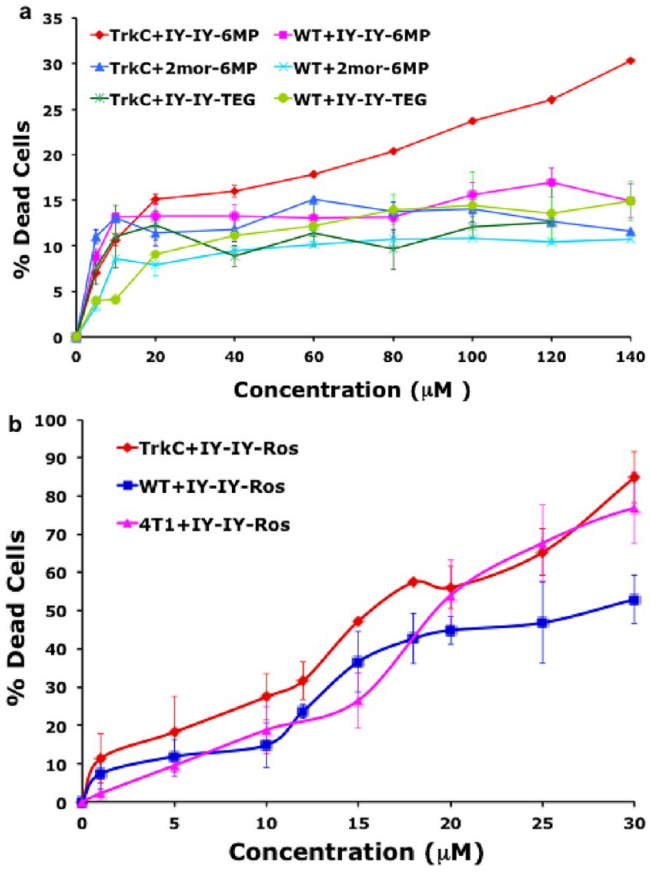


**Figure 2.** Antiproliferative assays. (a) **IY-IY-6MP** selectively targets TrkC expressing cells (red) and not the parent line (**WT**, purple; data are shown with controls that demonstrate both of the **IY-IY** amino acid side-chains and the **6MP** fragment were essential for cytotoxicity). (b) **IY-IY-Ros** shows more cytotoxicity for the TrkC-expressing cells, **TrkC** (red) and **4T1** (purple), than non-TrkC-expressing cells, **WT** (blue). Error bars were based on three runs.

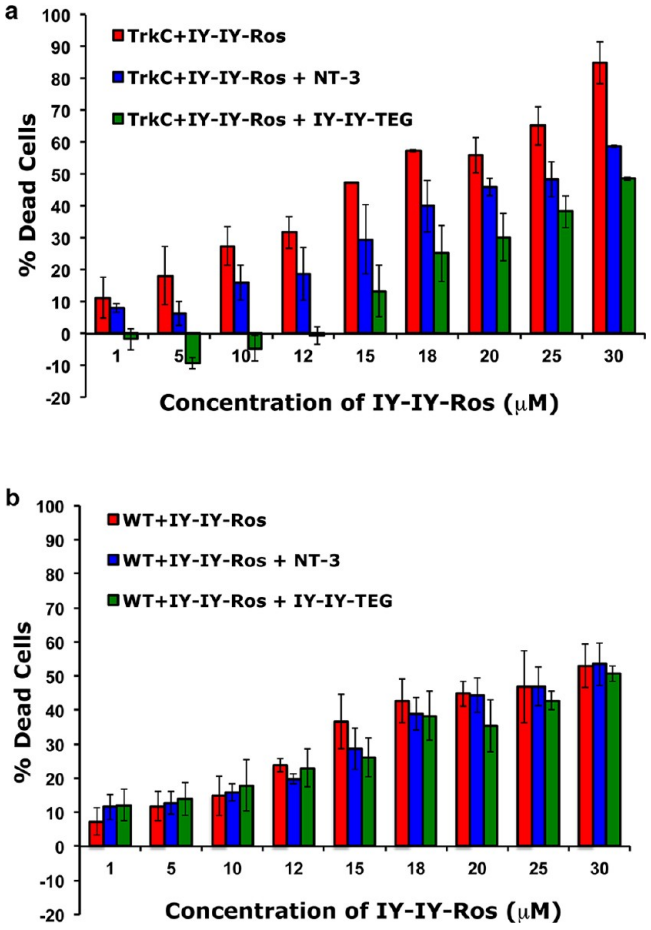


**Figure 3.** Dose-dependent reduction of **IY-IY-Ros** cytotoxicity (red) in competition with the TrkC ligands NT-3, 3.5 nM (blue), or **IY-IY-TEG**, 20 $\mu$M (green), which occurs for (a) **TrkC** cells but not for (b) **WT** cells. The concentrations of NT-3 and **IY-IY-TEG** were kept constant throughout the experiments.

Specifically, a triazine-bridged compound **IY-IY-TEG** was used as a basis for this study (Figure 1). That compound was previously shown to bind TrkC-expressing cells and elicit agonistic effects but only in synergy with the parent TrkC ligand, a protein growth factor called NT3.[7] Side-chain pharmacophores (shown in red) in **IY-IY-TEG** and related molecules were shown to be pivotal to binding.

The central hypothesis here is that the partial agonist activity of the **IY-IY** motif could be overwhelmed by incorporating a highly cytotoxic fragment in the same molecule, leading to selective binding and internalization for TrkC-expressing cells. 6-Mercaptopurine (**6MP**)[8] and a rosamine (**Ros**, a highly cytotoxic fluorescent probe)[9] were used for their cell-killing effects. We theorized that permeation of **IY-IY-6MP** and **IY-IY-Ros** into cells would be disfavored because of size and polarity effects, *unless* active targeting was involved. To evaluate our hypothesis, cytotoxicities of **IY-IY-6MP** toward a cell line that does not express TrkC (wild-type NIH3T3 abbreviated to **WT**)[10,11] were compared with the same cell line stably transfected with TrkC (NIH3T3 + TrkC abbreviated to **TrkC**; expression levels 10–20 $\times$ $10^3$ receptors cell$^{-1}$).

Figure 2a shows cytotoxicity data for **IY-IY-6MP**; the red line is for the TrkC-expressing cells (**TrkC**), and the purple one is for **WT** NIH3T3 cells. *The conjugate is more cytotoxic toward cells that express TrkC.* All the other data in Figure 2a are for controls that, in the event, support this conclusion. Thus, the nontargeted analogue **2mor-6MP** (where the IY-IY motif is substituted by morpholine; Figure 1) is not significantly cytotoxic to the **TrkC** or **WT** cells and neither is the targeted but non-cytotoxic compound **IY-IY-TEG**.

It was impossible to determine an $IC_{50}$ for **IY-IY-6MP** because the concentrations required exceeded the solubility of the compound. A relatively high amount was needed in solution because the cytotoxicities of $N^9$-alkylated derivatives of **6MP** are less than the parent system ($ED_{50}$ = 50 $\mu$M for **6MP**, 400 $\mu$M for $N^9$-butyl, and 600 $\mu$M for $N^9$-ethyl in Chinese hamster ovary cells).[12] For this reason, the focus of the study shifted to **IY-IY-Ros**; the rosamine fragment of this molecule is significantly more cytotoxic ($IC_{50}$ 0.39 $\pm$ 0.22 $\mu$M[9] for **Ros** vs 2.79 $\pm$ 0.69 $\mu$M[8] for **6MP**; both for MCF-7, human breast cancer cell line).

Figure 2b compares cytotoxicity data for **IY-IY-Ros** on the TrkC cells (**TrkC**; $IC_{50}$ = 15.80 $\pm$ 0.18 $\mu$M) and on a murine breast cancer line that also expresses TrkC (**4T1**; $IC_{50}$ = 19.88 $\pm$ 2.53 $\mu$M) with that for the non-TrkC expressing NIH3T3 cells (**WT**; $IC_{50}$ = 27.58 $\pm$ 1.38 $\mu$M). More cell death occurred for the TrkC-expressing cells than for the non-TrkC-expressing ones when ligand concentrations above about 18 $\mu$M were used. Cytotoxicity data for an analogue with the *inverted* amino acid sequence **YI-YI-Ros** was used as a negative control (the **YI-YI** motif does not bind TrkC-expressing cells).[7] The cytotoxicity of **YI-YI-Ros**


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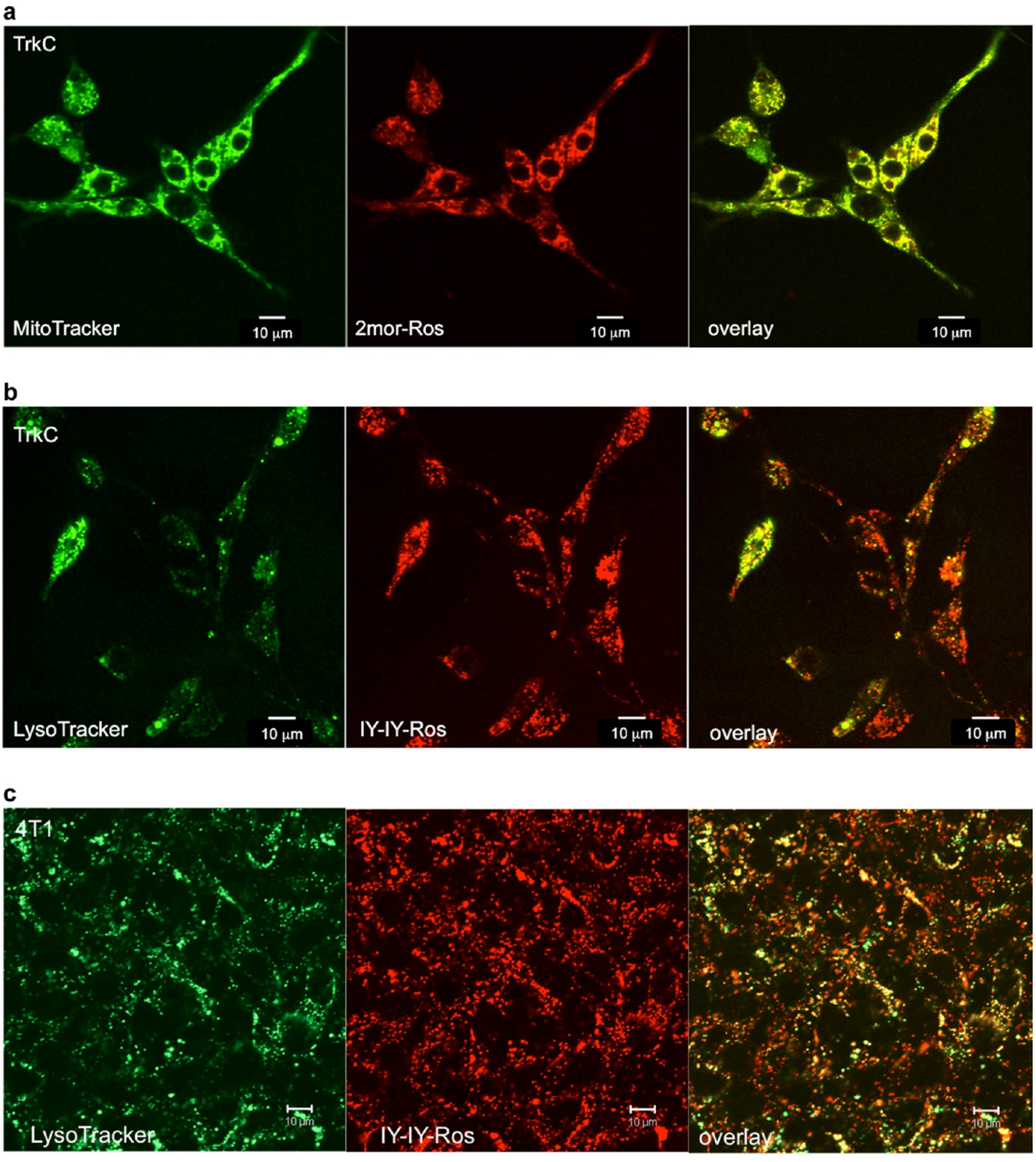


**Figure 4.** (a) **2mor-Ros** and MitoTracker colocalize in **TrkC** cells, whereas (b) **IY-IY-Ros** colocalizes with LysoTracker in the same cells and (c) in murine **4T1** breast cancer cells that also express TrkC.

with respect to the **TrkC** and **WT** cells was almost the same. Moreover, these data were also almost identical to that for **IY-IY-Ros** on the **WT** cells ($IC_{50}$ = 25.80 ± 2.24 and 25.79 ± 1.79 $\mu$M); these data further support the targeting effect of the **IY-IY** motif.

Further evidence for the targeting effect of the **IY-IY** motif was obtained from competition studies (Figure 3). The cytotoxicity of **IY-IY-Ros** was reduced in a dose-dependent way by natural protein (NT3) and by synthetic (**IY-IY-TEG**) ligands but only for the **TrkC** (Figure 3a) and not for the **WT** (Figure 3b) cells. Moreover, no competition was observed for the control with the inverted sequence **YI-YI-Ros** (see Figure S1 in the Supporting Information).

Complexes of NT3 with TrkC are internalized by cells and localize in the lysosome.[6] Conversely, the rosamine dye corresponding to **Ros** permeates into cells and accumulates in the mitochondria.[9] Experiments were performed in the current study


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to probe if the rosamine fragment *with* the bridging triazine (**2mor-Ros**) was imported in the same way as the dye *without* that fragment (**Ros**). Figure 4a shows the distribution of the MitoTracker label in **TrkC** cells, distribution of **2mor-Ros** in the same cell, and the degree of overlay. These data indicate that **2mor-Ros** localizes in the mitochondria, just as the parent dye does.

When the **Trk** cells were exposed to **IY-IY-Ros**, this *targeted* (cytotoxic) dye was internalized within 30 min. Fluorescence microscopy indicated the dye colocalized with LysoTracker (Figure 4b) and not with MitoTracker; that is, *it accumulated in the same intracellular compartment as the TrkC-NT3 complex and not in the same place as the rosamine dye*. When another TrkC-expressing line, murine 4T1 cells, was treated with **IY-IY-Ros**, then this targeted (cytotoxic) dye also localized in the lysosome (Figure 4c). These data support the hypothesis that the **IY-IY** motif binds the TrkC receptor and is internalized.

The featured targeted compounds **IY-IY-6MP** and **IY-IY-Ros** are *not* well suited for in vivo studies, for different reasons. The water solubility and cytotoxicity of **IY-IY-6MP** are inadequate for in vivo work. For **IY-IY-Ros**, the reasons are different; while these studies were in progress, our collaborator Dr. Hong Boon Lee tested the parent rosamine and found it caused severe weight loss in animals with inadequate concurrent reduction in tumor mass (personal communication). Consequently, studies to use the same targeting entities in conjunction with other cytotoxic compounds are underway.

Only a few small molecules are known for their active targeting effects; the important ones are as follows. Folic acid targets the folate receptor[13] expressed on many human cancers.[14] Mimics of the RGD peptide[15] target integrins,[16,17] and riboflavin-based compounds have been used to target its receptor expressed on some tumor types.[18,19] Bile acids target the vitamin D receptor in colon cancer, and this has been exploited.[20] Lectin-based molecules[21] have been employed to direct therapeutic agents to the asialoglycoprotein receptor in the liver,[22] and cholestenoic acid, a ligand for the liver X receptor,[23] also has value. γ-Amino-*n*-butyric acid (GABA) or the GABA derivative baclofen targeting the $GABA_B$ receptor have been used in a novel approach for pancreatic cancer.[24] Overall, there is a great deal more interest in antibody−drug conjugates than in targeting small molecule−drug conjugates.

Given the exquisite affinities of antibodies (mAbs) for antigens, it is easy to understand why mAbs have attracted so much interest in targeting approaches. However, we postulated above that affinity *and* cell permeability are both important, and large proteins such as mAbs tend not to permeate into cells, whereas small molecules can. Selective cytotoxicity studies of the kind performed here show the net effect of affinity *and* cell permeability.

Medicinal chemistry has been heavily biased toward the discovery and syntheses of cytotoxic compounds, whereas development of small molecule ligands for active targeting has received much less attention. We suggest design and testing of small molecule ligands for active targeting of cancer cells; indeed, for specific cell types in general, it is both important and, so far, somewhat neglected.

## ■ ASSOCIATED CONTENT

### Supporting Information

Synthetic procedures for **IY-IY-6MP**, **2mor-6MP**, **IY-IY-Ros**, **YI-YI-Ros**, and **2mor-Ros**. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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