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Double-targeting Using A TrkC-Ligand Conjugated To BODIPY-based PDT Agent

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Abstract

A molecule **1** (IY-IY-PDT) was designed to contain a fragment (IY-IY) that targets the TrkC receptor, and a photosensitizer that acts as an agent for photodynamic therapy (PDT). Molecule **1** had sub-micromolar photocytotoxicities to cells that were either engineered to stably express TrkC (NIH3T3-TrkC) or that naturally express high levels of TrkC (SY5Y neuroblastoma lines). Control experiments showed **1** is not cytotoxic in the dark, and has significantly less photocytotoxicity towards cells that do not express TrkC (NIH3T3-WT). Other controls featuring a similar agent **2** (YI-YI-PDT) which is identical and isomeric with **1** except that the targeting region is scrambled (a YI-YI motif, see text) showed **1** is considerably more photocytotoxic than **2** on TrkC⁺ cells. Imaging live TrkC⁺ cells after treatment with a fluorescent agent **1** (IY-IY-PDT) proved that **1** permeates into TrkC⁺ cells and localizes in the lysosomes. This observation indirectly indicates agent **1** enters the cells via the TrkC receptor. Consistent with this, the dose-dependent PDT effects of **1** can be competitively reduced by the natural TrkC ligand, neurotrophin NT3.

Keywords

TrkC; tumor targeting; photodynamic therapy; BODIPY; neurotrophins

Introduction

In oncology, the main problem with most FDA approved drugs is that they have poor therapeutic indices, *ie* they kill healthy tissue almost as effectively as tumors. In response there are many contemporary efforts to perturb biochemical pathways that are selectively upregulated in cancerous cells, *ie molecular* target-oriented approaches.¹ Kinase inhibitors are target-oriented in this way because they inhibit enzymes that are critical to the survival of cancer cells.²⁻⁴ Another form of targeting features agents that selectively bind to certain types of *cells* and deliver imaging or cytotoxic agents inside. Most current research on *cellular* targeting involves antibodies; this strategy is useful but usually does not lead to intracellular delivery.^{5,6} Conversely, some small molecules *can* be used for cell-based targeting and intracellular delivery;⁷⁻¹¹ the problem is how to design and validate substances that will do this. Yet another form of targeting is in photodynamic therapy (PDT). PDT is *physically* targeted insofar as only the tissue illuminated by the light source is vulnerable to production of reactive oxygen species from a photosensitizer and triplet oxygen. One problem with PDT is how to localize the required photosensitizer in tumor tissue.

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Any strategy that involves *two* of the approaches outlined above might be called “doubly-targeted”. For instance, a PDT agent conjugated to a small molecule cell-targeting unit might preferentially localize in tumor cells, giving a first level of targeting. A second level of targeting could then be achieved by limiting the light excitation source and consequently generation of reactive oxygen species to that area. There is, in fact, very little in the literature about cell-targeted PDT agents,^{12–14} and, to the best of our knowledge,¹⁵ nothing on such agents featuring BODIPY-based photosensitizers.¹⁶

Recently, we reported on a novel small molecule system for targeting cells that overexpress the TrkC receptor.¹⁷ That study featured two conjugates with cytotoxic compounds (6-mercaptopurine and a rosamine derivative), neither of which are PDT active. We found the potency of the conjugates to be somewhat restricted by inadequate solubility or cytotoxicity of the cargo. This paper reports conjugates of the same cell-targeting agent, based on Tyr and Ile side-chains (IY-IY), to a diiodo-BODIPY PDT sensitizer reported by Nagano *et al.*¹⁸ and us.¹⁹ Thus the featured conjugate **1** (which can be more informatively called IY-IY-PDT) can target TrkC⁺ cells and be physically-targeted by the regions exposed to light. We had believed this is the first example of double-targeting involving a BODIPY-based PDT agent, but we recently became aware of similar work by Ng *et al* in a paper that now accompanies this.

Results and Discussion

Compound 1 And Cell Lines Used

The targeting unit IY-IY has non-cytotoxic groups attached to the triazine entity; it weakly synergizes with the natural TrkC neurotrophin-ligand, NT3, enhancing cell survival but not differentiation.²⁰ Overexpression of TrkC receptors is associated with some forms of cancer (*eg* neuroblastoma,²¹ medulloblastoma,²² and breast cancer²³), and with melanoma,^{24–28} the

latter being particularly interesting because lesions near the tissue surface can be most effectively radiated by UV light. Conversely, compounds with the inverted side-chain sequence, *ie* YI-YI, did not show this activity. Thus IY-IY-PDT is the featured cell-targeting agent, and compound **2**, or YI-YI-PDT, can be used as a negative control. Compound **2** is an excellent control being isomeric with IY-IY-PDT but unable to target the TrkC receptor. Scheme 1 shows the synthetic route that was used to prepare compounds **1** and **2**. Basically, this entails copper-mediated 2+3 cycloaddition^{29,30} of the azido-diiodoBODIPY **B**, which has apparently not been made before, with the alkyne **A** featured in our earlier studies.²⁰

Photophysical properties for the conjugates **1** and **2** were measured in DMSO and in DMEM medium; the data are presented in Figure 1 (and S1 and Tables S1 of the supporting). The absorption maximum of the BODIPY core for these molecules is in the region of 540 nm. We judged this to be perfectly fine for the planned *ex vivo* studies. As the project progresses after this paper, a more conjugated agent with a longer λ_{max} will be required for *in vivo* work since tissue is far more permeable to longer wavelength light.³¹

Another strategy used to test for TrkC targeting in this work featured similar cell lines that do and do not express TrkC. Wild type NIH3T3 fibroblast cells (NIH3T3-WT) do not express TrkC,³² and these could be compared with genetically modified forms of the same cell line stably transfected with TrkC at approximately 20,000 copies per cell (NIH3T3-TrkC, kindly supplied by Dr David Kaplan, The Hospital for Sick Children, Toronto). We also tested a human neuroblastoma cell line, SY5Y that is TrkC-positive.^{22, 33}

Light And Dark Cytotoxicities

Figure 2 shows data that demonstrates IY-IY-PDT is only cytotoxic to NIH3T3-TrkC cells when illuminated (>480 nm source with fluence rate 12.2 mW/cm² for 10 min), whereas this agent is not cytotoxic in the dark. The cells survive under the same conditions when a photosensitizer was not added (data not shown). A significant PDT effect was also observed for IY-IY-PDT on the NIH3T3-TrkC cells as compared with the non-targeted agent YI-YI-PDT, (IC₅₀ 0.35 μ M and >2 μ M, respectively). These experiments were performed by treating the cells with **1** or **2**, washing them to remove the unbound agent, then illuminating. Observation of some PDT effect for the negative control YI-YI-PDT can be attributed to non-specific endocytosis; consistent with this light induced cell-death was also observed when the same experiment was performed using NIH3T3-WT cells (see Figure 6 below and Figure S2).

The PDT effect of **1** on NIH3T3-TrkC cells was dose dependent (Figure 3, red bars). In experiments also shown in Figure 3 the cells were simultaneously treated with a constant concentration of either the native ligand NT3 (green bars), or control compound **A** (or IY-IY; blue bars) comprised of the same targeting agent but lacking the diiodo-BODIPY entity. Data for the two competition experiments shows a “breakpoint” at around 0.2 μ M of **1**; below this, the two TrkC ligands that do not bear a PDT agent (*ie* 3.5 nM of NT3, and 20 μ M of **A**) overwhelmed the affinity of IY-IY-PDT to the cells so the green and blue bars do not show much cell death, whereas there were significant cytotoxicities in the experiments without these competing agents (red bars). Above that breakpoint the effects of binding the targeted PDT agent are evident for the blue and green bars because the concentration of the PDT agent overwhelmed that of the innocuous competing controls.

Imaging Of Internalization Via Confocal Microscopy

NIH3T3 cells were treated with a supernatant containing 0.5 μ M IY-IY-PDT for 2 h; the cells were washed (3 x PBS buffer), then imaged. Figure 4 shows that the weak I₂-BODIPY fluorescence becomes localized in punctates, but only for the cells that are transfected with

TrkC. No intracellular fluorescence was observed under these conditions for the negative control YI-YI-PDT (second row), or for TrkC⁻ cells treated with the featured agent IY-IY-PDT.

The images in Figure 4 do not show strong fluorescence because emission from IY-IY-PDT is intrinsically weak. However, bright images could be obtained by increasing the concentration and the incubation time from 2 h to 12 h, and these data are shown in Figure 5. The {higher magnification} images in Figure 5 include experiments with *LysoTracker Red* showing that IY-IY-PDT localizes in the lysosome, and not in the mitochondria and endoplasmic reticulum. It is known that when NT3 binds the TrkC receptor the complex is internalized and also localized in the lysosome.³⁴ The fact that IY-IY-PDT is processed in the same way is therefore circumstantial evidence that it enters the cells via the same receptor, TrkC.

Photocytotoxicities On The SY5Y Neuroblastoma Cell Line

Figure 6 shows that IY-IY-PDT is photocytotoxic to the TrkC⁺ neuroblastoma line SY5Y. We were unable to find a neuroblastoma line that does not express TrkC, so this plot also shows the photocytotoxicity of the same agent on NIH3T3-TrkC and NIH3T3-WT for comparison. The observed photocytotoxicity of IY-IY-PDT was significantly greater for the cells that express TrkC than that observed for the non-targeted YI-YI-PDT control on the SY5Y cells.

Conclusions

The featured agent IY-IY-PDT showed significantly higher photocytotoxicities ($IC_{50} = 0.35 \pm 0.06 \mu M$ NIH3T3-TrkC; $IC_{50} = 0.15 \pm 0.02 \mu M$ SY5Y) than similar conjugates we prepared featuring 6-mercaptopurine and a rosamine in place of the PDT agent.¹⁷ On the other hand, the non-targeted ligand YI-YI-PDT is considerably less cytotoxic.

In general, IC values can be used to calculate *ex vivo* therapeutic indices.³⁵ However, for IY-IY-PDT on TrkC⁺ cells relative to TrkC⁻ ones, this was impractical because the conjugate had such a low photocytotoxicity to the TrkC⁻ cells, which made it difficult to determine even an IC_{10} value. Furthermore, the lack of dark cytotoxicity is consistent with *in vivo* data that we have obtained in collaboration (in preparation); specifically, in the dark, IY-IY-PDT is not toxic in mice at concentrations of 100 mg/Kg, 12 days post injection. These observations point to the considerable potential for selective killing of TrkC⁺ tumor cells, and indicate further studies to monitor the effects of IY-IY-PDT on TrkC⁺ tumors *in vivo*, and pharmacokinetic analyses of this agent, are justifiable. Work towards these goals is in progress in our group.

Consideration of *The Human Protein Atlas*³⁶ reveals neuroblastoma tends to express high levels of TrkC, and several other tumor types (notably breast and melanoma) express lower, but significant TrkC levels. These tumors may therefore be suitable to test the targeting effects of IY-IY ligands.

Patients afflicted with the same type of cancer may have genetically different tumor cell types that express *different* levels of TrkC. Consequently, a goal for this type of research could be a form of personalized medicine of the following kind. More fluorescent cell-targeting conjugates similar to IY-IY-PDT would be used to identify and locate TrkC⁺ cancers, cytotoxic materials like IY-IY-PDT are employed to reduce the tumor mass, and the afflicted area is then re-imaged to gauge success. The next step towards reaching this goal will be to modify the PDT agents, for the reasons below.

Tissue is significantly more permeable to near-IR light than it is to radiation of shorter wavelengths.^{31, 37–39} Consequently, it will be important to use PDT agents that absorb at longer wavelengths when this work progresses to the *in vivo* stage because in that case tumors can be addressed in deeper tissue. The BODIPY fragment in IY-IY-PDT has a UV absorption maximum of around 540 nm in DMEM (one of the culture media used in this work). That absorption wavelength is suitable for *ex vivo* studies such as the ones described here. Alternatives to the diiodo-BODIPY fragments that could be used for *in vivo* studies include aza-BODIPYs,^{40–44} and styryl-BODIPYs^{45–47} as featured in the accompanying paper by Ng *et al.*

Experimental Procedures

Supporting Information available: synthesis and characterization data for all compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Cell Culture

NIH3T3-TrkC cells were cultured on 75 cm² culture flasks in Dulbecco's Modified Eagle Medium/nutrient mixture F-12 (DMEM/F12, Sigma Chemical, St. Louis, MO) including G418 (200 mg/mL, GIBCO) supplemented with 10 % NBGS (newborn calf serum, GIBCO). Wild-type NIH3T3 cells were cultured on 75 cm² culture flasks in DMEM/F12, does not include G418, supplemented with 10 %. SY5Y cells were cultured on 75 cm² culture flasks in DMEM/F12 supplemented with 10 % FBS. All cells were cultures in a humidified incubator at 37 °C with 5 % CO₂ and 95 % air.

Fluorescence Microscopy

Subcellular localization was measured on living NIH3T3 cells using a Zeiss 510 META NLO Multiphoton Microscope System consisting of an Axiovert 200 MOT microscope. Throughout, digital images were captured with a 40x/1.3 oil objective with the following filter sets: (i) for diiodo-BODIPY dyes used in this paper the excitation wavelength was 488 nm and the emission BP used was 500–530 nm; (ii) for LysoTracker® red, the excitation was at 543 nm and the emission BP was 565–615 nm; (iii) ER-Tracker™ Blue-White DPX, the excitation was at 740 nm and the emission BP was 435–485 nm; (iv) MitoTracker® Deep Red FM, the excitation was at 595 nm and the emission BP was 575–640 nm.

Sequential optical sections (Z-stacks) from the basal-to-apical surfaces of the cell were acquired. Digital image acquisition was initiated approximately 1 μm below the basal surface of the cells and optical slices were collected at 0.5 μm steps through their apical surface using a high numerical objective lens (C-APO 63X/1.2 W CORR D=0.28M27). These wide-field images were subjected to deconvolution using Intelligent Imaging Innovations (3I) software.

Subcellular Localization

NIH3T3-TrkC cells were incubated with IY-IY-PDT, 1 μM, for 12 h at 37 °C. After the cells were washed with PBS, LysoTracker Red (Life Technology, 500 nM) was added and the cells were incubated for 30 min at 37 °C. For the study using MitoTracker and ER Tracker, the markers were used at 100 nM and the cells were incubated for 15 min. In all cases, the cells were washed again with PBS before imaging.

Photoinduced Cytotoxicity Assay

Approximately 10,000 NIH3T3-TrkC cells/well or 7000 cells/well for NIH3T3-WT, and SY5Y cells in culture medium containing 10% fetal bovine serum were seeded in a 96-well plate. Cells were allowed to adhere overnight before test compounds were introduced. IY-

IY-PDT or YI-YI-PDT stock solutions (0.02 M in DMSO) were diluted with protein-free medium (PFHM-II), 1 μ L stock solution/1 mL PFHM-II, to make master stock solutions. The master stock solutions were further diluted with PFHM-II to the desired final concentrations varying from 0.02 to 2 μ M were tested on the cells (less than 0.001 % DMSO contained in the final solutions). After 2 h of treatments, cells were washed with PBS then culture media without any additives was added (ACAS) before irradiation. Cells were irradiated with a light dose of 7.3 J/cm² from a broad-spectrum halogen light source and fluence rate of 12.2 mW/cm² and were further incubated for 24 h. Their viabilities were assessed through an MTT conversion assay.⁴⁸ Briefly, 20 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT (5 mg/mL, in Hank's balanced salt solution) were added and the cells were incubated for an additional 3.5 h. The medium was then removed, and 100 mL of DMSO was added to dissolve the formazan crystal formed. The optical density of each well (at 570 nm) was measured with a BioTek Synergy 4 Microplate Reader. The viability of each cell line in response to the treatment with tested compounds was calculated as: % dead cells = 100 – (OD treated/OD control) $\times$ 100.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
a UV-Vis and **b** fluorescence spectra (excited at 450 nm) of **1** and **2** (both at 5.5 μM) in DMEM medium.

Figure 2.

Cytotoxicities induced by IY-IY-PDT under light (red) and dark (blue) and scrambled negative control YI-YI- PDT under light (green) conditions for a cell line stably transfected with TrkC receptors. Throughout this paper, in the light experiments the cells were illuminated with a broad spectrum source, filtered to only deliver photons of >480 nm wavelength, at a flux of approximately 12.2 mW/cm² for 10 mins. Error bars were based on 3 runs.

Figure 3. Dose-dependent reduction of IY-IY-PDT photoinduced cytotoxicity (red) in competition with the TrkC ligands NT-3, 3.5 nM (green) or IY-IY, 20 μM (blue) on cells expressing TrkC. The concentration of NT-3 and IY-IY were kept constant throughout the experiments. Error bars were based on 3 runs.

a

b **Figure 4.**

a Confocal imaging of: *first row*, the featured targeting ligand on TrkC⁺ cells showing the compound is internalized; *second row*, the negative control YI-YI-PDT is not localized under the same conditions; *third row*, the featured agent is not observed in TrkC⁻ cells under the same conditions. **b** Quantitative indications of the fluorescence intensity in each of the three experiments described above (error bars from 100 cells).

Figure 5.
IY-IY-PDT colocalizes with *LysoTracker Red* in TrkC⁺ cells. Throughout, the concentration of the agent used was 1 μM.

Figure 6. Photoinduced cytotoxicity assays. IY-IY-PDT shows high cytotoxicity for the TrkC expressing cells, NIH3T3-TrkC (red) and SY5Y (blue) compared to non-TrkC cells, NIH3T3-WT (purple). The non-targeting ligand, YI-YI-PDT (green) is significantly less photocytotoxic. Error bars were based on 3 runs.