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TrkC-Targeted Kinase Inhibitors And PROTACs

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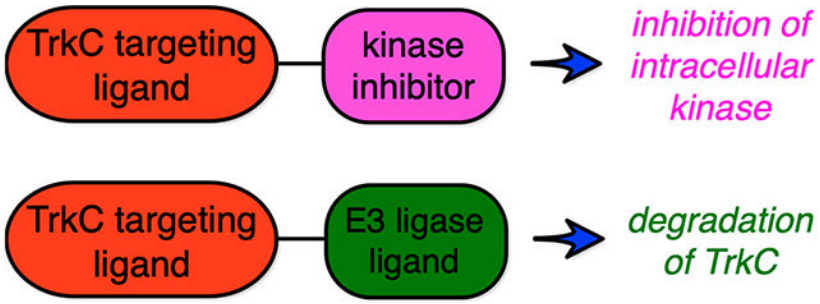
Abstract

A small molecule motif (IY–IY), which binds the tropomyosin receptor kinase C (TrkC), was used to deliver the promiscuous kinase inhibitor (KI) dasatinib into breast cancer. Conjugates with noncleavable (**1**) and cleavable (**2**) linkers were compared in cellular assays and shown to have more impact on the cell viabilities of $TrkC^+$ breast cancer cells over $TrkC^-$ epithelial cells. The IY–IY fragment was also used to recruit the E3 ligase cereblon, giving a potent proteolysis targeting chimeric (PROTAC) for TrkC degradation in metastatic breast cancer cells.

Graphical Abstract



Keywords

active targeting; TrkC receptors; chemotherapy; dasatinib; triple negative breast cancer cells; PROTACs

INTRODUCTION

Therapeutic indices of chemotherapeutics can be improved by delivering them into cancer cells via “active targeting”,[1] i.e., by conjugation with molecular fragments that bind cell surface receptors overexpressed in cancer cells. This approach leads to greater accumulation

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Supporting Information

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Synthesis and characterization of intermediates and key compounds **1–4**; procedures for biological assays (cytotoxicity assay, wound healing assay, Western blot, protein degradation); supplementary figures (compound stabilities, wound healing, cytotoxicity, protein degradation) (PDF)

The authors declare no competing financial interest.

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of drug in tumor over normal tissues, opening the therapeutic window. Active targeting is most commonly achieved via antibody–drug conjugates,[2] but small molecule approaches have significant advantage of deeper permeation into solid tumors.[3,4] Consequently, it seems logical to overlap active targeting of kinase inhibitors (KIs) with our interest in small molecules that binds TrkC.[5-12] TrkC is overexpressed on several metastatic cancer types, including neuroblastoma[13], glioblastoma, [14] breast cancer, [15] and melanoma.[16] Activation of TrkC promotes cell growth and metastasis in some forms of tumorigenesis.[17] We developed a small molecule fragment (IY–IY) that binds TrkC (K_d =112 nM)[11] and can be internalized into cells;[5] recently we demonstrated a conjugate of this with a cytotoxic cargo (DM4) had a better therapeutic index than the parent drug in a murine model.[11]

There are a few examples of active targeting being used for less cytotoxic chemotherapeutics. Small molecule targeting fragments are probably better suited than antibodies for this strategy because they can be made on larger molar scales. The less cytotoxic chemotherapeutic featured in this study is the kinase inhibitor dasatinib.

Dasatinib (Sprycel) was an approved drug for Philadelphia chromosome-positive (Ph+) chronic myeloid leukemia (CML) in 2006. Since then it has featured in over 150 clinical trials (clinicaltrials.gov) including an active Phase II study for breast cancer (). Treatment with dasatinib has side effects that can be severe for some patients,[18,19] and this restricts dose levels. At least some of those side effects can be attributed to poor selectivity within the kinome. In general, poor selectivity between kinases is a common problem with KIs because off-target interactions may lead to undesirable consequences; as of 2016, around 25% of FDA-approved KIs were known to bind ten or more targets.[20] Ultimately, active targeting may be a way to alleviate some of these off-target effects.

Recently we demonstrated active targeting of kinase inhibitors via conjugation with cyanine dyes that apparently bind all solid tumors.[21] Described here is uptake mediated by binding the IY–IY fragment of TrkC receptors applied to a KI (dasatinib).

PROTACs are a form of active targeting wherein a small molecule binds a target protein and delivers an E3-ligase ligand.[22,23] In PROTACs, small molecule targeting groups are invulnerable to E3-ligase mediated degradation before binding the target protein and facilitate catalysis; this would not be so for PROTACs based on antibodies. Thus, here we use the IY–IY fragment to deliver a kinase inhibitor via TrkC and also investigate how this same fragment could be used in PROTACs to degrade TrkC. Specifically, we report IY–IY–dasatinib conjugates (**2**) for actively targeting TrkC+ cancer cell to deliver the KI and a potent TrkC degrader (**4**) designed using the PROTACs concept. Consequently, we also report potentially the first TrkC PROTACs.

RESULTS AND DISCUSSION

Syntheses of IY–IY–Dasatinib Conjugates.

Crystal structures of dasatinib in complex with kinases show the —OH group is exposed to solvent (*eg* c-Src and Lyn; Figure S1). These observations suggest that hydroxyl is an

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appropriate attachment point to couple targeting entities without interfering with binding of dasatinib to kinase targets.

Linker fragments to connect the IY–IY and KI fragments could be designed to be robust or labile in cells, and neither strategy is clearly superior. Pharmacokinetics of systems containing robust linkers is easier to assess and tune, but this approach requires the conjugates to bind the target kinase. Conversely, if a cleavable linker is used then free KI (or KI derivative) may be released thereby circumventing any adverse effects the IY–IY fragment had on binding of the KI to the kinase, but unfavorable rates of cleavage are a possibility. For these reasons, this work features two IY–IY–dasatinib conjugates, one containing a noncleavable linker (**1**) and the other comprising a cleavable one (**2**).

Scheme 1 outlines the synthesis developed for the robust conjugate **1**. Highlights of this procedure include conversion of dasatinib to a known azide,[24] followed by copper-mediated Huisgen cycloaddition of that to an alkyne-functionalized IY–IY derivative, **IY–IY-TEG**, that was reported in another study.[6]

Scheme 2 outlines preparation of the disulfide-linked, conjugate **2**. Intracellular glutathione and other thiols are anticipated to liberate the dasatinib fragment in this system once it is internalized into the cell. Thus, **2** was accessed from a carboxylic acid derivative of dasatinib[25] coupled to a commercially available amino disulfide; subsequent amine deprotection, coupling with succinic anhydride to give a carboxylic acid terminus, and amide coupling to an amino IY–IY derivative[12] led to the final product (Scheme 2). Unlike IY–IY, the inverted sequence YI–YI does not bind TrkC.[7,8,11] Consequently, a negative control with the inverted side chain sequence, i.e., **3**, was made in a similar fashion.

Stabilities, Kinase Inhibition, and Cellular Effects of Conjugates 1 and 2.

Stabilities of conjugates **1–3** in 1:1 mouse serum/PBS were determined via reverse phase HPLC (Figure S2). All three conjugates were stable under these conditions after 48 h incubation at 37 °C. These experiments imply that even the disulfide conjugate **2** in blood would be relatively stable if delivered by *iv* injection.

Monitoring c-Src pTyr levels via Western blot required selection of an appropriate $TrkC^+$ cell line. MDA-MB-231 triple negative breast cancer cells have been reported to express TrkC,[26] but confirmatory gels are not shown in this literature. However, two different batches of MDA-MB-231 cells (from American Type Culture Collection, ATCC) were tested with TrkC-mAbs from two different sources, and throughout we were unable to detect TrkC. TrkC was detected though, in another triple negative metastatic breast cancer line, Hs578t (Figure S3), which is consistent with previous reports.[11,17]

Figure 1a shows blotting to monitor the levels of c-Src in Hs578t when treated with conjugates **1–3**. Cells were incubated with the compounds for 18 h before lysis and analyses. Conjugate **2** exhibited inhibited production of p-Src *as effectively as* **das**, whereas **1** and **3** showed significantly less inhibition. Insignificant TrkC expression was observed in our MDA-MB-231 cells (see above), so these were used in negative control experiments. In these cells, all three compounds inhibited p-Src slightly (there appeared to be no difference

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for **1–3** when the probe was used at 100 nM, and only were minor differences at 50 nM), but all were *less* effective than **das** (Figure 1b). These observations are consistent with internalization and delivery of the conjugates, particularly **2**, into the $TrkC^+$ Hs578t cells, but lesser into the MDA-MB-231 where TrkC levels are low, at most. Observation of more p-Src inhibition for the labile disulfide-containing conjugate **2** implies liberation of modified dasatinib is a superior strategy to use than the robust linker in **1**.

Wound healing assays were also performed on the two cell lines featured above further to test the functionality of conjugate **2**. Phosphorylation of Src is a positive regulator of cell migration,[27,28] so recovery rates are expected to be slower for cells treated with **das** and its conjugates compared to untreated groups. In the current study, cell suspensions were applied to 24-well plate with inner "culture-insert wells" (Ibidi) to create the artificial wound. Cells were allowed to attach overnight, followed by removal of inserts and addition of compounds. Wound recoveries were imaged by time lapse microscope, and scratch closure speeds were measured. Hs578t cells treated with **2** exhibited the slowest closure speed among all conjugates, but faster than **das** (Figure 2). In contrast, treatment with **IY–IY-TEG** had no effect on cell migration compared to the DMSO control (Figure 2); thus, this experiment showed no evidence that the IY–IY fragment activates TrkC to induce or suppress cell migration. However, treatment of MDA-MB-231 cells with all three conjugates gave similar recovery rates that are unambiguously slower than the DMSO control but faster than **das** (Figure S4). These results further confirm a delivery of IY–IY active targeting relies on TrkC overexpression and disulfide cleavage to observe the effect of the KI drug cargo.

Having confirmed the functionalities of conjugate **2**, we next examined whether our active targeting strategy can improve selectivity for $TrkC^+$ cancerous over $TrkC^-$ normal cells. Hs578t, a triple negative cell line, and MCF-10A, a nontumorigenic breast epithelial cell line that does not overexpress TrkC, were selected for comparisons in a cytotoxicity assay. In the $TrkC^+$ cancer cell line Hs578t, conjugate **2** exhibited a similar cytotoxicity to **das**, whereas noncleavable conjugate **1** and the nontargeting control with an inverted side chain sequence **3** were less toxic (Figure 3). This observation supports the previous assertion that, among the conjugates studied, IY–IY active targeting and a thiol-sensitive, disulfide linkage is crucial to suppress cell viability in $TrkC^+$ cells. In the noncancerous cell line MCF-10A, however, **2** is the least toxic of **das** and **1–3** (Figure 3), indicating binding to the TrkC receptor is important. Cytotoxicity in MDA-MB-231 was also tested, but no substantial difference is observed among all four compounds (Figure S5); this is in accord with lower TrkC expression levels for MDA-MB-231 compared with Hs578t. Overall, the cell viability for the compound **2** was less in the $TrkC^+$ Hs578t cells than the TrkC-deficient MDA-MB-231 line. When a normal vascular endothelial cell line, HUVEC, was tested (Figure S5), **2** proved to be less cytotoxic than **das**, and of similar potency to **1**. Overall, these results suggested IY–IY active targeting can improve the selectivity for $TrkC^+$ cancer cells over normal cells.

IY–IY Based PROTACs For TrkC. Several E3 ligand/ligase pairs have been reported for PROTACs, and two, nutlin-3a/ MDM2 and pomalidomide (**pom**)/cereblon(CRBN), were tested here. Thus, **IY–IY-PEG(n)-nutlin** ($n = 3$, **10**; $n = 5$, **11**) and **IY–IY-pom** (**4**) were

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prepared by azide–alkyne cycloaddition of **IY–IY-TEG** to known pom-azide[29,30] or nutlin-azide[31] (Schemes 3 and S1).

When the nutlin-based PROTACs (**10** and **11**) incubated with a TrkC-transfected cell line, NIH3T3-TrkC, only **11** induced significant degradation at 40 μM (Figure S6), and this took 24 h (Figure S7) to be clearly observable. Incomplete degradation and long times might be a consequence of significantly higher TrkC expression levels in transfected cells over natural cell lines.

For the natural TrkC$^+$ cell line, Hs578t, almost no degradation was observed when incubated with **11** at up to 10μM. However, the PROTAC based on pomalidomide, **4**, induced potent TrkC degradation at 1–10 μM, with an estimated DC_{50} (50% protein degradation conc.) of 0.1–1.0 μM (Figure 4). This observation is consistent with reports that imply nutlin-based PROTACs tend to be less effective than those comprising **pom**/CRBN pairs.[22]

CONCLUSIONS

In summary, the TrkC targeted conjugate **2** preferentially delivered dasatinib into TrkC$^+$ cancer cells over normal cells and matched the activity of dasatinib in cell viability and pTyr blotting assays. The cleavable disulfide bond connecting dasatinib to the IY–IY targeting fragment was important to the activity of the conjugate in all the assays used (viability, pTyr, and wound healing). Conjugate **2** has the potential to improve the selectivity and therapeutic index of the promiscuous kinase inhibitor dasatinib. The IY–IY fragment was also useful for development of the first potent TrkC degrader **4** comprising this ligand bound to pomalidomide to recruit CRBN for degradation. TrkC PROTACs may provide opportunities for alternative therapeutic strategies in diseases where that receptor is important.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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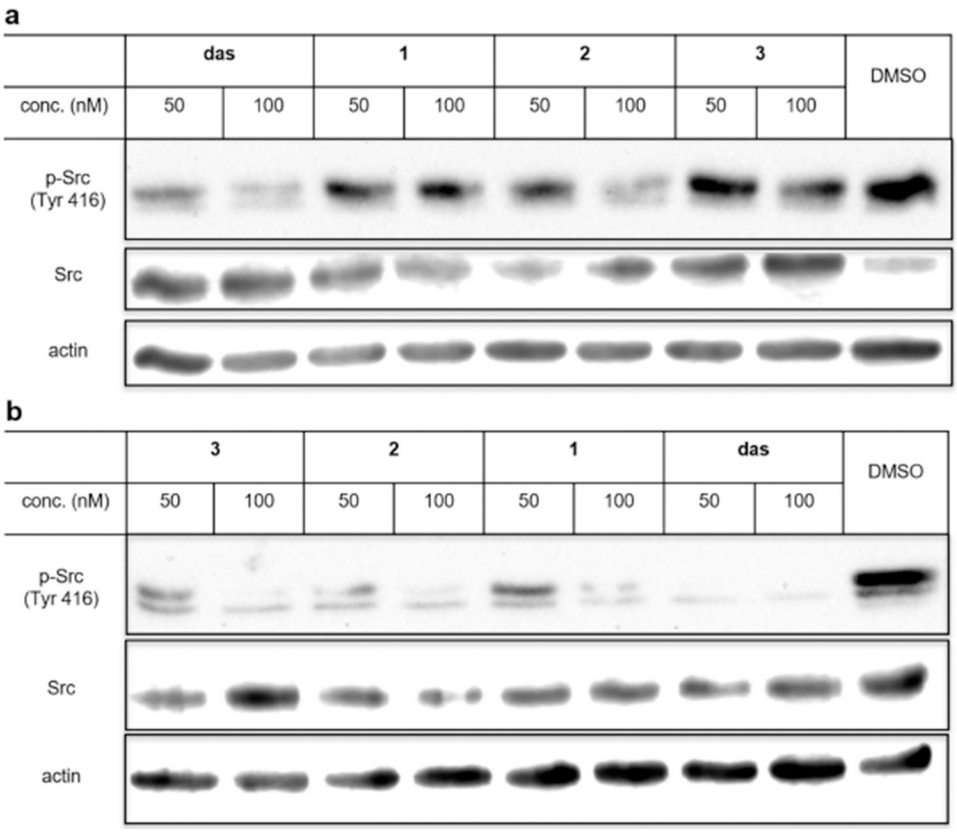

Figure 1.

Western blot analyses of the inhibitory effect of p-Src treated with das and conjugates 1–3 in (a) Hs578t and (b) MDA-MB-231 cells.

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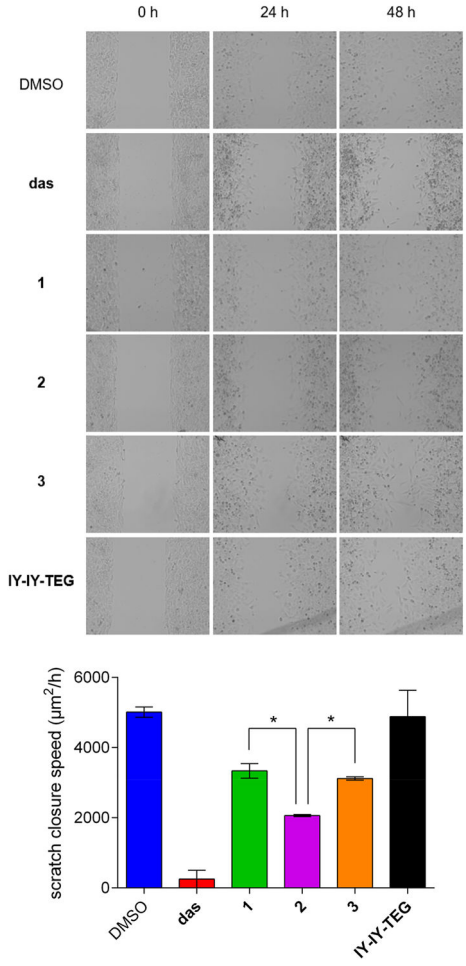

Figure 2.
Wound healing for Hs578t cells treated with das and conjugates 1, 2, and 3. *, $p < 0.01$ using one-way ANOVA.

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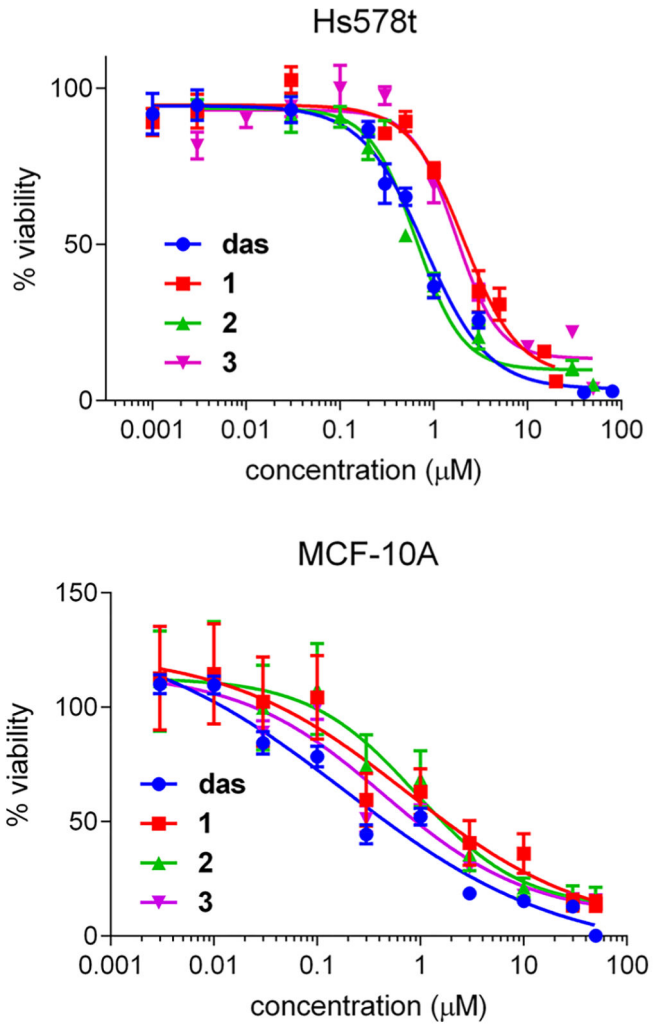

Figure 3.
Cytotoxicity assay on Hs578t (TrkC^{+}) and MCF-10A (TrkC^{-}, noncancerous) cells.

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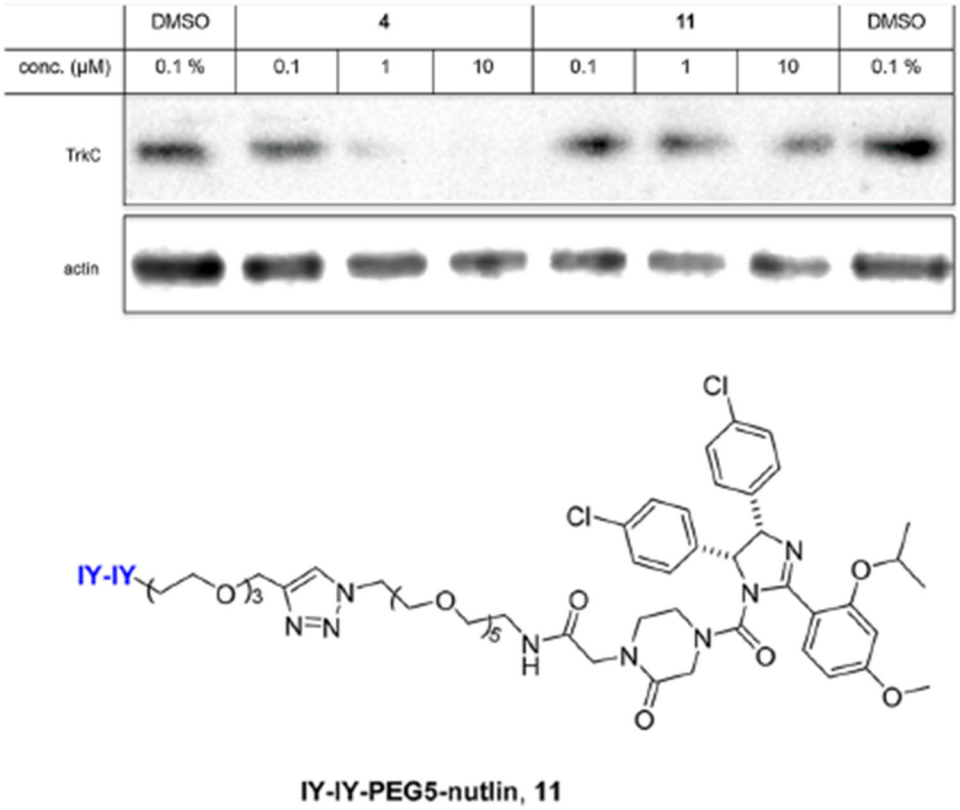

Figure 4.

TrkC levels in Hs578t cell lysates after 24 h incubation with IY–IY-pom (4) and IY–IY-PEG5-nutlin (11).

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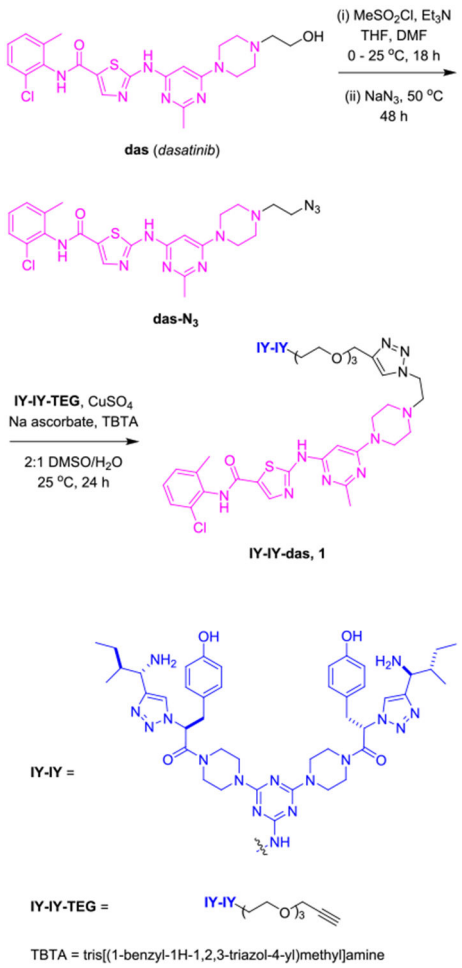

Scheme 1.
Synthesis of IY–IY–das (1) with a Robust Linker

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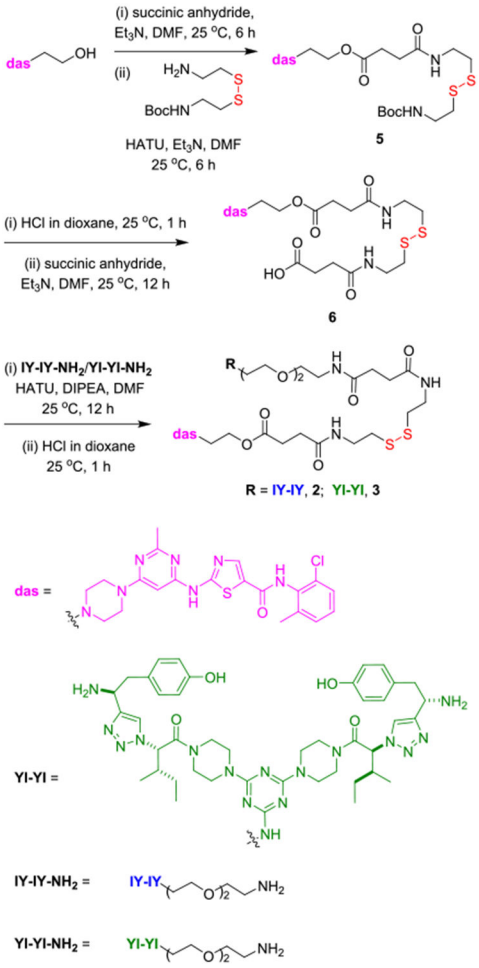

Scheme 2.

Synthesis of IY–IY-SS-das (2) and YI–YI–SS-das (3), Having Thiol-Labile Linkers

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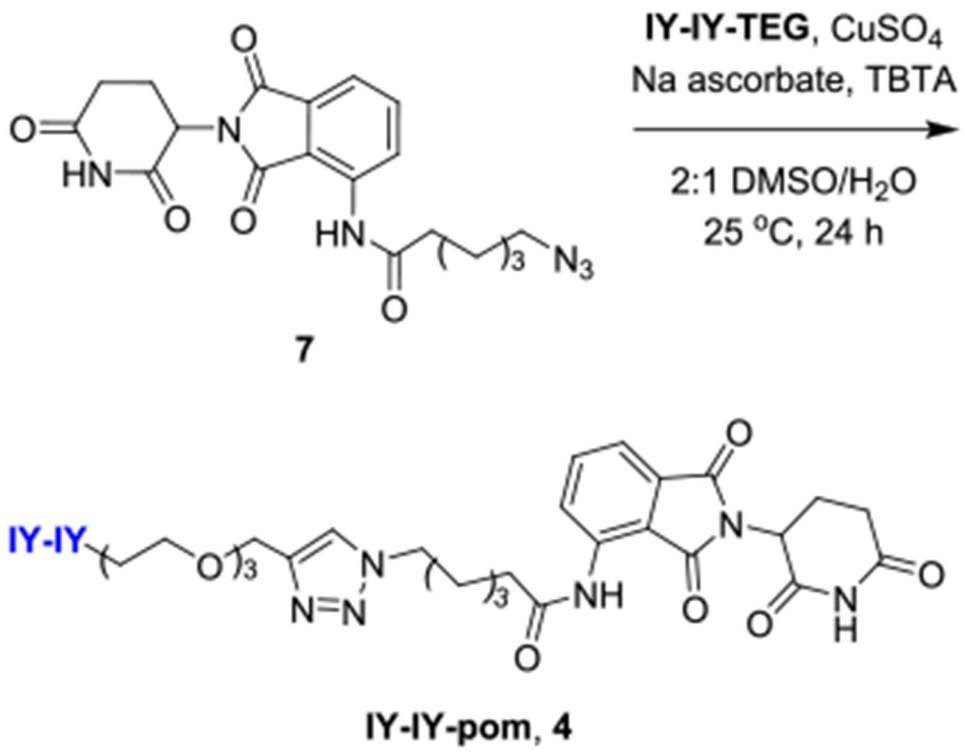

Scheme 3.
Synthesis of IY–IY-pom (4) as TrkC PROTAC