**Design, Synthesis, and Biological Evaluations of Tumor-Targeting Dual-Warhead Conjugates for a Taxoid–Camptothecin Combination Chemotherapy**

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**ABSTRACT:** Novel tumor-targeting dual-warhead conjugates, 2 (DW-1) and 3 (DW-2), which consist of a next-generation taxoid, 1 (SB-T-1214), and camptothecin as two warheads, self-immolative disulfide linkers for drug release, biotin as the tumor-targeting moiety, and 1,3,5-triazine as the tripod splitter module, were designed and synthesized. The potency of 2 was evaluated against MX-1, MCF-7, ID8, L1210FR (BR+), biotin receptor overexpressed) and WI38 (BR−, normal) cell lines in the absence and presence of glutathione (GSH), which is an endogenous thiol that triggers drug release inside the cancer cells. With the GSH and resuspension protocol, 2 exhibited IC_{50} values of 3.22–9.80 nM against all BR+ cancer cell lines, and 705 nM against WI38. Thus, there was a two orders of magnitude higher selectivity to cancer cells. Also, a clear cooperative effect was observed for the taxoid–camptothecin combination when two drugs were delivered to the cancer cells specifically in the form of a dual-warhead conjugate.

## INTRODUCTION

Over the past several decades, combination chemotherapy has been serving as a primary treatment option for many types of cancers. Compared to the use of a single cytotoxic agent, the use of two or more properly selected agents in combination can lead to a decrease in systemic toxicity and an increase in efficacy due to synergistic or cooperative effects of the drugs on the tumor eradication. Furthermore, a sequential treatment with chemotherapeutic agents bearing different mechanisms of action has been shown to avert drug resistance and lead to synergistic enhancement of efficacy.

Despite the profound advantages of combination chemotherapy, the lack of tumor-specificity continues to be a serious issue for cancer treatment. In the past decade, significant advancement has been made on the development of tumor-targeted drug delivery systems (TTDDs), especially for monoclonal antibody–drug conjugates (ADCs) and small molecule drug conjugates (SMDCs). However, only one such tumor-targeting dual-drug conjugate has been reported for the combination of mitomycin C and desacetylvinblastine using folate as the tumor-targeting module. Thus, there is an unmet need for the development of efficacious TTDDs amenable to anticancer drug combinations. Because a TTDD delivers anticancer drugs through receptor-mediated endocytosis (RME), it should be able to circumvent multidrug resistance caused by ABC transporter efflux pumps such as P-glycoprotein in the cancer cell membrane, which is one of the beneficial features of TTDDs.

We set out to design a versatile TTDDs platform for two-drug combinations and construct “dual-warhead guided molecular missiles” for tumor-targeted combination chemotherapy. Such a TTDD should have single pharmacokinetics for the delivery of two different drugs to tumor because this conjugate is a single molecule, which can get around potentially complicated treatment regimen to deal with two different pharmacokinetic parameters for two drugs. This is a salient feature of the dual-warhead TTDDs.

For the selection of two drugs (warheads) for the novel “dual-warhead molecular missiles”, we focused on the combination of a microtubule-stabilizing agent (taxanes) and a topoisomerase I inhibitor (camptothecin and its analogues) in this study. One of the primary reasons for the selection of this combination is the fact that these two classes of anticancer drugs possess clearly different and well-defined molecular targets to investigate their synergistic or additive effects. Combinations of paclitaxel or docetaxel (taxane) with topotecan or irinotecan (camptothecin analogue) have been extensively studied for preclinical and clinical drug development, exhibiting synergistic or additive effects depending on the...
sequence of drug administration. For taxane anticancer agents, we have been developing highly potent next-generation taxoids which exhibit 2–3 orders of magnitude greater potency than paclitaxel and docetaxel against multidrug-resistant and paclitaxel-resistant cancer cell lines and tumors. Thus, we have used one of these next-generation taxoids for combination with camptothecin in this study. We report here the design, synthesis, and biological evaluations of novel tumor-targeting dual-warhead conjugates, bearing a next-generation taxoid, and camptothecin as warheads, and biotin as the tumor-targeting module (Figure 1).

We designed a versatile platform, consisting of 1,3,5-triazine as the key tripod splitter module, self-immolative linkers with tetraethylene glycol diamine spacers to improve water solubility, and a propargylamine arm for the attachment of second warhead module. Then, novel dual-warhead conjugate, was synthesized based on this platform using "click" chemistry to attach the camptothecin module with a self-immolative linker and a tetraethylene glycol diamine spacer (Figure 2). Another dual-warhead conjugate, bearing the camptothecin module with a simple ester linkage, was also synthesized to secure stepwise drug release (Figure 2). In addition to these dual-warhead conjugates, we also designed two more drug conjugates using the same platform, wherein one of the warheads was replaced with a surrogate, i.e., phenol. Thus, a conjugate with taxoid 1 and phenol, and another with camptothecin and phenol, were synthesized in order to properly compare the efficacy of dual-warhead vs single-warhead in the same conjugate scaffold (Figure 3).

**RESULTS AND DISCUSSION**

**Overexpression of Biotin Receptor in Cancer Cells.** All living cells depend on vitamins for survival, but cancer cells require certain vitamins substantially more than most normal cells do in order to sustain their rapid cell growth and enhanced proliferation. Thus, receptors for vitamins are overexpressed on the cell surfaces of cancer cells to maintain sufficient vitamin uptake. Therefore, these cell surface vitamin receptors serve as useful biomarkers for tumor-targeting drug delivery. The folate receptor (FR) has been studied extensively for its overexpression in cancer cells and solid tumors, as well as the relevant target for tumor-directed drug delivery, as exemplified by a highly promising SMDC, "vintafolide." In addition to the FR, recent studies have disclosed that the biotin receptor (BR) is overexpressed even more than the FR in a good number of cancer cell lines. Thus, the BR has emerged as a promising biomarker for tumor-targeted drug delivery, and we have successfully employed biotin as a tumor-targeting module for small-molecule-based as well as single-walled carbon nanotube-based drug conjugates. In these previous studies, we designed, synthesized, and used several fluorescent and fluorogenic probes for the BR by employing fluorescein isothiocyanate (FITC), fluorescein, and coumarin. Among those fluorescent probes, biotin-NH-NH-FITC was successfully used to verify efficient receptor-mediated endocytosis (RME), targeting the BR. In the present study, we designed fluorescent probe 7, which is more water-soluble than biotin-NH-NH-FITC, for the assessment of the BR overexpression level in several cancer cell lines to select appropriate cancer cell lines for evaluation of biotin–drug conjugates, targeting the BR.

Probe 7 was synthesized through coupling of FITC with N-biotinyl-NH-PEG3-(CH2)2NH2, which was readily prepared from biotin and commercially available 11-azido-3,6,9-trioxanuclidean-1-amine through amide coupling, followed by Staudinger reduction of the resulting azide (Scheme 1).

**Assessment of the Expression Level of Biotin Receptor in Cancer Cell Lines Using Fluorescent Probe 7.** Although Russell-Jones et al. reported a dozen cell lines overexpressing the BR (BR+), most of those cell lines are from murine cancers. Accordingly, we used probe 7 to assess the BR expression levels in several human cancer cell lines by means of flow cytometry and confocal fluorescence microscopy (CFM). Internalization of probe 7 into MX-1 (breast), MCF-7 (breast), ID8 (ovary), and W138 (lung fibroblast, normal) cell lines via RME was monitored at 0, 1, and 3 h periods. Previously studied murine leukemia cell lines L1210FR (BR+) and L1210 (BR−) were also used for comparison. As Figure 4 clearly indicates, the BR is highly expressed in MCF-7 and ID8 at the same level as known BR+++ cell line, L1210FR, while...
the expression level is a little lower in MX-1 (BR++). As anticipated, the BR expression level is negligible in human normal cell line, WI38, and known BR- cell line, L1210. The flow cytometry histograms as well as the corresponding CFM images of probe 7 internalization into ID8 and MCF-7 are shown in Figure 5 as typical examples.

We also performed the assessment of the BR expression level in additional human breast cancer cell lines and found that these cell lines also overexpress the receptor: BT-20 (BR+++), LCC6-WT (BR+++), LCC6-MDR (BR+), MDA-MB 231 (BR+++), and SkBr3 (BR+++). We found that the BR and FR are equally overexpressed in BT-20, LCC6-WT, MDA-MB 231, and SkBr3 cell lines, while the FR is more expressed than the BR in LCC6-MDR and MCF-7 cell lines (see Supporting Information).

**Synthesis of the Key Components of Tumor-Targeting Drug Conjugates with “Smart” Linkers.** We have developed novel mechanism-based “smart” disulfide linkers, which can be conjugated to a cytotoxic warhead on one end and a tumor-targeting module on the other end. These “smart” linkers were designed to be stable during circulation in bloodstream but readily cleavable inside the tumor microenvironment.20 These “smart” linkers consist of a 2-sulphydrylphenylacetic acid component and a sulfanylalkanoic acid component, and the former is connected to a cytotoxic agent and the latter to a tumor-targeting module or a splitter unit linked to a tumor-targeting module (see Figure 2). In this study, 4-sulfanylpentanoic acid was used as the sulfanylalkanoic acid component, since it has been shown37 that linkers bearing 4-disulfanylpentanoic acid moiety are more stable than disulfanylpropanoic acid moiety, which was used in our previous studies.11,20,32 This “smart” disulfide linker was designed to be self-immolative for releasing a cytotoxic warhead through disulfide−thiol exchange reaction with endogenous thiols, represented by glutathione (GSH), followed by facile benzothiolactonization.11,20,38 The phenyl moiety was placed to electronically direct the cleavage of the disulfide linkage by intracellular thiol and entropically drive thiolactonization.11,20 Thus, when the tumor-targeting module navigates the drug-conjugate to the target biotin receptors on the tumor surface, the whole conjugate is internalized via receptor-mediated endocytosis (RME).11,20 Then, an intracellular thiol-triggered cascade drug-release takes place through thiolactonization. Because it has been shown that the glutathione level in tumor tissues (2−8 mM) is more than 1000 times higher than that in bloodstream (1−2 μM),39,40 glutathione and other endogenous intracellular thiols would act as excellent tumor-specific agents to trigger the drug release.11,32,41

The preparation of bifunctional disulfide linker unit 11 and its use for the synthesis of coupling-ready (14T, 14P) or “click”-ready constructs (15C, 15P) of drug-linker conjugates with taxoid 1, camptothecin (CPT), and phenol are illustrated in Scheme 2. First, 4-sulfanylpentanoic acid (9) was prepared by ring-opening substitution of γ-valerolactone (8) with hydrobromic acid and thiourea, followed by basic hydrolysis.
The thiol–disulfide exchange reaction of 9 with 4,4′-dipyridinedisulfide, followed by TIPS protection of the carboxylic acid moiety, gave TIPS pyridinyldisulfanylpentanoate 10 in 92% yield for two steps. Another thiol–disulfide exchange reaction of 10 with 2-sulfanylphenylacetic acid afforded 11 in 68% yield. The coupling reactions of 11 with 1, CPT, and phenol in the presence of DIC and DMAP gave the corresponding drug–disulfide linker constructs 12T, 12C, and 12P, respectively, in 57–79% yields. TIPS groups of 12T, 12C, and 12P were removed with HF-pyridine to afford 13T, 13C, and 13P, respectively.

Then, the esterification of 13T and 13P with N-hydroxysuccinimide (HOSu) gave the corresponding coupling-ready taxoid-linker construct 14T and phenol-linker construct 14P, respectively, as activated esters in high yields. Finally, the DIC coupling of 13C and 13P with 1-amino-11-azido-3,6,9-trioxauundecane afforded the corresponding “click”-ready drug-linker constructs, 15C and 15P, respectively, in good yields.

To investigate the effects of sequential drug release on the potency of dual warhead drug conjugates, CPT-linker construct 18, bearing a simple ester linker, was designed to secure that the taxoid is released prior to CPT inside the cancer cell.

Figure 5. Internalization of probe 7 monitored by flow cytometry and CFM: (A) flow cytometry analysis of 7 in ID8 at 0 h (red, control), 1 h (green), and 3 h (blue); (B,C) CFM images of 7 in ID8 at 1 and 3 h, respectively; (D) flow cytometry analysis of 7 in MCF-7 at 0 h (red, control), 1 h (green), 3 h (blue); (E,F) CFM images of 7 in MCF-7 at 1 and 3 h, respectively.

Scheme 2

Reagents and conditions: (i) thiourea, 48% HBr, 100 °C, 24 h; (ii) KOH, H2O, 100 °C, 24 h, 68%; (iii) 4,4′-dipyridinedisulfide, CH3CH2OH, 80 °C, 2 h; (iv) i-Pr3SiCl, Et3N, CH2Cl2, 0 → 25 °C, 24 h, 92%; (v) (2-sulfanylphenyl)acetic acid, THF, −10 °C, 3 h, 68%; (vi) 1 or CPT or phenol, DIC, DMAP, CH2Cl2, 25 °C, 24 h, 57–79%; (vii) 70% HF in pyridine, CH3CN/pyridine (1:1), 0 → 25 °C, 16 h, 85–91%; (viii) HOSu, EDC, THF/pyridine, 25 °C, 36 h, 70–84%; (ix) H2N-PEG3-(CH2)2N3, DIC, CH2Cl2, 25 °C, 10 h, 56–71%.
Scheme 3 illustrates, succinic anhydride (16) was reacted with 
H$_2$N-PEG$_3$-(CH$_2$)$_2$N$_3$ to give 17, which was coupled to CPT in 
the presence of DIC and DMAP to give 18 in good yield.

Design and Synthesis of Tumor-Targeting Dual-Warhead Conjugates. Design and Synthesis of Dual-Warhead Conjugates and Their Single-Warhead Surrogates. Among possible tripod splitter modules, we chose 1,3,5-triazine for novel dual-warhead conjugates with three arms. Also, we decided to implement water-soluble linker components by incorporating poly(ethylene glycol) oligomers, i.e., PEG$_3$, as well as the use of “click chemistry” to introduce the third arm at the end of the synthesis. Thus, dual-warhead conjugates, 2 and 3, as well as their single-warhead surrogates, i.e., 4 and 5, were designed for construction by incorporating those components and strategies (see Figure 2).

The syntheses of conjugates 2, 3, and 4 are illustrated in Scheme 4. First, cyanuric acid (19) was reacted with N-Boc-ethylenediamine (1.0 equiv) in the presence of N,N-diisopropylethylamine (DIPEA) at 0 °C, followed by the subsequent substitution reaction with biotinyl-NH-PEG$_3$-NH$_2$ (6) (1.0 equiv) in the presence of DIPEA at 40 °C to give 20 in moderate yield for 2 steps. The reaction of 20 with excess propargylamine in the presence of DIPEA at 50 °C afforded fully functionalized triazine 21 in 69% yield. The deprotection of 21 with TFA gave 22, which was coupled with taxoid-linker-OSu$_{14T}$ in the presence of pyridine at 25 °C to afford 23 in 73% yield. Then, 23 was subjected to three “click” reactions with azides 15C, 18, and 15P in the presence of cupric sulfate and ascorbic acid at 25 °C to give the corresponding conjugates, 2, 3, and 4, respectively, in 73–92% yields.

For the synthesis of the single-warhead surrogate 5 bearing camptothecin, triazine 22 was reacted with phenol-linker-OSu$_{14P}$ in the presence of pyridine at 25 °C to afford 24 in 52% yield. Then, the “click” reaction of 24 with azide 15C under the standard conditions gave 5 in 54% yield (Scheme 5).

Effect of Equimolar Combinations of Taxoid 1 and Camptothecin on Cytotoxicity against Cancer Cell Lines. The effect of the combinations of taxoid 1 and camptothecin (1:1) on cytotoxicity was examined using three different timings for drug mixing against human breast carcinoma (MX-1 and MCF-7), murine ovarian (ID8), and murine leukemia (L1210FR) cell lines. Taxoid 1 and camptothecin were also used as a single agent for comparison. The results are summarized in Table 1. As Table 1 shows, the cytotoxicity of taxoid 1 was 148–846 times stronger than camptothecin against four cancer cell lines examined (entries 1 and 2). The most significant and interesting observation was the fact that the order of exposure to these two drugs, i.e., (a) simultaneous
exposure (entry 3), (b) taxoid first for 24 h, followed by camptothecin (entry 4), and (c) camptothecin first for 24 h, followed by taxoid (entry 5), exhibited a marked difference in the potency. When cancer cells were exposed to camptothecin first for 24 h, followed by equimolar concentrations of taxoid (entry 5), drastic increases in the IC₅₀ values as compared to those values for simultaneous exposure (entry 3) were observed. These IC₅₀ values (entry 5) were even higher than those for camptothecin alone except for that against the ID8 cell line (entry 2). The results clearly indicate that these two drugs act antagonistically in this case, and camptothecin appears to block the action of taxoid 1. This is probably ascribed to the cell cycle control, e.g., synchronization, by camptothecin, which causes detrimental effects on the action of taxoid 1.⁴² In sharp contrast, when cancer cells were exposed to equimolar mixture of the two drugs (entry 3) or to taxoid 1 first for 24 h, followed by equimolar concentrations of camptothecin (entry 4), IC₅₀ values were essentially the same as those for taxoid 1 alone within errors (entry 1). The results indicate that in these two cases, these two drugs act at least cooperatively and taxoid 1 might be potentiating camptothecin.²¹

**Biological Evaluation of Dual-Warhead Drug Conjugates.** *Internalization of Dual-Warhead Conjugate 2 by CFM.* We have shown the efficient internalization of fluorescence-labeled biotin probe 7 into several cancer cell lines via RME (see Figures 4 and 5). We have also previously reported the proofs for highly efficient RME of a biotin-linker-(taxoid-fluorescein) conjugate¹¹ and a (biotin)₅–SWNT–(taxoid-fluorescein)₅₇ conjugate.³² In this study, we examined the receptor-mediated internalization of dual-warhead conjugate 2 by taking advantage of the intrinsic fluorescence of camptothecin using CFM. ID8 and MCF-7 cancers were incubated with conjugate 2 (10 μM) at 37 °C for 10 h. After ample washing of the drug media, the cells were analyzed by CFM. As Figure 6 shows, the intense signature blue fluorescence of camptothecin was observed in both cancer cells, confirming the anticipated efficient internalization of conjugate 2.

![Figure 6](image-url)
Biological Evaluations of Dual-Warhead Conjugates.

Cytotoxicities of novel dual-warhead conjugates, 2 and 3, as well as their single-warhead surrogates, 4 and 5, were evaluated in two ways against four BR(+) cancer cell lines, MX-1, MCF-7, ID8 and L1210FR, as well as normal human lung fibroblast cell line, WI38, using the standard MTT assay. Results are summarized in Tables 2 and 3. Taxoid 1 and camptothecin were also examined for comparison (Table 2, entries 1 and 2, respectively).

In the first experiment, MX-1 (BR+), MCF7 (BR++), ID8 (BR+++), and L1210FR (BR++++) cancer cells were incubated with conjugate 2–5 for 72 h, and the corresponding IC50 values were determined. As Table 2 shows, the cytotoxicities of conjugates 2 and 3 based on their IC50 values were found to be in a range of 19–65 nM (entries 3 and 4), which makes a sharp contrast with that against normal cell line WI38 (IC50 742–868 nM) (entries 3 and 4). The results indicate that these conjugates were selectively internalized into BR(+) cancer cells via RME, and the drug was partially released inside cancer cells. Single-warhead surrogate 4 showed a similar potency as that of conjugate 3 (entry 5), while the other surrogate 5 did not exhibit appreciable cytotoxicity (MX-1 and ID8) or very weak potency (MCF-7, WI38), with L1210FR as an exception (entry 6). For conjugates 2–4 bearing taxoid 1 as a warhead, the cytotoxicity level appeared to be markedly reduced as compared to that for free taxoid 1. Because we have confirmed that these four cancer cell lines overexpress BR (see Figures 3 and 4) and also observed marked selectivity for BR+ cancer cell lines as compared to normal WI38 cell line (BR−), the internalization of conjugate 2–5 via RME should not be the major issue for these cancer cell lines. Thus, it is very likely that the level of GSH (and/or other endogenous thiols) is not sufficient to release the warhead(s) via disulfide linker cleavage under in vitro conditions. This situation makes a sharp contrast to that in vivo where there are sufficient endogenous thiols (e.g., GSH) in tumors, which are under hypoxic conditions.

To clarify these points, glutathione ethyl ester (GSH-OEt) (6 mol equiv to a conjugate) was added to the resuspended cancer cells after the cells had been incubated with a conjugate for 24 h, followed by thorough washing with phosphate-buffered saline (PBS), and further incubated for 48 h in the second experiment (Table 3). It should be noted that the resuspended cancer cells only included a conjugate internalized in the first 24 h period. As Table 3 shows, this protocol, including washing of cells and addition of GSH-OEt, did not make any appreciable difference in the cytotoxicity of these conjugates against WI38 normal fibroblast cells, based on the comparison of the results in Table 2 with those in Table 3. Conjugate 2 showed IC50 values of 3.2–9.8 nM against four BR+ cancer cell lines, but that against WI38 was 742 nM (entry 1). Thus, there is two order of magnitude difference in cytotoxicity between cancer cells and normal cells, which is unambiguously attributed to the highly efficient receptor-targeting by conjugate 2. There was also considerable difference in potency between conjugate 2 and conjugate 3 (entries 1 and 2). This difference is attributable to full release of both taxoid 1 and camptothecin in conjugate 2 via cleavage of a disulfide linkage versus insufficient release of camptothecin in conjugate 3, due to a more stable ester linkage between the linker and the drug, under the conditions examined. The results clearly indicate that there were synergistic or cooperative effects between taxoid 1 and camptothecin when these two drugs were delivered to cancer cells simultaneously and released inside cancer cells. Because surrogate 4, bearing taxoid 1 and phenol, exhibited a similar but systematically slightly weaker potency that of conjugate 3 (entry 3), it is reasonable to assume that the release of camptothecin occurred to some extent under the experimental conditions examined.

It appears that all four BR+ cancer cell lines are highly drug resistant to camptothecin and the potency was further decreased substantially when camptothecin and phenol were delivered to MX-1, MCF-7, and ID8 cells via RME and released inside cancer cells (Table 3, entry 4) as compared to that observed in the standard MTT assay of camptothecin (Table 2, entry 2). The results may indicate that phenol interferes with the action of camptothecin in those cancer cells. Interestingly, surrogate 5 exhibited higher potency than camptothecin against L1210FR leukemia cell line (IC50 319 nM in Table 2 and 177 nM in Table 3 for 5 vs 510 nM for CPT in Table 2).
results may suggest that phenol has cytotoxic effect on this leukemia cell line.

**CONCLUSION**

We have designed and successfully synthesized novel tumor-targeting dual-warhead conjugates, 2 and 3, which consist of taxoid 1 and camptothecin as two warheads, self-immolative disulfide linkers for drug release, biotin as the tumor-targeting moiety, and 1,3,5-triazine as the tripod splitter module. Conjugates 2 and 3 incorporated tetraethylene glycol diamine moieties to increase the water solubility of the conjugates. The functionalized 1,3,5-triazine-based TTDDS platform is versatile and applicable to a variety of two-drug combinations as well as the use of various tumor-targeting modules. For the introduction of the second warhead-linker module to the 1,3,5-triazine splitter module, a propargylamine spacer was introduced first to the splitter module. Then, its acetylene moiety was used for click reaction, with the second drug-linker construct bearing an ω-azido group to install the second warhead module as the third arm.

Since biotin was used as the tumor-targeting moiety, we screened the expression levels of the biotin receptor (BR) in various human breast cancer cell lines, MX-1, MCF-7, LCC6-WT, LCC6-MDR, MDA-MB 231, and SKBr3, which had not been reported previously in addition to the known BR-overexpressing cancer cell lines, ID8 (human ovary) and L1210FR (murine leukemia), by means of flow cytometry and confocal fluorescence microscopy (CFM). Then, we confirmed that these human breast cancer cell lines overexpress BR at variable levels. Also, we confirmed that there was no appreciable expression of BR in L1210 (murine leukemia) and WI38 (normal, human lung fibroblast) cell lines.

The potencies of conjugates, 2 and 3, were evaluated against MX-1, MCF-7, ID8, L1210FR, and WI38. It was found that the amount of endogenous thiols such as glutathione (GSH) was insufficient to trigger the drug release via cleavage of the self-immolative disulfide linkers under in vitro assay conditions (this is not an issue in vivo). Accordingly, glutathione ethyl ester (GSH-OEt) (6 equiv to a conjugate) was added after the cells were incubated with 2 or 3 for 24 h, thoroughly washed with saline (PBS), and resuspended. Then, the cells were further incubated for 48 h. Thus, only the conjugate molecules internalized into the cancer cells in the first 24 h were treated with GSH-OEt in this protocol. The addition of GSH-OEt showed remarkable effect, as anticipated, and conjugate 2 exhibited IC₅₀ values of 3.22–9.80 nM against all BR+ cancer cell lines, while that against normal cell line, WI38, was 705 nM. Thus, there were two orders of magnitude difference in selectivity to cancer cells, which was impressive. Also, the potency of 1 was 2–3 times as high as those of 3 and surrogate conjugate 4, which clearly indicates the synergistic or cooperative effect of the taxoid–camptothecin combination. It is noteworthy that this substantial enhancement in potency in this combination was observed only when these two drugs were internalized via RME.

The IC₅₀ values observed for conjugate 2 were 3.5–7.3 times larger than that of taxoid 1. The observed reduced potencies can be ascribed to insufficient internalization of 1 to those cells in the first 24 h, which can be attributed to (a) a saturation phenomenon for BR-binding and internalization, depending on the expression level of BR on the cell surface and (b) slow recycling of the receptors in the first 24 h. This is, however, not an issue in vivo.

Further studies along this line, as well as the evaluation of the efficacy of 2 and 3 in vivo are actively underway in our laboratory.

**EXPERIMENTAL SECTION**

**Caution.** Taxoids and camptothecins have been identified as potent cytotoxic agents. Thus, these drugs and all structurally related compounds and derivatives must be considered as mutagens and potential reproductive hazards for both males and females. Appropriate precautions, such as the use of gloves, goggles, labware, and fume hood, must be taken while handling the compounds at all times.

**General Methods.** ¹H and ¹³C NMR spectra were measured on a Varian 300, 400, or 500 MHz spectrometer or a Bruker 500 MHz NMR spectrometer. Melting points were measured on a Thomas–Hoeover capillary melting point apparatus and are uncorrected. TLC was performed on Sorbent Technologies aluminum-backed Silica G TLC plates (Sorbent Technologies, 200 μm, 20 cm × 20 cm), and column chromatography was carried out on silica gel 60 (Merck, 230–400 mesh ASTM). Purity was determined with a Shimadzu L-2010A HPLC HT series HPLC assembly, using a Kinetex PFP column (4.6 mm × 100 mm, 2.6 μm) with acetonitrile–water gradient solvent system. Two analytical conditions were used and noted as a part of the characterization data and purity for literature unknown compounds, i.e., HPLC (1), flow rate 0.4 mL/min, a gradient of 15 → 95% acetonitrile for the 0–12 min period; HPLC (2), flow rate 0.5 mL/min, a gradient of 5–95% acetonitrile for the 0–12 min period and 95% acetonitrile for the 11–15 min period. All new compounds had >95% purity. High resolution mass spectrometry analysis was carried out on an Agilent LC-UV-TOF mass spectrometer at the Institute of Chemical Biology and Drug Discovery, Stony Brook, NY or at the Mass Spectrometry Laboratory, University of Illinois at Urbana–Champaign, Urbana, IL.

**Materials.** The chemicals were purchased from Sigma-Aldrich, Fisher Scientific, and VWR International and used as received or purified before use by standard methods. Tetrahydrofuran was freshly distilled from sodium and benzophenone. Dichloromethane was also distilled immediately prior to use under nitrogen from calcium hydride. 10-Deacetylbaiccanin III was obtained from Indena, SpA, Italy. 2,2′-Dipyridyl disulfide,¹¹ (2-sulfanylphenyl)acetic acid,¹² taxoid 1,²⁵ 1-amino-11-azido-3,6,9-trioxadecane,¹⁶ N-Boc-ethylenediamine,¹⁶ N-biotinyl-PEG₃-diamine (6),¹⁶ and 4-sulfanylpenanoic acid (9)³⁷,¹¹ were prepared by literature methods. 3-(4,5-Dimethylisoozal-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. Biological materials including RPMI-1640 and DMEM cell culture media, fetal bovine serum, NuSerum, PenStrep, and TrypLE were obtained from Gibco and VWR International, and used as received for cell-based assays.

**Biotin-NH-PEG₃(CH₂)₃-NH-FITC (7).** To a solution of fluorescein isothiocyanate (FITC) (20 mg, 0.0502 mmol) and N-biotinyl-3,6,9-tioxadecamethylene-1,11-diamine (30 mg, 0.0717 mmol) in DMF (0.6 mL) was added disopropylethylamine (DIEPA) (14 μL). The mixture was allowed to react for 48 h at room temperature in the dark with stirring, and the reaction mixture was directly loaded onto a silica gel column. Purification of the reaction mixture by column chromatography on silica gel using 15% CH₂OH in CH₃Cl as eluent gave probe 7 (27 mg, 68% yield) as an orange solid; mp 191–192 °C. ¹H NMR (500 MHz, CD₃OD) δ 1.40 (m, 2H), 1.62 (m, 2H), 1.69 (m, 2H), 2.19 (t, J = 7.4 Hz, 2H), 2.67 (d, J = 7.2 Hz, 1H), 2.88 (dd, J = 5.0, 12.7 Hz, 1H), 3.15 (m, 1H), 3.32 (2H), 3.51 (t, J = 5.2 Hz, 2H), 3.59 (2H), 3.63 (m, 2H), 3.68 (s, 4H), 3.71 (t, J = 5.2 Hz, 2H), 3.82 (bs, 2H), 4.26 (dd, J = 4.5, 7.9 Hz, 1H), 4.45 (dd, J = 4.5, 7.9 Hz, 1H), 6.55 (dd, J = 2.4, 8.8 Hz, 2H), 6.67 (d, J = 2.4 Hz, 2H), 6.69 (d, J = 8.8 Hz, 2H), 7.16 (d, J = 8.3 Hz, 1H), 7.77 (d, J = 7.8 Hz, 1H), 7.95 (bs, 1H), 8.15 (d, J = 7.7 Hz, 1H). ¹³C NMR (125 MHz, CD₃OD) 7.21, 12.43, 15.43, 21.71, 24.82, 27.47, 27.73, 30.77, 34.72, 38.32, 39.05, 43.49, 45.89, 54.97, 59.61, 61.35, 68.58, 69.24, 69.32, 69.56, 69.61, 101.54, 109.74, 112.02, 123.99, 128.46, 140.43, 152.40, 153.32, 157.89, 164.11, 169.25, 174.15, 180.88. HRMS (TOF) for C₃₀H₄₉N₅O₁₀S₂+.
To a solution of 9 (709 mg, 5.29 mmol) in ethanol (20 mL) was added 2,2'-dipyridyl disulfide (7.0 g, 31.7 mmol) in ethanol (100 mL), and the mixture was refluxed at 80 °C for 2 h. The reaction mixture was cooled to 25 °C, and column chromatography on silica gel with hexanes/ethyl acetate (1:4) as eluent gave product by column chromatography on silica gel with hexanes/ethyl acetate (3:1) as eluent gave 10 (2.71 g, 92% yield for 2 steps), as a colorless oil. 1H NMR (600 MHz, CDCl3) δ 1.17 (d, J = 6.8 Hz, 3H), 1.49 (t, J = 7.5 Hz, 2H), 1.88 (m, 1H), 2.18 (m, 1H), 2.33 (m, 2H), 2.87 (m, 1H), 4.00 (dd, J = 4.5, 16.5 Hz, 1H), 4.02 (d, J = 22.0 Hz, 1H), 4.17 (dd, J = 9.2, 16.5 Hz, 1H), 5.21 (m, 2H), 5.38 (d, J = 17.5 Hz, 1H), 5.64 (dd, J = 17.2 Hz, 1H), 7.21 (d, J = 2.5 Hz, 1H), 7.26 (m, 4H), 7.66 (t, J = 7.5 Hz, 1H), 7.85 (t, J = 8.0 Hz, 1H), 7.92 (d, J = 8.0 Hz, 1H), 8.27 (d, J = 8.5 Hz, 1H), 8.35 (s, 1H). 13C NMR (125 MHz, CDCl3) δ 76.0, 118.55, 177.18, 20.39, 20.98, 22.55, 23.53, 30.94, 31.17, 32.58, 38.59, 45.82, 121.12, 127.11, 127.67, 127.77, 127.92, 128.05, 128.13, 128.23, 128.27, 128.42, 129.69, 129.72, 129.94, 130.04, 130.40, 130.54, 130.59, 130.86, 131.06, 132.56, 132.63, 134.65, 137.09, 137.44, 145.46, 146.20, 148.83, 152.30, 153.82, 157.27, 167.33, 169.91, 172.91. HRMS (TOF) for C59H52NO12S2Si+ calcd, 879.3364; found, 879.3368 (Δ = 0.3 ppm).

Phenol-(SS-Linker)-OTIPS (12P). To a solution of phenol (135 mg, 1.434 mmol), 11 (545 mg, 1.195 mmol), and DMAP (14 mg, 0.119 mmol) in CH2Cl2 (15 mL) was added DIC (210 µL, 1.314 mmol), and the mixture was allowed to react for 10 h at room temperature with stirring. The reaction was quenched with saturated NH4Cl (10 mL), and the reaction mixture was diluted with water (5 mL) and extracted with CH2Cl2 (20 mL × 3). The combined organic layers were dried over MgSO4 and concentrated in vacuo to afford a yellow oil. Purification of the crude product by column chromatography on silica gel with 3% ethyl acetate in hexanes as eluent gave 12P (305 mg, 79% yield) as a colorless oil. 1H NMR (400 MHz, CDCl3) δ 1.19 (d, J = 6.8 Hz, 3H), 1.48 (m, 1H), 1.53 (m, 1H), 1.77 (d, J = 8.2 Hz, 1H), 7.84 (d, J = 4.8 Hz, 1H). 1H NMR spectrum was consistent with the reported data. 41

To a solution of tricamptothecin (158 mg, 0.434 mmol), 10 (489 mg, 1.072 mmol), and DMAP (53 mg, 0.431 mmol) in CH2Cl2 (20 mL) was added DIC (210 µL, 1.314 mmol), and the mixture was allowed to react for 10 h at room temperature with stirring. The reaction was quenched with 10% citric acid (10 mL), and the reaction mixture was diluted with water (10 mL) and extracted with CH2Cl2 (20 mL × 3). The combined organic layers were washed with a saturated solution of CuSO4 (60 mL), water (60 mL), and brine (3 × 20 mL). The organic layer was dried over MgSO4 and concentrated in vacuo to afford a clear oil. Purification of the crude product by column chromatography on silica gel with hexanes/ethyl acetate (1:4) as eluent gave 13T (516 mg, 91% yield) as a white solid. 1H NMR (500 MHz, CDCl3) δ 1.02 (m, 2H), 1.16 (m, 2H), 1.18 (m, 3H), 1.29 (s, 6H), 1.39 (s, 9H), 1.70 (s, 3H), 1.75 (s, 3H), 1.76 (s, 3H), 1.80 (m, 2H), 1.89 (bs, 1H), 1.93 (s, 1H), 1.94 (m, 2H), 2.39 (m, 2H), 2.42 (s, 3H), 3.03 (m, 1H), 3.83 (m, 1H), 3.96 (dd, J = 1.7, 16.8 Hz, 1H), 4.15 (d, J = 1.7, 16.8 Hz, 1H), 4.20 (d, J = 8.4 Hz, 1H), 4.34 (d, J = 8.5 Hz, 1H), 4.45 (m, 1H), 5.00 (m, 1H), 5.18 (d, J = 7.1 Hz, 1H), 5.70 (d, J = 7.2 Hz, 1H), 6.25 (m, 1H), 6.32 (m, 1H), 7.33 (m, 3H), 7.51 (t, J = 7.8 Hz, 2H), 7.63 (t, J = 7.5 Hz, 1H), 7.84 (d, J = 7.8 Hz, 1H), 8.14 (d, J = 7.1 Hz, 2H), 8.66 (bs, 1H). HRMS (TOF) for C59H52NO12S2Si+ calcd, 879.3364; found, 879.3362 (Δ = 0.2 ppm).

Camoepthecin-Me-SS-Linker-COOH (13C). To a cooled solution of 12C (192 mg, 0.244 mmol) in CH3CN–pyridine (1:1) (50 mL) at 0 °C was added 70% HF/pyridine (5 mL) dropwise, and the mixture was allowed to gradually warm to room temperature over 5 h with stirring. The reaction was quenched with 10% citric acid (10 mL), and the reaction mixture was diluted with water (10 mL). The reaction mixture was extracted with ethyl acetate (20 mL × 2) and washed with saturated CuSO4 (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried over MgSO4 and concentrated in vacuo to afford a yellow oil. Purification of the crude product by column chromatography on silica gel with hexanes/ethyl acetate (1:4) as eluent gave 13C (516 mg, 91% yield) as a white solid. 1H NMR (500 MHz, CDCl3) δ 1.02 (m, 2H), 1.16 (m, 2H), 1.18 (m, 3H), 1.29 (s, 6H), 1.39 (s, 9H), 1.70 (s, 3H), 1.75 (s, 3H), 1.76 (s, 3H), 1.80 (m, 2H), 1.89 (bs, 1H), 1.93 (s, 1H), 1.94 (m, 2H), 2.39 (m, 2H), 2.42 (s, 3H), 3.03 (m, 1H), 3.83 (m, 1H), 3.96 (dd, J = 1.7, 16.8 Hz, 1H), 4.15 (d, J = 1.7, 16.8 Hz, 1H), 4.20 (d, J = 8.4 Hz, 1H), 4.34 (d, J = 8.5 Hz, 1H), 4.45 (m, 1H), 5.00 (m, 1H), 5.18 (d, J = 7.1 Hz, 1H), 5.70 (d, J = 7.2 Hz, 1H), 6.25 (m, 1H), 6.32 (m, 1H), 7.33 (m, 3H), 7.51 (t, J = 7.8 Hz, 2H), 7.63 (t, J = 7.5 Hz, 1H), 7.84 (d, J = 7.8 Hz, 1H), 8.14 (d, J = 7.1 Hz, 2H), 8.66 (bs, 1H). HRMS (TOF) for C59H52NO12S2Si+ calcd, 1136.4342; found, 1136.4365 (Δ = 2.0 ppm).
to a solution of succinic anhydride (183 mg, 1.834 mmol) and 1-ethyl-3-(3-dimethylamino)propyl)carbodiimide hydrochloride (EDC, 100.0 g, 0.485 mmol), and the mixture was allowed to react for 36 h at room temperature with stirring. The reaction mixture was cooled to 0 °C and saturated NH4Cl (10 mL), extracting the organic layers with saturated CuSO4 (50 mL), followed by 2% CH3OH in CH2Cl2. The organic layers were washed with saturated Na2SO4 and concentrated in vacuo and directly loaded on a silica gel column. Purification of the crude product by column chromatography on silica gel with 9:1 CH2Cl2/MeOH eluent gave 1T (389 mg, 72% yield) as an oil.

Phenol-(SS-Linker)-PEG3-(CH2)2N3 (15P). To a solution of 1TP (505 mg, 0.497 mmol) and N-n-hexyloxycarbimid (HOSu) (0.160 g, 1.32 mmol) in THF-pyridine (1:1) (90 mL) was added 1-ethyl-3-(3-dimethylamino)propyl)carbodiimide hydrochloride (EDC-HCl) (100.0 g, 0.485 mmol), and the mixture was allowed to react for 36 h at room temperature with stirring. The reaction mixture was cooled to 0 °C and saturated NH4Cl (10 mL), extracting the organic layers with saturated Na2SO4 and concentrated in vacuo and directly loaded on a silica gel column. Purification of the crude product by column chromatography on silica gel with 9:1 CH2Cl2/MeOH eluent gave 15T (377 mg, 73% yield) as a white solid.

Phenol-(SS-Linker)-NH-PEG3-(CH2)2N3 (15C). To a solution of 14T (185 mg, 0.345 mmol), and DMAP (105 mg, 0.834 mmol) in CH2Cl2 (60 mL) was added DCC (35 μL, 0.229 mmol), and the mixture was allowed to react for 4 h at room temperature with stirring. The reaction mixture was concentrated in vacuo and directly loaded on to a silica gel column. Purification of the reaction mixture by column chromatography on silica gel with 2% CH3OH in CH2Cl2 as eluent gave 15C (107 mg, 71% yield) as a pale-white solid; mp 104 °C. H NMR (500 MHz, CDCl3) δ 0.00 (d, J = 6.5 Hz, 1H), 1.74 (m, 4H), 2.04 (m, 1H), 2.21 (m, 1H), 2.33 (m, 1H), 2.82 (m, 1H), 3.34 (m, 1H), 3.40 (m, 2H), 3.49 (m, 2H), 3.61 (m, 2H), 3.69 (m, 10H), 4.04 (d, J = 16.5 Hz, 1H), 4.22 (dd, J = 4.0, 16.5 Hz, 1H), 5.31 (s, 2H), 5.42 (d, J = 17.3 Hz, 1H), 5.66 (dd, J = 14, 17.3 Hz, 1H), 5.98 (m, 1H), 7.25 (m, 3H), 7.72 (m, 2H), 7.89 (t, J = 7.4 Hz, 1H), 7.98 (d, J = 8.1 Hz, 1H), 8.30 (m, 1H), 8.42 (s, 1H). 13C NMR (100 MHz, CDCl3) δ 57.57, 121.47, 125.88, 127.92, 128.42, 129.41, 129.47, 134.70, 137.54, 144.55, 146.36, 147.61, 148.80, 152.27, 155.29, 167.32, 170.20, 171.14, 172.05, 172.12. HRMS (TOF) for C23H24NO6S2+ calcd, 383.1820; found, 383.1823 (Δ = 0.2 ppm).

Phenol-(SS-Linker)-PEG3-(CH2)2N3 (15P). To a solution of Phenol-(SS-Linker)-OSu (14P) (505 mg, 0.497 mmol) and 1-amino-11-azido-3,6,9-trioxaundecane (400 g, 1.834 mmol) in dichloromethane (3 mL) was added triethylamine (25 μL, 0.183 mmol), and the mixture was allowed to react for 16 h at room temperature with stirring. The reaction mixture was concentrated in vacuo to afford a yellow oil. Purification of the crude product by column chromatography on silica gel with 2% CH3OH in CH2Cl2 as eluent gave 15P (107 mg, 71% yield) as a pale-white solid; mp 104 °C. H NMR (500 MHz, CDCl3) δ 0.00 (d, J = 6.5 Hz, 1H), 1.74 (m, 4H), 2.04 (m, 1H), 2.21 (m, 1H), 2.33 (m, 1H), 2.82 (m, 1H), 3.34 (m, 1H), 3.40 (m, 2H), 3.49 (m, 2H), 3.61 (m, 2H), 3.69 (m, 10H), 4.04 (d, J = 16.5 Hz, 1H), 4.22 (dd, J = 4.0, 16.5 Hz, 1H), 5.31 (s, 2H), 5.42 (d, J = 17.3 Hz, 1H), 5.66 (dd, J = 14, 17.3 Hz, 1H), 5.98 (m, 1H), 7.25 (m, 3H), 7.72 (m, 2H), 7.89 (t, J = 7.4 Hz, 1H), 7.98 (d, J = 8.1 Hz, 1H), 8.30 (m, 1H), 8.42 (s, 1H). 13C NMR (100 MHz, CDCl3) δ 57.57, 121.47, 125.88, 127.92, 128.42, 129.41, 134.70, 137.54, 144.55, 146.36, 147.61, 148.80, 152.27, 155.29, 167.32, 170.20, 171.14, 172.05, 172.12. HRMS (TOF) for C23H24NO6S2+ calcd, 383.1820; found, 383.1823 (Δ = 0.2 ppm).
white solid; mp 100–101 °C. 1H NMR (500 MHz, CDCl3) δ 0.99 (t, 
J = 7.5 Hz, 3H), 2.20 (m, 1H), 2.29 (m, 1H), 2.51 (m, 2H), 2.88 (m, 
2H), 3.36–3.45 (m, 6H), 3.55 (m, 2H), 3.60 (m, 2H), 3.63 (m, 6H), 
5.28 (m, 2H), 5.38 (d, J = 17.1 Hz, 1H), 5.68 (d, J = 17.1 Hz, 
1H), 6.31 (bs, 1H), 7.30 (s, 1H), 7.66 (t, J = 7.6 Hz, 1H), 7.84 (t, J = 7.8 
Hz, 1H), 7.93 (d, J = 7.8 Hz, 1H), 8.25 (d, J = 8.6 Hz, 1H), 8.42 (s, 
1H). 13C NMR (125 MHz, CDCl3) δ 75.8, 29.20, 30.57, 37.70, 39.31, 
49.90, 50.63, 66.99, 69.67, 69.79, 70.16, 70.49, 70.54, 70.62, 76.12, 
96.39, 119.90, 128.00, 128.13, 128.42, 129.66, 130.64, 131.11, 146.08, 
146.19, 148.82, 152.34, 157.37, 164.56, 170.74, 178.71, 193. MS (TOF) 
for C27H20N2O4O6 calc. 649.2617; found, 649.2622 (Δ = 0 ppm).

HPLC (2): t = 10.8 min, purity >98%.

2-Chloro-4-(11-biotinylamino-3,6,9-trioxadecylidene)lamino-6-
(2-N-Boc-aminoethyldiene)-1,3,5-triazine (20). To a cooled 
solution of cyanuric chloride (19) (414 mg, 2.282 mmol) in THF 
(20 mL) at 0 °C was added a solution of N-Boc-ethylenediamine (366 
mg, 2.282 mmol) and DIPEA (0.6 mL, 3.432 mmol) in THF (2 mL), 
and the mixture was stirred for 3 h at room temperature. Then, a 
solution of TFA (794 mg, 6.57 mmol) in THF (2 mL) was added and 
refluxed for 16 h. The reaction mixture was concentrated in vacuo 
to afford a red oil. Purification of the crude product by column chromatography on silica gel (eluent: 7% CH2OH in CH2Cl2) gave 
20 (645 mg, 42% yield) as a white solid; mp 65–65 °C. 1H NMR 
(300 MHz, CDCl3) δ 1.34 (dt, J = 6.6, 12.6 Hz, 2H), 1.41 (s, 9H), 
1.61 (m, 1H), 2.21 (t, J = 7.2 Hz, 2H), 2.69 (d, J = 12.6 Hz, 1H), 2.91 (dd, 
J = 5.0, 12.6 Hz, 1H), 3.20 (m, 3H), 3.37 (m, 4H), 3.54 (m, 4H), 3.63 (m, 
10H), 4.25 (dd, J = 4.4, 8.0 Hz, 1H), 4.48 (dd, J = 4.4, 8.0 Hz, 1H).

13C NMR (100 MHz, CDCl3) δ 25.5, 27.4, 28.1, 28.4, 35.4, 
39.0, 39.7, 42.0, 40.3, 40.6, 54.3, 54.2, 55.6, 60.2, 62.0, 69.2, 69.8, 
70.19, 70.21, 78.7, 157.2, 164.7, 165.8, 168.1, 168.8, 174.7. HRMS (TOF) 
calc for C27H21ClN2O5S, 690.3159; found, 690.3157 (Δ = 
–0.3 ppm). HPLC (2): t = 10.0 min, purity >98%.

4-(11-Biotinylamino-3,6,9-trioxadecylidene)-6-(2-N-Boc-
aminoethyldiene)-2-propargylamino-lamino-1,3,5-triazine (21). To a solution of 20 (794 mg, 1.151 mmol) and DIPEA (0.4 mL, 2.300 mmol) 
in THF (12 mL) was added propargylamine (362 
20 μmol) in THF (2 mL), and the mixture was stirred for 12 h at 50 °C. Reaction was 
monitored by ESI-MS, and additional propargylamine was added as 
needed. The reaction mixture was cooled to 25 °C and concentrated in vacuo 
to afford an orange oil. Purification of the crude product by column chromatography on silica gel (eluent: 7% CH2OH in CH2Cl2) gave 
21 (560 mg, 69% yield) as a light-yellow solid; mp 66–68 °C. 1H NMR 
(300 MHz, CDCl3) δ 1.41 (m, 2H), 1.42 (s, 9H), 1.63 (m, 
4H), 2.20 (t, J = 7.5 Hz, 2H), 2.52 (t, J = 2.4 Hz, 1H), 2.69 (d, J = 12.6 
Hz, 1H), 2.91 (dd, J = 5.0, 12.6 Hz, 1H), 3.19 (m, 3H), 3.36 (t, J = 5.4 
Hz, 4H), 3.54 (t, J = 5.4 Hz, 4H), 3.64 (m, 10H), 4.10 (bs, 2H), 4.27 
(dd, J = 4.4, 8.0 Hz, 1H), 4.47 (dd, J = 4.4, 8.0 Hz, 1H). 13C NMR 
(125 MHz, CDCl3) δ 27.01, 28.49, 29.64, 29.39, 30.94, 36.89, 40.55, 
41.21, 41.50, 41.56, 57.16, 61.77, 63.52, 70.74, 71.19, 74.40, 71.44, 
71.73, 71.76, 158.76, 166.27, 170.36. HRMS (TOF) calc for 
C24H20ClN2O5S, 709.3814; found, 709.3814 (Δ = 0 ppm). HPLC (2): 
t = 10.0 min, purity >98%.

6-(2-Aminoethyldiene)-4-(11-biotinylamino-3,6-
9-trioxadecylidene)-2-propargylamino-lamino-1,3,5-triazine TFA 
Salt (22). To a solution of 21 (400 mg, 0.564 mmol) in CH2Cl2 
(6 mL) was added TFA (0.5 mL, 5.657 mmol), and the mixture was stirred at 
25 °C for 48 h. The reaction mixture was concentrated in vacuo to 
afford a yellow oil, which was triturated with either (20 mL) and the 
crude product (TFA salt) crushed out. The resulting solid was washed with ether (20 mL × 4) to afford 22 (408 mg, 100% yield) as a white 
product; 1H NMR (400 MHz, CDCl3) δ 1.42 (m, 2H), 1.63 (m, 
4H), 2.20 (t, J = 7.2 Hz, 2H), 2.54 (m, 1H), 2.69 (d, J = 12.6 Hz, 1H), 2.92 
(dd, J = 5.1, 12.6 Hz, 1H), 3.36 (t, J = 5.4 Hz, 4H), 3.54 (t, J = 5.4 Hz, 
4H), 3.64 (m, 10H), 4.10 (bs, 2H), 4.30 (dd, J = 4.4, 8.0 Hz, 1H), 4.49 
(dd, J = 4.4, 8.0 Hz, 1H), 4.68 (bs, 2H); 13C NMR (100 MHz, 
CDCl3) δ 25.46, 28.09, 28.34, 29.38, 35.33, 38.26, 38.94, 39.64, 39.95, 
55.60, 60.25, 61.99, 69.17, 69.80, 69.95, 70.16, 112.74, 115.24, 118.15, 
121.62, 161.58, 161.93, 164.68, 174.81. MS (ESI) m/z 6093 (M+).
Dual-Warhead Conjugate 3. To a solution of 23 (12 mg, 6.95 μmol), 18 (5 mg, 6.95 μmol), and ascorbic acid (1.4 mg, 7.70 μmol) in THF (0.5 mL) was added an aqueous solution of CuSO₄·5H₂O (2 mg, 7.70 μmol) (0.1 mL). The mixture was allowed to react for 10 h at room temperature with stirring, and the reaction mixture was diluted with water (5 mL) and extracted with CH₂Cl₂ (5 mL × 3). The combined organic layers were concentrated in vacuo to afford a milky white solid. The crude product was purified by column chromatography on silica gel (eluent: 10% MeOH in CH₂Cl₂) to afford 3 (12 mg, 54% yield) as an off-white solid; mp 143–145 °C. H NMR (500 MHz, CDCl₃) δ 0.89 (m, 2H), 0.98 (m, 3H), 1.01 (t, J = 7.5 Hz, 3H), 1.07 (m, 2H), 1.15 (s, 3H), 1.16 (s, 3H), 1.25 (m, 6H), 1.40 (m, 2H), 1.40 (s, 9H), 1.60 (m, 4H), 1.64 (s, 3H), 1.73 (s, 3H), 1.74 (m, 3H), 1.75 (s, 3H), 1.86 (m, 2H), 1.90 (m, 3H), 2.01 (m, 1H), 2.20 (m, 7H), 2.38 (s, 3H), 2.42 (m, 2H), 2.53 (m, 1H), 2.68 (d, J = 12.8 Hz, 1H), 2.88 (m, 3H), 3.15 (m, 1H), 3.17 (bs, 2H), 3.34 (m, 3H), 3.39 (bt, J = 5.4 Hz, 2H), 3.43 (m, 4H), 3.52 (m, 6H), 3.62 (m, 10H), 3.73 (m, 2H), 3.80 (m, 1H), 3.83 (t, J = 7.3 Hz, 2H), 4.02 (d, J = 16.7 Hz, 1H), 4.06 (m, 1H), 4.20 (m, 2H), 4.28 (m, 2H), 4.38 (m, 1H), 4.48 (m, 2H), 4.54 (d, J = 6.7 Hz, 3H), 1.43 (t, J = 7.7 Hz, 2H), 7.18 (m, 2H), 7.20 (m, 2H), 7.34 (m, 4H), 7.40 (d, J = 7.4 Hz, 1H), 7.83 (d, J = 7.7 Hz, 4H), 8.35 (bs, 1H). 13C NMR (125 MHz, CDCl₃) δ 19.75, 25.47, 28.09, 28.39, 31.72, 32.27, 35.73, 38.74, 39.00, 39.48, 46.50, 55.62, 61.97, 69.18, 69.55, 69.89, 70.18, 70.21, 120.99, 125.64, 127.67, 128.05, 128.27, 129.95, 130.34, 130.75, 131.21, 131.60, 130.97, 131.38, 146.86, 150.95, 164.73, 170.23, 174.75. HRMS (TOF): for C₁₁₆H₁₅₂N₁₇O₃₁S₁₃, found, 2973.987 (C₂H₅NO₃S₁₃) calculated, 2973.987 (C₂H₅NO₃S₁₃).

Dual-Warhead Conjugate with Camptothecin and a Surrogate Warhead, 4. To a solution of 23 (20 mg, 11.6 μmol), 14P (6.7 mg, 11.6 μmol), and ascorbic acid (2 mg, 12.7 μmol) in THF (1 mL) was added an aqueous solution of CuSO₄·5H₂O (3.2 mg, 12.7 μmol) (0.25 mL). The mixture was stirred at 25 °C for 15 h, and the reaction mixture was lyophilized. Purification of the lyophilized reaction mixture by column chromatography on silica gel (eluent: 10% MeOH in CH₂Cl₂) gave 4 (21 mg, 79% yield) as a colorless solid; mp 125–127 °C. H NMR (500 MHz, CDCl₃) δ 0.90 (m, 3H), 0.92 (m, 3H), 0.97 (m, 3H), 0.98 (m, 3H), 1.01 (t, J = 7.5 Hz, 3H), 1.07 (m, 2H), 1.15 (s, 3H), 1.16 (s, 3H), 1.25 (m, 6H), 1.40 (m, 2H), 1.40 (s, 9H), 1.60 (m, 4H), 1.64 (s, 3H), 1.73 (s, 3H), 1.74 (m, 3H), 1.75 (s, 3H), 1.86 (m, 2H), 1.90 (m, 3H), 2.01 (m, 1H), 2.20 (m, 7H), 2.38 (s, 3H), 2.42 (m, 2H), 2.53 (m, 1H), 2.68 (d, J = 12.8 Hz, 1H), 2.88 (m, 3H), 3.15 (m, 1H), 3.17 (bs, 2H), 3.34 (m, 3H), 3.39 (bt, J = 5.4 Hz, 2H), 3.43 (m, 4H), 3.52 (m, 6H), 3.62 (m, 10H), 3.73 (m, 2H), 3.80 (m, 1H), 3.83 (t, J = 7.3 Hz, 2H), 4.02 (d, J = 16.7 Hz, 1H), 4.06 (m, 1H), 4.20 (m, 2H), 4.28 (m, 2H), 4.38 (m, 1H), 4.48 (m, 2H), 4.54 (d, J = 6.7 Hz, 3H), 1.43 (t, J = 7.7 Hz, 2H), 7.18 (m, 2H), 7.20 (m, 2H), 7.34 (m, 4H), 7.40 (d, J = 7.4 Hz, 1H), 7.83 (d, J = 7.7 Hz, 4H), 8.35 (bs, 1H). Cell Culture. All cell lines were obtained from ATCC unless otherwise noted. Cells were cultured in RPMI-1640 cell culture medium (Gibco) or DMEM culture medium (Gibco), both supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS), 5% (v/v) NuSerum, and 1% (v/v) penicillin and streptomycin (PenStrep) at 37 °C in a humidified atmosphere with 5% CO₂, L1210 and L1210FR (a gift from Dr. Gregory Russell-Jones, Access Pharmaceuticals Pty Ltd., Australia) cells were grown as a suspension in supplemented RPMI-1640. LCC6-MDR, LCC6-WT, MCF-7, MDA MB 231, MX-1, and ID8 cells were cultured as monolayers on 100 mm tissue culture dishes in a supplemented RPMI-1640 cell culture medium, and WT-38 as a monolayer in a supplemented DMEM cell culture medium. Cells were harvested, collected by centrifugation at 850 rpm for 5 min, and resuspended in fresh culture medium. Cell cultures were routinely divided by treatment with trypsin (Trypsin, Gibco) as needed every 2–4 days and collected by centrifugation at 850 rpm for 5 min, and then resuspended in fresh cell culture medium.
containing varying cell densities for subsequent biological experiments and analysis.

**Incubation of Cells with Fluorescent Probe 7.** Cell suspensions (3 mL) at 5 × 10^5 cells/mL were added to each individual well of 96-well plates and subsequently incubated overnight in the appropriate cell culture media. The cell culture media was replaced with 10 mM solutions of 7 in cell culture media (3 mL). The cells were then incubated with 7 for 1 and 3 h at 37 °C. In the case of leukemia cell lines (L1210 and L1210-FPR), probe 7 (1 mM) in DMSO (30 μM) was added directly into fresh cell suspensions to give the final concentration of 10 μM, and incubated for similar time intervals. After incubation, the cells were removed by treating with trypsin (as needed), washed twice with PBS, collected by centrifugation, and resuspended in 150 μL for imaging.

**Flow Cytometry Analysis of the Cells Treated with Probe 7.** Flow cytometry analysis of the cells treated with probe 7 was performed with a flow cytometer, FACSCalibur, operating at a 488 nm excitation wavelength and detecting 530 nm emission wavelength with a 30 nm bandpass filter (515–545 nm range). Cells treated as described above were resuspended in 0.5 mL of PBS. Approximately 10000 cells were counted for each experiment using CellQuest 3.3 software (Becton Dickinson), and the distribution of FITC fluorescence was analyzed using WinMDI 2.8 freeware (Joseph Trotter, Scripps Research Institute).

**Confocal Microscopy Imaging of the Cells Treated with Probe 7 and Conjugate 2.** Cells treated as described above were resuspended in 150 μL of PBS after each experiment and dropped onto an uncoated microscope slide with coverslip (MatTek Corp.). Confocal fluorescence microscopy (CFM) experiments were performed using a Zeiss LSM 510 META NLO two-photon laser scanning confocal microscope system, operating at a 488 nm excitation wavelength and at 527 ± 23 nm detecting emission wavelength using a 505–550 nm bandpass filter. Images were captured using a Zeiss Achromat 63x/1.2 water (corr) objective. Images for conjugate 2 (Figure 6) were obtained using the camera mode with a filter set of 350 ± 25 nm excitation wavelength and 420 nm long pass emission wavelength. Acquired data were analyzed using LSM 510 META software.

**In Vitro Cytotoxicity Assays.** The cytotoxicities of taxoid 1 and camptothecin were evaluated for single-agent administrations as well as for time-dependent administrations of equimolar combinations against various cancer cell lines by means of the standard quantitative colorimetric MTT assay.12 The inhibitory activity of each compound is represented by the IC_{50} value, which is defined as the concentration required for inhibiting 50% of the cell growth. Cells were harvested, collected, and resuspended in 100 μL of cell culture medium (RPMI-1640) at a concentrations ranging from 0.5 to 1.5 × 10^5 cells per well in a 96-well plate. For adhesive cell types, cells were allowed to adhere to the 96-well plate. After the addition of the first drug solution, the cells were incubated at 37 °C for 24 h before drug treatment. Two sets of serial dilutions of equimolar taxoid and camptothecin in sterile DMSO were added using the cell culture media. The cell culture media was replaced with 100 μL of PBS after each experiment and dropped onto an uncoated slide with coverslip (MatTek Corp.). Cytotoxicities (IC_{50}, nM) for conjugates 2–5 were evaluated in a similar manner. In DMSO stock solutions, each conjugate was diluted to a series of concentrations in cell culture medium to prepare test solutions. After removing the old medium, these test solutions were added to the wells in the 96-well plate to give the final concentrations ranging from 0.5 to 5000 nM (100 μL), and the cells were subsequently cultured at 37 °C for 24 h. Then, the second drug solution was added, followed by incubation for additional 48 h at 37 °C.

**ACKNOWLEDGMENTS**

This research was supported by a grant from the National Institutes of Health (CA103314 to I.O.). The authors are grateful to Dr. Anne Savitt and Rebecca Roweuhl for their valuable help with cell culture preparations at the Cell Culture and Hybridoma Facility of Stony Brook University, as well as Dr. Guo-Wei Tian at the Central Microscopy Imaging Center of Stony Brook University for his technical assistance with confocal fluorescence microscopy. We thank Dr. Béla Ruzsicska for his helpful discussions on mass spectrometric analyses.

**ABBREVIATIONS USED**

ABC, ATP-binding cassette; ADC, antibody drug conjugate; BR, biotin receptor; BT-20, human breast carcinoma cell line; CEM, confocal fluorescence microscopy; CPT, camptothecin; DIC, N,N-diisopropylcarbodiimide; Dipe, N,N-diisopropylthalamine; DMEM, Dulbecco’s Modified Eagle Medium; DW, dual-warhead; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; FITC, fluorescein isothiocyanate; FR, folate receptor; GSH, glutathione; GSH-OEt, glutathione ethyl ester; HOSt, N-hydroxysuccinimide; ID8, murine ovarian cancer cell line; LCC6, human breast carcinoma cell line; L1210, murine leukemia cell line; L1210FR, engineered murine leukemia cell line expressing folate receptors; MDA-MB 231, human breast carcinoma cell line; MCF-7, human breast carcinoma cell line; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MX-1, human breast carcinoma cell line; RME, receptor-mediated endocytosis; RPMI-1640, Roswell Park Memorial Institute medium; SkBr3, human breast
adenocarcinoma cell line; SMDC, small-molecule drug conjugate; SW, surrogate-warhead; SWNT, single-walled carbon nanotube; TTDDS, tumor-targeted drug delivery system

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