Journal of Medicinal Chemistry

Article

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

Jacob G. Vineberg, Edison S Zuniga, Anushree Kamath, Ying-Jen Chen, Joshua D Seitz, and Iwao Ojima

J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/jm500631u • Publication Date (Web): 05 Jun 2014

Just Accepted

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

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

Jacob G. Vineberg[†], Edison S. Zuniga[†], Anushree Kamath[‡], Ying-Jen Chen[†], Joshua D. Seitz[†], Iwao Ojima[†,‡,*1]

[†]*Department of Chemistry, Stony Brook University, Stony Brook, NY 11794-3400*

[‡]*Institute of Chemical Biology and Drug Discovery, Stony Brook University, Stony Brook, NY 11794-3400*

RECEIVED DATE (to be automatically inserted after your manuscript is accepted if required according to the journal that you are submitting your paper to)

* To whom correspondences should be addressed. Phone 631-632-1339; fax 631-632-7942; e-mail, iwao.ojima@stonybrook.edu

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 re-suspension 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.

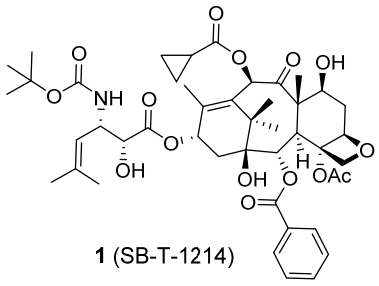
INTRODUCTION

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.[1] 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.[1, 2]

Despite the profound advantages of combination chemotherapy, the lack of tumor-specificity continues to be a serious issue for cancer treatment. In the last decade, significant advancement has been made on the development of tumor-targeted drug delivery systems (TTDDSs), especially for monoclonal antibody-drug conjugates (ADCs),[3-9] and small molecule drug conjugates (SMDCs).[7, 10-15] 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.[10, 16] Thus, there is an unmet need for the development of efficacious TTDDSs amenable to anticancer drug combinations. Since a TTDDS 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,[17-19] which is one of the beneficial features of TTDDSs.

We set out to design a versatile TTDDS platform for two-drug combinations, and construct "dual-warhead guided molecular missiles[20]" for tumor-targeted combination chemotherapy. Such a TTDDS would have only single pharmacokinetics for the delivery of two different drugs to tumor since 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 analogs) 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 analog) have been extensively studied for preclinical and clinical drug development, exhibiting synergistic or additive effects depending on the sequence of drug administration.[21-24] 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.[25-30] 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, **1** (SB-T-1214),[28] and camptothecin as warheads, and biotin as the tumor-targeting module.



1 (SB-T-1214) camptothecin

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,

2 (DW-1), 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, **3** (DW-2), 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, **4** (SW-Tax) and another with camptothecin and phenol, **5** (SW-CPT) were synthesized in order to properly compare the efficacy of dual-warhead vs single-warhead in the same conjugate scaffold (Figure 3).

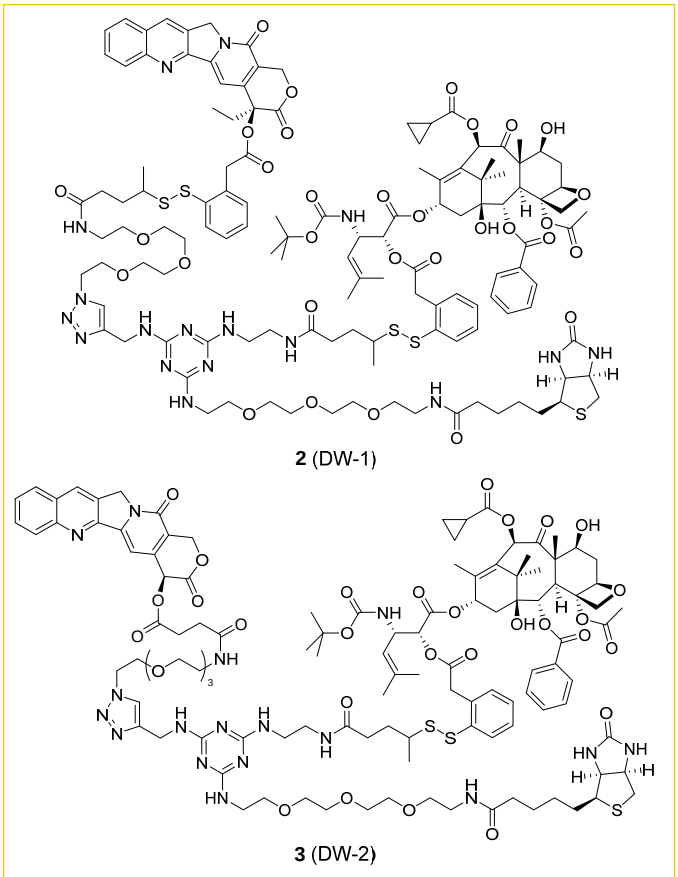


Figure 2. Chemical structures of dual-warhead conjugates **2** and **3**

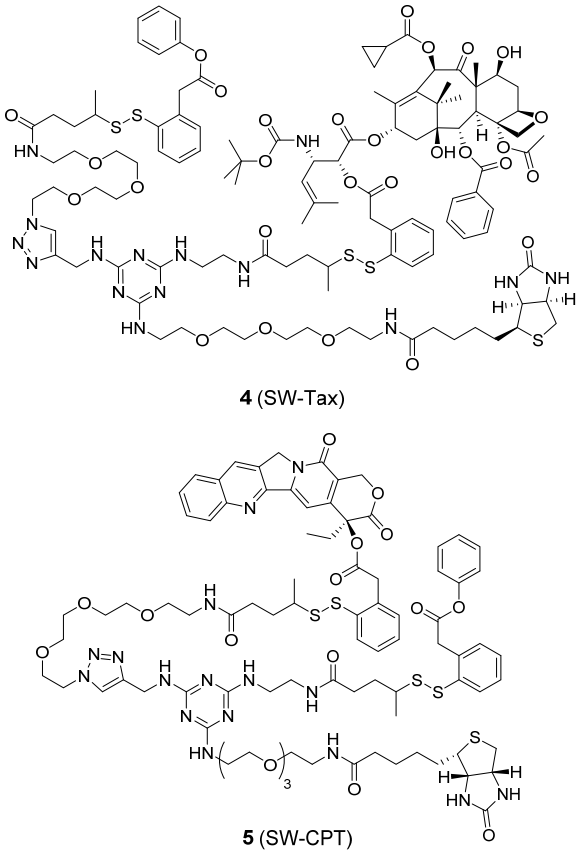


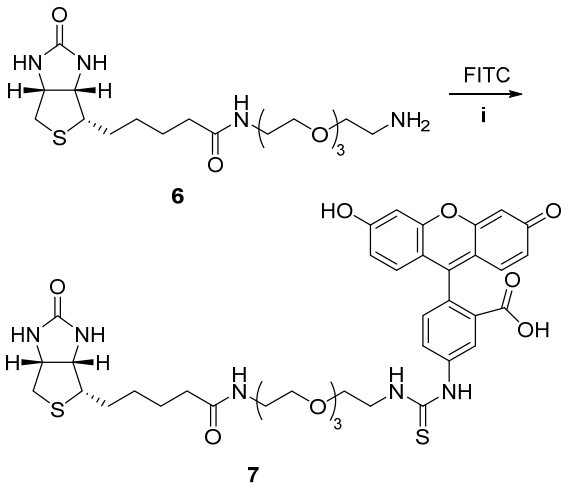
Figure 3. Chemical structures of conjugates **4** and **5** bearing a surrogate warhead.

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.[12, 15, 31] 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,[12] as exemplified by a highly promising SMDC, "vintafolide".[10] 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.[15] 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.[11, 20, 32, 33] 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.[11, 20, 32-34] Among those fluorescent probes, biotin-NHNH-FITC was successfully used to verify efficient receptor-mediated endocytosis (RME), targeting the BR.[11] In the present study, we designed fluorescent probe **6**, which is more water-soluble than biotin-NHNH-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 **6** was synthesized through coupling of FITC with *N*-biotinyl-NH-PEG_3-$(CH_2)_2NH_2$, which was readily prepared from biotin and commercially available 11-azido-3,6,9-trioxaundecan-1-amine through amide coupling, followed by Staudinger reduction of the resulting azide (Scheme 1).[35, 36]

Scheme 1[a]



[a]Reagents and conditions: (i) DIPEA, DMF, dark, 25 °C, 48 h, 68%.

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.[15] 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 WI38 (lung fibroblast, normal) cell lines via RME was monitored at 0, 1 and 3 h periods. Previously studied[15] 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.

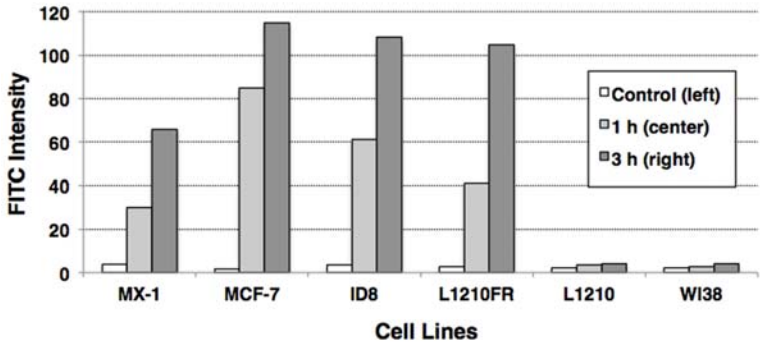


Figure 4. Internalization of probe **7** into various cell lines at 0, 1 and 3 h at 37 ºC. Fluorescence intensity is a geometric mean of values obtained from a flow cytometry histogram for each cell line, which is provided in the Supporting Information.

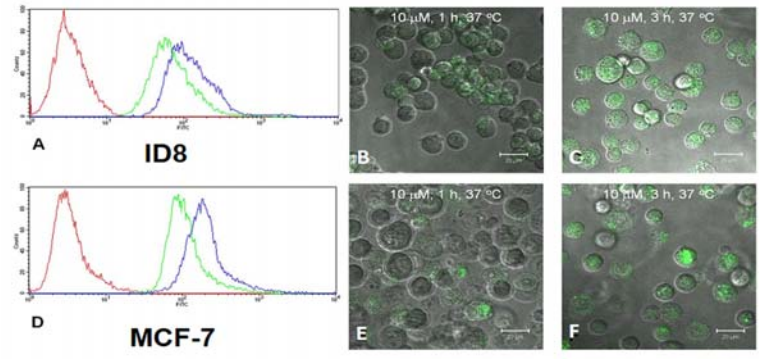


Figure 5. Internalization of probe **7** monitered by flow cytometry and CFM: (A) flow cytometry analysis of **7** in ID8 at 0 h (red, control), 1 h (green), and 3 h (blue); (B) and (C) CFM images of **7** in ID8 at 1 h and 3 h, respectively; (D) flow cytometry analysis of **7** in MCF-7 at 0 h (red,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

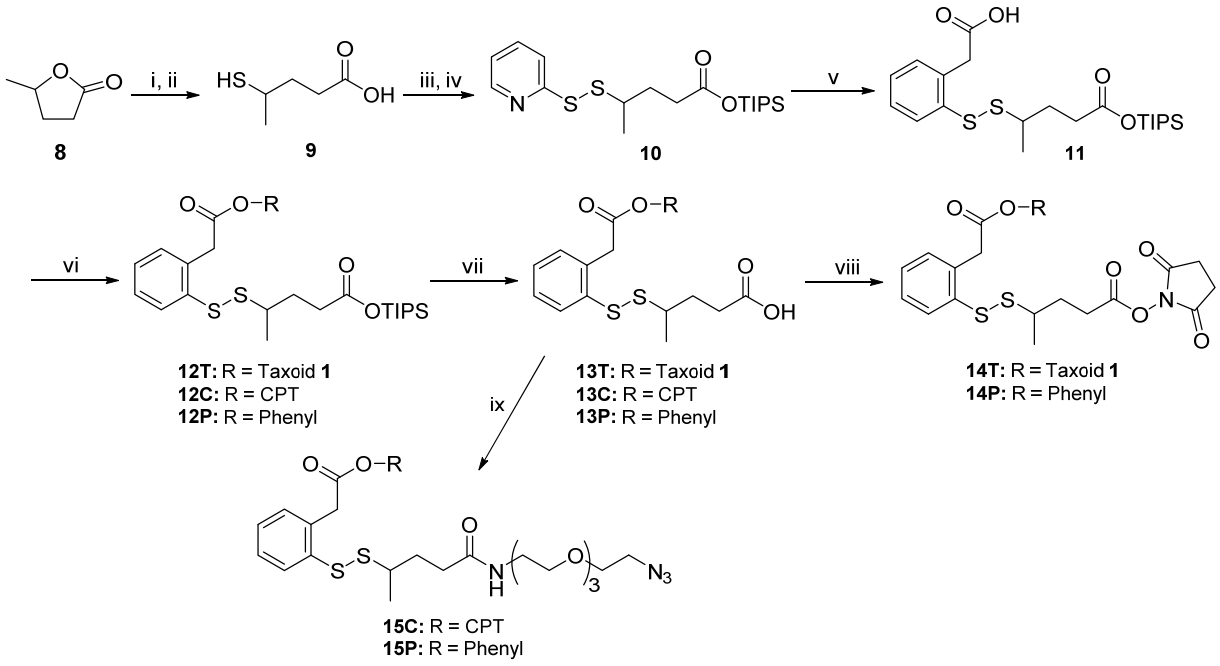
We also performed the assessment of the BR expression level in additional human breast cancer cell lines and found that these cell lines also overexpress the receptor: BT-20 (BR^{++}), LCC6-WT (BR^{++}), LCC6-MDR (BR^{+}), MDA-MB 231 (BR^{++}), and SkBr3 (BR^{++}) (see Supporting Information). It was also 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 blood stream, but readily cleavable inside the tumor microenvironment.[20] These "smart" linkers consist of a 2-sulfhydrylphenylacetic 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 shown[37] 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 thiolactionization.[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. Since it has been shown that the glutathione level in tumor tissues (2-8 mM) is more than 1,000 times higher than that in blood stream (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.

Scheme 2[a]

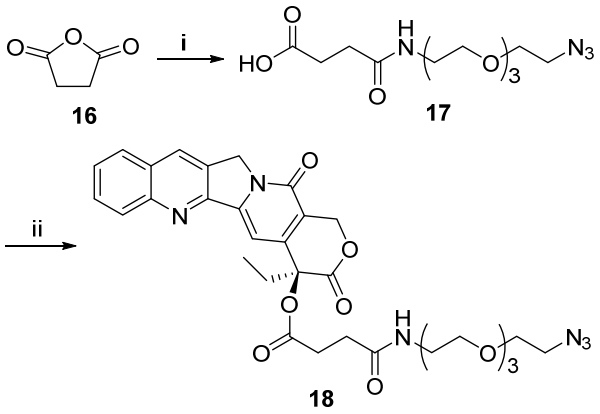


[a]Reagents and conditions: (i) thiourea, 48% HBr, 100 °C, 24 h; (ii) KOH, H_2O, 100 °C, 24 h, 68%; (iii) 4,4'-dipyridinedisulfide, CH_3CH_2OH, 80 °C, 2 h; (iv) *i*-Pr_3SiCl, Et_3N, CH_2Cl_2, 0→25 °C, 24 h, 92%; (v) (2-sulfanylphenyl)acetic acid, THF, -10 °C, 3 h, 68%; (vi) **1** or CPT or phenol, DIC, DMAP, CH_2Cl_2, 25 °C, 24 h, 57-79%; (vii) 70% HF in pyridine, CH_3CN/pyridine (1:1), 0→25 °C, 16 h, 85-91%; (viii) HOSu, EDC, THF/pyridine, 25 °C, 36 h, 70-84%.; (ix) H_2N-PEG_3-$(CH_2)_2N_3$, DIC, CH_2Cl_2, 25 °C, 10 h, 56-71%.

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-trioxaundecane afforded the corresponding "click"-ready drug-linker constructs, **15C** and **15P**, respectively, in good yields.

Scheme 3[a]



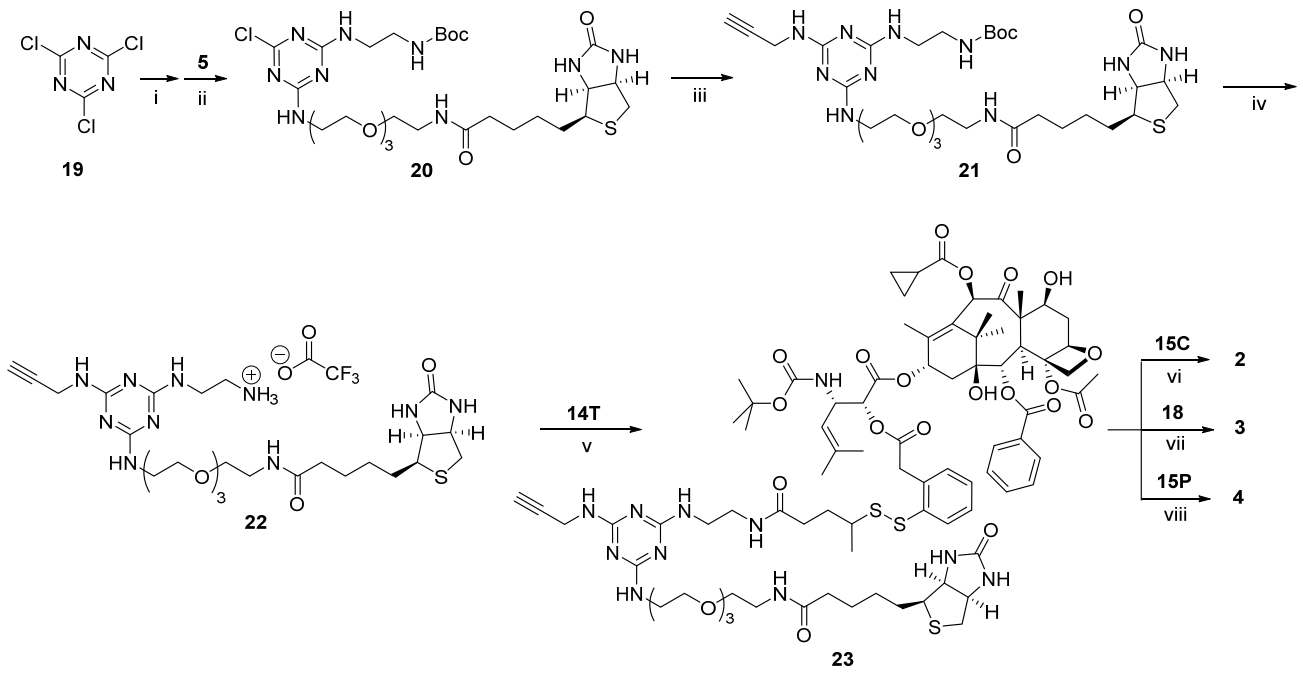
[a]Reagents and conditions: (i) H_2N-PEG_3-$(CH_2)_2N_3$, Et_3N, CH_2Cl_2, 25 °C, 16 h, 67%; (ii) CPT, DIC, DMAP, CH_2Cl_2, 25 °C, 36 h, dark, 72%.

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 polyethylene 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 ºC, followed by the subsequent substitution reaction with biotinyl-NH-PEG$_3$-NH$_2$ (**6**) (1.0 equiv.) in the presence of DIPEA at 40 ºC to give **20** in moderate yield for 2 steps. The reaction of **20** with excess propargylamine in the presence of DIPEA at 50 ºC 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 ºC 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 ºC to give the corresponding conjugates, **2**, **3** and **4**, respectively, in 73-92% yields.

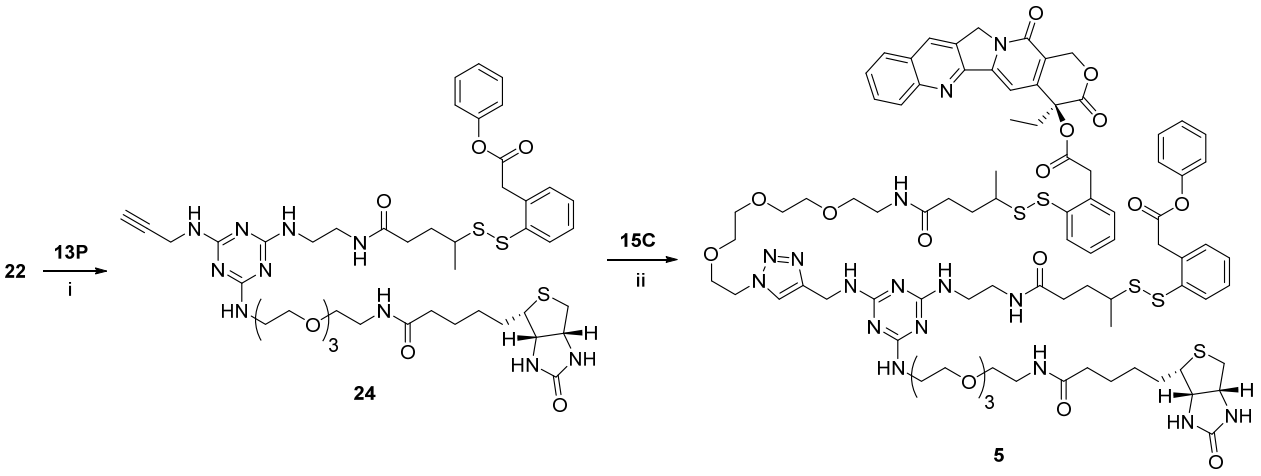
Scheme 4[a]



[a]Reagents and conditions: (i) *N*-Boc-ethylenediamine, DIPEA, 0 ºC; 3 h (ii) DIPEA, 40 ºC, 16 h, 43% for two steps; (iii) propargylamine, DIPEA, THF, 50 ºC, 12 h, 69%; (iv) TFA, CH_2Cl_2, 25 ºC, 24 h, 99%; (v) CH_2Cl_2/pyridine, 25 ºC, 36 h, 73%; (vi) $CuSO_4$•$5H_2O$, ascorbic acid, THF/H_2O/CH_2Cl_2, 25 ºC, 14 h, dark, 92%; (vii) $CuSO_4$•$5H_2O$, ascorbic acid, THF/H_2O/CH_2Cl_2, 25 ºC, 10 h, dark, 54%; (viii) $CuSO_4$•$5H_2O$, ascorbic acid, THF/H_2O, 25 ºC, 15 h, 79%.

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 ºC 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).

Scheme 5



[a]Reagents and conditions: (i) CH_2Cl_2/pyridine, 25 ºC, 10 h, 52%; (ii) $CuSO_4$•$5H_2O$, ascorbic acid, THF/H_2O/CH_3OH, 25 ºC, 4 h, dark, 54%.

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 **1** (entry 5), drastic increases in the IC_{50} values as compared to those values for simultaneous exposure (entry 3) were observed. These IC_{50} 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), IC_{50} 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]

Table 1. Cytotoxicities (IC_{50}, nM) of Taxoid 1, Camptothecin (CPT), and Their Equimolar Combinations against Cancer Cell Lines Overexpressing Biotin Receptor (BR+)

Entry	Drug	Time	MX-1[a]	MCF-7[b]	ID8[c]	L1210FR[d]
1[e]	**1**	72 h	2.01 ± 0.52	0.44 ± 0.18	1.11 ± 0.26	2.14 ± 1.30
2[e]	CPT	72 h	1,700 ± 200	65.1 ± 12.3	474 ± 101	510 ± 139
3[f]	**1**:CPT (1:1)	72 h	1.88 ± 0.14	0.40 ± 0.09	1.02 ± 0.17	4.14 ± 0.46
4[g]	**1** then CPT	24 h 48 h	1.98 ± 0.29	0.33 ± 0.10	0.96 ± 0.14	3.51 ± 0.27
5[h]	CPT then **1**	24 h 48 h	>5,000	79.2 ± 12.4	110 ± 12	949 ± 116

[a]Human breast carcinoma cell line (BR+); [b]Human breast carcinoma cell line (BR+); [c]Murine ovarian carcinoma cell line (BR+); [d]Murine lymphocytic leukemia cell line (BR+); [e]Cells were incubated with a drug for 72 h at 37 °C; [f]Cells were incubated with an equimolar mixture of two drugs for 72 h at 37 °C; [g]Cells were incubated with **1** at a given concentration for 24 h at 37 °C. Then, an equimolar amount of CPT was added and the cells were incubated for additional 48 h at 37 °C. [h]Cells were incubated with CPT at a given concentration for 24 h at 37 °C. Then, an equimolar amount of **1** was added and the cells were incubated for additional 48 h at 37 °C.

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) conjugate[11] and a $(biotin)_n$-SWNT-$(taxoid\text{-}fluorescein)_m$ 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**.

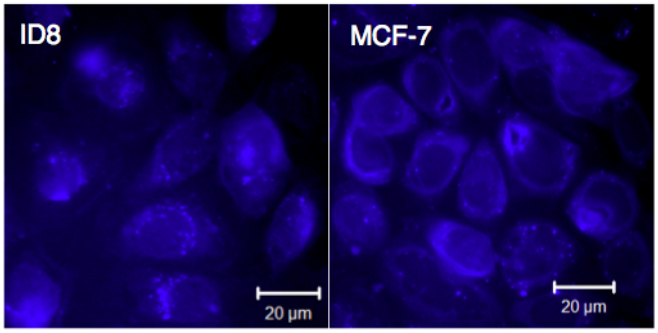


Figure 6. Confocal fluorescence microscopy images showing internalization of conjugate **2** in ID8 (left) and MCF-7 (right) after incubation at 37 °C for 10 h.

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 IC_{50} values were determined. As Table 2 shows, the cytotoxicities of conjugates **2** and **3** based on their IC_{50} 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 (IC_{50} 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**. Since 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.

In order to clarify these points, glutathione ethyl ester (GSH-OEt) (6 mole equiv. to a conjugate) was added to the re-suspended 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 re-suspended 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 IC_{50} 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. Since surrogate **4**, bearing taxoid **1** and phenol, exhibited a similar but systematically slightly weaker potency that that of conjugate **3** (entry 3), it is reasonable to assume that the release of campothecin 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 (IC_{50} 319 nM in Table 2 and 177 nM in Table 3 for **5** vs. 510 nM for CPT in Table 2). The results may suggest that phenol has cytotoxic effect on this leukemia cell line.

Table 2. Cytotoxicities (IC_{50}, nM) of Conjugates 2-5 against BR+ and BR- Cell Lines

Entry	Conjugate/Drug	MX-1[a]	MCF-7[b]	ID8[c]	L1210FR[d]	WI38[e]
1[f]	**1**	2.01 ± 0.52	0.44 ± 0.18	1.11 ± 0.26	2.14 ± 1.30	5.89 ± 2.38
2[f]	CPT	1700 ± 200	65.1 ± 12.3	474 ± 101	510 ± 139	786 ± 309
3[f]	**2**	51.7 ± 5.1	19.0 ± 3.4	23.4 ± 4.7	39.1 ± 9.1	742 ± 166
4[f]	**3**	65.1 ± 9.7	21.1 ± 2.9	25.6 ± 3.9	42.5 ± 8.9	868 ± 264
5[f]	**4**	66.9 ± 4.4	23.1 ± 1.8	26.7 ± 4.2	50.9 ± 15.9	941 ± 298
6[f]	**5**	>5,000	1,260 ± 172	>5,000	319 ± 139	1,440 ± 364

[a-d]See captions for cell lines in Table 1; [e]Human lung fibroblast cell line (BR-); [f]Cells were incubated with a drug or a conjugate at 37 °C for 72 h.

Table 3. Cytotoxicities (IC_{50}, nM) of Conjugates 2-5 in the Presence of GSH-OEt after Internalization

Entry	Conjugate	MX-1[a]	MCF-7[b]	ID8[c]	L1210FR[d]	WI38[e]
1	**2**	9.8 ± 1.4	3.2 ± 0.8	5.7 ± 1.0	7.40 ± 3.48	705 ± 114
2	**3**	20.8 ± 2.2	9.4 ± 1.3	10.3 ± 1.7	13.7 ± 1.5	782 ± 132
3	**4**	23.6 ± 4.6	9.9 ± 2.0	15.6 ± 3.0	14.4 ± 4.2	850 ± 100
4	**5**	>5000	538 ± 105	2,390 ± 405	177 ± 33	1230 ± 219

*Cells were initially incubated with conjugates **2-5** for 24 h, followed by washing of the drug media with PBS, then addition of GSH-OEt (6 equiv. to a conjugate) for drug release and additional incubation at 37 °C for 48 h.
[a-e]See captions for cell lines in Table 1.

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, 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 cojugate) was added after the cells were incubated with **2** or **3** for 24 h, thoroughly washed with saline (PBS), and re-suspended. 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 IC_{50} 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 IC_{50} 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. ^{1}H and ^{13}C 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-Hoover capillary melting point apparatus and are uncorrected. TLC was performed on Sorbent Technologies aluminum-backed Silica G TLC plates (Sorbent Technologies, 200 μm, 20 x 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 x 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-Deacetylbaccatin III was obtained from Indena, SpA,

Italy. 2,2'-Dipyridyldisulfide,[11] (2-sulfanylphenyl)acetic acid,[11] taxoid **1**,[25] 1-amino-11-azido-3,6,9-trioxaundecane,[35] *N*-Boc-ethylenediamine,[43] *N*-biotinyl-PEG_3-diamine[36] and 4-sulfanylpentanoic acid (**8**)[37, 41] were prepared by literature methods. 3-(4,5-Dimethylthiazol-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-PEG_3-$(CH_2)_2$-NH-FITC (7). To a solution of fluorescein isothiocyanate (FITC) (20 mg, 0.0502 mmol) and *N*-biotinyl-3,6,9-trioxaundecamethylene-1,11-diamine (30 mg, 0.0717 mmol) in DMF (0.6 mL) was added diisopropylethylamine (DIPEA) (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% CH_3OH in CH_2Cl_2 as eluent gave probe **7** (27 mg, 68% yield) as an orange solid: mp 191-192 °C; ^{1}H NMR (500 MHz, CD_3OD) 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 = 12.7 Hz, 1H), 2.88 (dd, J = 5.0, 12.7 Hz, 1H), 3.15 (m, 1H), 3.32 (m, 2H), 3.51 (t, J = 5.2 Hz, 2H), 3.59 (m, 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, 1.7 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) 7.21, 12.43, 15.43, 21.71, 24.82, 27.47, 27.73, 30.77, 34.74, 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, 164.11, 169.25, 174.15, 180.88; HRMS (TOF) for $C_{39}H_{46}N_5O_{16}S_2^+$ calcd: 808.2681. Found: 808.2691 (Δ = 1.2 ppm). HPLC (1): t = 9.1 min, purity >97%.

Triisopropylsilyl 4-(pyridin-2-yldisulfanyl)pentanoate (10).[41] To a solution of **9** (709 mg, 5.29 mmol) in ethanol (20 mL) was added 2,2'-dipyridinedisulfide (7.0 g, 31.7 mmol) in ethanol (100 mL),

and the mixture was refluxed at 80 ºC for 2 h. The reaction mixture was cooled to 25 ºC and concentrated *in vacuo* to afford a yellow precipitate. The crude product was purified by column chromatography on silica gel to afford a mixture of 4-(pyridin-2-yldisulfanyl)pentanoic acid and pyridine-2-thiol. To a cooled solution of this mixture in dichloromethane (40 mL) at 0 ºC was added triethylamine (2.1 mL, 15.1 mmol) and chlorotriisopropylsilane (1.93 mL, 9.04 mmol). The mixture was allowed to react for 24 h with stirring and concentrated *in vacuo* to afford a crude product as an oil. Purification of the crude product by column chromatography on silica gel with hexanes/ethyl acetate (2:1) as eluent gave **10** (2.71 g, 92% yield for 2 steps), as a colorless oil: ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.06 (m, 21 H), 1.34 (d, J = 6.8 Hz, 3H), 1.89 (m, 1H), 2.00 (m, 1H), 2.51 (m, 2H), 3.03 (m, 1H), 7.06 (ddd, J = 1.0, 4.8, 7.4 Hz, 1H), 7.62 (dt, J = 1.5, 8.2 Hz, 1H), 7.72 (d, J = 8.2 Hz, 1H), 8.44 (d, J = 4.8 Hz, 1H). ^{1}H NMR spectrum was consistent with the reported data.[41]

2-(5-Oxo-triisopropylsiloxypentan-2-yl)disulfanylphenylacetic acid (11).[41] To a cooled solution of **10** (300 mg, 0.752 mmol) in tetrahydrofuran (1.5 mL) at -10 ºC was added (2-sulfanylphenyl)acetic acid (130 mg, 0.752 mmol) in tetrahydrofuran (1.5 mL), and the mixture was allowed to react for 2 h at room temperature with stirring. The reaction mixture was concentrated *in vacuo* to afford a yellow oil. Purification the crude produce by column chromatography on silica gel with hexanes/ethyl acetate (3:1) as eluent gave **11** (166 mg, 68% yield) as an orange oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.06 (m, 21H), 1.25 (d, J = 6.8, 3H), 1.81 (m, 1H), 1.92 (m, 1H), 2.44 (m, 2H), 2.90 (m, 1H), 3.90 (s, 2H), 7.24 (m, 2H), 7.32 (m, 1H), 7.82 (d, J = 7.7 Hz, 1H); HRMS (TOF) for $C_{22}H_{37}O_4S_2Si^+$ calcd: 457.1897, found: 457.1892 (Δ = -1.1 ppm).

Taxoid-(SS-Linker)-OTIPS (12T).[41] To a solution of taxoid **1** (600 mg, 0.703 mmol), **11** (350 mg, 0.768 mmol), and 4-*N*,*N*-dimethylaminopyridine (DMAP) (38 mg, 0.308 mmol) in CH_2Cl_2 (13 mL) was added *N*,*N*'-diisopropylcarbodiimide (DIC) (135 μL, 0.877 mmol), and the mixture was allowed to

react for 24 h at room temperature with stirring. The reaction was quenched with saturated NH_4Cl (10 mL), and the reaction mixture was diluted with water (20 mL x 3), washed with brine (20 mL x 3), dried over $MgSO_4$, and concentrated *in vacuo* to afford a yellow oil. The crude product was dissolved in ether (20 mL) and the insoluble urea byproduct was removed by gravity filtration on a filter paper. Purification of the crude product by column chromatography on silica gel with hexanes/ethyl acetate (3:1) as eluent gave **12T** (697 mg, 77% yield), as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.97 (m, 2H), 1.06 (d, J = 7.2 Hz, 18H), 1.14 (m, 5H), 1.25 (s, 6H), 1.30 (d, J = 6.8 Hz, 3H), 1.33 (s, 9H), 1.66 (s, 3H), 1.69 (s, 3H), 1.72 (s, 3H), 1.76 (m, 2H), 1.85 (m, 1H), 1.90 (s, 3H), 1.93 (m, 2H), 2.30 (m, 2H), 2.35 (s, 3H), 2.42 (m, 2H), 2.53 (m, 2H), 2.59 (m, 1H), 2.94 (m, 1H), 3.81 (d, J = 7.2 Hz, 1H), 3.94 (dd, J = 1.7, 16.8 Hz, 1H), 4.10 (dd, J = 1.7, 16.8 Hz, 1H), 4.18 (d, J = 8.4 Hz, 1H), 4.30 (d, J = 8.4 Hz, 1H), 4.42 (m, 1H), 4.79 (d, J = 8.4 Hz, 1H), 4.92 (s, 1H), 4.96 (d, J = 7.2 Hz, 1H), 5.07 (d, J = 8.4 Hz, 1H), 5.67 (d, J = 7.2 Hz, 1H), 6.19 (t, J = 8.7 Hz, 1H), 6.28 (s, 1H), 7.26 (m, 3H), 7.47 (t, J = 7.9 Hz, 2H), 7.60 (t, J = 7.4 Hz, 1H), 7.80 (d, J = 7.4 Hz, 1H), 8.11 (d, J = 7.1 Hz, 2H); HRMS (TOF) for $C_{67}H_{94}NO_{18}S_2Si^+$ calcd: 1292.5676. Found: 1292.5676 (Δ = 0 ppm).

Camptothecin-(SS-Linker)-OTIPS (12C). To a solution of camptothecin (158 mg, 0.431 mmol), **10** (489 mg, 1.072 mmol), and DMAP (53 mg, 0.431 mmol) in CH_2Cl_2 (20 mL) was added DIC (73 μL, 0.474 mmol), and the mixture was allowed to react for 16 h at room temperature with stirring. The reaction mixture was cooled to 0 ^{o}C and the urea by-product was removed via filtration. The filtrate was concentrated *in vacuo* to give a green solid. Purification of the crude product by column chromatography on silica gel with 1.5% CH_3OH in CH_2Cl_2 as eluent gave **12C** (192 mg, 57% yield) as an off-white solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.98 (t, J = 7.5 Hz, 3H), 1.05 (m, 21H), 1.45 (d, J = 6.8 Hz, 2H), 1.85 (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 (d, 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); ^{13}C NMR (125 MHz, $CDCl_3$) δ 7.60, 11.85, 17.78, 20.39, 20.98, 22.55, 23.53, 30.94, 31.78, 32.96, 38.59, 45.82, 49.92, 53.51, 67.13, 96.26, 120.18, 127.62, 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, 130.89, 131.06, 132.56, 132.63, 134.65, 137.09, 137.44, 145.60, 146.20, 148.83, 152.30, 153.82, 157.27, 167.33, 169.91, 172.91; HRMS (TOF) for $C_{42}H_{51}N_2O_7S_2Si^+$ calcd: 787.2901; found: 787.2897 (Δ = -0.5 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 CH_2Cl_2 (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 NH_4Cl (10 mL), and the reaction mixture was diluted with water (5 mL), extracted with CH_2Cl_2 (20 mL x 3). The combined organic layers were dried over $MgSO_4$ 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** (505 mg, 79% yield) as a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.06 (m, 21H, TIPS), 1.30 (d, J= 6.8 Hz, 3H), 1.85 (m, 1H), 1.93 (m, 1H), 2.42 (m, 2H), 2.96 (m, 1H), 4.11 (s, 2H), 7.10 (d, J=7.4 Hz, 2H), 7.21 (t, J = 7.4 Hz, 1H), 7.26 (t, J = 7.3 Hz, 1H), 7.31 (t, J = 7.3 Hz, 2H), 7.36 (t, J = 7.4 Hz, 2H), 7.82 (d, J = 7.9, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 11.89, 17.79, 20.57, 30.99, 33.03, 39.41, 46.11, 121.48, 125.84, 127.65, 128.27, 129.36, 130.23, 130.83, 133.38, 137.67, 150.79, 169.40, 173.01; HRMS (TOF) for $C_{28}H_{41}O_4S_2Si^+$ calcd: 533.2210. Found: 533.2222 (Δ = 2.3 ppm).

Taxoid-(SS-Linker)-COOH (13T).[41] To a solution of **12T** (649 mg, 0.502 mmol) in a 1:1 mixture of CH_3CN-pyridine (1:1) (50 mL) and cooled to 0 °C under nitrogen was added HF/pyridine (6.5 mL), slowly, and the mixture was stirred for 24 h at room temperature. The reaction was quenched with 10%

citric acid (10 mL), and the reaction mixture was diluted with ethyl acetate (20 mL). The reaction mixture was extracted with ethyl acetate (20 mL x 2), washed with saturated $CuSO_4$ (3 x 20 mL) and brine (3 x 20 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to afford a clear oil. Purification of the crude produce by column chromatography on silica gel with hexanes/ethyl acetate (1:4) as eluent gave **13T** (516 mg, 91% yield) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) 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 (bt, 1H), 1.93 (s, 3H) 1.94 (m, 2H), 2.39 (m, 2H), 2.42 (s, 3H), 2.56 (m, 1H), 3.03 (m, 1H), 3.83 (m, 1H), 3.96 (dd, J = 1.7, 16.8 Hz, 1H), 4.15 (dd, 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, 3H), 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 for (TOF) $C_{58}H_{74}NO_{18}S_2^+$ calcd: 1136.4342. Found: 1136.4365 (Δ = 2.0 ppm).

Camptothecin-Me-SS-Linker-COOH (13C). To a cooled solution of **12C** (192 mg, 0.244 mmol) in CH_3CN-pyridine (1:1) (30 mL) at 0 °C was added 70% HF/pyridine (3 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), the reaction mixture was diluted with water (10 mL). The reaction mixture was extracted with CH_2Cl_2 (3 x 20 mL), and the combined organic layers were washed with saturated $CuSO_4$ (60 mL), water (60 mL), and brine (60 mL). The organic layers were dried over $MgSO_4$ and concentrated *in vacuo* to give a green solid. Purification of the crude product by column chromatography on silica gel with 1% CH_3OH in CH_2Cl_2 as eluent gave **13C** (131 mg, 85% yield) as a pale-green solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.91 (t, J = 7.5 Hz, 3H), 1.05 (dd, J = 6.8, 8.4 Hz, 3 H), 1.53 (m, 1H), 1.70 (m, 1H), 2.18 (t, J = 7.5 Hz, 2H), 2.20 (m, 2H), 2.88 (m, 1H), 4.05 (d, J = 16.5 Hz, 1H), 4.20 (dd, J = 4.0, 16.5 Hz, 1H), 5.30 (m, 2H), 5.46 (m, 2H), 7.10 (s, 1H), 7.27 (t, J = 7.5 Hz, 1H), 7.35 (m, 2H), 7.74

(m, 2H), 7.91 (t, J = 7.3 Hz, 1H), 8.15 (d, J = 8.0 Hz, 1H), 8.23 (d, J = 8.0 Hz, 1H), 8.70 (s, 1H), 12.10 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 8.00, 20.13, 20.24, 30.69, 30.79, 30.86, 31.29, 31.35, 38.57, 45.75, 45.83, 50.70, 55.40, 66.78, 76.61, 95.61, 119.37, 127.95, 127.97, 128.17, 128.46, 128.89, 129.00, 129.57, 129.78, 129.86, 130.16, 130.84, 131.70, 131.96, 133.11, 133.16, 137.59, 137.63, 145.48, 146.41, 148.43, 152.71, 156.98, 167.51, 169.84, 174.22; HRMS (TOF) for $C_{33}H_{31}N_2O_7S_2^+$ calcd: 631.1567; found: 631.1561 (Δ = -0.9 ppm).

Phenol-(SS-Linker)-COOH (13P). To a cooled solution of **12P** (505 mg, 0.947 mmol) in CH_3CN-pyridine (1:1) (80 mL) at 0 ºC, was added 70% HF/pyridine (5 mL), and the mixture was stirred for 6 h at room temperature. The reaction was quenched with 10% citric acid and extracted with ethyl acetate (50 mL x 3). The combined organic layers were washed with saturated $CuSO_4$ (50 mL x 3) and brine (50 mL x 3), dried over $MgSO_4$, and concentrated *in vacuo* to afford a pale-yellow oil. Purification of the crude product by column chromatography on silica gel with 50% ethyl acetate in hexanes as eluent gave **14P** (314 mg, 88% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.29 (d, J = 6.8 Hz, 3H, CH_3), 1.86 (m, 1H), 1.95 (m, 1H), 2.42 (m, 2H), 2.94 (m, 2H), 4.12 (s, 2H), 7.11 (d, J = 7.5 Hz, 2H), 7.21 (t, J = 7.5 Hz, 1H), 7.26 (m, 1H), 7.31 (t, J = 7.5 Hz, 2H), 7.35 (t, J = 7.5 Hz, 2H), 7.81 (d, J = 7.5 Hz, 1H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 20.48, 30.35, 31.03, 39.42, 45.90, 121.47, 125.88, 127.80, 128.31, 129.39, 130.45, 130.94, 133.53, 137.51, 150.77, 169.50, 178.29; HRMS (TOF) for $C_{19}H_{21}O_4S_2^+$ calcd: 377.0876. Found: 377.0888 (Δ = 3.2 ppm).

Taxoid-(SS-Linker)-OSu (14T). To a solution of **13T** (500 mg, 0.441 mmol) and *N*-hydroxysuccinimide (HOSu) (0.160 g, 1.32 mmol) in THF-pyridine (1:1) (9 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (0.100 g, 0.485 mmol), and the mixture was allowed to react for 36 h at room temperature with stirring. The reaction was quenched with saturated NH_4Cl (10 mL), washed with brine (3 x 20 mL), dried over $MgSO_4$, and concentrated *in vacuo* to afford

a yellow oil. Purification of the crude product by column chromatography on silica gel with hexanes/ethyl acetate (1:1) as eluent gave **14T** (376 mg, 70% yield) as a sticky white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.98 (m, 2H), 1.12 (m, 2H), 1.14 (s, 3H), 1.25 (m, 3H), 1.31 (dd, J = 4.8, 6.8 Hz, 3H), 1.33 (s, 2H), 1.34 (s, 9H), 1.66 (s, 3H), 1.71 (s, 3H), 1.73 (s, 3H), 1.86 (t, J = 13.8 Hz, 1H), 1.90 (s, 3H), 1.97 (m, 2H), 2.32 (m, 2H), 2.35 (s, 3H), 2.53 (m, 1H), 2.64 (t, J = 7.0 Hz, 2H), 2.83 (s, 4H), 2.99 (m, 1H), 3.80 (d, J = 7.2 Hz, 1H), 3.97 (dd, J = 1.7, 16.8 Hz, 1H), 4.08 (dd, J = 1.7, 16.8 Hz, 1H), 4.13 (m, 1H), 4.18 (d, J = 8.4 Hz, 1H), 4.30 (d, J = 8.4 Hz, 1H), 4.42 (m, 1H), 4.81 (m, 1H), 4.92 (s, 1H), 4.96 (d, J = 8.5 Hz, 1H), 5.09 (d, J = 8.5 Hz, 1H), 5.67 (d, J = 7.2 Hz, 1H), 6.18 (t, J = 8.5 Hz, 1H), 6.28 (s, 1H), 7.30 (m, 3H), 7.47 (t, J = 7.8 Hz, 2H), 7.60 (t, J = 7.4 Hz, 1H), 7.79 (d, J = 8.2 Hz, 1H), 8.11 (d, J = 7.5 Hz, 2H). ^{13}C NMR (500 MHz, $CDCl_3$) δ 9.19, 9.39, 9.58, 13.04, 13.75, 14.23, 14.87, 18.54, 19.15, 20.40, 22.25, 22.45, 23.51, 25.61, 25.75, 26.70, 28.24, 30.26, 30.66, 35.45, 35.51, 38.80, 42.29, 43.20, 45.60, 58.48, 64.40, 71.83, 72.16, 75.01, 75.22, 75.49, 76.42, 79.31, 80.97, 84.52, 119.98, 128.03, 128.48, 128.66, 129.29, 130.21, 130.64, 130.69, 131.00, 132.40, 133.45, 133.63, 154.94, 156.89, 167.01, 168.16, 169.10, 169.64, 170.19, 170.21, 175.12, 204.18; HRMS (TOF) for $C_{62}H_{77}N_2O_{20}S_2^+$ calcd: 1233.4506. Found: 1233.4496 (Δ = -0.8 ppm). HPLC (2): t = 14.1 min, purity >98%.

Phenol-(SS-Linker)-OSu (14P). To a solution of **13P** (154 mg, 0.410 mmol) and HOSu (0.141 g, 1.23 mmol) in CH_2Cl_2 (1.6 mL) was added EDC•HCl (94 mg, 0.493 mmol) in CH_2Cl_2 (1 mL), and the mixture was allowed to react for 16 h at room temperature with stirring. The reaction was quenched with saturated NH_4Cl (10 mL), and the reaction mixture was diluted with water (10 mL), extracted with CH_2Cl_2 (20 mL x 3) and washed with brine (20 mL x 3). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo* to afford a yellow oil. Purification the crude product by column chromatography on silica gel with 50% ethyl acetate in hexanes as eluent gave **14P** (162 mg, 84% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.30 (d, J = 6.8 Hz, 3H), 1.98 (m, 1H), 2.06 (m, 1H),

2.66 (t, J = 7.8 Hz, 2H), 2.82 (bs, 4H), 3.00 (m, 1H), 4.13 (s, 2H), 7.10 (d, J = 7.5 Hz, 2H), 7.21 (m, 1H), 7.26 (m, 1H), 7.34 (m, 4H), 7.82 (d, J = 7.5 Hz, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 20.41, 25.60, 28.31, 30.28, 39.44, 45.60, 121.50, 125.88, 127.92, 128.42, 129.41, 130.56, 130.94, 133.57, 137.43, 150.76, 168.16, 169.01, 169.46; HRMS (TOF) for $C_{23}H_{24}NO_6S_2^+$ calcd: 474.1040. Found: 474.1044 (Δ = 0.8 ppm).

Camptothecin-(SS-Linker)-NH-PEG$_3$-(CH$_2$)$_2$N$_3$ (15C). To a solution of **13C** (110 mg, 0.175 mmol) and 1-amino-11-azido-3,6,9-trioxaundecane (76 mg, 0.349 mmol) in CH_2Cl_2 (60 mL) was added DIC (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% CH_3OH in CH_2Cl_2 as eluent gave **15C** (103 mg, 71% yield) as a pale-white solid: mp 104-105 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.00 (m, 3H), 1.18 (d, J = 6.5 Hz, 3H), 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 = 1.4, 17.3 Hz, 1H), 5.98 (m, 1H), 7.25 (m, 5H), 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); ^{13}C NMR (400 MHz, $CDCl_3$) δ 7.57, 14.20, 20.60, 20.73, 21.05, 23.50, 31.21, 31.42, 31.82, 33.50, 38.56, 39.10, 42.15, 46.36, 49.95, 50.67, 53.44, 60.39, 67.15, 69.74, 70.03, 70.23, 70.56, 70.61, 70.69, 96.30, 120.20, 120.29, 127.89, 128.01, 128.17, 128.19, 128.24, 128.46, 129.71, 130.62, 130.69, 131.12, 131.15, 131.21, 133.07, 137.55, 145.59, 146.22, 148.86, 152.32, 157.29, 167.32, 170.20, 171.14, 172.05, 172.12; HRMS (TOF) for $C_{41}H_{47}N_6O_9S_2^+$ calcd: 831.2840; found: 831.2838 (Δ = -0.2 ppm). HPLC (2): t = 12.4 min, purity >95%.

Phenol-(SS-Linker)-PEG$_3$-(CH$_2$)$_2$N$_3$ (15P). To a solution of **13P** (149 mg, 0.396 mmol) and 1-amino-11-azido-3,6,9-trioxaundecane (259 mg, 1.19 mmol) in CH_2Cl_2 (1 mL) was added DIC (67 μL, 0.436 mmol), and the mixture was stirred for 10 h at room temperature. The reaction mixture was concentrated *in vacuo* to give a yellow oil. Purification of the crude product by column chromatography on silica gel with 1.5% CH_3OH in CH_2Cl_2 as eluent gave **15P** (0.127 g, 56%), as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.34 (d, J = 6.8 Hz, 3H), 1.95 (m, 2H), 2.17 (t, J = 7.6 Hz, 1H), 2.57 (m, 1H), 2.94 (m, 1H), 3.40 (m, 2H), 3.49 (t, J = 5.3 Hz, 2H), 3.75 (m, 12H),

4.17 (s, 2H), 6.02 (bs, 1H), 7.14 (m, 2H), 7.25 (t, J = 7.5 Hz, 1H), 7.35 (m, 3H), 7.40 (t, J = 7.5 Hz, 2H), 7.82 (d, J = 7.5 Hz, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 20.88, 23.53, 31.31, 33.55, 39.44, 46.48, 50.69, 61.79, 69.78, 70.07, 70.24, 70.59, 70.64, 70.73, 72.50, 121.51, 125.94, 127.97, 128.24, 129.43, 130.87, 131.21, 133.86, 137.62, 150.76, 169.81, 172.17; HRMS (TOF) for $C_{27}H_{37}N_4O_6S_2^+$ calcd: 577.2149; found: 577.2154 (Δ = 0.9 ppm).

5-Aza-16-azido-8,11,14-trioxa-4-oxohexadecanoic acid (17). To a solution of succinic anhydride (**16**) (183 mg, 1.834 mmol) and 1-amino-11-azido-3,6,9-trioxaundecane (400 g, 1.834 mmol) in dichloromethane (3 mL) was added triethylamine (25 μL, 0.1834 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% CH_3OH in CH_2Cl_2 as eluent gave **17** (389 mg, 67% yield) as a colorless liquid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.54 (m, 2H), 2.67 (m, 2H), 3.40 (m, 2H), 3.45 (m, 2H), 3.55 (t, J = 5.0 Hz, 2H), 3.63 (m, 2H), 3.68 (m, 8H), 6.75 (bs, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 30.35, 30.99, 39.51, 50.68, 69.63, 69.93, 70.14, 70.44, 70.57, 70.65, 172.71, 175.29; HRMS (TOF) for $C_{12}H_{23}N_4O_6^+$ calcd: 319.1612; found: 319.1622 (Δ = 3.2 ppm).

20-(5-Aza-16-azido-8,11,14-trioxa-4-oxohexadecanoyl)camptothecin (18). To a solution of camptothecin (40 mg, 0.115 mmol), **17** (157 mg, 0.345 mmol), and DMAP (105 mg, 0.115 mmol) in CH_2Cl_2 (6 mL) was added DIC (30 μL, 0.127 mmol), and the mixture was allowed to react for 36 h at room temperature with stirring. The reaction mixture was cooled to 0 ºC and the precipitated urea by-product was removed by filtration. The filtrate was concentrated *in vacuo* to afford a yellow oil. Purification of the crude product by column chromatography on silica gel with 3% CH_3OH in CH_2Cl_2 as eluent gave **18** (53 mg, 72% yield) as an off-white solid: mp 100-101 ºC; ^{1}H NMR (500 MHz, $CDCl_3$) 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); ^{13}C NMR (500 MHz, $CDCl_3$) δ 7.58, 29.40, 30.67, 37.70, 39.31, 49.90, 50.63, 66.99, 69.67, 69.97, 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, 176.54, 170.78, 171.93; HRMS (TOF) for $C_{32}H_{37}N_6O_9^+$ calcd: 649.2617. Found: 649.2622 (Δ = 0.8 ppm). HPLC (2): t = 10.8 min, purity >98%.

2-Chloro-4-(11-biotinylamino-3,6,9-trioxaundecyl)amino-6-(2-*N*-Boc-aminoethyl)amino-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 **7** (954 mg, 2.282 mmol) and DIPEA (0.6 mL, 3.423 mmol) in THF (80 mL) was added to the reaction mixture 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% CH_3OH in CH_2Cl_2) gave **20** (645 mg, 42% yield) as a white solid; mp 63-65 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 1.34 (dt, J = 6.6, 12.6 Hz, 2H), 1.41 (s, 9H), 1.61 (m, 4H), 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), 4.29 (dd, J = 4.4, 8.0 Hz, 1H), 4.48 (dd, J = 4.4, 8.0 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 25.5, 27.4, 28.1, 28.4, 35.4, 39.0, 39.7, 40.2, 40.3, 40.6, 53.4, 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) calcd for $C_{28}H_{49}ClN_9O_7S^+$: 690.3159. Found: 690.3157 (Δ = -0.3 ppm). HPLC (2): t = 10.0 min, purity >98%.

4-(11-Biotinylamino-3,6,9-trioxaundecyl)amino-6-(2-*N*-Boc-aminoethyl)amino-2-propargylamino-1,3,5-triazine (21). To a solution of **20** (794 mg, 1.151 mmol) and DIPEA (0.4 mL, 2.302 mmol) in THF (12 mL) was added propargylamine (362 μL, 6.600 mmol), 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 the crude product by column chromatography on silica gel (eluent: 7% CH_3OH in CH_2Cl_2) gave **21** (560 mg, 69% yield) as a light yellow solid: mp 66-68 °C; ^{1}H NMR (300 MHz, CD_3OD) 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); ^{13}C NMR (125 MHz, CD_3OD) δ 27.01, 28.94, 29.64, 29.93, 30.94, 36.89, 40.55, 41.21, 41.50, 41.56, 57.16, 61.77, 63.52, 70.74, 71.19, 71.40, 71.44, 71.73, 71.76, 158.76, 166.27, 176.30; HRMS (TOF) calcd for $C_{31}H_{53}N_{10}O_7S^+$: 709.3814; found: 709.3814 (Δ = 0 ppm). HPLC (2): t = 10.0 min, purity >98%.

6-(2-Aminoethyl)amino-4-(11-biotinylamino-3,6,9-trioxaundecyl)amino-2-propargylamino-1,3,5-triazine TFA salt (22). To a solution of **21** (400 mg, 0.564 mmol) in CH_2Cl_2 (6 mL) was added TFA (0.5 mL, 6.57 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 ether (20 mL) and the crude product (TFA salt) crashed out. The resulting solid was washed with ether (20 mL x 4) to afford **22** (408 mg, 100% yield) as a white solid; ^{1}H NMR (400 MHz, CD_3OD) 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); ^{13}C NMR (100 MHz, CD_3OD) 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* 609.3 $(M+H)^+$.

4-(11-Biotinylamino-3,6,9-trioxaundecyl)amino-2-propargylamino-6-[2-(taxoid-SS-Linker)amidoethyl]amino-1,3,5-triazine (23). A solution of **14T** (704 mg, 0.564 mmol) and **22** (408 mg, 0.564 mmol) in CH_2Cl_2-pyridine (4:1) (10 mL) was allowed to react at 25 °C for 36 h. The reaction was quenched by saturated NH_4Cl (10 mL), and the reaction mixture was extracted with CH_2Cl_2 (20 mL x 3). The combined organic layers were washed with brine (5 mL x 3), dried over $MgSO_4$, and concentrated *in vacuo* to afford a yellow oil. Purification of the crude product by column chromatography on silica gel (eluent: 8% CH_3OH in CH_2Cl_2) gave **23** (806 mg, 83% yield) as a white solid: mp. 164-166 °C; ^{1}H NMR

(500 MHz, CD_3OD) δ 0.86 (m, 2H), 0.98 (m, 2H), 1.17 (s, 6H), 1.26 (d, J = 6.8 Hz, 3H), 1.41 (s, 9H), 1.43 (m, 2H), 1.63 (m, 4H), 1.65 (s, 3H), 1.70 (m, 1H), 1.73 (s, 3H), 1.76 (s, 3H), 1.79 (m, 1H), 1.87 (m, 1H), 1.91 (s, 3H), 2.20 (t, J = 7.5 Hz, 2H), 2.25 (m, 4H), 2.38 (s, 3H), 2.45 (m, 2H), 2.65 (bs, 1H), 2.69 (d, J = 12.8 Hz, 1H), 2.86 (m, 1H), 2.90 (dd, J = 5.0, 12.8 Hz, 1H), 3.18 (m, 1H), 3.34 (m, 2H), 3.35 (t, J = 5.4 Hz, 2H), 3.52 (t, J = 5.4 Hz, 2H), 3.54 (m, 2H), 3.63 (m, 12H), 3.84 (d, J = 7.2 Hz, 1H), 4.02 (dd, J = 1.5, 16.7 Hz, 1H), 4.10 (d, J = 16.7 Hz, 1H), 4.17 (m, 2H), 4.20 (d, J =8.4 Hz, 2H), 4.28 (dd, J = 4.4, 8.0, 1H), 4.30 (dd, J = 6.5, 10.6 Hz, 1H), 4.47 (dd, J = 4.4, 8.0 Hz, 1H), 4.8-4.9 (m, 3H), 4.99 (d, J = 8.9 Hz, 1H), 5.26 (bs, 1H), 5.67 (d, J = 7.2 Hz, 1H), 6.13 (bt, J = 8.5 Hz, 1H), 6.45 (s, 1H), 7.26 (m, 1H), 7.32 (m, 2H), 7.50 (t, J = 7.9 Hz, 2H), 7.63 (t, J = 7.5 Hz, 1H), 7.78 (m, 1H), 8.12 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 5.17, 5.21, 6.42, 7.76, 9.80, 10.42, 11.08, 14.64, 16.99, 17.03, 18.36, 19.26, 19.70, 22.09, 22.20, 22.87, 22.98, 24.79, 25.51, 25.76, 28.62, 28.75, 30.31, 30.39, 31.67, 31.78, 32.76, 33.52, 35.48, 36.36, 37.08, 40.60, 43.35, 44.07, 46.71, 53.00, 55.32, 57.65, 59.38, 66.59, 67.25, 67.32, 67.57, 68.39, 68.98, 72.32, 72.57, 72.72, 73.46, 75.09, 76.50, 78.35, 81.85, 117.21, 124.96, 125.41, 125.68, 127.15, 127.42, 128.50, 130.57, 130.85, 131.02, 134.71, 138.61, 153.50, 162.08, 163.61, 166.30, 167.46, 171.12, 172.18, 201.18. HRMS (TOF) for $C_{84}H_{116}N_{11}O_{22}S_3^+$ calcd: 1726.7453. Found: 1726.7432 (Δ = -1.2 ppm). HPLC (1): t = 12.0 min, purity >98%.

Dual-warhead conjugate 2. To a solution of **23** (40 mg, 0.0232 mmol) and ascorbic acid (4 mg, 0.0255 mmol) in THF (1 mL) was added **15C** (19 mg, 0.0232 mmol) in CH_2Cl_2 (1 mL) first, followed by an aqueous solution of $CuSO_4{\cdot}5H_2O$ (6.4 mg, 0.0244 mmol) (1 mL). The mixture was allowed react at room temperature for 14 h, and the reaction mixture was diluted with water (5 mL) and extracted with CH_2Cl_2 (10 mL x 3). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel (eluent: 10% MeOH in CH_2Cl_2) to give **2** (54 mg, 92% yield) as an off-white solid: mp 138-139 °C; ^1H NMR (500 MHz, CD_3OD) δ 0.92

(m, 5H), 1.03 (m, 9H), 1.20 (s, 6H), 1.31 (m, 6H), 1.43 (s, 9H), 1.63 (m, 6H), 1.67 (s, 3H), 1.76 (s, 3H), 1.79 (s, 3H), 1.94 (s, 3H), 2.08 (t, J = 7.5 Hz, 1H), 2.24 (m, 8H), 2.40 (s, 3H), 2.47 (m, 1H), 2.71 (t, J = 12.8 Hz, 2H), 2.78 (m, 1H), 2.86 (m, 1H), 2.91 (dd, J = 5.5, 12.8 Hz, 1H), 3.19 (m, 1H), 3.26 (m, 1H), 3.37 (m, 2H), 3.45 (m, 2H), 3.63 (m, 24H), 3.81 (m, 6H), 4.03 (m, 3H), 4.08 (m, 2H), 4.10 (d, J = 16.7 Hz, 1H), 4.31 (m, 3H), 4.52 (m, 2H), 4.66 (m, 2H), 5.00 (t, J = 9.0 Hz, 2H), 5.32 (s, 2H), 5.35 (s, 1H), 5.48 (d, J = 16.7 Hz, 1H), 5.60 (d, J = 16.7 Hz, 1H), 5.70 (m, 1H), 6.15 (bt, J = 8.5 Hz, 1H), 6.47 (s, 1H), 7.30 (m, 6H), 7.41 (d, J = 8.2 Hz, 1H), 7.53 (m, 2H), 7.65 (m, 2H), 7.74 (m, 3H), 7.92 (t, J = 8.0 Hz, 1H), 7.94 (bs, 1H), 8.08 (d, J = 8.0 Hz, 1H), 8.14 (d, J = 7.5 Hz, 2H), 8.27 (d, J = 8.5 Hz, 1H), 8.61 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 7.99, 8.77, 8.85, 10.24, 13.16, 14.22, 14.25, 18.35, 20.08, 20.16, 20.47, 20.54, 20.74, 21.90, 23.00, 25.74, 25.96, 26.80, 28.51, 30.69, 30.89, 31.54, 31.64, 32.84, 34.87, 35.56, 38.51, 38.88, 41.14, 45.86, 46.69, 49.74, 50.72, 55.90, 57.96, 59.66, 61.50, 66.78, 69.52, 69.61, 69.98, 70.00, 70.07, 70.14, 70.19, 76.60, 77.21, 78.60, 80.00, 84.06, 95.59, 97.62, 119.37, 120.70, 125.38, 127.85, 128.17, 128.46, 128.87, 129.00, 130.17, 130.38, 130.83, 131.65, 131.96, 132.01, 133.03, 133.81, 133.89, 136.45, 137.33, 137.59, 137.63, 139.66, 139.97, 145.45, 145.49, 148.43, 151.93, 152.71, 155.39, 156.98, 157.24, 163.17, 165.59, 167.51, 169.27, 169.85, 170.07, 170.63, 171.73, 172.60, 178.58, 203.01; HRMS (TOF) for $C_{125}H_{162}N_{17}O_{31}S_5^+$ calcd: 2557.0221; found: 2557.0207. (Δ = -0.5 ppm). HPLC (1): t = 11.3 min, purity >97%.

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 $CuSO_4$-$5H_2O$ (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 CH_2Cl_2 (5 mL x 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 CH_2Cl_2) to afford **3** (12 mg, 54%

yield) as an off-white solid: mp 143-145 °C; ^{1}H NMR (500 MHz, CD_3OD) 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, 2H), 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, 3H), 4.28 (m, 2H), 4.38 (m, 1H), 4.48 (m, 3H), 4.91 (bs, 2H), 4.98 (t, J = 9.0 Hz, 1H), 5.26 (m, 1H), 5.31 (m, 2H), 5.45 (d, J = 16.7 Hz, 1H), 5.58 (d, J = 16.7 Hz, 1H), 5.66 (t, J = 6.7 Hz, 1H), 6.12 (bt, J = 8.5 Hz,1H), 6.43 (s, 1H), 6.45 (bs, 1H), 7.22 (m, 1H), 7.30 (m, 2H), 7.40 (s, 1H), 7.50 (t, J = 8.2 Hz, 2H), 7.63 (t, J = 7.4 Hz, 1H), 7.72 (m, 2H), 7.79 (m, 1H), 7.86 (t, J = 7.4 Hz, 1H), 7.93 (bs, 1H), 8.06 (d, J = 8.0 Hz, 1H), 8.11 (d, J = 7.8 Hz, 2H), 8.18 (d, J = 8.0 Hz, 1H), 8.61 (bs, 1H); ^{13}C NMR (125 MHz, CD_3OD) 6.70, 7.77, 7.82, 9.02, 12.41, 17.25, 19.61, 20.95, 21.88, 24.70, 25.09, 25.48, 25.58, 27.39, 28.09, 28.33, 28.33, 28.76, 30.75, 31.22, 35.34, 36.12, 38.04, 38.94, 43.20, 46.67, 50.26, 57.94, 60.24, 61.97, 66.32, 67.46, 69.03, 69.17, 69.84, 69.92, 69.95, 70.03, 70.20, 71.03, 73.25, 73.41, 74.90, 75.30, 75.59, 76.06, 76.41, 77.68, 78.07, 79.10, 80.95, 84.41, 87.85, 88.10, 96.77, 119.15, 119.81, 127.87, 128.00, 128.28, 128.41, 128.60, 129.74, 130.01, 130.62, 133.16, 137.31, 146.26, 146.96, 148.27, 157.59, 166.18, 167.97, 168.90, 170.05, 170.13, 170.92, 171.06, 172.63, 173.67, 173.72, 203.80; HRMS (TOF) calcd for $C_{116}H_{152}N_{17}O_{31}S_3^+$ calcd: 2374.9997. Found: 2374.9980 (Δ = -0.7 ppm). HPLC (1): t = 10.5 min, purity >96%.

Dual-warhead conjugate with a taxoid 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 $CuSO_4$-$5H_2O$ (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% CH_3OH in CH_2Cl_2) gave **3** (21 mg, 79% yield) as a colorless

solid: mp 125-127 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.90 (m, 3H), 1.02 (bt, J = 8.0 Hz, 2H), 1.18 (s, 3H), 1.27 (s, 3H), 1.33 (d, J = 6.8 Hz, 6H), 1.29 (m, 2H), 1.37 (s, 9H), 1.43 (m, 2H), 1.49 (m, 2H), 1.66 (m, 4H), 1.69 (s, 3H), 1.75 (s, 3H), 1.78 (m, 2H), 1.93 (s, 3H), 2.17 (t, J = 7.4 Hz, 2H), 2.29 (m, 1H), 2.39 (s, 3H), 2.54 (m, 2H), 2.73 (d, J = 12.6 Hz, 1H), 2.90 (m, 2H), 2.92 (dd, J = 5.0, 12.6 Hz, 1H), 3.15 (m, 1H), 3.41 (t, J = 5.0 Hz, 2H), 3.44 (t, J = 5.4 Hz, 2H), 3.49 (t, J = 5.4 Hz, 2H), 3.69 (m, 28H), 3.77 (t, J = 5.0 Hz, 1H), 3.83 (m, 1H), 4.17 (s, 2H), 4.20 (d, J = 8.4 Hz, 1H), 4.21 (m, 2H), 4.31 (d, J = 8.4 Hz, 1H), 4.33 (m, 1H), 4.42 (m, 1H), 4.51 (m, 1H), 5.00 (m, 2H), 5.16 (bs, 1H), 5.70 (d, J = 7.2 Hz, 1H), 6.06 (s, 1H), 6.20 (bt, J = 8.5,1H), 6.34 (s, 1H), 7.14 (d, J = 7.4 Hz, 1H), 7.23 (t, J= 7.4 Hz, 1H), 7.34 (m, 4H), 7.40 (t, J =8.0 Hz, 2H), 7.50 (t, J = 7.8 Hz, 2H), 7.63 (t, J = 7.4 Hz, 1H), 7.81 (bs, 1H), 7.83 (d, J = 7.7 Hz, 1H), 8.13 (d, J = 7.7 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 9.17, 9.33, 9.63, 13.06, 14.81, 17.50, 18.53, 20.01, 20.43, 20.87, 22.07, 22.48, 23.51, 25.76, 26.61, 27.90, 28.26, 31.30, 33.54, 35.48, 39.12, 43.21, 45.88, 46.47, 50.68, 53.51, 55.49, 58.41, 60.24, 61.75, 69.67, 69.78, 70.05, 70.15, 70.22, 70.26, 70.32, 70.41, 70.58, 70.63, 70.67, 70.69, 70.71, 71.99, 72.49, 75.11, 75.47, 76.40, 78.90, 79.15, 79.83, 81.00, 84.48, 115.39, 117.71, 120.04, 121.50, 125.93, 127.95, 128.23, 128.37, 128.66, 128.84, 128.28, 129.60, 130.17, 130.85, 130.94, 131.20, 133.67, 133.85, 137.64, 150.75, 155.07, 162.94, 163.23, 166.97, 168.37, 169.80, 172.19, 173.39, 174.95, 204.09; HRMS (TOF) for $C_{111}H_{152}N_{15}O_{28}S_5^+$ calcd: 2302.9529; found: 2302.9480 (Δ = -2.1 ppm). HPLC (1): t = 11.0 min, purity >98%.

4-(11-Biotinylamino-3,6,9-trioxaundecyl)amino-6-[2-(camptothecin-SS-Linker)-amidoethyl]amino-2-propargylamino-1,3,5-triazine (24). A mixture of **22** (19 mg, 26.2 μmol), **14P** (12 mg, 26.2 μmol) in CH_2Cl_2 (1 mL) and pyridine (1 mL) was stirred at room temperature for 10 h, and the reaction mixture was concentrated *in vacuo* to give a yellow oil. Purification of the crude product by column chromatography on silica gel (eluent: 4% CH_3OH in CH_2Cl_2) gave **24** (13 mg, 52% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.29 (d, J = 6.8 Hz, 3H), 1.43 (t, J = 7.7 Hz, 2H), 1.66 (m,

4H), 1.84 (m, 1H), 1.92 (m, 1H), 2.22 (m, 2H), 2.56 (bs, 1H), 2.72 (d, J = 12.8 Hz, 1H), 2.90 (m, 2H), 2.92 (dd, J = 5.0 12.8 Hz, 1H), 3.19 (m, 1H), 3.38 (t, J = 5.4 Hz, 2H), 3.44 (bs, 2H), 3.56 (t, J = 5.4 Hz, 2H), 3.66 (m, 14H), 4.15 (bs, 2H), 4.20 (s, 2H), 4.30 (dd, J = 4.4, 8.0 Hz, 1H), 4.49 (dd, J = 4.4, 8.0 Hz, 1H), 7.14 (d, J = 7.8 Hz, 2H), 7.25-7.45 (m, 6H), 7.83 (d, J = 7.8 Hz, 1H), 8.55 (bs, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.57, 25.47, 28.09, 28.39, 31.27, 32.97, 35.35, 38.74, 39.00, 39.68, 46.00, 55.62, 60.22, 61.97, 69.18, 69.55, 69.84, 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, 134.01, 137.38, 148.66, 150.95, 164.73, 170.23, 174.75. HRMS (TOF) for $C_{45}H_{63}N_{10}O_8S_3^+$ calcd: 967.3987; found: 967.3991 (Δ = 0.4 ppm).

Dual-warhead conjugate with campothecin and a surrogate warhead, 5. To a solution of **24** (13 mg, 13.5 μmol), **15C** (11 mg, 13.5 μmol), and ascorbic acid (2.6 g, 14.9 μmol) in THF (1 mL) was added a solution of $CuSO_4$•$5H_2O$ (3.7 mg, 14.9 mmol) in MeOH-H_2O (1:1) (1 mL). The mixture was stirred at 25 °C for 4 h, and the reaction mixture was concentrated *in vacuo* and extracted with CH_2Cl_2 (10 mL x 3). The combined organic layers were dried over $MgSO_4$, and concentrated *in vacuo* to give a yellow solid. Purification of the crude product by column chromatography on silica gel (eluent: 8% CH_3OH in CH_2Cl_2) gave conjugate **5** (13 mg, 54% yield) as a white solid: mp 127-128 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.90 (t, J = 7.5 Hz, 3H), 1.03 (dd, J = 6.8, 10.8, 2H), 1.20 (d, J = 6.8 Hz, 3H), 1.24 (s, 2H), 1.30 (m, 2H), 1.49 (m, 4H), 1.59 (m, 1.59), 1.71 (m, 2H), 1.84 (m, 1H), 2.07 (m, 4H), 2.17 (m, 4H), 2.57 (d, J = 12.8 Hz, 1H), 2.81 (m, 2H), 2.95 (m, 1H), 3.10 (m, 1H), 3.14 (m, 3H), 3.15 (m, 4H), 3.25 (m, 2H), 3.39 (t, J = 5.4 Hz, 4H), 3.43-3.49 (m, 20H), 3.77 (t, J = 5.0 Hz, 2H), 4.06 (m, 2H), 4.13 (m, 1H), 4.17 (m, 2H), 4.30 (m, 1H), 4.46 (m, 4H), 5.31 (m, 2H), 5.45 (d, J = 17.0 Hz, 1H), 5.50 (d, J = 17.0 Hz, 1H), 6.37 (s, 1H), 6.43 (s, 1H), 7.09 (s, 1H), 7.12 (d, J = 7.6 Hz, 2H), 7.24-7.44 (m, 9H), 7.73 (m, 3H), 7.88 (m, 4H), 7.90 (t, J = 8.1 Hz, 1H), 8.14 (d, J = 8.5 Hz, 1H), 8.23 (d, J = 8.5 Hz, 1H), 8.69 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 7.99, 20.06, 20.14, 20.51, 25.70, 28.51, 28.67, 30.69, 31.59, 32.83, 33.02, 35.56, 36.06,

45.85, 45.92, 46.18, 49.07, 49.66, 50.72, 55.41, 55.90, 59.65, 61.49, 66.78, 69.26, 69.52, 69.63, 69.99, 70.07, 70.14, 70.18, 70.22, 95.62, 119.36, 122.06, 126.41, 127.85, 128.04, 128.18, 128.46, 128.87, 129.00, 129.60, 129.73, 130.06, 130.17, 130.84, 131.66, 131.90, 131.96, 133.03, 133.83, 137.40, 137.63, 145.50, 146.42, 148.44, 150.91, 152.71, 156.99, 163.17, 166.15, 166.21, 167.53, 169.85, 171.74, 171.89, 172.58, 173.27; HRMS (TOF) for $C_{86}H_{109}N_{16}O_{17}S_5^+$ calcd: 1797.6755; found: 1797.6705 (Δ = -2.8 ppm). HPLC (1): t = 8.6 min, purity >97%.

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 ºC in a humidified atmosphere with 5% CO_2. LCC6-MDR, LCC6-WT, MCF-7, 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. 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 WI-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 (TrypLE, Gibco) as needed every 2-4 days and collected by centrifugation at 850 rpm for 5 min, and 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 x 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 μM solutions of **7** in cell culture media (3 mL). The cells were then incubated with **7** for 1 and 3 h at 37 ºC. In the case of leukemia cell lines (L1210 and L1210FR), probe **6** (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 re-suspended in 150 μL for imaging.

Flow Cytometry Analysis of the Cells Treated with Probe 7. Flow cytometry analysis of the cells treated with probe **6** 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 re-suspended in 0.5 mL of PBS. Approximately 10,000 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 re-suspended in 150 μL of PBS after each experiment, and dropped onto an uncoated microslide 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 C-Apochromat 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.[44] 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 re-suspended in 100 μL cell culture medium (RPMI-1640) at a concentrations ranging from 0.5-1.5 x 10^4 cells per well in a 96-well plate. For adhesive cell types, cells were allowed to descend to the bottom of the wells overnight, and a fresh medium (RPMI-1640) was added to each well upon removal of the old medium.

For the MTT assay of the time-dependent administrations of equimolar amounts of taxoid **1** and camptothecin in a sequential manner, cells were re-suspended in 200 μL medium with 8,000 to 10,000 cells per well of a 96-well plate and 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 medium. The residual medium in each well were aspirated and the different drug solutions were added to each well of every column of the 96-well plate. After the addition of the first drug solution, the cells were incubated at 37 °C for 24 h. Then, the second drug solution was added, followed by incubation for additional 48 h at 37 °C.

The 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 5,000 nM (100 μL), and the cells were subsequently cultured at 37 °C for 72 h. For the leukemia cell lines, cells were harvested, collected, and resuspended in the test solutions ranging from 0.5 to 5,000 nM (100 μL) at 0.5 to 0.8 x 10^4 cells per well in a 96-well plate and subsequently incubated at 37 °C for 72 h.

In another series of experiments, cells were incubated with a conjugate at 37 °C for 24 h and the drug medium was removed. Then, treated cells were washed with PBS, and GSH-OEt (6 equiv. to a

cojugate) in the cell culture medium (200 μL) was added to the wells. These cells were incubated at 37 °C for additional 48 h, i.e., the total incubation time was 72 h.

For all experiments, after removing the test medium, the fresh solution of MTT in PBS (40 μL of 0.5 mg MTT/mL) was added to the wells, and the cells were incubated at 37 ºC for 3 h. The MTT solution was then removed, and the resulting insoluble violet formazan crystals were dissolved in 0.1 N HCl in isopropanol with 10% Triton X-100 (40 μL) to give a violet solution. The spectrophotometric absorbance measurement of each well in the 96-well plate was run at 570 nm using a Labsystems Multiskan Ascent microplate reader. The IC_{50} values and their standard errors were calculated from the viability-concentration curve using Four Parameter Logistic Model of *Sigmaplot*. The concentration of DMSO per well was ≤1% in all cases. Each experiment was run in triplicate.

ASSOCIATED CONTENT

Supporting Information Available: ^{1}H and ^{13}C spectra of new compounds. FACS data for the expression level of biotin and folate receptors in additional human breast cancer cell lines. This material is available free of charge via the Internet at https://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

* Phone 631-632-1339; fax 631-632-7942; e-mail, iwao.ojima@stonybrook.edu

ACKNOWLEDGEMENTS

This research was supported by a grant from the National Institutes of Health (CA 103314 to I.O.). The authors are grateful to Dr. Anne Savitt and Ms. Rebecca Rowehl 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. The authors would like to 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; CFM, confocal fluorescence microscopy; CPT, camptothecin; DIC, *N*,*N'*diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; 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; HOSu, *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 overexpressing 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.

REFERENCES

1. Zimmermann, G. R.; Lehar, J.; Keith, C. T. Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discov Today* **2007,** 12, 34-42.

2. Rocha Lima, C.; Catapano, C. V.; Pacheco, D.; Sherman, C.; Oakhill, G.; Chaudhry, M.; Freeman, K. D.; Green, M. R. A phase I study of sequential administration of escalating doses of intravenous paclitaxel, oral topotecan, and fixed-dose oral etoposide in patients with solid tumors. *Cancer* **2004,** 100, 2671-2679.

3. Zolot, R. S.; Basu, S.; Million, R. P. Antibody–drug conjugates. *Nature Rev. Drug Discov.* **2013,** 12, 259-260.

4. Ducry, L.; Stump, B. Antibody-drug conjugates: Linking cytotoxic payloads to monoclonal antibodies. *Bioconj. Chem.* **2010,** 21, 5–13.

5. Alley, S. C.; Okeley, N. M.; Senter, P. D. Antibody–drug conjugates: targeted drug delivery for cancer. *Curr. Opin. Chem. Biol.* **2010,** 14, 529–537.

6. Carter, P. J.; Senter, P. D. Antibody-drug conjugates for cancer therapy. *Cancer J.* **2008,** 14, 154-169.

7. Jaracz, S.; Chen, J.; Kuznetsova, L. V.; Ojima, I. Recent advances in tumor-targeting anticancer drug conjugates. *Bioorg. Med. Chem.* **2005,** 13, 5043-5054.

8. Chen, J.; Jaracz, S.; Zhao, X.; Chen, S.; Ojima, I. Antibody-based toxin conjugates for cancer therapy. *Exp. Opin. Drug Deliv.* **2005,** 2, 873-890.

9. Ojima, I.; Geng, X.; Wu, X.; Qu, C.; Borella, C.; Xie, H.; Wilhelm, S.; Leece, B.; Bartle, L.; Goldmacher, V.; Chari, R. Tumor-specific novel taxoid-monoclonal antibody conjugates. *J. Med. Chem.* **2002,** 45, 5620-5623.

10. Vlahov, I. R.; Leamon, C. P. Engineering folate-drug conjugates to target cancer: from chemistry to clinic. *Bioconjug. Chem.* **2012,** 23, 1357-1369.

11. Chen, S.; Zhao, X.; Chen, J.; Kuznetsova, L.; Wong, S. S.; Ojima, I. Mechanism-based tumor-targeting drug delivery system. Validation of efficient vitamin receptor-mediated endocytosis and drug release. *Bioconjug. Chem.* **2010,** 21, 979-987.

12. Leamon, C. P. Folate-targeted drug strategies for the treatment of cancer. *Curr. Opin. Investig. Drugs* **2008,** 9, 1277-1286.

13. Kuznetsova, L.; Chen, J.; Sun, L.; Wu, X.; Pepe, A.; Veith, J. M.; Pera, P.; Bernacki, R. J.; Ojima, I. Syntheses and evaluation of novel fatty acid–second-generation taxoid conjugates as promising anticancer agents. *Bioorg. Med. Chem. Lett.* **2006,** 16, 974-977.

14. Bradley, M. O.; Webb, N. L.; Anthony, F. H.; Devanesan, P.; Witman, P. A.; Hemamalini, S.; Chander, M. C.; Baker, S. D.; He, L.; Horowitz, S. B.; Swindell, C. S. Tumor targeting by covalent conjugation of a natural fatty acid to taxol. *Clin. Cancer Res.* **2001,** 7, 3229-3238.

15. Russell-Jones, G.; McTavish, K.; McEwan, J.; Rice, J.; Nowotnik, D. Vitamin-mediated targeting as a potential mechanism to increase drug uptake by tumours. *J. Inorg. Biochem.* **2004,** 98, 1625-1633.

16. Vlahov, I. R.; Santhapuram, H. K.; Wang, Y.; Kleindl, P. J.; You, F.; Howard, S. J.; Westrick, E.; Reddy, J. A.; Leamon, C. P. An assembly concept for the consecutive introduction of unsymmetrical disulfide bonds: synthesis of a releasable multidrug conjugate of folic acid. *J. Org. Chem.* **2007,** 72, 5968-5972.

17. Bissery, M. C.; Nohynek, G.; Sanderink, G. J.; Lavelle, F. Docetaxel (Taxotere): a review of preclinical and clinical experience. Part I: Preclinical experience. *Anticancer Drugs* **1995,** 6, 339-355.

18. Fojo, A. T.; Ueda, K.; Slamon, D. J.; Poplack, D. G.; Gottesman, M. M.; Pastan, I. Expression of a multidrug-resistance gene in human tumors and tissues. *Proc. Natl. Acad. Sci. U S A* **1987,** 84, 265-269.

19. Gottesman, M. M.; Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann. Rev. Biochem.* **1993,** 62, 385-427.

20. Ojima, I. Guided molecular missiles for tumor-targeting chemotherapy – Case studies using the second-generation taxoid as warheads. *Acc. Chem. Res.* **2008,** 41, 108-119.

21. Madden, T.; Newman, R. A.; Antoun, G.; Johansen, M. J.; Ali-Osman, F. Low-level taxane exposure increases the activity of topoisomerase I targeted agents. *Proc. Am. Assoc. Cancer Res.* **1998,** 39, 527.

22. Bahadori, H. R.; Green, M. R.; Catapano, C. V. Synergistic interaction between topotecan and microtubule-interfering agents. *Cancer Chemother. Pharmacol.* **2001,** 48, 188-196.

23. Murren, J. R.; Peccerillo, K.; DiStasio, S. A.; Li, X.; Leffert, J. J.; Pizzorno, G.; Burtness, B. A.; McKeon, A.; Cheng, Y. Dose escalation and pharmacokinetic study of irinotecan in combination with paclitaxel in patients with advanced cancer. *Cancer Chemother. Pharmacol.* **2000,** 46, 43-50.

24. Kwong, M. S.; Bleickardt, E.; Murren, J. R. Camptothecin and taxane regimens for small-cell lung cancer. *Oncology (Williston Park, N.Y.) [2002, 16(9 Suppl 9):33-38]* **2002,** 16 33-38.

25. Ojima, I.; Slater, J.; Michaud, E.; Kuduk, S.; Bounaud, P.; Vrignaud, P.; Bissery, M.; Veith, J.; Pera, P.; Bernacki, R. Syntheses and structure-activity relationships of the second-generation antitumor taxoids: Exceptional activity against drug-resistant cancer cells. *J. Med. Chem.* **1996,** 39, 3889-3896.

26. Ojima, I.; Slater, J.; Kuduk, S.; Takeuchi, C.; Gimi, R.; Sun, C.; Park, Y.; Pera, P.; Veith, J.; Bernacki, R. Syntheses and structure-activity relationships of taxoids derived from 14β-hydroxy-10-deacetylbaccatin III. *J. Med. Chem.* **1997,** 40, 267-278.

27. Ojima, I.; Wang, T.; Miller, M. L.; Lin, S.; Borella, C.; Geng, X.; Pera, P.; Bernacki, R. J. Syntheses and structure-activity relationships of new second-generation taxoids. *Bioorg. Med. Chem. Lett.* **1999,** 9, 3423-3428.

28. Ojima, I.; Chen, J.; Sun, L.; Borella, C.; Wang, T.; Miller, M.; Lin, S.; Geng, X.; Kuznetsova, L.; Qu, C.; Gallagher, G.; Zhao, X.; Zanardi, I.; Xia, S.; Horwitz, S.; Clair, J. S.; Guerriero, J.; Bar-Sagi, D.; Veith, J.; Pera, P.; Bernacki, R. Design, synthesis, and biological evaluation of new-generation taxoids. *J. Med. Chem.* **2008,** 51, 3203-3221.

29. Ojima, I.; Das, M. Recent advances in the chemistry and biology of new generation taxoids. *J. Nat. Prod.* **2009,** 72, 554-565.

30. Kuznetsova, L.; Sun, L.; Chen, J.; Zhao, X.; Seitz, J.; Das, M.; Li, Y.; Veith, J. M.; Pera, P.; Bernacki, R. J.; Xia, S.; Horwitz, S. B.; Ojima, I. Synthesis and biological evaluation of novel 3'-difluorovinyl taxoids. *J. Fluor. Chem* **2012,** 143, 177-188.

31. Lu, Y.; Low, P. S. Folate-mediated delivery of macromolecular anticancer therapeutic agents. *Adv. Drug Deliv. Rev.* **2002,** 43, 675–693.

32. Chen, J.; Chen, S.; Zhao, X.; Kuznetsova, L. V.; Wong, S. S.; Ojima, I. Functionalized single-walled carbon nanotubes as rationally designed vehicles for tumor-targeted drug delivery. *J. Am. Chem. Soc.* **2008,** 130, 16778-16785.

33. Ojima, I.; Zuniga, E. S.; Berger, W. T.; Seitz, J. D. Tumor-targeting drug delivery of new-generation taxoids. *Future Med. Chem.* **2012,** 4, 33-50.

34. Ojima, I. Tumor-targeting drug delivery of chemotherapeutic agents. *Pure & Appl. Chem.* **2011,** 83, 1685-1698.

35. Schwabacher, A. W.; Lane, J. W.; Schiesher, M. W.; Leigh, K. M.; Johnson, C. W. Desymmetrization reactions: Efficient preparation of unsymmetrically substituted linker molecules. *J. Org. Chem.* **1998,** 63, 1727-1729.

36. Borcard, F.; Godinat, A.; Staedler, D.; Blanco, H. C.; Dumont, A. L.; Chapuis-Bernasconi, C.; Scaletta, C.; Applegate, L. A.; Juillerat, F. K.; Gonzenbach, U. T.; Gerber-Lemaire, S.; Juillerat-Jeanneret, L. Covalent cell surface functionalization of human fetal osteoblasts for tissue engineering. *Bioconjug. Chem.* **2011,** 22, 1422-1432.

37. Widdison, W. C.; Wilhelm, S. D.; Cavanagh, E. E.; Whiteman, K. R.; Leece, B. A.; Kovtun, Y.; Goldmacher, V. S.; Xie, H.; Steeves, R. M.; Lutz, R. J.; Zhao, R.; Wang, L.; Blattler, W. A.; Chari, R. V. Semisynthetic maytansine analogues for the targeted treatment of cancer. *J. Med. Chem.* **2006,** 49, 4392-4408.

38. Ojima, I. Use of fluorine in the medicinal chemistry and chemical biology of bioactive compounds – A case study on fluorinated taxane anticancer agents. *ChemBioChem* **2004,** 5, 628-635.

39. Kigawa, J.; Minagawa, Y.; Kanamori, Y.; Itamochi, H.; Cheng, X.; Okada, M.; Oisho, T.; Terakawa, N. Glutathione concentration may be a useful predictor of response to second-line chemotherapy in patients with ovarian cancer. *Cancer* **1998,** 82, 697-702.

40. Zheng, Z. B.; Zhu, G.; Tak, H.; Joseph, E.; Eiseman, J. L.; Creighton, D. J. N-(2-hydroxypropyl)methacrylamide copolymers of a glutathione (GSH)-activated glyoxalase I inhibitor and DNA alkylating agent: Synthesis, reaction kinetics with GSH, and in vitro antitumor activities. *Bioconjug. Chem.* **2005,** 16, 598-607.

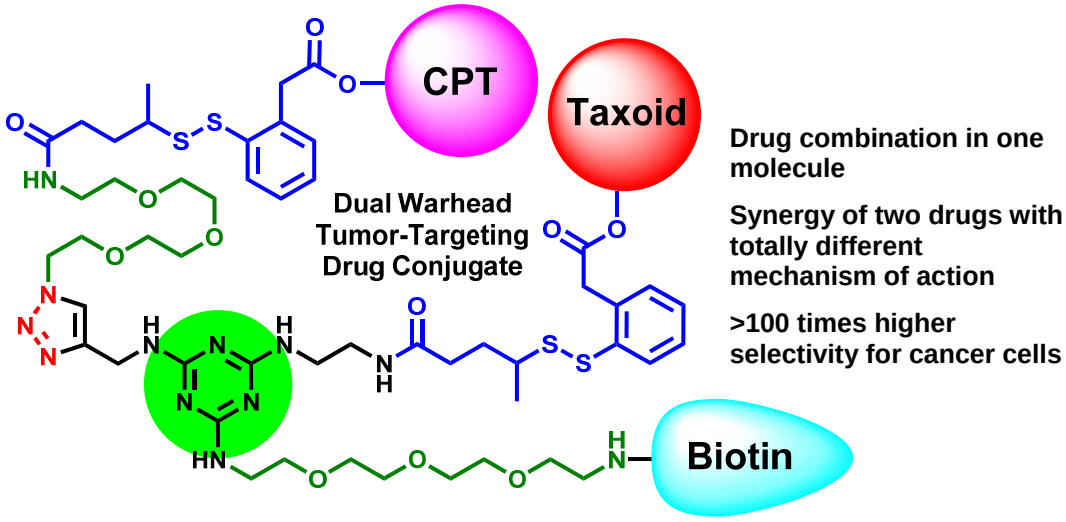
41. Banerjee, P. S.; Zuniga, E. S.; Ojima, I.; Carrico, I. S. Targeted and armed oncolytic adenovirus via chemoselective modification. *Bioorg. Med. Chem. Lett.* **2011,** 21, 4985-4988.

42. Pouillart, P.; Weiner, R.; Schwarzenberg, L.; Misset, J. L.; Oldham, R.; Amiel, J. L.; Mathe, G. Combination chemotherapy based on a model of cell recruitment by partial synchronization. *Med Pediatr. Oncol.* **1975,** 1, 123-134.

43. Mistry, S. N.; Baker, J. G.; Fischer, P. M.; Hill, S. J.; Gardiner, S. M.; Kellam, B. Synthesis and in vitro and in vivo characterization of highly beta1-selective beta-adrenoceptor partial agonists. *J. Med. Chem.* **2013,** 56, 3852-3865.

44. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. method* **1983,** 65, 55-63.

Table of Contents (TOC) graphic



Drug combination in one molecule

Synergy of two drugs with totally different mechanism of action

>100 times higher selectivity for cancer cells