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Dual-acting histone deacetylase-topoisomerase I inhibitors

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ABSTRACT

Current chemotherapy regimens are comprised mostly of single-target drugs which are often plagued by toxic side effects and resistance development. A pharmacological strategy for circumventing these drawbacks could involve designing multivalent ligands that can modulate multiple targets while avoiding the toxicity of a single-targeted agent. Two attractive targets, histone deacetylase (HDAC) and topoisomerase I (Topo I), are cellular modulators that can broadly arrest cancer proliferation through a range of downstream effects. Both are clinically validated targets with multiple inhibitors in therapeutic use. We describe herein the design and synthesis of dual-acting histone deacetylase–topoisomerase I inhibitors. We also show that these dual-acting agents retain activity against HDAC and Topo I, and potently arrest cancer proliferation.

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Current chemotherapeutic options for the treatment of cancer are often plagued by debilitating side effects and off-target toxicities. While other pharmacological options, such as gene or immunotherapies, are attaining increasing viability for researchers, most clinical options still center on traditional small molecule chemotherapy. There is considerable interest in designing novel small molecule agents that retain efficacy, while increasing the specificity toward the target of choice, thereby reducing side effects. While single-target drugs remain a popular design endpoint, there has been a recent surge of interest toward multivalent ligand design. It is thought that these drugs could possess a greater therapeutic advantage, by modulating multiple targets and avoiding the side effects of any single agent. Additionally, multivalent ligands are not expected to face the inherent pharmacokinetic and pharmacodynamics disadvantages of administering two or more separate drugs, a common liability that may complicate the outcome of traditional combination therapy.[1] The benefit of drugs with multiple targets relative to the conventional combination therapies has only begun to be elucidated, and these therapies are becoming increasingly common across a variety of pharmacological applications.[1–5]

Cancer offers a unique opportunity for the design of a multifunctional drug due to the multiple pathways contributing to the disease state. One promising pathway for tumor growth inhibition is that of epigenetic and protein acetylation state modulation by histone deacetylases (HDACs). HDACs function within a pathway that was originally discovered to alter the acetylation of histone proteins, leading to a more condensed nucleosome and decreased transcription.[6,7] The counterpart enzyme, histone acetyltransferase (HAT), has the opposite effects; acetylating histones and upregulating transcription.[8] The proposed cancer-promoting mechanism of HDAC involves transcriptional silencing of tumor suppressors via deacetylation of nucleosomes containing tumor suppressor genes.[9,10] However, recent evidence has shown HDAC involvement in the deacetylation of important non-histone regulatory proteins such as p53,[11] E2F,[12] and tubulin.[13] HDACs inhibitors (HDACi) have been shown to cause growth arrest, differentiation, and apoptosis in cancer cells.[14–16] Two HDACi, **SAHA** (Vorinostat) (Fig. 1) and **FK-228** (Romidepsin), have been approved by the FDA for the treatment of cutaneous T-cell lymphoma,[17,18] thus opening the door for HDACi as viable therapeutic agents.[19,20] For these reasons, HDACs remain an attractive target for small molecule inhibition.

Another proven anticancer target is topoisomerase I (Topo I). The Topo I enzyme relieves the torsional strain on DNA during DNA replication by cutting one strand of the DNA double helix and passing one strand over the other.[21,22] Due to the inherent need for rapid replication in cancer, inhibitors of topoisomerases result in DNA strand breaks, cell cycle arrest, and apoptosis.[23–27] Many small molecule inhibitors of Topo I have proven clinically

Abbreviations: HDAC, histone deacetylase; HAT, histone acetyltransferase; HDACi, histone deacetylase inhibitors; **SAHA**, suberoylanilide hydroxamic acid; TSA, trichostatin A; Topo I, topoisomerase I class of enzymes.

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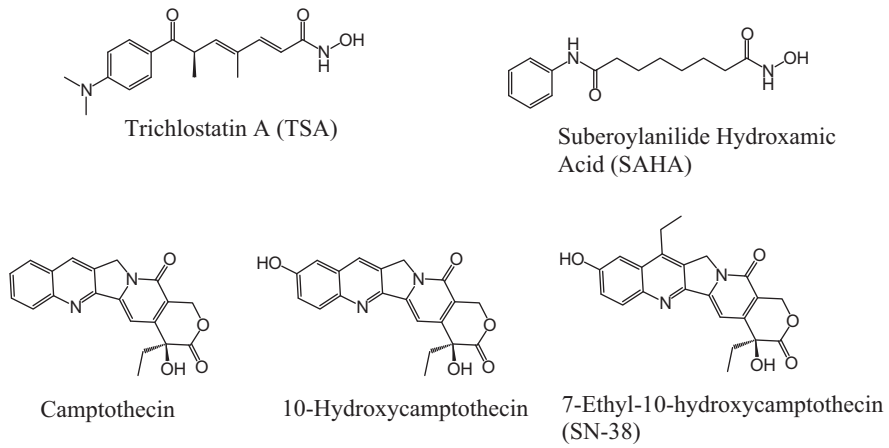


Figure 1. Representative HDAC and Topo I inhibitors.

effective and are currently FDA-approved for cancer chemotherapy.[25] Since both HDAC and Topo I enzymes are localized to the nucleus, the opportunity for dual inhibition from a single agent is a promising possibility. Creating a dual-acting HDAC–Topo I inhibitor could prove beneficial for many reasons. First, HDACi have been shown to act synergistically with Topo I inhibitors, resulting in enhanced apoptosis in cancer.[28] Also, since both enzymes are nuclear-localized, dual-acting agents may have better therapeutic indices.

Using fused-frameworks design approach,[1] we have previously, described dual-acting agents derived from an anthracycline, a topoisomerase II (Topo II) inhibitor and **SAHA** analogs, prototypical HDACi. A subset of these dual-acting HDAC–Topo II inhibitors inhibited Topo II and HDAC activities more potently compared to parent anthracycline and **SAHA**, respectively.[29] Furthermore, a lead compound from this series was equipotent to daunorubin against selected breast, lung and prostate cancer cell lines. As a follow-up to our work on dual-acting HDAC–Topo II inhibitors, we have designed and synthesized dual-acting HDAC–Topo I inhibitors derived from the camptothecin ring system and the linker region of **SAHA**-like HDACi. We show here that an alternative designed multiple ligand approach, merged-frameworks strategy,[1b] proved successful in the design of HDAC–Topo I inhibitors. We present evidence here that these compounds retain inhibitory activities against both target enzymes and inhibit the proliferation of selected cancer cell lines.

The camptothecin family of Topo I inhibitors are potent anticancer drugs that form a ternary complex at the interface of the cleavage complex, inhibiting dissociation of Topo I from DNA. We chose 10-hydroxycamptothecin and 7-ethyl-10-hydroxycamptothecin (**SN-38**) (Fig. 1) as the Topo I inhibiting templates for the design of the proposed dual-acting HDAC–Topo I inhibitors due to their promising activity against a range of tumor types and the presence of a functionalizable phenolic group at their C-10 position. Also, both templates have demonstrated more potency and less toxicity than camptothecin.[30–32] From structure–activity relationship (SAR) studies on camptothecins, substitution at the 10-hydroxy group has been found to be tolerable,[33] so we used this position as the point of attachment for the HDACi moiety. We have already reported the suitability of 1,2,3-triazole ring as a surface recognition cap group-linking moiety in **SAHA**-like HDAC inhibitors.[34] These studies showed cap group-dependent preference for five to six methylene linkers. In the designed dual-acting compounds, the linker region of **SAHA**-like HDACi is coupled through a triazole moiety to the camptothecin template, which in turn is anticipated to act as an aromatic surface recognition cap group essential for HDAC

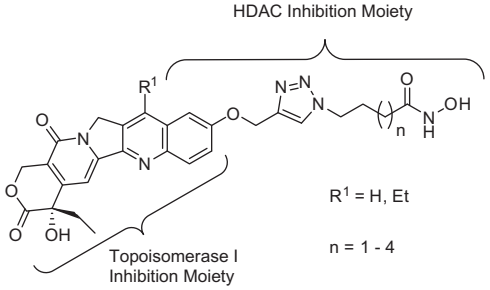
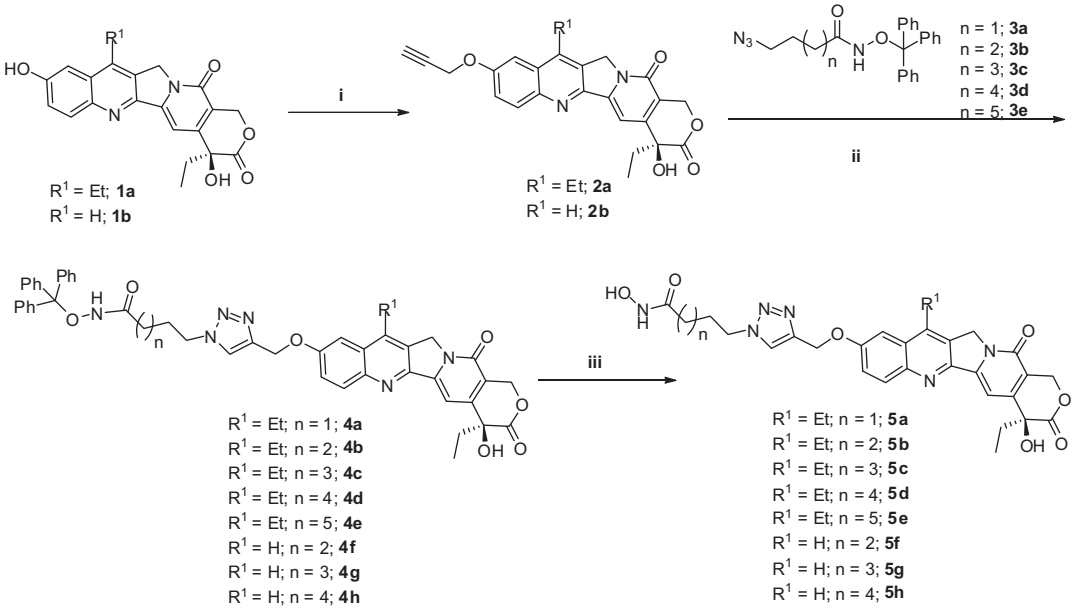


Figure 2. Designed dual-acting HDAC–Topo I inhibitors.

inhibition while also retaining its Topo I inhibition activity (Fig. 2). We introduced variations into the linker region to test the linker length-dependent potency of the resulting dual-acting agents. Additionally, incorporation of the triazole ring into compound design helped to simplify synthesis and SAR studies.

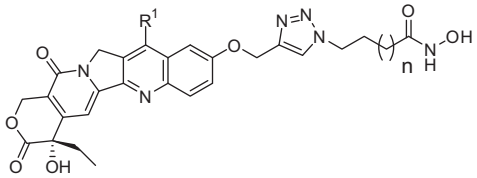
The reaction route to all the designed compounds is shown in Scheme 1. The phenolic OH-group of 7-ethyl-10-hydroxycamptothecin **1a** and 10-hydroxycamptothecin **1b** was alkylated with propargyl bromide to yield the corresponding alkyne intermediates **2a** and **2b**, respectively. Cu-catalyzed Huisgen cycloaddition[35] with known azido intermediates **3a–e**[34,36] afforded trityl-protected compounds **4a–h**. Subsequent TFA deprotection of **4a–h** yielded the desired compounds **5a–h** in good yields with minimal purification required.

Building on our previous observations about the linker length-dependent potency of aryltriazolyl HDACi,[34,36] we first synthesized and evaluated the anti-HDAC activity of 7-ethylcamptothecin-derived compounds **5a–e** against HeLa cell nuclear extract HDACs as described previously with a slight modification.[34] Briefly, camptothecin has a fluorescence emission (excitation $\lambda = 370$ nm, emission $\lambda = 434$ nm) close to the wavelength (460 nm) of the fluorescence generated by the HDAC enzyme cleavage of its fluorogenic substrate. To circumvent this potential interference, controls containing the same concentration of the test compound without the enzyme were used, and the background fluorescence of these controls were subtracted from the experimental fluorescence readings. Compound **5a**, an analog with a three methylene linker separating the triazole ring and the hydroxamate moiety, has no measurable anti-HDAC activity at concentrations as high as 10 μM (Table 1). The inactivity of **5a** may be due to the fact that its linker region is too short to effectively position its hydroxamate



Scheme 1. Synthesis of dual-acting HDAC–Topo I inhibitors. Reagents and conditions: (i) propargyl bromide, K_2CO_3, DMSO, rt, 48 h; (ii) compounds **3a–e**, CuI, THF:DMSO:Hunig's base 10:1:0.1; (iii) TFA, thioanisole, CH_2Cl_2, 0 °C.

Table 1
In vitro HDAC inhibition activity of novel HDAC–Topo I inhibitors



Compound	*n*	R^1	HeLa[a] IC_{50} (nM)	HDAC 1[b] IC_{50} (nM)	HDAC 6[b] IC_{50} (nM)	HDAC 8[b] IC_{50} (nM)
5a	1	$-CH_2CH_3$	ND	ND	85 ± 34	1726 ± 577
5b	2	$-CH_2CH_3$	155.4	NT	NT	NT
5c	3	$-CH_2CH_3$	120.7	129 ± 33	42 ± 6	ND
5d	4	$-CH_2CH_3$	64.65	50 ± 7	36 ± 5	ND
5e	5	$-CH_2CH_3$	212.3	369 ± 111	75 ± 34	2599 ± 475
5f	2	–H	144.5	116 ± 40	260 ± 40	ND
5g	3	–H	112.2	NT	NT	NT
5h	4	–H	56.2	37 ± 7	81 ± 26	1046 ± 316
SN-38	—	—	ND	NT	NT	NT
SAHA	—	—	65.0	38 ± 2	27 ± 2	1989 ± 156

ND—Nondeterminable within tested range, 1 nM–10 μM; NT—not tested.
[a] HeLa nuclear extract. Each value is obtained from three independent experiments.
[b] Data obtained through contract arrangement with BPS Bioscience (San Diego, USA; www.bpsbioscience.com). Assays were performed in duplicates at each concentration and data reported with standard error.[29,34c]

moiety within the active site while maintaining the crucial surface residue contacts. Conversely, compounds **5b–e** displayed linker length-dependent HDAC inhibitory activities with compound **5d**, analog with six methylene linkers, having inhibition activities comparable to **SAHA** (Table 1). The anti-HDAC activities of these compounds followed linker length-dependence similar to what we observed for other aryltriazolyl HDACi.[34,36] This result suggests that the camptothecin ring could function as a cap group, facilitating HDAC inhibition perhaps through interactions with the enzyme surface residues.

In order to elucidate the contribution of the ethyl group at the C-7 of 7-ethylcamptothecin on HDAC inhibition activity, we synthesized camptothecin-derived compounds **5f–h**, analogs with four, five and six methylene linkers, respectively. Our choice of this linker range is based on the foregoing observation that this range conferred the optimum activity to the 7-ethylcamptothecin derived compounds **5b–d**. A comparison of the anti-HDAC activities of **5b–d** and **5f–h**, against the HeLa cell nuclear extract HDACs, reveals that pairs with the same linker length have nearly identical HDAC inhibition activity (Table 1). These results suggest that the presence or lack thereof of the ethyl group at the C-7 of camptothecin ring system has no significant effect on the inhibition of HeLa cell nuclear extract HDACs. As expected, the standard Topo I inhibitor—**SN-38**—had no measurable HDAC inhibition activity.

To obtain additional evidence for the specific mode of HDAC inhibition, isoform selectivity was investigated by testing selected

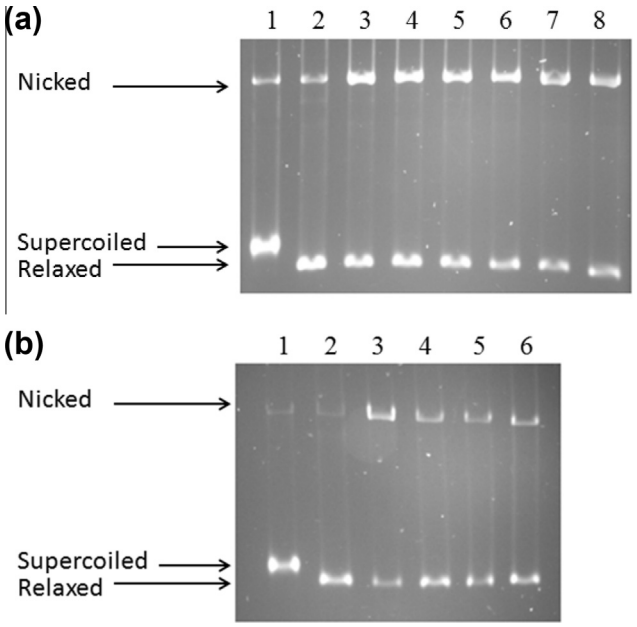


Figure 3. Topoisomerase I-induced plasmid relaxation assay: (a) (lane 1) pBR322 plasmid DNA, (lane 2) DNA and Topo I, (lanes 3–8) DNA, Topo I, and 50 μM (3) **SN-38**, (4) **5a**, (5) **5b**, (6) **5c**, (7) **5d**, (8) **5e**; (b) (lane 1) pBR322 plasmid DNA, (lane 2) DNA and Topo I, (lanes 3–6) DNA, Topo I, and 50 μM: (3) **SN-38**, (4) **5f**, (5) **5g**, (6) **5h**.

Table 2
Whole cell cytotoxicity activity against DU-145 prostate cancer cells, as determined by MTS assay after 72 h

Compound	*n*	R^1	IC_{50} (μM)
5a	1	$-CH_2CH_3$	6.27
5b	2	$-CH_2CH_3$	4.25
5c	3	$-CH_2CH_3$	2.05
5d	4	$-CH_2CH_3$	3.11
5e	5	$-CH_2CH_3$	3.51
5f	2	–H	2.50
5g	3	–H	1.95
5h	4	–H	2.03
SN-38	—	—	0.11
SAHA	—	—	2.12

All values are mean of two experiments performed in triplicate as measured by the MTS assay (Promega).

compounds against purified HDAC1, HDAC6, and HDAC8. The pattern of the anti-HDAC activities of these compounds against HDAC 1 and HDAC 6 mirrored what was observed for the HeLa cell nuclear extract HDACs with one exception. Specifically, the three methylene-linked compound **5a** is inactive against HDAC 1 while it maintains low nanomolar and micromolar IC_{50}'s against HDAC 6 and HDAC 8, respectively (Table 1). In general, these dual-acting agents are more selective for HDAC 6 with modest or no activity against HDAC 8. The preference for HDAC6 over HDAC1 could further explain the inactivity of **5a** against HeLa nuclear extract, which is a rich source of HDACs 1 and 2.[37]

We performed a cell-free DNA plasmid relaxation assay, according to a literature protocol, in order to determine the Topo I inhibition activity of these HDAC–Topo I inhibitors.[38,39] In this assay, a supercoiled plasmid is incubated with Topo I in the presence or absence of Topo I inhibitors. Reactions are terminated by addition of SDS, which denatures Topo I. Reaction mixtures are then electrophoresed in an agarose gel and DNA is visualized using a nucleic acid dye. Stabilized cleavage complexes that are covalently bound to DNA will inhibit migration of DNA in the gel significantly more, relative to unbound, relaxed DNA. **SN-38** was used as a positive control for Topo I inhibition and drugs were dosed at 50 μM. The 7-ethylcamptothecin compounds **5a–e** inhibited Topo I, evidenced by both the reduction of relaxed plasmid and increase in nicked plasmid compared to uninhibited Topo I, with no apparent drop in activity compared to **SN-38** (Fig. 3a). Similarly, camptothecin compounds **5f–h** inhibited Topo I with similar activities to each other, but are less active relative to **SN-38** (Fig. 3b). The enhanced Topo I inhibitory activities of **5a–e** relative to **5f–h** is not unexpected as **SN-38**, the template for **5a–e**, is a more potent Topo I inhibitor compared to 10-hydroxycamptothecin, the template for **5f–h**.[40,41] These results, taken together with the HDAC inhibition data, showed that these dual-acting HDAC–Topo I inhibitors could function to inhibit either target enzyme and merging of the two inhibiting moieties does not preclude the activities of either parent compound significantly.

To examine the effects of these dual-acting inhibitors on cancer cell proliferation, they were screened against the DU-145 prostate cancer cell line and the inhibition of cell viability was measured. **SN-38** and **SAHA** were used as positive controls with **SN-38** potently inhibiting DU-145 viability in the mid-nanomolar range, while **SAHA**'s IC_{50} was higher, in the low micromolar range. The bifunctional compounds **5a–h** showed linker length dependent anti-proliferative activities with a five methylene linker proving to be optimum for cytotoxicity among the 7-ethylcamptothecin compounds (Table 2, comparing **5a–e**). Compound **5a**, with the shortest linker of three methylenes, possessed the least potent activity against DU-145. Conversely, the camptothecin compounds **5f–h** displayed indistinguishable cytotoxic activity. Comparatively, most of the 7-ethylcamptothecin compounds are generally less active than their camptothecin congeners. One exception is compound **5c** which showed cytotoxicity activity that is identical to that of **5g**, its direct camptothecin analog. More importantly, the micromolar IC_{50} values suggest that HDAC inhibition may be the dominating mode of antiproliferative activities of compounds **5a–h**.

Since the anticancer activities displayed by these dual-acting HDAC–Topo I inhibitors against DU-145 appeared to be largely driven by HDACi-based mechanisms, we profiled the contribution of intracellular HDAC inhibition through the level of $p21^{waf1}$ expression.[34b] Compounds **5c** and **5g** were used, premised on the fact that they are representative examples from each of the two Topo I

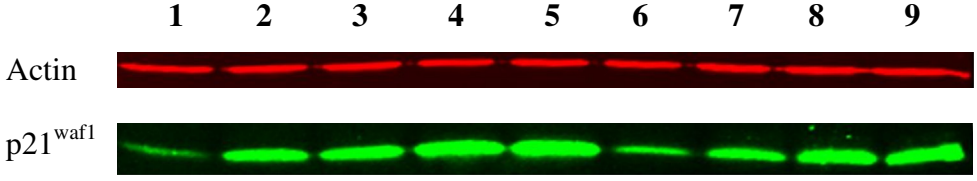


Fig. 4. Western blot probing for actin and p21 in the DU-145 cell line. Lanes: (1) control, (2) **SAHA**, 2.5 μM, (3) **SAHA** 5.0 μM, (4) **SN-38**, 0.1 μM, (5) **SN-38** 0.5 μM, (6) **5c**, 2.5 μM, (7) **5c**, 5.0 μM, (8) **5g**, 2.5 μM, (9) **5g**, 5.0 μM.

inhibiting templates with identical linker lengths and anticancer activities. Inhibitors were dosed at concentrations near the determined IC_{50}'s in DU-145 (Table 2) and $p21^{waf1}$ expression was probed via immunoblot (Fig. 4). Equivalent protein loading was demonstrated using an anti-actin antibody (Fig. 4, top). Both **SAHA** and **SN-38** resulted in marked upregulation of $p21^{waf1}$ expression levels with 24 hour treatment (Fig. 4, bottom, lanes 2–5). Gratifyingly, we observed that the dual-acting compounds **5c** and **5g** resulted in substantial upregulation of $p21^{waf1}$ expression in a concentration-dependent manner with **5g** causing upregulation at levels comparable to **SN-38** (Fig. 4, bottom panel, lanes 6–9). These results suggest that compounds **5c** and **5g** derived their cytotoxic activity, in part, through HDAC inhibition. It is unclear at present how much of the $p21^{waf1}$-dependent anticancer activity is contributed by each inhibiting moiety as both **SAHA** and **SN-38** significantly increased $p21^{waf1}$ expression. Subsequent investigation into the expression levels of other cellular markers could clarify the driving force behind the cellular effects observed.

A new class of dual-acting HDAC–Topo I inhibitors has been created from camptothecin and **SAHA**-like templates. Two types of camptothecin templates were used and both were connected through their 10-hydroxy moieties to alklyltriazolyl hydroxamates that we have shown possess enhanced HDAC inhibition activity.[34] Results from cell-free and whole cell studies showed that these compounds possess inhibition activities against both target enzymes and inhibit the growth of DU-145 prostate carcinoma cells. Relative to the camptothecin standard **SN-38**, the functionalization of the 10-hydroxy moiety presented no observable deleterious effect on the Topo I inhibition by 7-ethyl-10-hydroxycamptothecin-derived conjugates **5a**–**e** and only minor attenuation in the inhibitory activities of 10-hydroxycamptothecin-derived conjugates **5f**–**h** at the concentration tested (50 μM). Despite their potent Topo I inhibition activities in cell-free DNA plasmid relaxation assays, these compounds displayed anticancer activities against DU-145 cells at levels more comparable to the HDACi standard **SAHA**. One plausible explanation for this observation is that the functionalization of the 10-hydroxy moiety may negatively impact the binding of these conjugates to Topo I as crystallographic evidence suggests that the 10-hydroxy group is involved in a hydrogen bonding interaction with a water molecule oxygen at the Topo I active site.[42] Alternatively, the ability of these conjugates to interact with other tumor growth-inhibiting secondary targets of camptothecins[43,44] may be compromised.

Overall, these compounds show promise as potent anticancer agents with the potential to broadly arrest tumor growth by inhibiting two essential enzymes. Ongoing efforts in our laboratory are on the design of a second generation conjugates which retain the 10-hydroxy moiety of camptothecinin order to better understand the mechanism of antiproliferative activity of this class of compounds.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.108.

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