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Discovery of Novel Multiacting Topoisomerase I/II and Histone Deacetylase Inhibitors

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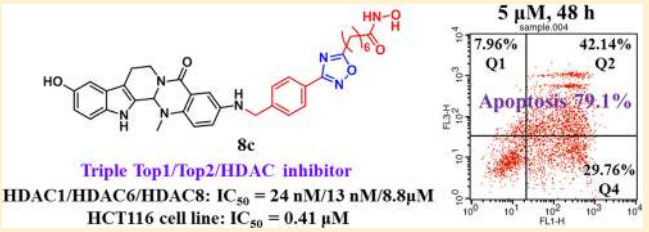
Supporting Information

ABSTRACT: Designing multitarget drugs remains a significant challenge in current antitumor drug discovery. Because of the synergistic effect between topoisomerase and HDAC inhibitors, the present study reported the first-in-class triple inhibitors of topoisomerase I/II and HDAC. On the basis of 3-amino-10-hydroxylevodiamine and SAHA, a series of hybrid molecules was successfully designed and synthesized. In particular, compound **8c** was proven to be a potent inhibitor of topoisomerase I/II and HDAC with good antiproliferative and apoptotic activities. This proof-of-concept study also validated the effectiveness of discovering triple topoisomerase I/II and HDAC inhibitors as novel antitumor agents.



KEYWORDS: *Evodiamine derivatives, topoisomerase I, topoisomerase II, histone deacetylase, antiproliferative activity*

Over the last two decades, drug discovery has been primarily focused on the development of single-target drugs with high potency and selectivity.[1] However, not all the diseases are amenable to this one disease−one target approach. Also, these drugs are not as effective as expected for the treatment of complex diseases, such as cancer. Recent evidence has shown that most single-target drugs are plagued by toxic side effects and development of resistance. To overcome these problems, designing a single drug molecule that interacts with multiple targets simultaneously and specifically is gaining considerable interests in drug discovery.[2−5]

Cancer offers a unique opportunity for the multivalent ligand design because of the complicated target networks and multiple cellular pathways contributing to the disease state. Epigenetic control, such as protein acetylation and deacetylation state, is regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs). Targeting HDACs represents one of the most popular approaches for tumor growth inhibition.[6,7] HDACs regulate the acetylation of a diverse range of histone and nonhistone proteins, controlling the transcription and regulation of genes as well as cell proliferation, migration, death, and angiogenesis, and have been recognized as important molecular targets for cancer treatment.[8,9] Two HDAC inhibitors (HDACi), SAHA (vorinostat) (Figure 1) and FK228 (romidepsin), have been approved by FDA for the treatment of the cutaneous T-cell lymphoma (CTCL).[10,11] In many reports, HDACi can synergistically enhance the inhibitory effect of other antitumor agents, such as tubulin,[12] Hsp90,[13] EGFR,[14] and topoisomerase[15−17] inhibitors, to suppress proliferation and induce apoptosis in tumor cells. The synergistic effects of HDAC inhibitors with other antitumor agents can be used as a useful strategy to design multifunctional inhibitors to simultaneously interact with multiple targets with high potency and low toxicity. Among these targets, topoisomerase (Top1 and/or Top2) is a good starting point for multivalent ligand design.[8,18] It was reported that the resistance to Top2 inhibitors is often concomitant with a rise in the level of Top1 expression and vice versa.[19] In this regard, a single molecule able to inhibit Top1/Top2/HDAC could be helpful to prevent mechanism-based drug resistance and show more powerful antitumor activity. However, the discovery of a single molecule targeting three proteins remains a significant challenge, and no HDAC1/Top1/Top2 triple inhibitors have been reported up to date.

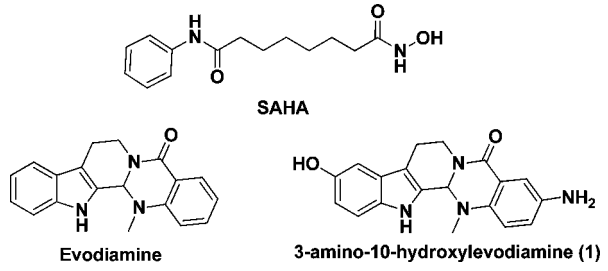


Figure 1. Chemical structure of SAHA, evodiamine, and dual Top1/Top2 inhibitor **1**.

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Evodiamine (Figure 1) is a quinazolinocarboline alkaloid isolated from the fruits of traditional Chinese herb *Evodiae fructus* (Chinese name: Wu-Chu-Yu) with diverse biological activities.[20] In our previous study, a number of evodiamine derivatives were designed and synthesized.[21,22] Among them, 3-amino-10-hydroxylevodiamine (**1**) showed excellent antitumor activity against a variety of cancer cell-lines with good *in vivo* potency in xenograft nude mice. Moreover, compound **1** was proven to be a dual Top1/Top2 inhibitor by *in silico* target identification in combination with biological assays. Inspired by these results, compound **1** and SAHA can be used as good templates to design triple HDAC/Top1/Top2 inhibitors.

As depicted in Figure 2, a series of novel evodiamine−SAHA hybrids were rationally designed and synthesized as triple-targeting antitumor agents. First, using a molecular hybridization strategy, compound **1** and SAHA were merged into a new hybrid molecule (**7**). From structure−activity relationship studies on evodiamine derivatives,[21−23] substitution at the 3-amino group was tolerable. Thus, SAHA was attached at this position. Meanwhile, because of the presence of large hydrophobic patches at the HDAC surface rim, conjugating SAHA with hydrophobic antitumor agent **1** may generate potent HDAC inhibitors.[24] Second, 1,2,4-oxadiazoles and 1,3,4-oxadiazoles were introduced as a proper spacer between the evodiamine scaffold and the zinc binding group of SAHA (compounds **8a−c** and **9a−c**). Oxadiazole was chosen as the spacer because it is a drug-like privileged structure in many therapeutic drugs and always used as a flat, aromatic linker to place substituents in the appropriate orientation for ligand binding.[25] Moreover, introduction of 1,3,4-oxadiazole ring was proven to be an effective strategy to modulate lipophilicity and pharmacokinetic profiles.[25] Third, our previous study indicated that the C-10 hydroxyl group of evodiamine was important in maintaining antitumor potency. In order to validate the importance of this hydroxyl group in the newly designed hybrid molecules, a series of 10-methoxyl derivatives (**10a−d**) were also designed and synthesized.

The synthetic routes of the target compounds are shown in Schemes 1−4. The commercially available compound **2** was reacted with monomethyl suberate to give intermediate **4**,[26] which was further oxidized by pyridinium chlorochromate (PCC) to afford aldehyde **5**. Treatment of compounds **5** and **1** via reductive amination reaction yielded the key intermediate **6**, which was reacted with freshly prepared hydroxylamine methanol solution to give the target compound **7** (Scheme 1).

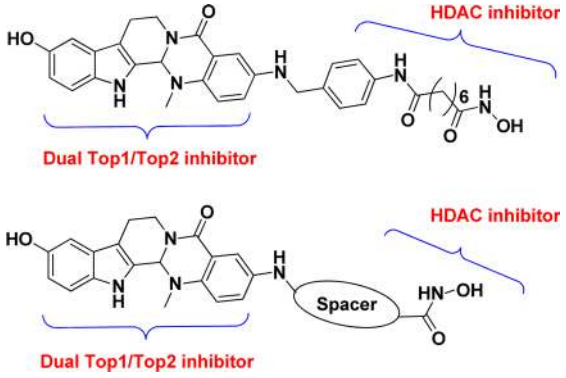
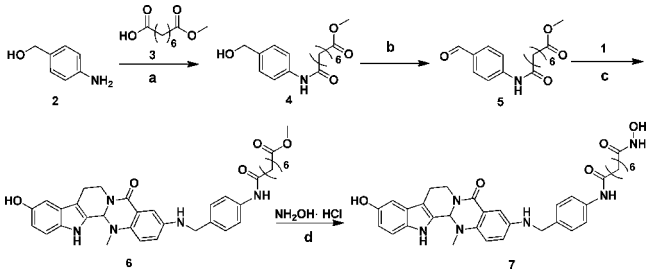


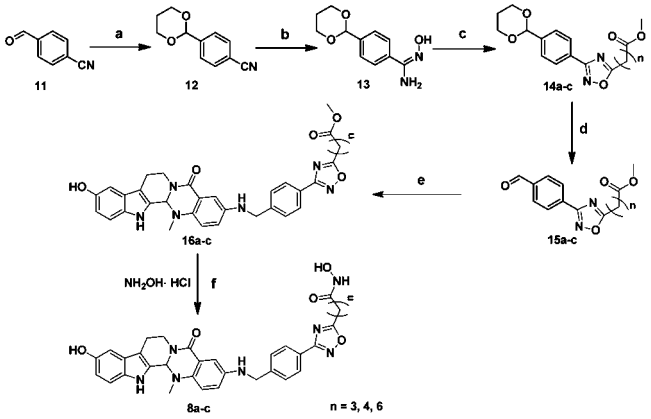
Figure 2. Design of triple-acting Top1/Top2/HDAC inhibitors.

Scheme 1. Chemical Synthesis of Compound 7[a]



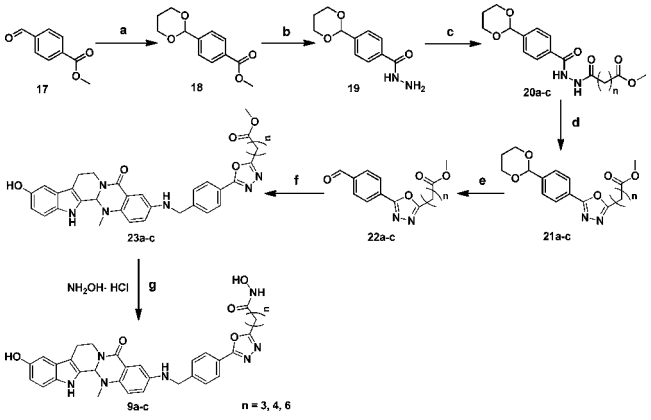
[a]Reagents and conditions: (a) HBTU (*O*-benzotriazole-*N*,*N*,*N*,*N*-tetramethyl-uronium-hexafluorophosphate), Et_3N, MeCN, rt, 2 h, 76%; (b) PCC, rt, 2 h, 95%; (c) $NaBH_3CN$, THF, rt, 3 h, 46%; (d) KOH, CH_3OH, 40 °C, 45 min, 89%.

Scheme 2. Chemical Synthesis of Compounds 8a−c[a]



[a]Reagents and conditions: (a) propane-1,3-diol, toluene, reflux, 6 h, 95%; (b) $NH_2OH{\cdot}HCl$, $NaHCO_3$, CH_3OH, reflux, 2 h, 76%; (c) $HO_2C(CH_2)_nCO_2CH_3$ (n = 3, 4, 6), HBTU, DIPEA, DMF, microwave, 191 °C, 2 min; (d) $Fe(HSO_4)_3$, acetone/H_2O = 1:1, reflux, 1 h, 85−86%, over two steps; (e) **1**, $NaBH_3CN$, CH_3OH, rt, 3 h, 45%; (f) KOH, CH_3OH, 45 °C, 45 min, 90−92%.

Scheme 3. Chemical Synthesis of Compounds 9a−c[a]



[a]Reagents and conditions: (a) propane-1,3-diol,toluene, reflux, 6 h, 93%; (b) $N_2H_4{\cdot}H_2O$, rt, 24 h, 73%; (c) $HO_2C(CH_2)_nCO_2CH_3$ (n = 3, 4, 6), HBTU, Et_3N, DMF, rt, 2 h, 72%; (d) *p*-toluenesulfonyl chloride, Et_3N, THF, reflux, 8 h, 78%; (e) $Fe(HSO_4)_3$, acetone/H_2O = 1:1, reflux, 1 h, 59−69%; (f) **1**, $NaBH_3CN$, CH_3OH, rt, 3 h; (g) KOH, CH_3OH, 45 °C, 45 min, 84−91%, over two steps.

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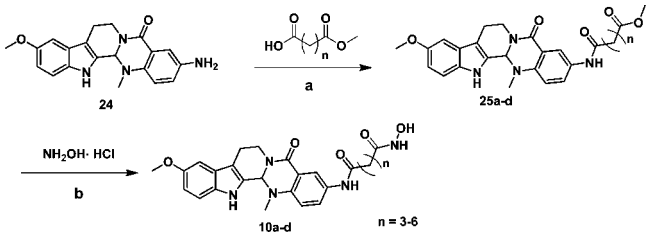
Scheme 4. Chemical Synthesis of Compounds 10a−d[a]



[a]Reagents and conditions: (a) HBTU, Et_3N, DMF, rt, 2 h, 63−75%; (b) KOH, CH_3OH, 45 °C, 45 min, 83−90%.

Similar strategy was applied for the synthesis of 1,2,4-oxadiazole-based conjugates (**8a−c**) (Scheme 2). Acetalization of the 4-formylbenzonitrile **11** afforded the propanediol-protected compound **12**, which was treated by hydroxylamine hydrochloride in the presence of $NaHCO_3$ and methanol to give compound **13** in good yield. Condensation of compound **13** with one of three carboxylic acid monomethyl esters under microwave conditions afforded compounds **14a−c**. Deprotection of 1,3-propanediol group of **14a−c** yielded the requisite aldehydes **15a−c** using freshly prepared $Fe(HSO_4)_3$ solution. Then, the synthesis of target compounds **8a−c** was similar to that of compound **7**. Synthesis of compounds **9a−c** was summarized in Scheme 3. Protection of aldehyde **17** with 1,3-propanediol gave the intermediate **18**, which was reacted with hydrazine hydrate to yield compound **19**. Acylation of **19** provided the amides **20a−c**, which were cyclized to give key intermediates **21a−c**. Then, compounds **9a−c** were synthesized using conditions similar to that of compounds **8a−c**. As depicted in Scheme 4, compounds **10a−d** were prepared from 3-amino-10-methoxylevodiamine (**24**) by a two-step synthesis via the condensation and ammonolysis reaction.

Initially, we tested the inhibitory activity of the target compounds against human recombinant HDAC1 enzyme using the assay as previously described by Bradner et al.[27] Generally, most compounds showed good to excellent HDAC1 inhibitory activity (Table 1). For example, compound **7** was highly active against HDAC1 (IC_{50} = 30 nM). Interestingly, all the 1,2,4-oxadiazole derivatives (**8a−c**) potently inhibited HDAC1 with IC_{50} values in the nanomolar range. Moreover, it was observed that the HDAC1 inhibitory activity was enhanced with the increasing of the carbon chain length (**8c** > **8b** > **8a**, **9c** > **9b** > **9a**, and **10d** > **10b** > **10a**) with the exception of compound **10c**. Compounds **9c** and **10d** exhibited good anti-HDAC1 activity with the IC_{50} values of 89 and 64 nM, respectively. In particular, compound **8c**, the best HDAC1 inhibitor (IC_{50} = 24 nM), showed comparable activity to SAHA (IC_{50} = 23 nM). To obtain evidence for the HDAC isoform selectivity, the inhibitory activity of compound **8c** was examined against selected recombinant HDAC2, HDAC3, HDAC6, and HDAC8 (Table 2). Compound **8c** displayed nanomolar activity against HDAC2 (IC_{50} = 275 nM), HDAC3 (IC_{50} = 71 nM), and HDAC6 (IC_{50} = 13 nM). In contrast, its activity against HDAC8 (IC_{50} = 2.5 μM) was significantly decreased. As compared with SAHA, compound **8c** was more potent against HDAC6 and HDAC8.

Table 1. *In Vitro* HDAC1 Inhibition and Antitumor Activity of Target Compounds

compd	HDAC1 IC_{50} (nM)	MDA-MB-231 IC_{50} (μM)	HCT116 IC_{50} (μM)	HLF IC_{50} (μM)
1	NT[a]	0.49 ± 0.02	0.43 ± 0.03	0.47 ± 0.03
SAHA	23 ± 2.1	7.4 ± 0.65	3.7 ± 0.30	3.0 ± 0.33
7	30 ± 4.5	14 ± 1.0	2.1 ± 0.26	13 ± 1.5
8a	321 ± 21	16 ± 1.9	3.5 ± 0.39	6.7 ± 0.48
8b	190 ± 13	5.9 ± 0.43	3.7 ± 0.28	8.2 ± 0.75
8c	24 ± 3.2	2.3 ± 0.14	0.41 ± 0.03	1.3 ± 0.10
9a	>10000	>168	11 ± 2.4	>168
9b	93 ± 5.7	30 ± 3.4	4.9 ± 0.32	26 ± 3.0
9c	89 ± 7.9	5.7 ± 0.61	9.7 ± 1.2	6.1 ± 0.55
10a	527 ± 45	191 ± 20	31 ± 3.1	97 ± 10
10b	275 ± 23	36 ± 3.1	22 ± 2.1	62 ± 5.6
10c	>10000	58 ± 4.5	35 ± 4.1	68 ± 6.7
10d	64 ± 5.5	54 ± 5.7	20 ± 2.3	30 ± 3.8

[a]NT = not tested.

Table 2. Inhibitory Activity of HDAC6 and HDAC8

compd	HDAC2 IC_{50} (nM)	HDAC3 IC_{50} (nM)	HDAC6 IC_{50} (nM)	HDAC8 IC_{50} (μM)
8c	275 ± 52	71 ± 6.4	13 ± 1.7	2.5 ± 0.29
SAHA	174 ± 70	56 ± 4.6	23 ± 2.1	8.8 ± 0.70

Previously, compound **1** and other evodiamine derivatives were identified as dual Top1/Top2 inhibitors.[22] Top1- and Top2α-mediated pBR322 DNA relaxation assays with purified Top1 and Top2α were used to evaluate the inhibitory activity of the target compounds. Camptothecin (CPT, Top1 inhibitor) and etoposide (Eto, Top2 inhibitor) were employed as positive controls. As shown in Figure 3A, all the tested compounds were found to be active against Top1-mediated DNA relaxation. Most of them exhibited comparable or superior Top1 inhibitory activity to CPT. The 1,2,4-oxadiazole derivatives (**8a−c**) showed higher Top1 inhibitory activity than CPT. The best inhibition was observed for compound **8c** containing the six methylene alkyl chain. In contrast, the 10-methoxyl derivatives (**10a−c**) showed weak inhibitory activity, and compound **10c** was almost inactive. Similarly, all the tested compounds were active against Top2 (Figure 3B). As compared to Eto, compounds **7**, **8b−c**, **10a**, and **10c** showed stronger Top2 inhibitory activity. Among them, compound **7** was the most active one. Interestingly, the activity of 1,2,4-oxadiazole derivatives was dependent on the length of the

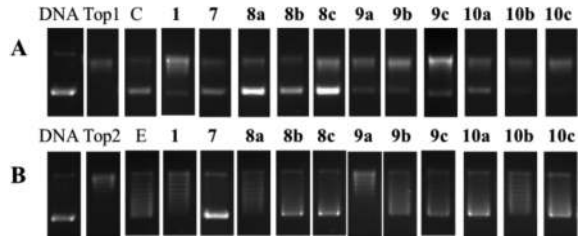


Figure 3. Top1 and Top2 inhibitory activity of target compounds. (A) Inhibition of Top1 relaxation activity at 50 μM. Lane 1, supercoiled plasmid DNA; Lane 2, DNA + Top1; Lane 3, DNA + Top1 + CPT (50 μM); Lanes 4−14, DNA + Top1 + compounds (**1**, **7**, **8a−c**, **9a−c**, and **10a−c** at 50 μM). (B) Inhibition of Top2 relaxation activity at 50 μM. Lane 1, supercoiled plasmid DNA; Lane 2, DNA + Top2; Lane 3, DNA + Top2 + Eto (50 μM); Lanes 4−14, DNA + Top2 + compounds (**1**, **7**, **8a−c**, **9a−c**, and **10a−c** at 50 μM). The assay was repeated 3 times.

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methylene chain. Reducing the alkyl chain to three carbon atoms (**8a**) resulted in decrease of Top2 inhibitory activity.

On the basis of the above results, the hybrid compounds derived from **1** and SAHA were proven to be triple-targeting inhibitors of HDAC/Top1/Top2. Particularly, compound **8c** generally exhibited the highest activity. Furthermore, we investigated the antiproliferative activities of the target compounds against MDA-MB-231 (breast cancer), HCT116 (colon cancer), and HLF (liver cancer) cell lines using parent compounds **1** and SAHA as reference drugs. The antitumor activity was determined using the standard MTT assay.[21] As shown in Table 1, most of the target compounds showed moderate to good antiproliferative activity against all the three cell lines. Moreover, these compounds showed better activity against HCT116 than the other two cell lines. Increasing the length of the methylene chain of 1,2,4-oxadiazole derivatives led to the improvement of antiproliferative activities, which was closely correlated to their anti-HDAC activities. This trend was also observed in 1,3,4-oxadiazole and 10-methoxyl derivatives, respectively. In particular, compound **8c** showed the most potent antitumor activity with IC_{50} values in the range of 0.41 to 2.29 μM. For the HCT116 cell line, compound **8c** was significantly more active than the reference drug SAHA and comparable to lead compound **1**. Thus, it was subjected to an apoptotic assay for further evaluation. Apoptosis was evaluated by annexin V test.[22] As shown in Figure 4, compound **8c**

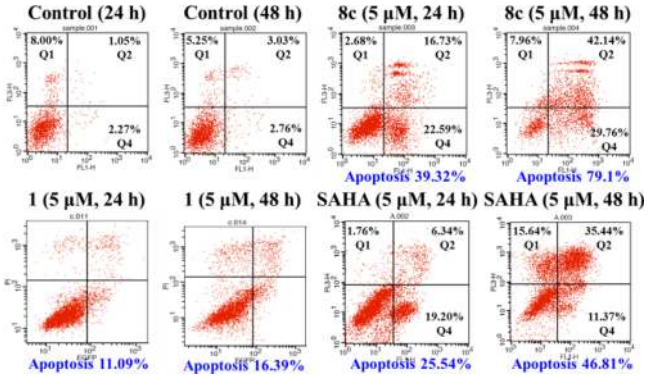

Figure 4. Cell apoptosis induced by compound **1**, **8c**, and SAHA. HCT116 cells were treated with DMSO and 5 μM compounds for 24 and 48 h, respectively. Apoptosis was examined by flow cytometer (n = 3).

exhibited strong pro-apoptotic activity in the HCT116 cell line. After treating with 5 μM compound for 24 and 48 h, the percentage of apoptotic cells for compound **8c** was 39.32% and 79.1% (Q2 + Q4), respectively. It displayed a much higher apoptosis level than compound **1** and SAHA (11.09% and 16.39% for **1** and 25.54% and 46.81% for SAHA, respectively). This result demonstrated that combination topoisomerase and HDAC inhibitor in one single molecule led to synergy at the cellular level. On the basis of the above results, compound **8c** was subjected for further evaluation of cellular HDAC inhibition. The effect of the compound **8c** on the acetylation level of histone 3 is shown in Figure 5. Exposure to compound **8c** for 24 and 48 h induced a hyperacetylation of histone 3 in the HCT116 cell line.

In summary, a series of novel evodiamine/SAHA hybrids were rationally designed and synthesized on the basis of synergistic effect observed between topoisomerase and HDAC inhibitors. They were identified as the first-in-class triple

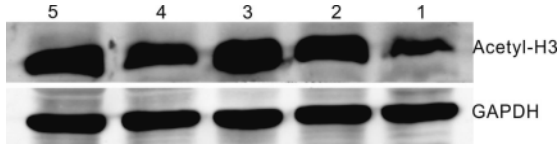

Figure 5. Western blot probing for acetylated histones H3 in the HCT116 cell line after 24 h treatment with compounds. Lanes: (1) control, (2) SAHA, 2.5 μM, (3) SAHA, 5.0 μM, (4) **8c**, 2.5 μM, and (5) **8c**, 5 μM.

inhibitors of Top1/Top2/HDAC. Notably, compound **8c** was proven to be a potent inhibitor of Top1/Top2/HDAC, which also showed good antiproliferative activities and remarkable apoptotic effect. Taken together, the present study provided a proof-of-concept study for discovering inhibitors simultaneously targeting Top1/Top2/HDAC. Further evaluation and optimization of the evodiamine/SAHA hybrids are in progress.

■ ASSOCIATED CONTENT

Supporting Information

Chemical synthesis and structural characterization of the target compounds; protocols of biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

§S.H. and G.D. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Top1, topoisomerase I; Top2, topoisomerase II; HDAC, histone deacetylase; HDACi, HDAC inhibitors; PCC, pyridinium chlorochromate; HBTU, *O*-benzotriazole-*N*,*N*,*N*,*N*-tetramethyl-uronium-hexafluorophosphate; CPT, camptothecin; Eto, etoposide

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