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Hybrids from Farnesylthiosalicylic Acid and Hydroxamic Acid as Dual Ras-Related Signaling and Histone Deacetylase (HDAC) Inhibitors: Design, Synthesis and Biological Evaluation

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A novel series of hybrids was designed and synthesized by combining key elements from farnesylthiosalicylic acid (FTS) and hydroxamic acid. Several 3,7,11-trimethyldodeca-2,6,10-trien-1-yl) thio)benzamide derivatives, particularly those with branched and linear aliphatic linkers between the hydroxamic zinc binding group (ZBG) and the benzamide core, not only displayed significant antitumor activities against six human cancer cells but also exhibited histone deacetylase (HDAC) inhibitory effects in vitro. Among them, *N*-(4-(hydroxyamino)-4-oxobutyl)-2-(((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6, 10-trien-1-yl)-thio)benzamide (**8d**) was the most potent, with IC_{50} values of 4.9–7.6 μM; these activities are eight- to sixteen-fold more potent than FTS and comparable to that of suberoylanilide hydroxamic acid (SAHA). Derivative **8d** induced cell cycle arrest in the G0/G1 phase, inhibited the acetylation of histone H3 and α-tubulin, and blocked Ras-related signaling pathways in a dose-dependent manner. The improved tumor growth inhibition and cell-cycle arrest in vitro might result from the dual inhibition. These findings suggest dual inhibitors of Ras-related signaling pathway and HDAC hold promise as therapeutic agents for the treatment of cancer.

Ras proteins have long been considered drug targets for cancer therapy since they were first identified and characterized 30 years ago.[1] A large percentage of all cancer types either express one of the mutationally activated Ras isoforms or harbor a chronically activated Ras isoform.[2,3] Therefore, the inhibition of excessive activated Ras proteins or Ras signal pathway could revert malignant cells to a nonmalignant phenotype and cause tumor regression both in vitro and in vivo.[4] For instance, Ras inhibitor farnesylthiosalicylic acid (FTS, salirasib) exhibits antiproliferative effects in several cancer cell lines and has recently been evaluated in a phase II clinical study in patients with solid tumors.[5] However, FTS by itself does not induce differentiation or a complete cell-cycle arrest.[6] Therefore, FTS as a single-target drug has exhibited limited therapeutic efficacy.

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are enzymes that control reversible acetylation and deacetylation of histone. In addition, these enzymes have been shown to modulate other proteins such as transcription factors NF-κB and p53.[7] It has been well documented that HDACs are overexpressed in many cancers, such as human liver, colon, breast, and others,[8] and aberrant HDAC pathways are believed to promote cancer growth and metastasis.[9,10] HDAC inhibitors (HDACis) are thought to represent a new class of potential small-molecule cancer therapeutics.[11] A number of HDACis have been developed to date that are generally grouped into four chemical classes: hydroxamic acids, benzamides, cyclic tetrapeptides, and short-chain fatty acids. Suberoylanilide hydroxamic acid (SAHA, vorinostat, Figure 1) was the first HDACi approved in 2006 by the US Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL).[12] FK228 (romidepsin), a cyclic tetrapeptide, was approved for CTCL and peripheral T-cell lymphoma (PTCL) therapy in 2009 and 2011, respectively.[13] Another hydroxamic acid, PXD101 (belinostat), was approved for the treatment of PTCL in 2014.[14] In addition, several hydroxamic acid HDACis, such as PCI-24781 (abexinostat), ITF2357 (givinostat), BYK408740 (resminostat), and LBH589 (panobinostat), are now at various stages of clinical trials as drug candidates against various cancers.[15–17]

Importantly, FTS combined with HDACi valproic acid (VPA) has been shown to synergistically block Ras-related signal pathways, inhibit histone deacetylation, and prevent proliferation of cancer cells.[6] In addition, recent studies have shown that Ras protein inhibitor selumetinib in combination with HDACi vorinostat exerts potent antitumor effects, including synergistic inhibition of cell proliferation, G1 cell-cycle arrest, and induction of apoptosis.[18] Moreover, the combination of HDACi VPA and tyrosine kinase inhibitor imatinib (gleevec) downregulates expression of Bcr-Abl and Bcl-2 in chronic myeloid leukemia and enhances apoptosis.[19] Since Bcr-Abl is known to indirectly activate Ras proteins,[20] it is plausible that the combined inhibitory effect of imatinib and VPA might operate in part through inhibition of Ras pathways.[6,20] Therefore,

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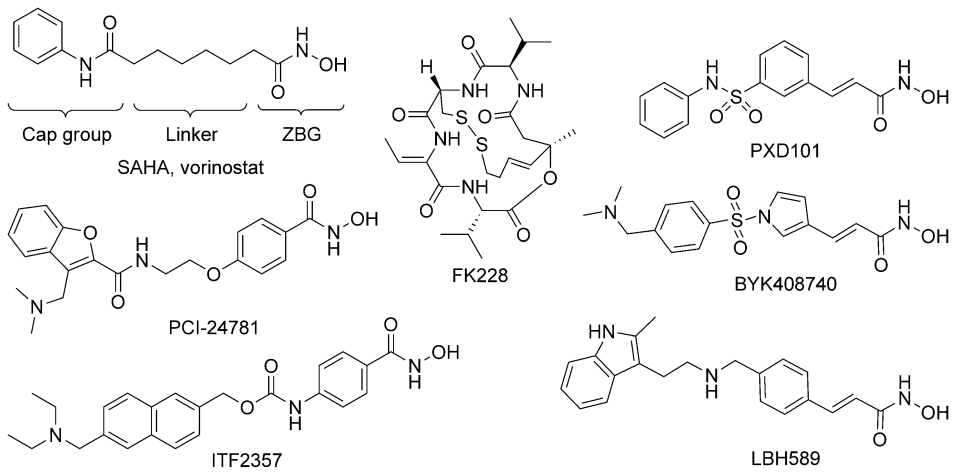


Figure 1. Structures of clinically approved or representative hydroxamic acid-based HDAC inhibitors in clinical trials.

development of novel antitumor agents with dual Ras-related signaling and HDAC inhibitory effects could be of pharmaceutical importance in the treatment of cancers. Indeed, a number of dual-target inhibitors have recently seen success as an attractive strategy of drug therapy,[21] including RTK/HDAC dual inhibitors that have demonstrated potential ability to overcome tumor recurrence and drug resistance.[22,23]

It is known that an HDACi consists of three domains: a zinc-binding group (ZBG) that chelates with the zinc ion to form a complex; a cap group, generally a hydrophobic and aromatic group, linked to the ZBG that fits into the tubular pocket; a saturated or unsaturated linker domain, composed of linear or cyclic structures that connect the ZBG and the cap group.[24] In an attempt to develop novel dual inhibitors as antitumor agents, we have designed and synthesized a series of novel hybrids by introducing the ZBG group, the hydroxamic acid fragment, into the carboxyl group of FTS via alkane or aromatic ring linkers (Figure 2). We hypothesize that FTS/hydroxamic acid hybrids might efficaciously inhibit both the Ras-signaling pathway and the deacetylation of histone. Herein, we report the synthesis and biological evaluation of these hybrids for their inhibitory activities against HDACs and in cancer cell lines, the blockade effects of cell cycle, the expression of Ras-related signal pathways and the acetylation of histone in vitro.

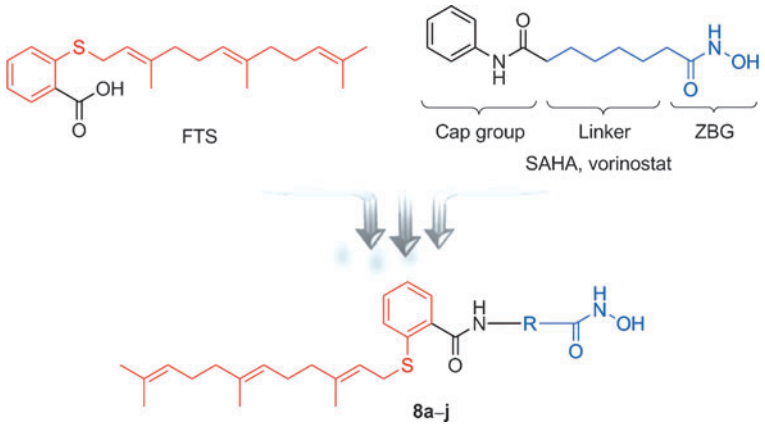
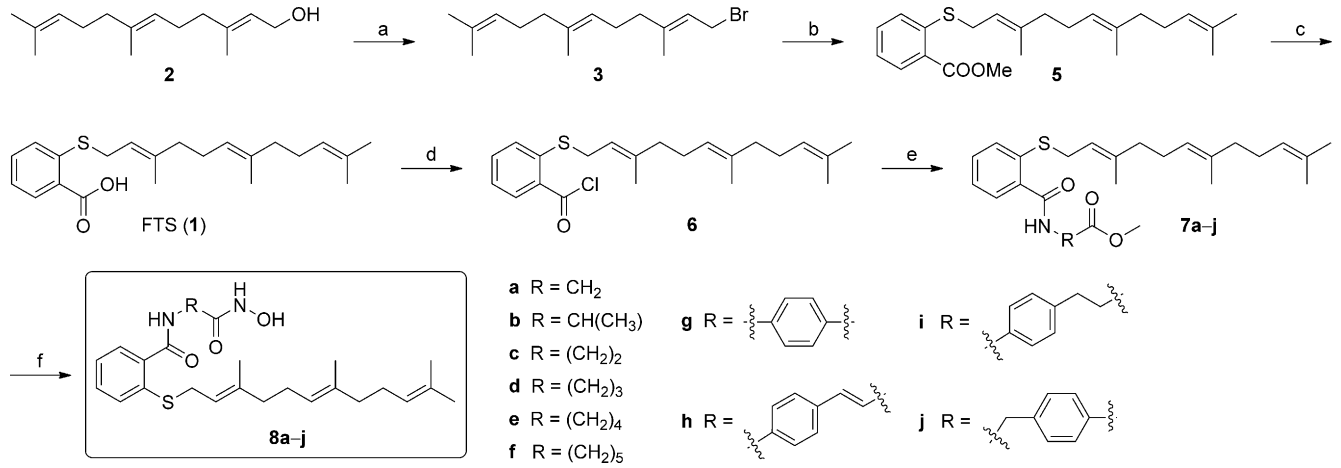


Figure 2. Design of hybrids **8a–j** from farnesylthiosalicylic acid (FTS) and suberoylanilide hydroxamic acid (SAHA).

The general route for the synthesis of the target compounds **8a–j** is shown in Scheme 1. As we reported previously,[25] farnesol **2** was treated with phosphorus tribromide to form farnesyl bromide **3**, which was then reacted with methyl thiosalicylate **4** in the presence of potassium carbonate to provide methyl farnesylthiosalicylicate **5**. The parent compound, FTS (**1**), was obtained via hydrolysis of **5** in aqueous sodium hydroxide. Subsequently, FTS was treated with oxalyl chloride to give (*E,E*)-farnesylthiosalicyl chloride **6**, which then reacted with differently substituted $NH_2RCOOMe$ in the present of triethylamine to provide intermediates **7a–j**. Finally, esters **7a–j** were converted to hydroxamic acid derivatives **8a–j** with NH_2OK in methanol. Final products **8a–j** were purified by column chromatography, and their structures were characterized by infrared and ^1H NMR spectroscopy, mass spectrometry, and elemental analysis. All compounds were of determined to be >95% purity by high-performance liquid chromatography (HPLC).

The inhibitory activities of synthesized compounds against human hepatocellular carcinoma cells (SMMC-7721 and HepG2), human breast cancer cells (MCF-7), human lung cancer cells (H460), and human bladder carcinoma cells (EJ) were evaluated in vitro in MTT assays. FTS and SAHA were used as positive controls. The IC_{50} values of **7d**, **8a–j** against the six human cancer cell lines are listed in Table 1. Most of the target compounds displayed better antiproliferative activity against the tested cells than FTS. Compound **8d** ($R=(CH_2)_3$) was the most potent, exhibiting antiproliferative activities with IC_{50} values of 4.93–7.61 μM, which are comparable to those of SAHA ($IC_{50}=5.46$–7.85 μM) and eight- to sixteen-fold more potent than FTS ($IC_{50}=48.6$–98.5 μM) in vitro. All target compounds were also tested against HeLa cell nuclear extract, a rich source of HDACs, for their HDAC inhibitory potencies,[26] using SAHA as a positive control. Similar to the antiproliferative activity, compound **8d** was the most active analogue, with an IC_{50} value of 0.48 μM against the nuclear extract.

The structure–activity relationship (SAR) studies of the series confirm the importance of the hydroxamic acid and also revealed that the length of the linker has significant impacts on the inhibitory activities against the cell growth and HDACs. For instance, compound **7d** (the corresponding ester of **8d**) only



Scheme 1. Synthesis of the target compounds **8a–j**. *Reagents and conditions*: a) PBr_3, *n*-hexane, pyridine, ether, 0 °C→RT, 4 h, 76%; b) methyl 2-mercaptobenzoate (**4**), K_2CO_3, CH_3CN, 50 °C, 6 h, 82%; c) aq NaOH (1 N), MeOH, 60 °C, 10 h, 90%; d) oxalyl chloride, CH_2Cl_2, RT, 1 h; e) $NH_2RCOOMe$, Et_3N, CH_2Cl_2, 0 °C→RT, 2 h, 65–86%; f) NH_2OK, MeOH, 5–10 h, RT, 65–75%.

Table 1. In vitro inhibitory activity of compounds **7d** and **8a–j** against five human cancer cell lines and HDAC.[a]

Compd	IC_{50} [μM]					
	SMMC-7721	HepG2	MCF-7	H460	EJ	HDAC[b]
FTS	66.7 ± 5.49	98.5 ± 5.10	48.6 ± 3.42	51.9 ± 4.38	49.2 ± 3.85	ND
SAHA	5.46 ± 0.66	6.17 ± 0.81	7.85 ± 0.89	ND	ND	0.56 ± 0.06
7d	> 25	22.4 ± 2.16	> 25	> 25	> 25	> 10
8a	7.23 ± 0.65	8.50 ± 1.02	12.1 ± 1.09	11.5 ± 1.28	10.3 ± 1.31	2.4 ± 0.37
8b	9.87 ± 0.80	9.34 ± 0.86	8.07 ± 0.95	10.1 ± 1.05	12.4 ± 1.10	1.7 ± 0.22
8c	6.46 ± 0.73	5.79 ± 0.65	8.04 ± 0.71	7.29 ± 0.60	7.52 ± 0.83	0.85 ± 0.07
8d	4.93 ± 0.55	5.89 ± 0.81	7.15 ± 0.63	6.06 ± 0.77	5.81 ± 0.61	0.48 ± 0.05
8e	11.8 ± 0.88	16.2 ± 1.62	15.1 ± 1.44	13.7 ± 1.50	10.5 ± 1.24	1.5 ± 0.16
8f	21.3 ± 1.76	> 25	> 25	19.2 ± 2.01	22.9 ± 1.93	3.3 ± 0.29
8g	> 25	> 25	> 25	> 25	> 25	> 10
8h	10.3 ± 1.06	8.65 ± 0.69	11.5 ± 1.23	7.97 ± 0.81	9.49 ± 1.00	0.77 ± 0.08
8i	> 25	23.8 ± 2.05	> 25	> 25	> 25	4.6 ± 0.52
8j	10.2 ± 1.12	6.95 ± 0.81	10.5 ± 1.16	9.02 ± 1.62	8.87 ± 0.97	0.92 ± 0.08

[a] The inhibitory effects were determined by using an MTT assay; data are expressed as the mean ± SD of three independent experiments performed in triplicate. Cell lines: hepatocellular carcinoma (SMMC-7721 and HepG2), breast cancer (MCF-7), lung cancer (H460), and bladder carcinoma (EJ). [b] For HDAC inhibition, compounds were evaluated against HeLa cell nuclear extract. Abbreviations: FTS: farnesylthiosalicylic acid; SAHA: suberoylanilide hydroxamic acid; ND: not detected.

showed weak cell growth inhibitory activity, confirming that the hydroxamic acid moiety plays an important role in the antitumor activity of this series of FTS derivatives. Interestingly, among the alkyl linker series (**8a–f**), a trend was apparent where the antiproliferative potency initially increased and then deceased with the elongation of the linker. Compounds **8c** and **8d** with the linker comprised of two and three carbon units, respectively, showed greater growth inhibitory activity against all five tumor cell lines than the other compounds, with **8d** exhibiting the most potent activity in the series. A similar trend of linker-length-dependency was observed against the HeLa cell nuclear extract (Table 1). Again, **8d** showed the highest potency within the series. This might be because **8d**, which has the appropriate linker chain, could be more effective in forming hydrogen bonds between the NH or OH group of the ZBG hydroxamic acid moiety with amino acid residues near the HDAC zinc binding pocket. In general, compounds with aliphatic linker chain showed slightly higher antitumor potency than compounds with aryl linkers (except **8j**). Further investigation about the precise SAR of these compounds is ongoing.

Encouraged by the inhibitory activity of this series of compounds against HeLa cell nuclear extracts, which mainly contains HDAC1 and HDAC2, we further tested a selected group of compounds (**8b–d,j**) against several HDAC enzymes. The in vitro inhibitory activities against HDAC enzymes were assessed employing SAHA as the positive control. The potency of these compounds reported herein were obtained by measuring the fluorescence-based HDAC biochemical activity using recombinant human HDAC1, HDAC6, and HDAC8 enzymes, and the IC_{50} values are summarized in Table 2. Most of these compounds exhibited significant HDAC1, HDAC6, and HDAC8 inhibition. In particular, compound **8d** exhibited the most potent inhibitory activity against both HDAC1 and HDAC8, with IC_{50} values of 0.058 μM and 0.19 μM, respectively, which are similar to or even higher than those of SAHA. Compound **8d** is three-fold more potent than SAHA against HDAC1. Since **8d** showed the highest potency in inhibition of both cell growth and HDACs, it was advanced for further evaluation.

Cell-cycle checkpoints are check mechanisms that verify whether the processes at each phase of the cell cycle have

Table 2. Inhibitory activities of compounds **8b–d, j** against different HDAC isoforms.[a]

Compd	IC_{50} [μM]		
	HDAC1	HDAC6	HDAC8
SAHA	0.19 ± 0.03	0.076 ± 0.01	0.33 ± 0.05
8b	0.18 ± 0.02	0.41 ± 0.05	0.52 ± 0.06
8c	0.11 ± 0.01	0.20 ± 0.03	0.35 ± 0.04
8d	0.058 ± 0.008	0.23 ± 0.02	0.19 ± 0.02
8j	0.27 ± 0.03	0.36 ± 0.04	0.22 ± 0.03

[a] Inhibition was determined using a fluorescence-based assay with recombinant human enzymes; data are expressed as the mean ± SD of three independent experiments performed in triplicate.

been precisely completed before entering into the next phase, therefore ensuring the fidelity of cell division. The G1 phase checkpoint, also called the restriction point, is susceptible to arrest if environmental conditions, such as inhibition of HDAC and cellular protein synthesis, cause cellular stress and make division impossible.[27,28] Here, we investigated whether the potent antiproliferative activity and HDAC inhibition by **8d** resulted from the induction of cell-cycle arrest. The cellular DNA content was analyzed by flow cytometric analysis in propidium iodide (PI) stained cells to detect changes in the cell-cycle distribution (Figure 3a). The analysis revealed a marked change in the cell-cycle profile of **8d**-treated SMMC-7721 cells (Figure 2b). Compared with the control cells treated with DMSO, when SMMC-7721 cells were treated with increasing concentrations of **8d** (1.0 and 5.0 μM), the mean percentage of cells in the G0/G1 phase increased from 30.1% to 39.8% and 51.3%, respectively, and the percentages of cells in the S and G0/G1 phases decreased concomitantly. This cell-cycle arrest at the G0/G1 phase caused by treatment with **8d** occurred in a dose-dependent manner (Figure 3b).

Given that the inhibition of HDACs by hybrid **8d** enhanced the tumor cell antiproliferative activity, the effects of **8d** on the levels of acetylation of histone H3 and tubulin were determined by immunoblotting assays using histone H3 and α-tubulin as negative controls (Figure 4). SMMC-7721 cells were incubated with the vehicle alone, FTS, SAHA, FTS + SAHA, or **8d** (5.0 and 10 μM). Similar to the control, FTS did not affect the expression levels of Ac-histone H3 or Ac-α-tubulin. As expected, SAHA induced increases in the expression levels of Ac-H3 and Ac-α-tubulin without affecting the levels of non-acetylated histone H3 and α-tubulin. The combination of FTS and SAHA showed increased expression levels than SAHA alone, suggesting inhibition of both pathways. Importantly, **8d** at 5 μM significantly increased the amounts of Ac-α-tubulin and Ac-histone H3, which were higher than SAHA alone and comparable to FTS and SAHA together (Figure 4b). This effect was even more pronounced at 10 μM, suggesting that **8d** could dose-dependently inhibit HDACs.

To investigate whether the antitumor activities of **8d** result from the function of the FTS moiety of the hybrids, we examined the inhibitory effects of **8d** on the expression of Ras-related signaling in SMMC-7721 cells. In this study, the cells were incubated with the vehicle alone, SAHA, FTS, SAHA + FTS, or **8d** (5.0 and 10 μM). The expression and phosphorylation levels of the Ras-related signal events, Akt and ERK1/2, were determined by immunoblotting assays using β-actin as the negative

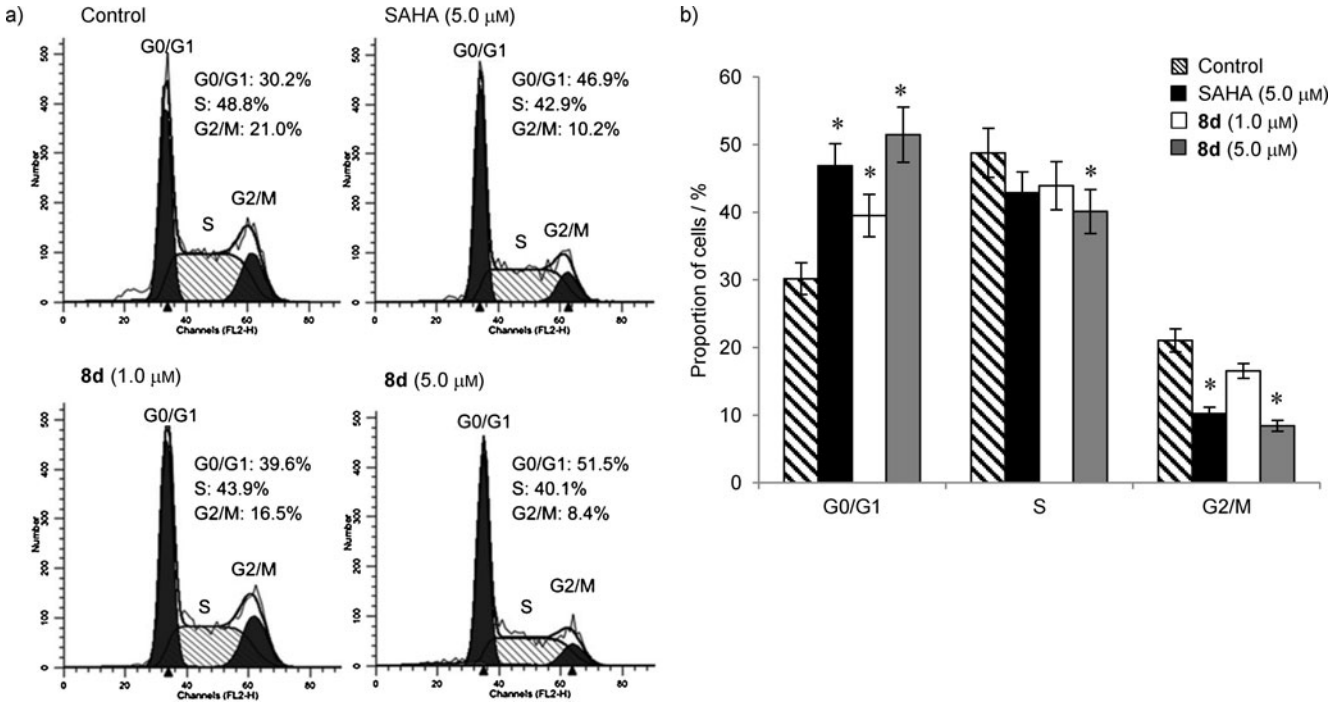


Figure 3. The effects of compound **8d** on cell-cycle progression in SMMC-7721 cells. a) Cells were treated with vehicle (control), SAHA, or different doses of **8d** and stained with propidium iodide, followed by flow cytometry analysis. b) Representative histograms; data are expressed as the mean ± SD of three independent experiments. * $P < 0.01$ versus control.

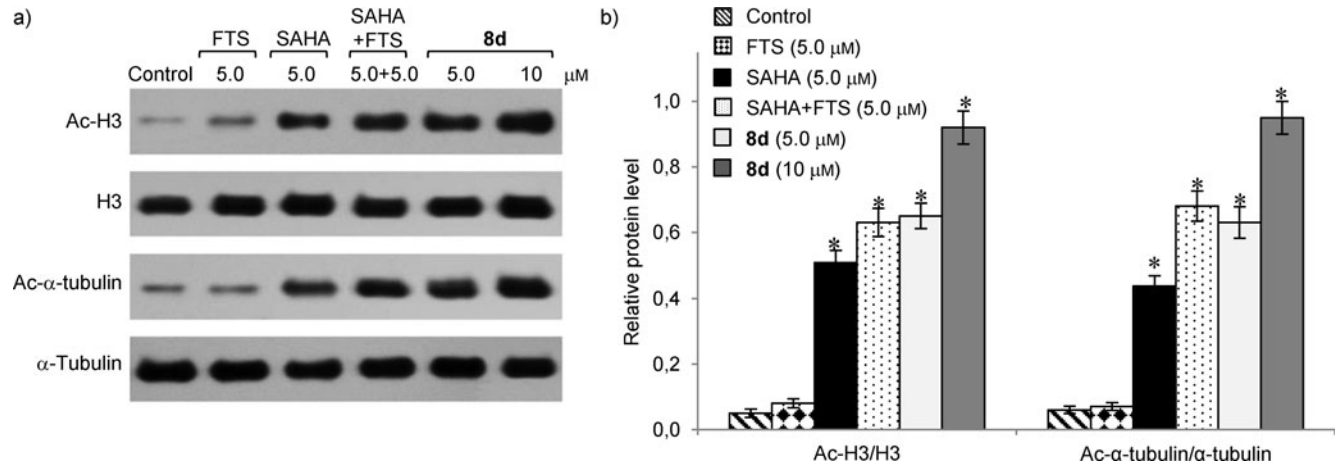


Figure 4. Immunoblot analysis of the expression of the acetylation for histone H3 and tubulin in vitro. a) SMMC-7721 cells were treated with FTS, SAHA, FTS + SAHA, or **8d** for 48 h at the indicated concentrations. Cell lysates were prepared and subjected to immunoblot analysis using acetyl-histone H3 (Ac-H3), histone H3, acetyl-α-tubulin, and α-tubulin antibodies. Histone H3 and α-tubulin were used as controls. b) Quantitative analysis; the relative levels of Ac-H3/H3, Ac-α-tubulin/α-tubulin were determined by densimetric scanning. Data are expressed as the mean ± SD of three independent experiments. * $P < 0.01$ versus control.

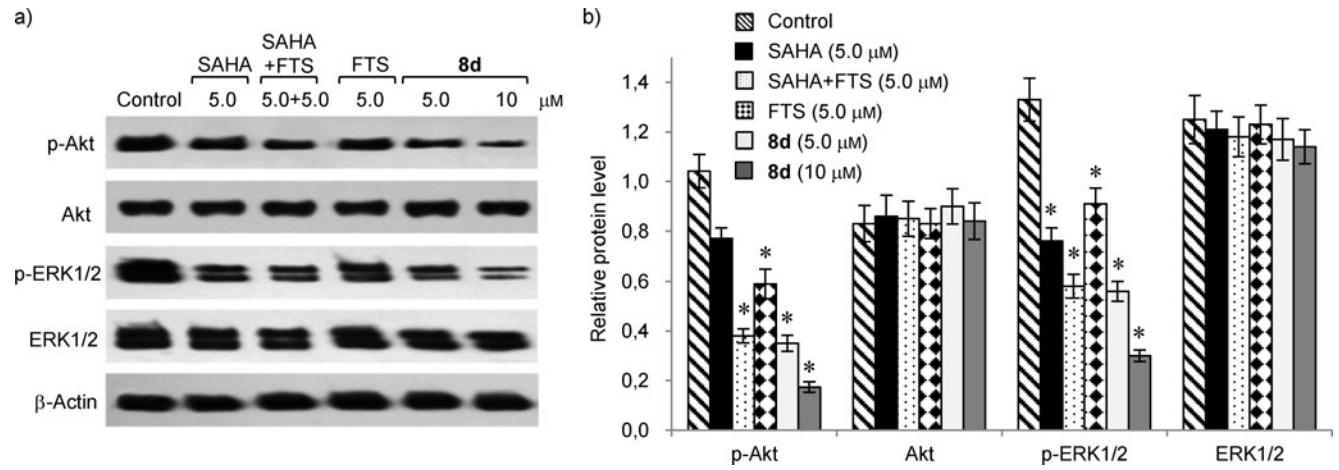


Figure 5. Immunoblot analysis of the expression of the Ras-related signal events in vitro. a) SMMC-7721 cells were treated with vehicle (control), SAHA, SAHA + FTS, FTS, or **8d** for 48 h at the indicated concentrations, and their lysates were subjected to immunoblot analysis using anti-Akt, antiphospho-Akt (Ser473), anti-ERK1/2, antiphospho-ERK1/2 (Thr202/Tyr204), and anti-β-actin antibodies, respectively. β-Actin was used as the control. b) Quantitative analysis; the relative levels of each signaling event to control β-actin were determined by densimetric scanning. Data are expressed as the mean ± SD of three independent experiments. * $P < 0.01$ versus control.

control. As shown in Figure 5, the expression of free Akt and ERK1/2 remained at similar levels with these treatments. While FTS treatment decreased the levels of phospho-Akt and phospho-ERK1/2 in SMMC-7721 cells, SAHA also led to decreases, although to a lesser extent. This result suggests that SAHA might also modulate Ras-related signaling, possibly via an indirect mechanism. The combination of these two compounds further decreased the expression levels of phospho-Akt and phospho-ERK1/2. Furthermore, the levels of phospho-Akt and phospho-ERK1/2 under treatment with **8d** at 5.0 μM resulted in obvious decreases that were comparable to FTS and SAHA combined but greater than FTS or SAHA alone. At 10 μM, compound **8d** caused an even more significant decrease than SAHA and FTS together. These results suggest that **8d** effectively inhibits the Ras-related signaling pathway. Therefore, the increased antitumor potency of **8d** compared with FTS might be attributed to the concomitant inhibition of both HDAC and Ras-related signaling in a synergistic manner.

In conclusion, using a structure-based design strategy, we have designed and synthesized a novel series of hybrids by conjugating FTS and hydroxamic acid with different linkers as dual Ras-related signaling pathway and HDAC inhibitors, and evaluated their biological activities in vitro. It was found that several hybrids, particularly **8b–e** and **8j**, not only displayed significant antitumor activities against five human cancer cells, but also showed HDAC inhibitory effects. Compound **8d** demonstrated the highest anticancer potency against cancer cell lines with IC_{50} values of 4.93–7.61 μM, which are eight- to six-

teen-fold more potent than those of FTS (IC_{50} = 48.6–98.5 μM) and similar to those of SAHA (IC_{50} = 5.46–7.85 μM). Compound **8d** also exhibited the most potent inhibitory activity against HDAC1 and HDAC8. Based on cell-cycle analysis, **8d** arrested SMMC-7721 cells at the G0/G1 phase in a concentration-dependent manner. Furthermore, immunoblot analysis revealed that **8d** dose-dependently inhibited histone H3 and α-tubulin acetylation, and Ras-related signaling pathways simultaneously. The concomitant inhibition of both HDAC and Ras-related signaling in a synergistic manner might help explain the increased antitumor potency of **8d** compared with FTS. Together, our findings provide the basis for the design of hybrids as new dual inhibitors for the treatment of human cancers.

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Keywords: antitumor agents · farnesylthiosalicylic acids · histone deacetylases · hydroxamic acids · ras-related signaling pathways

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