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Histone deacetylase and microtubules as targets for the synthesis of releasable conjugate compounds

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ABSTRACT

Design and synthesis of an HDAC inhibitor and its merger with three tubulin binders to create releasable conjugate compounds is described. The biological evaluation includes: (a) in vitro reactivity with glutathione, (b) antiproliferative activity, (c) cell cycle analysis and (d) quantification of protein acetylation. The cellular pharmacology study indicated that the HDAC-inhibitor-drug conjugates retained antimitotic and proapoptotic activity with a reduced potency.

The anticancer agents in clinical use suffer from severe dose-limiting toxicities, but in any case chemotherapy continues to be the primary systemic treatment of cancer. As a consequence there is a need for innovative approaches to design anticancer drugs with reduced toxicity[1,2] and improved efficacy. Recently, the use of targeted agents in combination with conventional cytotoxic treatment[3] has been proposed as a promising strategy for optimizing antitumor therapies. In particular, histone deacetylase inhibitors (HDAI) are potential antitumor agents (Fig. 1 reports two representative samples), because their biological effects include inhibition of cell proliferation and induction of apoptosis. These effects result in a significant antitumor activity in 'in vivo' models. HDAC inhibitors[4] enhance histone acetylation, thus inducing chromatin relaxation and modulation of gene expression, and reversing the epigenetic changes usually found in tumor cells.[5] In addition, the hydroxamic acid-based pan-HDAC inhibitors are known to modulate the acetylation status of non-histone proteins, including α-tubulin. Since tubulin acetylation may be implicated in regulation of microtubule stability, the combination of microtubule-binding agents with HDAC inhibitors may have a synergistic effect. In a recent paper[6] a combined treatment of Ark2 and KLE endometrial cancer cells with TSA (**5**)/paclitaxel (**3**) (Fig. 1) is reported to cause synergistic inhibition of cell growth and induction of apoptosis.

On the base of this finding we thought to exploit the higher levels of glutathione present in cancer cells (1 mM intracellular concentration compared to micromolar extracellular concentration)[7] by designing novel disulfide containing hybrid compounds[8] that are activated selectively to release effective amounts of chemotherapeutic agents (HDAC inhibitor and tubulin binders) into cancer cells (Scheme 1). Reduced glutathione could secure reduction of the disulfide bond, with subsequent release of the chemotherapeutic agents by nucleophilic attack of the thiol group (thiolate anion) at the ester function with formation of a thiolactone. As tubulin-binder building blocks we selected thiocolchicine,[9] paclitaxel and cephalomannine in order to have agents representative of both classes: inhibitors of tubulin polymerisation and stabilizers of microtubules).[10] Thiocolchicine (**1**) offers the possibility to remove the acetyl group to obtain *N*-deacetylthiocolchicine **2** possessing an amino group that can be easily acylated.[11] Paclitaxel and cephalomanninne could be functionalized at the C2′–OH group.[12] 4,4′-Dithiobutyric acid was selected as a proper linker because of the possibility of enhancing the release of the therapeutic drugs by formation of a five-membered thiolactone.[13]

For our purpose we designed the structure of compounds that could be effective as HDAC inhibitor with the specific structural characteristics that are required for the interaction with the enzyme active site.[14]

In particular we were guided by the need for an anchor point suitable to introduce a spacer that warrants the obtainment of the conjugate compounds. The general structure of the designed

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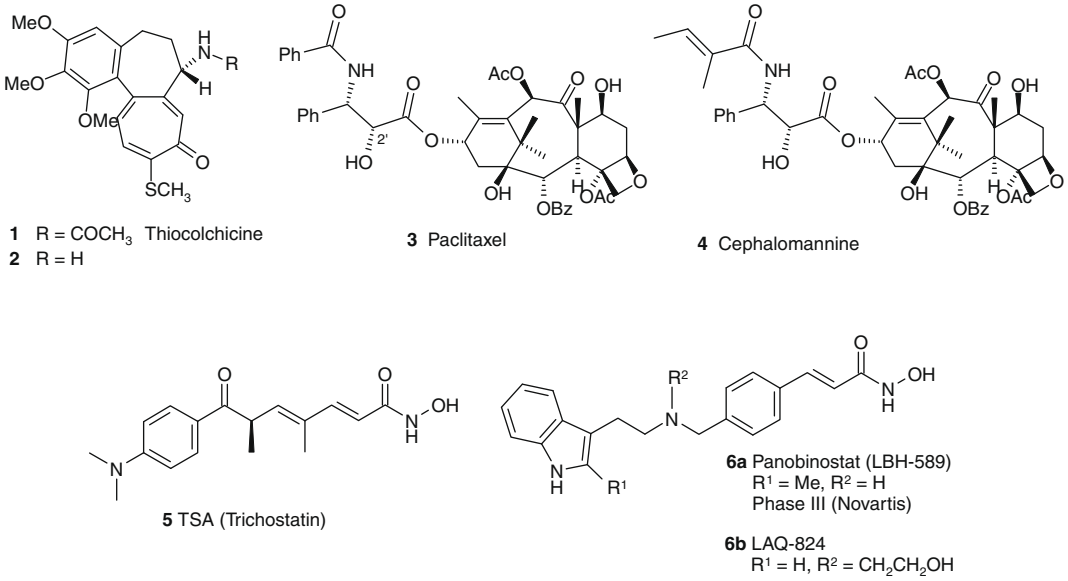
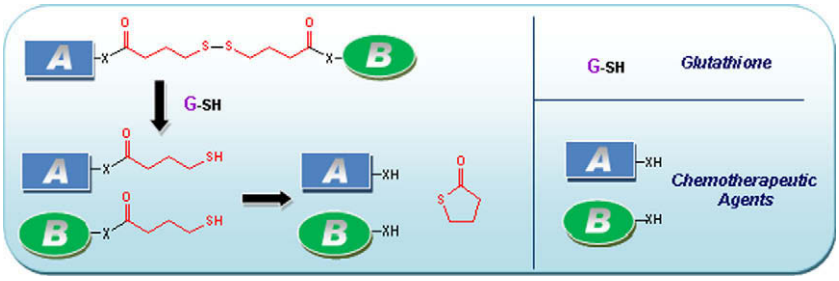


Figure 1. Example of (a) tubulin-binders and (b) HDAC inhibitors.



Scheme 1. Design of novel molecules and their expected reactivity with glutathione.

HDAC inhibitors is reported in Figure 2 and was suggested by analogy with an established HDAC inhibitor, Panobinostat (LBH589, Novartis) **6a**, now in clinical trial.

In our first plan, we studied the use of LBH589 (**6a**) that we prepared according the reported procedure but we had several problems in the formation of the dimeric structure due to the presence of the indole nitrogen.

As consequence we designed the structure where the indole nucleus is removed and replaced by a phenyl group. We picked out compound **19** as our first goal. This was prepared in a five-step sequence using benzylamine and methyl ester **8** (Scheme 2) of 4-formylcinnamic acid (**7**) as building blocks. The crucial reductive amination step furnished compound **9** permitting the formation of the required hydroxamic acid by subsequent manipulation of the ester function.[15] In particular this was realized by base-catalyzed ester hydrolysis and condensation reaction with protected hydroxylamine (NH_2OTHP) in the presence of EDC and HOBt to give compound **16** that was finally deprotected by camphorsulfonic acid (CSA) to give **19**. In a general attempt to obtain the desired

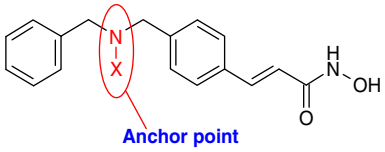
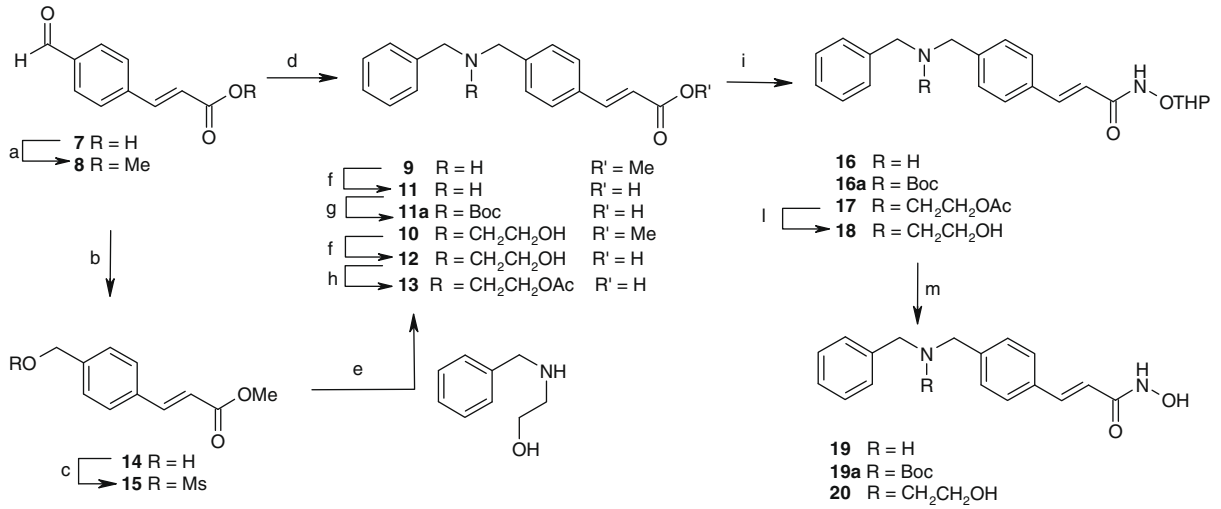
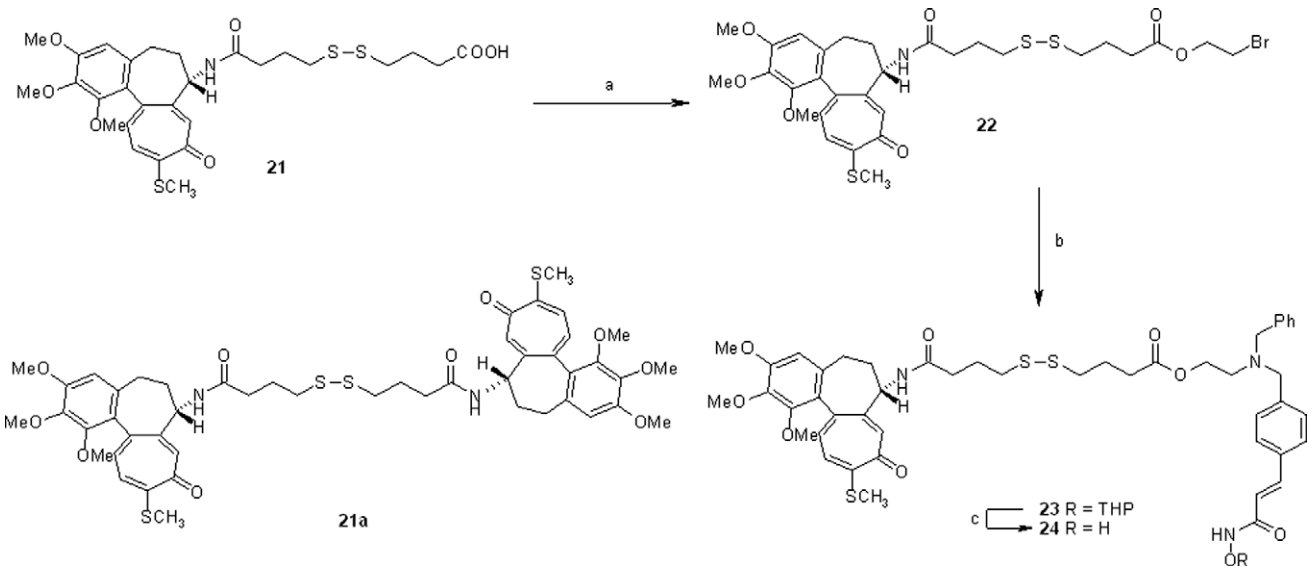


Figure 2. General structure of designed HDAC inhibitors.

hybrids we planned to use the benzylic NH nitrogen for the introduction of the dithiodiacyl spacers to take in the second scaffold (tubulin binder unities). Surprisingly and without any plausible explanation, acylation of **19** and **16** with 4,4′-dithiobutyric acid proved tricky and the corresponding amide could not be isolated. For this reason we realised the opportunity to move the acylation point out from the encumbered nitrogen and far from the disulfide moiety. Compounds **18** and **20** were therefore synthesized according to Scheme 2. Compound **10** was prepared from **7** by $NaBH_4$ reduction in the presence of Dowex1-x8,[16] formation of the mesyl derivative **15** and final reaction with 2-benzylamino ethanol in the presence of DIPEA. Manipulation of the carboxylic function required: (a) ester hydrolysis and successively (b) protection of the hydroxyl group as acetate to give compound **13**. Reaction of **13** with NH_2OTHP in the presence of EDC and HOBt furnished compound **17**, that was converted into compound **18** by potassium carbonate hydrolysis of the acetate ester. The need to have at hand compound **20** for biological evaluation drove us to submit compound **18** to reaction with CSA in MeOH. In this case the critical purification step was carried out by filtration on SPE ISOLUTE PE-AX (quaternary amine functionalized silica). We used compound **11** as starting material for the preparation of reference compounds **16a** and **19a**. We explored different strategies for the preparation of the disulfide hybrid. After many unsuccessful trials we realized that only the THP-protected hydroxylamides **16** and **18**, could be used for a simple and efficient synthesis of the desired compounds. For the preparation of the *N*-deacetylthiocolchicine heterodimer derivative **24** (Scheme 3), reaction of *N*-deacetylthiocolchicine (**2**)

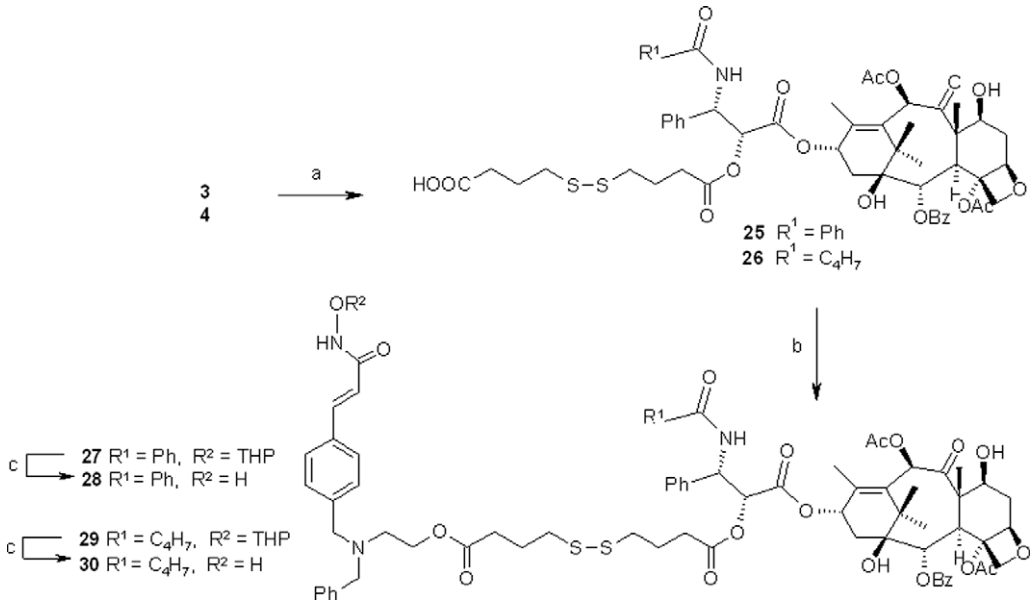


Scheme 2. Reagents: (a) $SOCl_2$, MeOH; (b) $NaBH_4$, Dowex-8, THF; (c) MsCl, Et_3N, DMAP, THF; (d) $PhCH_2NH_2$, $NaBH_3CN$, CH_3COOH, MeOH; (e) DIPEA, CH_2Cl_2; (f) KOH, MeOH; (g) $(Boc)_2O$, K_2CO_3; (h) CH_3COCl, TEA, DMAP, THF; (i) NH_2OTHP, EDC, HOBt, DMF; (l) K_2CO_3, MeOH, H_2O; (m) CSA (Amberlite IR120 for **16a**), MeOH.



Scheme 3. Reagents: (a) $HOCH_2CH_2Br$, DMAP, DCC, CH_2Cl_2; (b) **16**, K_2CO_3, CH_3CN; (c) CSA, MeOH; (d) 4,4′-dithiobutyric acid, DMAP, DCC, CH_2Cl_2.

with 4,4′-dithiobutyric acid furnished the monoamide derivative **21** that was subsequently reacted with 2-bromoethanol to give compound **22.** The last compound gave rise to N-alkylation of intermediate **16** and the hybrid structure **23** was obtained after 20 h at 60 °C. Finally, removal of the THP protective group from the hydroxamic acid function by means of camphorsulfonic acid (CSA) furnished the target compound **24**. Purification of **24** was possible by scavenging of CSA with Amberlist A-21 (basic resin). Spectroscopic data reported in the Experimental section (Supplementary data) fully support the complex structure. For the preparation of target compounds **28** and **30** (Scheme 4) we preferred to exploit the higher reactivity of the hydroxyl group at position 2′ of the taxanes building blocks (**3** and **4**, Fig. 1). The easily obtained 2′-dithiodibutyroyl-paclitaxel **25** and cephalomannine **26** were condensed with the *N*-hydroxyethyl **18** in the presence of DCC and DMPA. Removal of the THP protective group by CSA furnished the target compounds that were purified by Amberlist A-21 treatment and characterized. We first investigated from a chemical point of view if glutathione is really able to cleave the disulfide bond of the conjugate compounds **24**, **28** and **30**. Solution of these (1.8 μM, acetone/water 1:1) were treated with L-glutathione (1.8 μM) for 6 h and the mixtures was directly analysed by HRES-I-FT-MS. The experiment is far from the idea to reproduce the cellular medium but the detected ionic species offer a qualitative evidence of the release of the drugs (Table 1). The antiproliferative activity of the tested antimicrotubule agents (thiocolchicine **1**, paclitaxel **3** and cephalomannine **4**), HDAC inhibitor moieties **19** and **20**, *N*-Boc derivatives **16a** and **19a** and conjugate compounds was evaluated under the same conditions following 72 h-exposure in ovarian carcinoma cells (IGROV-1) (Table 2). All tested compounds exhibited a significant antiproliferative activity in a submicromolar range apart from *N*-Boc derivative **16a** and **19a**. However, all conjugated compounds were characterized by a substantial reduction of activity, as compared with free antimicrotubule agent most evident in the paclitaxel conjugate (**28**). Among the tested conjugates, the thiocolchicine analogue (**24**) was the most effective and therefore used for additional cellular and biochemical pharmacological studies. Again, the compound **24** exhibited a reduction of potency as compared to thiocolchicine (**1**) and its deacetyl derivative (**2**). A comparable reduction of potency was found with the thiocolchi-



Scheme 4. Reagents: (a) 4,4′-dithiobutyric acid, DMAP, DCC, CH_2Cl_2; (b) **18**, DMAP, DCC, CH_2Cl_2; (c) CSA, MeOH.

Table 1
HRESI-FT-MS detection of ionic species after treatment with glutathione

	Detected species
24	**HDACIn**-H **Tio**-$CO(CH_2)_3$-SS-$(CH_2)_2CO$-**Tio** **Tio**-$CO(CH_2)_3$-SS-**G** **G**-SS-**G**
28	**HDACIn**-H **Pacli**-H **G**-SS-**G**
30	**HDACIn**-$CO(CH_2)_3$-SS-**G** **Cepha**-H **Cepha**-$CO(CH_2)_3$-SS-**G** **G**-SS-**G** **HDACIn**-H

Table 2
Antiproliferative activity

Compounds	IC_{50} (μg/ml)	IC_{50} (μM)
1	0.005 ± 0.002	0.012 ± 0.005
2		0.023 ± 0.002
3	0.05 ± 0.01	0.06 ± 0.01
4	0.250	0.30
19	0.14 ± 0.01	0.48 ± 0.03
20	0.06 ± 0.01	0.018 ± 0.03
16a	1.4	0.53
19a	41	19.1
21		0.019 ± 0.001
24	0.07 ± 0.014	0.078 ± 0.015
28	1.0	0.72
30	1.0	0.74
21a		0.050 ± 0.001

cine dimmer. In contrast, no reduction of the cytotoxic potency was observed with the compound **21**. The cell cycle analysis of cells treated with thiocolchicine or its conjugate (**24**) at equitoxic concentrations (IC_{80}) indicated similar pattern of perturbation that was consistent with a G2/M arrest as expected for antimicrotubule agents (Fig. 3a–c). The comparable effects of thiocolchicine and the derivative **24** on cell cycle examined at 24 h reflected the antimicrotubule activity of these compounds, because under the same conditions the HDAC inhibitor **20** induced only marginal perturbations. The drug ability to induce mitotic arrest was also documented by morphological evidence of mitotic cells (Fig. 3d). Treated cells also exhibited large sub-G1 peaks, which were consistent with the presence of dead cells. Indeed, at equitoxic concentrations (IC_{80}) used in these experiments, thiocolchicine and its conjugate (**24**) exhibited a comparable ability to induce apoptosis (52 ± 2% and 45 ± 3%, respectively, as detected by the TUNEL assay). Western blot analysis performed in ovarian carcinoma cells

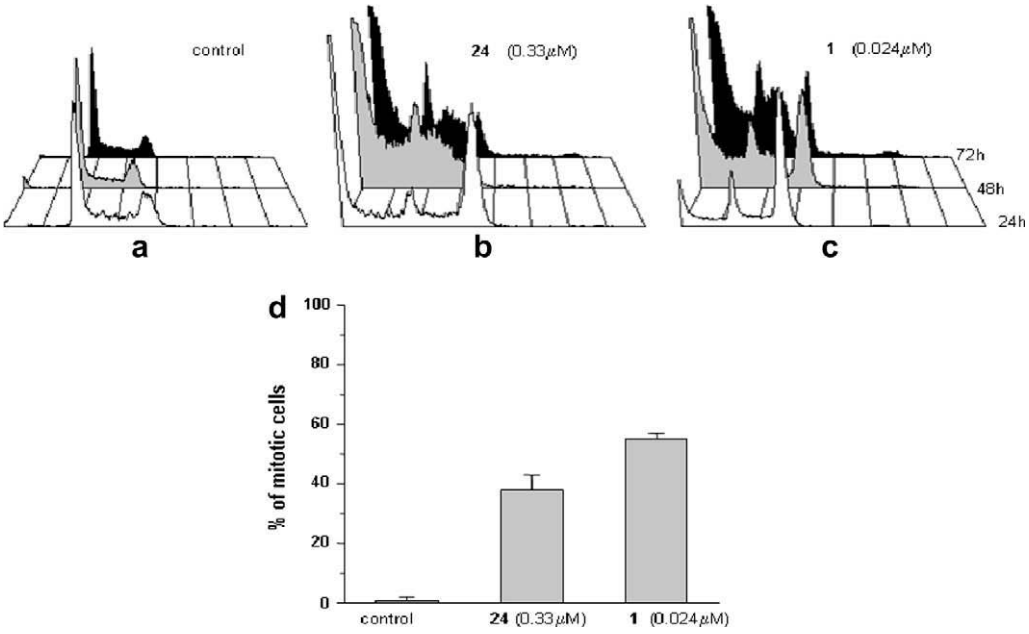


Figure 3. Cell cycle analysis.

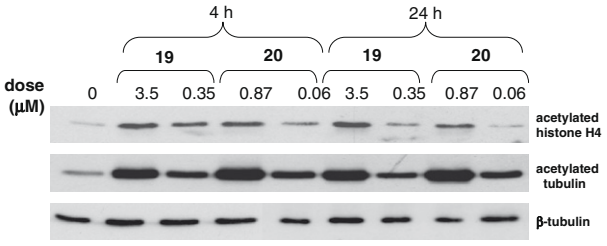


Figure 4. Western blot analysis of protein acetylation in IGROV-1 cells heated with HDAC inhibitors. Analysis was performed after 24 h exposure. β-Tubulin is shown as control of protein loading.

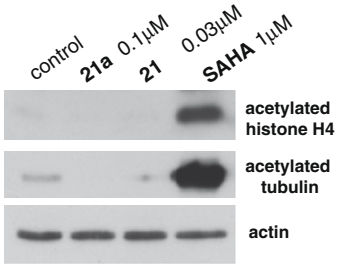


Figure 6. Western blot analysis of protein acetylation in IGROV-1 cells treated with **21**, **21a** and **SAHA** (Suberoylanilide hydroxamic acid).

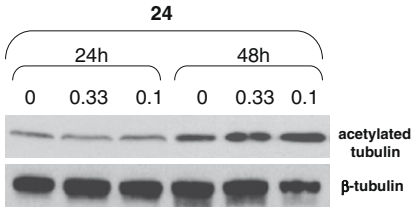


Figure 5. α-Tubulin acetylation in IGROV-1 cells following 24-h exposure to conjugate **24**. β-Tubulin is shown as control of protein loading.

indicated that HDAC inhibitor moiety (**19** and **20**) used for conjugation was able to enhance acetylation of both histone H4 and α-tubulin (Fig. 4). This early event, already evident at 4 h, was clearly dose-dependent. In cells treated with the conjugate **24**, tubulin acetylation was a delayed event, detectable only at 48 h (Fig. 5), thus suggesting a sluggish release of the HDAC inhibitor. We cannot rule out the possibility that the thiocolchicine derivative containing the mercapto-acylated chain may have HDAC inhibitory activity. However, the marginal effects of thiocolchicine dimer **21a**, which is expected to generate the thiol-containing monomer, do not support this interpretation (Fig. 6). The rationale for the synthesis of the novel prodrugs described in the present study stems from the combination of two moieties, antimicrotubule agent and HDAC inhibitor, each affecting different cellular targets and able to act synergistically when released simultaneously inside the tumor cells. All the tested conjugates, prepared using various antimicrotubule agents, exhibited an appreciable reduction of the cytotoxic potency. The cellular/molecular basis of the disappointing result is unclear, because in vitro glutathione was able to promote the release of the drug moieties. A plausible explanation of this behaviour could be an inefficient reduction of S–S bond and slow release of the HDAC inhibitor as suggested by a delayed increase of the tubulin acetylation in cells treated with **24** (Fig. 3). In addition, since the acetylation of protein substrates by the HDAC inhibitory moiety is dose-dependent and an effective HDAC inhibition could be achieved at higher concentration (~1 μM) than that released by the conjugate at the tested concentration (~0.3 μM), it is likely that the intracellular level of the HDAC inhibitor is inadequate to produce the expected synergistic interaction. Alternatively, and perhaps concomitantly, a reduced tubulin interaction of the conjugate itself or of the released mercapto-acylated thiocolchicine **21** could account for the lower potency as compared with free drug. This interpretation is consistent with the marked reduction (~10-fold) of the potency of the paclitaxel conjugate **28**. Indeed, substituents at the 2′-position are expected to impair drug-microtubule interactions. Finally, it should be noted that the large molecular size of the conjugates may impair their ability to penetrate cell membrane. Thus a low membrane permeability could contribute to the observed reduction of potency. If this is

the case, antimicrotubule agents with lower molecular weight may be more appropriate in this approach. In spite of the reduction of the cytotoxic potency, we retain that the dynamics of the release of the conjugate compounds described in the present study have to be investigated in order to take out any possible suggestion that could be useful in the design of new anticancer compounds. At the same time, the therapeutic advantages of these conjugates should be documented by in vivo models.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.09.075.

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