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Trithiocarbonates—Exploration of a new head group for HDAC inhibitors

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Abstract—Inhibition of histone deacetylases class I/II enzymes is a new, promising approach for cancer therapy. In the present study, we disclose a new structural class of HDAC inhibitors with the trithiocarbonate motif. A clear structure–activity-relationship was obtained for the cap-linker motif and the putative Zn^{2+} complexing head group. Selected analogs display potent inhibition of HDAC enzymatic activity and a cellular potency comparable to that of suberoylanilide hydroxamic acid (SAHA), recently approved for treatment of patients with advanced cutaneous T-cell lymphoma.

Tumor development and progression are controlled by both, genetic and epigenetic events. Unlike genetic events, epigenetic aberrations such as DNA methylation and core histone acetylation can be reversed with small molecule effectors.[1] In this regard, the DNA methyltransferase (DNMT) inhibitor 5-aza-3′-deoxycytidine (Decitabine®) and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA, Zolinza®) are drugs approved for the treatment of patients with myelodysplastic syndrome and cutaneous T-cell lymphoma,[2] respectively.

The reversible acetylation and deacetylation of lysine residues within core histone proteins H2A/B, H3, and H4 is catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. HDAC isoenzymes belonging to class I and II are part of multiprotein complexes and clear evidence has been provided linking overexpression or dysregulated function to cancer pathophysiology.[3] HDAC inhibitors affect transcriptional regulation, inducing or repressing genes involved in differentiation, proliferation, cell cycle regulation, protein turnover, and apoptosis. HDAC inhibiting agents are highly divergent in structure and comprise short chain fatty acids, hydroxamic acids, cyclic tetrapeptides/peptolides, ketones, and benzamides.[4] Various agents are currently in clinical development, namely the hydroxamate analogs LBH589, PXD101, CRA024781, and SAHA, the benzamide analogs MS275 and MGCD0103, the cyclic peptolide Depsipeptide/FK228, and finally the butyrate analog valproic acid (VPA). As shown for SAHA and the natural product trichostatin A, the hydroxamic acid head group complexes the Zn^{2+} in the active site, thereby inhibiting the enzyme in a substrate competitive manner.[5] Although the structural basis of HDAC inhibition is well established, the identification of new head groups bearing drug like properties proved to be difficult.[6]

Compounds with a free thiol head group were described as potent HDAC inhibitors,[7] but in general cellular activity was very weak.[8] Depsipeptide is a remarkable exception by acting as a prodrug, liberating the reduced sulfhydryl group only intracellular.[9] Different head groups containing sulfur include thiocarboxylates,[10] thioglycolamides[11], and α-thio-substituted acetyl compounds[12] have been described.

During our program to identify suitable starting points for the development of HDAC inhibitors, a high throughput screening (HTS) campaign delivered amongst other hits the *tert*-butyl-phenacetyl trithiocarbonates **1a** and **2a** (Fig. 1). These compounds exhibited a complete, submicromolar inhibition of a HDAC isoenzyme mixture derived from HeLa cervical carcinoma

Keywords: Trithiocarbonates; Histone deacetylase; HDAC inhibitors; Novel head group.
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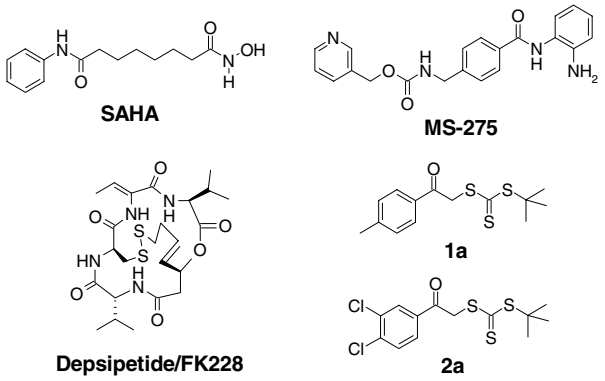


Figure 1. HDAC inhibitors from literature and identified by HTS.

nuclei, containing HDAC isoenzymes 1, 2, 3, 5, and 8 (Table 1).[13] Their anti-proliferative/cytotoxic activity,[14] however, was found to be rather moderate.

To gain knowledge about the structural requirements for HDAC inhibition of such trithiocarbonates, these compounds were hypothetically compartmentalized in a first approach into three parts: an aromatic moiety on the left part of the molecule, which was considered to be the cap-linker part of the HDAC inhibitor, a central moiety consisting of carbonyl-methylene-trithiocarbonate, which was regarded as being the zinc binding head, and an alkyl substituent on the right end of the molecule, which can be seen as an affix to the head and which might contribute to the activity by interaction within the cavity underneath the binding site known from structural elucidation from the HDAC isoenzymes HDAC8,[15] HDAH,[16] and HDLP[5] (Fig. 2).

To evaluate their potential as HDAC inhibitors, first, the bulky and lipophilic *tert*-butyl substituent (head-affix, Fig. 2) was replaced with various alkyl and aryl substituents (**1a**–**f**; **2a**–**e**). All compounds were accessible

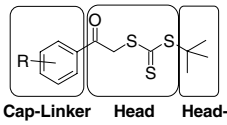
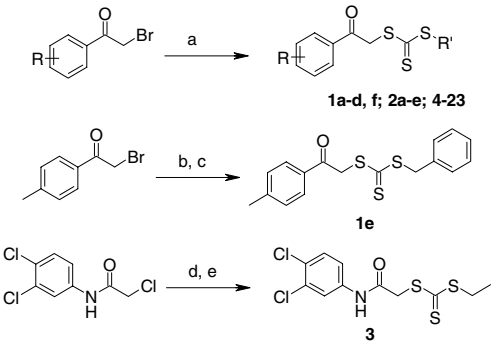


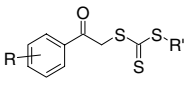
Figure 2. Schematic segmentation of the trithiocarbonates into typical HDAC inhibitor fragments.[2a]



Scheme 1. General preparation of trithiocarbonates and analogs. Reagents and conditions: (a) R'SNa (or R'SH, NaH, THF, 0 °C, 15 min), CS_2, THF, rt, 0.5 h; then phenacyl bromide, THF or EtOAc, rt, 0.5–16 h; (b) KSAc, THF, 40 °C, 2 h; (c) CS_2, 1 M NaOH, THF, rt, 16 h; then BnBr, THF, rt, 2 h (16%); (d) KSAc, THF, 55 °C, 48 h; (e) CS_2, 1 M NaOH, THF, 0 °C to rt, 16 h; then EtBr, THF, rt, 2 h (2%).

from its corresponding phenacyl bromide through conversion with the requisite sodium trithiocarbonate (Scheme 1), which was prepared in situ from the sodium thiolate (from the thiol and NaH or commercially available) and carbon disulfide in THF.[18] Only the synthesis of the benzyl trithiocarbonate **1e** required a variation in the synthetic sequence as phenacyl bromide was first converted into a thioacetate. Saponification, direct addition of carbon disulfide to the resulting thiolate, and subsequent substitution with benzyl bromide furnished **1e** (Scheme 1).

Table 1. In vitro activity for alkyl phenacyl trithiocarbonates



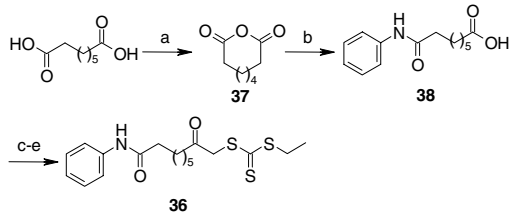
Compound	R	R′	HDAC pIC_{50}[a]	HeLa prolif pIC_{50}[b]
SAHA			7.1	5.7
MS-275			4.9	5.8
1a	4-Me	*t*-Bu	6.6	4.5
1b	4-Me	Me	6.4	5.5
1c	4-Me	Et	6.2	5.4
1d	4-Me	*i*-Pr	5.9	4.6
1e	4-Me	Bn	6.6	4.6
1f	4-Me	C_2H_4Ph	6.5	4.4
2a	3,4-Cl_2	*t*-Bu	6.9	5.0
2b	3,4-Cl_2	Me	7.0	5.2
2c	3,4-Cl_2	Et	7.1	5.1
2d	3,4-Cl_2	*i*-Pr	7.0	4.6
2e	3,4-Cl_2	Ph	7.0	4.7

[a] Biochemical HDAC assay.[13,17]

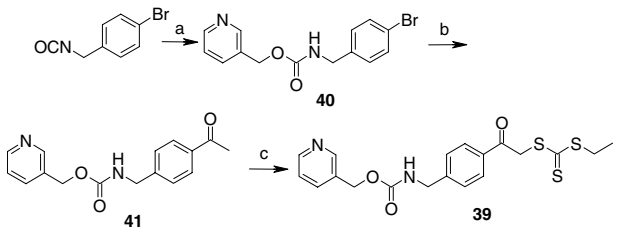
[b] Anti-proliferative, cytotoxic activity.[14,17]

The resulting compounds were evaluated for their inhibitory activity in the HeLa nuclear extract containing a mixture of HDACs as well as for their cellular activity in the HeLa proliferation/cytotoxicity assay.[13,14] The results demonstrated that substitution of the *tert*-butyl group was well tolerated and a range of different alkyl and aryl substituents retained HDAC inhibition (Table 1). All compounds exhibited inhibition of HDAC activity with pIC_{50} = 5.9–7.1. In contrast, the anti-proliferative/cytotoxic activity in general was enhanced for small alkyl substituents like methyl and ethyl (**1b** and **c**; **2b** and **c**). For compound **1c**, additionally a K_i value of 7 μM was determined in respective enzyme kinetic studies with recombinant HDAC1 isoenzyme from a Lineweaver–Burk plot, showing a substrate competitive mode-of-action.[19] Thus, and for the superior chemical stability in aqueous media of the ethyl trithiocarbonate over the methyl trithiocarbonate (data not shown), the ethyl substituent was chosen as test substituent for further evaluations.

With the ethyl moiety in hand as a suitable head affix, the ability of the trithiocarbonate to act as a HDAC inhibiting head compared with the hydroxamate of SAHA was probed. For this reason, an ethyl trithiocarbonate was attached to the core structure of SAHA in a short synthesis (Scheme 2). The conversion of the carboxylic acid **38** into the acetyltrithiocarbonate **36** via the diazo-intermediate was low yielding, but provided sufficient amounts for initial testing.[20,21]



Scheme 2. Preparation of trithiocarbonate analog of SAHA. Reagents and conditions: (a) Ac_2O, 160 °C, 2 h (99%); (b) $PhNH_2$, THF, rt, 0.5 h (50%); (c) $(COCl)_2$, DMF, $CHCl_3$, rt, 1 h; (d) $(Me_3Si)CHN_2$, CH_3CN, rt, 2 h, then HBr (33% in AcOH), rt, 1 h; (e) EtSC(=S)SNa, THF, rt, 18 h (4%, 3 steps).



Scheme 3. Preparation of trithiocarbonate analog of MS-275. Reagents and conditions: (a) 3-pyridinyl-CH_2OH, CH_2Cl_2, rt, 6 h (74%); (b) $CH_2C(OEt)Sn(n\text{-}Bu)_3$, $Pd(dppf)Cl_2$, DMF, μw, 80 °C, 3.5 h (39%); (c) NaHMDS, THF, −78 °C, 15 min, then TMSCl, −78 °C, 15 min, then Br_2, −78 °C, 30 min, then EtSC(=S)SNa, −78 °C to rt, 2 h (19%).

For comparing the trithiocarbonate with other HDAC inhibiting head groups, an analog of MS-275 bearing a trithiocarbonate instead of the amino-benzamide was also prepared (Scheme 3). 4-Bromo-benzyl isocyanate was reacted to the required carbamate **40**. Stille-coupling of using tributyl(1-ethoxyvinyl)-tin[22] resulted directly in the acetyl compound **41** instead of the expected vinyl-enolether. Thus, **41** was brominated via NaHMDS-deprotonation, in-situ silylenolether-formation and conversion with bromine at low temperature.[23] The reaction mixture was directly treated with a solution of ethyl sodium trithiocarbonate and **39** could be obtained after aqueous work-up and purification using preparative HPLC.

Interestingly, the direct SAHA analog **36** was found to inhibit HDAC activity as potent as SAHA itself, albeit with little cellular activity (Table 2). The lacking cellular activity might be explained by a high lipophilicity and high protein binding. The trithiocarbonate analog of MS-275 **39** inhibited HDAC significantly higher than MS-275 itself (Table 2). In contrast to MS-275, the HDAC activity was completely inhibited by **39**, which is most likely explained by a HDAC isoenzyme inhibition profile distinct to MS-275.

The next focus laid on the evaluation of the influence on activity of substituents at the phenyl ring (cap-linker, Fig. 2). Thus, a series of substituted phenyl moieties was prepared to investigate this question. In a straightforward synthesis, different phenacyl bromides were converted with a solution of sodium ethyl trithiocarbonate[24] to yield the trithiocarbonates **4**–**23** in good yields (84–96%; Scheme 1). As described for **1e**, the acetamide **3** was not immediately accessible, but could be synthesized via its thioacetate (Scheme 1).

As seen for the variation of the alkyl substitution, a wide range of substituents at the phenyl moiety retained their HDAC inhibitory potential (Table 3). An aniline acetamide (**3**) instead of a phenacyl residue was still tolerated, but lost significantly its HDAC inhibitory activity. Electron donating (e.g., **1c**, **7**, **8**, **23**, **13**, and **15**) and electron withdrawing substituents (e.g., **2c**, **6**, **16**–**18**, **20**, and **22**) had only marginal influence on the HDAC inhibition.

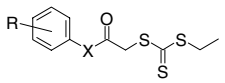
The position of the substitution, however, had dramatic effects on the HDAC activity. Subtle differences between *meta*- or *para*-substitution were observed (e.g., for OMe: **7**/**8**; for NHAc: **16**/**17**), slightly favoring substitution in *para*-position. Introduction of substituents in *ortho*-po-

Table 2. In vitro activity for SAHA and MS-275-like trithiocarbonates

Compound	HDAC pIC_{50}[a]	HeLa pIC_{50}[b]
SAHA	7.1	5.7
36	7.3	40% at 25 μM[c]
MS-275	4.9	5.8
39	7.4	4.7

[a] Biochemical HDAC activity assay.
[b] Anti-proliferative, cytotoxic activity.
[c] Inhibition at highest test concentration.

Table 3. In vitro activity for ethyl phenacyl trithiocarbonates

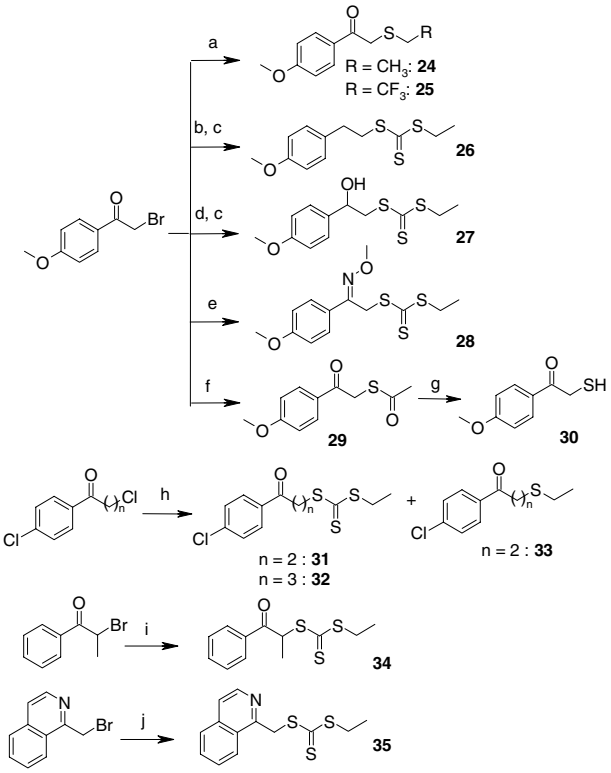


Compound	R	X	HDAC pIC_{50}[a]	HeLa pIC_{50}[b]
1c	4-Me	Bond	6.2	5.4
2c	3,4-Cl_2	Bond	7.1	5.1
3	3,4-Cl_2	NH	5.6	0% at 25 μM[c]
4	2-Me	Bond	7% at 32 μM[c]	5.0
5	H	Bond	5.7	5.3
6	4-Cl	Bond	6.0	5.3
7	4-MeO	Bond	6.3	5.6
8	3-MeO	Bond	5.9	5.3
9	2-MeO	Bond	6% at 50 μM[c]	4.8
10	3,4-$(MeO)_2$	Bond	5.4	5.3
11	2,5-$(MeO)_2$	Bond	6% at 50 μM[c]	4.8
12	2,4-$(MeO)_2$	Bond	3% at 50 μM[c]	4.9
13	3,4-(OC_2H_4O)	Bond	6.3	5.1
14	2,3-(OC_2H_4O)	Bond	4.5	4.8
15	3,4-(OCH_2O)	Bond	6.3	5.2
16	3-AcHN	Bond	6.2	5.0
17	4-AcHN	Bond	6.4	5.7
18	4-NC	Bond	6.3	5.1
19	3-EtOC(O)NH	Bond	6.7	5.7
20	4-F_2HCO	Bond	6.1	5.4
21	4-$MeS(O)_2NH$	Bond	5.7	4.4
22	4-$H_2NS(O)_2$	Bond	6.2	4.7
23	4-Et_2N	Bond	5.7	5.5

[a] Biochemical HDAC activity assay.
[b] Anti-proliferative, cytotoxic activity.
[c] Inhibition at highest test concentration.

sition, however, resulted in a decrease of HDAC inhibition by about 100 times. This effect was found for mono-substituted (e.g., for Me, **1c/4** and for MeO **7/9**) as well as for bis-substituted phenyl-moieties (e.g., for $(MeO)_2$, **10/11/12** and for (OC_2H_4O), **13/14**).

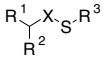
Finally, variations around the central unit (head, Fig. 2) were investigated. Compounds were synthesized (Scheme 4) from phenacyl bromides or similar precursors in short sequences. Thioethers **24** and **25** were obtained in quantitative yield from the thiols and 4-methoxyphenacylbromide using potassium carbonate and ethanol. 4-Methoxyphenacylbromide was partially reduced to the alcohol or completely reduced to the methylene, and the crude products were used without purification for the conversion into the trithiocarbonates **27** and **26**. In situ formation of the oxime, which resulted in one single stereoisomer, and conversion with sodium ethyl trithiocarbonate gave direct access to **28**. Facile displacement of the bromide with potassium thioacetate delivered the corresponding thioacyl analog **29**, which was easily hydrolyzed to the free thiol **30** (Scheme 4).[10a] Sodium iodide promoted displacement of 1-(4-chloro-phenyl-)-1-oxo-4-chloro-butane ($n = 3$) with sodium ethyl trithiocarbonate yielded the expected product **32**, while the conversion of the corresponding chloro-propane ($n = 2$) under the same reaction conditions resulted exclusively in thioether **33**, probably via substitution and elimination of the trithiocarbonate in a retro-Michael-type reaction.[25] Compounds **34** and **35** were accessible using similar reaction conditions (Scheme 4).

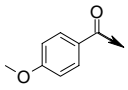
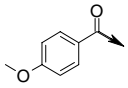
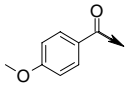
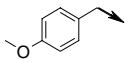
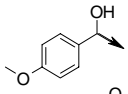
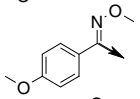
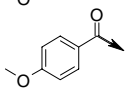
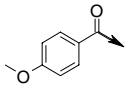
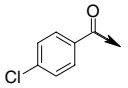
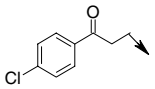
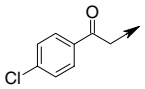


Scheme 4. Preparation of ethyl phenacyl trithiocarbonates and structural analogs. Reagents and conditions: (a) HSEt or $HSCH_2CF_3$, K_2CO_3, EtOH, rt, 20 h (99% for **24** and for **25**); (b) $BH_3{\cdot}NH_2t$-Bu, $AlCl_3$, CH_2Cl_2, 0 °C, 1 h (99%); (c) EtSC(=S)SNa, THF, 50 °C, 3 h (51% for **26**; 15% for **27**); (d) $NaBH_4$, MeOH, 0 °C, 15 min (99%); (e) $NH_2OCH_3{\cdot}HCl$, NEt_3, 4 Å MS, THF, rt, 1 d; then EtSC(=S)SNa, THF, rt, 3 h (30%, only one stereo-isomer); (f) KSAc, THF, 40 °C, 24 h (99%); (g) 1 M NaOH, MeOH, rt, 1 h (84%); (h) EtSC(=S)SNa, NaI, DMF, 50 °C, 48 h (87% for **31**; 0% for **32**; 74% for **33**); (i) EtSC(=S)SNa, THF/EtOAc, rt, 0.5 h (99%); (j) EtSC(=S)SNa, NEt_3, THF, rt, 0.5 h (98%).

The results for the modification around the central unit of the molecule (head, Fig. 2) clearly indicated a sharp SAR in this part of the molecule (Table 4). As described before,[12] the ethylthioether **24** showed no HDAC inhibition in contrast to the trithiocarbonate **7**. A more electron deficient thioether such as the trifluoroethylsulfide **25** did neither result in any HDAC activity. Very remarkable is the difference in activity between the trithiocarbonate **7**, the thioacetate **29** and its thiol analog **30**. While thiol **30** and thioacetate **29** analog, which might be considered as a prodrug of **30**,[10] inhibited the HDAC nuclear extract with submicromolar activity ($pIC_{50} = 6.3$ and 6.9), almost no cellular activity was observed. In contrast, the trithiocarbonate **7** inhibited the HDAC nuclear extract with comparable activity ($pIC_{50} = 6.3$), yet retaining cellular cytotoxic activity ($pIC_{50} = 5.6$) at concentrations inhibiting cellular HDAC activity and inducing histone hyperacetylation (see below). These observations indicate that trithiocarbonates act as HDAC inhibitors themselves

Table 4. In vitro activity of ethyl phenacyl trithiocarbonates and structural analogs



Compound	R^1	R^2	X	R^3	HDAC pIC_{50}[a]	HeLa pIC_{50}[b]
7		H	SC(=S)	Et	6.3	5.6
24		H	Bond	Et	31% at 32 μM[c]	0% at 25 μM[c]
25		H	Bond	CH_2CF_3	21% at 32 μM[c]	0% at 25 μM[c]
26		H	SC(=S)	Et	16% at 0 μM[c]	0% at 25 μM[c]
27		H	SC(=S)	Et	11% at 50 μM[c]	0% at 25 μM[c]
28		H	SC(=S)	Et	4% at 32 μM[c]	0% at 50 μM[c]
29		H	Bond	Ac	6.3	4.6
30		H	Bond	H	6.9	4.7
6		H	SC(=S)	Et	6.0	5.3
32		H	SC(=S)	Et	5% at 50 μM[c]	0% at 25 μM[c]
33		H	Bond	Et	5% at 50 μM[c]	0% at 25 μM[c]
5		H	SC(=S)	Et	5.7	5.3
34		CH_3	SC(=S)	Et	3% at 50 μM[c]	5.3
35		H	SC(=S)	Et	8% at 30 μM[c]	4.7

[a] Biochemical HDAC activity assay.
[b] Anti-proliferative, cytotoxic activity.
[c] Inhibition at highest test concentration.

and not as a prodrug for the corresponding thiols. However, it cannot be excluded from these data that the trithiocarbonate eventually serves as a substrate in the active site of the enzyme.

The carbonyl moiety in the head group was also found to be essential for HDAC inhibition as its reduction product **26** or the corresponding alcohol **27** was inactive, too. The attempt to replace the carbonyl group by a methyl-oxime **28** or to mimic the carbonyl's donating properties by its incorporation into a heterocycle, such as **35**, was also not successful and did neither yield in active compounds. The distance between carbonyl moiety and trithiocarbonate also seems to be of great importance for HDAC inhibition, as when the methylene unit of **6** was replaced by a propylene linker, the resulting compound **32** lost its HDAC activity. The ethylene compound **31** was not accessible under standard conditions and the corresponding thioether **33** was, as one would expect, inactive. Additional substitution of the central methylene unit with a methyl substituent resulted in the inactive compound **34**.

Finally, compound **7** was studied in more detail for its HDAC isoform inhibition profile. While the nuclear extract containing a mixture of several HDAC isoenzymes was inhibited in the submicromolar range (pIC_{50} = 6.4), all tested isoenzymes were inhibited in the low micromolar range (rHDAC1: pIC_{50} = 5.4; rHDAC3: pIC_{50} = 5.5; rHDAC6: pIC_{50} = 5.9; rHDAC8: pIC_{50} = 4.8). Therefore, the higher inhibitory activity of **7** in the nuclear extract assay must be explained by another, not tested HDAC isoform(s) or defined HDAC containing complexes not represented in purified enzyme preparations. The most potent inhibition was observed with HDAC6, which is not present in the HeLa nuclear extract. Inhibition of isoenzymes HDAC1 and HDAC3 was similar, while HDAC8 was significantly less potently inhibited. HDAC inhibition in a cellular context through trithiocarbonates was also demonstrated with **7** as a representative example by induction of histone H3 hyperacetylation at pIC_{50} = 5.7 in a high-content screening assay[19] and inhibition of cellular HDAC enzymatic activity in vital Hela cells with pIC_{50} = 5.3. These target related data correlated well with the inhibition of proliferation of several cancer cell lines (HeLa-cervical carcinoma pIC_{50} = 5.6; A549-lung cancer: pIC_{50} = 5.2; RKO-colon cancer: pIC_{50} = 5.7).

We have demonstrated that trithiocarbonates are potent, cellular active HDAC inhibitors. Based on the inhibition of a HDAC isoenzyme mixture, a clear SAR picture could be established for the cap-linker and head groups. In enzyme kinetic studies with the recombinant HDAC1 isoenzyme, a substrate competitive mode-of-action of **1c** was determined. This result as well as the potent activity of chimeric SAHA analogs **36** and the narrow SAR of the trithiocarbonate motif makes it highly likely, that this motif complexes Zn^{2+} in the active site of HDAC class I and II enzymes. Surprisingly and in contrast to the hydroxamate and benzamide head groups, an extension of the head group is compatible with HDAC inhibitory activity. As exemplified by the phenyl or ethyl–phenyl residues in **1f** and **2e**, even bulky moieties are potent submicromolar inhibitors with reasonable cellular activity. Defined modifications in the cap-linker moiety as exemplified by analogs **7**, **17**, and **19** lead to a cellular activity in the Alamar blue proliferation assay comparable to that of SAHA (Table 3).

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17. *Experimental details:*
(a) Biochemical HDAC assay: HDAC activity was isolated from HeLa nuclear extracts according to a method originally described by Dignam et al.[13a] Fractions from the purification were analyzed by Western blotting and for specific activity in the biochemical enzyme assay. The HDAC enzyme activity assay was performed as described by Wegener et al.[13b] Briefly, 40 µl HeLa cell nuclear extract, 29 µl enzyme buffer (15 mM Tris–HCl, pH 8.1, 0.25 mM EDTA, 250 mM NaCl, 10% v:v glycerol; for recombinant HDAC isoenzymes, 0.1 mg/ml bovine serum albumin (BSA) was also added), and 1 µl compound solutions were added per well of a microtiter plate. The reaction was started by addition of 30 µl substrate (Ac-NH-GGK(Ac)-AMC final 25 µM/Thermo Biosciences, Ulm/Germany). After incubation for 90 min at 30 °C, reaction was terminated by adding 25 µl stop solution (50 mM Tris–HCl, pH 8, 100 mM NaCl, 0.5 mg/ml trypsin, 2 µM TSA). After 40-min incubation at room temperature, fluorescence was measured using a Wallac Victor 1420 multilabel counter (exeitation $\lambda = 355$ nm, emission $\lambda = 460$ nm).
(b) Tumor cell line and proliferation assay: the human tumor cell line HeLa (cervical cancer/CCL-2) was derived from ATCC (Promochem, Germany). The cell line was cultivated at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 vol% fetal calf serum. The anti-proliferative activity of compounds was evaluated by using the Alamar blue (Resazurin) cell viability assay.[14] HeLa cells were seeded into 96-well flat bottom plates at 1.000 cells/well to allow proliferation during 72 h cultivation with test compound. Twenty-four hours after seeding, 1 µl of each of the compound dilutions was added per well ($n = 4$). After incubation for 72 h at 37 °C in a humidified atmosphere containing 5% carbon dioxide, cell viability was determined by addition of 20 µl of 90 mg/l Resazurin solution (Sigma) and subsequent measurement of fluorescence at excitation ($\lambda = 544$ nm) and emission ($\lambda = 590$ nm).
(c) Data analysis: IC_{50} values from concentration-effect curves were calculated by means of non-linear regression analysis using the program GraphPad Prism (Version 4.0). In general, results are given as mean values of independent experiments.
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