

Effect of inhibitors of histone deacetylase on the induction of cell differentiation in murine and human erythroleukemia cell lines

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Histone deacetylase (HDAC) inhibitors are a novel class of promising anti-cancer agents. Little information is available on the capacity of structurally different HDAC inhibitors to induce terminal cell differentiation in different cell types in relation to enzyme inhibition and subtype selectivity. Consequently, the aim of this study was to provide a comprehensive comparison of these effects. New biarylalanine inhibitors of HDAC were synthesized and compared to a series of standard inhibitors from different laboratories. Chromatographically purified rat liver and immunoprecipitated FLAG-tagged recombinant human HDACs were used as sources of HDAC activity. Enzyme inhibition was studied using a fluorescent substrate and its conversion was monitored by high-performance liquid chromatography. The ability to induce cell differentiation was compared in murine (Friend DS-19) and human (K562) erythroleukemic cell lines, and was quantified by benzidine staining. Inhibition of cell proliferation was evaluated by cell counting. All HDAC inhibitors were identified as potent inhibitors of erythroleukemic cell proliferation. However, we observed a complex pattern of differentiation induction: structurally similar inhibitors resulted in disparate activity profiles, whereas similar profiles were detected within distinct structural classes. Among the newly synthesized biarylalanine compounds, a 3'-methoxy derivative was

identified as a very effective inducer of terminal cell differentiation. We conclude that investigation of subtype selectivity of selected HDAC inhibitors does not provide a clear link between selectivity and the observed cellular activity profile. The predictive value of *in vitro* HDAC inhibition assays for identifying anti-proliferative compounds has been emphasized. *Anti-Cancer Drugs* 16:635–643 © 2005 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2005, 16:635–643

Keywords: benzidine staining, cell differentiation, erythroleukemia, histone deacetylase, hydroxamic acid, K562, murine Friend leukemic cell

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Sponsorship: Funding by the Deutsche Forschungsgemeinschaft (Ju295/2) is gratefully acknowledged. C. G. was supported by the Verein zur Förderung der Krebsforschung in Deutschland eV.

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Received 9 November 2004 Revised form accepted 29 March 2005

Introduction

Histone deacetylase (HDAC) inhibitors are a relatively new class of potential drugs for the treatment of hyperproliferative, as well as parasitic diseases [1,2]. Inhibition of HDAC leads to hyperacetylation of chromatin which is usually associated with transcriptional activation of a number of genes [3,4]. One of the important downstream events of histone hyperacetylation is increased expression of the cyclin-dependent kinase (cdk) inhibitory protein p21/WAF1/CIP1 resulting in cell cycle arrest [5]. The dysregulated recruitment of HDACs by oncogenic fusion proteins was identified as a general mechanism for the pathogenesis of leukemia [6,7]. Therefore, HDAC is viewed as an attractive target for anti-cancer drug development [8,9]. There are 11 subtypes known for the 'classical' zinc-dependent HDACs [10], but little is known about the subtype selectivity of established inhibitors or the biological consequences of such an inhibition.

We had previously proposed a general model for HDAC inhibitors that was derived from the potent natural product inhibitors trapoxin B (TPX) and trichostatin A (TSA) [11], and had accordingly developed the first simple potent inhibitors of HDAC [12]. Structure-activity studies have led to the improved inhibitor BIP1 with a biphenylalanine instead of a phenylalanine moiety [13]. Here, we present a set of substituted biphenylalanines BIP2–6 that were synthesized in order to elucidate whether HDAC inhibitory activity is dependent on electronic properties in the biaryl moiety. We report *in vitro* inhibition of rat liver HDAC activity as well as anti-proliferative and differentiation-inducing effects in murine Friend leukemic cells (MELC) and the human K562 cell line. We also present a comparison of a series of structurally diverse standard HDAC inhibitors in these two erythroleukemic cell lines. Finally, selected inhibitors were tested for their subtype selectivity concerning HDACs 1, 3 and 6.

Materials and methods

Chemicals

For further modification of our biphenylalanine lead compound we used the strategy that was used previously for similar analogs [13]. As a new route for structural variations within this strategy 4-bromophenylalanine methyl ester was converted to a building block with a trityl-protected hydroxamate function that was successfully subjected to palladium-mediated biaryl coupling. This resulted in substituted biphenylalanines which were deprotected with trifluoroacetic acid/triethylsilane in order to obtain the desired hydroxamic acids BIP2–6 (see Fig. 1). We have used boronic acids as the electrophilic coupling partners and $\text{Pd}(\text{OAc})_2/\text{P}(\text{o-tol})_3$ as the catalyst. The biaryls were isolated with yields ranging from 43 to 80%. This catalytic system has been used previously for the synthesis of biarylalanine-containing di- and tripeptides [14]. Purity and identity were assured using IR and NMR spectroscopy as well as elemental analyses. TSA, Scriptaid, SAHA and MS275 were purchased from EMD Biosciences (San Diego, CA). MD85 [12,15], M344, M360 [16] and BIP1 [13] were synthesized according to the literature. M344 is also commercially available (Alexis, Lausen, Switzerland or EMD Biosciences: histone deacetylase inhibitor III).

In vitro enzyme inhibition

Rat liver HDAC was partially purified essentially as described earlier [17,18]. An ammonium sulfate precipitation step was included for further purification prior to chromatography [19]. This preparation is commercially available (EMD Biosciences or Alexis). For testing, stock solutions of the inhibitors (1 mg/ml in ethanol for TSA and 12 mM in DMSO for all other inhibitors) were diluted with enzyme buffer (15 mM Tris-HCl, pH 7.9, 0.25 mM EDTA, 10 mM NaCl, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol).

The assay was performed as described earlier [20]. Briefly, 12 μl of a solution of the substrate (4.682 mg/ml in ethanol) and 24 μl of a solution of the internal standard 7-hydroxycoumarin (3.66 mg/ml DMSO) were combined and brought to a total volume of 1 ml with enzyme buffer. An aliquot of 10 μl of this stock solution was added to a mixture of 100 μl (approximately 90 U/ml; 1 U: 1 pmol/min) of rat enzyme preparation (at 4°C) and 10 μl of inhibitor dilution. After 15 min at 4°C the mixture was incubated for 90 min at 37°C. Then, the reaction was stopped by addition of 72 μl of 1 M HCl/0.4 M sodium acetate and 800 μl of ethyl acetate. After centrifugation (10 000 g, 5 min), 200 μl of the upper phase was dried under a stream of nitrogen. The residue was dissolved in 600 μl of the chromatography eluent and 20 μl was injected via an autosampler onto the high-performance liquid chromatography (HPLC) system. In a modification of the previously published assay [17,18,20], 10.5 μM

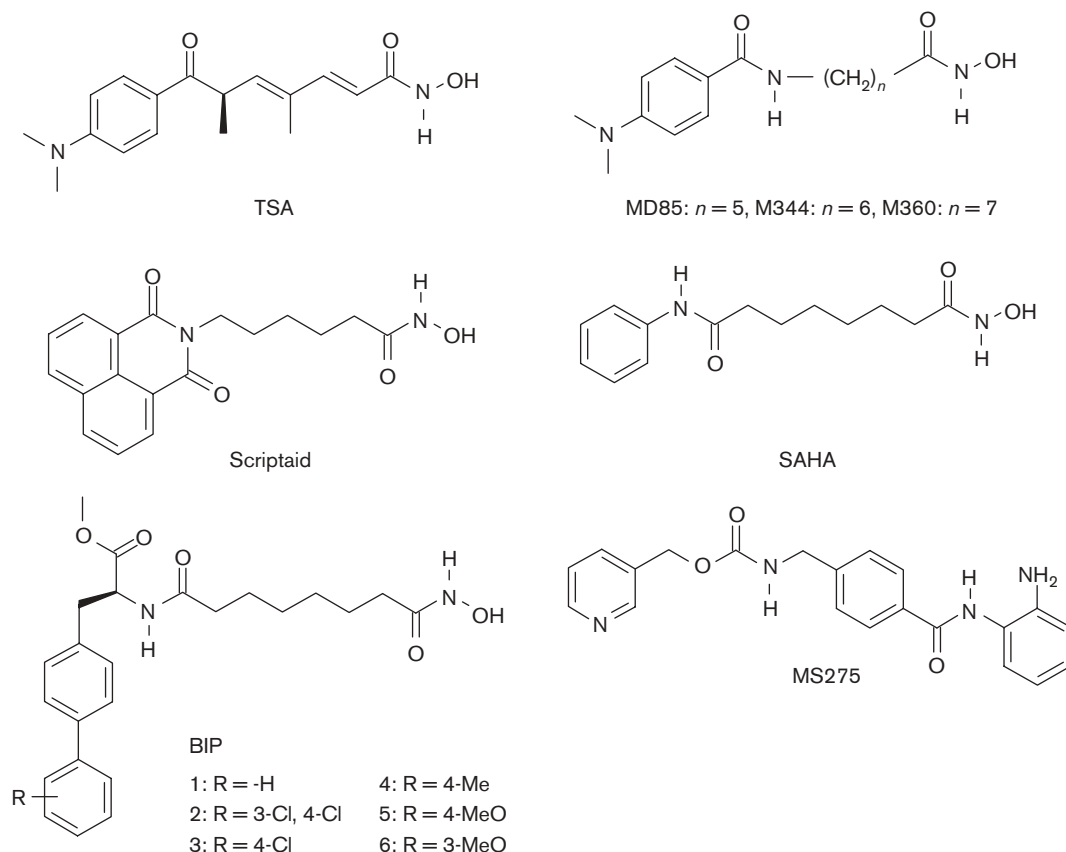
substrate was used here instead of 3.5 μM , resulting in increased precision (data not shown). The amount of remaining substrate was calculated relative to the substrate control incubated without enzyme (each as quotient of the peak area of the substrate divided by the peak area of the internal standard). A Shimadzu RF 535 was used as fluorescence detector for HPLC and a LiChrosorb RP 18-5 μm (125 \times 3 mm; Knauer, Berlin, Germany) column was used for separation. The Biomol HDAC fluorescent activity assay kit was used according to the manufacturer's instructions. FLAG-HDAC immunoprecipitation and assays were performed as published previously [21]

Induction of terminal cell differentiation

MEL DS19 murine erythroleukemia cells (MELC) were maintained in DMEM containing 100 U/ml penicillin G sodium and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate supplemented with 10% fetal bovine serum (Greiner, Frickenhausen, Germany) at 37°C in a 5% CO_2 atmosphere. K562 human erythroleukemia cells (obtained from the Tumorbank, German Cancer Research Center, Heidelberg, Germany) were maintained in RPMI medium containing 100 U/ml penicillin G sodium and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate supplemented with 10% fetal bovine serum (Greiner) at 37°C in a 5% CO_2 atmosphere. To test compounds for potential to induce cell differentiation, log-phase cells were used. Serial dilutions of compounds were prepared in 24-well plates (Falcon) using 1 ml medium/well. If compounds were dissolved in DMSO, control wells contained the same amount of solvent (generally 2 $\mu\text{l}/\text{ml}$ medium, 0.1% final concentration). Subsequently, the cell suspension was added to the wells (1 ml/well, 2×10^4 cells/ml; final cell concentration 1×10^4 cells/well). After 72 (MELC) or 96 h (K562), respectively, the experiment was evaluated. Cell numbers were counted using a Casy 1 TTC flow cytometer (Schärfe System, Reutlingen, Germany). The proliferation of treated cells was expressed as percent proliferation in comparison with the solvent control.

Differentiated K562 and Friend leukemic cells accumulate hemoglobin. Therefore, the induction of cell differentiation was determined by benzidine staining according to the literature [22]. To 50 μl of cells suspension, an equal volume of benzidine solution (2 mg/ml in 0.5 M acetic acid containing 2% H_2O_2) was added. Within 5 min the hemoglobin-containing cells stain blue. Benzidine-positive and -negative cells were counted under the microscope in a hemocytometer, and the percentage of positive cells was calculated. All compounds were first tested at 10 and 50 μM final concentrations. According to the activity/toxicity profile, a range of concentration was chosen for a dose-response analysis. In selected cases, dose-response experiments were repeated at the same concentrations and deviations were below 5%.

Fig. 1



Chemical structures of tested HDAC inhibitors.

Histone hyperacetylation

K562 cells (1×10^5 cells/ml in 20 ml) were incubated in RPMI for 24 h and then treated with BIP6 ($1.56 \mu\text{M}$) or MS275 ($0.625 \mu\text{M}$), respectively, for 12 h. Control cells obtained DMSO (0.1% final concentration). Isolation of histones, quantification of protein and acid-urea-Triton (AUT) PAGE analyses were performed as described previously [23].

Results

HDAC inhibition

The series of biarylalanine compounds BIP1–5 was tested for inhibition of a partially purified rat liver HDAC employing the fluorogenic substrate developed in our group [17,18] and enzyme inhibitory properties were compared. The substituents of these novel derivatives were selected in order to determine with a limited number of compounds whether inhibitory activity was dependent on electronic properties of the aromatic ring [24].

As BIP1 was the most potent inhibitor (see Table 1), an unfavorable steric repulsion in the 4'-position of

the substituted compounds BIP2–5 was assumed. For substituted derivatives, the activity increased from BIP2 to BIP5, indicative for a beneficial effect of electron-donating substituents. Consequently, the 3'-methoxy analog BIP6 was synthesized, but this compound again was less active than the most potent parent compound BIP1 (see Table 1). For comparison, we included several standard HDAC inhibitors from our and other labs. We investigated TSA [25], the trichostatin analogs MD85 [12,15], M344 and M360 [16] that were synthesized previously in our group, Scriptaid [26], the so-called hybrid polar drug SAHA [27] which is in clinical trials, and the benzamide MS275 [28,29] (see Fig. 1). All IC_{50} values summarized in Table 1 (except for MS275) were determined under identical assay conditions. In contrast to published data, we could not detect inhibitory activity of MS275 using our methodology [30] or the commercially available Biomol kit. A similar lack of inhibition has been reported recently [31] and it was speculated by that group that *in vitro* inhibition by MS275 is dependent on certain cofactors that are required in the enzyme preparation.

Table 1 *In vitro* rat liver HDAC inhibition in comparison with inhibition of cell proliferation of Friend murine and K562 human erythroleukemia cells by HDAC inhibitors

Compound	HDAC inhibition (IC ₅₀ , μM ± SD ^a)	MEL inhibition (IC ₅₀ , μM ^b)	K562 inhibition (IC ₅₀ , μM ^b)
BIP1	0.29 ± 0.03 ^c	0.64	0.04
BIP2	0.70 ± 0.04	1.92	1.14
BIP3	0.53 ± 0.03	0.46	0.31
BIP4	0.45 ± 0.06	0.64	0.23
BIP5	0.41 ± 0.03	0.58	0.20
BIP6	0.63 ± 0.06	3.27	0.71
TSA	0.01 ± 0.001 ^d	0.04	0.05
MD85	0.22 ± 0.02 ^d	9.51	2.20
M344	0.12 ± 0.01 ^d	1.00	0.34
M360	0.20 ± 0.02	3.97	1.80
Scriptaid	0.06 ± 0.02 ^d	9.30	11.8
SAHA	0.17 ± 0.03 ^d	0.89	0.61
MS275	NI ^e /4.8 ^f /130 ± 18 ^g	1.24	0.31

Referenced inhibition data (except note f) was generated in our laboratory under identical conditions.

^aMean values of half-maximal inhibitory concentrations (IC₅₀) ± SD were computed from four experiments.

^bIC₅₀ values were generated from testing six to eight concentrations in duplicate.

^cData taken from [13].

^dData taken from [19].

^eNo inhibition at 100 μM.

^fData taken from [29].

^gData using the Biomol assay.

Inhibition of proliferation and induction of terminal differentiation

The novel HDAC inhibitors were further tested for the anti-proliferative and differentiation-inducing potential of murine Friend erythroleukemic cells and human K562 erythroleukemia cells. Friend cell differentiation has been a standard model for the investigation of HDAC inhibitors in many studies [16,32–34]. The aim of our current studies was a comparison of effects in the murine cell line with those in a human erythroleukemia cell line. In addition, the above-mentioned reference inhibitors were tested for comparative reasons. Cell differentiation was detected by an accumulation of hemoglobin which is easily visualized by benzidine staining. Figure 2 shows the profiles of cellular activity, and Table 1 summarizes IC₅₀ values for inhibition of HDAC and cellular proliferation.

All HDAC inhibitors demonstrated dose-dependent inhibition of cell proliferation in both leukemic cell lines. Overall, the K562 cell line was more sensitive to the growth inhibitory potential of the test compounds than the Friend cell line and all compounds except Scriptaid were more toxic for the human cell line. We noticed that IC₅₀ values from the HDAC *in vitro* test were of the same order of magnitude as the ones obtained in the anti-proliferative tests. The biggest differences were seen with Scriptaid, which was more than 100-fold less potent in the cellular systems. TSA was identified as the most potent compound in all three assays, but the unsubstituted biarylalanine BIP1 was equally effective in the human K562 cell line.

For the induction of terminal cell differentiation, the compounds can be arranged in three groups depending on

the maximum percentage of benzidine-positive cells. In the Friend cell line, weak induction (less than 30% benzidine-positive cells) was monitored with the first group, the halogenated biaryls BIP2 and BIP3, the trichostatin analogs MD85 and M360, Scriptaid, and MS275. BIP2, MD85 and Scriptaid were weak inducers in the K562 cell line as well. The second group, consisting of the methyl biaryl BIP4, its methoxy analogs BIP5 and BIP6, TSA, and SAHA, showed a moderate differentiation induction potential in 30–50% of the surviving Friend cells. The percentage of benzidine-positive cells for BIP4 was similar in K562 cells. BIP5 was less effective in the human cell line, whereas SAHA, MS275 and the 3'-methoxy compound BIP6 were identified as potent inducers of differentiation in K562 cells (above 50%). The unsubstituted biarylalanine BIP1 and the aminoheptanoate analog of TSA (M344) were found to most potently induce terminal cell differentiation in Friend cells (above 50%) which was reduced somewhat for BIP1 and strongly (below 30%) for M344 in the human cell line.

Histone hyperacetylation

With compound BIP6 and the reference compound MS275 we additionally investigated the potential to induce hyperacetylation in K562 cells by AUT gel electrophoresis as described in the literature [22,23]. As outlined above, both compounds belong to the most potent group to induce differentiation in K562 cells. Both compounds led to accumulation of hyperacetylated histone H4 when K562 cells were treated for 12 h with 1.56 μM BIP6 or with 0.625 μM MS275, respectively (Fig. 3).

HDAC subtype selectivity

We have previously shown that the aminoheptanoate M344 (good inducer of MELC differentiation) is selective for HDAC6 (3-fold) [30]. On the other hand, the aminooctanoate M360 (little induction of differentiation) was slightly selective for HDAC1 [30] (see Table 2). In this report, we additionally investigated the aminocaproate MD85, and the biaryls BIP2 (little induction of differentiation) and BIP6 (good induction of differentiation) for inhibition of immunoprecipitated FLAG-tagged HDAC1 and HDAC6, using a radioactively labeled histone peptide fragment as the substrate. Data for inhibition of HDAC3 is additionally presented for all compounds.

Discussion

Here, we report the synthesis of novel biarylalanine HDAC inhibitors, which do not exceed the potency of the unsubstituted lead compound in its *in vitro* HDAC-inhibitory potency. As neither electron-donating nor electron-withdrawing substituents lead to an increase, we assume that additional substitutions of the biaryl

moiety in BIP1 are not favorable for inhibitory activity due to steric hindrance. Possibly, the region of the enzyme that the biphenyl group binds to is quite restricted in the tolerance of inhibitors. As HDAC1, in particular, is thought to be important for mediation of cancer cell proliferation [35] and no structural data for that subtype is available, BIP1 might be an interesting candidate for docking experiments in homology models of

human HDACs [36] that can be derived from the structure of the homologous bacterial histone-deacetylase-like-protein (HDLP) [37] or human HDAC8 [38].

All HDAC inhibitors that were investigated in this study were identified as strong anti-proliferative drugs in erythroleukemic cells, which emphasizes their potential as anti-cancer drugs. Especially interesting is the activity

Fig. 2

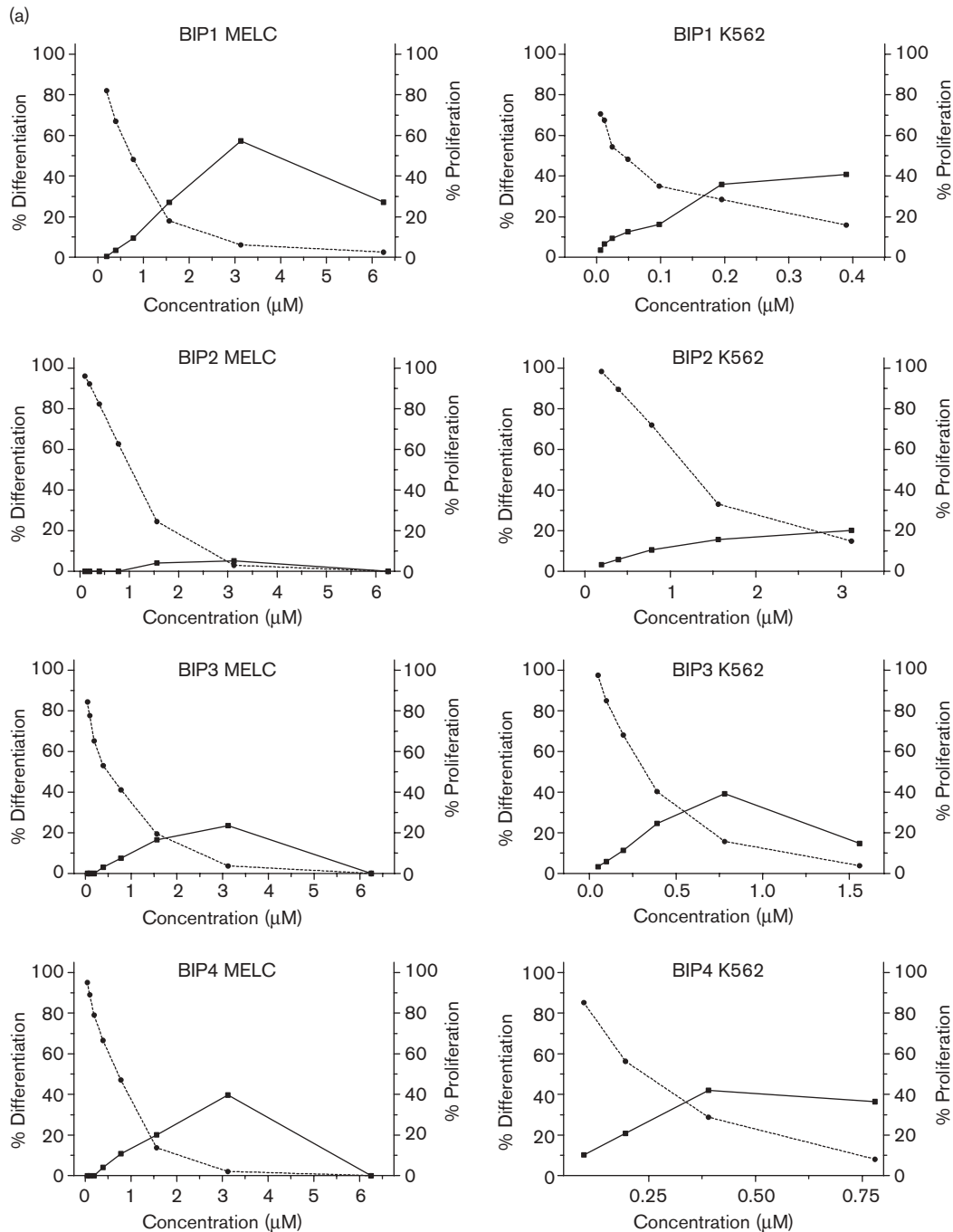
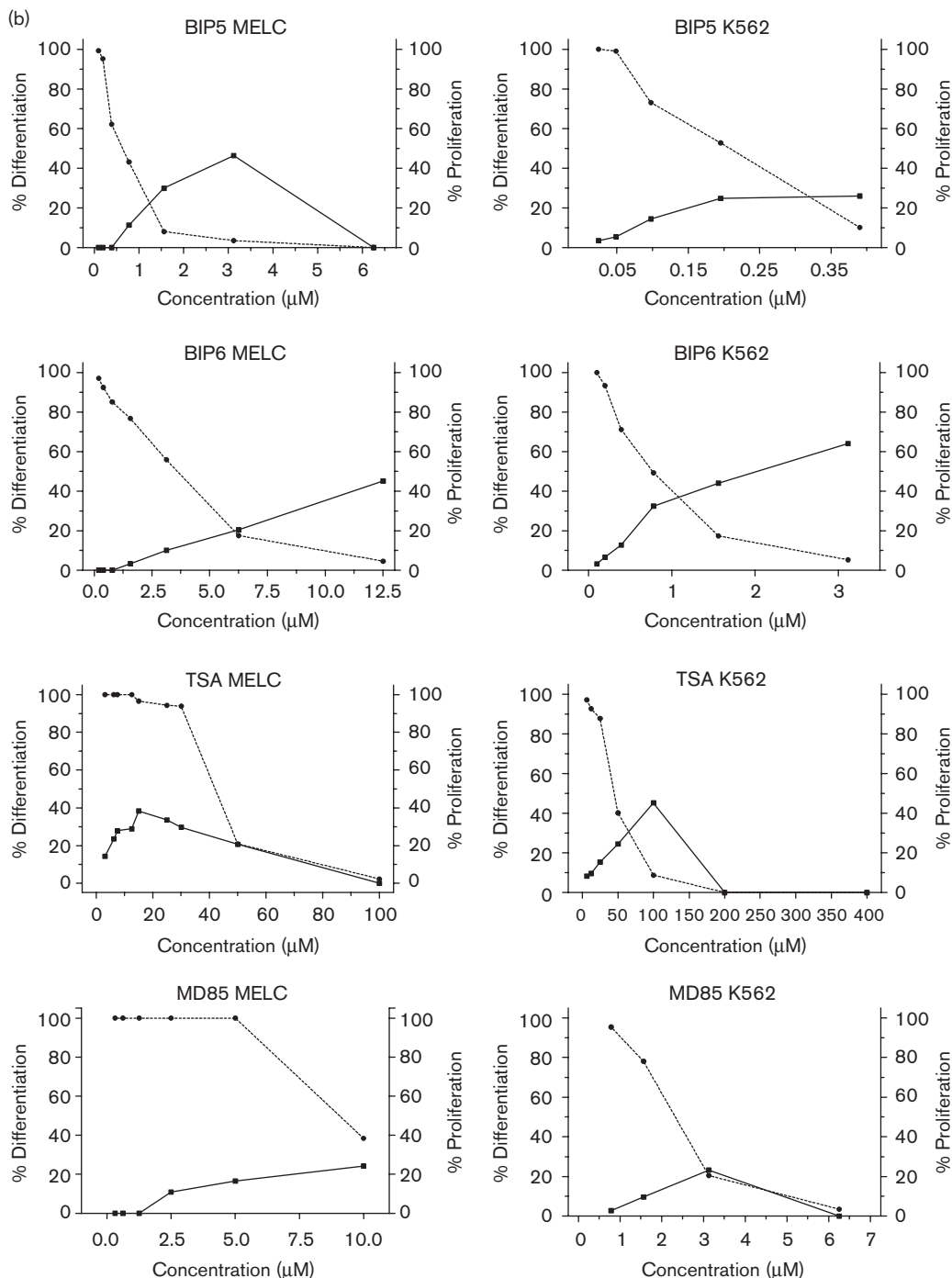


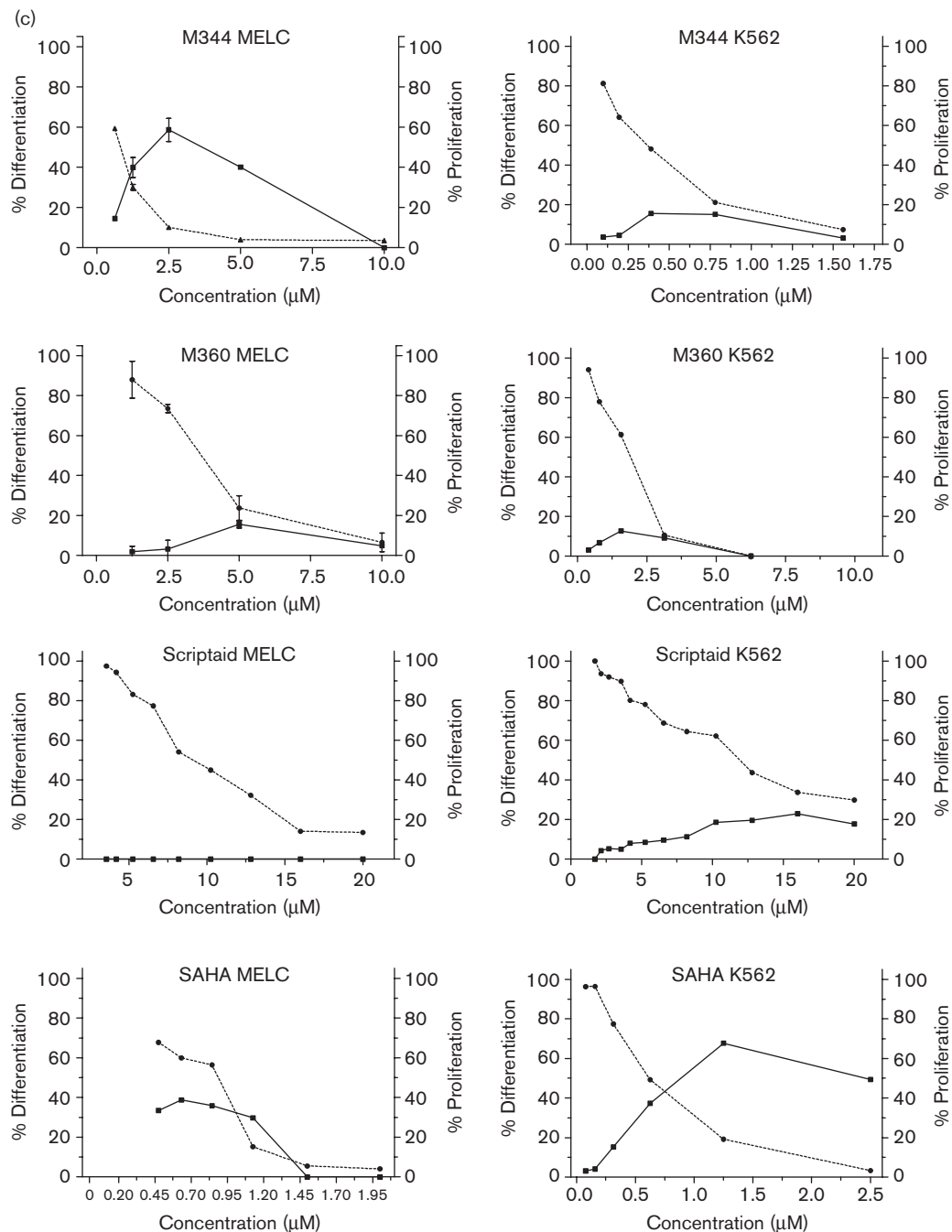
Fig. 2 Continued



of the biphenylalanine BIP1 that is as potent as TSA in the human erythroleukemic K562 cell line. For most compounds the cellular activity was observed in the same concentration range as for enzyme-inhibitory activity, which supports the use of HDAC *in vitro* assays as screening tools for drug discovery. Therefore, we and others have also developed homogeneous assays for the

determination of HDAC activity [39,40]. The lack of correlation with MS275 has been reported by another group before [31]. As MS275 shows *in vitro* enzyme inhibition in some papers in the low micromolar range which we and others could not reproduce, its activity might depend strongly on the enzyme preparation.

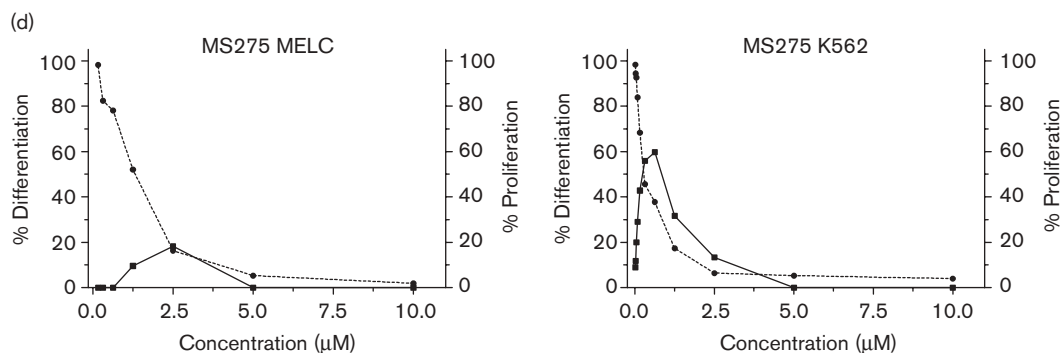
Fig. 2 Continued



The patterns of induction of differentiation are more complex as the maximum levels of terminal cell differentiation achieved in the surviving cell population range from 0 to 68% among the various inhibitors. Not only are there differences between structurally very similar inhibitors in one cell type (e.g. M344 versus M360 in Friend cells), but some of the inhibitors have entirely different profiles of activity in the two erythroleukemic cell lines (e.g. M344 or MS275). The reasons

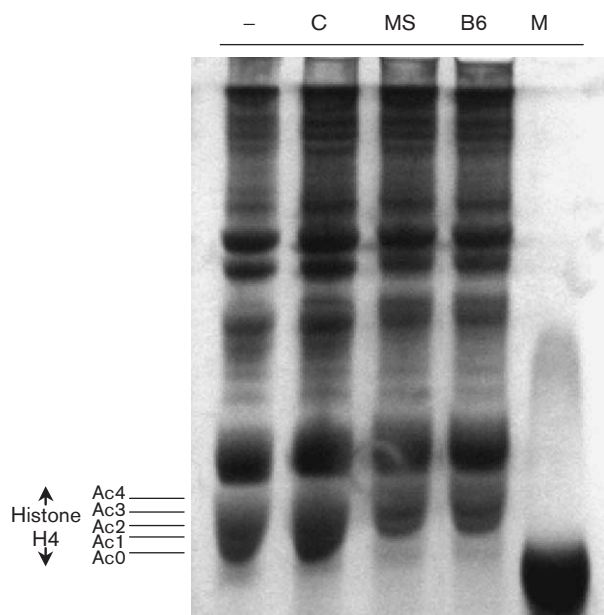
for these observations remain unclear. A HDAC subtype selectivity of the various inhibitors is a possible explanation, and docking experiments with MD85, M344 and M360 in a homology model of human HDAC1 [36] supported the possibility of such a selectivity. Consequently, we have identified a difference in selectivity regarding HDAC1 and HDAC6 for M344 and M360 [30]. In this group of trichostatin analogs, stronger inhibition of HDAC6 is associated with stronger anti-

Fig. 2 Continued



Induction of terminal cell differentiation (squares and solid lines) and inhibition of proliferation (circles and dotted lines) by HDAC inhibitors in Friend (MELC) and K562 cells. The graphs for the MELC results for TSA, MD85, M344, M360 [16], BIP1 and SAHA [13] are taken from our previous work. Reproduced with permission. © 1999 and 2002 American Chemical Society.

Fig. 3



Acetylation status of histone H4 in K562 cells treated with BIP6 (B6, 1.56 μM) or MS275 (MS, 0.625 μM) for 12 h. A '-' represents untreated cells, C stands for the solvent control treated with 0.1% DMSO. Ac0-4: number of acetylated lysines. M: marker (cytochrome *c*). See Materials and methods for details.

proliferative activity and more potent induction of Friend cell differentiation (see Fig. 2). Additionally, the HDAC6-selective M344 is also a better inducer of γ -globin expression [41] and leads to a more pronounced radiosensitization of cancer cells [42]. On the other hand, in the biaryl series, the weak inducer of differentiation (BIP2) is selective towards HDAC6, whereas the potent inducer (BIP6) is selective for HDAC3. TSA is unselective among the three subtypes, whereas TPX inhibits

Table 2 HDAC subtype selectivity

Inhibitor	FLAG-HDAC1 (IC ₅₀ , nM ^a)	FLAG-HDAC3 (IC ₅₀ , nM ^a)	FLAG-HDAC6 (IC ₅₀ , nM ^a)
BIP2	400	310	100
BIP6	2540	790	2500
MD85	390	390	330
M344	250 ^b	480	90 ^b
M360	110 ^b	410	170 ^b
TSA ^c	2-6	2	9
TPX ^c	0.1-10	100	360
Butyrate ^c	0.1 mM	0.1 mM	6 mM

^aMean values of half-maximal inhibitory concentrations (IC₅₀) were generated from testing four to six concentrations in duplicate.

^bData taken from [30].

^cData taken from [43,44].

mainly HDAC1. The weak inhibitor butyrate leads to stronger inhibition of HDAC1 and HDAC3 (Table 2). Larger numbers of inhibitors have to be tested on more subtypes to further elucidate possible links between subtype selectivity and a profile of biological activity.

A differential downstream response to the gene activation induced by distinct inhibitors in the two cell lines may explain the varying results between Friend and K562 cells. We have shown before that, for example, BIP1 [13] or M344 [16] induce histone hyperacetylation at relevant concentrations, but an interaction with additional targets is also possible. As an example, BIP1 inhibits cell proliferation in the K562 cell line at 7-fold lower concentrations as compared to the inhibition in the enzyme assay.

Conclusion

Structural variations of a biphenylalanine HDAC inhibitor BIP1 have identified its 3'-methoxy analog BIP6 as a very good inducer of terminal cell differentiation in the K562 human erythroleukemic cell line. Compound BIP1 has an anti-proliferative potency in K562 cells that reaches that

of the benchmark TSA. Based on these results, further modifications of its structure besides 3'- or 4'-substitution are planned. The first comprehensive comparison of standard HDAC inhibitors in two cellular differentiation models further reveals striking differences among the various inhibitors and in the action of some of the inhibitors on different cell lines. This will stimulate mechanistic studies aimed at the elucidation of the molecular basis of action of these promising drug candidates. So far there is no clear link between HDAC subtype selectivity and good induction of differentiation.

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