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Chemistry

Potent cytodifferentiating agents related to hexamethylenebisacetamide

(hydroxamic acids/erythroleukemia/colon carcinoma/HL-60/protein kinase C)

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ABSTRACT **Bishydroxamic acids are effective inducers of differentiation in murine erythroleukemia cells. Flexible analogs of suberic acid bisdimethylamide are ≈100 times as active as the parent compound or hexamethylenebisacetamide. They also induce differentiation of human promyelocytic leukemia cells (HL-60) and a subclone of human colon carcinoma cells (HT-29-U4). Some rigid bishydroxamic acids with benzene rings in the spacers are even more active toward murine erythroleukemia cells but show curious biological differences. In contrast to the flexible molecules, those with benzene spacers show poor activity toward HL-60 cells; they also have different geometric requirements, and they are not additive with hexamethylenebisacetamide in their effect. It is likely that rigid bishydroxamic acids, with a benzene ring spacer, induce differentiation by a different mechanism in spite of their chemical resemblance to the flexible bisamide and bishydroxamic acid inducers.**

Since the discovery by Friend *et al.* (1) that murine erythroleukemia cells (MELC) can be induced to differentiate by the addition of rather high concentrations of dimethyl sulfoxide (DMSO) to the culture medium, we have been pursuing a number of research lines to expand on this finding. Our first work showed that many other polar solvents are also effective and that amides in particular are more effective than is DMSO (2–5). However, the concentrations required to induce a significant fraction of the cells to differentiate were still quite high.

The high concentrations required could mean many things, but they might indicate the need to bind more than one molecule of polar solvent to two (or more) receptor sites. If this were true, and if the receptor sites were close, more effective differentiating agents could result from linking two polar solvent-like molecules together by means of a spacer. There was no other evidence for this but, in fact, linking two amide groups with six methylene groups affords three much-improved differentiating agents, hexamethylenebisacetamide (HMBA; Fig. 1, compound **1**), suberic acid bisdimethylamide (compound **2**) and suberic acid bismethylamide (compound **3**). The six-carbon spacer chain is the best in both types of amides, nitrogen linked and carbon linked, but good activity is also seen with five- and seven-carbon spacers (3, 4).

Since that discovery, HMBA has been extensively investigated. It has been shown to induce the differentiation of many other tumor cell lines (6). It has even had some success in clinical trials against certain cancers (7, 8). It works not by killing the cancer cells but by inducing them to differentiate and to express characteristics of the normal nontransformed counterpart. This is a promising approach to cancer therapy, potentially without many of the disadvantages of cytotoxic agents. However, HMBA is not an ideal drug prospect.

Rapid deacetylation of the compound, and rapid renal clearance, mean that the biological lifetime of HMBA is short (7, 9). Since induction of differentiation in MELC requires relatively high drug concentrations, of the order of 5 mM, and exposure to the agent over a relatively prolonged period, quite high doses of HMBA are needed to achieve blood levels with potential clinical effectiveness. In none of the patients studied to date were blood levels achieved much in excess of 1 mM (7–9). Clearly a more potent compound is needed. Some improvement in potency has been achieved with selected trisamides, but the change is not large (5). When bisamides are made with additional polar groups in the middle of a longer chain, increases in activity are seen, but they are at most 10-fold (5).

From the extensive structure–activity relations developed in testing many compounds, it seemed likely that the two polar amide groups were binding to two receptor-like sites in the target cell. Amide binding might involve coordination to a bound metal ion, or it might involve simple hydrogen bonding to the receptor site. In either case, it seemed possible that a hydroxamic acid group would bind more strongly. Provided that the geometry is correct, the extra hydroxyl group of a hydroxamic acid might participate in additional hydrogen bonds; furthermore, hydroxamic acids are much superior to amides as potential metal ion ligands. Appropriate bishydroxamic acids have indeed proven to be potent cytodifferentiating agents in the MELC assay.

METHODS

Materials. We have described compounds **1**, **2**, and **3** (Fig. 1) previously (3, 4). The known compounds **6**, **8**, **9**, **10**, and **11** were prepared from the corresponding acid chlorides with hydroxylamine and purified by crystallization from methanol or acetone. They showed the expected H NMR and mass spectra and elemental analysis values. Compounds **4**, **5**, **7**, **12–15**, **19**, and **20** (Figs. 1 and 2) were prepared similarly and also fully purified and characterized. Compound **16** was prepared from *O*-benzylacethydroxamic acid by alkylation with hexamethylene dibromide, then removal of the benzyl groups by hydrogenation. Compounds **17** and **18** were prepared as described (10).

Cells. MELC 745A-DS19 were maintained as described previously (4). HL-60 human leukemia cells, derived from peripheral blood leukocytes of a patient with acute promyelocytic leukemia (11), were maintained as described elsewhere (12). The human colon carcinoma cell line U4, a

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Abbreviations: MELC, murine erythroleukemia cells; DMSO, dimethyl sulfoxide; HMBA, hexamethylenebisacetamide; NBT, nitro blue tetrazolium; PKC, protein kinase C.

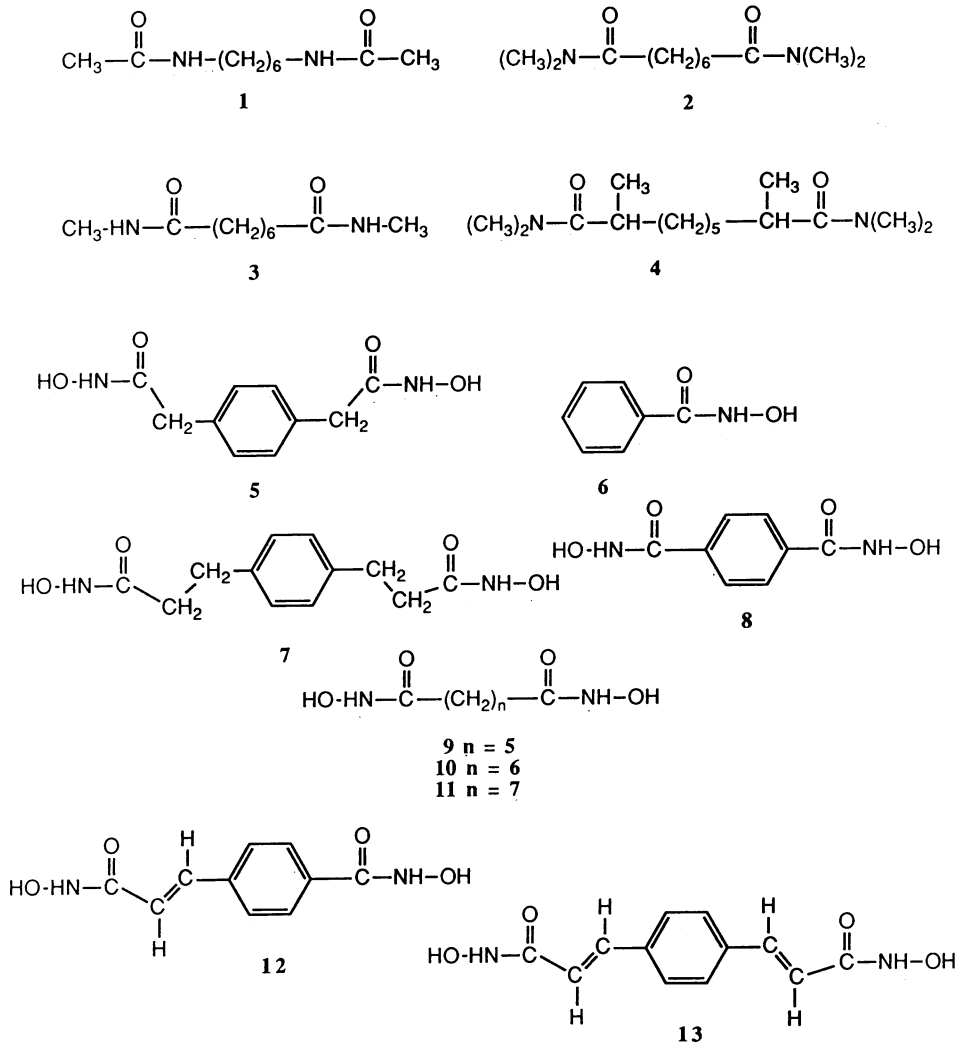

FIG. 1. Compounds active for induced differentiation of MELC.

subclone of HT-29, was maintained as described by Hafez *et al.* (13).

Assays for Differentiation. Induced differentiation of MELC was assayed by determining the proportion of cells that accumulated hemoglobin (benzidine-reactive cells) (2). Induced differentiation of HL-60 cells was assayed by determining the proportion of cells that developed the capacity to reduce nitroblue tetrazolium (NBT) (12). In studies with MELC or with HL-60 cells, cell density was determined using a Coulter Counter (5). Inducer-mediated differentiation of HT-29 cells was evaluated by determining the proportion of cells that had lost the expression of a malignancy marker

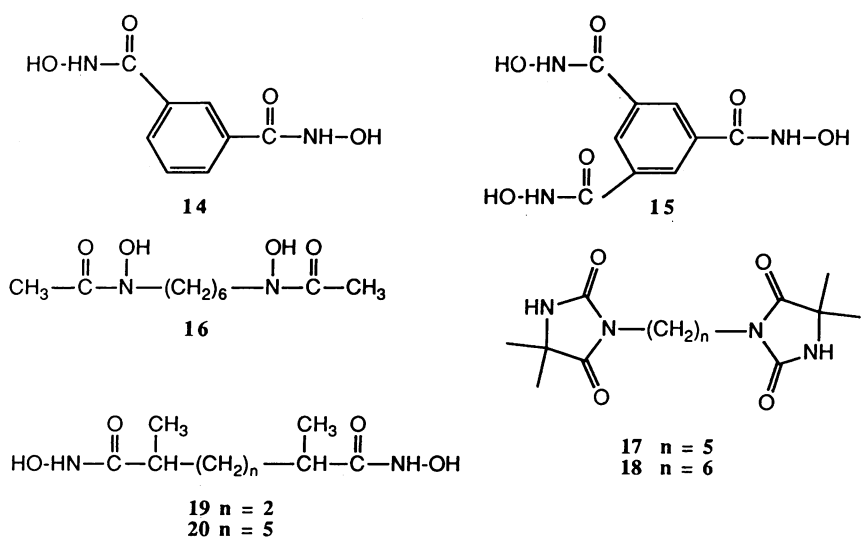

FIG. 2. Some compounds inactive in the MELC differentiation assay.

(14). This was ascertained with a monoclonal antibody, 29-15, that binds to a cell surface epitope expressed on the majority of invasive colon carcinomas of the Dukes' B_2, C, or D histopathology classes but not on benign adenomas (14). Loss of antigen expression correlates with loss of anchorage-independent growth (14) and loss of oncogenicity (15). In the U4 subclone of HT-29 cells, 75% of cells constitutively display the 29-15 epitope (see Table 2).

To test the efficacy of compounds related to HMBA as inducers of differentiation, each compound was dissolved in culture medium (compounds **1–3, 6, 16,** and **19**), DMSO (compounds **4, 5, 7, 8,** and **9–15**), or ethyl alcohol (compounds **17** and **18**) and added to the final concentration indicated. The final concentration of DMSO in culture medium did not exceed 7.0 mM for any of the active compounds tested. This is a concentration far below that at which DMSO is itself active as a differentiation inducer (optimum, 280 mM). At concentrations of DMSO of 70 mM or below there is no detectable effect on cell growth or differentiation of MELC and no detectable additive effect with suboptimal concentrations of HMBA (see Table 3). Neither of the compounds requiring ethyl alcohol for solution (compounds **17** and **18**) proved to be active as an inducer of differentiation. Ethyl alcohol itself, at concentrations up to 0.1%, had no effect on cell growth or differentiation of the cell lines tested, and this concentration exceeded that required for dissolution of these compounds.

Additivity of Pairs of Compounds. The differentiation-inducing additivity of several compounds with HMBA was tested by adding each compound to a MELC culture, over a range of suboptimal concentrations, both separately and together with suboptimal concentrations of HMBA. The effectiveness of these combinations was assayed by determining the proportion of benzidine-reactive cells after 5 days of culture.

RESULTS AND DISCUSSION

MELC Differentiation Is Strongly Promoted by Hydroxamic Acids. As shown in Table 1, suberic bishydroxamic acid (Fig. 1, compound **10**) is >100 times as effective in inducing MELC differentiation as is HMBA (compound **1**) or suberic bisdimethylamide (compound **2**). Similar but diminished activity is seen with the analogous five- and seven-methylene hydroxamic acids (compounds **9** and **11**), just as in the bisamide series. Again as in the bisamide series, activity falls off with much shorter or much longer chains. The dimeric bishydroxamic acid (compound **10**) is considerably more active than is its half molecule, butyrohydroxamic acid. The detailed geometry is important; the bishydroxamic acid (Fig. 2, compound **16**) related to HMBA is not active. Furthermore, the methyl substituents in bishydroxamic acids (compounds **19** and **20**) block activity, but those in the related bisamide (compound **4**) do not.

Compounds with very different properties are produced when some flexibility is removed by incorporating a benzene ring into the spacer region. Terephthalic bishydroxamic acid (compound **8**) is almost as active as is compound **10**. It is more active than simple benzohydroxamic acid (compound **6**), indicating that both hydroxamic acid groups participate in promoting differentiation. The *m*-bishydroxamic acid (compound **14**) is not active, so adding an additional group to compound **6** in the wrong position does not help, apparently interfering with insertion of the phenyl ring into what must be a hydrophobic region. As expected from this, the 1,3,5-trishydroxamic acid (compound **15**) is not active either.

At first sight, these results suggest that flexible inducers, such as compound **10**, bind in an extended conformation mimicking the geometry of compound **8**. However, other evidence (discussed below) suggests strongly that the flexible compound **10** and the rigid compound **8** do not act at the same sites. If this is true, the geometric preferences of the rigid benzene-linked series need not reflect the preferred geometries of the flexible molecules.

Table 1. Induction of differentiation of MELC

Compound	Conc., μM	Benzidine-reactive cells, %
None	—	<1 ± 1
1	5,000	>95
2	5,000	>95
3	5,000	>95
4	1,250	80
5	300	20
6	160	15
7	120	25
8	80	90
9	40	90
10	30	>95
11	20	75
12	5	60
13	4	65
14	40–2,500	0
15	20–60	0
16	10–2,500	0
17	120–10,000	0
18	120–4,000	0
19	20–2,500	0
20	20–2,500	0

Concentrations (Conc.) are reported as optimal (single values) or as range tested. Optimal concentration, final concentration in culture medium at which the highest proportion of differentiated cells as assayed by percentage benzidine-reactive cells was observed; range tested, given for compounds that did not induce differentiation, with the maximum being the value at which substantial inhibition of cell growth occurred.

Adding an extra methylene group to compound **8** between the benzene ring and each hydroxamic acid group, to produce the bishydroxamic acid derivative of *p*-phenylenebisacetic acid (compound **5**), causes substantial loss of activity. With two more carbons the activity is restored; that is, the bishydroxamic acid derived from *p*-phenylenebispropionic acid (compound **7**) is somewhat more active, measured as optimal concentration. The corresponding unsaturated derivative (compound **13**) derived from *p*-phenylenebisacrylic acid is the most active of this series. The shorter compound **12** is also quite active, even though it has the same formal length as the less-active compound **5**.

These at first curious results are sensible if there is a significant geometric requirement in order to achieve cooperative double binding of the two hydroxamic acid groups with their intervening benzene ring connection. In compound **8** the two carbonyl carbons lie in a straight line with the benzene ring axis; in compound **5** both lie at an angle of 109.5° relative to that axis, while in compound **7** they can again lie on a straight line, and in compounds **12** and **13** they must.

The effectiveness of compounds **8, 12,** and **13** suggests that the receptor is to some degree flexible, such that the two binding sites can accommodate hydroxamic acid groups separated by somewhat different length spacers. However, there are still serious structural and geometric requirements. In contrast to the group of amides, in which the fully methylated compound **2** is active, the N-methyl derivative of butyrohydroxamic acid is considerably less active than the unmethylated compound. Similarly, N-methylation or O-methylation of the bishydroxamic acids greatly diminishes their activity. Furthermore, as mentioned above, the bishydroxamic acid (compound **16**) related to HMBA is inactive.

Thus, not all bishydroxamic acids of the same length are equally effective.

The Rigid Bishydroxamic Acids and the Flexible Analogues Have Different Biological Properties. As shown in Tables 1 and 2, the several compounds assayed for inducer activity against each of the three cell lines (MELC, HL-60, and HT-29) appear to fall into three classes: those that induce all three cell lines (compounds **1–4** and **10**), those that induce only MELC (such as compound **13**), and those that induce only HL-60 cells (compounds **17** and **19**). Flexible molecules such as compounds **1–4** and **10** are effective in all three systems. Rigid bishydroxamic acids (compounds **8** and **13**) induce MELC but not HL-60 cells. The bishydantoin (compound **17**) induces HL-60 cells but not MELC.

The difference between the two groups of bishydroxamic acids might, in principle, be simply a matter of spacer thickness. If the region between two receptor sites in MELC could accommodate a benzene ring, but that region in HL-60 cells were narrower and could not, then the flexible molecules might be active in HL-60 cells not because of their flexibility but because of their slimness. However, this idea does not fully explain other differences between the two series.

The optimal length is different in the two series of bishydroxamic acids. The most effective rigid compound, **13**, has 10 carbons in the spacer, 8 of them in a line. The length is greater than that of the optimal 6-methylene-group spacer in the flexible series. More striking are the results of studies addressing the additive effects of HMBA and other compounds (Table 3). Mixtures of a suboptimal concentration of HMBA and a range of suboptimal concentrations of one of the additional compounds were tested for their ability to induce differentiation of MELC. As might be expected for compounds that work in the same way, HMBA (compound **1**) and compounds **2** or **3** are additive in their effects. This is also true for HMBA with compound **10**, so the flexible bishydroxamic acid and the flexible bisamide can replace each other at appropriate concentrations. However, the rigid bishydroxamic acids, compounds **8**, **12**, and **13**, are not additive with HMBA.

This is a striking and surprising result; it indicates that the two classes of compounds induce differentiation in two different ways that are mutually exclusive. For instance, they may act at two different groups of sites on the same receptor molecule, each inducing a different conformational change, or the sites for the two classes could be on different target molecules. This idea of two different receptor loci could also explain the difference in chain length optima. Further evidence bearing on this point comes from studies on protein kinase activation.

Table 2. Induction of differentiation of HL-60 and HT-29 cells

Compound	HL-60 cells		HT-29 cells	
	Conc., μM	NBT positive, %	Conc., μM	Malignancy marker, %
None	—	<2 ± 2	—	75 ± 7
1	3,000	35	2,000	6
2	3,000	70	5,000	11
3	3,000	50	5,000	2
4	1,250	62	1,250	9
8	20–2,500	0	125	19
10	10	8	60	3
12	10	5	ND	ND
13	4–125	0	1–200	65
17	2,500	90	ND	ND
19	2,500	8	310–10,000	73
20	20–2,500	0	31–1,000	73

See legend to Table 1. ND, not determined.

Table 3. Additivity of compounds in combination with HMBA as inducers of differentiation of MELC

Compound	Conc., μM	Additive, nonadditive
2	1000–3000	Additive
3	1000–3000	Additive
8	30–80	Nonadditive
10	5–20	Additive
12	3–7	Nonadditive
13	2–4	Nonadditive

HMBA was added to the culture medium to a final concentration of 2000 μM. At this concentration of HMBA alone, the proportion of benzidine-reactive MELC induced after 5 days was 60% (range, 55–65%). Assay for benzidine reactive cells was performed after 5 days of culture. Over the range of concentrations used, each compound was consistently additive or nonadditive with HMBA. At least four different concentrations, within the range indicated, were tested for each compound. Each study was performed at least twice. A compound is denoted "additive" in combination with HMBA if the observed percent benzidine-reactive cells from exposure to the mixtures was equal to or greater than the sum of the percent benzidine-reactive cells from exposure to the same concentrations of inducers added separately. A compound is denoted nonadditive if induction is less than predicted from exposure to the same concentrations of inducers added separately.

Melloni *et al.* (16, 17) report that HMBA causes transient translocation of protein kinase C (PKC) activity to the plasma membrane followed by a progressive decrease in the activity of the β isozyme of PKC (PKCβ) in MELC. MELC that respond more rapidly to HMBA also display higher levels of PKCβ activity (17). We have recently found that HMBA may have a direct effect on PKC activity. Included in the enzyme assay, HMBA causes an increase in the activity of one of the minor isozymes of PKC, with little or no effect on the activity of the more abundant α isozyme, PKCα. The effect on PKC is maximal at the concentration of HMBA (5000 μM) optimal for inducing MELC differentiation. In view of the well-established role of PKC in cellular differentiation (18), this activation of a PKC isozyme may be a clue to the mechanism by which HMBA induces differentiation. The flexible bishydroxamic acid, compound **10**, also activates PKC at its differentiation-inducing concentration, but a rigid bishydroxamic acid (compound **13**) does not. These findings, taken together with the present data, support the idea that compound **13** has a different site of action than the flexible inducers (compounds **1–4** and **9–11**). The conclusion that two such similar classes of molecules can induce differentiation by apparently different mechanisms is certainly unexpected but strongly supported by our observations. The nature of this difference remains to be elucidated.

CONCLUSIONS

It seems clear that the group of flexible bishydroxamic acids are promising for further study as inducers of differentiation of transformed cells. Compound **10** seems to be a more effective analog of HMBA, and HMBA has shown effectiveness in inducing differentiation in transformed cell lines of diverse origins (6) and to a limited extent even in clinical trials (7, 8). Although other structures continue to be examined, the activity already seen in these bishydroxamic acids is of great interest. If they induce cytodifferentiation of other cancer cells at similar low concentrations, and show no untoward side effects, they could be of clinical importance.

The precise mode of action of HMBA, and of these additional more-powerful analogues, remains to be elucidated. The flexible molecules activate one of the isozymes of

PKC *in vitro* (15, 16), but we do not yet know how this activation occurs or its role in cellular differentiation. The mode of action of rigid bishydroxamic acids, such as compound **13**, remains more of a mystery, as is the apparent cell type selectivity of compounds **13** and **17**.

With the availability of these highly active compounds, it should prove easier to study their modes of action. In addition to their clinical potential, the bishydroxamic acids and the interesting differences among structural types that they display may prove to be useful tools in the study of the biology of cellular growth and differentiation.

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