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Second generation hybrid polar compounds are potent inducers of transformed cell differentiation

(cell cycle/pRB/p21/differentiation therapy/c-*myb*)

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ABSTRACT **Hybrid polar compounds, of which hexamethylenebisacetamide (HMBA) is the prototype, are potent inducers of differentiation of murine erythroleukemia (MEL) cells and a wide variety of other transformed cells. HMBA has been shown to induce differentiation of neoplastic cells in patients, but is not an adequate therapeutic agent because of dose-limiting toxicity. We report on a group of three potent second generation hybrid polar compounds, diethyl bis-(pentamethylene-*N*,*N*-dimethylcarboxamide)malonate (EMBA), suberoylanilide hydroxamic acid (SAHA), and *m*-carboxycinnamic acid bis-hydroxamide (CBHA) with optimal concentrations for inducing MEL cells of 0.4 mM, 2 μM, and 4 μM, respectively, compared to 5 mM for HMBA. All three agents induce accumulation of underphosphorylated pRB; increased levels of p21 protein, a prolongation of the initial G_1 phase of the cell cycle; and accumulation of hemoglobin. However, based upon their effective concentrations, the cross-resistance or sensitivity of an HMBA-resistant MEL cell variant, and differences in c-*myb* expression during induction, these differentiation-inducing hybrid polar compounds can be grouped into two subsets, HMBA/EMBA and SAHA/CBHA. This classification may prove of value in selecting and planning prospective preclinical and clinical studies toward the treatment of cancer by differentiation therapy.**

In previous reports (1–4), we have identified a group of hybrid polar compounds that are inducers of differentiation of a variety of transformed cells. Hexamethylenebisacetamide (HMBA) is the prototype of these compounds and has been used to investigate the mechanism of action (5) and to evaluate the therapeutic efficacy of hybrid polar compounds in the control of cancer by differentiation therapy (6). In this regard, a phase II clinical trial was conducted using HMBA to treat patients with myelodysplastic syndrome or acute myelogenous leukemia (6). Of 28 patients, 9 achieved a complete or partial remission lasting from 1 to 16 months. These clinical studies also provided direct evidence that HMBA induces the differentiation of transformed cells in patients. In four separate courses of treatment with HMBA, a patient with myelodysplastic syndrome and the monosomy 7 karyotype marking the malignant clone of bone marrow blast cells, achieved peripheral blood granulocyte counts that approached normal levels. More than 80% of these morphologically mature granulocytes carried the chromosomal marker characteristic of the malignant clone, providing clear evidence that the clinical response was consequent to maturation within the malignant clone. These phase II studies, however, also showed that HMBA is not a satisfactory therapeutic agent, owing to severe thrombocytopenia, which limits the amount of drug that can be administered. Furthermore, because continuous exposure is required for induction, and the biological half-life of HMBA in the patient is very short (about 1.5 hr), HMBA must be administered by continuous infusion to maintain a clinical effect. These factors, taken together, have inspired a continued search for additional hybrid polar compounds effective at significantly lower concentration and that might avoid the clinical side effects that limit the use of HMBA.

In this study we report on three new "second generation" hybrid polar agents that, on a molar basis, are up to 2000-fold more active than HMBA as inducers of differentiation of murine erythroleukemia (MEL) cells. MEL cells have proved of value in predicting the effectiveness of hybrid polar compounds in inducing the differentiation of other transformed cell lines and fresh human tumor cells (for review see ref. 7). The three new compounds characterized are diethyl bis-(pentamethylene-*N*,*N*-dimethylcarboxamide)malonate (EMBA), suberoylanilide hydroxamic acid (SAHA), and *m*-carboxycinnamic acid bis-hydroxamide (CBHA). These compounds appear to be representative of at least two sets of closely related alternative compounds, distinguished by shared biological properties. EMBA, a carboxyamide compound, is most similar in its structure and biological properties to the acetamide HMBA, although it is active at about 1/10th the molarity. SAHA and CBHA are hydroxamic acids that share certain biologic properties and an approximately 2000-fold greater potency on a molar basis than HMBA.

MATERIALS AND METHODS

Cell Culture. MEL cells (DS19) (23, 24), the vincristine-resistant variant, VCR-C15 (8, 9), and the HMBA-resistant R1 cells (10) were maintained in minimal essential medium containing 10% fetal calf serum. Cultures were initiated from cells in logarithmic growth at 10^5 cells/ml. Cell density, benzidine-reactivity, and commitment to terminal differentiation were assayed as described (11). Cells were synchronized with respect to cell cycle by elutriation (12).

Hybrid Polar Compounds. HMBA (1) was obtained from Aldrich. We have described (2) the preparation and characterization of EMBA (Table 1). SAHA was prepared from suberoyl dichloride by reaction with one equivalent of aniline and then hydrolysis to form the monoamide monoacid. The carboxyl group was then activated with carbonyl diimidazole, and reaction with hydroxylamine formed SAHA. CBHA was prepared from the bis acid chloride of *m*-carboxycinnamic acid and hydroxylamine. All compounds were purified by chromatography, and had the expected proton NMR and mass spectra.

Immunoblot Analysis. MEL cells grown under the conditions described for each experiment were recovered by cen-

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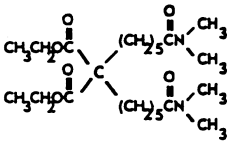
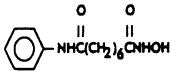
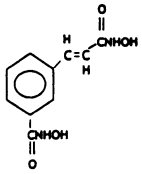
Abbreviations: HMBA, hexamethylenebisacetamide; MEL, murine erythroleukemia; EMBA, diethyl bis-(pentamethylene-*N*,*N*-dimethylcarboxamide)malonate; SAHA, suberoylanilide hydroxamic acid; CBHA, *m*-carboxycinnamic acid bis-hydroxamine.

Table 1. Differentiation activity of second generation hybrid polar agents in MEL cells (DS19)

Agent		Optimal concentration, μM	Benzidine positivity, %
	HMBA	5000	90–95
	EMBA	400	90–95
	SAHA	2	80–85
	CBHA	4	70–75

trifugation at 800 × *g* for 10 min and total cell extracts were prepared by lysing 10^7 cells in 250 μl of SDS/polyacrylamide sample buffer, boiled for 5 min, sheared through a 26-gauge needle, centrifuged to remove debris, and applied to SDS/10% or SDS/15% polyacrylamide gels. Electrophoretic transfers of proteins from the SDS/polyacrylamide gels were carried out as described by Towbin *et al.* (13) and modified by Burnette (14). The antibody binding was detected using enhanced chemiluminescence (Amersham). Antibodies used in the immunoblot analysis were obtained from the following sources: anti-pRB antibody (no. 14001) was obtained from PharMingen; anti-p21 antibody (M-19) was obtained from Santa Cruz Biotechnology; and anti-myb type I antibody (catalogue no. 05–175) was obtained from Upstate Biotechnology (Lake Placid, NY). Filters were exposed to Kodak Biomax film and the resulting film scanned using an LKB 2202 Ultrascan Densitometer.

RESULTS

Hybrid Polar Compounds. Three second generation hybrid polar compounds EMBA, SAHA, and CBHA (Table 1) were selected for further structure/function study from our laboratories' portfolio of more than 600 novel synthesized hybrid polar compounds, based upon their potency as inducers of MEL cell differentiation and structural features that might impart distinct and improved biological (and perhaps clinical) characteristics. EMBA was synthesized in a search for novel bisamides that would be more potent inducers of differentiation than the bisacetamide, HMBA, and the equipotent biscarboxamide (3). EMBA is distinctly more active (optimal concentration 0.4 mM) (Fig. 1*B*) than HMBA (optimal concentration 5 mM) (Fig. 1*A*), and like HMBA it induces a high proportion of MEL cells (DS19) to differentiate (Table 1) and HL-60 cells as well (data not shown). EMBA displays a broad concentration range of activity without significant cytotoxicity, as determined by cell proliferation (Fig. 1*B*) and trypan blue exclusion (data not shown).

As a class, the group of hydroxamic acid derivatives are significantly more potent than either HMBA or EMBA (3). We have previously described the preparation and properties of the prototypic bishydroxamic acid, suberoyl bishydroxamic acid (SBHA) (3, 4), which, at its optimal effective concentration of 30 μM, is 160-fold more active than HMBA. SAHA was designed to explore the possibility that both ends of the inducer need not carry the hydroxamic group and that activity may be

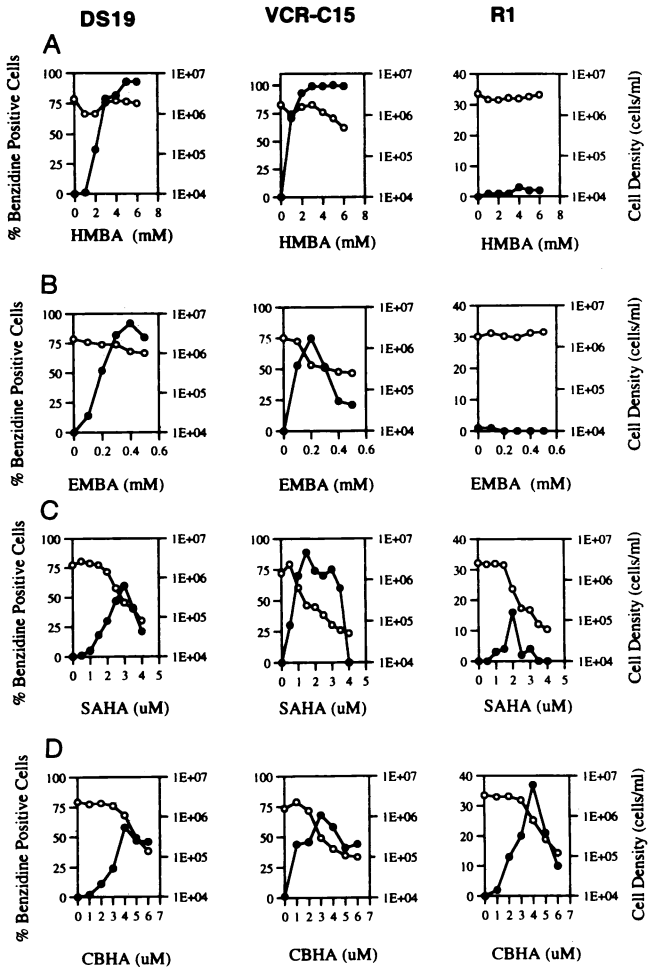


FIG. 1. Determination of optimal inducing concentration of HMBA (*A*), EMBA (*B*), SAHA (*C*), and CBHA (*D*) for induction of MEL cell differentiation. MEL cells were exposed to increasing concentrations of compound for 5 days at which time the cell density (○) and benzidine positivity (●) were determined.

enhanced if one end has a hydrophobic residue. As shown in Table 1, this is the case; SAHA, active at 2 μM, is more than 15-fold more active than SBHA (Fig. 1*C*). CBHA is yet another relative of SBHA in which, in this case, the apolar linker between the two hydroxamate groups is designed to be more rigid. This modification also retains the improved activity characteristic of the class of hydroxamic acid derivatives (Table 1). Both SAHA and CBHA share a relatively narrow effective dose range and an abrupt fall in viable cell count across that range (Fig. 1 *C* and *D*).

Variant MEL cell lines with either enhanced or decreased sensitivity to hybrid polar differentiation-inducing compounds (8, 10) have previously been used in the biological evaluation of these agents. HMBA and the three second generation agents were assayed for their activities as inducers of differentiation with two of these variant cell lines derived from the parental MEL cell, DS19: VCR-C15 (8), a cell line with increased sensitivity to HMBA and R1, a cell line selected for resistance to HMBA (10). DS19, VCR-C15, and R1 cells were cultured with each compound across a range of concentrations to determine the optimal dose for induction of differentiation and the dose effect on cell growth. VCR-C15 cells are sensitive to all three agents at a somewhat lower concentration than are the parental DS19 cells, a property these new agents share with HMBA (Fig. 1). Of the three new agents, R1 cells display distinct cross-resistance to EMBA, whereas these HMBA-resistant cells are more sensitive to induction by SAHA and

CBHA. HMBA and EMBA were also distinguished from SAHA and CBHA in their effects on cell proliferation of DS19, VCR-C15, and R1 at the concentrations optimal for inducing differentiation of these cell lines (Fig. 1). The effect of EMBA on cell proliferation is similar to that of HMBA. SAHA and CBHA profoundly inhibit cell proliferation at the optimal inducing concentrations. Thus, the effect on cell proliferation and the responsiveness of R1 cells appears to establish at least two subsets of hybrid polar compounds—those which profoundly inhibit cell proliferation and are more active as inducers of differentiation in the R1 cell variant (SAHA and CBHA) and those that do not initially inhibit cell proliferation and are much less active as inducers of the R1 cell variant (HMBA and EMBA).

Effect of Cytodifferentiation Agents on Cell Cycle Progression. We have previously shown that HMBA-induced differentiation of MEL cells is associated with a transient prolongation of the first G_1, which follows the passage of cells through a cell cycle in the presence of the inducer (15). To determine whether this response is a general response or limited to HMBA-induced differentiation, a G_1-enriched population of DS19 MEL cells was prepared by elutriation and then cultured with no addition or with the addition of 5 mM of HMBA, 0.4 mM of EMBA, 2 μm of SAHA, or 4 μM of CBHA (Fig. 2). Cells cultured without inducer have completed the first cell cycle and approximately 40% of the cells have already entered the second S phase within 12 hr after onset of culture. Less than 20% of these untreated cells are in the G_1 phase after 15 hr. Cells exposed to HMBA or EMBA were predominately (>80%) in G_1 at 12 hr, and remained in G_1 through 15 hr of culture, by which time some cells were beginning to move into S. Cells cultured with SAHA and CBHA appear to traverse the initial cell cycle at a slower rate than those cells cultured with no addition, HMBA, or EMBA. These cells also arrest in the subsequent G_1 phase as evidenced by the increasing proportion of cells in G_1 from 9 to 15 hr of culture.

Effect of Cytodifferentiation Agents on Proteins Regulating Cell Cycle Progression. HMBA-induced differentiation of MEL cells is accompanied by an increase in the level of inhibitors of G_1 to S transition, including the underphosphorylated form of the retinoblastoma gene product (pRB) (16, 17) and the cyclin-dependent kinase inhibitor, p21 (ref. 18, F. Civoli, unpublished observation). We asked whether the induction of differentiation by EMBA, SAHA, and CBHA is also associated with changes in the levels of pRB and p21 (Fig. 3). DS19 cells were cultured with HMBA, EMBA, SAHA, or CBHA and protein extracts prepared at 48 hr. Each of the inducers caused both an increase in the total amount of pRB protein and an increase in the underphosphorylated form of the protein. The level of p21 protein was also determined in DS19 cells at 48 hr of culture with HMBA, EMBA, SAHA, or CBHA (Fig. 3). Each agent induced an increase in the level of p21 protein.

We and others have previously demonstrated that down-regulation of the positive G_1- to S-phase regulator, c-*myb*, is associated with and apparently critical for HMBA-induced differentiation of MEL cells (19–21), so that the expression of c-*myb* protein was also studied during induction with EMBA, SAHA, and CBHA (Fig. 3). Following 48 hr of DS19 cell culture with HMBA and EMBA, the level of c-*myb* protein decreased compared to control. In contrast, SAHA and CBHA initiated differentiation without decreasing c-*myb* protein expression, consistent with the proposal that there are at least two functional subsets within the family of hybrid polar inducers of differentiation.

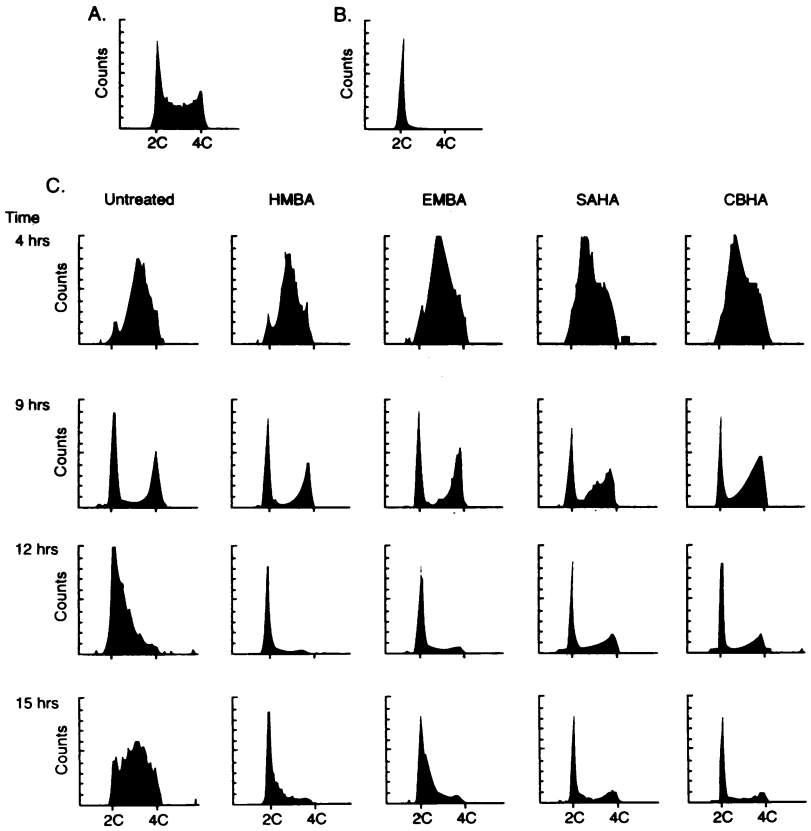


FIG. 2. Effect of HMBA, EMBA, SAHA, and CBHA on cell cycle progression. The DNA content was determined by flow cytometry after nuclear DNA had been stained with propidium iodide. G_1 cells correspond to 2C and G_2 cells to 4C. Exponentially growing MEL cells were separated by elutriation (*A*), the cell fraction was enriched for G_1 phase cells (*B*), and cultured without inducer (untreated) or with 5 mM of HMBA, 0.4 mM of EMBA, 2 μM of SAHA, and 4 μM of CBHA (*C*). Cells were harvested after 4, 9, 12, and 15 hr of culture.

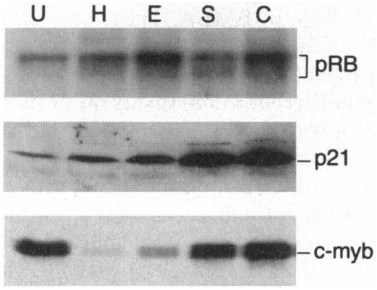


FIG. 3. Effect of HMBA, EMBA, SAHA, and CBHA on the expression of pRB, p21, and c-*myb* protein MEL DS19. Cellular protein extracts were prepared from uninduced cells (U) and MEL DS19 cells cultured with 5 mM of HMBA (H), 0.4 mM of EMBA (E), 2 μM of SAHA (S), and 4 μM of CBHA (C). Protein extracts were prepared after 2 days of culture and immunoblots were done as described in the text.

DISCUSSION

This study reports on the identification and characterization of three second generation hybrid polar compounds, EMBA, SAHA, and CBHA, which are more potent, on a molar basis, than HMBA as inducers of MEL cell erythroid differentiation. This feature, in the case of HMBA, has proved of value in predicting broader effectiveness for differentiation induction and even for potential clinical effectiveness (5). Stowell *et al.* (22) following the reports of synthesis of SAHA (4) showed that it is a potent inhibitor of AXC rat prostate cancer cell proliferation and changes cell morphology suggestive of differentiation.

The evidence indicates that although all four compounds (including HMBA) share certain common structural features, there are differences between them in terms of their biological effects, suggesting that the broad group of hybrid polar compounds can be divided into at least two subsets, based upon effective concentration range, the sensitivity and resistance of selected MEL cell variants, and the intracellular molecular pathways targeted. Both HMBA and EMBA are effective in the millimolar range (5 mM for HMBA and 0.4 mM for EMBA) whereas SAHA and CBHA are active in the micromolar range (2 μM for SAHA and 4 μM for CBHA), suggesting that other features might distinguish these two classes from each other as well. HMBA and EMBA induce MEL cell differentiation with less inhibition of cell proliferation than SAHA and CBHA. Among other features is the shared cross-resistance to induction of the R1 MEL cell line variant, which was selected for resistance to HMBA (12). R1 cells exhibit resistance to EMBA, but are sensitive to induction by the putative group SAHA/CBHA. In molecular terms, the induction of differentiation by HMBA and by EMBA is characterized by suppressed expression of c-*myb*, whereas SAHA/CBHA induce differentiation without this molecular response. Several lines of evidence have suggested that, in the case of HMBA, the late (48 hr) suppression of c-*myb* expression is an integral component of the induction process (19, 20). The identification of agents which, though initiating other events such as hemoglobin production and cell cycle perturbations in common with HMBA, fail to utilize the c-*myb*-related mechanism, suggests that significant differences in pathway controls have yet to be elucidated.

A major reason for exploring the actions of novel hybrid polar agents is to design more clinically useful differentiation-inducing compounds. The identification of subset differences in biologic activity may hold promise for the development of more potent hybrid polar differentiating agents with reduced toxicity.

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