Advances in Brief

The Histone Deacetylase Inhibitor, CBHA, Inhibits Growth of Human Neuroblastoma Xenografts *in Vivo*, Alone and Synergistically with *All-Trans* Retinoic Acid[1]

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Abstract

Histone deacetylase inhibitors (HDACIs) inhibit the growth of a variety of transformed cells in culture. We demonstrated previously that the hybrid-polar HDACI m-carboxycinnamic acid bis-hydroxamide (CBHA) induces apoptosis of human neuroblastoma *in vitro* and is effective in lower doses when combined with retinoids. The current study investigates the effect of CBHA on the growth of human neuroblastoma *in vivo*, both alone and in combination with *all-trans* retinoic acid (atRA), using a severe combined immunodeficiency-mouse xenograft model. CBHA (50, 100, and 200 mg/kg/day) inhibited growth of SMS-KCN-69n tumor xenografts in a dose-dependent fashion, with 200 mg/kg CBHA resulting in a complete suppression of tumor growth. The efficacy of 50 and 100 mg/kg CBHA was enhanced by the addition of 2.5 mg/kg atRA. This dose of atRA was ineffective when administered alone. Treatment was accompanied by mild weight loss in all groups except the lowest dose of CBHA. Our results suggest HDACIs alone or combined with retinoids may have therapeutic utility for neuroblastoma.

Introduction

Neuroblastoma, the most common extracranial solid tumor of childhood, accounts for more than 15% of cancer-related deaths in this age group (1). The high mortality of advanced-stage disease has spurred active investigation of new agents with therapeutic potential. Recently, a series of HDACIs[4] (2), at micromolar concentrations, has been shown to induce differentiation and/or apoptosis of a variety of transformed cell lines (reviewed in Ref. 3). We have demonstrated previously that the HDACI, CBHA, causes apoptosis of neuroblastoma cells *in vitro* (4). HDACs together with histone acetyltransferases (*e.g.,* CBP/p300) regulate the acetylation state of nucleosomal histones, thereby altering chromatin structure (5, 6). It has been proposed that these structural changes regulate the transcription of target genes causing the induction of differentiation or apoptosis in transformed cells.

HDACs and histone acetyltransferases are components of multiprotein complexes associated with gene promoters. The discovery of the RA receptor/HDAC complex (7) provides a rationale for combining RAs and HDACIs. The *in vitro* effects of RAs on neuroblastoma include programmed cell death, growth arrest, and/or terminal differentiation (8, 9). Recently, we reported an enhanced inhibitory effect on neuroblastoma cells in culture when HDACIs were combined with RAs (10).

In this study we examine the effect of the HDACI, CBHA, on neuroblastoma *in vivo*, both alone and in combination with atRA, using a human neuroblastoma xenograft model in SCID mice. Our data show that CBHA inhibits tumor growth in mice in a dose-dependent fashion. In addition, CBHA and atRA synergistically inhibit tumor growth, rendering lower doses of CBHA effective.

Materials and Methods

Cell Culture and Drug Stocks. The SMS-KCN-69n cell line was provided by Drs. J. Biedler, R. Ross, and B. Spengler (Fordham University, Bronx, NY). Cells were grown in a 1:1 mixture of RPMI 1640 and Ham's F-12 media with 10% FCS, penicillin, and streptomycin incubated at 37°C and 5% CO_2. Cells were maintained in log-phase growth and harvested with 0.125% trypsin and 0.02% EDTA in HBSS. Stock solutions of CBHA and atRA were prepared in 100% DMSO and stored at −20°C. atRA was protected from light at all times.

Implantation of SMS-KCN-69n Neuroblastoma Xenografts. SMS-KCN-69n cells were screened in the Memorial Sloan-Kettering Monoclonal Antibody Core Facility to assure freedom from infectious agents. Cells were grown to sufficient numbers in culture, trypsinized as described above, washed three times in PBS, and then resuspended in Matrigel basement membrane matrix (Sigma Chemical Co.) at a concentration of $\sim 2 \times 10^7$ cells/ml. Female SCID mice 4–6 weeks of age (Taconic) were anesthetized by methoxyflurane inhalation before shaving and skin decontamination with 100% ethanol. Approximately 2×10^6 cells in 0.1 ml of matrix were implanted in the right flank via s.c. injection. Mice were housed in barrier conditions on a 12-h light/dark cycle, with food and water supplied *ad libitum*. Tumors were allowed 7 days to engraft.

CBHA and atRA Preparation and Administration. Mice with palpable tumors, all of roughly equivalent size (6.5 ± 0.09 mm $\times 5.1 \pm 0.07$ mm, or 91 ± 2.7 mm^3), were randomly assigned to one of eight treatment groups, with nine mice in each group. CBHA was administered in a dose of 50 mg/kg, 100 mg/kg, or 200 mg/kg, both alone and in combination with atRA, at a fixed dose of 2.5 mg/kg. One group received atRA alone, and a control group received vehicle (DMSO) alone. Drug stocks were appropriately diluted to keep injection volume at a constant 1 μl/g body weight for all mice. For groups receiving combination treatments, CBHA and atRA were maintained as separate stock solutions in DMSO and mixed daily immediately before injection. Drugs were administered for 21 days via daily i.p. injection. Animals were weighed, with dosages adjusted accordingly, and tumors were measured in two dimensions using calipers three times per week. Tumor volume was calculated using the formula: Tumor Volume = Length $\times$ (Width)^2 $\times$ $\pi/6$.

The study concluded on the 21st day of treatment, when the tumor size of the control group necessitated killing. A single mouse from each group was sent alive to a veterinary pathologist at the Research Animal Resource Center of Cornell University Medical College and Memorial Sloan-Kettering Cancer Center for complete tissue necropsy. The remaining animals were killed by CO_2 asphyxiation. Tumors, blood, spleens, livers, and brains were harvested for additional analysis. Tissues were divided, with portions fixed in formalin,

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Fig. 1. s.c. growth of the SMS-KCN-69n neuroblastoma xenograft in SCID mice treated with daily i.p. injections of vehicle (*Control*), atRA alone, or CBHA (50, 100, or 200 mg/kg) alone or combined with atRA. The dose of atRA was 2.5 mg/kg. *Bars,* mean tumor volume ± SE. Measurements were obtained every 2–3 days during the 3-week treatment course (total of nine measurements). Tumor volumes for days 1, 6, 11, 16, and 21 are shown. Tumors were implanted 7 days before the start of treatment. Treatment commenced on day 1, when the average tumor volume was 91 ± 2.7 mm^3.
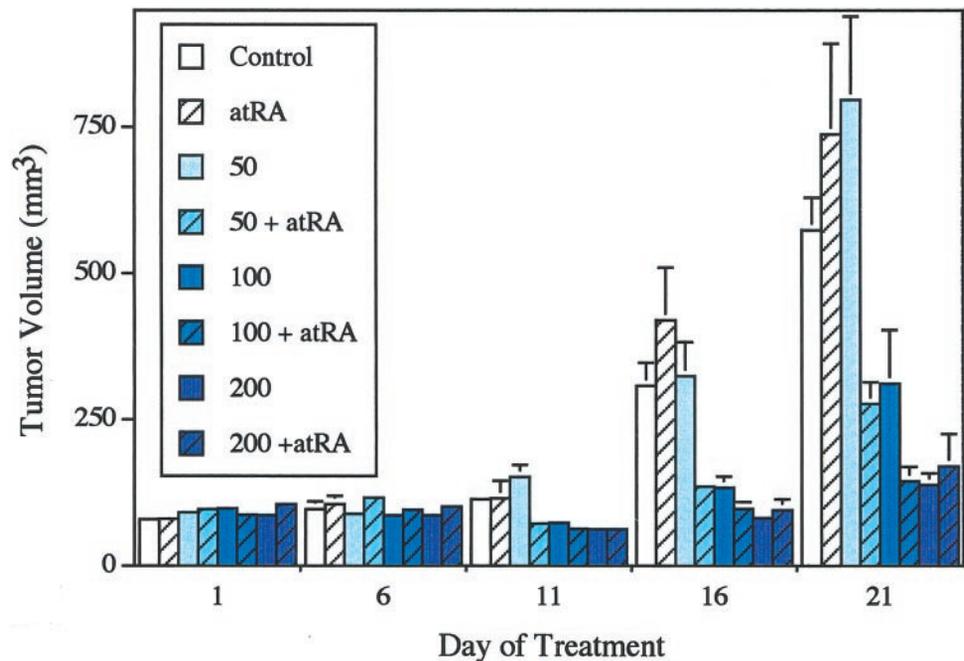
frozen in OCT media, frozen in liquid nitrogen, or homogenized for isolation of histones.

Statistical Analysis. To compare differences between groups with respect to tumor volume over time, a permutation test was used (11). The null hypothesis for this test is that the growth rates in the two groups are equal. The statistic used to test this hypothesis is the squared differences between mean tumor volumes in each group summed overall time points. This statistic reflects the amount by which the two treatment groups are different with respect to average tumor volume over time: the greater the differences between the trajectories of the average tumor volumes of the two treatment groups, the greater the value of the statistic. All possible permutations of the treatment group affiliations were computed. For each possible permutation, the test statistic was calculated. The P of the permutation test corresponds to the proportion of test statistics from the permutation distribution that was more extreme than the test statistic observed from the experiment**.**

To assess synergy between CBHA and atRA, a permutation test was again used. Synergistic inhibition of tumor growth is demonstrated when the inhibitory effect of a given dose combination on the average log tumor volume is greater than the sum of the inhibitory effects of the two drugs administered separately. We describe this relationship through the equation

$$\text{Avg } (V \mid A = a, C = c) < v + \{\text{avg } (V \mid A \ a, C = 0) - v\}$$

$$+ \{\text{avg } (V \mid A = 0, C = c) - v\}$$

where V is the log tumor volume, a and c represent the doses of atRA and CBHA respectively, and *v* is the average log tumor volume in the control group.

The nonparametric Wilcoxon rank-sum test was used to determine whether a group's change in weight from day 1 to day 21 was different from control. ANOVA was used to detect any differences in hematological parameters.

Isolation and Immunoblot Analysis of Histones. Histones from tumor, liver, and spleen samples (*n* = 2/group) were isolated as described previously (2). Acetylated histone H3 was assayed by Western blotting as described previously (4, 12).

Results

CBHA Suppresses Growth of Human Neuroblastoma Xenografts Alone and Synergistically with atRA. The effect of CBHA, both alone and in combination with atRA, in inhibiting the growth of human neuroblastoma *in vivo* was tested using the N-*myc*-amplified SMS-KCN-69n cell line xenografted s.c. in SCID mice.

Tumor-bearing mice were assigned randomly (*n* = 9/group) to receive one of three doses of CBHA (50, 100, or 200 mg/kg) alone or with atRA (2.5 mg/kg).[5] On a per-kilogram basis, the dose of atRA corresponds to a safe dose cited in pediatric pharmacokinetic literature (13). Drugs were administered by daily i.p. injection. Tumor volumes were measured and compared with controls receiving vehicle or atRA alone (Fig. 1).

Administration of 100 mg/kg and 200 mg/kg CBHA resulted in reductions of average final tumor volume of ~50% (*P* = 0.02) and 75% (*P* < 0.0001), respectively, compared with tumors in vehicle-treated control animals (Fig. 1). Treatment with high-dose CBHA (200 mg/kg) resulted in near total suppression of tumor growth. Neither low-dose CBHA (50 mg/kg) nor atRA used alone inhibited tumor growth.

The combination of 50 mg/kg CBHA with 2.5 mg/kg atRA led to a 52% reduction of final tumor volume, as compared with control (*P* = 0.0002; Fig. 1). The inhibition of growth by this combination was synergistic, in that its effect was greater than the sum of the effects of the two drugs administered separately (*P* = 0.01). The addition of atRA to 100 mg/kg CBHA caused greater inhibition of tumor growth, as compared with 100 mg/kg CBHA used as a single agent. Of the animals treated with 100 mg/kg CBHA, the final average tumor volume was reduced an additional 54% in the presence of atRA (*P* < 0.0001 *versus* control; Fig. 1). The inhibitory effect of 100 mg/kg CBHA combined with atRA was comparable with the maximal effect observed using 200 mg/kg CBHA alone. The enhanced inhibition of growth by this combination approached, but did not reach, significant synergy (*P* = 0.07). The addition of atRA to the highest dose of CBHA (200 mg/kg) caused no additional tumor growth inhibition; synergy was not detected (*P* = 0.19).

In addition to the overall growth inhibition noted in treated mice, some animals demonstrated tumor regression, *i.e.,* final tumor size was less than at the outset of treatment. Tumors regressed in one of nine mice treated with 100 mg/kg and two of nine mice treated with 100 mg/kg CBHA combined with atRA. Tumors regressed in three of nine mice treated with 200 mg/kg and in four of nine mice treated with

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[5] L.Z. He, personal communication.

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200 mg/kg CBHA combined with atRA. Tumor regression was not seen in mice treated with 50 mg/kg CBHA either alone or in combination with atRA.

Toxicity of the Higher Doses of CBHA, Alone or in Combination with atRA, was Limited to Mild Weight Loss. The general condition and weights of the mice were noted throughout the treatment course as a marker of toxicity. Also, serum chemistries, complete blood counts, and full tissue necropsies were performed at the conclusion of treatment. The general appearance of drug-treated mice was indistinguishable from that of vehicle-treated controls, with the exception of the two groups receiving 200 mg/kg CBHA. These animals had disheveled fur and soft stools, suggestive of skin and gastrointestinal toxicity. The addition of atRA seemed to confer little or no greater clinical toxicity than CBHA alone. No animals died as a result of treatment, except for one vehicle-treated animal that sustained a vascular injury during injection.

All drug-treated groups either gained less weight than the vehicle-treated group or had mild weight loss during the treatment period (Table 1). The differences in weight changes compared with control reached statistical significance in all groups except the group treated with 50 mg/kg CBHA alone. Differences in tumor size may have contributed to the differences in weight, but this could not be quantified because all weights were determined with tumors *in situ*.

Necropsy consisted of gross and histological examination of at least 26 organs or tissues, including a general chemistry panel on one animal/group and complete blood counts in three animals/group. All groups, including vehicle-treated controls, displayed mild, chronic peritonitis and right hind leg myositis proximate to the injection sites. No other gross, histological, or serological pathology was found. There were no specific histological correlates to the clinical weight, fur, and stool changes noted in the high-dose treatment groups. There were no statistically significant differences in WBC count, hemoglobin and hematocrits, or platelet counts between the various groups (Fig. 2).

CBHA Causes the Accumulation of Acetylated Histones in Tumors of Treated Mice. As a marker of the biological activity of CBHA, we assayed tumors excised from treated mice for acetylated histones by Western blotting with antibodies to acetylated histone H3. Administration of CBHA resulted in the accumulation of acetylated histones in tumor samples (Fig. 3). When normalized for variation in protein loading, there appeared to be a dose response such that increasing the dosage of CBHA led to an increased intensity of signal.

Discussion

We have reported previously that the HDACIs, in micromolar concentrations, induce the differentiation or apoptosis of transformed cell lines (3), including neuroblastoma (4). The current investigation reports on the marked growth-inhibitory activity of the HDACI,
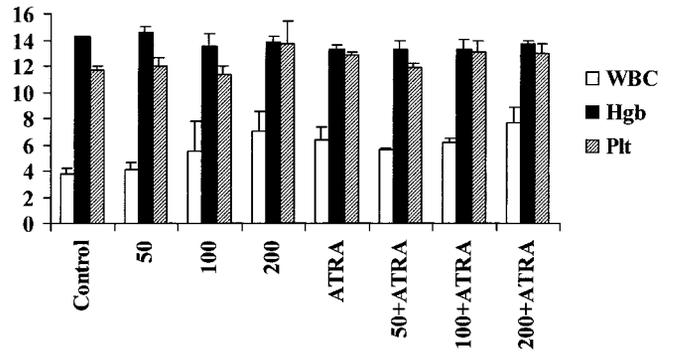

Fig. 2. Effect of treatment with CBHA and atRA on hematopoiesis in SCID mice bearing SMS-KCN-69n human neuroblastoma xenografts. Complete blood counts were obtained upon death on the final day of treatment for 3 or 4 animals from each group. P was not significant for each parameter across all groups by ANOVA (see text). WBC count (*WBC*) is expressed in thousands, hemoglobin (*Hgb*) as gm/dl, and platelets (*Plt*) as the value $\times 10^5$.

CBHA, on human neuroblastoma xenografts of the SMS-KCN-69n cell line in SCID mice. The finding of increased levels of acetylated histones in tumor samples from mice treated with CBHA supports the hypothesis that inhibition of HDAC leads to growth inhibition in neuroblastoma. Toxicity was limited to mild weight loss and fur changes at the higher CBHA dosage (200 mg/kg). When combined with low-dose atRA, lower doses of CBHA (100 mg/kg) resulted in growth inhibition comparable with that achieved with 200 mg/kg CBHA. Synergy between these two classes of agents—RAs and HDACIs—was demonstrated by combining noninhibitory doses of CBHA (50 mg/kg) and atRA (2.5 mg/kg) and achieving a statistically significant 52% reduction in final tumor volume compared with control.

Combination therapies for human tumors using HDACIs and RAs are currently under active investigation (14). *In vitro*, the responsiveness of neuroblastoma cells to RAs has been described (8, 9, 15), and clinical evidence now suggests a role for retinoids in high-risk patients as well (16). Although an earlier trial of 13-*cis* RA was discouraging (17), a more recent randomized, controlled trial demonstrated improved outcome after myeloablation and bone marrow transplant in patients who received 13-*cis* RA *versus* no additional treatment (16). The nuclear receptor complex through which RAs exert their biological effects has recently been characterized (7) and was found to contain HDAC. It is possible that HDAC inhibition potentiates the derepression of retinoid responsive genes in the presence of ligand. Indeed, RA-induced differentiation of leukemia cell lines can be enhanced by the addition of an HDACI (14, 18, 19). Remission in a retinoid-resistant APL patient was reported after adding an HDACI to atRA therapy (20). In neuroblastoma, we have demonstrated *in vitro*

Table 1 *Group average weight changes for the eight experimental groups*

Weights were obtained three times/week throughout the treatment course. The beginning and ending weights are shown, as well as the average change in grams and percentages.

CBHA (mg/kg)	RA (mg/kg)	Start weight[a]	Final weight[a]	Change (%)[b]	P[c]
0	0	23.8 ± 0.76	26 ± 0.84	2.2 (9)	
0	2.5	26.1 ± 0.77	26.3 ± 0.80	0.2 (0)	0.04
50	0	22.7 ± 0.85	24.4 ± 0.71	1.7 (7)	0.81
50	2.5	26.7 ± 1.04	25.6 ± 0.75	−1.1 (−4)	<0.01
100	0	28.2 ± 1.34	27.9 ± 1.32	−0.3 (−1)	<0.01
100	2.5	25.3 ± 0.67	24.6 ± 0.58	−0.7 (−2)	<0.01
200	0	27.1 ± 0.9	24.9 ± 0.79	−2.2 (−8)	<0.01
200	2.5	27.2 ± 0.8	25.4 ± 0.78	−1.8 (−6)	<0.01

[a] All values are presented as the mean ± SE in grams for each treatment group. Initial and final body weights refer to the weight of the mice at the start of treatment and at the end of the study (day 21), respectively, with tumors *in situ*.

[b] Weight change is listed as the difference between the group mean final and the initial weights in grams with the percent change in parentheses.

[c] The P indicates whether the distribution of average weight change was significantly different from that of control. Derived by the nonparametric Wilcoxon rank-sum test.
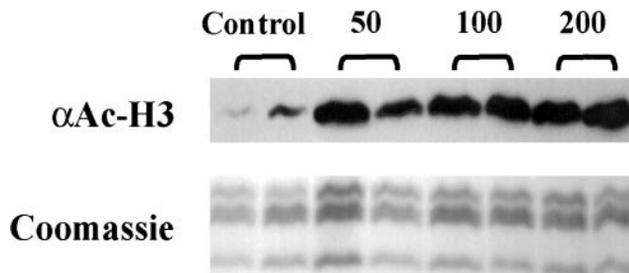
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Fig. 3. Accumulation of acetylated core histone H3 in SMS-KCN-69n neuroblastoma xenografts after administration of CBHA. Tumors were excised from mice on the final day of treatment, ~2 h after the final i.p. injection of vehicle (*Control*) or 50, 100, or 200 mg/kg CBHA (*column headings*), and histones were isolated. Acetylation of histone H3 was analyzed by Western blotting of histone samples using antibodies to acetylated histone H3 (*top panel*). Two separate tumors are shown for each dose. Coomassie Blue-stained polyacrylamide gel of the histone samples (*bottom panel*) controls for protein loading variation.

that HDACIs and RAs inhibit the growth of cultured cells in markedly lower concentrations when combined (10).

Extensive clinical experience with RAs in children has accumulated, making these compounds an available modality for the treatment of refractory neuroblastoma (13). The results of the current investigation suggest that HDACIs, alone or in combination with retinoids, may be safe and effective agents for the treatment of neuroblastoma disease in children.

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