ABSTRACT

Inhibitors of histone deacetylase (HDAC) have been shown to have both apoptotic and differentiating effects on various tumor cells. M-carboxycinnamic acid bis hydroximide (CBHA) is a recently developed hybrid polar compound structurally related to hexamethylene bisacetamide. CBHA is a potent inhibitor of HDAC activity. CBHA induces cellular growth arrest and differentiation in model tumor systems. We undertook an investigation of the effects of CBHA on human neuroblastoma cell lines in vitro. When added to cultures of a panel of neuroblastoma cell lines, CBHA induced the accumulation of acetylated histones H3 and H4, consistent with the inhibition of HDAC. Concentrations of CBHA between 0.5 μM and 4 μM led to apoptosis in nine of nine neuroblastoma cell lines. Apoptosis was assessed by DNA fragmentation analysis and the appearance of a sub-G1 (<2N ploidy) population by flow cytometric analysis. The addition of a caspase inhibitor (benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) completely abrogated CBHA-induced apoptosis in three of three cell lines. The addition of cycloheximide greatly reduced CBHA-induced apoptosis, suggesting that apoptotic induction was dependent on de novo protein synthesis. In addition, CBHA induced the expression of both CD95 (APO-1/Fas) and CD95 ligand within 12 h. The effect of CBHA on human neuroblastoma cells suggests that this agent and structurally related synthetic hybrid polar compounds have therapeutic potential for the treatment of this malignancy.

INTRODUCTION

Neuroblastoma is derived from the neural crest and affects children early in life. It is the most common extracranial solid tumor in the pediatric age group (1), accounting for >15% of cancer-related deaths in children (2). Surgery alone has been found to be efficacious in so-called “low-risk” tumors. However, “high-risk” disease involves the use of multiple cytotoxic drugs, with 4-year survival rates between 12% and 40% (2).

Several interesting observations have been made in high-risk neuroblastoma tumors. First, conventional multiagent chemotherapy often results in dramatic reductions in tumor mass after initial treatment, suggesting a strong primary apoptotic response (3). Second, histopathology reveals that, in many cases, neuroblastoma tumors are composed of both pathogenic undifferentiated neuroblasts and neuronal lineage cells at various stages of differentiation. In addition, other neural crest cell types, such as melanocytes and glial cells, may be detected in these tumors (4). These observations suggest that neuroblastoma tumors, even those considered high risk, retain the biochemical pathways mediating both programmed cell death (apoptosis), as well as multilineage terminal differentiation (5–7).

Many children die of recurrent disease despite intensive chemo- therapeutic regimens and, to date, cytodifferentiation therapies have proven disappointing (8). For these reasons, new and different classes of agents are being investigated in neuroblastoma to improve outcome.

Most studies of neuroblastoma involve analyses of tumor-derived cell lines. Many neuroblastoma cell lines have a mixed population of tumor-derived cell types that have been subcloned into three identifiable morphological groups (9, 10). Of these, the neuritic or N-type neuroblastoma cell is the most relevant to neuroblastoma disease. N-type cells express several markers and chromosomal abnormalities frequently found in undifferentiated neuroblastoma tumor isolates (1) and have retained the potential to form xenograft tumors in immuno-deficient rodent models (11). In addition, recent studies have demonstrated that, similar to undifferentiated neuroblastoma tumors, N-type neuroblastoma cell lines undergo apoptosis by CD95-dependent and CD95-independent mechanisms in response to a variety of cytotoxic drugs (12, 13). These cell lines have also been shown to terminally differentiate into sympathetic neurons and lose tumorigenic potential in response to a variety of cytodifferentiation agents (e.g., retinoic acid and its derivatives; Refs. 14–17).

HDACs enzymes that affect eukaryotic chromatin structure, have been shown recently to be a target for potent hydroxamic acid-based hybrid polar inducers of transformed cell differentiation (18). In addition to chromatin remodeling, the HDAC enzyme complex has been linked with several important regulatory pathways for growth and differentiation. For example, HDAC is associated with the retinoblastoma protein pRB (19, 20) and members of the c-myc family (21, 22). In addition, HDAC is the active mediator of the retinoid receptor repressor complex (23–26). Several compounds (e.g., butyrates) have been shown to inhibit HDAC activity with low potency, resulting in the accumulation of acetylated histones and an increase in transcriptionally active chromatin (27, 28). Butyrates have been shown to be relatively weak inducers of tumor cell apoptosis or differentiation (29–34). These compounds require relatively high concentrations (effective concentration in the millimolar range) and are known to influence other metabolic pathways (35–38). Thus, the links between the HDAC enzyme complex and the induction of apoptosis or differentiation remain unresolved.

The recent identification of more potent inhibitors of HDAC, such as TSA (39–41), trapoxin (42), and CBHA (18), has fostered studies that suggest a role for the HDAC-associated complex in induced tumor cell differentiation or apoptosis. These inhibitors have been shown to induce differentiation in certain colonic tumors and leukemic cell lines (18, 40, 43, 44) and promote apoptosis in T lymphocytes and other colonic tumor cell lines (45–47). TSA was found to synergize with derivatives of retinoic acid to promote cellular differentiation of leukemias that exhibit retinoid resistance (24, 25). CBHA is a member of a recently synthesized family of hybrid polar compounds that have been shown to be inhibitors of HDAC (18) and potent inducers of transformed cell growth arrest and terminal differentiation.
at micromolar (4 \mu M) concentrations (48). There has been a long-standing interest in cytodifferentiation therapy in neuroblastoma because of the ability to induce differentiation in vitro with multiple agents (10, 14, 15, 17). In addition, these tumors are found to contain cells in various states of differentiation in vivo (4), and spontaneous maturation and involution of neuroblastoma has been documented (6).

The present study was designed to investigate the effects of CBHA in neuroblastoma. We found that CBHA induced apoptosis in nine of nine cell lines investigated. It was observed that 0.5–4 \mu M CBHA induced dose-dependent decreases in cell viability and caspase-dependent apoptosis in all neuroblastoma cell lines investigated. Additional studies demonstrated that CBHA-induced apoptosis was inhibited by cycloheximide and that CBHA caused the rapid induction of the CD95/CD95-L system. These results suggest that CBHA and other members of these hydroxamic acid-based hybrid polar compounds are possible therapeutic agents in the treatment of neuroblastoma.

**MATERIALS AND METHODS**

**Cell Culture.** Cloned subpopulations of N- and S-type neuroblastoma cells [SK-N-Be(2)-M17, SK-N-Be(2)C, SK-N-SY5Y, SK-N-SH (EP1), BE(1)n, LA1–55n, LA1–5 s, and SMS-KCN (69n)] were kindly provided by Dr. J. Biedler, Dr. R. Ross, and B. Spengler (Fordham University, Bronx, NY). The cell line SK-N-ER was established at Memorial Sloan-Kettering Cancer Center from a patient with metastatic neuroblastoma. Cells were grown in a 1:1 mixture of RPMI 1640 and F-12 medium supplemented with 10% FCS and incubated at 37°C and 5% CO2. They were maintained in log phase growth and harvested with 0.125% trypsin and 0.02% EDTA in HBSS. CBHA was prepared as described previously (48). The compound was maintained as a 100-mM stock in 100% DMSO. In all assays, appropriate dilutions of DMSO alone were used as a solvent control. All cultures were seeded and allowed to adhere for 24 h before the addition of CBHA. Cells were quantified and assessed for viability on a hemacytometer by the trypan blue (Sigma Chemical Co.) method. The individual sensitivity of each cell line was consistent over the course of 12 months of experimentation, suggesting that factors such as culture media, different sources of FCS, and passage number did not affect the results.

**Incubation with zVAD-fmk and Cycloheximide.** To inhibit caspase activity, neuroblastoma cells were preincubated with 60 \mu M zVAD-fmk (Calbiochem) for 3 h at 37°C before the addition of CBHA. Cells were preincubated with 0.5 \mu g/ml cycloheximide (Sigma Chemical Co.) for 1 h at 37°C before the addition of CBHA to assess the effect of inhibition of protein synthesis.

**Flow Cytometry.** Cells were harvested by trypsinization, and cell suspensions were washed twice in PBS, then fixed in 80% ice-cold ethanol and stored at −20°C until analysis. Before analysis, cell suspensions were digested by DNase-free RNase (Boehringer Mannheim) for 20 min at 37°C and then stained in a propidium iodide solution (50 \mu g/ml) containing 0.1% Triton X-100 overnight in the dark. Cell cycle analysis was performed with a FACScan equipped with a FACStation running CellQuest software (Becton Dickinson, San Jose, CA). Debris was eliminated from analysis using a forward angle light scatter threshold trigger. Cell doublets and other clumps were removed using analysis gates on either fluorescence pulse width or height versus pulse area (integral). Data for 1–2 \times 10^6 single cells were collected/
RESULTS

Accumulation of Acetylated Histones H3 and H4 in Neuroblastoma Cell Lines Cultured with CBHA. Recently, CBHA was shown to be an inhibitor of HDAC, resulting in accumulation of acetylated histones in MEL cells (18). To determine whether CBHA induces acetylated histone accumulation in neuroblastoma, four cell lines [SMS-KCN (69n), SK-N-BE(2)-M17, LA1–55n, and SK-N-ER] were cultured with 2 μM CBHA for 4 h. Acetylated histones H3 and H4 were detected in the extracts of all four neuroblastoma cell lines cultured with CBHA, whereas little or no acetylated histones H3 and H4 are detected in cultures without this agent (Fig. 1).

Cell Proliferation Assays. Treatment of MEL cells with CBHA (4 μM) results in: (a) prolongation of G1 phase of the cell cycle; (b) increased expression of p21(WAF1); (c) underphosphorylation of the retinoblastoma protein pRB; and (d) terminal differentiation (48). Nine neuroblastoma cell lines were separately cultured with CBHA. In the LA1–55n cell line, culture with CBHA in the submicromolar range (0.25–0.5 μM) for 72 h resulted in a decrease in accumulation of viable cells (Fig. 2A). The KCN-69n line yielded similar results (Fig. 2B). For the cell line SK-N-ER, this effect was seen only at concentrations >0.25 μM (Fig. 2C). In the low-micromolar dose range (1–3 μM), there was a sharp decline in viable cell number for all three cell lines after 24 h of culture with CBHA. Culture of nine of nine human neuroblastoma cell lines (SK-N-BE(2)-M17, SK-N-BE(2)C, SK-N-SYS5Y, SK-N-SH (EP1), BE(1)m, LA1–55n, LA1–5s, SMS-KCN (69n), and SK-N-ER) with CBHA lead to extensive cell death within 24–72 h. The LD₅₀ in all nine cell lines ranged between 1 μM and 4 μM. Analysis of these results reveal that all neuroblastoma cell lines are sensitive to the effects of CBHA. Morphological examination of cultures 24–48 h after CBHA (1–2 μM) culture revealed extensive cellular debris with membrane blebbing and morphological appearances characteristic of apoptosis (data not shown).

In the three cell lines LA1–55n, KCN-69n, and SK-N-ER, 2 μM CBHA was observed to greatly reduce cell viability after 72 h of exposure. Studies were performed to determine the duration of drug exposure necessary to induce these effects. Thus, the three neuroblastoma cell lines were cultured with CBHA for varying durations. Results of this analysis (Fig. 3) revealed that only 12–24 h of culture with CBHA was necessary to reduce cell viability to the same level as observed with continuous 72-h exposure. This suggests that an irreversible process is initiated within 12–24 h of CBHA culture, which is sufficient to trigger its effects on neuroblastoma cell lines.

CBHA Causes Progressive Accumulation of Hypodiploid (sub-Gⱽ) Nuclei. FACs analysis of propidium iodide-stained nuclei was performed to evaluate the effects of CBHA on cell cycle and to assess the cells for the presence of a hypodiploid or sub-Gⱽ fraction, consistent with DNA fragmentation. Three neuroblastoma cell lines were exposed to 2 μM CBHA for 12, 24, 36, and 48 h. Analysis revealed a progressive accumulation of the hypodiploid fraction for KCN-69n cells cultured with CBHA between 12 h and 48 h (Fig. 4A). Specific sub-Gⱽ was calculated for each cell line and was found to increase up to 82% by 48 h of culture (Fig. 4B). The initial time course titration studies (Fig. 2) demonstrated that CBHA in the nanomolar range resulted in reduced cell number by 72 h. This result suggested that the neuroblastoma cells were undergoing either growth inhibition or...
increased cell death as compared with controls. FACS analysis was performed on neuroblastoma cell lines treated with CBHA at concentrations below LD50 (0.25–1.0 μM) to determine whether there was an increase in the population of cells in the sub-G1 fraction. An example of this analysis is presented for the cell line SK-N-ER, where it was observed that there was a dose-dependent increase in the specific sub-G1 fraction (Fig. 4C). The FACS analysis also showed that there are no statistically significant changes in the G1-S-G2 phases of the cell cycle. These results confirm that CBHA induces cell death in neuroblastoma in a dose-dependent manner.

CBHA Causes DNA Fragmentation. To further clarify the nature of the sub-G1 fraction induced in response to CBHA, four cell lines were cultured with this agent for 12–36 h, at which time total DNA was extracted and analyzed for the appearance of canonical nucleo-
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Table 1. Experimental apoptosis (% cells attributable to CBHA (specific apoptosis), apoptosis percentage was calculated as: (experimental apoptosis (%) − spontaneous apoptosis in medium (%)) × 100 (12, 13). Analyzed cells included: (a) untreated (data not shown); (b) zVAD-fmk alone (○); (c) CBHA alone (■); and (d) CBHA + zVAD-fmk (□). Untreated cell cultures in log phase growth routinely contained 3–6% apoptotic cells in each of the three cell lines. Data are presented for KCN-69n (1 μM CBHA), SK-N-ER (2 μM CBHA), and LA-1-55n (1 μM CBHA).

Fig. 5. Analysis of DNA ladders from neuroblastoma cultures treated with CBHA. Genomic DNA was isolated from four untreated (+) and 2 μM CBHA (+)-induced neuroblastoma cell lines (36-h drug exposure). DNA was subjected to agarose gel electrophoresis (1% agarose/TBE), stained with ethidium bromide for 30 min, and visualized on short-wave UV light box. M, marker DNA, 1-kb ladder (Life Technologies, Inc.).

Fig. 6. zVAD-fmk inhibition of CBHA-induced apoptosis. Three neuroblastoma cell lines were preincubated for 3 h with 60 μM zVAD-fmk resuspended in normal tissue culture media. After preincubation, a 10 × CBHA stock was added at 1:10 (v/v), and cultures were maintained at 37°C for 48 h. Apoptosis was assessed by FACS analysis of propidium iodide-stained nuclei (sub-G1 faction). To assess the percentage of apoptotic cells attributable to CBHA (specific apoptosis), apoptosis percentage was calculated as: [experimental apoptosis (%) − spontaneous apoptosis in medium (%)] × 100 (12, 13). Analyzed cells included: (a) untreated (data not shown); (b) zVAD-fmk alone (○); (c) CBHA alone (■); and (d) CBHA + zVAD-fmk (□). Untreated cell cultures in log phase growth routinely contained 3–6% apoptotic cells in each of the three cell lines. Data are presented for KCN-69n (1 μM CBHA), SK-N-ER (2 μM CBHA), and LA-1-55n (1 μM CBHA).

Fig. 7. zVAD-fmk restores normal growth to sub-LD50 CBHA-treated neuroblastoma cells. Three neuroblastoma cell lines were preincubated with zVAD-fmk, as described in Fig. 6. CBHA was then added to cultures for 72 h at a final concentration of 0.25, 0.5, or 1.0 μM. Cells were harvested and assayed for viability, as described in Fig. 2. Representative data are presented for SK-N-ER (1.0 μM CBHA; ■), KCN-69n (0.5 μM CBHA; ○), and LA-1-55n (0.5 μM CBHA; □).

Fig. 8. Inhibition of protein synthesis abrogates apoptosis. The cell lines KCN-69n (■) and LA-1-55n (□) were preincubated for 1 h with 0.5 μg/ml of cycloheximide and supplemented with normal media (control) or media containing CBHA. Cells were maintained for 48–72 h, counted for viability, and assayed for the accumulation of the sub-G1 cell fraction by FACS analysis. Specific apoptosis was calculated (see Fig. 6).
CELL LINES WITHIN 12–24 H OF CBHA CULTURE. OTHER CD95-L SPECIES IT WAS OBSERVED THAT CD95-L Expression COULD BE INDUCED IN ALL THREE OBSERVED TO BE NEGATIVE FOR CD95-L EXPRESSION (FIG. 9). IN TREATED CELL LINES (LA1–55n AND SK-N-ER), WHEREAS KCN-69n WAS CD95-L POLYPEPTIDE COULD BE DETECTED AT LOW LEVELS IN TWO OF THREE.

CD95 (APO-1 OR FAS) IS A 45-KDA MEMBER OF THE TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY. THIS CELL SURFACE RECEPTOR, ALONG WITH ITS COGNATE LIGAND CD95-L, FORMS AN IMPORTANT SYSTEM OF APOPTOSIS IN NEUROBLASTOMA CELLS. IN THOSE STUDIES, BOTH CD95 AND CD95-L INDUCTION PRECEDED CASPASE-DEPENDENT APOPTOSIS (13). SIMILARLY, WE HAVE SHOWN THAT CBHA INDUCES AN APOPTOTIC EFFECT IN ALL NINE HUMAN NEUROBLASTOMA CELL LINES INVESTIGATED AT MICROMOLAR CONCENTRATIONS (LD₅₀ RANGE, 1–4 μM). HOWEVER, DOSE-DEPENDENT INCREASES IN APOPTOSIS WERE DETECTED IN CELL LINES CULTURED WITH CBHA IN THE NANOMOLAR RANGE. THIS RANGE OF EFFECTIVE CONCENTRATIONS IS CONSISTENT WITH PREVIOUS STUDIES IN WHICH CBHA WAS SHOWN TO INDUCE TERMINAL DIFFERENTIATION IN MEL CELLS (48).

DISCUSSION

THESE INVESTIGATIONS HAVE FOUND THAT THE HYDROXAMIC ACID-BASED HYBRID POLAR COMPOUND CBHA INDUCES NINE OF NINE HUMAN NEUROBLASTOMA CELL LINES TO UNDERGO APOPTOSIS. CBHA-INDUCED APOPTOSIS WAS CASPASE MEDIATED, INHIBITED BY CYCLOHEXIMIDE, AND ASSOCIATED WITH INCREASED EXPRESSION OF THE CD95/CD95-L SYSTEM.

CBHA HAS BEEN SHOWN TO INHIBIT HDACs (18). IN THE PRESENT STUDY, THIS AGENT WAS FOUND TO CAUSE AN ACCUMULATION OF ACETYLATED HISTONE H3 AND HISTONE H4. EARLIER STUDIES SHOWED THAT BUTYRATE, AN INHIBITOR OF HDAC (28), CAN INDUCE APOPTOSIS AND/OR DIFFERENTIATION OF VARIOUS SOLID TUMOR AND HEMATOPOIETIC CELL TYPES (29–34, 55, 56). BUTYRATE WAS REPORTED TO INHIBIT CELL GROWTH AND INCREASE DIFFERENTIATION IN TWO HUMAN NEUROBLASTOMA CELL LINES (57, 58), AND INDUCTION OF PROGRAMMED CELL DEATH WAS OBSERVED IN ANOTHER AT MILLIMOLAR CONCENTRATIONS OF BUTYRATE (37). AT THESE RELATIVELY HIGH CONCENTRATIONS, IT HAS BEEN SUGGESTED THAT BUTYRATE MAY ALTER OTHER CELLULAR MACHINERY, IN ADDITION TO INHIBITING HDAC (35–38). THE HALF-LIFE OF THIS FATTY ACID IN VIVO IS VERY SHORT AND HAS LIMITED ITS EFFECTIVENESS AS A THERAPEUTIC AGENT (59–61). WE HAVE SHOWN THAT CBHA INDUCES AN APOPTOTIC EFFECT IN ALL NINE HUMAN NEUROBLASTOMA CELL LINES INVESTIGATED AT MICROMOLAR CONCENTRATIONS (LD₅₀ RANGE, 1–4 μM). HOWEVER, DOSE-DEPENDENT INCREASES IN APOPTOSIS WERE DETECTED IN CELL LINES CULTURED WITH CBHA IN THE NANOMOLAR RANGE. THIS RANGE OF EFFECTIVE CONCENTRATIONS IS CONSISTENT WITH PREVIOUS STUDIES IN WHICH CBHA WAS SHOWN TO INDUCE TERMINAL DIFFERENTIATION IN MEL CELLS (48). FURTHERMORE, THE PRESENT DATA SHOW THAT BRIEF EXPOSURE TO CBHA (4 h) LEADS TO THE ACCUMULATION OF ACETYLATED HISTONES, WHICH WAS ALSO DEMONSTRATED IN MEL CELLS (18).

OUR DATA ALSO REVEAL THAT THE IRREVERSIBLE COMMITMENT TO PROGRAMMED CELL DEATH WAS ACHIEVED WITHIN 12–24 h OF DRUG EXPOSURE, CONSISTENT WITH THE ACTIVATION OF A PROAPOPOTIC CASCADE. THE CASPASE ENZYMES ARE CONSIDERED KEY EFFECTOR MOLECULES THOUGHT TO BE REQUIRED FOR MOST APOPTOTIC PATHWAYS (51). IN HUMAN NEUROBLASTOMA CELLS, THEY HAVE BEEN SHOWN TO BE NECESSARY FOR THE CYTOTOXIC EFFECTS OF ANTICANCER AGENTS (62). OUR DATA INDICATE THAT INHIBITION OF CASPASE ACTIVITY LEADS TO AROBATION OF CBHA-INDUCED APOPTOSIS.


Indeed, the chemotherapeutic agents described above all have been used clinically in the treatment of neuroblastoma. They are distinct from each other in their known mechanisms of intracellular activity, but all have been shown to induce apoptosis via the CD95/CD95-L system. This observation emphasizes the importance of CD95-dependent apoptotic induction in the treatment of neuroblastoma. Several pathways (including the generation of ceramide, activation of the c-jun NH₂-terminal kinase/stress-activated protein kinase-dependent stress pathway, and the accumulation of p53) have been implicated in the induction of the CD95 system, leading to apoptosis (63–66). The mechanism regulating the increased expression of these proteins by CBHA in neuroblastoma is an intriguing question to be explored. It would be useful to have multiple modalities by which to induce and...
maintain CD95 sensitivity in tumors. HDAC inhibition may affect the expression of these genes by chromatin alteration and transcriptional activation (67). Alternatively, HDAC inhibition may induce other intracellular pathways that then activate the death receptor pathway.

The multidrug resistance gene, MDR, and the multidrug resistance-associated protein, MRP, have been implicated in chemoresistance and poor outcome in neuroblastoma (68, 69). The drug efflux mechanism is associated with many of the agents used in the treatment of this disease, including the drugs discussed above. In previous studies, MDR positive, vincristine-resistant MEL cells were shown to be sensitive to the effects of CBHA (5). Preliminary studies in our laboratory with the vincristine-resistant neuroblastoma cell line BE(2)C-VCR suggest that these cells are susceptible to CBHA. The investigation of this agent in the context of MDR-amplified neuroblastoma cell lines will be important.

Combination therapies with HDAC inhibitors and retinoids have recently become an area of keen interest and investigation. In vitro studies have shown neuroblastoma cell lines to be responsive to the effects of retinoic acid (14, 15), yet retinoid therapies in patients have proven discouraging (8). Recent work has shown that in the absence of retinoic acid ligand, the RAR and RXR nuclear retinoid acid receptor families are associated with a protein repressor complex, which includes SMRT/mSin3 and HDAC (26). Indeed, several recent studies demonstrated that the addition of HDAC inhibitors enhances retinoic acid-induced differentiation in leukemic model systems (23–26). There is a recent study of a patient with recurrent retinoid-resistant acute promyelocytic leukemia who achieved complete clinical and cytogenetic remission after the addition of a HDAC inhibitor to all-trans-retinoic-acid therapy (70). Current investigation in our laboratory suggests that CBHA can enhance retinoid sensitivity in neuroblastoma cell lines, and we have initiated studies of CBHA and the drug combination on tumor xenografts.

Our results suggest that CBHA and other potent HDAC inhibitors may have therapeutic potential in the treatment of neuroblastoma.

REFERENCES


Hybrid Polar Histone Deacetylase Inhibitor Induces Apoptosis and CD95/CD95 Ligand Expression in Human Neuroblastoma

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