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

Richard D. Glick, Steven L. Swendeman, Dennis C. Coffey, Richard A. Rifkind, Paul A. Marks, Victoria M. Richon, and Michael P. La Quaglia

Abstract

Inhibitors of histone deacetylase (HDAC) have been shown to have both apoptotic and differentiating effects on various tumor cells. M-carboxycinnamic acid bishydroxamide (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 \( \mu M \) and 4 \( \mu M \) 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 biochemistry underlying both neoplastic and neuronal differentiation (5–7).

Many children die of recurrent disease despite intensive chemotherapeutic 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 immunodeficient 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 euchromatic 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., butyrate) 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). Butyrate has 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.

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4 The abbreviations used are: HDAC, histone deacetylase; CBHA, M-carboxycinnamic acid bishydroxamide; TSA, trichostatin A; CD95-L, CD95 ligand; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; FACS, fluorescence activated cell sorting; MEL, murine erythroleukemia.
at micromolar (4 μM) 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 μM 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 (EPI), 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 μM zVAD-fmk (Calbiochem) for 3 h at 37°C before the addition of CBHA. Cells were preincubated with 0.5 μg/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 μg/ml) containing 0.1% Triton X-100 overnight in the dark. Cell cycle analysis was performed with a FACSscan 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 × 10^6 single cells were collected/
centrifuged at 16,000 ×g for 15 min. The supernatant was then mixed with an equal volume of sample buffer, which was prepared in buffer containing 10 mM Tris-HCl (pH 6.5), sodium bisulfite, 1% Triton X-100, and proteinase inhibitors. Lysate buffer was then added to the cells in the amount of 25 

### 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 

### RESULTS

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Fig. 3. Effect of noncontinuous CBHA culture. CBHA (2 

### Cell Proliferation Assays.

Treatment of MEL cells with CBHA (4 

### Immunoblot Analysis of Isolated Histones.

Nuclei were isolated by lysis in buffer containing 10 mM Tris-HCl (pH 6.5), sodium bisulfite, 1% Triton X-100, 10 mM MgCl₂, 8.6% sucrose, and Dounce homogenization. Histones were isolated by acid extraction, as described previously (18). Isolated histones (2.5 

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### Immunohistochemistry.

Immunohistochemistry was performed with a mouse monoclonal antibody to acetylated histone H3 and a rabbit polyclonal antibody to acetylated histone H4. Tissue sections were treated with 0.1% Triton X-100 in PBS for 15 min and incubated with the primary antibody for 1 h at room temperature. The sections were then washed three times with PBS and incubated with a biotinylated secondary antibody for 30 min. The sections were then reacted with peroxidase-conjugated streptavidin for 15 min. The reaction was visualized with diaminobenzidine and counterstained with hematoxylin.

### Discussion

Overall, the experiments described here indicate that CBHA is a potent inhibitor of HDAC activity and that it can induce apoptosis in neuroblastoma cells. The results support the hypothesis that HDAC inhibitors may be useful in the treatment of neuroblastoma.

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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-
somal DNA ladders. Monomeric, as well as multimeric nucleosomal ladders (average approximate monomeric length, 180 bp), were observed in DNA isolated from all four cell lines by 36 h of culture (Fig. 5). The nucleic acid from untreated control cell lines had high molecular weight genomic DNA. The detection of substantial sub-G₁ fractions combined with the presence of DNA ladders suggests that CBHA triggers an apoptotic pathway in the neuroblastoma cell lines. Apoptosis was also assessed by the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) staining method (50) with fluorescent microscopic analysis and yielded results consistent with DNA fragmentation (data not shown).

Caspase Inhibition Abrogates CBHA-induced Cell Death. Induction of the caspase pathway is one of the hallmarks of apoptosis (51). Furthermore, doxorubicin-induced apoptosis in neuroblastoma cells has previously been shown to involve activation of the caspase cascade (13). The agent zVAD-fmk, described previously as a broad range caspase inhibitor (12, 13), was used to assess whether CBHA-induced apoptosis was caspase dependent. Cells were cultured with CBHA in the presence or absence of zVAD-fmk. FACS analysis (propidium iodide-stained nuclei) of three cell lines revealed little or no accumulation of sub-G₁ DNA fragments in the presence of the caspase inhibitor (Fig. 6). ZVAD-fmk alone had no detectable effect on cell growth or viability (data not shown). These results suggest that the cell death induced by CBHA is dependent on activation of the caspase cascade.

To evaluate whether sublethal concentrations of CBHA caused increased cell death alone or growth inhibition, three cell lines were cultured for 48 h in the presence or absence of CBHA and in the presence or absence of zVAD-fmk. It was observed that in the presence of zVAD-fmk, overall cell number and viability was similar in cultures with or without CBHA (Fig. 7). For each cell line, control cultures and zVAD-fmk + CBHA-treated cultures underwent at least three to four cell divisions based on calculated doubling times (data not shown). This observation suggests that neuroblastoma cells continue to proliferate in the presence of both compounds. Furthermore, immunoblot analyses of extracts from control and CBHA-cultured cell lines revealed that there was no apparent change in the levels of expression of the cell cycle inhibitors p16INK4A, p21waf1, or p27kip1 (data not shown). These observations are consistent with CBHA causing an increase in cell death, even at low concentrations.

Cycloheximide Abrogates CBHA-induced Apoptosis. Cycloheximide was used to determine whether CBHA-induced apoptosis was dependent on protein synthesis. Two cell lines, KCN-69n and
CBHA Induces Both CD95 and CD95-Ligand in Neuroblastoma

LA1–55n, were incubated with CBHA in the presence or absence of cycloheximide. Cycloheximide suppressed the apoptotic response induced by CBHA (Fig. 8). These results suggest that CBHA activates an apoptotic pathway that is dependent on protein synthesis.

CBHA induces both CD95 and CD95-Ligand in Neuroblastoma. 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 apoptotic initiation (52). It was recently observed that the induction of apoptosis in neuroblastoma cell lines with chemotherapeutic agents (e.g., doxorubicin) is associated with the accumulation of CD95/CD95-L (13). A time-course analysis of three neuroblastoma cell lines was performed, and extracts were assayed for the expression of both CD95 and CD95-L by immunoblotting. It was found that CD95 expression was induced within 12–24 h of treatment with CBHA (Fig. 9A) in three of three cell lines. CD95-L is an N-linked glycoprotein with a reported molecular weight of \( M_w = 40,000 \) (52, 53). The 40-kDa CD95-L polypeptide could be detected at low levels in two of three untreated cell lines (LA1–55n and SK-N-ER), whereas KCN-69n was observed to be negative for CD95-L expression (Fig. 9B). In addition, it was observed that CD95-L expression could be induced in all three cell lines within 12–24 h of CBHA culture. Other CD95-L species (molecular weight range, \( M_w = 37,000–44,000 \)) were observed in the immunoblots, and these may be due to variations in N-linked glycan density, as reported previously (54). Thus, both CD95 and CD95-L were induced in 12–24 h in all three cell lines. The temporal pattern of expression for these proteins is consistent with the results of the growth and flow cytometric analyses, where declines in cell viability and increases in the sub-G1 fraction were apparent 12–24 h after the addition of CBHA to the culture.

**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 induce 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 cells 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 (LD_{50} range, 1–4 \( \mu M \)). Moreover, 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 proapoptotic 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 abrogation of CBHA-induced apoptosis.

Recently, Fulda et al. (13, 62) demonstrated the importance of the CD95/CD95-L system in the apoptotic response to chemotherapeutic agents, such as doxorubicin, cisplatinum, and VP-16, in neuroblastoma cells. In those studies, both CD95 and CD95-L induction preceded caspase-dependent apoptosis (13). Similarly, we have shown that CBHA induces CD95 and CD95-L in neuroblastoma cells and observed that the time course of induction of apoptosis parallels that of induction of CD95.

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

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Fig. 9. CBHA induces the expression of CD95 (A) and CD95 ligand (B). Control and CBHA-induced cultures of neuroblastoma cell lines LA1–55n, SK-N-ER, and KCN-69n were harvested at 12, 24, 36, and 48 h. For CD95, the leukemic cell line KG-1 was used as an expression control. For CD95-L, the colon carcinoma cell line SW620 was used as an expression control. Protein extracts were prepared, separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose for immunoblotting. The resulting blots were blocked in 5% powdered milk and incubated with primary antibody for 1 h at room temperature anti-Fas (sc-715-G) (A) or anti-Fas-L (sc-6237) (B) at 1:2000. As controls for protein loading, all lanes were stained with Ponceau S before immunostaining and judged to have approximately equivalent protein loading for each lane.

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maintain CD95 sensitivity in tumors. HDAC inhibition may affect the expression of these genes by chromatin alternation 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 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 have demonstrated that the addition of HDAC inhibitors enhances retinoid 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


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