Favorable Neuroblastoma Genes and Molecular Therapeutics of Neuroblastoma

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ABSTRACT

Purpose and Experimental Design: Neuroblastoma (NB) is a common pediatric solid tumor that exhibits a striking clinical bipolarity: favorable and unfavorable. Favorable NB genes (EPHB6, EFNB2, EFNB3, NTRK1, and CD44) are genes whose high-level expression predicts favorable NB outcome, and forced expression of these genes inhibits growth of unfavorable NB cells. In this study, we investigated whether favorable NB gene expression could be augmented in unfavorable NB cells by chemical compounds and whether an increased expression of these genes was associated with suppression of NB growth and metastasis.

Results: We found that inhibitors of DNA methylation [5-aza-2'-deoxycytidine (5AdC)], histone deacetylase (HDAC) [4-phenylbutyrate (4PB)], and proteasome (MG262) enhanced the expression of favorable NB genes in NB cell lines and inhibited the growth of these cells in vitro ($P < 0.0005$). The growth-inhibitory effects of 5AdC and 4PB in vitro were in part due to caspase-dependent cell death and inhibition of DNA synthesis. Administration of 5AdC and/or 4PB also suppressed growth of subcutaneous NB xenografts in nude mice ($P < 0.001$), which was accompanied by enhanced favorable NB gene expression and an increase in apoptosis. Moreover, 4PB suppressed bone marrow and liver metastases of NB cells in severe combined immunodeficient/Beige mice ($P = 0.007$ and $P = 0.008$, respectively). The growth-suppressive activity of HDAC inhibitors on NB was further confirmed by the efficacy of trichostatin A, a potent and specific HDAC inhibitor.

Conclusions: Collectively, these observations further emphasize the link between the elevated favorable NB gene expression and a benign phenotype of NB.

INTRODUCTION

Neuroblastoma (NB) accounts for 7% to 10% of all childhood cancers and causes approximately 15% of deaths in children with cancer. The tumor is unique because of its propensity to exhibit either a favorable or an unfavorable phenotype. Favorable NBs undergo regression or maturation even without treatment or can be cured by surgical removal or without adjuvant chemotherapy. Unfavorable NBs, on the other hand, exhibit unrestrained growth despite the most intensive treatment. Our previous study (1) has shown that high-level expression of transcripts encoding the EPHB6 receptor or its ligands, ephrin-B2 and ephrin-B3 (EPHB6, EFNB2, and EFNB3, respectively), predicts favorable disease outcome of NB. Conversely, their low-level expression forecasts an adverse outcome. Moreover, the expression patterns of these genes differ among individual NB cases. As yet, if one of these genes is expressed at high levels, disease outcome of the patient is favorable, suggesting that a good NB disease outcome is a consequence of high-level expression of at least one of these genes (1). These observations thus suggest that enhanced expression of such a gene (EPHB6, EFNB2, or EFNB3) in an otherwise unfavorable NB may suppress its malignant phenotype. In fact, forced expression of EPHB6 in NB cell lines derived from unfavorable NB (with or without MYCN amplification) results in growth suppression in vitro and in vivo (1). EFNB2 and EFNB3 also exhibit a similar growth-suppressive activity in vitro against NB cells with or without MYCN amplification.4 These clinical and biological characteristics of EPHB6, EFNB2, and EFNB3 have led us to define them as “favorable neuroblastoma genes” (1), which also include those with similar characteristics such as NTRK1 (TrkA) and CD44 (2–5).

This study was designed to investigate whether favorable NB gene expression could be augmented in unfavorable NB cells by chemical compounds and whether such a change in gene expression is associated with suppression of NB growth and metastasis. We found that inhibitors of DNA methylation and histone deacetylase (HDAC) enhanced favorable NB gene expression in unfavorable NB cells. In addition, these compounds inhibited growth of NB cells in vitro and in vivo and suppressed NB metastases in vivo. Similarly, a proteasome...
inhibitor enhanced favorable NB gene expression in unfavorable NB cells and inhibited growth of these cells in vitro. Together with previous gene transfer studies (1, 2, 5), which have shown that forced expression of favorable NB genes in unfavorable NB cells results in growth suppression, this study further emphasizes the correlation between the elevated expression of favorable NB genes and a benign phenotype of NB.

MATERIALS AND METHODS

Neuroblastoma Cell Lines. The NB cell lines (SY5Y, IMR5, CHP134, and Nb69) were grown in RPMI 1640 supplemented with 5% fetal bovine serum and 1% OPI (GIBCO, Grand Island, NY). SY5Y was a gift from Dr. Robert Ross (Fordham University, Bronx, NY). IMR5 (a clone of IMR32), CHP134, and Nb69 were provided to us by Dr. Roger H. Kennett (Department of Biology, Wheaton College, Wheaton, IL; a former faculty member of Department of Human Genetics, The University of Pennsylvania School of Medicine).

Quantitative Reverse Transcription-Polymerase Chain Reaction. RNAs were isolated from NB cell lines and tumors using the RNeasy kit (Qiagen, Valencia, CA). Experimental procedures for quantitative reverse transcription-polymerase chain (RT-PCR) reaction have been described in detail elsewhere (6–8), and results of the method were shown to be consistent with those obtained by Northern blot analysis (6, 7). Conditions for polymerase chain reaction and cycles and of primer sequences of EPHB2, EFNB2, EFNB3, and NTRK1 have been described previously (8). Other primer sequences were as follows: CD44, 5′-CCGCTATGTCCAGAAAGGAG-3′ and 5′- TCCAGGGACTGTCTTCGTCT-3′; CDKN1A, 5′-GAACACCAC TGGAGGGTACT-3′ and 5′-CA GTCACACATGTCCTCT-3′; and VEGF, 5′-CACATAGGAGATGACCTC-3′ and 5′-CCGCTTCGGTTGCACAT-3′.

Preparation of 5-Aza-2′-deoxycytidine, 4-Phenylbutyrate, Trichostatin A, and MG262. 5-Aza-2′-deoxycytidine (5AdC; Fluka) was dissolved in acetic acid/H2O (1:1) at a concentration of 40 mg/mL as the stock solution. The stock solution was then diluted in PBS, filter-sterilized, and frozen at −20°C. Sodium 4-phenylbutyrate (4PB; Aldrich) was prepared as a solution was then diluted in PBS, filter-sterilized, and frozen at −20°C. Trichostatin A (TSA; Calbiochem, La Jolla, CA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 40 mg/mL as the stock solution and then filter-sterilized and frozen at −20°C. The stock solution was diluted in cell culture medium or PBS to achieve the desired concentration for in vitro and in vivo experiments. MG262 (Calbiochem) was dissolved in DMSO at 10 mmol/L and stored at −20°C until use.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide and Bromodeoxyuridine Incorporation Assays. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed essentially as described in our previous study (1). Bromodeoxyuridine (BrdUrd) incorporation assays (a nonradioactive alternative to [3H]thymidine incorporation assay to measure DNA synthesis) were performed according to the instructions of the BrdUrd incorporation assay kit (Roche).

Mouse Xenograft Studies. IMR5 or CHP134 cells were suspended in Matrigel (Calbiochem) and injected subcutaneously in the flank of nude mice (10⁷ cells/0.25 mL/mouse). Treatment with 5AdC (0.25 mg/kg/d), 4PB (1 g/kg/d), or a combination of both was started when the tumor volume reached 0.2 to 0.25 cm³ in size. Animals in the control group were given saline. The total amount of drugs or saline given daily was divided into two intraperitoneal injections (2 injections per day, 7 days per week). The difference in tumor size between the control group and the treatment group was assessed by a t test. A similar procedure was used in the TSA study, in which the dose of TSA was used as 10 mg/kg/d, and the control group was given 10% DMSO in PBS.

Histologic Analysis of Human Neuroblastoma Xenografts. Paraffin-embedded tissue blocks were prepared from human NB xenografts. Hematoxylin and eosin-stained tumor sections were then subjected to microscopic examination. The high power (×100) oil immersion was used to evaluate the number of karyorrhexis (apoptotic cells) present in random fields, and the total numbers were recorded. A t test was used to assess whether the values of the two groups were significantly different. P values of <0.05 are considered statistically significant.

Mouse Metastasis Model. SY5Y cells were genetically marked with green fluorescent protein (GFP) by transfection of pLEIN cDNA (Clontech, Palo Alto, CA) into SY5Y using a retrovirus-mediated gene transfer procedure. Severe combined immunodeficient (SCID)/Beige mice received injection with SY5Y-GFP (10⁷ cells/0.3 mL PBS/mouse) via the tail vein. The intraperitoneal 4PB treatment (1 g/kg/d) began 3 days later. The control group was injected with saline. After 3 weeks of 4PB treatment, the 4PB dose was reduced to 0.5 g/kg/d to minimize drug toxicity. Bone marrow cells were prepared by flushing them out from mouse tibia and femur with PBS. The resultant cell preparations were filtered through a 70 μm nylon mesh and analyzed by FACScan (Becton Dickinson, San Jose, CA) to detect GFP-positive cells. The difference in variables between the control group and treatment group was assessed by a t test.

RESULTS

Inhibitors of DNA Methylation and Histone Deacetylation Enhance the Expression of Favorable Neuroblastoma Genes in Neuroblastoma Cell Lines and Supress Growth of These Cells. All NB cell lines available to date are derived from unfavorable NB and express low levels of favorable NB genes. We thus first examined whether inhibitors of DNA methylation (5AdC) and histone deacetylase (4PB) could augment the expression of favorable NB genes in NB cell lines in vitro. We included two MYCN-amplified NB cell lines (IMR5 and CHP134) and two NB cell lines without MYCN amplification (SY5Y and Nb69) in this analysis. Fig. 1A and B show the patterns of favorable NB gene expression after 4 days of drug treatment. The expression of EPHB6 and CD44 was markedly increased by 5AdC and/or 4PB in the NB cell lines. This observation was consistent with a recent finding that CD44 expression was enhanced in NB cell lines by 5AdC (9). The same treatments result in an enhancement of EFNB2 expression in MYCN-amplified lines (IMR5 and CHP134), but not in NB cell lines without MYCN amplification (SY5Y and Nb69). In addition, 4PB treatment enhanced EFNB3 expression in IMR5 and SY5Y, but not in the other cell lines.
NTRK1 expression was markedly enhanced in CHP134 cell lines by the 5AdC and/or 4PB treatment. The other cell lines also responded to the drug treatment by increasing their NTRK1 expression, but to a lesser extent. We also examined the expression of CDKN1A, encoding p21 WAF1 as a control, because inhibitors of HDAC and DNA methylation are known to enhance the expression of CDKN1A transcripts (10–12). As shown in Fig. 1, NB cell lines expressed CDKN1A at moderate levels, and treatment with 5AdC and/or 4PB further enhanced CDKN1A expression in these cells. Collectively, these results show that 4PB and/or 5AdC can enhance the expression of several, if not all, favorable NB genes in a given NB cell line. In addition, 4PB in general has a more prominent effect than 5AdC on the enhancement of favorable NB gene expression in NB cell lines.

To gain further insight into the effect of 5AdC and 4PB on the enhancement of favorable NB gene expression, we examined the expression of these genes in a time course study using IMR5 and SY5Y cells at days 1, 2, and 4 of the drug treatment (Fig. 1C). These results recapitulated the data presented in Fig. 1A and B. Moreover, the results showed that each favorable NB gene had a unique pattern of time-dependent expression in response to 4PB and/or 5AdC treatment and that the enhancement of favorable NB gene expression in response to drug treatment also differed among the NB cell lines examined (Fig. 1C; see Discussion).

Previous gene transfer studies have shown the growth-suppressive function of favorable NB genes on NB cell lines (1, 2, 5). We therefore next examined whether 5AdC and 4PB suppress the growth of NB cells in vitro. As shown in Fig. 1D, 5AdC and/or 4PB exhibited a significant growth-suppressive effect on all four NB cell lines examined. In general, 4PB showed a more potent growth-suppressive effect than 5AdC, and the effect of 4PB and 5AdC was additive. Although 4PB and/or 5AdC enhanced the expression of several, if not all, favorable NB genes in a given NB cell line, the same treatment inhibited the growth of all cell lines examined (Fig. 1D). This finding is consistent with our clinical observation that not all of the favorable NB genes are expressed at high levels in a given favorable NB. However, when one of these genes is highly expressed, the patient outcome is favorable (1). Under the conditions described in Fig. 1, we observed little neurite extension, an indicator of neuronal differentiation, in the drug-treated NB cell lines, with the exception of moderate morphologic changes in CHP134 (data not shown). In addition, we found that 5AdC and/or 4PB increased the expression of neurofilament...
isoforms in IMR5 and CHP134, but not in SY5Y and Nb69 (data not shown).

The In vitro Growth Suppression of Neuroblastoma Cells by 5-Aza-2′-deoxycytidine and 4-Phenylbutyrate Involves Caspase-Dependent Cell Death and Inhibition of DNA Synthesis. Mechanistically, there are at least two ways by which 5AdC and 4PB suppress NB growth: acceleration of cell death and inhibition of cell proliferation. We therefore examined the effect of benzoyloxycarbonyl-Val-Ala-Asp (OMc) fluoromethylketone (Z-VAD-FMK) (a general inhibitor of caspases) on the growth suppression mediated by 5AdC and 4PB. As shown in Fig. 2A and B, the viability of IMR5 (MYCN-amplified cell line) and SY5Y (MYCN normal cells) was significantly better (P < 0.0005) in the presence of Z-VAD-FMK. These observations suggest that caspase-dependent cell death is involved in the growth suppression of NB cells by 5AdC and 4PB. We next examined whether 5AdC and 4PB inhibited DNA synthesis of NB cells. As shown in Fig. 2C and D, 4PB and the 4PB/5AdC combination significantly inhibited DNA synthesis in IMR5 and SY5Y cells as measured by BrdUrd incorporation, whereas 5AdC alone had a lesser effect on DNA synthesis. These results thus indicate that the 5AdC and 4PB treatment leads to inhibition of DNA synthesis by NB cells.

Inhibitors of DNA Methylation and Histone Deacetylase Suppress Growth of IMR5 Xenografts In vivo. The enhancement of favorable NB gene expression by 5AdC and 4PB and the growth-suppressive effect of these compounds in vitro led to the idea that chemical agents that increase the expression of favorable NB genes may suppress the malignant phenotype of NB. In an attempt to test this idea, we next evaluated the efficacy of 5AdC and 4PB on NB in vivo using mouse xenograft models. Nude mice bearing subcutaneous IMR5 tumors were treated with 4PB, a combination of 5AdC and 4PB, or saline as control. As shown in Fig. 3A, at day 18, there was a statistically significant difference in average tumor size between the control and both the 5AdC + 4PB-treated group (P = 0.0006) and the 4PB-treated group (P = 0.0005). However, there was no apparent advantage of the combination of 5AdC + 4PB over treatment with 4PB alone.

The Growth-Suppressive Effect of 5-Aza-2′-deoxycytidine and/or 4-Phenylbutyrate In vivo Is Accompanied by Enhanced Favorable Neuroblastoma Gene Expression. To further test the relationship between favorable NB gene expression and NB growth suppression by 5AdC/4PB treatment, we examined the expression of favorable NB genes in 21 NB xenografts derived from the experiment shown in Fig. 3A. These include nine tumors of the saline control group, seven tumors of the 5AdC/4PB-treated group, and five tumors of the 4PB-treated group. Fig. 3B showed representative data on 12 of the 21 tumors examined. Because all nine control tumors exhibit the same gene expression pattern, we show representative data for two control tumors in Fig. 3B. Six mice that responded well to the treatment (mice 42, 40, 13, 41, 33, and 11; namely, those whose tumors did not grow >0.45 cm3 in volume) showed elevated levels of NTRK1, EFNB3, and/or CD44 expression. Interestingly, the tumors of mice 42, 40, 13, and 41 also expressed low levels of angiogenic factor (vascular endothelial growth factor) as compared with control mice (Fig. 3B). We did
not detect an increase in the expression of EPHB6 or EFN B2 in
tumors of the treated mice, suggesting that the microenviron-
ment in which host-tumor interaction occurs may affect the
expression of these genes. In addition, we found CDKN1A
expression in all tumors examined. There was no significant
difference in CDKN1A expression levels between the control
and drug-treated groups (data not shown), suggesting that the

\[ \text{5 X. Tang, M. Robinson, D. Kim, and N. Ikegaki, unpublished obser-
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**Fig. 3 A**, 5AdC and 4PB inhibit growth of IMR5 xenografts in vivo.
IMR5 cells were injected subcutaneously into the flank of nude mice
\((10^7 \text{ cells/mouse})\). The drug treatment or saline injection was started via
intraperitoneal route when tumors reached 0.2 to 0.25 cm\(^3\) in volume.
There were two experimental groups: one group was treated with the
combination of 5AdC and 4PB \((n = 7)\), and the other was treated with
4PB alone \((n = 9)\). The control group included 14 mice. The dose of
5AdC was 0.25 mg/kg/d, and the dose of 4PB was 1g/kg/d. Body weight
of the animals was monitored every other day, and the amount of the
drugs given was adjusted accordingly. **B**, Favorable NB gene expression
is enhanced in the IMR5 xenografts treated with 5AdC and/or 4PB. The
control or drug-treated tumors analyzed in this experiment were the
IMR5 xenografts grown in nude mice shown in Fig. 3. Quantitative
RT-PCR was used to measure the expression levels of the indicated
genes. GAPD was used as an internal standard.

**Fig. 4** 4PB suppresses bone marrow and liver metastases of SY5Y-
GFP in SCID/Beige mice. **A**, Fluorescence-activated cell-sorting anal-
ysis of bone marrow samples taken from the saline control and 4PB-
treated SCID/Beige mice injected with SY5Y-GFP. One representative
each from the 4PB-treated and control groups is shown. **B**, Average liver
weights of saline control, 4PB- treated, and normal (no SY5Y-GFP
injected) mice are shown to indicate the difference in tumor load in liver.

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} \text{vations.} \]
growth suppression of IMR5 xenografts in vivo by these compounds was unlikely to be due to CDKN1A. It should be mentioned that there were eight tumors that responded well to treatments, but only six tumors (in mice 42, 40, 13, 41, 33, and 41) expressed elevated levels of known favorable NB genes. This observation suggests that unidentified favorable NB genes may be activated in the two remaining tumors.

**Increase in Apoptosis in Neuroblastoma Xenografts Treated with 5-Aza-2'-deoxycytidine and 4-Phenylbutyrate.**

In an attempt to understand whether the NB xenografts undergo apoptosis and/or differentiation in response to 5AdC and 4PB treatment, we next performed histologic analyses on these tumors based on the International NB Pathology Classification or the Shimada system, in which karyorrhexis represents apoptotic cells (13). The eight drug-treated NB xenografts were derived from the experiment described in Fig. 3 (mice 11, 13, 24, 31, 33, 40, 41, and 42). The 10 saline-treated control NB xenografts were derived from a separate experiment, in which tumors were collected when they reached 0.45 to 0.5 cm³ in volume. Necrosis occurs when the tumor becomes any larger, and this interferes with accurate histologic analyses. We have found that all NB xenografts, regardless of treatment, were undifferentiated. However, there was a significant difference in numbers of apoptotic cells between the treated (30.0 ± 12.9) and control (16.8 ± 7.9) groups (P = 0.016; see Materials and Methods).

**4-Phenylbutyrate Suppresses Metastases of SY5Y Cells in Severe Combined Immunodeficient/Beige Mice.**

Metastatic NB has remained a major challenge in treatment of unfavorable NB. Therefore, it was of interest to investigate whether HDAC and/or DNA methylation inhibitors suppress NB metastases. Because the 5AdC/4PB combination did not have an advantage over 4PB treatment alone in suppressing the growth of subcutaneous NB xenografts (Fig. 3A), we focused on the effect of 4PB on NB metastases in a mouse metastasis model. The mouse metastasis model included tail vein injection of GFP-labeled SY5Y cells into SCID/Beige mice. The 4PB treatment began 3 days later. We chose SY5Y cells in this experiment because the pattern of its metastases in SCID/Beige mice resembles that of NB in humans. Mice of both control and 4PB-treated groups were sacrificed and subjected to autopsy when they showed clear distress due to tumor bearing. The average life span of the control mice was 30.7 ± 1.44 days (n = 8), and that of the 4PB-treated mice was 32.6 ± 1.21 days (n = 7). Although small, such a difference was statistically significant (P = 0.017). The reduced SY5Y metastases were also evident in the 4PB-treated group in comparison with the control group. SY5Y-GFP cells that metastasized to the bone marrow of control mice ranged from 2.33% to 13.24% (average, 5.84%) of total mononucleated cells, whereas SY5Y-GFP in the bone marrow of 4PB-treated mice ranged from 0.45% to 3.5% (average, 1.47%), and such a difference was also statistically significant (P = 0.007; Fig. 4A). In addition, liver metastases, as measured by tumor load (or gain of liver weight), were suppressed in the 4PB-treated mice. As shown in Fig. 4B, the average liver weights of the control, 4PB- treated, and normal (no NB cell injected) mice were 4.24 ± 0.8, 2.71 ± 1.1, and 1.2 ± 0.1 g, respectively. The difference in liver weight between the saline control and the 4PB-treated groups was statistically significant (P = 0.008). It should be mentioned that although Fig. 5 TSA inhibits growth of NB cells in vitro and in vivo. Caspase-dependent cell death is in part responsible for the growth-inhibitory effect of TSA on IMR5 (A) and SY5Y (B) cells in vitro. IMR5 and SY5Y cells were grown in culture in the presence of various concentrations of TSA with or without 25 μmol/L Z-VAD-FMK as indicated. After 2 days of treatment, the cells were subjected to MTT assay. C, TSA inhibits the growth of CHP134 xenografts in vivo. Nude mice received injection in the flank with 10⁶ CHP134 cells suspended in 0.25 mL of Matrigel per mouse. When tumors grew to approximately 0.2 cm³ in size, the TSA treatment was started at a dose of 10 mg/kg/d. TSA was given to the animals via intraperitoneal injection. Animals of the control group were given vehicle control (10% DMSO in PBS).
the average body weight of 4PB-treated mice ($n = 7$) decreased from $20.39 \pm 2.07$ to $18.26 \pm 2.25$ g during the study period, this difference was not statistically significant ($P = 0.09$). The average body weight of the saline control group ($n = 8$) increased slightly from $19.96 \pm 1.56$ to $20.46 \pm 1.26$ g.

**Trichostatin A, a Potent and Specific Histone Deacetylase Inhibitor, Suppresses Neuroblastoma Cell Growth In vitro and In vivo.** To further explore the therapeutic potential of HDAC inhibitors for the treatment of unfavorable NB, we examined the efficacy of TSA. TSA is one of the most specific and potent HDAC inhibitors known to date (14). In addition, TSA has a good $t_{1/2}$ of 14.5 hours in mouse (15). As yet, its in vivo efficacy for human cancer, especially pediatric malignancies, has not been well investigated. As shown in Fig. 5A and B, TSA showed a potent growth-suppressive effect on both IMR5 and SY5Y cells in vitro, and its effect was in part due to caspase-dependent cell death. Furthermore, TSA showed a statistically significant growth-suppressive effect on CHP134 xenografts grown in nude mice at the dose of 10 mg/kg/d (Fig. 5C). This was 100-fold less than that of 4PB (Fig. 3). During the course of the TSA treatment, the average weight of mice increased, indicating that TSA had little systemic toxicity to the mice at this dose.

**Proteasome Inhibitor Enhances the Expression of Favorable Neuroblastoma Genes and Suppresses Growth of Neuroblastoma Cell Lines In vitro.** To further evaluate the idea that chemical compounds that can enhance the expression of favorable NB genes in unfavorable NB cells may also suppress the growth of these cells, we examined the effect of proteasome inhibitors because proteasome inhibitors have been shown to inhibit growth of a murine NB cell line in vitro (16). In addition, proteasome inhibitors might enhance the expression of favorable NB genes by inhibiting degradation of transcriptional factors that positively regulate their expression and/or that of RNA-binding proteins that stabilize their transcripts. As shown in Fig. 6A, a boronic acid-based proteasome inhibitor, MG262, enhanced the expression of EPHB6 and CD44 in IMR5 and SY5Y cell lines. CDKN1A expression was enhanced only in SY5Y cells by MG262. The MG262 treatment resulted in a slightly reduced expression of EFNB3 and NTRK1 and no change in the expression of EFNB2 in both SY5Y and IMR5 cells (data not shown). Nonetheless, MG262 showed a significant growth-suppressive effect on both IMR5 and SY5Y cell lines (Fig. 6B and C). In addition, Z-VAD-FMK partially inhibited the growth-suppressive effect of MG262 in both IMR5 and SY5Y, suggesting that its effect involves caspase-dependent cell death (Fig. 6B and C).

**DISCUSSION**

Our previous study suggests that the differential expression of favorable NB genes in favorable and unfavorable NB together with their growth-suppressive effects in part constitutes an underlying mechanism for the clinical bipolarity of NB (1). Subsequent observations made by others also support this idea (2, 5). This study was designed to test whether chemotherapeutic agents that reactivate favorable NB genes in unfavorable NB and/or augment the expression of these genes also suppress the malignant phenotype of these tumors. The results presented in

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**Fig. 6** A proteasome inhibitor, MG262, enhances the expression of favorable NB genes and suppresses the growth of NB cells in vitro. A. A boronic acid-based proteasome inhibitor, MG262, enhanced EPHB6 and CD44 expression in SY5Y and IMR5. IMR5 and SY5Y cells were grown in the absence or presence of MG262 (2.5 μmol/L) for 2 days. Quantitative RT-PCR was used to measure the expression levels of the genes of interest in the indicated NB cell lines. GAPD was used as an internal standard. The growth-suppressive effect of MG262 on IMR5 (B) and SY5Y cells (C) in vitro is partly dependent on caspase-dependent cell death. MTT assay was used to assess the growth-inhibitory effect of MG262 in the presence and absence of 25 μmol/L Z-VAD-FMK.
this study are consistent with this hypothesis and provide a proof in principle for a therapeutic approach for NB.

DNA methylation and histone deacetylation could occur at the promoter sites of favorable NB genes in unfavorable NB, leading to transcriptional silencing of these genes. However, it is equally possible that the low expression of these genes is a consequence of the secondary effect of gene silencing that has occurred at the loci encoding for positive transcriptional regulators and/or mRNA stabilizing factors for favorable NB genes. Based on the time course experiment (Fig. 1C), both mechanisms might contribute to the down-modulation of favorable NB gene expression. For example, the former might be likely for EPHB6 in SY5Y cells because HDAC inhibition quickly up-modulates EPHB6 expression, whereas the latter might be likely for EFNB3 and CD44 because the enhanced expression of these genes requires a longer time than EPHB6 (Fig. 1C). It has been reported that the CpG islands of the CD44 gene are not methylated in a small cohort of primary NB specimens (9). Although this observation has to be confirmed by a large-scale study, it suggests an indirect mechanism for the attenuated expression of CD44 in vivo.

Interestingly, our gene expression study on the IMR5 xenografts treated with 5AdC and/or 4PB (Fig. 3B) suggests that there are additional members of the favorable NB gene family and that inhibitors of gene silencing can reactivate these genes. In fact, our recent results suggest that this is the case. In addition, as shown in Fig. 3B, the four xenografts derived from the treated mice (mice 42, 40, 13, and 41) expressed high levels of favorable NB genes (EFNB3 and NTRK1) and, conversely, low levels of an angiogenic factor (vascular endothelial growth factor). It remains to be seen whether there are functional interactions among the products of favorable NB genes and those of apoptotic and angiogenic genes, leading to a benign NB phenotype as has been suggested (2).

In summary, chemical or biological agents that can increase favorable NB gene expression in unfavorable NB may be excellent therapeutics for this malignant tumor. Furthermore, a better understanding of molecular characteristics of favorable NB gene products, how these products exhibit tumor-suppressive activities, and how these proteins interact with each other may have an impact on the development of therapeutic strategies for unfavorable NB.

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