The Combined Therapeutic Effects of Bortezomib and Fenretinide on Neuroblastoma Cells Involve Endoplasmic Reticulum Stress Response

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Abstract

Purpose: The proteasome inhibitor bortezomib inhibited cell growth and angiogenesis in neuroblastoma. Bortezomib has been shown to induce synergistic activity when combined with other antineoplastic agents. Here we have investigated the antitumor activity of bortezomib in combination with fenretinide, a synthetic retinoid, against neuroblastoma cells.

Experimental Design: Different neuroblastoma cell lines were tested for sensitivity to bortezomib and fenretinide, given alone or in different dose-dependent and time-dependent combination schedules. Cell proliferation, cell viability, and apoptosis were evaluated by measuring 3H-thymidine incorporation, trypan blue staining, DNA fragmentation, and western blot analysis. Angiogenesis was assessed by the chick embryo chorioallantoic membrane assay. An orthotopic neuroblastoma mouse model was used to examine in vivo sensitivity.

Results: Each compound alone was able to induce a dose-dependent inhibition of cell proliferation, with a significant enhanced antiproliferative effect for the drugs used in combination. This inhibition was characterized by marked G2-M and G1 cell cycle arrest with nearly complete depletion of S phase. Bortezomib and fenretinide in association triggered an increased apoptosis through activation of specific genes of the endoplasmic reticulum stress compared with either drug tested alone. Tumor-bearing mice treated with bortezomib plus fenretinide lived statistically significantly longer than mice treated with each drug alone. Histologic evaluation and chorioallantoic membrane analysis of primary tumors showed that the combined therapeutic activity of bortezomib and fenretinide rested upon antitumor and antiangiogenic mechanisms.

Conclusions: These findings provide the rationale for the development of a new therapeutic strategy for neuroblastoma based on this pharmacologic combination.

The multicatalytic ubiquitin-proteasome pathway represents the major nonlysosomal proteolytic system of all eukaryotic cells (1). Protein substrates, ubiquitinated and degraded by the proteasome system, regulate many physiologic cellular processes, such as cell cycle, apoptosis, signal transduction, DNA transcription, and antigen processing (2). Alterations in this cellular regulatory machinery lead to pathologic phenotypes, including cancer. Due to its pivotal role in neoplastic growth and metastasis, the ubiquitin-proteasome pathway became a promising target for cancer therapy (3).

Bortezomib, a modified boronic dipeptide, selectively and reversibly inhibits the chymotryptic site of the 26S proteasome, inducing the degradation of proteins critically involved in the regulation of cell proliferation and survival. Bortezomib is the first proteasome inhibitor to be approved for clinical use as an antineoplastic agent. (4). Indeed, bortezomib has proven to be efficacious and well tolerated in many clinical trials (5).

The sensitivity of pediatric tumors to proteasome inhibitors has not been extensively investigated but is coming of age (6, 7). To determine the maximum tolerated dose, dose-limiting toxicity, and pharmacodynamics of bortezomib, a phase I study was done in children with recurrent or refractory solid tumor and (8) with refractory leukemia (9). Recently, testing of bortezomib has been started in childhood cancer preclinical models, according to the Pediatric Preclinical Testing Program (10). Our previous work has highlighted the ability of...
bortezomib to cause cell growth arrest, induce apoptosis, and inhibit the angiogenic process in human neuroblastoma (11). Indeed, metastatic neuroblastoma remains a clinical challenge despite advances in multimodal therapy (12). The retinoid compound 13-cis-retinoic acid has proven to be efficacious in prolonging event-free survival of these patients after autologous hematopoietic stem cell transplant (13). Retinoids are a class of compounds known to induce both terminal differentiation and apoptosis in neuroblastoma cells and its high safety profile in humans, has prompted clinicians to design several phase I and phase II studies. We show that this promising pharmacologic combination acts via interference with the unfolded protein response of the endoplasmic reticulum by triggering apoptosis and inhibiting neuroblastoma-induced angiogenesis. The effects of the drug combination were detected at concentrations nontoxic to normal cells and achievable in vivo. Bortezomib plus fenretinide had, in vivo, both antitumor and antiangiogenic effects that translated into significantly prolonged survival of treated animals. We believe that this report unravels the major mechanisms of the antineuroblastoma activity of the combination of bortezomib and fenretinide, and provides a sound conceptual background for the quick design of clinical studies aimed at testing the efficacy of these drugs in neuroblastoma patients.

On the basis of these findings, in the present report we have investigated for the first time the sensitivity of human neuroblastoma to bortezomib when delivered with fenretinide to tumor cells in vitro and in vivo. We show that this promising pharmacologic combination acts via the unfolded protein response of the ER by triggering apoptosis and inhibiting neuroblastoma-induced angiogenesis.

Materials and Methods

Chemicals. Bortezomib [PS-341; Velcade] was kindly provided by Millenium Pharmaceuticals Inc. and used as previously described (11). Fenretinide (N-4-hydroxyphenyl retinamide) was kindly provided by Dompé and dissolved in absolute ethanol at a final concentration of 10 mmol/L and stored at -20°C until used. For in vitro experiments, fenretinide was diluted in complete medium to contain <0.1% ethanol immediately before use. For in vivo experiments, fenretinide was diluted in a sterile 0.9% (weight/volume) NaCl solution containing 1.65 mg/ml bovine serum albumin (Sigma) and 5% absolute ethanol (volume for volume) as reported (23). Fenretinide dilutions were freshly prepared once a week, protected from light, and kept at 4°C.

Thapsigargin, propidium iodide, pan-caspase inhibitor (Z-VAD-FMK), caspase-4 inhibitor (Z-YVAD-FMK), and c-Jun-NH2-kinase (JNK) inhibitor (SP600125) were purchased from Sigma.

Cell lines and culture conditions. The following human neuroblastoma cell lines were used: GI-LI-N, HTLA-230, SH-SY5Y, and ACN (11). Human skin fibroblasts from healthy donors served as controls. All cells were grown in Dulbecco’s modified medium (Sigma) as described (11, 14).

Cell proliferation assay. All neuroblastoma cell lines were plated (8-12 × 10^4 per well) in 96-well plates in complete medium and were treated in quadruplicate with different concentrations of either bortezomib (0-20 nmol/L) or fenretinide (0-100 μmol/L), alone or in combination for 24 h. The cells were then incubated overnight with 0.5 μCi (0.0185 MBq) ^3H-thymidine (Amersham Bioscience) and were processed for liquid scintillation counting (Packard Instruments Company) as described (11, 24).

Analysis of DNA synthesis by pulse labeling with bromodeoxyuridine. Exponentially growing neuroblastoma cells (SH-SY5Y and GI-LI-N; 1.5-3 × 10^4 cells/25 cm² flask) were treated for 24 h with 5 nmol/L bortezomib and 2.5 μmol/L fenretinide, given alone or in combination. At the end of treatment, the cells were pulse labeled with 10 μmol/L bromodeoxyuridine (Sigma) for 30 min as previously described (24). Bivariate distributions of bromodeoxyuridine amounts (FITC) versus DNA content (propidium iodide) were assessed by flow cytometry with the use of a FACS Calibur device (Becton Dickinson) as previously described (11, 24).

Cell viability assay. GI-LI-N and SH-SY5Y cells were plated in 12-well plates (1.5-2.5 × 10^5 cells per well) and treated for 24 h as described above. In some experiments, the cells were treated for 1 h with 50 μmol/L pan-caspase inhibitor (Sigma) and 5 μmol/L caspase-4 inhibitor (Sigma) before bortezomib and/or fenretinide administration. Then, the cells were harvested, washed with complete medium, and incubated with trypan blue (0.04%; Sigma) for 1 min at 37°C as reported (11, 24).

Cell death assay. Cultured neuroblastoma cells were treated with bortezomib and fenretinide, given alone or in combination as above, and then processed for propidium iodide staining as reported (11, 24). A Cell Death Detection ELISAO kit (Roche Diagnostics GmbH, Roche Applied Science) and a human Annexin V-FITC kit (Bender MedSystem) were also used according to the manufacturers’ instructions.

Mitochondrial membrane potential assay. Detection of the mitochondrial permeability transition event was assayed by MitOPT kit (Immunochemistry Technologies) according to the manufacturer’s instructions and as described (11). The analysis was done with the

Translational Relevance

Because approximately half of neuroblastoma-bearing children present with metastatic disease at diagnosis, and a large proportion of them have a grim prognosis, novel therapeutic approaches are urgently warranted.

In the present report, we have investigated for the first time the sensitivity of human neuroblastoma to bortezomib, a reversible inhibitor of the 26S proteasome that has been widely investigated for its antitumor activity in different human malignancies, administered with fenretinide, a synthetic retinoid that, thanks to its high efficacy in triggering apoptosis in neuroblastoma cells, has prompted clinicians to design several phase I and phase II studies. We show that this promising pharmacologic combination acts via interference with the unfolded protein response of the endoplasmic reticulum by triggering apoptosis and inhibiting neuroblastoma-induced angiogenesis. On the basis of these findings, in the present report we have investigated for the first time the sensitivity of human neuroblastoma to bortezomib when delivered with fenretinide to tumor cells in vitro and in vivo. We show that this promising pharmacologic combination acts via the unfolded protein response of the ER by triggering apoptosis and inhibiting neuroblastoma-induced angiogenesis.
use of either a FACSScan (Becton Dickinson) or a 96-well fluorescence plate reader (SPECTRAFluor Plus, TECAN Austria GmbH).

Measurement of cytosolic Ca\(^{2+}\). Free cytosolic calcium was measured with the membrane-permeable, Ca\(^{2+}\)-sensitive fluorescent dye Fluo-4 AM (Molecular Probes; ref. 25). GI-LI-N and SH-SYSY cells were grown on 96-well microplates (20,000 cells per well) for 48 h. The cells were then washed with PBS and incubated with 100 μL PBS containing 4 μmol/L Fluo-4 AM, 10 mM L-glutamate, and 2 mM L-probenecid. After 1 h, the cells were washed and treated with PBS containing 2 mM L-probenecid with or without 1 mM L-thapsigargin, 5 mM L-bortezomib, 2.5 μmol/L fenretinide, or a combination of bortezomib and fenretinide at the same concentrations. The cells were then analyzed immediately after Fluo-4 fluorescence intensity in a microplate reader (FluoStar Galaxy; BMG Labtech GmbH) equipped with 485 nm excitation and 520 nm emission filters. Alteration in the cytosolic free Ca\(^{2+}\) concentration was determined in a time-dependent manner until 4 h, and the increase in Fluo-4 AM fluorescence was normalized to the initial background-subtracted fluorescence. Intracellular calcium levels were also determined after 24 h of treatments with the use of flow cytometry with the Ca\(^{2+}\)-sensitive fluorochrome Calcium Green-1 AM (Molecular Probes).

Western blot analysis. Total cell lysates were prepared and analyzed by western blot analysis as described earlier (11). Briefly, GI-LI-N cells were lysed with Cell Extraction Buffer (BioSource International) plus protease inhibitor cocktail (Sigma). To obtain subcellular fractions, the cells were processed with the Qproteome Cell Compartment kit according to the manufacturer’s instructions (Qiagen) and were resuspended with the above-mentioned lysis buffer. Protein lysates (50 μg per lane) were resolved on SDS 10% to 15% polyacrylamide gels and were transferred to nitrocellulose membranes. The membranes were then incubated with mouse monoclonal antibodies against caspase-3 and caspase-9 (Cell Signaling Technology), Bip/glucose-regulated protein GRP78 (BD Bioscience, Pharmingen), GADD153 (Santa Cruz Biotechnology), or rabbit monoclonal antibodies against phospho-eukaryotic initiation factor 2α, inositol-requiring enzyme 1α (IRE 1α), and c-Jun (Cell Signaling Technology); and poly(ADP)ribose polymerase (PARP) (Abcam) or polyclonal antibodies against eukaryotic initiation factor 2α, cytochrome c, stress-activated protein kinase/p-JNK, phospho–stress-activated protein kinase/JNK, and phospho-c-Jun (Cell Signaling Technology), caspase-10 (Abcam), caspase-4 (BIOMOL International-LP), and caspase-2 (MBL). Peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were used as secondary antibodies (Upstate and Santa Cruz Biotechnology, respectively). Immune complexes were visualized with the use of a Supersignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer’s instructions, and were normalized to internal controls (mouse monoclonal antibody against α-tubulin or a rabbit antibody against glyceraldehyde-3-phosphate dehydrogenase (Sigma)). Protein levels were quantified by scanning densitometry of the autoradiography films and normalized over (ratio) the housekeeping protein levels.

In vivo therapeutic studies. All experiments involving animals have been reviewed and approved by the licensing and ethics committee of the National Cancer Research Institute, Genoa, Italy and by the Italian Ministry of Health. All the in vivo experiments were done with the use of 10 mice per group and were repeated at least twice with similar results. GI-LI-N cells were injected in the capsule of the left adrenal gland as described (24, 26). Tumors were allowed to grow for 14 d, and mice were then randomly assigned to four groups and treated with bortezomib and fenretinide administered individually or in combination, or with saline solution (control group). Bortezomib (0.75 or 1 mg/kg) and fenretinide (1 or 1.5 mg/kg) were i.v. injected twice a week for a total of 6 wk, with a 3-d interval between injections. Body weight and general physical status of the animals were recorded daily until they were judged to be in discomfort by animal caretakers. Specifically, once showing signs of poor health (i.e., abdominal dilatation, dehydration, paraplegia, severe weight loss) the veterinarian (M. Cillii) from the Animal Research Facility euthanized the mice following anesthesia with xilene (Xilor 2%; Bio98 Srl), and the day of euthanasia was recorded as the day of death.

Histologic analysis of mouse orthotopic tumors. Histologic evaluation of primary tumor tissues was done after the 5th wk of treatment. Briefly, orthotopic tumor-bearing mice (2 of 10 mice per group) were anesthetized with xilene and killed by cervical dislocation. Tumor masses were collected, split in two samples, and then processed for either paraffin or optimum cutting temperature compound (Miles Chemical Co.) embedding as described (11, 24). Paraffin-embedded tissue sections (3 μm thick) were stained with primary antibodies against the proliferation antigen Ki67 (clone MB-1; Dako), the 57-kDa human neuroblastoma-specific antigen NB84a (clone NB84a; Dako), factor VIII (clone F8/86; Dako), and the smooth muscle actin (SMA) (clone 1A4; Dako), and for terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) analysis with the in situ Cell Death Detection kit (Roche) according to the manufacturer’s instructions. Sections of cryopreserved (optimum cutting temperature–embedded) tissue were immunostained with anti-α-smooth muscle actin (EastEnders endothelial cell actin) molecule DC31 (clone SC-1506, Santa Cruz Biotechnology). Binding of the primary antibodies was detected with tetramethylrhodamine isothiocyanate–conjugated rabbit anti-mouse (Sigma) or green FITC-conjugated anti-rabbit goat-anti-mouse (Caltag Laboratories-Invotrogen) immunogoldulin for immunofluorescence analysis, and with horseradish peroxidase–conjugated horse anti-goat immunoglobulin G (Vector Laboratories) for immunohistochemical analysis. Endothelial and neuroblastoma cell apoptosis was quantified as described (11, 24).

Microvessel density. Microvessel area in tumor sections stained with CD31 antibody was assessed independently by two investigators (B. Nico and D. Ribatti) with the use of a Quantimet 5000 computerized image analysis system (Leica) as previously described (11, 24).

Chick embryo chorioallantoic membrane assay. Chick embryo chorioallantoic membrane (CAM) assays were done according to Ribatti et al. (27). Briefly, growing CAMs (10 eggs per group) were treated by grafting 2 mm\(^3\) biopsy fragments from xenograft tumors onto the CAM, and then were treated with PBS or with 5 mmol/L bortezomib or 2.5 μmol/L fenretinide given alone or in combination. The CAMs were examined daily until day 12 of incubation, when they were photographed in ovo with a stereomicroscope equipped with a camera and image analysis system (Olympus Italia). The CAMs were then processed for light microscopy, and the angiogenic response was evaluated as described (11, 24).

Statistical analysis. Results are expressed as mean values with 95% confidence intervals. All in vitro data are from at least three independent experiments. The statistical significance of differential findings between experimental and controls groups was determined by ANOVA with Tukey’s multiple comparison test in Graph-Pad Prism 3.0 software (GraphPad Software, Inc.). These findings were considered significant if two-tailed P values were <0.05. Isobologram analysis and dose-reduction index calculation have been done with CalcuSyn software program (Biosoft). Survival curves have been constructed with the Kaplan-Meier method. All the in vivo experiments were done at least twice with similar results. A P value of <0.05 was considered statistically significant with the use of Petos’s log-rank test in StatsDirect 0.1 statistical software (CamCode).

Results

Combined treatment with bortezomib and fenretinide inhibits neuroblastoma cell proliferation. Different neuroblastoma cell lines were tested for their sensitivity to bortezomib and fenretinide in vitro by measuring [H]-thymidine incorporation. The doses of bortezomib necessary to inhibit neuroblastoma cell proliferation were nearly three logs lower than those of fenretinide [mean bortezomib half maximal inhibitory
concentration (IC\textsubscript{50}) at 24 h: 11 nmol/L; mean fenretinide IC\textsubscript{50} at 24 h: 6 \mu mol/L) as shown in Fig. 1A and B.

To determine whether bortezomib and fenretinide in combination had additive or synergistic activity, different concentrations of both drugs were added to neuroblastoma cells. From the dose-response curves, the concentrations at which GI-LI-N cell proliferation was inhibited to 90% (ED\textsubscript{90}), 75% (ED\textsubscript{75}), and 50% (ED\textsubscript{50}) of control were calculated. Figure 1C shows the isobologram generated from these data. When compared with the three theoretical lines representing additive effect, the value from the combination treatment did not decrease dramatically below the lines, indicating mild synergistic or summation activity. Similar results have been obtained also for SH-SY5Y, HTLA-230, and ACN cells (data not shown).

When GI-LI-N cells were treated for 24 h with 5 nmol/L bortezomib and 2.5 \mu mol/L fenretinide in combination, they underwent a significantly decreased cell viability compared with incubation with either drug (Fig. 1D). These drug concentrations were chosen because they represent likely half the IC\textsubscript{50} on neuroblastoma cells. Moreover, the antiproliferative effects deriving from the combined treatment were independent of the timing of drug administration. Indeed, preincubation for 1 h of tumor cells with fenretinide followed by the addition of bortezomib for 23 h or vice versa, or the simultaneous administration of both drugs led to similar inhibition of neuroblastoma cell proliferation. This inhibition was statistically significant in comparison with that achieved with the use of either drug alone. Similar results were obtained with the SH-SY5Y, HTLA-230, and ACN neuroblastoma cell lines (data not shown). In contrast, bortezomib plus fenretinide did not alter the growth rate of both normal diploid human fibroblasts (Fig. 1D) and human peripheral blood cells (data not shown), showing that the combined treatment specifically induced the dose-dependent death of neuroblastoma tumor cells without toxicity to nontransformed cells. To gain insight in future setting of therapeutic schedules, dose-reduction index (28) values were calculated from the isobologram curves. The dose-reduction index values for bortezomib were 2.3-fold to 8.2-fold and, for fenretinide were 3.5-fold to 7.7-fold, indicating that the dose of both bortezomib and fenretinide may be reduced until 8 and 7 times, respectively, to give the same results with the doses for each drug alone.

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**Fig. 1.** Effects of BTZ and HPR on the growth of human neuroblastoma cell lines. Four neuroblastoma cell lines were cultured in the presence of various concentrations of either BTZ (1-20 nmol/L; A) or HPR (0-10 \mu mol/L; B) for 24 h. Data, derived from four independent experiments, are expressed as mean percentage of \textsuperscript{3}H-thymidine incorporation from quadruplicated wells as compared with that of control untreated cells. Error bars, mean ± 95% CIs. C, isobologram analysis of dose-response curves (expressed in nmol/L) were done with the CalcuSyn software program; inhibition of cell proliferation of 50% (+: ED\textsubscript{50}), 75% (+: ED\textsubscript{75}), and 90% (circled dot: ED\textsubscript{90}). Points, values from the combination treatment. D, GI-LI-N cells were treated with 5 nmol/L BTZ and 2.5 \mu mol/L HPR, administered alone or in combination (HPR + BTZ), for 24 h to evaluate \textsuperscript{3}H-thymidine incorporation as above. Human fibroblasts were treated only with the combination (HPR + BTZ). The effects of 1-h preincubation with HPR followed by the addition (23 h) of BTZ (HPR→BTZ) or vice versa (BTZ→HPR) have been also reported (***, \(P < 0.001\)). BTZ, bortezomib; HPR, fenretinide; 95% CI, 95% confidence interval.
Next, we studied the cell cycle kinetics in drug-treated cells pulse labeled with bromodeoxyuridine to determine DNA synthesis. The combined treatment dramatically reduced S phase and caused a G2-M and G1 phase arrest of the cell cycle in a statistically significant manner (Supplementary Fig. S1).

Bortezomib and fenretinide induce cell death and apoptosis in neuroblastoma cells. To investigate whether the cellular growth arrest generated by the combined drug treatment was due to cell death, we tested the viability of GI-LI-N cells by trypan blue staining. Figure 2A shows that, at 24 h, both fenretinide and bortezomib induced a percentage of trypan blue–positive cells statistically significant over control cells. When administered together, bortezomib and fenretinide induced a greater and statistically significant cell death with respect to that obtained with the individual drug treatments.

Propidium iodide staining was done to measure DNA fragmentation. As revealed in Fig. 2B, and with similar statistics to that reported in A, the combined drug treatment resulted in an increased number of apoptotic cells compared with treatments with individual drugs. Results were confirmed by detecting phosphatidylserine exposure (data not shown).

Both trypan blue dye exclusion assay and propidium iodide staining showed that pretreatment for 1 h of neuroblastoma cells with a pan-caspase inhibitor significantly restored cell viability from bortezomib-plus-fenretinide–mediated cell death and apoptosis at 24 h (Supplementary Fig. S2A and B). A partial recovery of cell survival was also obtained after pretreatment with an inhibitor of caspase-4 that resides in the ER and is one of the critical proapoptotic executioners of the ER stress response (29).

Thus, we analyzed whether the intrinsic apoptotic signaling pathway was involved in this bortezomib-plus-fenretinide–induced death of neuroblastoma cells by studying some mitochondria-related events (30). The percentage of cells with polarized mitochondria decreased after 18-h treatment, and this reduction was statistically significant with respect to that obtained with either agent alone (Fig. 2C).

Combination of bortezomib and fenretinide induces ER stress response and sensitizes neuroblastoma cells to apoptosis. We next investigated whether calcium homeostasis could be affected by bortezomib-plus-fenretinide–mediated apoptosis on neuroblastoma cells. By using as positive control thapsigargin, an inhibitor of sarcoplasmic/ER calcium ATPase, to trigger ER stress–mediated apoptosis by calcium leakage from ER into the cytosol, we found that our pharmacologic combination did not induce substantial alterations in intracellular calcium concentration over the single agents as early apoptotic event (within the 1st 4-h treatment; Fig. 2D).

GI-LI-N cells were then processed by western blot analysis. As reported in Fig. 3A, the combined treatment increased and induced expression of the ER stress proteins, the prosurvival chaperon GRP78/Bip, and the proapoptotic transcription

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**Fig. 2.** BTZ and HPR reduce neuroblastoma cell survival. Cell survival (A) and apoptosis (B) of GI-LI-N cell were determined by the trypan blue exclusion method and PI staining, respectively, after 24 h of culture in the presence of 5 nmol/L BTZ and 2.5 μmol/L HPR given alone or in combination. Results are expressed as mean values from three independent experiments; error bars, 95% CIs. C, to assess the effects of BTZ and HPR on mitochondrial membrane permeability, GI-LI-N cells were treated for 18 h as above, harvested, and analyzed for changes in mitochondrial membrane potential. Results are expressed as the mean percentage of cells with polarized mitochondria from four independent experiments; error bars, 95% CIs. D, measurement of cytosolic Ca2+ was done after 4-h treatment of neuroblastoma cells as above. Thapsigargin (1 μmol/L) was used as positive control. Results are expressed as mean fold increase of intracellular calcium concentration of treated cells with respect to untreated cells from five independent experiments; error bars, 95% CIs. *, P < 0.05; **, P < 0.01; ***, P < 0.001. PI, propidium iodide.
factor C/EBP homologous protein/GADD153 (31): this activity was higher than that detected when bortezomib or fenretinide was tested alone. On the other hand and in line with previous reports (22, 32), phosphorylation of eukaryotic initiation factor 2α, a key event in translational suppression, was transiently up-regulated by both the single agents and the combination of the two drugs (data not shown). Inositol-requiring enzyme 1α, one of the three distinct signaling pathways triggered in response to ER stress, has been recognized to activate the JNK pathway, possibly by direct phosphorylation of downstream targets (31, 33). In addition, the JNK is one of the principal executioners of the proapoptotic arm of ER stress response (29). We thus evaluated whether the active form (i.e., phosphorylation) of JNK was required for bortezomib-plus-fenretinide–mediated apoptosis. After the combined treatment, neuroblastoma cells displayed increased expression of the inositol-requiring enzyme 1α protein, leading to a strong induction and activation of JNK as well as of its target gene, c-jun. Moreover, both trypan blue dye exclusion assay and propidium iodide...
staining showed that pretreatment for 1 h of neuroblastoma cells with a JNK inhibitor significantly restored cell viability from bortezomib-plus-fenretinide–mediated cell death and apoptosis at 24 h (Supplementary Fig. S2A and B).

The apoptotic response to the combined bortezomib-plus-fenretinide effects on neuroblastoma cells was proved by the involvement of different caspases, such as caspase-4 (Fig. 3A). In addition, bortezomib plus fenretinide triggered the release of cytochrome c into the cytosol more potently than the individual drug treatments (Fig. 3B) and, consequently, the cleavage of caspase-9 was induced (Fig. 3A), indicating that, together with mitochondrial damage (Fig. 2C), the intrinsic apoptotic pathway was also involved in this gene activation cascade (34, 35). The level of procaspase-3 was also decreased in response to the combination treatment at 24 h together with a concomitant increase of its cleaved fragments. Finally, cleavage of poly(ADP)ribose polymerase seemed more evident in the combined treatment when compared with that caused by each single drug. Both caspase-2 and caspase-10 protein levels were not influenced by fenretinide and bortezomib (Fig. 3A).

Combined antitumor activity of bortezomib and fenretinide against human neuroblastoma xenografts. GI-LI-N cells were orthotopically injected into the adrenal gland of nude mice to mimic a highly angiogenic and aggressive pattern of human neuroblastoma disease as reported (24, 26).

Bortezomib, used at the maximal tolerated dose of 1 mg/kg, was found to produce significant inhibition of tumor growth in several different human xenograft models, including neuroblastoma (11, 29, 36, 37). On the other hand, fenretinide was usually given through oral route at high dosage (120-360 mg/kg) but with poor bioavailability (21) and, only recently, it has been developed as i.v. emulsion formulation, achieving high fenretinide plasma levels associated with better tolerability (38). By preliminary experiments, 1.5 mg/kg of fenretinide, emulsified in a bovine serum albumin–saline solution (23) and injected i.v., had good pharmacokinetics and was well tolerated (data not shown).

We therefore started to treat mice with 1 mg/kg bortezomib and 1.5 mg/kg fenretinide, delivered in combination or as a single agent by i.v. injection twice a week for 6 wk. This schedule was chosen because the 20S proteasome activity had been found to decrease and recover its normal function as long as at least 72 h separate the bortezomib doses (4). Unfortunately, these combined bortezomib-plus-fenretinide doses generated a systemic cumulative toxicity that led us to stop the treatment at the 4th wk even though surviving mice lived for >140 d (Fig. 4A). Next, we reduced the bortezomib dose to 0.75 mg/kg first (Fig. 4B) and the fenretinide dose to 1 mg/kg later (Fig. 4C), and we observed that in both schedules the two drugs, administered together, caused a significantly higher increase in life span compared with mice treated with either drug alone. Noteworthy, according to the dose reduction index values reported above, a better survival rate was observed after treatment with the lowest combined doses (Fig. 4C). No side effects were detected throughout these experiments.

Immunofluorescence analysis was carried out on tissue sections derived from orthotopic tumor-bearing mice sacrificed 1 d after the 5th wk of treatment. As shown in Fig. 5, Ki67 staining revealed that bortezomib plus fenretinide was able to statistically reduce neuroblastoma cell proliferation compared with individual drug treatment. This cellular inhibition translated into significant induction of tumor cell apoptosis as shown by double staining of xenograft sections with TUNEL and NB84a antibody, a specific marker of human neuroblasto-
toma cells. Furthermore, apoptosis was not detected in normal tissues, such as the kidneys, heart, lung, liver, and spleen (data not shown).

Combined effects of bortezomib and fenretinide on neuroblasto-
toma-triggered angiogenic responses. Next, we studied whether the antiangiogenic potential of both bortezomib and fenretinide (11, 39, 40) was increased when the drugs were administered in combination. Double staining of tumor tissue sections with TUNEL and antibodies against factor VIII or smooth muscle α-actin indicated that the combined treatment resulted in a noticeable induction of apoptosis of both tumor endothelial cells and pericytes in comparison with that found in tissue sections derived from mice, either untreated or treated with each single agent (Fig. 5). Moreover, a marked reduction of intratumoral vessel counts in tumors treated with bortezomib plus fenretinide compared with control mice and mice treated with the single drugs was observed (Fig. 6A and C).

To further validate these findings, we did the CAM assay, in which CAMs were implanted with tumor xenografts derived from mice orthotopically injected with GI-LI-N cells. After 96 h from the start of tumor specimen treatments, macroscopic observations unveiled fewer allantoic vessels that converged toward the implant in CAM treated with combined bortezomib plus fenretinide than in CAM treated with saline solution and in CAMs treated with either drug alone (Fig. 6B). These observations were confirmed by morphometric assessment of microvessel area (Fig. 6D).

Because we showed that part of the observed therapeutic effects, obtained through the combined treatment, depend, at least in part, on an antiangiogenic activity, we did experiments to investigate whether our treatment could also affect, in vitro and in vivo, the production of vascular endothelial growth factor (VEGF) molecule, being the main proangiogenic factor involved in neuroblastoma-induced angiogenesis. To this purpose, we determined the physiologic production of VEGF on a panel of neuroblastoma cells (SH-SY5Y, HTLA-230, GI-LI-N, ACN). Although all the neuroblastoma cells analyzed produced VEGF, even if at different levels (data not shown), we focused our attention on GI-LI-N cells, being those chosen for the orthotopic in vivo model. Supplementary Fig. S3A shows that only the combination of bortezomib and fenretinide down-modulated significantly the production of VEGF compared with control cells. To rule out the possibility that the decreased VEGF production could simply be a reflection of the cytotoxicity of the drugs, the raw data were normalized for the number of viable cells. The relevance of these results has been further confirmed by in vivo assaying of the effects of the combined treatment on VEGF expression. In this case, tissue sections of primary tumor masses, derived from untreated or treated mice, were excised on day 36 from the beginning of the treatment and were then stained for VEGF (C) expression. As shown, either bortezomib or fenretinide induced a significant decrease of VEGF-positive cells. Once again, the simultaneous administration of both drugs resulted in a stronger effect (B).
**Discussion**

The concept that proteasome itself has been selected as a target for cancer therapy comes from several studies that showed how proteasome inhibitor molecules represent excellent drug candidates, being able to trigger apoptosis in leukemic cells without affecting nontransformed cells (3). In this work, we have shown for the first time an increased antitumor efficacy of bortezomib on human pediatric neuroblastoma when administered in combination with fenretinide, one of the most promising synthetic retinoids tested in the clinic as a chemopreventive agent (41) but whose chemotherapeutic effects are still limited by low bioavailability in its oral formulation (16, 17).

Despite the current advances in treatment options, the clinical prognosis of aggressive neuroblastoma remains dismal. For these reasons, combination chemotherapy represents, so far, one of the major successes in oncology research, characterized by acceptable systemic toxicity and appreciable efficacy.

Herein, we show that the ability of both bortezomib and fenretinide to interfere with specific phases of the cell cycle (i.e., G2-M phases mainly for bortezomib and G1 phase mainly for fenretinide; refs. 3, 11, 42, 43) was markedly potentiated when the drugs were combined together for cellular treatment. This alteration relied on a significant block of cell cycle progression that drove neuroblastoma cells to enter the death route. This observed cell death was mainly due to apoptosis, in agreement with the apoptotic potential of either drug alone (11, 14, 22, 32). Caspase cascade, implicated in this cellular death, included the ER membrane–resident caspase-4, leading

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**Fig. 4.** BTZ and HPR induce in vivo antitumor activity. After 14 d from orthotopic Gl-LI-N cell inoculation, mice i.v. received 1 mg/kg BTZ and 1.5 mg/kg HPR individually or in combination (A; n = 8); 0.75 mg/kg BTZ and 1.5 mg/kg HPR individually or in combination (B; n = 8); 0.75 mg/kg BTZ and 1 mg/kg HPR individually or in combination (C; n = 16); or saline solution every 3 d for 6 wk. Survival of mice was monitored daily. Inset, absolute mean body weight in grams. Experiments have been done twice with similar results. P values were calculated through analysis of variance with Tukey’s multiple comparison test, and survival curves were compared with the use of Peto’s log-rank test.
us to hypothesize the involvement of a persistent ER stress response. On the contrary, subcellular calcium homeostasis was not disturbed by combined bortezomib plus fenretinide, at least, used at these low doses, even if it represents one of the major steps of ER stress–mediated apoptosis (29, 37). Furthermore, after treatment with the pharmacologic combination, neuroblastoma cells with polarized mitochondria statistically decreased, meaning that the intrinsic cell death pathway should be involved as well.

ER stress–mediated apoptosis is characterized by the activation and/or induction of either the most protective ER chaperone component of ER stress response, GRP78, or the critical executioners of the proapoptotic arm of ER stress response, such as GADD153, CASP4, and phospho-JNK (29). Here, we found that GRP78, GADD153, CASP4, and phospho-JNK were all induced or up-regulated when neuroblastoma cells were treated with bortezomib plus fenretinide, and this activity was more consistent with respect to the single drugs. Moreover, the intrinsic apoptotic route took place after cytochrome c release from depolarized mitochondria membrane, confirming that the antitumor and proapoptotic efficacy of each single agent (22, 29, 44) was increased when they were used in combination.

We showed that low doses of bortezomib and fenretinide reduced in vivo neuroblastoma growth through both antitumor and angiostatic effects when the two drugs were delivered in combination to orthotopically implanted neuroblastoma mice. Based on these data, we assumed that the increased antineoplastic efficacy of the combined therapy stems from a dual mechanism of action that affects tumor cells as well as the cells belonging to the tumor vasculature. Therefore, even the angiostatic activity reported to characterize both drugs (11, 40) was potentiated in this combined therapy. Note-worthy, it should be evidenced that either bortezomib and fenretinide doses chosen for the in vivo experiments are lower than those so far used in preclinical trials (11, 21, 37), ameliorating antineoplastic effects without increasing, or at least leaving unaltered, systemic toxicity.

In the light of what we have shown, the association of bortezomib with fenretinide gains more efficacy in triggering
antitumor effects on neuroblastoma cells when compared with the results obtained with the single monotherapy. This observed anticancer effectiveness derives from the inhibition of cell proliferation and the induction of apoptosis of tumor environment, including both tumor and vascular cells, and provide the rationale to draw new therapeutic strategy for treating human neuroblastoma.

Fig. 6. Effects of BTZ and HPR on angiogenesis in vivo. A, immunohistochemical analysis of tissue sections from the orthotopic tumors as in Fig. 6, stained for CD31 (to show endothelial cells). Cell nuclei were stained with DAPI. Results are also reported as the mean percentage of microvessel density obtained in three independent experiments (C). B, chick CAMs were implanted with tumor xenografts derived from mice injected orthotopically with GI-LI-N cells. Macroscopically, after treatment with BTZ and HPR, administered alone or in association, fewer blood vessels invaded xenografts than those observed in xenografts treated with saline solution. D, microvessel area was also calculated for the different treatments, from three independent experiments. Error bars, 95% CIs. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
Therapeutic Efficacy of Combined Therapy

The Combined Therapeutic Effects of Bortezomib and Fenretinide on Neuroblastoma Cells Involve Endoplasmic Reticulum Stress Response

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