Molecular Cancer Therapeutics

Targeting the Inhibitor of Apoptosis Proteins as a Novel Therapeutic Strategy in Medulloblastoma

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Abstract

Medulloblastoma is the most common malignant brain tumor of childhood. Novel therapeutic strategies are urgently needed to overcome cytotoxic resistance. We hypothesized that antiapoptotic signals contribute to resistance and that treatment with proapoptotic agents could increase the efficacy of conventional therapies. A PCR array was used to assess the status of the apoptotic signaling pathway in medulloblastoma cells after treatment with cytotoxic chemotherapy. Treatment with cisplatin led to the upregulation of antiapoptotic signals, including inhibitor of apoptosis proteins (IAP), in medulloblastoma cells. We subsequently investigated the synergistic effect of a small-molecule IAP inhibitor, LBW242, in combination with cisplatin and/or radiotherapy in three human medulloblastoma cell lines and 5 short term primary patient medulloblastoma cultures. The addition of LBW242 to chemotherapy resulted in significantly increased antitumor activity with a similar effect observed in combination with radiotherapy. Measurement of caspase-8 and -9 activity indicated that the synergy resulted from induction of both the intrinsic and extrinsic apoptotic pathways. Apoptosis was confirmed by Annexin V staining and activation of caspases 3/7. Xenograft models were used to evaluate the mechanism of action and efficacy *in vivo*. The combination therapy significantly reduced the tumor burden in a medulloblastoma xenograft model and TUNEL analysis in a medulloblastoma orthograft confirmed *in vivo* induction of apoptosis. These findings support the strategy of targeting IAPs in combination with cytotoxic therapy as a novel treatment strategy for patients with medulloblastoma. *Mol Cancer Ther; 11(12); 2654–63. ©2012 AACR.*

Introduction

Novel therapies for medulloblastomas are urgently needed. Medulloblastomas remain the most common malignant brain tumor of childhood, accounting for 20% of all pediatric CNS tumors (1). While survival rates have improved, treatment options are limited, necessitating the development of new therapeutic strategies. Two fundamental problems exist. First, patients with high-risk features, such as metastatic disease, unfavorable biologic features, or relapsed disease have poor to dismal clinical outcomes (2–6). Second, radiotherapy, which is the mainstay of current treatment regimens, results in substantial long-term morbidities including endocrinopathies, impaired cognition, vasculopathies, and the risk of second malignant brain tumors (7). While the addition of chemotherapy to treatment regimens has allowed for reductions in radiation doses for lower risk patients, attempts to eliminate radiation therapy have resulted in inferior outcomes (8).

Recent data suggests that activation of antiapoptotic signaling pathways may be a mechanism that leads to treatment resistance in medulloblastoma. Apoptosis is the specific, ubiquitous mechanism by which cells undergo a highly regulated death and an important mechanism by which cytotoxic drugs and radiation eliminate tumor cells (9, 10). Overexpression of antiapoptotic molecules or defective apoptotic signaling may result in resistance to cytotoxic therapies (11, 12). Many of the oncogenic pathways that have been implicated in the tumorigenesis of medulloblastomas directly modulate the apoptotic machinery. For example, a subset of medulloblastoma are driven by mutations in the sonic hedgehog (SHH) pathway, which has been shown to result in the prevention of cellular apoptosis (13). Other antiapoptotic signals, such as miR-21, have been shown to be universally upregulated across all medulloblastoma subgroups (4). The overexpression of antiapoptotic proteins has recently been shown to be critical to the development of medulloblastomas in a mouse model (14) and the efficacy of

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radiotherapy has been shown to depend on activation of the apoptotic pathway (15). In medulloblastoma samples, high levels of antiapoptotic proteins have been shown to correlate with tumor grade and poor outcomes (16, 17). Together, these data provide a compelling rationale for targeting the apoptotic pathway in medulloblastoma; however, this novel approach has not yet been tested as a therapeutic strategy.

We hypothesized that dysregulation of the apoptotic pathway in medulloblastoma cells may contribute to treatment resistance. We therefore sought to determine whether specific targeting of the apoptotic pathway in medulloblastomas may represent a rational therapeutic strategy, with the prospect of enhancing chemo- and radiotherapy responsiveness and improving patient outcome.

We show here that cytotoxic therapies lead to upregulation of antiapoptotic signals in medulloblastoma cells, including the inhibitor of apoptosis protein (IAP) cIAP-2. We and others have previously described a novel, small-molecule, orally bioavailable second mitochondria-derived activator of caspases (SMAC) mimetic, LBW242, which binds tightly to the BIR3 domain of IAPs at nanomolar concentrations, prevents binding to caspases, and overcomes the inhibitory effect of IAPs (18–21). We have previously shown that LBW242 crosses the blood–brain barrier and achieves high micromolar concentrations in tumors (21, 22). Combination with proapoptotic therapies led to an induction of apoptosis and suppression of tumor growth in glioblastoma models (21, 22). We show here that the addition of LBW242 successfully enhanced the proapoptotic and antitumor effects of radiotherapy and chemotherapy in medulloblastoma cell lines and primary patient tumor samples. *In vivo* treatment with LBW242 and cytotoxic chemotherapy led to increased levels of apoptosis resulting in a pronounced antitumor effect.

Targeting the apoptotic pathways in medulloblastomas offers a unique opportunity to develop novel therapeutic strategies that overcome tumor resistance and reduce the toxicity of conventional therapies. These results are readily translatable to clinical trial and offer the potential for improved treatment outcomes for patients with medulloblastoma.

Materials and Methods

Cell lines and reagents

Human medulloblastoma cell lines Daoy, D283, and UW228 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life technologies) with 10% fetal calf serum incubated at 37°C in 5% CO_2. Daoy cells were obtained from the American Type Culture Collection where they were authenticated by short tandem repeat (STR) profiling and passaged for less than 6 months. UW228 were obtained from Queensland Children's Tumor Bank (Royal Children's Hospital, Brisbane, Australia) and identified as a unique cell line by STR profiling on May 14, 2012. D283 cells were obtained from D. Binger

(Duke University, Durham, NC) and authenticated by STR profiling on May 14, 2012. UW228 and D283 cells were passaged for less than 6 months from the receipt of original stocks. Short-term primary cultures were prepared from fresh medulloblastoma samples finely minced with a scalpel and cultured in DMEM/F12, 10% FBS, penicillin/streptomycin, and Fungizone (Invitrogen) until explant growth was established. Explants were then trypsinized and separated into single-cell suspensions. Once a monolayer culture had been established and successfully cryopreserved, subsequent passages were grown in DMEM/F12, 10% FBS without antibiotics. Cell were passaged and used for experiments to a maximum of passage 10.

Drugs and irradiation

LBW242 was generously provided by Novartis Pharma. Stock solutions of LBW242 were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO), stored at −20°C, and diluted in fresh medium immediately before use. Cisplatin and vincristine (Pfizer; Fig. 1D) were obtained from the Prince of Wales Hospital pharmacy (Randwick, NSW) and diluted in saline before use. Cells were irradiated using an XRAD320 biological irradiator, calibrated to deliver the indicated doses.

RT-PCR and apoptosis array

RNA was extracted from irradiated Daoy and cisplatin-treated Daoy, D283, and UW228 cells with Trizol reagent (Life technologies) following manufacturer's protocol. cDNAs were synthesized from 1 μg of RNA using random hexanucleotide primers and Moloney murine leukemia virus reverse transcriptase. The TaqMan Human Apoptosis Array was conducted using the Applied Biosystems 7900HT Fast Real-Time PCR System as per manufacturer's protocol with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control. For independent replication, gene expression was determined by real-time PCR using TaqMan method for Daoy RNA and SYBR Green for D283 and UW228 RNA. Primer sequences were searched for homology with sequences in the NCBI human genomic database using the BLAST tool to ensure specificity. *Gus-B* gene was used as an internal control for cisplatin-treated samples and GAPDH for irradiated samples. PCR was conducted with a Prism 7900 Sequence Detection System (Applied Biosystems), and the level of target gene expression was determined using the $\Delta\Delta C_t$ method.

Western blotting

Cells were lysed, protein extracted, and separated by SDS-PAGE. After Western transfer, membranes were probed with rabbit anti-cIAP-2 monoclonal antibody (diluted 1:500), rabbit anti-XIAP monoclonal antibody (diluted 1:1,000) from Cell Signaling, goat anti-CIAP-1 polyclonal antibody (1:500) from RD Systems, followed by HRP-conjugated anti-rabbit antibody (1:2,000; Cell Signaling) and horseradish peroxidase-conjugated anti-goat
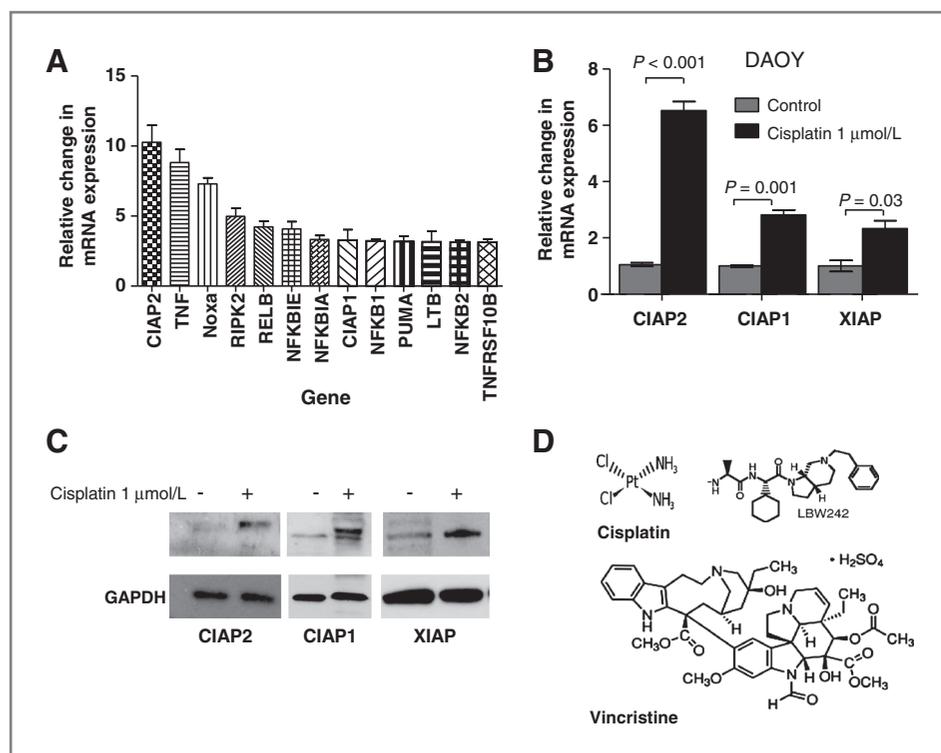
Figure 1. Treatment with cisplatin increases antiapoptotic gene expression in medulloblastoma cells. Daoy cells were treated with cisplatin and gene expression of the apoptotic pathway measured with a PCR array. A, 13 genes in the apoptotic pathway showed more than 3 times increase in expression (measured in triplicate, mean ± SEM). Change in expression of cIAP-2, cIAP-1, and XIAP were confirmed independently by PCR (B) and Western blot (C) in Daoy cells. Data points, mean ± SEM. P values = unpaired t-test (2-tailed). D, chemical structures of LBW-242, cisplatin, and vincristine.

antibody (1:2,000; Cell Signaling). Protein bands were visualized with SuperSignal (Pierce). The membranes were reprobed with anti-GAPDH antibody (Abcam) as a loading control.

Resazaurin cytotoxicity assay

In vitro drug sensitivity was assessed with a resazaurin (Sigma-Aldrich) assay. Cells were treated with LBW242 ± vincristine or cisplatin and the fraction surviving was calculated for each drug concentration. Synergy was evaluated by the method of Chou and Talalay (23) and the data subsequently analyzed by the median effect method using CalcuSyn software (Biosoft). Combination index (CI) values were calculated for each drug combination, where synergy is indicated by a CI less than 1, additive where CI is equal to 1, and antagonism where CI is more than 1.

Clonogenic assays

Hundred to five hundred cells were plated into 6-well plates and incubated for 24 hours. Cell were then treated with LBW242 and followed by cytotoxic drug treatment or radiation 6 to 8 hours later and incubated for further 10 days. Cells were then washed with PBS, fixed, and stained for 20 minutes with 0.5% crystal violet:methanol (1:1; Sigma-Aldrich). Colonies were counted and expressed as a percentage of untreated control.

Annexin V and propidium iodide assay

A total of 7.5×10^4 Daoy cells were plated in a 25-cm flask of medium, cultured for 24 hours, treated with the indicated dose of drugs or irradiation, incubated for an additional 3 days, collected for analysis, washed twice in cold PBS, and resuspended in 100 μL of Annexin-binding buffer containing A total of 2.5 μL of Annexin V-FITC and 2 μL propidium iodide (BD Biosciences), in accordance with the manufacturer's instructions. Before fluorescence-activated cell sorting (FACS) analysis, an additional 400 μL of binding buffer was added to the cell suspension. FACS analysis was conducted on CellQuest (BD Bioscience), and gated to exclude cellular debris; 10,000 events were collected for each sample.

Caspase activity assays

Caspases 8, 9, and 3/7 were measured using individual Caspase glo assays (Promega) in accordance with the manufacturer's protocol. Briefly, 1,000 cells per well were added to a 96-well plate in 100 μL of media, cultured for 24 hours, and then treated with the indicated concentrations of the drugs and incubated for 72 hours. 100 μL of labeling reagent were added to each well and luminescence measured after 3 hours on a plate reading luminometer.

Caspase inhibition assay

Daoy medulloblastoma cells were plated at 1,000 cells per well in a 96-well plate and incubated for 24 hours. Cells were then treated with cisplatin (1 μmol/L), LBW-242 (10 μmol/L), and a combination of both drugs in the presence or absence of caspase inhibitors [Z-IEDT-FMK, Z-DEVD-FMK (BD Biosciences) or Z-LEHD-FMK (R&D systems)] for 72 hours before determining survival using a resazaurin assay (Sigma-Aldrich).

Tumor cell line xenografts

Daoy tumor cells were harvested in log phase, resuspended in PBS, and injected subcutaneously or orthotopically in 6 to 8 week old BalbC nu/nu mice (ARC). Mice were anaesthetised with isoflourane and injected with 1.0×10^6 cells subcutaneously on the hind flank or orthotopically by injecting 1.5×10^5 cells in 5 μL PBS through a 27-gauge needle over 1 minute, 2 mm lateral, and 2 mm posterior to the bregma at 3 mm below the dura.

In vivo treatment

Treatment of subcutaneously xenografted mice commenced when tumor reached 50 mm³ by caliper measurement. Drugs were administered by intraperitoneal (i.p.) injection with LBW242 25 mg/kg/dose daily for 14 days ± cisplatin (2 mg/kg) or vincristine (0.2 mg/kg) for the first 5 days. Tumors were measured every second day and mice euthanized when tumors reached 1,000 mm³. Treatment of orthotopically xenografted mice commenced when mice displayed symptoms of tumor burden (i.e., head tilting, circular movements, and weight loss), and were treated with 25 mg/kg LBW242 i.p. ± 2 mg/kg cisplatin i.p. for 72 hours and then euthanized. Brains were removed and fixed in 10% buffered formalin before paraffin embedding.

All animal studies were conducted under protocols approved by the University of New South Wales Animal Care and Ethics Committee.

In situ cell death detection

Apoptosis was detected in tissue sections using an *In situ* cell death detection kit, POD (Roche) as per manufacturer's instructions. Briefly, formalin-fixed, paraffin-embedded sections of brain tissue were dewaxed and rehydrated by heating to 60°C followed by washing in xylene and rehydrating through a graded series of ethanol. Slides were then pretreated by microwave irradiation for 2 minutes in 0.1 mol/L citrate buffer, pH 6.0 (Sigma-Aldrich). TUNEL reaction mix was applied for 60 minutes at 37°C before addition of POD converter and DAB substrate. Slides were counterstained with hematoxylin and mounted with glycerol. The total number of apoptotic cells was quantitated in 5 fields of view in sections from 3 individual tumors for each treatment group.

Statistical analysis

Data were analyzed with GraphPad Prism 5 using an unpaired Student t test. All tests are two tailed. P values less than 0.05 were considered to be statistically significant. Results are displayed as mean ± SEM.

Results

Cytotoxic therapy upregulates antiapoptotic signals

Tumor cells are subject to a variety of proapoptotic signals, which are counterbalanced by a number of antiapoptotic mechanisms. We hypothesized that the treatment of medulloblastoma cells with cytotoxic therapies may lead to upregulation of antiapoptotic signaling

mechanisms that directly counterbalance the proapoptotic effects of the therapy. To elucidate the effects of cytotoxic therapy on the apoptotic signaling pathway, we treated the Daoy medulloblastoma cell line with cisplatin—a critical cytotoxic component of standard medulloblastoma chemotherapy regimens. To comprehensively assess the effects of the cytotoxic treatment on apoptosis signaling, we measured changes in mRNA expression of a broad array of pro- and antiapoptotic mediators using a 93 chamber RT-PCR format as previously described (24). Cisplatin was found to directly increase, by greater than 3-fold, the RNA expression of 13 proteins in the apoptotic signaling pathway, with a greater than 10-fold increase in expression of cIAP-2 (Fig. 1A). cIAP-2 is one of 8 members of the IAP family of proteins that represent the final molecular safeguard against caspase activation, and hence act to prevent extrinsic or intrinsic apoptotic pathway–mediated cell death. An IAP protein with similar function, cIAP-1, was also upregulated following cisplatin treatment (Fig. 1A). An independent quantitative PCR analysis confirmed significantly increased expression of cIAP-2 following cisplatin treatment (Fig. 1B). Western blot confirmed that the enhanced transcriptional activity led to substantially increased protein levels of cIAP-2 48 hours following cytotoxic treatment (Fig. 1C). Furthermore, mRNA expression analysis indicated significantly increased transcript levels of cIAP-1 and XIAP following cisplatin treatment (Fig. 1B) and Western Blot showed similarly increased protein levels (Fig. 1C). We next independently assessed the effects of cisplatin on the 3 IAP proteins cIAP-1, cIAP-2, and XIAP in 2 additional medulloblastoma cell lines D283 and UW228. cIAP-2 was significantly increased in D283 and cIAP-1, and XIAP were significantly increased in UW228 following cytotoxic treatment (Supplementary Figs. S1A and S1B).

In view of the upregulation of IAPs following cytotoxic treatment in medulloblastoma cells, we next sought to explore whether using an IAP inhibitor may enhance the efficacy of standard therapy. To achieve this, we treated 3 medulloblastoma cell lines with a combination of cytotoxic chemotherapy and the small-molecule IAP inhibitor, LBW242 (Fig. 1D), at varying drug concentrations. We measured cellular viability after 72 hours incubation. As a single agent, LBW242 showed varying degrees of cytotoxicity, but, when combined with cisplatin, resulted in cytotoxicity over and above that observed with either LBW242 or cisplatin alone, in all the 3 cell lines tested (Fig. 2A–C). A similar effect was observed with the combination of vincristine and LBW242 in the Daoy cell line (Fig. 2D). To determine the type of interaction effect, a combinatorial index was calculated using Calcusyn software, as described. The interaction of LBW242 with cisplatin was synergistic in all cell lines at all dose levels tested (ED50, 75 and 90) apart from ED90 in the D283 cells, in which the interaction was additive (Table 1). Similarly, the combinatorial index indicated a synergistic interaction for LBW242 combined with vincristine in the Daoy cells at the 3 dose levels tested (Table 1).
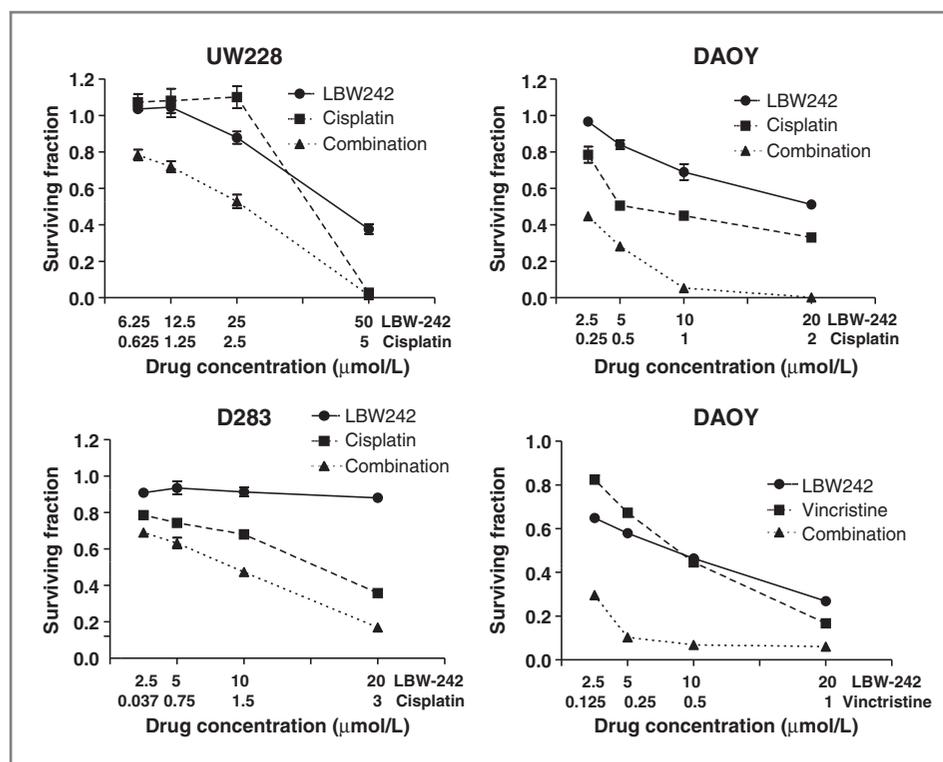
Figure 2. IAP inhibition increases the efficacy of cytotoxic chemotherapy. Medulloblastoma cells treated with LBW242 and cisplatin (A–C) or vincristine (D) at fixed ratios show enhancement of the effect of chemotherapy following addition of the IAP inhibitor. Experiments carried out in triplicate. Data points, mean ± SEM.

Enhancing the efficacy of cytotoxic chemotherapy may improve treatment outcomes, but it is the clinical use of high doses of radiotherapy that has the most profound long-term effects on survivors of childhood medulloblastoma. Because radiation therapy is known to activate the intrinsic apoptotic pathway, we postulated that the therapeutic strategy could be extended to the combination of

Table 1. Combinatorial indices (CI) for medulloblastoma cell lines and short-term cultures treated with LBW242 and cisplatin ([a]or vincristine) at ED50, 75 and 90

	Combinatorial index		
Cells cultured	ED50	ED75	ED90
Daoy	**0.05**	**0.2**	**0.69**
D283	**0.45**	**0.61**	0.98
UW228	**0.52**	**0.47**	**0.43**
Daoy[a]	**0.091**	**0.7**	**0.4**
R001	1.15	1.1	1.07
R029	1.15	**0.92**	**0.91**
R034	**0.36**	**0.44**	**0.59**
R060	**0.35**	**0.24**	**0.16**
R026	**0.73**	**0.68**	**0.64**

NOTE: CI was calculated using the Calcusyn program, where a CI < 1 (bold) indicates synergy, CI of 1 (±0.2) indicates an additive effect and CI > 1 indicates an antagonistic effect.

an IAP inhibitor with radiotherapy. We therefore assessed whether the treatment of medulloblastoma cells with radiotherapy also led to upregulation of antiapoptotic signals. PCR measurement showed that radiotherapy led to increased transcription of cIAP-1, cIAP-2, and XIAP (Supplementary Fig. S1C), with correspondingly increased protein levels (Fig. 3A). We next assessed the effect of radiation therapy on medulloblastoma cells that had been pretreated with LBW242. Both radiotherapy and LBW242 significantly decreased the formation of colonies as single agents in all 3 cell lines tested. A statistically significant further reduction in clonogenicity was observed when the cells were treated with the combination of irradiation and LBW242 (Fig. 3B–D). Supplementary Figure S2A shows representative photos of plates of UW228 colonies treated with LBW242 ± radiation. The combinatorial index was measured, and was either additive or synergistic all cell lines (Supplementary Table S1). Together, these data show that IAP inhibition can enhance the efficacy of cytotoxic chemo- and radiotherapy in medulloblastoma cells.

IAP inhibition is effective is primary patient-derived medulloblastoma specimens

It has been suggested that the prolonged passage of tumor cell lines in culture can result in genetic perturbations that result in different *in vitro* characteristics by comparison with *in situ* tumors. Therefore, to further extend our findings, we next tested the efficacy of the combination strategy on primary patient-derived

Figure 3. IAP inhibition enhances the activity of radiotherapy. A, representative Western blot of cIAP1, cIAP2, and XIAP in Daoy cells treated with 8 Gy irradiation for 72 hours. B–D, clonogenic assays in medulloblastoma cell lines (Daoy, UW228, D283) show enhancement of the effect of radiotherapy (2 Gy: Daoy, D283; 4 Gy: UW228) by addition of 1 μmol/L LBW242. Experiments carried out in triplicate; P values = unpaired t tests (2-tailed). Error bars, SEM.
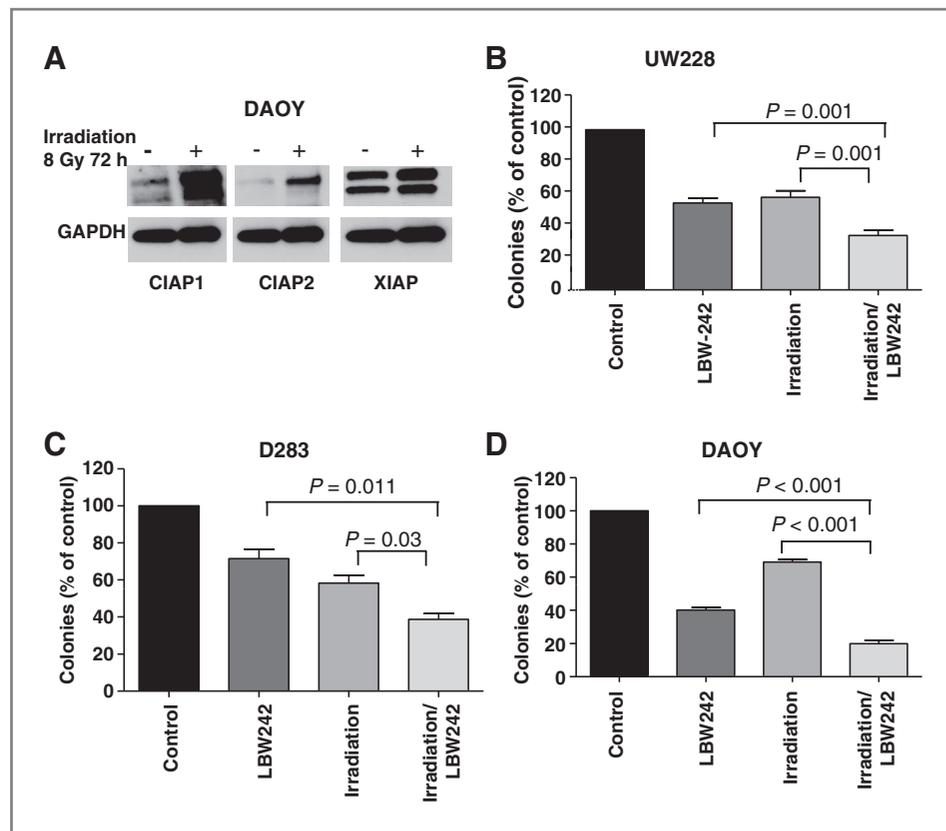
medulloblastoma specimens. Short-term medulloblastoma cultures were established from 5 primary patient samples taken at diagnosis (Supplementary Table S2). All experiments were carried out on primary cell cultures that had undergone fewer than 10 passages. The same combination of cytotoxic drug therapy (cisplatin) and LBW242 that had been used with medulloblastoma cell lines was used in the patient-derived cultures, with assessment of effect on cellular proliferation and apoptosis. As shown in Table 1, the combination therapy was additive or synergistic in all 5 primary patient samples tested. The relative ID50s for cisplatin and LBW242 used as single agents or in combination therapy are shown in Supplementary Table S3. As expected, the concentrations required for LBW242 and cisplatin were higher in the more resistant primary cell cultures, however, the addition of LBW242 reduced the required concentration of cisplatin approximately 2-fold across the range of cultures assessed.

LBW242 combines with cytotoxic drug treatment to activate the intrinsic and extrinsic apoptotic pathways

To confirm the mechanism of interaction between LBW242 and cytotoxic therapies, we next sought to interrogate the apoptotic signaling pathway. To determine whether the combinatorial effect was secondary to an induction of apoptosis, we used Annexin V staining of

medulloblastoma cells as a marker of activation of the apoptotic pathway. While both LBW242 and cisplatin triggered some degree of apoptosis as single agents, the combination therapy dramatically increased the number of cells undergoing programmed cell death (Fig. 4A). Similar results were seen with the combination of radiotherapy and LBW242 (Supplementary Fig. S2B). To confirm that the induction of cell death was due to activation of caspase-induced apoptosis, we next measured the activity of caspases 3 and 7. At 48 hours of treatment, the combination of either cisplatin or radiotherapy with LBW242 therapy led to a profound elevation of caspase activity, confirming the enhancement of the effector caspases 3/7 activity as the mode of apoptosis induction (Fig. 4B, Supplementary Fig. S2C).

Both chemotherapy and radiotherapy are known to activate the intrinsic apoptotic pathway. However IAPs inhibit the activity of both pathways, and recent evidence suggests that SMAC mimetics that overcome IAP inhibition can directly lead to extrinsic pathway activation via independent mechanisms (25). To further elucidate the mechanism of action we used caspase-8 and -9 activity as surrogate markers for extrinsic and intrinsic pathway activation, respectively. While neither cisplatin nor LBW242 alone led to substantial caspase activity, the combination therapy raised activity levels of both caspase-8 and -9 significantly, suggesting the efficacy is a result of activation of both the intrinsic and extrinsic
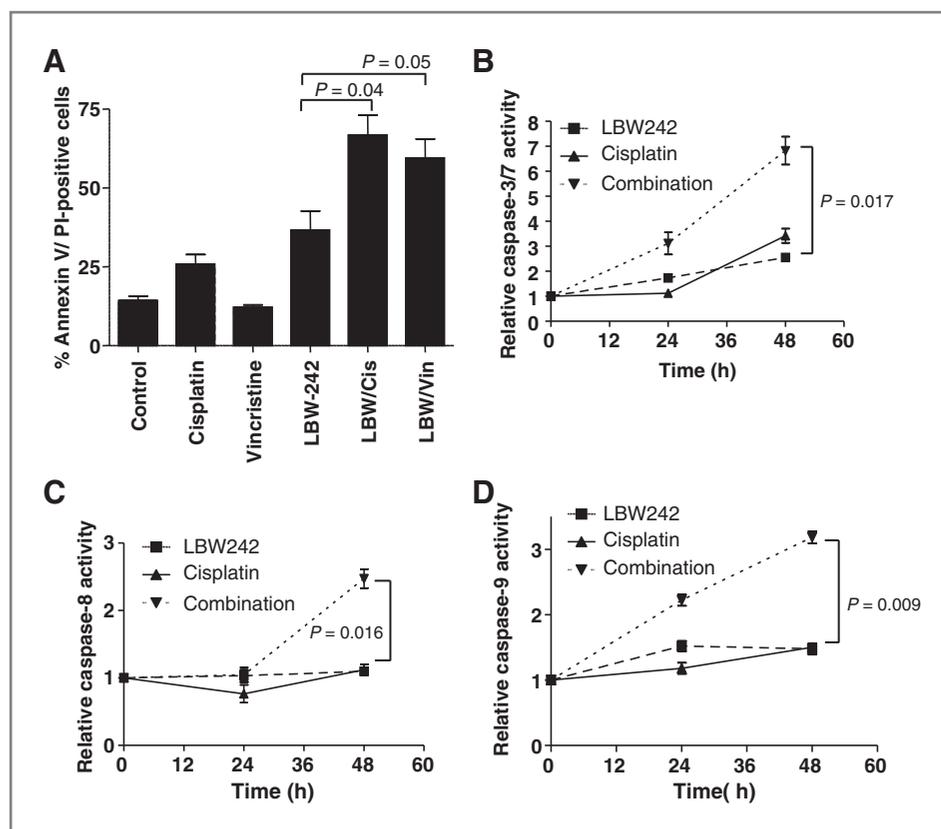
Figure 4. IAP inhibition combines with cytotoxic therapy to induce caspase activity and activate apoptosis. A, Daoy cells treated with 10 µmol/L LBW242 ± cisplatin (1 µmol/L) or vincristine (0.5 µmol/L) were quantitated after staining with Annexin V-fluorescein isothiocynate (FITC) and propidium iodide (PI). At 0, 24, and 48 hours following LBW242 (10 µmol/L) ± cisplatin (1 µmol/L), ELISA was used to detect caspase 3/7 (B), caspase-8 (C), and caspase-9 activity (D). *P* values compare the relative activity of LBW242 ± cisplatin at 48 hours. Data points, mean ± SEM, *P* values = unpaired *t* test (2-tailed).

apoptotic pathways (Figs. 4C, D). Similarly, while radiation therapy led to a slight increase in both caspase-8 and -9 activity, the addition of LBW242 led to significantly increased levels of both enzymes, indicating activation of both apoptotic pathways (Supplementary Figs. S2D and S2E).

To further validate the finding that both pathways are responsible for the induction of apoptosis, we next tested the ability of apoptosis inhibitors to rescue the cells from the effect of the combination treatment. The caspase-8 inhibitor Z-IETD-FMK, the caspase-9 inhibitor Z-LEHD-FMK, and the caspase-3/7 inhibitor Z-DEVD-FMK were each independently added to Daoy cells treated with cisplatin and LBW242. Caspase-3/7 inhibition led to the greatest rescue of the medulloblastoma cells, followed by caspase-9 and then caspase-8 inhibition (Supplementary Fig. S2F), consistent with the finding that activation of both intrinsic and extrinsic apoptotic pathways contribute independently to the proapoptotic effect of the combination therapy.

IAP inhibition combines with cytotoxic therapy in medulloblastoma xenografts

We next sought to extend these findings to an *in vivo* system using 2 independent medulloblastoma xenograft models. In the first model, Daoy cells were implanted subcutaneously in nude mice. Tumor burden was serially assessed by caliper measurement, and animals with estab-

lished tumors that reached 50 mm^3 in size were divided into treatment cohorts. One group was treated with cisplatin at 2 mg/kg/d by i.p. injection for 5 days, one group with LBW242 25 mg/kg/d i.p. for 14 days, one group with DMSO control, and the fourth group with LBW242 plus cisplatin. Consistent with the *in vitro* studies, while monotherapy with either LBW242 or chemotherapy alone had some effect, the animals treated with cisplatin and LBW242 had almost complete cessation of tumor growth that was sustained for the duration of the experiment (Fig. 5A). Tumor volume as measured at day 26 was significantly decreased in the combination group in comparison with animals treated with LBW242 alone. No significant toxicity was observed in any of the treatment groups.

To assess whether the combinatorial effect was due to the successful induction of apoptosis in the *in vivo* setting, we sought to mimic the clinical scenario using an orthograft model of medulloblastoma. Daoy cells were implanted intracranially in nude mice that were then treated with LBW242, cisplatin, DMSO, or combination therapy. Treatment was commenced when the animals started to display clinical signs of tumor progression. Following 3 days of treatment mice were sacrificed, and their tumors harvested, fixed, and examined for evidence of apoptosis using TUNEL staining. Tumors harvested from mice treated with either LBW242 or cisplatin alone showed minimal induction of apoptosis, however, those obtained from animals treated with combination therapy
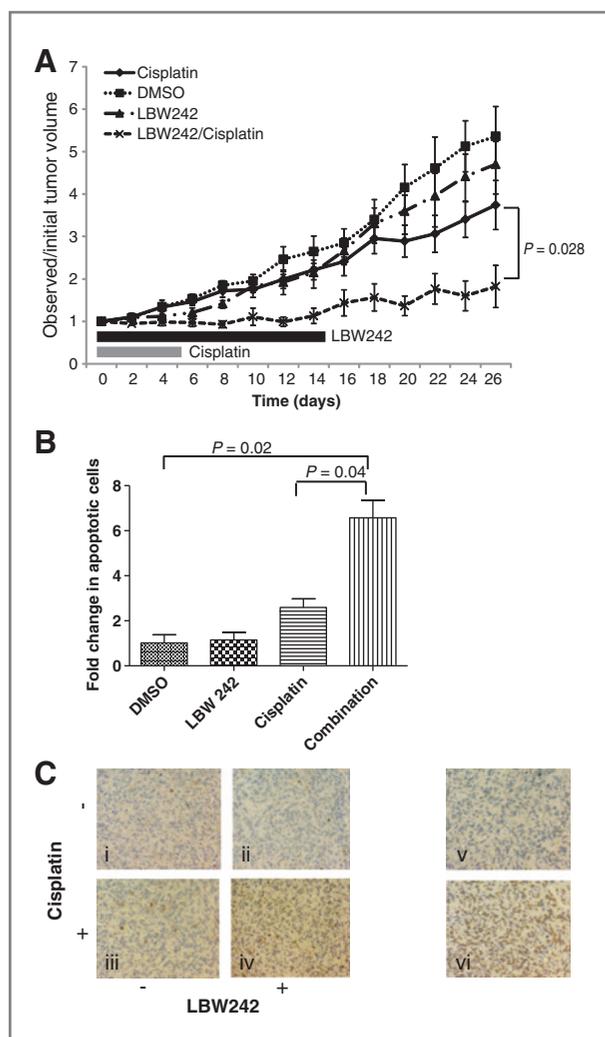
Figure 5. IAP inhibition induces apoptosis and enhances the effect of cytotoxic chemotherapy *in vivo*. A, Daoy medulloblastoma cells were injected in the hind flank of Balb c *nu/nu* mice. Cohorts of 6 to 8 animals were treated with LBW242 (25 mg/kg/d) for 14 days $\pm$ cisplatin (2 mg/kg/d) for 5 days. Data points, mean tumor volume $\pm$ SEM. Tumor volumes after 26 days treatment were compared with an unpaired *t* test (2-tailed). B, apoptosis was detected in formalin-fixed paraffin-embedded tumor tissue harvested from orthografts of mice treated with LBW242 $\pm$ cisplatin using a Roche In Situ Cell Death Detection Kit. Total numbers of apoptotic cells were quantitated in 5 fields of view in sections from 3 individual mice. Error bars represent SE; *P* values calculated by unpaired *t* test (2-tailed). C, representative images of tumor sections: mice treated with: (i) DMSO, (ii) LBW242 (10 mg/kg), (iii) cisplatin (0.1 mg/kg), (iv) LBW242/cisplatin combination, (v) unstained control, (vi) DNAse-treated positive control.

showed a statistically significant increase in apoptotic cells (Fig. 5B and C). To assess for nonspecific activity and to determine the potential for toxicity, we examined the surrounding normal brain tissue for increased TUNEL staining. There was no evidence of apoptosis induction in the normal brain tissue of any of the treatment groups (data not shown). These results show that IAP inhibition successfully enhances the effect of cytotoxic therapy in

xenograft models of medulloblastoma by overcoming resistance to the induction of apoptosis.

Discussion

To our knowledge, this is the first study to show the potential efficacy of targeting the IAP proteins as a therapeutic strategy in medulloblastoma. We have shown that cytotoxic therapies lead to upregulation of antiapoptotic signals, and that a small-molecule proapoptotic IAP inhibitor can enhance the efficacy of cytotoxic therapies *in vitro*, in primary patient samples, and in the *in vivo* setting. This novel treatment approach has the potential to overcome the intrinsic resistance of medulloblastoma cells to the effects of radiation therapy and chemotherapy and may ultimately improve patient survival. Moreover, by enhancing the efficacy of cytotoxic therapies, it offers the opportunity to reduce the dosage and dose intensity of cytotoxic therapies, particularly radiation therapy, and thus reduce short-term and long-term treatment side effects.

Increasing preclinical evidence suggests that perturbation of the apoptosis signaling pathway is a tumorigenic factor in medulloblastoma, may in some cases be a prognostic indicator, and can result in resistance to standard cytotoxic therapies and treatment failure. However, strategies that use novel compounds that specifically induce apoptosis to enhance the efficacy of cytotoxic therapies in medulloblastoma have not previously been tested. The preclinical rationale for developing this treatment has been shown in other malignant brain tumors in similar circumstances. Overcoming antiapoptotic mechanisms has been shown to be a potentially effective therapeutic strategy in highly resistant brain tumors such as glioblastoma multiforme (GBM). Indeed, the plausibility of targeting the IAPs was first shown using a peptide SMAC mimetic in an *in vivo* model of GBM (26). While peptides cannot be used clinically, we have previously shown that this same strategy can be used by using small-molecule IAP inhibitors that are more tractable for clinical translation (21, 22). The results here show the potential efficacy for this strategy in medulloblastoma—the most common malignant brain tumor in children. The small-molecule IAP inhibitor used in these studies is orally bioavailable and penetrates the blood–brain barrier, suggesting the potential for rapid translation to the clinic. Several small-molecule IAP inhibitors are currently undergoing early-phase testing in clinical trials in adult patients. The rationale now exists for extension to the pediatric population and pediatric medulloblastoma in particular.

Increasing attention has focussed in recent years on developing targeted therapies in medulloblastoma. In particular, research has focussed on implementing strategies that inhibit the pathways known to be particularly active tumorigenic drivers in specific medulloblastoma subtypes—namely the SHH pathway and the Wnt pathway. The advanced clinical development of specific inhibitors of these pathways has helped drive their investigation in early-phase clinical trials. The demonstration

that a small-molecule SHH inhibitor was able to eradicate widespread metastatic disease in a multiply relapsed patient has generated a high level of enthusiasm for this approach (27). However, the potential efficacy of these compounds is mitigated by the fact that tumors with SHH and WNT pathway activation represent only a minority of cases, and these patients already have favorable prognoses with standard therapy (3–5). The approach we have shown of targeting the apoptotic pathway offers an alternative targeted strategy that may be applicable more broadly to more resistant medulloblastoma subsets, including those with poorer prognoses. A variety of techniques have been used to divide medulloblastoma into different genetic subgroups, and thus help with clinical stratification (3–5). While IAP expression has not specifically been reported in these analyses, all subgroups have been shown to have activation of anti-apoptotic mechanisms (4). The ultimate initiation of apoptosis is understood to depend on the relative balance of pro- versus antiapoptotic signals within the cellular matrix (28). Thus, irrespective of which particular antiapoptotic proteins are overexpressed in any individual tumor cell, (or conversely, which proapoptotic signals are underexpressed) the addition of a proapoptotic small molecule to standard treatments may be sufficient to tip the balance in favor of apoptosis. Thus, the therapeutic strategy shown here may be applicable to the broad range of medulloblastoma phenotypes and subgroups. This is supported by our results showing that the combination therapy was additive or synergistic in 7 of 8 cell cultures tested, including cell lines and primary patient samples.

Importantly, the strategy that we have tested incorporates a novel agent with standard cytotoxic agents routinely used in medulloblastoma treatment regimens. It is likely that improved treatment outcomes will only be achieved via the development of rational combination therapies. Combinations that use 2 or more novel agents are difficult to translate to the clinic due to ethical barriers, toxicity concerns, and regulatory issues, particularly in the pediatric population. An approach, such as the one tested here that combines novel therapies with well-established cytotoxic therapies mitigates against these concerns. Furthermore, by using a combination that employs agents used as standard therapies, a clear path for translation to clinical trial in newly diagnosed patients can be easily defined. These results also offer potential for the evaluation of similar therapeutic strategies in other embryonal malignancies. It is notable that the activity of LBW242 in combination with cytotoxic chemotherapy has recently been shown in neuroblastoma—another common embryonal tumor of childhood (29).

In conclusion, we have shown a novel, targeted approach to enhance the efficacy of standard cytotoxic therapies in medulloblastoma. The use of a small-molecule, orally bioavailable IAP inhibitor results in increased levels of apoptosis and a greater antitumor effect of cytotoxic therapy in the *in vitro* and *in vivo* setting. The clinical development of IAP inhibitors ensures that this approach is readily translatable to the clinic with the potential to improve clinical outcomes and decrease long-term toxicities.

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