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 hypothesised that anti-apoptotic signals contribute to resistance and that treatment with pro-apoptotic agents could increase the efficacy of conventional therapies. A PCR array was used to assess the status of the apoptotic signalling pathway in medulloblastoma cells after treatment with cytotoxic chemotherapy. Treatment with cisplatin led to the upregulation of anti-apoptotic signals, including Inhibitor of Apoptosis Proteins (IAPs), 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 medulloblastoma patients.
Introduction

Novel therapies for medulloblastomas are urgently needed. Medulloblastomas remain the most common malignant brain tumor of childhood, accounting for 20% of all paediatric CNS tumors (1). While survival rates have improved, treatment options are limited, necessitating the development of new therapeutic strategies. Two fundamental problems exist. Firstly, patients with high risk features, such as metastatic disease, unfavourable biological features, or relapsed disease have poor to dismal clinical outcomes (2-6). Secondly, 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 anti-apoptotic signalling 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 anti-apoptotic molecules, or defective apoptotic signalling 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 demonstrated to result in the prevention of cellular apoptosis (13). Other anti-apoptotic signals, such as miR-21, have been shown to be universally up-regulated across all medulloblastoma subgroups (4). The over-expression of anti-apoptotic proteins has recently been shown to be critical to the development of medulloblastomas in a mouse model (14), and the efficacy of radiation therapy has been shown to depend on activation of the apoptotic pathway (15). In medulloblastoma samples high levels of anti-apoptotic 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 hypothesised 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 up-regulation of anti-apoptotic 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 pro-apoptotic 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 pro-apoptotic and anti-tumor 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 anti-tumor 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 medulloblastoma patients.
Materials and Methods

Cell Lines and reagents.

Human medulloblastoma cell lines Daoy, D283, and UW228 were cultured in DMEM (Life technologies) with 10% FCS incubated at 37°C in 5% CO₂. Daoy cell were obtained from ATCC 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) and identified as a unique cell line by STR profiling on 14/05/2012. D283 cells were obtained from D Binger, Duke University, NC, USA and authenticated by STR profiling on 14/05/2012. UW228 and D283 cells were passaged for less than 6 months from 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 trypsinised 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 utilized for experiments to a maximum of passage 10.

Drugs & Irradiation

LBW242 was generously provided by Novartis Pharma. Stock solutions of LBW242 were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis), stored at -20°C, and diluted in fresh medium immediately prior to use. Cisplatin and Vincristine (Pfizer) (Figure 1D) were obtained from Prince of Wales Hospital pharmacy (Randwick, NSW) and diluted in saline prior to 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, Grand Island, NY) 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 performed using the Applied Biosystems 7900HT Fast Real-Time PCR System as per manufacturer’s protocol with GAPDH as endogenous control. For independent replication gene expression was determined by real-time polymerase chain reaction (PCR) using Taqman method for Daoy RNA and SybrGreen 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, GAPDH for irradiated samples. PCR was performed with a Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA), and the level of target gene expression was determined using the ΔΔCt method.

Western Blotting

Cells were lysed, protein extracted and separated by SDS-page gel electrophoresis. After western transfer, membranes were probed with rabbit anti-cIAP-2 monoclonal antibody (diluted 1:500), rabbit anti-XIAP monoclonal antibody (diluted 1:1000) from Cell Signalling (Boston, MA), goat anti-CIAP-1 polyclonal antibody (1:500) from RD Systems (Minneapolis, MN) followed by horseradish peroxidase-conjugated anti-rabbit antibody (1:2000; Cell Signalling, Boston, MA) and horseradish peroxidase-conjugated anti-goat antibody (1:2000; Cell Signalling, Boston, MA). Protein bands were visualized with SuperSignal (Pierce, Rockford, IL). The membranes were re-probed with anti-GAPDH antibody (Abcam, Cambridge, UK) 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 data subsequently analysed by the median effect method using CalcuSyn software (Biosoft, Cambridge, UK). Combination index (CI) values were calculated for each drug combination, where synergy is indicated by a CI <1, additive where CI=1 and antagonism where CI>1.

Clonogenic assays

100-500 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 6-8 hours later and incubated for a 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, Propidium Iodide Assay

7.5 x 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 2.5 μL of Annexin V-FITC and 2 μL propidium iodide (BD Biosciences, San Jose, CA), in accordance with the manufacturer's instructions. Before FACS analysis, an additional 400 μL of binding buffer was added to the cell suspension. FACS analysis was performed 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, Madison, WI) in accordance with the manufacturer's protocol. Briefly, 1000 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 labelling 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 1000 cells / well in a 96 well plate and incubated for 24 hours. Cells were then treated with Cisplatin (1µM), LBW-242 (10µM) 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 prior to 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 6-8 week old BalbC nu/nu mice (ARC, Perth, WA). Mice were anaesthetised with isoflurane and injected with 1.0 x 10⁶ cells subcutaneously on the hind flank or orthotopically by injecting 1.5 x 10⁵ cells in 5 µL PBS through a 27-gauge needle over 1min, 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 calliper measurement. Drugs were administered by intraperitoneal (IP) 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 1000 mm³. Treatment of orthotopically xenografted mice commenced when mice displayed symptoms of tumor burden (i.e. head tilting, circular movements, weight loss), and were treated with 25mg/kg LBW242 IP +/- 2mg/kg cisplatin IP for 72 hours.
and then euthanized. Brains were removed and fixed in 10% buffered formalin prior to paraffin embedding.

All animal studies were performed 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, AG Switzerland) 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 pre-treated by microwave irradiation for 2 minutes in 0.1M citrate buffer, pH 6.0 (Sigma Aldrich). TUNEL reaction mix was applied for 60 min at 37°C prior to addition of POD converter and DAB substrate. Slides were counterstained with haematoxylin 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’s *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 up-regulates anti-apoptotic signals

Tumor cells are subject to a variety of pro-apoptotic signals, which are counterbalanced by a number of anti-apoptotic mechanisms. We hypothesised that treatment of medulloblastoma cells with cytotoxic therapies may lead to up-regulation of anti-apoptotic signalling mechanisms that directly counter-balance the pro-apoptotic effects of the therapy. To elucidate the effects of cytotoxic therapy on the apoptotic signalling 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 signalling we measured changes in mRNA expression of a broad array of pro- and anti-apoptotic 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 signalling pathway, with a greater than 10 fold increase in expression of cIAP-2 (Figure 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 up-regulated following cisplatin treatment (Figure 1A). An independent quantitative PCR analysis confirmed significantly increased expression of cIAP-2 following cisplatin treatment (Figure 1B). Western blot confirmed that the enhanced transcriptional activity led to substantially increased protein levels of cIAP-2 48 hours following cytotoxic treatment (Figure 1C). Furthermore, mRNA expression analysis indicated significantly increased transcript levels of cIAP-1 and XIAP following cisplatin treatment (Figure 1B) and Western Blot showed similarly increased protein levels (Figure 1C). We next independently assessed the effects of cisplatin on the three IAP proteins cIAP-1, cIAP-2 and XIAP in two 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 Figures 1A-B).
In view of the up-regulation of IAPs following cytotoxic treatment in medulloblastoma cells we next sought to explore whether utilising 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 (Figure 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 3 cells lines tested (Figure 2A-C). A similar effect was observed with the combination of vincristine and LBW242 in the Daoy cell line (Figure 2D). To determine the type of interaction effect, a combinatorial index was calculated utilising 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 three dose levels tested (Table 1).

Enhancing the efficacy of cytotoxic chemotherapy may improve treatment outcomes, but it is the clinical use of high doses of radiation therapy 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 an IAP inhibitor with radiation therapy. We therefore assessed whether the treatment of medulloblastoma cells with radiation therapy also led to upregulation of anti-apoptotic signals. PCR measurement showed that radiotherapy led to increased transcription of cIAP-1, cIAP-2 and XIAP (Supplementary Figure 1C), with correspondingly increased protein levels (Figure 3A). We next assessed the effect of radiation therapy on medulloblastoma cells that had been pre-treated with LBW242. Both radiation therapy and LBW242 significantly decreased the formation of colonies as single agents, in all three cell lines tested. A statistically significant further reduction in
clonogenicity was observed when cells were treated with the combination of irradiation and LBW242 (Figure 3B-D). Supplementary Figure 2A 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 1). Together, these data show that IAP inhibition can enhance the efficacy of cytotoxic chemo- and radio-therapy in medulloblastoma cells.

IAP inhibition is effective in 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 medulloblastoma specimens. Short term medulloblastoma cultures were established from 5 primary patient samples taken at diagnosis (Supplementary Table 2). All experiments were performed 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 utilised 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 3. 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 signalling 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 (Figures 4A). Similar results were seen with the combination of radiation therapy and LBW242 (Supplementary Figure 2B). 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 (Figure 4B, Supplementary Figure 2C).

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 caspase 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 apoptotic pathways (Figures 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 Figures 2D, E).

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 Figure 2F), consistent with the finding that activation of both intrinsic and extrinsic apoptotic pathways contribute independently to the pro-apoptotic 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 two independent medulloblastoma xenograft models. In the first model Daoy cells were implanted subcutaneously in nude mice. Tumor burden was serially assessed by calliper measurement, and animals with established tumors, that reached 50 mm³ in size, were divided into treatment cohorts. One group was treated with cisplatin at 2 mg/kg/day by IP injection for 5 days, one group with LBW242 25 mg/kg/day IP 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 (Figure 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.

In order 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 which 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 three 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 showed a statistically significant increase in apoptotic cells (Figures 5B, C). To assess for non-specific 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 any of the treatment groups (data not shown). These results demonstrate 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 demonstrate the potential efficacy of targeting the IAP proteins as a therapeutic strategy in medulloblastoma. We have shown that cytotoxic therapies lead to up-regulation of anti-apoptotic signals, and that a small molecule pro-apoptotic 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 signalling 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 employ 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 demonstrated in other malignant brain tumors in similar circumstances. Overcoming anti-apoptotic
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 demonstrated 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 employed utilising small molecule IAP inhibitors that are more tractable for clinical translation (21, 22). The results here demonstrate 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 bio-available 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 paediatric population, and paediatric 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 favourable 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 employed 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 anti-apoptotic signals within the cellular matrix (28). Thus irrespective of which particular anti-apoptotic proteins are over-expressed in any individual tumor cell, (or conversely, which pro-apoptotic signals are under-expressed) the addition of a pro-apoptotic small molecule to standard treatments may be sufficient to tip the balance in favour of apoptosis. Thus the therapeutic strategy demonstrated 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 out 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 employed in medulloblastoma treatment regimens. It is likely that improved treatment outcomes will only be achieved via the development of rational combination therapies. Combinations that utilise two or more novel agents are difficult to translate to the clinic due to ethical barriers, toxicity concerns and regulatory issues, particularly in the paediatric population. An approach, such as the one tested here, that combines novel therapies with well established cytotoxic therapies mitigates against these concerns. Further, by utilising 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 demonstrated in neuroblastoma – another common embryonal tumor of childhood (29).

In conclusion, we have demonstrated a novel, targeted approach to enhance the efficacy of standard cytotoxic therapies in medulloblastoma. The use of a small molecule, orally bio-
available IAP inhibitor results in increased levels of apoptosis and a greater anti-tumor effect of cytotoxic therapy in the \textit{in vitro} and \textit{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.

References


Table 1 Combinatorial indices (CI) for medulloblastoma cell lines and short term cultures treated with LBW242 and cisplatin (* or vincristine) at ED50, 75 and 90. CI was calculated using the Calcusyn program, where a CI < 1 indicates synergy, CI of 1 (+/- 0.2) indicates an additive effect and CI > 1 indicates an antagonistic effect.

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**Figure 1:** Treatment with cisplatin increases anti-apoptotic 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 > 3x 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.

**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 performed in triplicate. Data points = mean +/- SEM.

**Figure 3:** IAP inhibition enhances the activity of radiation therapy. (A) Representative western blot of cIAP1, cIAP2 and XIAP in Daoy cells treated with 8 Gy irradiation for 72hrs. (B-D) Clonogenic assays in medulloblastoma cell lines (Daoy, UW228, D283) show enhancement of the effect of radiation therapy (2 Gy: Daoy, D283; 4 Gy: UW228) by addition of 1uM LBW242. Experiments performed in triplicate; P values = unpaired t-tests (2-tailed). Error bars = SEM.

**Figure 4:** IAP inhibition combines with cytotoxic therapy to induce caspase activity and activate apoptosis. (A) Daoy cells treated with 10µM LBW242 +/- cisplatin (1 µM) or vincristine (0.5 µM) were quantitated after staining with Annexin V-FITC and propidium iodide (PI). At 0, 24 and 48 hours following LBW242 (10 µM) +/- cisplatin (1 µM), ELISA was used to detect (B) Caspase 3/7 (C) caspase 8 and (D) Caspase 9 activity. P values compare the relative activity of LBW242 +/- cisplatin at 48 hours. Data points = mean +/- SEM, P values = unpaired t-test (2-tailed).
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 6-8 animals were treated with LBW242 (25 mg/kg/day) for 14 days +/- Cisplatin (2 mg/kg/day) for 5 days. Data points = mean tumor volume, +/- 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 +/- 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 standard error; 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.
Figure 1

A

Relative change in mRNA expression

Gene

B

DAOY

Relative change in mRNA expression

Gene

C

Cisplatin 1uM - + - + - +

GAPDH CIAP2 CIAP1 XIAP

D

Cisplatin

Vincristine
Figure 2

**UW228**
- LBW242
- Cisplatin
- Combination

**DAOY**
- LBW242
- Cisplatin
- Combination

**D283**
- LBW242
- Cisplatin
- Combination

**DAOY**
- LBW242
- Vincristine
- Combination

Drug concentration (uM)
- UW228: 6.25, 12.5, 25, 50
- LBW242: 0.625, 1.25, 2.5, 5
- Cisplatin: 0.0, 0.25, 0.5, 1, 2, 5, 10, 20

Surviving fraction
- 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2

Drug concentration (uM)
- D283: 2.5, 5, 10, 20
- LBW242: 0.037, 0.75, 1.5, 3
- Cisplatin: 0.0, 0.125, 0.25, 0.5, 1, 2, 5, 10, 20

Surviving fraction
- 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2
Figure 3

A

Irradiation 8Gy 72 h

GAPDH
CIAP1 CIAP2 XIAP

B

UW228

Colonies (% of control)
P=0.001

Control LBW-242 Irradiation Irradiation/LBW242

C

D283

Colonies (% of control)
P=0.011

Control LBW242 Irradiation Irradiation/LBW242

D

DAOY

Colonies (% of control)
P<0.001

Control LBW242 Irradiation Irradiation/LBW242
Figure 4

A

% Annexin V/PI positive cells

Control | Cisplatin | Vincristine | LBW-242 | LBW/Cis | LBW/Vin

B

Relative caspase 3/7 activity

LBW242 | Cisplatin | Combination

C

Relative caspase 8 activity

LBW242 | Cisplatin | Combination

D

Relative caspase 9 activity

LBW242 | Cisplatin | Combination
Figure 5

A

![Graph showing observed/initial tumor volume over time for Cisplatin, DMSO, LBW242, and LBW242/Cisplatin treatments. The x-axis represents time in days (0-26), and the y-axis represents observed/initial tumor volume. The graph includes error bars and statistical significance (P=0.028).]

B

![Bar graph showing fold change in apoptotic cells for DMSO, LBW242, Cisplatin, and Combination treatments. The graph includes statistical significance (P=0.02 and P=0.04).]

C

![Images of tissue samples labeled i through vi. Images i and ii show Cisplatin treatment, images iii and iv show LBW242/Cisplatin treatment, and images v and vi show LBW242 treatment.]

Legend:

- Cisplatin
- DMSO
- LBW242
- LBW242/Cisplatin

Statistical significance:

- P=0.028
- P=0.04
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