Smac Mimetic LBW242 Sensitizes XIAP-Overexpressing Neuroblastoma Cells for TNF-α-Independent Apoptosis

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Abstract

Despite intensive treatment regimens, high-risk and late-stage neuroblastoma tends to have a poor survival outcome. Overexpression of the apoptotic regulator, X-linked inhibitor of apoptosis protein (XIAP), has been associated with chemotherapy resistance in several cancers including neuroblastoma. Here, we report preclinical evidence that XIAP offers an effective therapeutic target in neuroblastoma. Human and murine neuroblastoma cells were treated with the Smac mimetic LBW242 alone or in combination with cytotoxic drugs used clinically to treat neuroblastoma. Expression of XIAP protein, but not mRNA, was highly increased in neuroblastoma cells compared to healthy adrenal gland tissue, consistent with a posttranscriptional regulation of XIAP expression. Treatment with LBW242 sensitized human and murine neuroblastoma cells to chemotherapy-induced apoptosis, which was mediated by activation of both the intrinsic and extrinsic apoptosis pathways. Although Smac mimetics have been reported to stimulate TNF-α-induced apoptosis by degradation of cellular IAP (cIAP)-1/2, we found that LBW242-mediated sensitization in neuroblastoma cells occurred in a TNF-α-independent manner, despite induction of cIAP-1/2 degradation and TNF-α expression. Together, our findings show that XIAP targeting sensitizes neuroblastoma to chemotherapy-induced apoptosis, suggesting a novel therapeutic approach to treat this childhood malignancy. Cancer Res; 72(10); 1–12. ©2012 AACR.

Introduction

Neuroblastoma is the most common extracranial tumor during infancy and childhood originating from sympathetic nervous tissue. More than 50% of the patients initially present with metastatic stage IV disease. At the same time, outcome remains poor despite of intensive treatment regimens including megatherapy followed by blood stem cell rescue, differentiation therapy, and most recently also immunotherapy (1). Thus, the development of novel targeted therapy approaches is urgently needed (2, 3).

X-linked inhibitor of apoptosis protein (XIAP) is a major intrinsic cellular suppressor of apoptosis and XIAP overexpression has been shown in several malignancies. Deregulation of apoptosis is a hallmark of cancer and contributes to development and progression of various malignancies (4). Proteins of the IAP family are important regulators of apoptosis characterized by the presence of the highly conserved baculovirus IAP repeat (5). Overexpression of IAPs is associated with resistance against chemotherapy and poor outcome in several human cancers (6–8).

XIAP is the most potent and best characterized member of the IAP family and is thus considered to be a key regulator of apoptosis. XIAP directly inhibits the upstream caspase-9 and the downstream caspases-3 and -7 and, therefore, controls critical checkpoints of intrinsic and extrinsic pathways of apoptosis (9). According to its central role in regulation of apoptosis, XIAP expression is controlled at multiple levels. Transcriptional activation of XIAP expression is mainly regulated via the NF-κB pathway (10). Alternative translation initiation via an internal ribosomal entry site (IRES) located in the 5′ untranslated region of XIAP mRNA allows translational control of XIAP expression (11). At the protein level, XIAP activity is antagonized by at least 3 specific endogenous inhibitors (Smac/DIABLO, HtrA2/Omi, and XAF-1; refs. 12–14).

Second mitochondria-derived activator of caspase (Smac) promotes caspase activation after release from mitochondria in response to several apoptotic stimuli. Smac interacts with the BIR2 and BIR3 domains of XIAP via its N-terminal AVPI tetrapeptide motif. This motif has served as a structural template for the development of XIAP antagonists (Smac mimetics) that disrupt XIAP-caspase interactions (7, 15). Consequently, XIAP inhibition by Smac mimetics may represent a promising treatment strategy for malignant disease characterized by XIAP overexpression.

IAP inhibition by Smac mimetics results in induction of apoptosis and contributes to overcoming of resistance against chemotherapy in several hematologic malignancies and solid
tumors in vitro (7, 16). Blockade of XIAP-mediated caspase inhibition as well as induction of TNF-α expression following degradation of cellular IAP (cIAP) appear to contribute to the proapoptotic effect of Smac mimetics (17–19). The potential of Smac mimetics for the treatment of resistant malignancies is currently evaluated in several clinical trials (20).

Here, we show for the first time that XIAP expression is highly upregulated in neuroblastoma via transcriptional and posttranscriptional mechanisms and that targeted therapy with the Smac mimetic LBW242 results in sensitization of neuroblastoma cells against cytotoxic compounds currently used in neuroblastoma treatment regimens. These data establish a role of Smac mimetics as promising new agents in the treatment of resistant, thus challenging childhood malignancy.

Materials and Methods

Cell lines, patient samples

Cell lines were purchased from American Type Culture Collection or German Collection of Microorganisms and Cell Cultures (DSMZ). Informed consent was obtained from all patients and/or their guardians in accordance with the Declaration of Helsinki, and the investigation was approved by the ethics committee of the Charité - Universitätsmedizin Berlin (Berlin, Germany).

Chemical compounds, biologic reagents, and drugs

Smac mimetics LBW242 and LCL161 were generously provided by Novartis Pharma. Cytostatic drugs doxorubicin, etoposide, and vincristine were obtained from Sigma-Aldrich. Inhibitors targeting caspase-8 (Z-IETD; FMK007) and -9 (Z-LEHD; FMK008) and pan-caspase inhibitor (Z-VAD; FMK001) were purchased from R&D.

Cellular proliferation assays

Proliferation assays were conducted with Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer’s protocol. Cells were seeded in 96-well plates to adhere. Cytostatic drugs and LBW242 were added to the cells for an incubation period of 48 hours. Following incubation with WST-1 for 1 to 2 hours, absorbance was measured with an ELISA reader. IC50 and IC75 values were determined using in-house software (MS Excel).

Detection of apoptosis by flow cytometry

Cells were seeded in cell culture medium in 24-well plates to adhere. Cells were treated with the indicated reagents for 24 to 48 hours. Apoptosis was detected by Annexin V-allophycocyanin (APC; 550475; BD Pharmingen) and propidium iodide (PI; 1 mg/mL in ddH2O; Invitrogen) staining and flow cytometry. Detection of active caspase-3 was conducted using an FITC Active Caspase-3 Apoptosis kit (550480; BD Pharmingen) according to the manufacturer’s protocol.

Analysis of mitochondrial membrane potential (ΔΨm)

To measure the loss of the mitochondrial membrane potential (MMP), the cationic dye JC-1 (Invitrogen) was used according to the manufacturer’s protocol and detected by flow cytometry.

Detection of active caspases-8 or -9 with Caspase-Glo assays or green caspase staining kits

Active caspases-8 or -9 was detected using Caspase-Glo 8 and 9 assays (Promega) according to the manufacturer’s protocol or Green Caspase-8 or -9 Staining Kits (PromoKine) according to the manufacturer’s protocol and flow cytometry.

Protein extraction and Western blot analysis

Cell lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with complete protease inhibitor cocktail (Roche). Standard procedures for Western blotting were followed using the following antibodies: caspase-3 (#9662; Cell Signaling), cIAP-1 (AF8181; R&D), cIAP-2 (AF8171; R&D), PARP (#9542; Cell Signaling), and XIAP (ab21278; Abcam; all 1:1,000); and β-actin-peroxidase (A3854; Sigma-Aldrich; 1:10,000).

RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel) according to the manufacturer’s protocol. Equal amounts of total RNA was reverse-transcribed using oligo d(T)15 primers (Roche) and SuperScript III Reverse Transcriptase (Invitrogen) as per the manufacturer’s recommendations. Quantitative real-time PCR (qRT-PCR) was carried out using Fast SYBR Green Master Mix and a StepOnePlus instrument (Applied Biosystems) according to the manufacturer’s protocol. Specific amplification of cDNA was detected with SYBR Green and melt curve analysis. Relative expression of target genes was calculated as a ratio of the expression level of the target gene to that of 18S rRNA determined in the same sample using the comparative threshold (ΔΔCt) method.

Gene knockdown using antisense oligonucleotides

Cells were transfected for 48 to 72 hours in 24-well plates using DharmaFECT 2 (Thermo Scientific) and the transfection protocol as recommended from the manufacturer. For transient knockdowns, XIAP antisense (AS) oligonucleotide AEG35156 and scrambled control AEG35187 were generously provided by Aegera Therapeutics (21).

Microarray analysis

Data from Affymetrix HuEx1.0 arrays (GEO acc. no. GSE32664) were reanalyzed for XIAP expression. Exon level expression was recovered using Affymetrix power tools 1.10.0. In total, expression data from 101 primary neuroblastomas from the German and European clinical neuroblastoma trials were included in this study.

NXS2 mouse model of neuroblastoma

Syngeneic female A/J mice (6–8 weeks old) were obtained (Harlan Laboratories) and housed according to the German guide for the care and use of laboratory animals (Tierschutzgesetz). The NXS2 model was used as previously described (22). Briefly, primary tumors were induced by subcutaneous injection of 2 × 106 NXS2 cells. Cohorts of mice were treated 1 day
after induction of primary tumors 3 times a week with intraperitoneal administration of 0.9% NaCl or 1,000 µg/kg vincristine, oral administration of 40 mg/kg LCL161, or a combination of vincristine and LCL161. Primary tumor growth was analyzed by microcaliper, measurements and tumor volume was calculated according to the formula: \( \text{volume} = \left( \frac{1}{2} \times \text{length} \times \text{width} \right)^2 \). The weight of primary tumors was also determined after surgical removal.

**Statistics**

Combination indices (CI) for the cellular proliferation assays were calculated using the Chou–Talay method (23) to determine synergism (CI < 1), additivity (CI = 1), or antagonism (CI > 1), if combining LBW242 with cytostatic drugs. The CI was calculated according to the classic isobologram equation: \( \text{CI} = \left( \frac{d_1}{D_1} \right) + \left( \frac{d_2}{D_2} \right) \), where \( D_1 \) and \( D_2 \) represent the required doses of drug 1 and 2 to produce \( x \% \) effect and \( d_1 \) and \( d_2 \) the

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Figure 1. XIAP mRNA and protein expression in surgically removed neuroblastoma lesions and cell lines. XIAP exon 1 mRNA expression was analyzed by Affymetrix HuEx1.0 arrays in 101 patients with neuroblastoma (A). XIAP mRNA expression is significantly increased in relapsed patients (B) and in tumors with MYCN amplification (C). Amplif, amplification; CR, complete remission; wt, wild-type. D, expression of XIAP and survivin mRNA was analyzed by qRT-PCR in neuroblastoma cell lines and primary tumor samples and normalized to the respective expression in normal murine adrenal glands. Values represent the mean of 3 independent experiments ± SD. E, XIAP protein expression was analyzed by Western blot in neuroblastoma (NB) cell lines, primary tumor samples, and normal murine adrenal glands. MYCN +, MYCN-amplified tumor; **, \( P < 0.004 \).
required doses of drug 1 and 2 to produce the same effect if used in combination.

Statistical significance of differences between treatment groups was determined using a Student t test for in vitro data or a Wilcoxon rank-sum test for in vivo data. A 2-tailed P value ≤ 0.05 was considered as significant.

Results

Expression of XIAP in neuroblastoma

Microarray analysis of 101 tumor samples from children with neuroblastoma revealed consistent XIAP expression in all analyzed cases (Fig. 1A). XIAP mRNA expression was increased in patients suffering from relapse (P < 0.004) and tumors with MYCN amplification (Fig. 1B and C). To further examine regulation of XIAP expression in neuroblastoma, human [n = 2; Kelly (MYCN-amplified) and SK-N-SH (MYCN wild-type)] and murine [n = 2; NXS2 and C1300] neuroblastoma cell lines were analyzed by qRT-PCR for expression of XIAP mRNA. Murine adrenal glands (n = 5) served as a healthy tissue reference for basal expression levels, one of the main locations of neuroblastoma development (2). Interestingly, the analysis did not reveal relevant upregulation of XIAP mRNA expression in neuroblastoma cell lines compared with healthy adrenal gland tissue (Fig. 1D). In contrast, we found a 20- to 200-fold increase of survivin mRNA expression by qRT-PCR analysis, a well-known phenomenon in neuroblastoma (24). However,
Western blot analysis revealed a strong overexpression of the XIAP protein in all neuroblastoma cell lines and primary human neuroblastoma tissue samples (Fig. 1E). These results strongly suggest transcriptional and posttranscriptional upregulation of XIAP in neuroblastoma. On the basis of this observation, we analyzed the impact of XIAP inhibition by Smac mimetics as a strategy to improve the chemotherapeutic efficiency.

Inhibition of cell proliferation by combined treatment with LBW242 and chemotherapy in neuroblastoma

We first evaluated proliferation of murine (n = 2) and human (n = 2) neuroblastoma cells in the presence of increasing amounts of Smac mimetic LBW242 used as a monotherapy. Relevant inhibition of proliferation was only observed at very high concentrations of LBW242 in the micromolar range (Fig. 2A and Table 1). Because LBW242 is toxic to normal tissue at concentrations higher than 10 μmol/L (25), experiments addressing LBW242 combinations with cytotoxic therapy were carried out at 10 μmol/L. At this concentration, no relevant inhibition of cell proliferation was achieved by LBW242 alone.

Treatment of neuroblastoma cell lines with the topoisomerase II inhibitor etoposide showed a concentration-dependent inhibition of cell proliferation (0.1–5 μmol/L). Cotreatment with LBW242 was not able to enhance the antiproliferative potential of etoposide (Fig. 2B and Tables 1 and 2).

Doxorubicin inhibited the cell proliferation of neuroblastoma cell lines to a similar extent as etoposide in a concentration-dependent manner. Addition of LBW242 synergistically enhanced the antiproliferative effect of doxorubicin in all cell lines except NXS2 (Fig. 2C and Tables 1 and 2).

The vinca alkaloid vincristine revealed only a moderate effect on proliferation and the human neuroblastoma cell line Kelly was resistant. Here, addition of LBW242 resulted in a strong synergistic vincristine-induced inhibition of proliferation in all cell lines and was effective to overcome vincristine resistance in Kelly cells (Fig. 2D and Tables 1 and 2).

Sensitization of neuroblastoma cells for chemotherapy-induced apoptosis by LBW242

We next questioned whether LBW242-induced decrease in neuroblastoma cell proliferation is due to induction of apoptosis.

All cell lines tested were sensitive to etoposide-induced apoptosis. Combination with LBW242 resulted in a weak sensitization for etoposide only (Fig. 3A). Higher etoposide concentrations or LBW242 dose-escalation were not able to

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NOTE: CI values <1, =1, and >1 indicate synergism, additive effect, and antagonism, respectively.

Abbreviation: n.d., not determined.
further increase the LBW242 effect (Supplementary Fig. S1 and data not shown).

In 3 of the tested cell lines, only minor sensitization for doxorubicin-induced apoptosis was achieved by LBW242. In contrast to the other cell lines, the human neuroblastoma cell line SK-N-SH was highly susceptible for doxorubicin. In this cell line, LBW242 caused a distinct sensitization for doxorubicin-induced apoptosis (Fig. 3B).

According to the proliferation data, the most pronounced effect on apoptosis induction was observed for the combination of vincristine and LBW242. In all cell lines but NXS2 treatment with LBW242 resulted in a distinct sensitization for vincristine-induced apoptosis (Fig. 3C). Similar to etoposide, even escalation of LBW242 concentrations up to 40 \( \mu \text{mol/L} \) did not result in sensitization of NXS2 cells for vincristine-induced apoptosis (Supplementary Fig. S1).

To directly confirm a role of XIAP in the resistance of neuroblastoma cells against chemotherapy-induced apoptosis, we analyzed the effect of XIAP knockdown in vincristine-resistant Kelly cells. XIAP protein and mRNA expression was efficiently reduced by targeted knockdown using XIAP antisense oligonucleotide AEG35156 but not the scrambled control (AEG35187; Supplementary Fig. S2A and S2B). As expected,
XIAP Inhibition in Neuroblastoma

AEG35156 did not cause relevant cIAP-1 protein and mRNA knockdown (Supplementary Fig. S2C and S2D). XIAP knockdown by AEG35156 strongly enhanced apoptosis induction in Kelly cells, but no synergistic effect was detected in combination with vincristine (Supplementary Fig. S2E). Similarly, XIAP knockdown in SK-N-SH and SH-EP cells did result in strong induction of apoptosis alone and in addition to vincristine (Supplementary Figs. S3 and S4).

To further investigate the role of XIAP in the regulation of apoptosis in neuroblastoma cells, we selected the cell line Kelly as a model system. Comparison of apoptosis induction after 24 and 48 hours revealed that LBW242 enhanced vincristine-induced apoptosis in Kelly cells in a time-dependent manner (Fig. 3C and D).

Next, we decided to provide additional proof that LBW242 treatment results in stimulation of vincristine-induced apoptotic cell death. For this purpose, we determined cleavage of caspase-3 and its target PARP, which are critical hallmarks of apoptosis. Treatment with vincristine or LBW242 alone caused only weak cleavage of caspase-3 and PARP, correlating with the limited effect on induction of apoptosis. In contrast, simultaneous treatment of vincristine and LBW242 strongly induced cleavage of caspase-3 and PARP (Fig. 4A) in a time-dependent manner (Fig. 4B). Caspase-3 cleavage was almost completely inhibited by pan-caspase inhibitor Z-VAD (Fig. 4B), indicating that the effect of LBW242 is mediated by activation of upstream caspases.

Sensitization for vincristine-induced apoptosis by LBW242 was similarly reduced by inhibition of caspases-8 and -9, respectively. This finding was consistent with pan-caspase inhibition by Z-VAD, which completely abrogated the effect of LBW242 (Fig. 5A).

Activation of the intrinsic apoptosis pathway is mediated by complex mitochondrial processes resulting in the loss of MMP. Cotreatment of LBW242 and vincristine caused strong loss of MMP in a time-dependent manner indicating activation of the intrinsic apoptosis pathway (Fig. 5B). At the same time, the LBW242-mediated increase of vincristine-induced activation of caspase-9 was abrogated by caspase inhibition (Fig. 5C).

Furthermore, we show a time-dependent activation of the extrinsic apoptosis pathway reflected by activation of caspase-8 using LBW242 in combination with vincristine and again, this effect could be blocked by addition of the caspase inhibitor Z-VAD (Fig. 5D).

To confirm these findings in our Kelly model system, we investigated the effect of LBW242 on activation of intrinsic and extrinsic apoptosis pathways in 2 further human neuroblastoma cell lines. In SK-N-SH as well as SH-EP cells LBW242 significantly increased vincristine-induced activation of caspases-3, -8, and -9 (Supplementary Figs. S3 and S4).

Induction of TNF-α expression and degradation of IAPs by combined treatment with Smac mimetics and chemotherapy

Degradation of cIAP-1/2 and subsequent induction of TNF-α synthesis have been described as a molecular mechanism of Smac mimic–induced apoptosis in distinct cell types. Therefore, we explored whether LBW242 alone or in combination with vincristine results in degradation of cIAP-1/2 and induction of TNF-α expression in neuroblastoma cells.

Surprisingly, LBW242 alone did not cause significant degradation of cIAP-1/2 after 48 hours (Fig. 6A). Time course experiments revealed transient degradation of cIAP-1 (but not cIAP-2) after 30 minutes, but cIAP1 levels rapidly recovered thereafter (Fig. 6B). In contrast, combination of LBW242 and vincristine resulted in strong and sustained degradation of cIAP-1/2 after 48 hours (Fig. 6B).
cIAP-1/2 (Fig. 6A). As expected, no relevant XIAP degradation was induced by LBW242 in the presence and absence of vincristine. Similarly, only weak increase of TNF-α mRNA levels was detected following treatment with LBW242 or vincristine alone. In contrast, combined treatment of Kelly cells with LBW242 and vincristine resulted in an about 10-fold induction of TNF-α mRNA synthesis (Fig. 6C). Therefore, induction of apoptosis by combination of LBW242 and vincristine appears to be due to sustained degradation of cIAP-1/2 followed by induction of TNF-α expression.

Blockade of TNF-α by a specific monoclonal antibody (adalimumab; Humira) was not able to impede sensitization of Kelly cells for vincristine-induced apoptosis (Fig. 6D).

Consequently, treatment with LBW242 did not significantly sensitize Kelly cells for exogenous TNF-α (Fig. 6E). Induction of TNF-α expression was also detected after combination of LBW242 with etoposide (Supplementary Fig. S5). As LBW242 does not sensitize neuroblastoma cells for etoposide-induced apoptosis, this notion again supports the hypothesis that sensitization of neuroblastoma cells for chemotherapy by LBW242 does not occur in a TNF-α-dependent manner.

Again these findings were confirmed in 2 further human neuroblastoma cell lines. In SK-N-SH as well as SH-EP cells, TNF-α inhibition by adalimumab did not abrogate the LBW242-mediated sensitization for vincristine-induced apoptosis (Supplementary Figs. S3 and S4).

Figure 5. Influence of combined LBW242 and vincristine treatment on the activation of the intrinsic and extrinsic apoptosis pathways in neuroblastoma cells. MYCN-amplified Kelly cells were treated with the indicated concentrations of LBW242, vincristine (A–D), and 100 μmol/L caspase inhibitors (A, C, and D). The proportion of apoptotic cells was determined by flow cytometry 48 hours after treatment initiation (A). The proportion of cells with decreased MMP was determined by flow cytometry 24 or 48 hours after treatment initiation. Values represent the mean ± SD of 3 independent experiments (B). Active caspase-8 (C) or caspase-9 (D) was determined by luminometry 12, 24, or 48 hours after treatment. Luminescence in untreated cells was defined as 100%. Values represent the mean ± SD of 3 independent experiments (A and B) or triplicates (C and D). *P < 0.05. DMSO, dimethyl sulfoxide.
In Inhibition of tumor growth by LBW242 in a neuroblastoma mouse model

Finally, we evaluated the in vivo efficacy of LCL161, a closely related analogue of LBW242 (26), alone or in combination with vincristine in an NXS2 cell–based neuroblastoma mouse model established in our laboratory. In vivo treatment with LCL161 caused a strong reduction of tumor growth as determined by tumor volume and weight (Fig. 7A and B). LCL161 cooperated with vincristine resulting in a significant sensitization for vincristine-induced inhibition of primary neuroblastoma tumor growth in vivo (P < 0.05). Parallel analysis of body weight revealed no signs of treatment-related toxicity (data not shown).

These findings show antitumor activity of LCL161 together with vincristine in an in vivo neuroblastoma mouse model without detectable toxicity.

Discussion

Despite intensive chemotherapy regimens, the prognosis of children with stage IV neuroblastoma is poor (1, 2). The resistance against chemotherapy is most likely due to deregulation of apoptosis in neuroblastoma cells. Therefore, the development of strategies restoring the apoptotic program in resistant neuroblastoma cells may help to improve treatment of these patients.

There is accumulating evidence that XIAP plays a central role in the regulation of apoptosis sensitivity in cancer cells (5, 27–29). Here, we show for the first time that XIAP expression is highly elevated in neuroblastoma cells most likely contributing to the resistance against chemotherapy-induced apoptosis. Interestingly, our data indicate upregulation of XIAP expression by transcriptional and posttranscriptional mechanisms. Within a population of 101 patients with neuroblastoma, microarray data showed an association of increased XIAP mRNA expression with risk for relapse. On the other hand, comparing murine neuroblastoma cells and healthy adrenal gland tissue, we did detect highly increased XIAP protein expression but no regulation of XIAP mRNA. According to the role of n-myc as important transcriptional activator, XIAP mRNA expression is increased in MYCN-amplified tumors. In contrast, posttranscriptional regulation of XIAP expression appears to occur independent of MYCN amplification status. Posttranscriptional regulation of XIAP expression has been suggested in several hematologic and non hematologic malignancies (7, 27, 30–32) and this concept seems to hold up also for...
neuroblastoma. Posttranscriptional regulation of XIAP expression is controlled by alternative translation initiation via an IRES that is essential for maintenance of XIAP protein expression during cellular stress (11). Increased XIAP protein expression resulting from enhanced XIAP IRES activity has been shown during tumor progression and resistance to chemotherapy induced by interleukin-6 in cholangiocarcinoma (33). Overexpression of IRES-stimulating proteins might also contribute to increased XIAP IRES activity in childhood acute lymphoblastic leukemia (34–37).

Enhanced protein stability represents an alternative possible mechanism resulting in posttranscriptional upregulation of XIAP expression (31). Phosphorylation of XIAP by AKT (38) as well as binding of the IAP family member survivin to XIAP (39) have been shown to stabilize XIAP protein and to reduce proteasomal degradation of XIAP.

Targeting XIAP using Smac mimetics is a promising new strategy to overcome chemotherapy resistance in malignant cells. It becomes increasingly evident that the regulation of apoptosis by XIAP occurs in a cell type- and stimulus-dependent manner. Accordingly, the effect of LBW242 in neuroblastoma showed a distinct variation between different cell lines and cytostatic drugs used for induction of apoptosis.

Whereas LBW242 alone does not result in a relevant induction of apoptosis in neuroblastoma, LBW242 can be used as sensitizer to restore and enhance the responsiveness of neuroblastoma cells toward chemotherapy in vitro. Several Smac mimetics (including LBW242) have been shown to reduce tumor growth in mouse models of different malignant tumors (19, 25, 28, 40). Using an NXS2 cell–based neuroblastoma mouse model, we detected a significant sensitization for vincristine-induced inhibition of primary neuroblastoma growth by LCL161 (a closely related structural analogue of LBW242) in vivo. This again emphasizes the relevance of XIAP as therapeutic target for Smac mimetics in neuroblastoma.

The drugs used in our experiments are known to induce apoptosis by different mechanisms. LBW242 sensitized neuroblastoma cells to apoptosis induced by inhibition of mitotic spindle assembly (vincristine) or by intercalating DNA (doxorubicin). It remains unclear why apoptosis induction by inhibition of DNA repair (etoposide) was not stimulated by IAP inhibition using LBW242.

Recent reports suggest induction of necroptosis but not apoptosis by Smac mimetics under certain circumstances (41, 42). Nevertheless, sensitization of neuroblastoma cells for chemotherapy was mediated via activation of apoptosis, as indicated by cleavage of caspases-3, -8, -9 and PARP as well as blockade of cell death by caspase inhibition. Originally, the major proapoptotic effect of Smac mimetics was attributed to direct inhibition of XIAP-mediated blockade of caspase activation. Therefore, Smac mimetics were designed to bind to the BIR domains of IAPs to activate caspase-3/7 (BIR2; refs. 43, 44) or caspase-9 (BIR3; refs. 17–19, 40, 45, 46). As LBW242 targets the BIR3 domain, treatment of neuroblastoma cells resulted in caspase-9 activation. Interestingly, caspase-9 activation occurred after combination of LBW242 with vincristine only, but not by LBW242 alone. Notably also a dose-escalation of LBW242 up to 100 μmol/L did not result in measurable activation of caspase-9 (Supplementary Fig. S6A).

LBW242 binds to cIAP with much higher affinity than to XIAP (45), but in contrast to XIAP, cIAP-1/2 cannot inhibit caspases directly (47). Recent reports showed a different mechanism of apoptosis induction by Smac mimetics involving cIAP-1/2 (17–19). According to their response to Smac mimetics, tumor cells have been classified into 3 cell types (48). In sensitive tumor cells, Smac mimetics induce degradation of cIAP-1/2 that is followed by a stimulation of autocrine TNF-α synthesis. In addition, degradation of cIAP-1/2 results in sensitization against TNF-α–induced apoptosis, as in the absence of cIAP-1/2, complex-I detaches from the TNF-R1

![Figure 7. Effect of LCL161 and vincristine treatment on primary neuroblastoma tumor growth in vivo. Primary tumor growth after subcutaneous injection of NXS2 neuroblastoma cells in A/J mice was analyzed by microcaliper measurements and calculation of tumor volume (A) as well as tumor weight after resection (B). Values represent the mean ± SEM (A) or ±SD (B). *, P < 0.05.](image_url)
favoring the formation of a RIP1-dependent caspase-8–activating complex (complex-III; ref. 19). The second cell type does not respond to Smac mimetics alone and there is no induction of autocrine TNF-α synthesis. Nevertheless, a sensitization against TNF-α by Smac mimetics occurs, as addition of exogenous TNF-α results in apoptosis induction. Tumor cells of the third type do not respond to cotreatment with Smac mimetics and TNF-α.

In neuroblastoma, we did not detect relevant cIAP-1/2 degradation, TNF-α induction, or caspase-8 activation (nor induction of apoptosis) by LBW242 alone (Fig. 6A–C and Supplementary Fig. S6B). Furthermore, neuroblastoma cells were not sensitized for exogenous TNF-α by LBW242. Therefore, neuroblastoma cells appear to be nonresponsive to LBW242. Vincristine alone resulted in a weak increase in TNF-α synthesis, but no activation of caspase-8, nor relevant induction of apoptosis. In contrast, cotreatment with LBW242 and vincristine resulted in cIAP-1/2 degradation, TNF-α induction, and caspase-8 activation (as well as induction of apoptosis). Surprisingly, sensitization for vincristine-induced apoptosis by LBW242 was not dependent on TNF-α. Recent reports indicating that Smac mimetics induced stimulation of ripoptosome assembly followed by apoptosis in a TNF-α–independent manner provide a possible explanation for this phenomenon (42, 49).

In contrast to LBW242 treatment, XIAP knockdown resulted in significant induction of apoptosis, most likely by a more pronounced derepression of caspasess, but no synergistic effect with vincristine was detected.

In conclusion, our data emphasize the important role of XIAP mediating resistance against chemotherapy-induced apoptosis in neuroblastoma and we show a role for targeted therapy using Smac mimetics in combination with cytotoxic compounds. These findings provide an important baseline to further elaborate XIAP inhibition as a strategy to overcome resistance against chemotherapy in neuroblastoma in the clinical setting.

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

Authors’ Contributions
Conception and design: G. Eschenburg, H.N. Lode, P. Hundsdoerfer
Development of methodology: G. Eschenburg, P. Hundsdoerfer
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Eschenburg, P. Hundsdoerfer
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Eschenburg, A. Schramm, P. Hundsdoerfer
Writing, review, and/or revision of the manuscript: G. Eschenburg, A. Eggert, H.N. Lode, P. Hundsdoerfer
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Eschenburg, A. Eggert
Study supervision: G. Eschenburg, H.N. Lode, P. Hundsdoerfer

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