Topotecan Triggers Apoptosis in p53-Deficient Cells by Forcing Degradation of XIAP and Survivin Thereby Activating Caspase-3-Mediated Bid Cleavage

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ABSTRACT

The topoisomerase I inhibitor topotecan (TPT) is used in the therapy of different tumors including high-grade gliomas. We previously showed that TPT-induced apoptosis depends on p53 with p53 wild-type (wt) cells being more resistant because of p53-controlled degradation of topoisomerase I. Here, we show that p53-deficient (p53−/−) fibroblasts undergo excess mitochondrial apoptosis featuring H2AX phosphorylation, Bcl-xL decline, cytochrome c release, caspase-9/-3/-2 activation, and cleavage of Bid. In wt and apaf-1−/− cells, caspase-2 did not become activated and Bid was not cleaved. In addition, p53−/− cells cotreated with TPT and caspase-3 inhibitor showed neither caspase-2 activation nor Bid cleavage, implying that caspase-2 is processed downstream of the apoptosome by caspase-3. Although processing of caspase-9/-3/-2 was similar in wt and p53−/− cells, only p53−/− cells displayed active caspase-3. This was due to the proteasomal degradation of X-chromosome-linked inhibitor of apoptosis (XIAP) and survivin that inhibits caspase-3 activity. Accordingly, TPT-induced apoptosis in wt cells was increased after XIAP/survivin knock-down. Silencing of Bid led to reduction of TPT-triggered apoptosis. Data obtained with mouse fibroblasts could be extended to human glioma cells. In U87MG (p53wt) cells cotreated with TPT and pifithrin-α, or transfected with p53-siRNA, caspase-2 and Bid were significantly cleaved and XIAP/survivin was degraded. Furthermore, the knockdown of XIAP and survivin led to increased TPT-triggered apoptosis. Overall, the data show that p53-deficient/depleted cells are hypersensitive to TPT because they down-regulate XIAP and survivin, and thus amplify the intrinsic apoptotic pathway via caspase-3-mediated Bid cleavage. Therefore, in gliomas harboring wild-type p53, TPT-based therapy might be improved by targeted down-regulation of XIAP and survivin.

Topotecan (TPT) is a camptothecin derivative that belongs to the class of topoisomerase I (topoI) inhibitors. It is used in the therapy of different types of cancer including pediatric high-grade gliomas. After the formation of a DNA single-strand break, topoI remains covalently bound to the DNA phosphodiester backbone forming the topoI-DNA-cleavable complex (Nitiss and Wang, 1996), which is a reversible intermediate catalyzing the cleavage-religation reaction of the enzyme (Porter and Champoux, 1989). TopoI inhibitors such as TPT stabilize this complex preventing the religation of topoI-mediated single-strand breaks (Hertzberg et al., 1989). The cytotoxicity of topoI inhibitors is limited to the S phase of the cell cycle and is triggered by a collision of the replication fork with the inhibitor-stabilized cleavable complex. This results in blockage of fork movement and finally the formation of toxic DNA double-strand breaks (DSBs) (Hsiang et al., 1989; Goldwasser et al., 1996). These DSBs induce a checkpoint response by activation of upstream kinases like ataxia telangiectasia-mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), and DNA protein kinase, and subsequent phosphorylation of the histone variant H2AX. The phosphorylated H2AX (γH2AX) recruits different repair proteins to the site of DNA damage, like the Mre11/Rad50/Nbs1 complex, thereby activating DSB repair (Furuta et al., 2003).

ABBREVIATIONS: TPT, topotecan; IAP, inhibitor of apoptosis protein; DMEM, Dulbecco’s minimal essential medium; DSB, double-strand break; FBS, fetal bovine serum; mAab, monoclonal antibody; MEF, mouse embryonic fibroblast; pAb, polyclonal antibody; PIDD, p53-induced protein with a death domain; PI, propidium iodide; Pth, pifithrin-α; siRNA, small interfering RNA; topoI, topoisomerase I; XIAP, X-chromosome linked inhibitor of apoptosis; zVAD-fmk, benzoylxy-carbonyl-Val-Ala-Asp-fluoromethylketone; zDEVD-fmk, benzoylxy-carbonyl-Asp-Glu-Val-Asp-fluoromethylketone; zLEHD-fmk, benzoylxy-carbonyl-Leu-Glu-His-Asp-fluoromethylketone.

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Different parameters such as drug accumulation, the level of top1-DNA complex formation, the expression and activity of top1, and the level of Mdr-1, Bcl-2, and Bax were not found to be predictive for the sensitivity to top1 inhibitors or were disputed (Goldwasser et al., 1995; Davis et al., 1998; Schmidt et al., 2001; Blumenthal et al., 2004). This also applies to p53, for which we showed that it determines the sensitivity of glioma cells to TPT (Tomicic et al., 2005b).

In many cell systems p53 has been identified as a proapoptotic player, stimulating either the death receptor (Müller et al., 1998) or the mitochondrial apoptotic pathway (Miyashita and Reed, 1995). However, in some cell types, including mouse embryonic fibroblasts (MEFs), p53 preferentially acts in an antiapoptotic manner (Lackinger and Kaina, 2000; Christmann et al., 2005; Tomicic et al., 2005a). This is explained by a predominant role of p53 in DNA repair (Christmann et al., 2003). Using nontransformed MEFs and glioblastoma cell lines proficient or deficient for p53, we showed that cells lacking p53 are significantly more sensitive to TPT than their p53-proficient counterpart, indicating that lack of functional p53 sensitizes cells to topo1 poisons (Tomicic et al., 2005b). Because TPT-triggered sensitivity strongly differed between p53-proficient and p53-deficient cell lines, we hypothesized that p53 not only mediates the topo1 degradation in the cleavable complex (Tomicic et al., 2005b), but may also be responsible for differential activation of the apoptotic machinery in p53wt versus p53 mutated/deficient cells. Therefore, we extended our previous study by dissecting the TPT-induced apoptotic response in p53 wt and deficient cells and addressed the issues of caspase-2 processing, Bid cleavage, and cellular caspase inhibitors (IAPs). Data were obtained with MEFs and human glioma cells. Because TPT is used in clinical trials of pediatric high-grade gliomas that have a poor prognosis (Wagner et al., 2008), novel insights into the network of prosurvival and proapoptotic factors evoked by the drug might help to understand the mechanism of drug resistance that limits the success of therapy.

Materials and Methods

Cell Lines. MEF knockouts for Apaf-1 were kindly provided by Dr. Schuler (University of Essen, Essen, Germany) at passage 15. The wild-type (wt) cell line was a littermate to the apaf-1 null cell line (apaf-1/−), both proficient for p53 (Milosevic et al., 2003). The p53-deficient cell line cell line used (apaf-1/−; p53−/−) was generated from p53 knockout mouse embryos (C57Bl; The Jackson Laboratory, Bar Harbor, ME) as described previously (Lackinger and Kaina, 2000). Cells were grown in Dulbecco's minimal essential medium (DMEM) with high glucose and glutamine at 37°C in an atmosphere containing 7% CO2. The wt MEFs were grown in medium containing 15% fetal bovine serum (FBS), whereas apaf-1/− and p53−/− cells were grown in the same medium with 10% FBS. Caspase-2/− MEFs were kindly provided by Dr. Du (University of Cincinnati, Cincinnati, OH). They were immortalized by transformation with SV40 large T antigen (Shi et al., 2009) and were grown in DMEM with 10% FBS. U87MG human glioma cell line was provided by Dr. Weller (Department of Neurology, Medical School, University of Tübingen, Germany) (Wischhusen et al., 2003) and cultivated in DMEM with 10% FBS. Cells were mycoplasma-free and tested for their origin by reverse transcription-polymerase chain reaction.

Reagents, Drug Treatment, and Caspase Activity Assay. Topotecan hydrochloride (Hycamtin; GlaxoSmithKline, Welwyn Garden City, Hertfordshire, UK) was obtained from and prepared to stock solution (1 mg/ml) by the pharmacy of the University Medical Center (Mainz, Germany). In all experiments cells were exposed continuously to TPT. zVAD-fmk, zDEVD-fmk, and zLEHD-fmk were irreversible cell-permeable caspase inhibitors (Calbiochem, La Jolla, CA). Pifithrin-α (Sigma-Aldrich, Munich, Germany) was applied at working concentration of 30 μM. The Caspase Colorimetric Assay was conducted according to the manufacturer (R&D Systems, Wiesbaden, Germany).

Flow Cytometry. For monitoring drug-induced apoptosis and necrosis, annexin V-fluorescein isothiocyanate/propidium iodide (PI) double staining combined with flow cytometry were performed. Exponentially growing cells were treated with different concentrations of TPT for 48 or 96 h and thereafter subjected to analysis as described previously (Tomicic et al., 2002a). In brief, cells were fixed in ethanol, treated with 0.1 mg/ml RNase in PBS, and stained with PI, for flow cytometric determination of subG1 fraction.

Preparation of Cell Extracts and Western Blot Analysis. Whole-cell extracts for H2AX phosphorylation were prepared by direct lysis of the cells in preheated 1× loading buffer (Roti-Load 1; Roth, Karlsruhe, Germany), sonified, and separated by 10% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes, which were then incubated with mouse anti-pH2AX mAb (Millipore, Billerica, MA), diluted 1:500 in 5% nonfat dry milk, 0.2% Tween TBS, and incubated overnight at 4°C. For Western blot analysis with mouse anti-p53 mAb (Dianova, Hamburg, Germany) and anti-p21 mAb (Calbiochem), nuclear extracts were prepared. Whole-cell extracts for Western blot analysis with rabbit anti-Bid pAb, anti-Bcl-2 pAb (BD PharMingen, Heidelberg, Germany), mouse anti-Bcl-2 mAb, rabbit anti-Bax pAb, anti-ERK2 pAb (Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-Caspase-2 pAb (Neomarkers, Fremont, CA) were prepared by lysis in ice-cold sample buffer (25 mM Tris-HCl, pH 6.8, 1 mM EDTA, 5% glycerol, 2.5% 2-mercaptoethanol; 1 mM phenylmethylsulfonyl fluoride was added freshly), followed by sonication on ice. For Western blot analysis with anti-caspase-9/3 antibodies, rabbit anti-XIAP pAb, anti-survivin mAb, human-specific mouse anti-caspase-2 mAb, and rabbit anti-ERK1/2 pAb protein extracts were prepared according to the manufacturer (Cell Signaling Technology, Beverly, MA). Preparation of cytosolic extracts for immunoblotting with anti-cyt c pAb (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was described previously (Tomicic et al., 2002b). Protein-antibody complexes were detected by enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

siRNA Transfection. Human double-stranded p53-siRNA and the control nonsense siRNA (NON-siRNA) were ready-to-use (QIAGEN GmbH, Hilden, Germany), identical to mouse sequence. XIAP/survivin-siRNAs suitable for knockdown experiments in mouse and human cell lines and mouse-specific Bid-siRNA were synthesized as single-stranded oligonucleotides (MWG Biotech, Munich, Germany), with the following sequence: XIAP-si-up, aagugcuuacaguggaga; XIAP-si-low, uccucacagagaaagacauc; survivin-si-up, gguugguuccaucaaggct; survivin-si-low, gaggauaggaagacagcagc. The sequence for Bid-siRNA was as published (Ziporen et al., 2009). Single-stranded siRNAs were annealed according to the protocol (Metabion, Martinsried, Germany). Annealed siRNA oligonucleotides were transfected into wild-type or p53-deficient MEFs, or glioma cells depending on the experimental design by use of HiPerFect Transfection Reagent (QIAGEN) according to a protocol for primary MEFs. For six-well plates, the concentration of transfected siRNA was 20 nM. Gene silencing was verified 24 to 72 h after transfection.

Statistical Analyses. Statistical significance was verified by unpaired Student’s t test or, in case of significant differences between the standard deviations of the comparing variants, by alternate (Welch) t test of the statistical software GraphPadInStat v2.04a (Dr. F. Gotz, TSE Systems GmbH, Bad Homburg, Germany). The probability values were defined as follows: *, p < 0.05 (significant difference); **, p < 0.01 (very significant difference); ***, p < 0.001 (extremely significant difference).
Results

Cellular Sensitivity to TPT. First, we compared MEFs wild-type for p53, knockout for p53 (p53\(^{-/-}\)), and knockout for Apaf-1 (apaf-1\(^{-/-}\), proven to be p53-proficient; Supplemental Fig. 1) as to their killing response to TPT. To this end, we determined the level of apoptosis and necrosis by annexin V/PI flow cytometry. Cells were exposed to increasing concentrations of the drug and harvested 48 and 96 h later. In all experiments, necrosis (annexin V plus PI-stained cells) was marginal (<5%; data not shown), indicating apoptosis is the main route of cell death (Fig. 1A). p53 wt cells were quite resistant, showing at maximum 10% apoptosis in the dose range used. Even more resistant to TPT were apaf-1\(^{-/-}\) cells, displaying <4% apoptosis (similar to untreated controls) for the whole dose range tested. In contrast, p53\(^{-/-}\) MEFs were highly sensitive to TPT. They underwent excessive apoptosis, culminating in 80% after 96 h of exposure to 5 µg/ml drug (Fig. 1A, right).

H2AX Phosphorylation and Induction of DNA Double-Strand Breaks. Because TPT bound to topoisomerase I collides with the replication machinery, we determined the phosphorylation level of histone H2AX. As shown in Fig. 1B, in p53\(^{-/-}\) cells we observed strong phosphorylation of H2AX (γH2AX) by 4 h after addition of the drug, which still increased up to 20 h after TPT exposure. In contrast, wt and apaf-1\(^{-/-}\) cells showed only weak H2AX phosphorylation, indicating their ability to repair TPT-induced DNA damage and thereby preventing DSB formation. To substantiate that H2AX phosphorylation reflects DSB formation, we conducted neutral single-cell gel electrophoresis (“comet assay”) experiments. A significant induction of DSBs was observed only in p53\(^{-/-}\) fibroblasts, as determined 24 h after addition of the drug (Fig. 1C). We should note there were only a few apoptotic cells (tail separated from the head of the comet) in the samples, which were excluded from the analysis. From the data we infer that the high level of γH2AX in p53-deficient cells very likely results from the accumulation of DSBs.

Caspase-2 Activation in p53-Deficient Cells Is Mediated by Caspase-3. Caspase-2 can also be located in the nucleus and is supposed to be involved in the DNA damage response (Norbury and Zhivotovsky, 2004). Therefore, we elucidated whether caspase-2 was differently activated in p53-deficient cells, compared with the wt cells. Indeed, in the TPT-sensitive p53-deficient cells caspase-2 was significantly

Fig. 1. Induction of apoptosis, H2AX phosphorylation, and DNA double-strand breaks. A, exponentially growing cells were exposed for 48 or 96 h to TPT. Apoptosis (percentage of annexin V-positive cells) was determined by use of annexin V-fluorescein isothiocyanate/PI staining combined with flow cytometry. Necrosis (annexin V plus PI-positive cells) was <5% and is not shown. Data are the mean of three independent experiments ± S.D. B, cells were exposed to 1 µg/ml TPT for 4 or 20 h, lysed in 1× SDS denaturing buffer, and incubated with phospho-specific anti-γH2AX antibody. C, cells were treated for 2 and 24 h with 1 µg/ml TPT, and the neutral comet assay for determination of DSBs was performed. The data are the mean of three independent experiments ± S.D. Fifty randomly appearing nuclei were counted in each single experiment. *, p < 0.05.
processed: the small active cleavage fragment (p12) appeared 16 h after addition of TPT to the medium, and further accumulated up to 24 h, whereas it was only borderline detectable in wt cells (Fig. 2A).

During apoptosis, caspase-2 is generally activated by autolysis (CARD-mediated, dimerization-induced intrasubunit cleavage) but it can also be cleaved by caspase-3 (Krumbschnabel et al., 2009). Because caspase-2 cleavage was not observed in apaf-1$^{-/-}$ cells (Fig. 2A), we inferred that caspase-2 is presumably processed downstream of caspase-9 in the TPT-triggered apoptotic pathway and hypothesized that caspase-3 might be involved. To analyze whether caspase-3 plays a role in caspase-2 processing, we treated p53$^{-/-}$ cells with TPT and made use of a specific inhibitor of caspase-3, zDEVD-fmk (DEVD). As shown in Fig. 2B (upper panel), in the presence of zDEVD-fmk the active caspase-2 fragment (p12) was not formed, both in wt and p53-deficient cells, indicating that caspase-2 is cleaved by caspase-3 upon TPT treatment.

**Topotecan-Triggered Cleavage of Bid Is Mediated by Caspase-3.** Next, we determined whether the TPT-induced processing of caspase-2 results in activation of the proapoptotic BH3-only protein Bid, originally found to be a substate of caspase-8 (Li et al., 1998b), which was, however, not activated by TPT (Fig. 4A and Supplemental Fig. 2). As shown in Fig. 2A, 24 h after addition of TPT the pro-form of the Bid protein (22 kDa) almost completely disappeared in p53$^{-/-}$ but not in wt and apaf-1$^{-/-}$ cells, indicating possible cleavage of Bid by other caspases, e.g., by caspase-2 (Guo et al., 2002) or caspase-3 (Slee et al., 2000). The cleavage of Bid was abrogated in the presence of zDEVD-fmk (Fig. 2B, upper). In addition to these inhibitor experiments we transfected p53-deficient cells with a dominant-negative mutant of caspase-3 that yielded similar results, i.e., abrogation of Bid cleavage (Fig. 2B, lower). Because caspase-3 inhibition also prevented caspase-2 activation, the obtained data could still not rule out the possibility that caspase-2 was involved in Bid cleavage.

To determine whether Bid can be cleaved in the absence of caspase-2, we made use of caspase-2$^{-/-}$ MEFs. As shown in Fig. 2C, Bid was cleaved in caspase-2$^{-/-}$ cells (partial p53 inactivation) treated with TPT (lane 3). Moreover, in caspase-2$^{-/-}$ cells Bid cleavage was significantly inhibited by cotreatment with zDEVD-fmk (DEVD; lanes 5 and 8), indicating that caspase-3 is indeed involved in Bid cleavage. Because we learned from these experiments that Bid was much more effectively cleaved in cells deficient for p53, we additionally blocked the p53 trans-activating activity in caspase-2$^{-/-}$ MEFs by using a transcriptional inhibitor of p53, pifithrin-$\alpha$ (Pth), or knocked down p53 by transfection with p53-siRNA. In both cases cleavage of Bid in caspase-2$^{-/-}$ cells could be enhanced (Fig. 2C, lanes 4 and 7) compared with MEFs treated with nonsense-siRNA (non-si) plus TPT (lane 6). We also determined the enzymatic activity of important caspases in crude cytoplasmic extracts of caspase-2$^{-/-}$ cells after treatment with TPT. As shown in Fig. 2D, not caspase-8, and caspase-9 and caspase-3 were clearly activated in caspase-2$^{-/-}$ cells upon TPT exposure. In addition, similar to Bid cleavage in caspase-2$^{-/-}$ MEFs, caspase-3 was already significantly active in cells treated only with TPT. Its activity was significantly enhanced after cotreatment of cells with pifithrin-$\alpha$ and was completely inhibited by zDEVD-fmk. Overall, the data suggest that, in response to TPT, Bid is cleaved by activated caspase-3.

To find out whether Bid is involved in a late-amplification loop of the killing response triggered by TPT, we silenced Bid in p53-deficient MEFs by use of mouse-specific Bid-siRNA. Thereafter, cells were treated with TPT. As shown in Fig. 2E (left), Bid was down-regulated on protein level by $\sim$80% 24 to 72 h after Bid-siRNA transfection. Down-regulation of Bid led to significant reduction in TPT-induced apoptosis, determined as subG1 fraction 72 h after drug addition (Fig. 2E, right). From this we conclude that Bid cleavage is part of an intrinsic amplification loop in TPT-induced apoptosis.

**Signaling to Mitochondrial Pro- and Antiapoptotic Factors Is p53-Dependent.** To elucidate the role of additional factors involved in mitochondrial apoptosis upon TPT, we checked players known to be involved in mitochondrial permeabilization. As shown in Fig. 3A, we observed a clear stabilization of Bax in TPT-treated p53-proficient (wt and apaf-1$^{-/-}$) MEFs and a decline in Bcl-x$\text{L}$ in TPT-treated p53-deficient MEFs. The reduction in Bcl-x$\text{L}$ expression was not abrogated by the pan-caspase inhibitor zVAD-fmk (Fig. 3B, upper), indicating that it occurs upstream of caspase activation. Degradation of Bcl-x$\text{L}$ was blocked by the 26S proteasomal inhibitor MG132 (Fig. 3B, lower). We could also show that Bcl-2 remained unchanged in all cell lines (Supplemental Fig. 3). Collectively, the data indicate that upon TPT treatment in p53-proficient cells Bax stabilization and in p53-deficient cells Bcl-xL degradation is responsible for mitochondrial permeabilization induction.

**Cytocrome c Release and Caspase Activation.** Next, we determined cytochrome c release from mitochondria into the cytosol by use of purified ultracentrifuged cytosolic extracts. As measured 16 h after addition of TPT, cytochrome c was released in all cell lines (Fig. 3C). Caspase-9 was cleaved to a similar extent in both wt and p53-deficient cells (Fig. 3D, upper), which coincides with the similar early cytochrome c release observed in these cells. As expected, in TPT-treated apaf-1$^{-/-}$ cells caspase-9 activation was completely abrogated. Despite great sensitivity differences toward TPT, caspase-3 was processed almost to the same extent in wt and p53-deficient cells, although the processing occurred somewhat faster in p53$^{-/-}$ cells (Fig. 3D, lower). No cleavage products of caspase-3 were observed in apaf-1$^{-/-}$ cells, supporting a key role of the apoptosome in caspase-3 activation. The similar level of cytochrome c release and caspase-9 and caspase-3 cleavage in the resistant and sensitive wt and p53-deficient cells prompted us to determine the activity of caspases. We did not observe any caspase-8 activity in the cell lines used with and without TPT treatment and determined only slightly but insignificantly less activity of caspase-9 in the wt cells compared with p53$^{-/-}$ cells (Fig. 4A). A remarkable difference, however, was found for caspase-3, whose activity was low in wt cells and high in p53-deficient cells (Fig. 4A). From this we infer that, although caspase-3 was well processed in wt cells, it was not significantly active. The low caspase-3 activity in wt cells is in line with the low killing response of these cells.

**XIAP and Survivin in Fibroblasts and Effect of Their Down-Modulation via siRNA.** It is known that caspase-3 activity can be inhibited by a group of proteins that are collectively termed “inhibitors of apoptosis proteins” (IAPs).
**Fig. 1.** Caspase-2 processing, Bid cleavage, and caspase activity. A (upper), MEFs were exposed to 1 μg/ml TPT for 16 and 24 h; whole-cell extracts were prepared and subjected to Western blot analysis with anti-caspase-2 antibody. The 12-kDa cleavage fragment (p12) shows a complete processing of caspase-2. A (lower), MEFs were exposed to 1 μg/ml TPT for 16 and 24 h; whole-cell extracts were prepared and subjected to Western blot analysis with anti-Bid antibody. A 22-kDa protein (p22) represents the full-length Bid. B (upper), inhibition of caspase-2 processing and abrogation of Bid cleavage after cotreatment of wt and p53−/− cells for 24 h with TPT and a caspase-3 inhibitor (20 μM DEVD). ERK2, loading control. C, untreated control. B (lower), abrogation of Bid cleavage by transient transfection of dominant-negative caspase-3. Wt and p53−/− MEFs were transfected with a dominant-negative mutant of caspase-3 (DN-Csp3) and 24 h later exposed to 1 μg/ml TPT for another 24 h. Whole-cell extracts were isolated and subjected to Western blot analysis with anti-Bid antibody. A 22-kDa protein (p22) represents the full-length Bid. ERK2, loading control. C, cleavage of Bid in caspase-2−/− MEFs after exposure to 1 μg/ml TPT and in combination with a p53 inhibitor, Pth, or after transfection with p53-siRNA (p53-si); non-si, nonsense siRNA; ERK2, loading control. D, caspase-2−/− MEFs were exposed to 1 μg/ml TPT for 48 h and thereafter collected for caspase activity assay. Casp-3, caspase-3; Casp-8, caspase-8; Casp-9, caspase-9; DEVD, caspase-3 inhibitor; LEHD, caspase-9 inhibitor; Pth, Pifithrin-α.
Therefore, we considered the hypothesis that the enzymatic activity of caspase-3 observed in wt cells was blocked by these factors. We determined the expression of XIAP and survivin because both were shown to directly bind and inhibit caspase-3 (Deveraux et al., 1997, 1998; Li et al., 1998a, 1999). The protein level of XIAP and survivin in TPT-treated wt cells did not change, whereas in p53-deficient cells it almost completely vanished after 16 to 48 h of treatment with the drug (Fig. 4B, left). We should note that the basal level of XIAP and survivin in p53-deficient cells was even higher than in the wt cells. The data support the hypothesis that XIAP and survivin inhibit caspase-3 activity in wt cells, which implicates a p53-dependent involvement of XIAP and survivin in apoptosis regulation upon TPT. The TPT-triggered reduction in the protein level of XIAP and survivin in p53<sup>−/−</sup> cells was due to proteasomal degradation because the effect was reversed by MG132 (Fig. 4B, right).

To determine whether down-modulation of XIAP and survivin in wt MEFs bears biological consequences as to the endpoint cell death, we transfected cells with corresponding siRNA and checked the protein expression 24 and 48 h after transfection. As shown in Fig. 4C, 48 h after transfection both proteins were clearly down-regulated. Under the same conditions, we treated cells with TPT and determined the frequency of apoptosis. As shown in Fig. 4D, transfection of either XIAP or survivin siRNA elevated the apoptotic fraction by ~50%. This supports that XIAP and survivin block TPT-induced apoptosis in p53 wt cells, whereas their degradation in p53-deficient cells allows caspase-3 to be actively executing apoptosis.

### p53, Caspase-2, and Bid in Human Glioma Cells Treated with TPT

To ascertain whether the findings obtained with MEFs can be extended to human glioma cells, for which TPT is being used in therapy, key experiments were repeated with U87MG cells that are wt for p53. As expected, significant stabilization of p53 was observed after exposure to TPT (Fig. 5A). The experiment also shows that p53 protein can be down-regulated by transfection with p53-siRNA; this approach was used in further experiments.

As shown in Fig. 5B (upper), TPT treatment resulted in processing of caspase-2. Similar to MEFs, caspase-2 processing was more efficient when p53 activity was inhibited, which occurred by coexposure to Pth or by transfection with p53-siRNA. Caspase-2 processing was completely abrogated by zDEVD-fmk, indicating that also in glioma cells caspase-2 is cleaved by caspase-3. Furthermore, Bid was significantly

The data are the mean of two experiments done in triplicate ± S.D. *p < 0.05; **p < 0.01; ***p < 0.001. E (left), p53<sup>−/−</sup> cells were transfected with mouse-specific Bid-siRNA, and the silencing was monitored by Western blot analysis in the subsequent 24 to 72 h. E (right), 24 h after Bid-siRNA transfection cells were exposed to 1 μg/ml TPT for 72 h. subG1 fraction (%) represents apoptosis. The data are the mean of three independent experiments ± S.D. ***p < 0.001.
cleaved only in cells in which p53 was inhibited or downregulated. Bid cleavage was also inhibited by zDEVD-fmk (Fig. 5B, lower). Accordingly, caspase-3 activity in U87MG cells was relatively moderate. It could be significantly enhanced, however, by cotreatment with Pth or by p53-siRNA transfection (Fig. 5C). Cotreatment of p53-depleted cells with zDEVD-fmk reduced caspase-3 activity, showing that the effects are specific for caspase-3.

To determine whether the observed events bear relevance for the endpoint cell death, we determined the apoptotic fraction of U87MG cells after exposure to TPT with and without Pth treatment or p53-siRNA transfection. Both approaches resulted in a 50 to 80% increase in the fraction of apoptotic cells (Fig. 5D), confirming that p53 protects glioma cells against TPT-induced cytotoxicity.

**XIAP and Survivin in p53-Depleted Glioma Cells.** To clarify whether the low caspase-3 activity and moderate apoptotic frequency in U87MG cells exposed to TPT is related to XIAP and survivin, we determined their expression level (Fig. 6A). Similar to p53 wt MEFs, the expression of these proteins in p53-proficient U87MG cells did not change when they were treated with TPT. It was, however, reduced after cotreatment with Pth and when the cells were transfected with p53-siRNA, indicating that, in a p53-inactivated background, XIAP and survivin become degraded in response to the drug. This degradation was abrogated by MG132, indicating that in glioma cells proteasomal degradation is also involved. Under p53-depleted conditions, caspase-3 activation was fully achieved (see Fig. 5C). Collectively the data show that TPT-triggered degradation of XIAP and survivin...
in a p53-inactivated background is a phenomenon that can be extended to human glioma cells.

**Knockdown of XIAP/Survivin Supports Cell Death of TPT-Treated U87MG Glioma Cells.** To examine whether the events described above influence TPT-triggered cytotoxicity, we silenced XIAP and survivin in U87MG glioma cells. Both proteins were almost completely down-regulated 48 h after siRNA transfection (Fig. 6B). Under the same conditions, we treated cells with TPT and determined the apoptotic frequency. As shown in Fig. 6C, transfection of XIAP or survivin siRNA elevated the subG1 fraction of cells by 80% in case of XIAP siRNA, and 60% in case of survivin siRNA (last two columns). This suggests that XIAP and survivin are key prosurvival factors in p53 wt glioma cells, whereas their degradation in glioma cells harboring inactive p53 supports TPT-induced apoptosis.
finding demonstrating that caspase-2 processing occurs without functional PIDD (Manzl et al., 2009). We also show that inhibition of caspase-3 by zDEVD-fmk blocks caspase-2 processing and Bid cleavage. Similar results were obtained upon transfection of a dominant-negative mutant of caspase-3. TPT-triggered activation of caspase-2 in p53-deficient, but not in p53-proficient cells can be explained by the finding that caspase-2 is processed by caspase-3, which is activated only in p53-deficient cells due to down-regulation of XIAP and survivin. The data obtained with MEFs were extended to human glioma cells that also display caspase-3-mediated processing of caspase-2. Collectively, the results implicate a direct involvement of caspase-3 in caspase-2 processing.

Bid is a proapoptotic protein that, upon activation by cleavage, translocates to mitochondria and binds there as truncated Bid (Esposito, 2002). It can be cleaved by caspase-8 (Li et al., 1998b), caspase-2 (Guo et al., 2002), and caspase-3 (Slee et al., 2000). We demonstrate, by means of caspase-2−/− cells and caspase-3 inhibition, that upon TPT treatment caspase-3, rather than caspase-2, is involved in cleavage of Bid. Caspase-8 does not participate in our cell system in Bid cleavage because it was not active in TPT-treated cells. Thus, TPT seems not to activate the caspase-8-driven pathway. Similar findings were reported for the topoisomerase II inhibitor etoposide (Franklin and Robertson, 2007).

Because TPT has the ability to activate caspase-2, one might speculate that caspase-2 is required for triggering apoptosis. This would implicate that cells lacking caspase-2 are more resistant to TPT. This was not the case, however. Caspase-2−/− MEFs (Shi et al., 2009) were even more sensitive to TPT than the wt (data not shown). A large number of studies in knockout and knockdown cells and with other DNA-damaging agents (for review, see Krumschnabel et al., 2009) show that caspase-2 is not an apical caspase after DNA damage. Caspase-2 was recently shown to interact with DNA protein kinase and be involved in the maintenance of a G2/M DNA damage checkpoint that facilitates nonhomologous end joining (Shi et al., 2009). The relevance of this finding for topoisomerase inhibitor-induced cytotoxicity remains to be established. Nevertheless, the experiments with caspase inhibitors and transfection of dominant-negative mutants of caspase-9 and caspase-3 in p53−/− cells, which resulted in reduced TPT-induced apoptosis (Supplemental Fig. 4), suggest that the mitochondrial damage pathway involving caspase-9 and caspase-3, but not caspase-2, is crucially involved in TPT-induced apoptosis.

Another part of this work focused on the regulation of the mitochondrial damage pathway in TPT-treated cells. An unexpected finding was that, despite a great sensitivity difference between wt and p53-deficient cells and a high level of DNA damage (as determined by γH2AX) in p53-deficient cells, the TPT-triggered cytochrome c release and caspase-9 cleavage were nearly the same in these cells. This might be explained by upstream events: p53-regulated stabilization of Bax in wt cells on one side, and Bel-xL degradation in p53−/− cells on the other, leading to a similar Bel-xL/Bax ratio and comparable cytochrome c release. Accordingly, the cells showed similar caspase-9 and caspase-3 processing. Despite similar cleavage of caspase-9 and caspase-3, however, we found only caspase-3 enzyme activity in p53-deficient cells (both MEFs and glioma cells). This prompted us to study in more detail a possible involvement of IAPs, which are known

Fig. 6. Expression of XIAP and survivin, their knockdown and cell death in TPT-exposed U87MG cells. A, cells were treated with 1 μg/ml TPT for 32 h, and expression of XIAP and survivin was determined by use of anti-XIAP and antisurvivin antibody, respectively. B-Actin, loading control. Where indicated, cells were cotreated with 30 μM Pth or transfected with p53-siRNA (p53-si), or exposed to TPT in the presence of a proteasomal inhibitor (10 μM MG132). non-si, nonsense siRNA. B, exponentially growing U87MG cells were transfected with XIAP-siRNA or survivin-siRNA by use of HiPerFect Reagent. ERK1/2, loading control; C, untreated control; non-si, nonsense siRNA. C, U87MG cells were transfected with XIAP-siRNA or survivin-siRNA, and 24 h later were exposed to 1 μg/ml TPT for 48 h. Cells were collected for subG1 flow cytometry to determine the level of apoptosis. Data are the mean of three independent experiments ± S.D. **, p < 0.01; ***, p < 0.001.

Discussion

We previously showed that cells expressing p53 wt are more resistant to TPT than p53-deficient and mutated cells, which corresponded with the fate of the topol-cleavable complex (Tomicic et al., 2005b). Here, we extended this study assessing downstream pathways of apoptosis activated by TPT. First, we addressed caspase-2, which is the only caspase found in the nucleus. Its function has been disputed for almost a decade (Krumschnabel et al., 2009). We show that after TPT treatment caspase-2 is processed downstream of the apoptosome. It is more efficiently processed in p53-deficient than in p53-proficient cells. This implicates that it can be processed without p53-regulated PIDD induction (Lin et al., 2000) and probably without being in the PIDDosome (Tinel and Tsopp, 2004). The data indicate that caspase-2 cleavage occurs independent of p53 and probably independent of the PIDD protein. This is supported by a recent
To act as caspase-3 inhibitors, in TPT-triggered apoptosis. Key IAPs are XIAP and survivin that directly bind and inhibit caspase-3 (Deveraux et al., 1997, 1998; Li et al., 1998a, 1999). Thus, we considered the idea that the expression of these antiapoptotic factors could be changed upon TPT treatment in a p53-dependent fashion. Indeed, expression of XIAP and survivin was observed in p53 wt cells, which correlated to a lack of caspase-3 activity. In contrast, in p53-deficient cells XIAP and survivin became degraded upon TPT. Degradation was blocked by proteasomal inhibitor. This was found for TPT-treated p53−/− MEFs and p53-depleted U87MG glioma cells. The data suggest that proteasomal degradation of XIAP and survivin results in activation of caspase-3 enzyme activity and execution of apoptosis.

Caspase-2 becomes processed as a by-product of this activation. We should note that survivin, although it can act as a caspase-3 enzyme activity and execution of apoptosis.

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References
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