Small Molecule XIAP Inhibitors Enhance TRAIL-Induced Apoptosis and Antitumor Activity in Preclinical Models of Pancreatic Carcinoma

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Abstract

Evasion of apoptosis is a characteristic feature of pancreatic cancer, a prototypic cancer that is refractory to current treatment approaches. Hence, there is an urgent need to design rational strategies that counter apoptosis resistance. To explore X-linked inhibitor of apoptosis (XIAP) as a therapeutic target in pancreatic cancer, we analyzed the expression of XIAP in pancreatic tumor samples and evaluated the effect of small molecule XIAP inhibitors alone and in combination with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) against pancreatic carcinoma in vitro and in vivo. Here, we report that XIAP is highly expressed in pancreatic adenocarcinoma samples compared with normal pancreatic ducts. Small molecule XIAP inhibitors synergize with TRAIL to induce apoptosis and to inhibit long-term clonogenic survival of pancreatic carcinoma cells. In contrast, they do not reverse the lack of toxicity of TRAIL on nonmalignant cells in vitro or normal tissues in vivo, pointing to a therapeutically index. Most importantly, XIAP inhibitors cooperate with TRAIL to trigger apoptosis and suppress pancreatic carcinoma growth in vivo in two preclinical models, i.e., the chorioallantoic membrane model and a mouse xenograft model. Parallel immunohistochemical analysis of tumor tissue under therapy reveals that the XIAP inhibitor acts in concert with TRAIL to cause caspase-3 activation and apoptosis. In conclusion, our findings provide, for the first time, evidence in vivo that XIAP inhibitors prime pancreatic carcinoma cells for TRAIL-induced apoptosis and potentiate the antitumor activity of TRAIL against established pancreatic carcinoma. These findings build the rationale for further (pre)clinical development of XIAP inhibitors and TRAIL against pancreatic cancer. [Cancer Res 2009;69(6):2425–34]

Introduction

Pancreatic cancer is one of the leading causes of cancer deaths in the Western world with steadily rising numbers (1). Resistance of pancreatic cancer to even aggressive treatment regimens presents a major challenge in oncology (2). Because resistance to apoptosis, which is the cell’s intrinsic cell death program, contributes to treatment failure in pancreatic cancer (3), current attempts to improve the survival of pancreatic cancer patients will have to include strategies that target apoptosis resistance.

Apoptosis is critical for maintaining tissue homeostasis, and too little apoptosis may lead to cancer (4). Also, cell death by apoptosis has been implied to mediate therapy-induced cytotoxicity, e.g., after chemotherapy (5). Apoptosis pathways may be initiated through death receptors or mitochondria resulting in caspase activation (5). Ligation of death receptors, such as CD95 (APO-1/Fas), or agonistic tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) receptors by their cognate ligands results in caspase-8 activation, which induces direct cleavage of downstream effector caspases (6). The mitochondrial pathway is engaged by the release of apoptogenic factors, such as cytochrome c or second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis protein (IAP) binding protein (DIABLO) from mitochondria into the cytosol (7, 8). Cytochrome c triggers caspase-3 activation via formation of the apoptosome complex, whereas Smac/DIABLO promotes apoptosis by neutralizing IAPs (7). Inducing apoptosis in cancer cells by ligation of death receptors is of special interest for cancer therapy, because death receptors are directly linked to the intrinsic death program of the cell (6). The death-inducing ligand TRAIL is a prime candidate for clinical application, because it has been reported to induce apoptosis in a wide spectrum of cancer cells with no or minimal toxicity to normal human cells (9, 10). However, many human cancers, including pancreatic carcinoma, proved to be TRAIL resistant, e.g., because of high levels of IAPs, such as X-linked inhibitor of apoptosis (XIAP). XIAP prevents apoptosis at the effector phase by binding to and inhibiting activated caspase-3 and caspase-9 (11, 12). Because XIAP blocks apoptosis at the core of the apoptotic machinery, therapeutic modulation of XIAP can tackle a key control point in apoptosis resistance (11, 12).

Previously, we provided a proof of concept in an orthotopic glioblastoma model that Smac peptides, which neutralize XIAP, sensitize for TRAIL-induced killing in vivo (13). Because we recently found that the majority of pancreatic carcinoma cell lines are refractory to TRAIL despite expression of the essential components...
of the TRAIL signaling system (14), we evaluated the therapeutic potential of small molecule XIAP inhibitors to restore sensitivity of pancreatic cancer to TRAIL in the present study.

Materials and Methods

Cell culture and reagents. Pancreatic carcinoma cells were cultured in DMEM or RPMI 1640 (Life Technologies, Inc.), HCT116 and DLD1 colon carcinoma cells were cultured in McCoy’s 5A medium (Life Technologies, Inc.), and 3T3 fibroblasts and MM157 breast carcinoma cells were cultured in DMEM (Life Technologies, Inc.), as described (14). Medium was supplemented with 10% FCS (Biochrom), 1 mmol/L glutamine (Biochrom), 1% penicillin/streptavidin (Biochrom), and 25 mmol/L HEPES (Biochrom). Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors by ficoll separation (Biochrom) and cultured in RPMI 1640 supplemented with 30 units/ml of interleukin-2 (Biochrom). Upon purification, PBMC were stimulated with 2.5 μl/ml phytohemaggulatin (Sigma) for 24 h and allowed to proliferate for 6 d before the start of the experiment. All chemicals were purchased by Sigma unless indicated otherwise. XIAP inhibitor 1, XIAP inhibitor 2, and control compound correspond to compounds 2, 11, and 15, respectively, described by Oost and colleagues (15), and XIAP inhibitors 3 to 5 were described by Chao and colleagues (16) and were kindly provided by Idun Pharmaceuticals (now Pfizer, Inc.). XIAP inhibitors are capped tripeptides consisting of unnatural amino acids that were designed on the basis of the nuclear magnetic resonance (NMR) structure of a Smac peptide bound to the BIR3 domain of XIAP and bind to XIAP BIR3 with high-nanomolar affinities (15).

Determination of apoptosis, metabolic activity, and clonogenic survival. After stimulation with TRAIL (R&D Systems, Inc.), apoptosis was determined by fluorescence-activated cell sorting (FACS) analysis (FACSscan, BD Biosciences) of DNA fragmentation of propidium iodide–stained nuclei or by forward/side scatter (17, 18). Metabolic activity was assessed by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer’s instructions (Roche Diagnostics). For clonogenic assay, cells were seeded as single cells (0.05/cm2) in six-well plates for 24 h, treated with TRAIL for 24 h before medium was exchanged and recovered for additional 6 d before staining with 0.75% crystal violet, 50% ethanol, 0.25% NaCl, and 1.5% formaldehyde.

Determination of mitochondrial membrane potential and cytochrome c release. CMXRos (1 μmol/L; Molecular Probes) was used to measure the mitochondrial transmembrane potential. Cells were incubated for 30 min at 37°C in the presence of the fluorochrome and immediately analyzed by flow cytometry. CMXRos was recorded in fluorescence 3. Cytochrome c release was determined in permeabilized cells using mouse anti–cytochrome c monoclonal antibody (BD Biosciences) as described (19).

Caspase activity. Caspase activity was determined in living, nonfixed, nonlysed cells, as described (20), using the following caspase substrates conjugated to rhodamine R110: N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone-R110 (zDEVD-R110), N-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone-R110 (zVDVAD-R110), N-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone-R110 (zVDVAD-R110), N-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone-R110 (zDEVD-R110), N-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone-R110 (zVDVAD-R110), N-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone-R110 (zDEVD-R110) for all purchased from Molecular Probes. For inhibition of caspases the broad carbonyl-Leu-Glu-His-Asp-fluoromethylketone-R110 (zLEHD-R110; all Ile-Glu-Thr-Asp-fluoromethylketone-R110 (zIETD-R110), and N-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone-R110 (zLEHD-R110; all purchased from Molecular Probes). For inhibition of caspases the broad range caspase inhibitor zVAD.fmk (Bachem) and the relatively specific N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone-R110 (zDEVD-R110), N-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone-R110 (zVDVAD-R110), N-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone-R110 (zDEVD-R110) for all purchased from Molecular Probes. For inhibition of caspases the broad range caspase inhibitor zVAD.fmk (Bachem) and the relatively specific

Cell surface staining. To determine surface expression of TRAIL receptors, cells were incubated with mouse anti-human TRAIL-R1, mouse anti-human TRAIL-R2, mouse anti-human TRAIL-R3, mouse anti-human TRAIL-R4 (all from ApoTech Corporation) monoclonal antibodies for 30 min at 4°C, washed in PBS containing 1% FCS, incubated with rabbit anti–mouse FcαRI, IgG–biotin (BD Biosciences) for 20 min at 4°C in the dark, washed in PBS containing 1% FCS, incubated with streptavidin–PE (BD Biosciences) for 20 min at 4°C in the dark, and analyzed by flow cytometry. For detection of mouse TRAIL-R PE-labeled, hamster antimouse, TRAIL-R monoclonal antibody (eBioscience) was used.

Chorioallantoic membrane assay. Chorioallantoic membrane (CAM) assay was done as described previously (21). Briefly, 1 × 106 tumor cells were resuspended in 25 μl serum-free medium and 25 μl Matrigel matrix (BD Biosciences) and implanted on fertilized chicken eggs on day 8 of incubation using a silicone ring of 6 mm in diameter. Tumors were topically treated with 0.7 ng TRAIL in 15 μl PBS and/or 10 μmol/L XIAP inhibitor 2 or control compound, sampled with the surrounding CAM 4 d after seeding, fixed in 4% paraformaldehyde, paraffin embedded, cut in 5-μm sections, and analyzed by immunohistochemistry.

Xenograft mouse model. 1 × 106 PancTu1 cells were s.c. inoculated at left and right dorsal sides of 6-wk-old female NCBM-nt-nu mice (Janvier Laboratories). In the tumor regression model, treatment started 1 wk after tumor cell inoculation by daily i.p. injection of 50 μg isoleucine zipper-TRAIL (22) and/or XIAP inhibitor 5 for 2 cycles of 10 d. Tumor size was assessed twice weekly by caliper; the tumor volume was calculated according to the formula: 0.5 × L × W × T (L, length; W, width; T, thickness). Relative tumor growth ratio [treated versus control (T/C)] was calculated by tumor volume in treated group divided by tumor volume in control group. At the end of the experiment, tumors were recovered for histologic and pathologic analysis. Also, additional two to three mice were sacrificed 24 h after the second injection of TRAIL for histologic and pathologic analysis. Tumor tissue was analyzed by immunohistochemistry. Animal experiments were performed in accordance with relevant institutional and national regulations; research protocols were approved by relevant authorities.

Immunohistochemistry. Immunohistochemistry of XIAP was performed on 12 pancreatic ductal carcinoma and 8 normal pancreas, as previously described (23). Briefly, 2-μm-thick cryosections were immediately fixed in ice-cold acetone for 10 min, air-dried, and incubated for 1 h with mouse anti-XIAP monoclonal antibody with a dilution of 1:100 (clone 48, BD Bioscience). Control experiments using protein lysates of wild-type and XIAP knockout HCT116 colon carcinoma cells were performed to confirm the specificity of the antibody to detect XIAP (data not shown). Bound primary antibody was detected via EnVision antimmunoglobulins conjugated to peroxidase-labeled dextran polymer (EnVision, Dako). 3-Amino-9-ethyl-carbazole (Sigma) was used as substrate and hematoxylin as counterstain. Negative controls were performed by omitting the first step with primary antibody and yielded negative results. Results of immunohistochemistry were graded as follows: 1, no staining; 2, cytoplasmatic staining in 20% to 50% of cells; 3, cytoplasmatic staining in 50% to 80% of cells; 4, cytoplasmic staining in over 80% of cells. No nuclear staining was observed.

Immunohistochemical staining were performed with 1:1 hematoxylin and 0.5% or anti–Ki-67 (Dako) or antibody (Biocytex) antibody, as previously described (24). Apoptosis was assessed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL; Roche Diagnostics) according to the manufacturer’s instructions. Negative controls were performed by omitting the first step with primary antibody and yielded negative results. The percentage of positive cells was independently determined by two examiners.

Statistical analysis. Statistical significance was assessed by Student’s t test or Mann-Whitney U test, where appropriate, using Winstat (R. Fitch Software) or SPSS (SPSS GmbH Software) software. All statistical tests are two sided. Interaction between XIAP inhibitors and TRAIL was analyzed by the combination index (CI) method based on that described by Chou (25) using CalcuSyn software (Biosoft). CI values of <0.9 indicate synergism, 0.9 to 1.1 indicate additivity, and >1.1 indicate antagonism.

Results

Overexpression of XIAP in pancreatic carcinoma. To explore whether XIAP presents a therapeutic target in pancreatic cancer, we analyzed XIAP expression in pancreatic ductal adenocarcinoma samples. All cases of pancreatic carcinoma exhibited strong cytoplasmatic staining for XIAP in the majority of tumor cells, whereas normal pancreatic ductal cells displayed no XIAP staining (Fig. 1). This shows that XIAP is highly expressed in pancreatic carcinoma compared with normal pancreatic ducts, suggesting


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XIAP inhibitors enhance TRAIL-induced caspase activation and mitochondrial perturbations. Next, we explored molecular mechanisms that mediate the synergistic action of XIAP inhibitors and TRAIL. Because XIAP inhibitors 1 and 2 displayed similar activities to enhance TRAIL-induced apoptosis (Fig. 2), we used XIAP inhibitor 2 for these studies. XIAP inhibitor 2 significantly increased TRAIL-induced caspase activity compared with cells treated with TRAIL in the absence of XIAP inhibitor (Fig. 3A). To explore whether caspases are required for apoptosis induction, we used the broad-range caspase inhibitor zVAD.fmk and the relatively selective caspase-3 inhibitor zDEVD.fmk. Sensitization for TRAIL-induced apoptosis by XIAP inhibitor 2 was significantly blocked by zVAD.fmk or zDEVD.fmk (Fig. 3B). This shows that the XIAP inhibitor sensitizes pancreatic carcinoma cells for TRAIL-induced cell death in a caspase-dependent manner.

Next, we asked whether the XIAP inhibitor has an effect on the mitochondrial contribution during TRAIL-induced apoptosis. To address this question, we determined mitochondrial membrane potential and cytochrome c release in cells treated with TRAIL in the presence and absence of XIAP inhibitor 2. XIAP inhibitor 2 significantly enhanced TRAIL-induced loss of mitochondrial membrane potential and cytochrome c release from mitochondria (Fig. 3C and D). These findings show that the XIAP inhibitor promotes TRAIL-mediated caspase activation and mitochondrial perturbations.

XIAP inhibitors preferentially sensitize malignant over nonmalignant cells for TRAIL-induced killing. To test the broader and potential clinical relevance of our findings, we extended our studies to additional cancer cell lines and XIAP inhibitors. Distinct XIAP inhibitors cooperated with TRAIL to reduce viability of a panel of pancreatic carcinoma cell lines (Fig. 4A and Supplementary Fig. S1). Moreover, XIAP inhibitor 2, but not the control compound, synergized with TRAIL to reduce viability of colon and breast carcinoma cells (Fig. 4B and Supplementary Fig. S2; Supplementary Table S1). These results show that XIAP inhibitors enhance TRAIL-induced killing in various pancreatic cancer and other carcinoma cell lines.

Furthermore, we examined the effect of XIAP inhibitors on normal cells to test for potential side effects. Notably, XIAP inhibitors alone or in combination with TRAIL exerted no or minor cytotoxicity on different normal cell types, e.g., activated PBMCs and murine 3T3 fibroblasts, despite expression of the apoptosis-inducing TRAIL receptors on the surface of these cells (Fig. 4C and D). These data show that XIAP inhibitors specifically prime cancer cells of different tissues of origin for TRAIL-induced cell death with little toxicity to normal cells.

XIAP inhibitor enhances TRAIL-induced antitumor activity against pancreatic carcinoma in vivo. We then evaluated the antitumor activity of XIAP inhibitors, together with TRAIL in vivo, using the CAM model, an established in vivo tumor model (21, 26). PancTu1 pancreatic carcinoma cells were seeded on the CAM of chicken embryos and treated with TRAIL in the presence or absence of XIAP inhibitor 2 for 3 consecutive days. Importantly, the XIAP inhibitor significantly enhanced TRAIL-induced caspase-3 activation, apoptosis, and antiproliferative effects in pancreatic carcinoma in vivo (Fig. 5). This shows that the XIAP inhibitor cooperates with TRAIL in vivo to trigger apoptosis and inhibit growth of pancreatic cancer.

XIAP inhibitor cooperates with TRAIL to suppress tumor growth of established pancreatic carcinoma in a xenograft mouse model. Finally, we evaluated the antitumor activity of the
XIAP inhibitor and TRAIL against established pancreatic carcinoma in vivo in a tumor regression model of pancreatic cancer in xenograft-bearing mice. In this model, PancTu1 cells were s.c. injected into the left and right dorsal sides of nude mice and TRAIL treatment was initiated 1 week afterwards, when pancreatic carcinoma had been established. Strikingly, simultaneous treatment with XIAP inhibitor and TRAIL cooperated to suppress tumor growth with a T/C ratio of 27%, whereas either agent alone had no significant effect on tumor growth (Fig. 6A; compare with T/C ratio of 90% for treatment with TRAIL and vehicle and T/C ratio of 100% for treatment with XIAP inhibitor and vehicle). By comparison, the combination of TRAIL and the control compound that weakly binds to XIAP only showed a modest separation from the group treated with TRAIL and vehicle (Fig. 6A). Representative mice and

![Graphs and images](https://example.com/graphs.png)

**Figure 2.** Small molecule XIAP inhibitors synergize with TRAIL to trigger apoptosis in pancreatic carcinoma cells. A, PancTu1 (left) and PaTuII (right) pancreatic carcinoma cells were treated for 48 h with indicated concentrations of TRAIL and/or 10 μmol/L XIAP inhibitors, control compound, or DMSO. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei. Columns, mean of three independent experiments performed in triplicate; bars, SE. *, *P < 0.05; #, *P < 0.01, comparing XIAP inhibitors to solvent. B and C, PancTu1 (left) and PaTuII (right) cells were treated for 72 h with indicated concentrations of TRAIL and/or XIAP inhibitor 2 (top) or control compound (bottom). Metabolic activity was determined by MTT assay and is expressed as percentage of untreated controls. Mean of three independent experiments performed in triplicate with SD of <10%. D, PancTu1 (left) and PaTuII (right) cells were treated with indicated concentrations of TRAIL and/or 10 μmol/L XIAP inhibitors, control compound, or DMSO, and clonogenic survival was assessed by crystal violet staining. A representative experiment of three independent experiments.
tumors are shown for each therapy group in Fig. 6B. Similarly, analysis of tumor weight showed that TRAIL treatment was significantly more effective to reduce tumor burden in the presence of the XIAP inhibitor compared with treatment with TRAIL and vehicle (Fig. 6C).

In parallel, we monitored apoptosis in pancreatic carcinoma tissue in situ during the treatment course by immunohistochemistry. Pancreatic carcinoma treated with the combination of XIAP inhibitor and TRAIL displayed a significant increase in cells positive for cleaved caspase-3 and TUNEL and a reduction of Ki-67–positive cells compared with treatment with TRAIL and vehicle (Fig. 6D). These findings reflect, at the cellular level, in tumor tissue of xenograft-bearing mice that the XIAP inhibitor acts in concert with TRAIL to trigger apoptosis in pancreatic carcinoma in vivo. Of note, no signs of acute or delayed toxicity were observed during treatment or at the end of the experiment, as assessed by clinical observation, body weight, blood cell counts, and liver enzymes (Supplementary Fig. S3). By comparison, XIAP inhibitors and TRAIL exerted cross-species activity against murine glioblastoma cells (data not shown), indicating that XIAP inhibitors increase TRAIL-induced apoptosis in murine neoplastic, but not in nonneoplastic, cells. These findings show that the XIAP inhibitor strongly augments the antitumor activity of TRAIL against established pancreatic cancer in vivo, resulting in suppression of tumor growth without detectable toxicities to normal tissues, thus pointing to a therapeutic index.

Discussion

Because resistance of pancreatic cancer to apoptosis is a key factor preventing responses to therapies (3), efforts to improve the poor outcome of this disease will depend on successful strategies to target apoptosis resistance. Here, we show in vitro and in vivo that small molecule XIAP inhibitors prime pancreatic carcinoma cells for TRAIL-induced apoptosis and cooperate with TRAIL to suppress pancreatic tumor growth. Several independent lines of evidence support this conclusion. First, small molecule XIAP inhibitors synergize with TRAIL to induce apoptosis and to inhibit clonogenic survival of pancreatic carcinoma cells in vitro. Second, XIAP inhibitors act in concert with TRAIL to suppress the growth of established pancreatic cancer in two distinct in vivo models. This cooperative antitumor activity of XIAP inhibitors and TRAIL in vivo is accompanied by increased levels of cleaved caspase-3 and apoptosis in tumor tissue in situ, demonstrating at the cellular level...
Figure 4. XIAP inhibitors preferentially sensitize malignant over nonmalignant cells for TRAIL-induced killing. Panc-1 (A, left), DanG (A, middle) and ASPC-1 (A, right) pancreatic carcinoma cells, HCT116 (B, left) or DLD1 (B, middle) colon carcinoma cells, MM157 breast carcinoma cells (B, right), phytohemagglutinin-stimulated PBMC (C, left) or murine 3T3 fibroblasts (C, right) were treated for 72 h with indicated concentrations of TRAIL in the presence or absence of 10 μmol/L XIAP inhibitors, control compound, or DMSO (A and C) or indicated concentrations of XIAP inhibitor 2 (B). Metabolic activity was determined by MTT assay and is expressed as percentage of untreated controls (% of c.; A–C, right). Apoptosis was determined by light scatter changes and flow cytometry (C, left). A and C, columns, mean of three independent experiments performed in triplicate; bars, SE. \#, \( P < 0.05 \); *, \( P < 0.01 \), comparing XIAP inhibitors to solvent. B, mean of three independent experiments performed in triplicate with SD of <10%. D, surface expression of the four human TRAIL receptors TRAIL-R1 to TRAIL-R4 on PBMC or the single mouse TRAIL-R on murine 3T3 fibroblasts was determined by fluorescence-conjugated antibodies and flow cytometry (thin line, cells stained with isotype control; thick line, cells stained with anti-TRAIL receptor antibodies). Fluorescence intensity (x axis) is plotted against cell counts (y axis). A representative experiment of three independent experiments.
Figure 5. XIAP inhibitor enhances TRAIL-induced antitumor activity against pancreatic carcinoma in vivo. PancTu1 cells were seeded on the CAM of chicken embryos and treated for 3 d with 0.7 ng TRAIL and/or 10 μmol/L XIAP inhibitor 2 or control compound. The CAM was excised on day 4, fixed, and stained with H&E, anti–Ki-67 antibody, TUNEL, or an antibody recognizing cleaved caspase-3. Representative pictures are shown in A; scale bars, 800 μm (left) and 200 μm (right). Percentages of cells positive for cleaved caspase-3 (B), TUNEL (C), and Ki-67 (D). Columns, mean of 12 samples per group of two independent experiments; bars, SE. *, P < 0.01, comparing XIAP inhibitor to solvent.
Figure 6. XIAP inhibitor cooperates with TRAIL to suppress tumor growth of established pancreatic carcinoma in a xenograft mouse model. PancTu1 cells were s.c. inoculated at left and right dorsal site of mice. Treatment started 1 wk after tumor cell inoculation by i.p. injection of 50 μg TRAIL or buffer and/or 40 mg/kg XIAP inhibitor 5 or control compound for two cycles of 10 d, n = 10 tumors per treatment group. Mice were sacrificed on day 33. A, points, mean of tumor volumes; bars, SE. *, P < 0.01, comparing tumors treated with XIAP inhibitor 5 and TRAIL to tumors treated with PBS and TRAIL. Bar below x axis marks the TRAIL treatment period. Therapeutic efficacy of TRAIL and/or XIAP inhibitor was estimated by T/C ratio (%) at day 33 relative to vehicle-treated tumors. B, representative animals and tumors for each group; arrows, tumors. C, tumor weight is depicted by box plots; the line inside each box denotes median, and boxes at 25th and 75th percentiles denote error bars at minimum and maximum. **, P < 0.001, comparing tumors treated with XIAP inhibitor 5 and TRAIL to tumors treated with PBS and TRAIL. D, immunohistochemical analysis of H&E, cytokeratin, cleaved caspase-3, TUNEL, and Ki-67 stainings of tumors 24 h after the second TRAIL dose (day 10). Number of cells per high power field positive for cleaved caspase-3 (top left), TUNEL (top middle), and Ki-67 (top right). Columns, mean are depicted; bars, SE. *, P < 0.01; **, P < 0.001, comparing tumors treated with XIAP inhibitor 5 and TRAIL to tumors treated with PBS and TRAIL. Representative pictures at the bottom; scale bar, 100 μm.
the superiority of the combination therapy for full activation of apoptosis programs. Third, XIAP is overexpressed in pancreatic carcinoma samples compared with nonmalignant pancreatic ducts, indicating that XIAP is a suitable target for cancer cell–selective induction of apoptosis. However, no clear correlation between XIAP expression and TRAIL sensitivity was observed in a survey of pancreatic carcinoma cell lines (14), indicating that additional factors are involved in the regulation of TRAIL sensitivity in pancreatic cancer. Fourth, XIAP inhibitors do not reverse the relative resistance of nonmalignant cells in vitro and normal tissues in vivo to TRAIL, pointing to a therapeutic index.

**Sensitizer/inducer strategy for apoptosis-based therapy of pancreatic cancer.** Our findings highlight the significance of a sensitizer/inducer concept for targeting apoptosis pathways in pancreatic cancer. The success of recombinant TRAIL or agonistic TRAIL receptor antibodies that are presently evaluated in early clinical trials (27, 28) may be curtailed by primary or acquired resistance of many human cancers including pancreatic carcinoma to TRAIL. Hence, there is a need to design rational combinations that counteract resistance mechanisms and lower the threshold for TRAIL-triggered apoptosis. Our findings provide the first in vivo evidence in preclinical animal models of pancreatic cancer that XIAP inhibitors can act as "sensitizers" to potentiate the antitumor activity of TRAIL against established pancreatic carcinoma. Previously, the combination of XIAP antagonists or XIAP knockdown and TRAIL was evaluated in pancreatic cancer in vitro (14, 29), whereas Smac mimetics were tested in vivo as monotherapy in a model of pancreatic cancer and in other malignancies (29–31). Our results showing that XIAP inhibitors do not reverse the lack of toxicity of TRAIL to nonmalignant cells in vitro or to normal tissues in vivo point to a therapeutic window. Whereas the insensitivity of nonneoplastic cells to XIAP inhibitors and TRAIL is not due to a lack of the apoptosis-inducing TRAIL receptors on their surface, the identification of the underlying mechanism(s) remains subject to future investigations. The sensitizer/inducer concept of XIAP inhibitors and TRAIL is also of broader relevance for a variety of human cancers besides pancreatic cancer, e.g., colon or breast carcinoma, to potentiate the efficacy of TRAIL. In addition, we recently reported that targeting XIAP can increase radiosensitivity of several cancers including pancreatic carcinoma (20, 32). XIAP inhibitors tested in this report were designed on the basis of the NMR structure of a Smac peptide bound to the BIR3 domain of XIAP and bind to XIAP BIR3 with high-nanomolar affinities (15). Our results are consistent with a mechanism in which they enhance TRAIL-induced apoptosis by relieving XIAP-mediated repression of effector caspases, whereas they exhibit no single-agent toxicity against pancreatic carcinoma cells up to micromolar concentrations. Mechanism-based cytotoxicity of active analogues is also supported by our findings showing that the weak BIR3 binding of the close structural control analogue correlates with absent cellular and strongly reduced in vivo activity. By comparison, bivalent Smac mimetics as single agents were recently shown to kill cancer cells by stimulating autocrine TNFα signaling (33–36) and cooperate with TNFα to trigger apoptosis by switching TNFα signaling from cell survival to apoptosis (34, 37, 38).

Clinically, resistance of pancreatic cancer to current regimens is a major cause of treatment failure and largely due to defects in cell death programs (3). Our present study sets a novel paradigm of how apoptosis pathways can be targeted for the treatment of pancreatic cancer by developing in preclinical models the combination of XIAP inhibitors plus TRAIL as a new and efficient therapeutic strategy for pancreatic carcinoma. These findings provide the rationale for further (pre)clinical evaluation of XIAP inhibitors and TRAIL in pancreatic cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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