TRAIL-Induced Apoptosis Is Preferentially Mediated via TRAIL Receptor 1 in Pancreatic Carcinoma Cells and Profoundly Enhanced by XIAP Inhibitors

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Abstract

Purpose: We previously reported that small molecule X-linked inhibitor of apoptosis (XIAP) inhibitors synergize with soluble TRAIL to trigger apoptosis in pancreatic carcinoma cells. Because cancers may preferentially signal via 1 of the 2 agonistic TRAIL receptors, we investigated these receptors as a therapeutic target in pancreatic cancer in the present study.

Experimental Design: We examined TRAIL receptor expression and cytotoxicity of specific monoclonal antibodies to TRAIL-R1 (HGS-ETR1, mapatumumab) or TRAIL-R2 (HGS-ETR2, lexatumumab) and of TRAIL receptor selective mutants alone and in combination with small molecule XIAP inhibitors in pancreatic cancer cell lines, in primary specimens, and in a xenotransplant model in vivo.

Results: The majority of primary pancreatic carcinoma samples and all cell lines express one or both agonistic TRAIL receptors. Nine of 13 cell lines are more sensitive to mapatumumab-induced apoptosis, whereas lexatumumab requires cross-linking for maximal activity. Similarly, TRAIL-R1 selective mutants display higher cytotoxicity than TRAIL-R2 selective mutants. Small molecule XIAP inhibitors preferentially act in concert with mapatumumab to trigger caspase activation, caspase-dependent apoptosis, and suppress clonogenic survival. Also, primary cultured pancreatic carcinoma cells are more susceptible to mapatumumab than lexatumumab, which is significantly enhanced by a XIAP inhibitor. Importantly, combined treatment with mapatumumab and a XIAP inhibitor cooperates to suppress tumor growth in vivo.

Conclusions: Mapatumumab exerts antitumor activity, especially in combination with XIAP inhibitors against most pancreatic carcinoma cell lines, whereas lexatumumab requires cross-linking for optimal cytotoxicity. These findings have important implications for the design of TRAIL-based protocols for pancreatic cancer. Clin Cancer Res; 16(23); 5734–49. ©2010 AACR.

Pancreatic cancer is one of the leading causes of cancer deaths in the Western world (1). Resistance of pancreatic cancer to even aggressive treatment regimens presents a major challenge in oncology (2). Because evasion of apoptosis, the cell’s intrinsic cell death program, contributes to treatment failure in pancreatic cancer (3, 4), current attempts to improve the survival of pancreatic cancer patients will have to include strategies that target apoptosis resistance.

Apoptosis pathways may be initiated through death receptors or mitochondria, resulting in caspase activation (5). Ligation of death receptors such as tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors by their cognate ligands results in caspase-8 activation, which induces direct cleavage of downstream effector caspases such as caspase-3 (6). The mitochondrial pathway is engaged by the release of apoptogenic factors from mitochondria into the cytosol, that is, cytochrome c or second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis (IAP)-binding protein with low pl (DIABLO) (7). Cytochrome c triggers caspase-3 activation via formation of the multimeric apoptosome complex, whereas Smac/DIABLO promotes apoptosis by neutralizing “IAP” proteins (7).

The concept of triggering TRAIL receptors on the cell surface to elicit apoptosis in cancer cells is especially relevant for cancer therapy, as death receptors are directly linked to the cell death program (6). To this end, TRAIL is considered as a prime candidate for clinical application, because it has been reported to induce apoptosis in a panel.
of cancer cells without limiting toxicity to normal human cells (8). However, many human cancers including pancreatic carcinoma proved to be TRAIL-resistant because of high levels of IAP proteins such as X-linked inhibitor of apoptosis (XIAP) (9, 10), which prevents apoptosis at the effector phase by binding to and inhibiting activated caspase-3 and -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).

Although TRAIL signals to apoptosis via either of the apoptosis-inducing TRAIL receptors TRAIL-R1 and TRAIL-R2, it has initially been assumed that it is in particular TRAIL-R2 that plays a dominant role in initiating apoptosis (13). The higher expression of TRAIL-R2 on many cancer cell lines has been put forward as an argument to support this concept, although a clear relationship between receptor expression levels and the response to either TRAIL-R1 or TRAIL-R2 activating compounds has not been established (13). More recently, the concept that it is predominately TRAIL-R2 that mediates TRAIL-induced apoptosis has also been challenged by data showing that some cancers, for example, chronic lymphocytic leukemia (CLL), predominantly signal to cell death via TRAIL-R1 (14, 15).

We previously reported that inhibition of XIAP profoundly enhances TRAIL-induced apoptosis in pancreatic carcinoma in vitro and in vivo (16–18). Besides soluble TRAIL, specific TRAIL receptor antibodies have been developed for clinical application, which demonstrate promising activities in early clinical trials (13, 19–21). TRAIL-R1 monoclonal antibodies have already been administered to pancreatic carcinoma patients in a phase I clinical trial (22). However, the question which of the two agonistic TRAIL receptors is in fact better suited as a therapeutic target in pancreatic cancer has not yet been answered. So far, no parameters have been identified that can accurately predict upfront whether a given tumor responds better to TRAIL-R1 versus -R2 stimulation. This highlights the need to evaluate the efficacy of TRAIL-R1 and -R2 specific antibodies in preclinical models of pancreatic cancers. Therefore, we investigated the effects of fully human monoclonal antibodies that bind specifically to either TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab) in pancreatic carcinoma in the present study.

Materials and Methods

Cell culture and reagents

Pancreatic carcinoma cells were cultured in DMEM, DMEM/F12, or RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1 mmol/L of glucose (Biochrom), 1% penicillin/streptomycin (Biochrom), and 25 mmol/L of HEPES (Biochrom) as described (17). A culture was established from primary pancreatic carcinoma cells (ULLA) derived from a peritoneal metastasis of a 71-year-old female patient with pancreatic adenocarcinoma and subsequently used at low passage numbers. Genotypic characterization showed homozygous deletion of p16 and Smad4, single deletion of p53, loss of heterozygosity of LKB1, wild-type status for PRSS1, and normal expression of MLH1. TRAIL was purchased from R&D Systems, Inc. (Wiesbaden, Germany), and TRAIL receptor-specific mutants were described previously (14). The fully human agonist monoclonal antibodies against TRAIL-R1 and TRAIL-R2, mapatumumab and lexatumumab, respectively, were kind gifts from Human Genome Sciences (13). XIAP inhibitor 1, XIAP inhibitor 2, and control compound correspond to compounds 2, 11, and 15, respectively, described by Oost et al. (23). XIAP inhibitors 3 and 4 were described by Chao et al. (24) and were kindly provided by Idun Pharmaceuticals (now Pfizer, Inc., Groton, CT). XIAP inhibitors are capped tripeptides consisting of unnatural amino acids that were designed on the basis of the nuclear magnetic resonance structure of a Smac peptide bound to the BIR3 domain of XIAP and bind to XIAP BIR3 with high nanomolar affinities (23). The broad-range caspase inhibitor zVAD.fmk was purchased from Bachem (Heidelberg, Germany). All chemicals were purchased from Sigma unless indicated otherwise.

Determination of apoptosis, cell viability, and clonogenic survival

Apoptosis was determined by fluorescence-activated cell-sorting analysis (FACSscan; BD Biosciences, Heidelberg, Germany) of DNA fragmentation of propidium iodide-stained nuclei (25). Cell viability was assessed by MTI assay according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). For clonogenic assay, cells were seeded as single cells (0.02 × 105 cells/cm2) in 6-well plates for 24 hours, treated with TRAIL receptor antibodies
and/or XIAP inhibitor for 24 hours (PancTu1) or 3 hours (PaTu1) before medium was exchanged, and colonies were stained after an additional 10 days with crystal violet solution (0.75% crystal violet, 50% ethanol, 0.25% NaCl, and 1.57% formaldehyde).

Western blot analysis
Western blot analysis was performed as described (17) using the following antibodies: mouse anti-caspase-8 (ApoTech Corporation, Epalinges, Switzerland), rabbit anti-caspase-3 (Cell Signaling, Beverly, MA), rabbit anti-caspase-9 and mouse anti-XIAP from BD Biosciences (Heidelberg, Germany), rabbit anti-cIAP2 (Epitomics, Burlingame, CA), goat anti-cIAP1 and rabbit anti-survivin (R&D Systems, Inc.), or mouse anti-β-actin (Sigma) followed by goat-anti-mouse IgG or goat-anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany).

TRAIL receptor surface staining
To determine surface expression of TRAIL receptor, cells were incubated with mouse anti-human TRAIL-R1 to -R4 monoclonal antibodies (all from ApoTech Corporation) for 30 minutes at 4°C, washed in PBS containing 1% FCS, incubated with rabbit anti-mouse-F(ab')2 IgG/Biotin (BD Biosciences) for 20 minutes at 4°C in the dark, washed in PBS containing 1% FCS, incubated with streptavidin-PE (BD Biosciences) for 20 minutes at 4°C in the dark, and analyzed by flow cytometry.

Immunohistochemistry
Immunohistochemistry of TRAIL receptors was performed on 24 pancreatic ductal adenocarcinomas and 4 normal pancreata as previously described (26). Briefly, 2 μm thick cryosections were immediately fixed in ice-cold acetone for 10 minutes and air-dried and incubated for 1 hour with mouse monoclonal antibodies to TRAIL-R1 (clone HS101; IgG1 isotype), TRAIL-R2 (clone HS201; IgG1 isotype), TRAIL-R3 (clone HS301, IgG1 isotype), or TRAIL-R4 (clone HS401; IgG1 isotype), respectively, in a 1:100 dilution (Alexis, San Diego, CA). Bound primary antibody was detected via the REAL EnVision Detection System Peroxidase/DAB+ (K5007; Dako, Glostrup, Denmark), followed by hematoxylin counterstaining. TRAIL-R expression using the above-monoclonal antibodies yielded identical results in normal pancreata as obtained in a previous study by different antibodies to these receptors (26). Negative controls were carried out by omitting the first antibody and yielded negative staining results (Fig. 1A, a). Results of immunohistochemistry were scored as “negative,” “weakly positive,” “positive,” or “strongly positive,” respectively. In cases of staining heterogeneity within the target cell population, two modalities were allowed (e.g., “p/n”) and not further quantified.

Chorioallantoic membrane assay
Chorioallantoic membrane (CAM) assay was done as described previously (16). Briefly, 1 × 10⁶ tumor cells were resuspended in 10-μL serum-free medium and 10-μL Matrigel Matrix (BD Biosciences) and implanted on fertilized chicken eggs on day 8 of incubation. Tumors were topically treated with 0.25 μg of mapatumumab or lexatumumab diluted in 15-μL serum-free medium with or without 10 μmol/L of XIAP inhibitor daily for three days, sampled with surrounding CAM four days after seeding, fixed in 4% paraformaldehyde, paraffin embedded, cut in 5-μm sections, and hematoxylin/eosin (H&E) stained. Tumor area was measured from a representative photograph of each tumor and the percentage of cellular area of the whole tumor area was calculated using OPTIMAS 6.5.1 (Media Cybernetics, Bethesda, MD). In detail, the whole tumor area of each picture was marked as region of interest, including tumor cells, Matrigel, and CAM tissue. Color threshold was set for viable H&E-stained tumor cells and the threshold reaching area containing viable tumor cells was calculated and expressed as the percentage of region of interest.

Statistical analysis
Statistical significance was assessed by 2-sided Student’s t test, using Microsoft Excel (Microsoft Deutschland GmbH, Unterschleissheim, Germany). Interaction between XIAP inhibitors and TRAIL receptor antibodies was analyzed by the combination index (CI) method, as described by Chou (27), using CalcuSyn software (Biosoft, Cambridge, UK). CI less than 0.9 indicates synergism, 0.9 to 1.1 additivity, and greater than 1.1 antagonism.

Results
Recently, we reported that small molecule XIAP inhibitors synergize with TRAIL to trigger apoptosis in pancreatic carcinoma cells in vitro and in vivo (16). Because it is not known at present which of the two agonistic TRAIL receptors is superior as a therapeutic target in pancreatic carcinoma, we evaluated agonistic TRAIL-R1 and -R2 specific antibodies alone and in combination with XIAP inhibitors in the present study.

Expression of TRAIL receptors in pancreatic carcinoma
First, we explored the expression status of TRAIL receptors both in human primary pancreatic ductal adenocarcinoma samples and in normal pancreatic tissue by immunohistochemistry. Ducts of normal, noninflamed pancreata were consistently negative for all TRAIL receptors (not shown), confirming our previously published data (26). Expression data of pancreatic carcinomas are listed in Table 1. Of 24 carcinomas, twelve were, at least in part, induced for TRAIL-R1 expression compared with normal pancreatic ducts, seven of which were entirely positive for TRAIL-R1 expression (Fig. 1A,b). TRAIL-R2 was expressed in 18 of 24 carcinomas, twelve of which were entirely TRAIL-R2 positive, including one with strong TRAIL-R2 expression throughout (Fig. 1A,c). TRAIL-R3 was expressed in 14 of 24 carcinomas, often only in subsets of neoplastic growth.
cells and only once in a weak manner (Fig. 1A,e). TRAIL-R4 was most frequently induced in pancreatic carcinomas compared with normal pancreatic tissue (22/24 samples) (Fig. 1A,f). Regarding the TRAIL-receptor expression profile, there was no entirely TRAIL-R1 to -R4 positive case or an entirely TRAIL-R1 to -R4 negative case; however, four cases lacked both TRAIL-R1 and -R2 (Table 1). No obvious correlation between TRAIL receptor expression and grade of differentiation was observed (Table 1).

Next, we examined cell surface expression of TRAIL receptors in a panel of pancreatic carcinoma cell lines. All cell lines exhibited surface expression of the two agonistic TRAIL receptors TRAIL-R1 and -R2 [Fig. 1B and (17)]. TRAIL-R3 and TRAIL-R4 were expressed at low or undetectable levels in most cell lines except PaTu8988t, T3M4, ASPC1, and PaTuII that express considerable levels of TRAIL-R4 [Fig. 1B and (17)].

XIAP inhibitor preferentially cooperates with mapatumumab to reduce viability in pancreatic carcinoma cells

To gain insight into the regulation of TRAIL-induced apoptosis via TRAIL-R1 and TRAIL-R2 in pancreatic carcinoma cells, we analyzed the cytotoxicity of fully human monoclonal antibodies specifically directed against TRAIL-R1 (mapatumumab) and TRAIL-R2 (lexatumumab). Interestingly, mapatumumab was more potent to reduce cell viability than lexatumumab in the majority of pancreatic carcinoma cell lines (9/13 cell lines), whereas four cell lines (MiaPaCa2, PaTu8988t, PaTu8988s, ASPC1) were more susceptible to lexatumumab (Fig. 2A).

Next, we assessed the effect of TRAIL receptor–specific antibodies in combination with a small molecule XIAP inhibitor that binds to the BIR3 domain of XIAP (23). Of note, the XIAP inhibitor significantly enhanced loss of

Fig. 1. TRAIL receptor expression in pancreatic carcinoma. A, TRAIL receptor expression was analyzed by immunohistochemistry on frozen sections in 24 samples of pancreatic ductal adenocarcinoma and four samples of normal pancreas. Examples of a case of a moderately differentiated (case number 11; a–c) and a poorly differentiated (case number 15; d–f) ductal pancreatic adenocarcinoma are presented. Case 11 shows weak TRAIL-R1 expression in all tumor cells (b) and TRAIL-R2 expression at a clearly higher level (c) (a is the negative control). Case 15 expresses TRAIL-R1 only weakly in a minority of neoplastic cells (d) and has clear-cut TRAIL-3 expression in a subset of neoplastic cells (e) while featuring strong TRAIL-R4 expression (f). Scale bar in a, valid for a–c, corresponds to 240 μm; scale bar in d, valid for d–f, corresponds to 120 μm.
viability in combination with one of the agonistic TRAIL receptor antibodies in all cell lines investigated (Fig. 2A). The majority of pancreatic carcinoma cell lines (9/13 cell lines) were more susceptible to the combination of mapatumumab and the XIAP inhibitor than lexatumumab (Fig. 2A). In comparison, the four cell lines that were more responsive to treatment with lexatumumab alone (MiaPaCa2, PaTu8988t, PaTu8988s, and ASPC1) were also more sensitive to the combination of lexatumumab plus XIAP inhibitor (Fig. 2A).

Moreover, we simultaneously treated cells with mapatumumab and lexatumumab to test whether the concomitant stimulation of both agonistic TRAIL receptors results in additive, synergistic, or antagonistic cytotoxicity. The combined use of mapatumumab and lexatumumab neither acted in concert to reduce viability nor antagonized each other in the presence or absence of the XIAP inhibitor (Fig. 2A). The only exception were MiaPaCa2 cells, for which simultaneous treatment with mapatumumab and lexatumumab resulted in enhanced reduction of cell viability compared with treatment with mapatumumab and lexatumumab alone (Fig. 2A). Control experiments using a close structural analogue that weakly binds to XIAP (23) showed no cooperative interaction with either of the TRAIL

**Fig. 1.** (cont'd.) TRAIL receptor expression in pancreatic carcinoma. B, Surface expression of TRAIL receptors 1 to 4 on pancreatic carcinoma cell lines was determined by flow cytometry (thin line: isotype control, thick line: anti-TRAIL receptor antibodies). Fluorescence intensity (x-axis) is blotted against cell counts (y-axis). A representative experiment of three independent experiments is shown.
receptor antibodies (Supplementary Fig. 1). Together, this set of experiments demonstrates that the majority of pancreatic carcinoma cell lines are more susceptible to TRAIL-R1 than TRAIL-R2–specific antibodies, either as single agents or in combination with a small molecule XIAP inhibitor. For further studies, we selected PancTu1 and PaTuII pancreatic carcinoma cells as prototype cell lines, which preferentially respond to mapatumumab in combination with the XIAP inhibitor.

### Distinct XIAP inhibitors preferentially cooperate with mapatumumab to induce apoptosis in pancreatic carcinoma cells

To test the broader relevance of our findings, we extended our studies to additional, structurally modified XIAP inhibitors, which all bind to the same surface groove of the BIR3 domain of XIAP (23, 24). Distinct XIAP inhibitors profoundly enhanced mapatumumab-induced loss of viability, whereas they displayed a minor cooperative interaction with lexatumumab (Fig. 2B and Supplementary Fig. 2). The simultaneous stimulation with mapatumumab and lexatumumab resulted in a similar reduction of cell viability compared with stimulation with mapatumumab alone, either in the absence or in the presence of XIAP inhibitors (Fig. 2B and Supplementary Fig. 2). Dose titration studies of mapatumumab and XIAP inhibitor 3 revealed that the interaction of these two agents was highly synergistic (Fig. 2C and Table 2).

To confirm that cells die by apoptotic cell death, we assessed DNA fragmentation as a characteristic feature of apoptosis. Importantly, the addition of XIAP inhibitors profoundly increased mapatumumab-induced apoptosis in a dose- and time-dependent manner (Figs. 3 A and B and Supplementary Fig. 3). In comparison, no or only a slight augmentation of apoptosis was observed when the XIAP inhibitor was combined with lexatumumab (Fig. 3A and B). Furthermore, we performed colony assays to examine the long-term effects of the combination treatment. The XIAP inhibitor preferentially cooperated with mapatumumab to suppress colony formation compared with lexatumumab (Fig. 3C). The specificity of mapatumumab and lexatumumab for TRAIL-R1 and TRAIL-R2, respectively, was confirmed by RNAi-mediated knockdown (Supplementary Fig. 4). Together, this set of experiments demonstrates that inhibition of XIAP preferentially sensitizes pancreatic carcinoma cells for TRAIL-R1–mediated apoptosis, resulting in long-term suppression of clonogenic survival.

### Table 1. TRAIL receptor expression in primary pancreatic carcinoma samples

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**NOTE:** Results of immunohistochemistry were scored as negative, weakly positive, positive, strongly positive, or in cases of staining heterogeneity as p/n.

**Abbreviations:** n, negative; wp, weakly positive; p, positive; sp, strongly positive; dpc, ductal pancreatic carcinoma; pc, pancreatic carcinoma; c, cell.
Preferential activity of TRAIL-R1 selective mutants against pancreatic carcinoma cells

To further explore the susceptibility of pancreatic carcinoma cells toward TRAIL-R1 versus TRAIL-R2 stimulation, we used mutant forms of TRAIL that bind to TRAIL-R1 or TRAIL-R2 with high specificity (14). In combination with the XIAP inhibitor, the TRAIL-R1 selective mutant was more potent than the TRAIL-R2 selective mutant to reduce viability of PancTu1 and PaTull cells (Fig. 4A). These results confirm the findings with TRAIL receptor–specific antibo-

Fig. 2. XIAP inhibitors cooperate with TRAIL receptor antibodies to reduce viability of pancreatic carcinoma cells. A, Cells were treated for 48 hours with indicated concentrations of mapatumumab (Mapa), lexatumumab (Lexa), or both mapatumumab and lexatumumab (Mapa + Lexa) and/or 10 μmol/L of XIAP inhibitor 2 or DMSO. Cell viability was determined by MTT assay and is expressed as the percentage of untreated controls. B, PancTu1 (left panels) and PaTull (right panels) cells were treated for 48 hours with indicated concentrations of mapatumumab (upper panels), lexatumumab (middle panels), or mapatumumab and lexatumumab (lower panels) and/or a subtoxic concentration (10 μmol/L) of XIAP inhibitor 3 or DMSO. C, Cells were treated for 48 hours with indicated concentrations of mapatumumab and XIAP inhibitor 3. Cell viability was determined by MTT assay and is expressed as the percentage of untreated controls. Mean ± SEM values of 3 independent experiments performed in triplicate are shown; #, P < 0.05; *, P < 0.01 comparing XIAP inhibitors to solvent.
activation (Fig. 5A). Treatment with the XIAP inhibitor significantly cooperated with mapatumumab to enhance caspase cleavage of caspase-9 and -3 into active fragments than lexatumumab (Fig. 5A). Also, the XIAP inhibitor preferentially cooperated with mapatumumab to enhance caspase activation, demonstrating that loss of viability occurred in a caspase-dependent manner.

To gain insight into the activation of the TRAIL signaling cascade upon triggering of TRAIL-R1 or TRAIL-R2 and its modulation by XIAP inhibitors, we monitored cleavage of caspases by Western blotting. In both PancTu1 and PaTuII cells, mapatumumab alone was more potent to induce cleavage of caspase-9 and -3 into active fragments than lexatumumab (Fig. 5A). Also, the XIAP inhibitor preferentially cooperated with mapatumumab to enhance caspase activation (Fig. 5A). Treatment with the XIAP inhibitor alone did not initiate caspase cleavage (Fig. 5A), consistent with our findings that the concentration of XIAP inhibitor used in these experiments is subtoxic and insufficient to initiate apoptosis in the absence of an additional proapoptotic stimulus (Figs. 2 and 3). We also assessed caspase activity by enzymatic caspase assays. Similarly, the XIAP inhibitor preferentially acted in concert with mapatumumab to increase caspase activity compared with lexatumumab (Fig. 5B and Supplementary Fig. 5). To test the requirement of caspase activity for apoptosis induction, we used the broad-range caspase inhibitor zVAD.fmk. Addition of zVAD.fmk almost completely rescued loss of viability upon the combination treatment with mapatumumab or lexatumumab and XIAP inhibitor (Fig. 5C), demonstrating that loss of viability occurred in a caspase-dependent manner.

**Cross-linking increases lexatumumab’s activity when combined with XIAP inhibitor**

Because TRAIL-R2 has previously been reported to require cross-linking for its full activity (28), we next investigated whether a cross-linking agent augments the cytotoxicity of TRAIL receptor antibodies. In the presence of the XIAP inhibitor, cross-linking of lexatumumab significantly increased its cytotoxicity whereas cross-linking of mapatumumab did not alter its cytotoxicity (Fig. 4B). In the absence of the XIAP inhibitor, however, the addition of a cross-linker had no or a minor effect on the cytotoxicity of TRAIL receptor antibodies (Fig. 4B). These findings demonstrate that cross-linking enhances lexatumumab-induced cytotoxicity either alone or when XIAP is simultaneously neutralized.

**XIAP inhibitor preferentially cooperates with mapatumumab to trigger caspase activation**

To gain insight into the activation of the TRAIL signaling cascade upon triggering of TRAIL-R1 or TRAIL-R2 and its modulation by XIAP inhibitors, we monitored cleavage of caspases by Western blotting. In both PancTu1 and PaTuII cells, mapatumumab alone was more potent to induce cleavage of caspase-9 and -3 into active fragments than lexatumumab (Fig. 5A). Also, the XIAP inhibitor preferentially cooperated with mapatumumab to enhance caspase activation (Fig. 5A).

**Table 2. Synergistic induction of apoptosis by XIAP inhibitor and mapatumumab**

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CI was calculated as described in the Materials and Methods section for apoptosis induced by combined treatment of pancreatic cancer cells for 72 hours with indicated concentration of mapatumumab and 10 µmol/L of XIAP inhibitor 3. CI < 0.9 indicates synergism.

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No preferential activation of PI3K/Akt/mTOR or Raf/MEK/ERK survival pathways by mapatumumab or lexatumumab

Because TRAIL has been reported to stimulate survival signaling such as the PI3K/Akt/mTOR and Raf/MEK/ERK pathways besides the induction of apoptosis (29), we asked whether TRAIL-R1 and TRAIL-R2 may differentially activate these survival cascades in pancreatic carcinoma cells. To address this question, we monitored the phosphorylation status of both upstream and downstream components of the PI3K/Akt/mTOR pathway, using Akt as a target of PI3K, S6 ribosomal protein as a target of mTOR, and ERK as a component of the Raf/MEK/ERK pathway. Treatment with mapatumumab or lexatumumab did not increase phosphorylation of Akt, S6 ribosomal protein, or ERK compared with control cells treated with solvent (Supplementary Fig. 6). This indicates that the reduced cytotoxicity of lexatumumab over mapatumumab is not simply due to preferential activation of the PI3K/Akt/mTOR and/or Raf/MEK/ERK pathways by lexatumumab.

**Mapatumumab shows higher activity than lexatumumab against primary cultured pancreatic carcinoma cells**

To validate the results obtained in cell lines, we extended our studies to primary cultured pancreatic carcinoma cells derived from a pancreatic adenocarcinoma specimen. Primary cultured pancreatic carcinoma cells (ULA) express TRAIL-R1, -R2, and -R4 as well as cIAP1, cIAP2, XIAP, and survivin protein (Fig. 6A and B). At equimolar concentrations, mapatumumab was more potent than lexatumumab to reduce cell viability of primary cultured pancreatic carcinoma cells (Fig. 6C). Importantly, the addition of the XIAP inhibitor further enhanced mapatumumab-mediated loss of viability (Fig. 6C). These findings demonstrate that mapatumumab exerts higher cytotoxicity against primary cultured pancreatic carcinoma cells than lexatumumab alone or in combination with XIAP inhibitor.
Fig. 3. XIAP inhibitor preferentially cooperates with mapatumumab to induce apoptosis and to reduce colony formation. PancTu1 (left panel) and PaTuII (right panel) cells were treated with mapatumumab (Mapa), lexatumumab (Lexa), or both mapatumumab and lexatumumab (Mapa + Lexa), at indicated concentrations (A) or 3 μg/mL (B) and/or 10 μmol/L of XIAP inhibitor 3 or DMSO. Apoptosis was determined by fluorescence-activated cell-sorting analysis of DNA fragmentation of propidium iodide–stained nuclei. C, colony formation after treatment with mapatumumab or lexatumumab at 3 μg/mL (left panel) or 1 μg/mL (right panel) and/or 10 μmol/L of XIAP inhibitor 3 or DMSO was assessed by crystal violet staining. A representative experiment of three independent experiments is shown; #, P < 0.05; *, P < 0.01 comparing treatment with Mapa or Mapa and Lexa in the presence or absence of XIAP inhibitors.
Mapatumumab cooperates with XIAP inhibitor to suppress pancreatic carcinoma growth in vivo

Finally, we extended our studies to an in vivo setting, using the CAM model as an established in vivo tumor model that allows the assessment of antitumor activity in a 3-dimensional setting (16). Mapatumumab together with XIAP inhibitor significantly reduced tumor growth compared to untreated tumors (Fig. 6D). Also, the combined treatment with lexatumumab and the XIAP inhibitor exerted some antitumor activity compared with the control, although this did not reach statistical significance (Fig. 6D). This shows that although both mapatumumab and lexatumumab can act in concert with XIAP inhibitor to suppress pancreatic carcinoma growth in vivo, mapatumumab is significantly more potent than lexatumumab.

Discussion

TRAIL receptor agonists are currently evaluated in early clinical trials in a variety of tumors including pancreatic cancers (13, 19–22, 30). There is mounting evidence that individual cancers preferentially signal via one of the

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**Fig. 4.** XIAP inhibitor preferentially cooperates with TRAIL-R1 selective mutant to induce apoptosis, whereas cross-linking of TRAIL-R2 potentiates apoptosis. PancTu1 (left panels) and PaTull (right panels) cells were treated for 48 hours with wild-type soluble TRAIL, TRAIL-R1 selective mutant (R1-5), or TRAIL-R2 selective mutant (R2-6) or with mapatumumab (Mepa) and lexatumumab (Lexa) and/or 10 μmol/L of XIAP inhibitor 3 or DMSO (A). B, mapatumumab and lexatumumab were used in the absence or presence of cross-linker. Cell viability was determined by MTT assay and is expressed as the percentage of untreated controls. Mean ± SEM values of three independent experiments performed in triplicate are shown; *, \( P < 0.01 \).
agonistic TRAIL receptors (14, 15). In pancreatic carcinoma, however, it has not yet been explored whether one of the two proapoptotic TRAIL receptors is superior as a therapeutic target.

Here, we provide first evidence that the majority of pancreatic carcinoma cell lines and also primary cultured pancreatic carcinoma cells are more sensitive to TRAIL-R1 over TRAIL-R2 agonists, especially in combination with XIAP inhibitors, and that cross-linking is required for maximal activity of the TRAIL-R2 antibody lexatumumab. This conclusion is supported by two distinct approaches to trigger one of the agonistic TRAIL receptors: First, fully human monoclonal antibodies that specifically bind with a 1,000 times greater affinity to either TRAIL-R1 or TRAIL-R2 (13) and, second, TRAIL receptor-selective mutants (14). Data obtained in a panel of pancreatic carcinoma cell lines underline the generality of the results, and experiments using an established culture of clinical tumor material confirm the clinical relevance of the findings.

The molecular basis of the preferential signaling via one of the two agonistic TRAIL receptors in cancer cells that express both receptors is currently not exactly known. Although our data show no differential activation of cell survival signaling, that is, activation of the PI3K/Akt/mTOR or Raf/MEK/ERK pathways, upon triggering of TRAIL-R1 or TRAIL-R2, they reveal clear differences in the cross-linking requirements. Accordingly, cross-linking of TRAIL-R2 profoundly enhances its cytotoxicity either alone or when XIAP is concomitantly antagonized, whereas cross-linking of TRAIL-R1 has no or minimal additional effects. Thus, the relatively low susceptibility of pancreatic cancer cells to TRAIL-R2 antibodies can be overcome by increasing the cross-linking status of TRAIL-R2. Distinct cross-linking requirements have previously been reported for TRAIL-R1 and TRAIL-R2. It is, in particular, TRAIL-R2 that is described to require cross-linking to enhance its apoptosis-inducing activity (28, 31, 32) and that triggers apoptosis under certain conditions only when cross-linked (33). In vivo, antibody cross-linking can occur either via Fc receptors of immune effector cells (34) or via the complement system (35). This may account for some antitumor activity of lexatumumab and XIAP inhibitor that we observed in our in vivo experiments, as an inflammatory reaction to exogenous material of the CAM of chicken embryos has been reported (36) and Fc receptor-bearing cells have been

Fig. 5. XIAP inhibitor enhances TRAIL-induced activation of caspases. A, PancTu1 (upper panel) and PaTuII (lower panel) cells were treated for indicated times with 3 μg/mL of mapatumumab (Mepa) or lexatumumab (Lexa) and/or 10 μmol/L of XIAP inhibitor 3 or DMSO. Caspase activation was determined by Western blotting. Arrows indicate caspase cleavage fragments. For procaspase-8, short and long exposures of the blots are presented. A representative experiment of three independent experiments is shown.
described in the intraembryonic mesenchyme of chicken embryos (37). Hence, partial cross-linking of lexatumumab by the host’s immune system might occur in the CAM model in vivo. It will be interesting to investigate whether different TRAIL-R2 agonists that are under (pre) clinical evaluation differ in their ability to cross-link TRAIL-R2 and thus may vary in their anticancer activity.

Furthermore, our findings reveal that the susceptibility of pancreatic carcinoma cells to TRAIL-R1 or TRAIL-R2 ligation does not directly correlate with surface expression of the respective TRAIL receptors. This observation is in line with previous studies showing that membrane expression of agonistic TRAIL receptors does not directly link to the cell’s susceptibility toward TRAIL-R1 or TRAIL-R2 stimulation (14, 15, 28, 31, 38). For example, CLL cells were found to signal primarily via TRAIL-R1, although they express TRAIL-R1 and TRAIL-R2 at similar levels. Vice versa, lung, colon, and breast carcinoma cell lines, all with similar membrane levels of TRAIL-R1 and TRAIL-R2, displayed a higher sensitivity to TRAIL-R2 selective mutants (31). In comparison, the preferential response of ovarian, colon, and renal cell carcinoma cell lines to TRAIL-R2 antibodies was associated with higher surface expression levels of TRAIL-R2 (32, 39–41). Although a more widespread and higher expression of TRAIL-R2 has been reported in a number of studies, no clear relationship between TRAIL receptor levels and the response of cancer cells to targeting one or other of the agonistic TRAIL receptors has been established (13). Thus, the relative contribution of each of the agonistic TRAIL receptors to initiate apoptosis in cells that express both receptors is not a simple consequence of surface expression levels and might be determined by
intracellular regulators of apoptosis. This highlights the importance of functional (pre)clinical studies such as the present one to identify the TRAIL receptor subtype that may preferentially or exquisitely transmit the apoptotic signal in a given type of cancer.

From the translational perspective of targeting TRAIL receptors in pancreatic cancer, it is important to note that neoexpression of all TRAIL receptors was found in malignant versus nonmalignant pancreatic carcinoma tissues, consistent with our previous findings showing the absence of TRAIL receptors in normal, noninflamed pancreata (26). A recent immunohistochemical study similarly showed upregulation of TRAIL-R1 and TRAIL-R4 in pancreatic carcinoma tissue compared with the normal pancreas (42). Together, these findings suggest that the TRAIL receptor system may serve as a therapeutic target in pancreatic cancer.

Another important finding of this study is that simultaneous inhibition of XIAP enhanced TRAIL-R1- or TRAIL-R2-induced apoptosis in a highly synergistic manner. This is particularly relevant, as TRAIL receptor antibodies as monotherapy displayed limited antitumor activity in the majority of the pancreatic cancer cell lines investigated, in line with our previous results for soluble recombinant TRAIL (17). This indicates that combination regimens to enhance the therapeutic potential of TRAIL receptor agonists are required to ensure the success of TRAIL receptor agonists against pancreatic cancer. Previously, we demonstrated that neutralizing XIAP either by RNA interference-mediated knockdown or by small molecule inhibitors acted in concert with soluble recombinant TRAIL to induce apoptosis in pancreatic cancer in vitro and in vivo (16–18). In addition, XIAP small molecule antagonists that target the BIR2 domain of XIAP were reported to synergize with TRAIL in pancreatic cancer (17, 43). Compared with these earlier reports that focus on soluble recombinant TRAIL, the current study shows for the first time that the antitumor activity of TRAIL receptor–specific antibodies is profoundly enhanced by neutralizing XIAP in pancreatic cancer cells. These findings support the concept that simultaneous targeting of XIAP in combination with stimulation of the TRAIL pathway is a promising approach to augment the antitumor activity of TRAIL receptor agonists against pancreatic cancer. They also have important implications for the design of future clinical trials with TRAIL receptor antibodies for the treatment of pancreatic cancer, as mapatumumab and lexatumumab are already under clinical evaluation alone or in combination with chemotherapy (22). The clinical relevance of our findings is supported by
data obtained in primary cultured pancreatic carcinoma cells that were established from a tumor specimen of a patient with pancreatic adenocarcinoma. Although the analysis of primary material is so far restricted to one specimen, the data provide a first proof of concept that the reported findings are not only restricted to established cell lines but also relevant for patients’ derived primary tumor cells established in culture.

In conclusion, this preclinical evaluation of a rational combination of two novel classes of apoptosis-targeting drugs, that is, TRAIL receptor antibodies and XIAP inhibitors, in relevant preclinical in vitro and in vivo models of pancreatic cancer provides the molecular basis for the design of new combination therapies for the treatment of pancreatic cancer. This strategy may help to overcome apoptosis resistance of pancreatic cancer, one of the cancers with the worst prognosis.

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

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TRAIL-Induced Apoptosis Is Preferentially Mediated via TRAIL Receptor 1 in Pancreatic Carcinoma Cells and Profoundly Enhanced by XIAP Inhibitors

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