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ORIGINAL ARTICLE

Sensitization of pancreatic carcinoma cells for γ-irradiation-induced apoptosis by XIAP inhibition

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Resistance of pancreatic cancer to current treatments including radiotherapy remains a major challenge in oncology and may be caused by defects in apoptosis programs. Since 'inhibitor of apoptosis proteins' (IAPs) block apoptosis at the core of the apoptotic machinery by inhibiting caspases, therapeutic modulation of IAPs could tackle a key resistance mechanism. Here, we report that targeting X-linked inhibitor of apoptosis (XIAP) by RNA-interference-mediated knockdown or overexpression of second mitochondria-derived activator of caspase significantly enhanced apoptosis and markedly reduced clonogenic growth of pancreatic carcinoma cells upon γ-irradiation. Analysis of signaling pathways revealed that antagonizing XIAP increased activation of caspase-2, -3, -8 and -9 and loss of mitochondrial membrane potential upon γ-irradiation. Interestingly, inhibition of caspases also reduced the cooperative effect of XIAP targeting and γ-irradiation to trigger mitochondrial perturbations, suggesting that XIAP controls a feedback mitochondrial amplification loop by regulating caspase activity. Importantly, our data demonstrate for the first time that small molecule XIAP inhibitors sensitized pancreatic carcinoma cells for γ-irradiation-induced apoptosis, whereas they had no effect on γ-irradiation-mediated apoptosis of non-malignant fibroblasts indicating some tumor specificity. In conclusion, targeting XIAP, for example by small molecules, is a promising novel approach to enhance radiosensitivity of pancreatic cancer that warrants further investigation.
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Introduction

Pancreatic carcinoma is among the leading causes of cancer deaths in the Western World (Schneider *et al*., 2005). Since pancreatic carcinoma is a prototype cancer that is usually resistant to current treatment protocols, treatment of pancreatic cancer remains one of the most challenging problems in oncology (Schneider *et al*., 2005). This calls for novel strategies to overcome resistance of pancreatic carcinoma cells in order to improve the poor prognosis of this disease. Since pancreatic cancer cells become refractory to undergo apoptosis, the cell's intrinsic death program (Westphal and Kalthoff, 2003), current attempts to improve the survival of pancreatic cancer patients will have to include strategies that specifically target tumor cell resistance to apoptosis.

Cell death by apoptosis has been implied to mediate therapy-induced cytotoxicity, for example, following chemo- or radiotherapy (Fulda and Debatin, 2006a–c). Apoptosis pathways may be initiated through different entry sites, such as death receptors or mitochondria resulting in activation of effector caspases (Fulda and Debatin, 2006a–c). 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 proteins (IAPs)-binding protein with low isoelectric point from mitochondria into the cytosol triggering caspase-3 activation as a result of formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex (Saelens *et al*., 2004).

Radiation-induced apoptotic signaling can be initiated in different cellular compartments, for example the nucleus, cytosol or at the plasma membrane, which eventually fuel into a common effector phase of apoptosis characterized by activation of caspases as death effector molecules (Jendrossek and Belka, 2006). However, caspase-independent apoptosis and non-apoptotic modes of cell death upon irradiation may also be involved (Schmidt-Ullrich *et al*., 2000; Jendrossek and Belka, 2006).

Defects in cell death programs including apoptosis may contribute to radioresistance (Jendrossek and Belka, 2006). Apoptosis signaling may be disrupted by deregulated expression and/or function of antiapoptotic molecules, for example 'inhibitor of apoptosis proteins' (Fulda and Debatin, 2006c). IAPs such as X-linked inhibitor of apoptosis (XIAP), cellular inhibitor of apoptosis protein 1 (cIAP1), cIAP2, survivin or livin are expressed at high levels in many human cancers, which has been associated with treatment resistance, for example, resistance to radiotherapy (Salvesen and Duckett, 2002; Rodel *et al*., 2003; Cao *et al*., 2004; Lu

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et al., 2004). Since IAPs block apoptosis at the core of the apoptotic machinery by inhibiting caspases, therapeutic targeting of IAPs may overcome resistance. Therefore, there has been much interest in recent years to antagonize IAPs for radiosensitization of human cancers. To this end, survivin or XIAP antisense oligonucleotides were shown to enhance the efficacy of radiotherapy by reducing survival and increasing apoptosis in lung cancer cells (Cao *et al*., 2004; Lu *et al*., 2004). We recently reported that ectopic expression of Smac, an endogenous inhibitor of IAPs, enhanced γ-irradiation-induced apoptosis of cancer cells (Giagkousiklidis *et al*., 2005). However, the question whether or not inhibition of XIAP is an effective approach to overcome radioresistance of pancreatic cancer has not yet been explored. Searching for novel strategies to overcome radioresistance of pancreatic cancer, we investigated the role of XIAP in regulating cell death of pancreatic carcinoma cells in response to γ-irradiation in the present study.

Results

XIAP targeting and γ-irradiation cooperate to induce apoptosis and inhibit clonogenic growth of pancreatic carcinoma cells

To investigate the role of XIAP in regulating radioresistance of pancreatic carcinoma, we used two distinct approaches to target XIAP: first, we knocked down XIAP expression by RNA-interference (RNAi) and second, we overexpressed full-length Smac. Infection of PaTuII pancreatic carcinoma cells with the retroviral vector containing XIAP short hairpin RNA (shRNA) resulted in marked downregulation of XIAP protein compared to nonsense shRNA in different clones (Figure 1a). To monitor potential off-target effects of shRNA, we also assessed expression of other IAP proteins. No detectable alterations in Smac, cIAP1, cIAP2, survivin or livin expression were observed in PaTuII pancreatic carcinoma cells stably expressing XIAP shRNA (Figure 1a), demonstrating that stable suppression of XIAP by shRNA did not result in suppression or compensatory upregulation of other IAP members or Smac. Reh B-precursor cell leukemia cells were used as positive control for livin expression (Figure 1a). Importantly, knockdown of XIAP significantly increased γ-irradiation-induced apoptosis of PaTuII pancreatic carcinoma cells in a dose- and time-dependent manner compared to cells harboring nonsense shRNA as assessed by the analysis of DNA fragmentation (Figure 2a and Supplementary Figure 1 for kinetic analysis). Since sensitization for γ-irradiation-induced apoptosis was observed in several individual clones of PaTuII pancreatic carcinoma cells, in which XIAP was knocked down by shRNA (Figure 2a), it is unlikely that the sensitization for γ-irradiation-induced apoptosis provided by XIAP downregulation was simply the result of clonal selection.

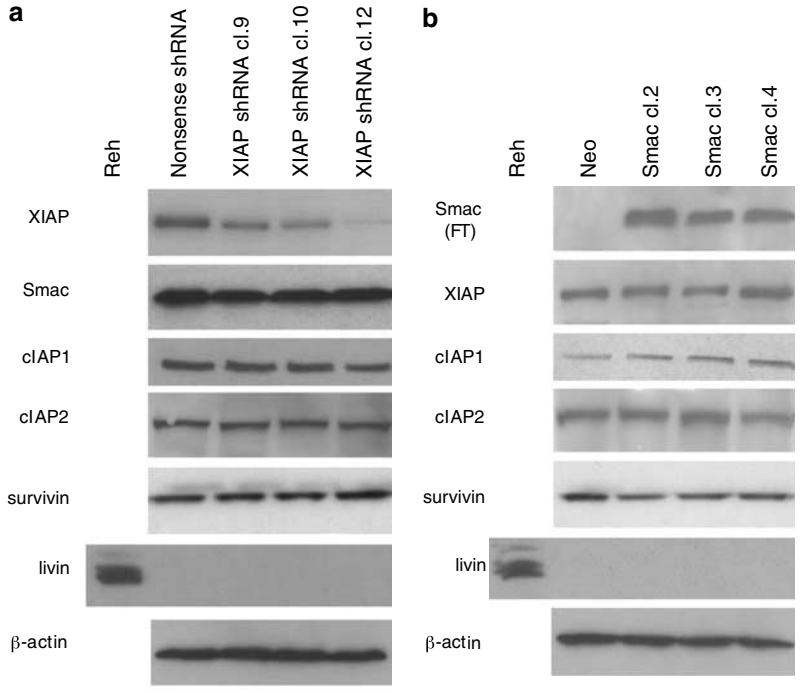
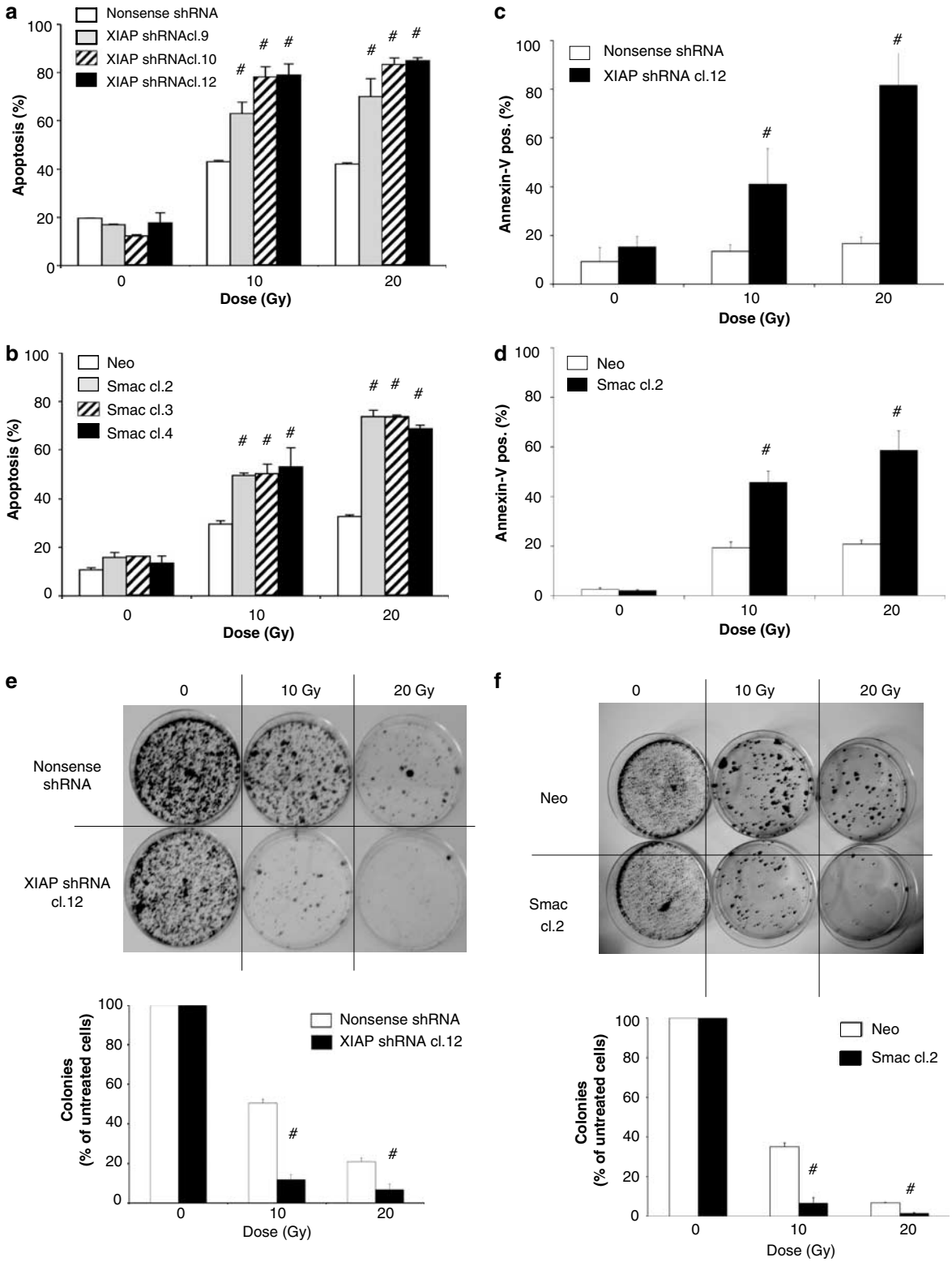


Figure 1 Downregulation of XIAP or overexpression of Smac in PaTuII pancreatic carcinoma cells. Protein expression of XIAP, cIAP1, cIAP2, survivin, livin, Smac, Flag-tagged Smac (Smac (FT)) and β-actin was assessed by western blot in stable clones of PaTuII pancreatic carcinoma cells transduced with nonsense shRNA or shRNA against XIAP (**a**) and in PaTuII pancreatic carcinoma cells transduced with full-length Smac cDNA or empty vector control (**b**). Reh B-precursor acute lymphocytic leukemia cells were used as positive control for livin expression. Abbreviations: cDNA, complementary DNA; cIAP1, cellular inhibitor of apoptosis protein 1; shRNA, short hairpin RNA; Smac, second mitochondria-derived activator of caspase; XIAP, X-linked inhibitor of apoptosis.

In the second approach to antagonize XIAP, PaTuII pancreatic carcinoma cells were engineered to overexpress full-length Smac, which localizes to mitochondria as determined by fluorescence microscopy, or empty vector as control (Figure 1b and data not shown for fluorescence microscopy). No detectable changes in XIAP, cIAP1, cIAP2, survivin or livin levels were found in clones overexpressing Smac compared to empty vector control (Figure 1b). In addition, control experiments showed that transduction of PaTuII pancreatic carcinoma cells with the corresponding control vectors, that is, nonsense shRNA (control for XIAP shRNA) or



Neo vector (control for Smac-containing vector), had no detectable effect on expression levels of XIAP or Smac compared to parental PaTuII pancreatic carcinoma cells (Supplementary Figure 2). Notably, overexpression of Smac significantly enhanced γ-irradiation-induced apoptosis compared to empty vector control cells (Figure 2b). Since we observed a significant increase in γ-irradiation-induced apoptosis in different clones overexpressing Smac (Figure 2b), this sensitization provided by Smac is unlikely to be due to clonal selection. Notably, knockdown of XIAP by shRNA or overexpression of Smac alone without concomitant γ-irradiation had no effect on spontaneous apoptosis of PaTuII pancreatic carcinoma cells (Figure 2). This is in line with results reported by our group and other investigators that silencing of XIAP or overexpression of Smac did not trigger apoptosis in the absence of an additional apoptotic stimulus in many cancer cells including pancreatic carcinoma (Holcik *et al*., 2000; Guo *et al*., 2002; Fulda *et al*., 2002; Cummins *et al*., 2004; Giagkousiklidis *et al*., 2005; Vogler *et al*., 2005, 2007). Together, these findings demonstrate that targeting XIAP either by shRNA-mediated knockdown of XIAP or by overexpression of Smac markedly enhanced γ-irradiation-induced apoptosis of PaTuII pancreatic carcinoma cells. For further experiments, we selected clone 12 of PaTuII pancreatic carcinoma cells, in which XIAP was knocked down by shRNA, and clone 2 of PaTuII pancreatic carcinoma cells overexpressing Smac.

To double confirm the sensitization effect of XIAP antagonism for γ-irradiation-induced apoptosis, we used annexin-V staining as a second method to determine apoptotic cell death (van Engeland *et al*., 1998). Downregulation of XIAP by shRNA significantly enhanced γ-irradiation-induced exposure of phosphatidylserine on the plasma membrane compared to nonsense shRNA, as shown by the increase in annexin-V positive cells (Figure 2c). Similarly, overexpression of Smac significantly increased the number of annexin-V positive PaTuII pancreatic carcinoma cells in response to γ-irradiation compared to vector control cells (Figure 2d).

Next, we explored whether targeting XIAP also has an effect on long-term survival of pancreatic carcinoma cells by performing clonogenic assays. Importantly, shRNA-mediated silencing of XIAP significantly reduced clonogenic growth of PaTuII pancreatic carcinoma cells upon γ-irradiation compared to control cells with nonsense shRNA (Figure 2e). Similarly, ectopic expression of Smac cooperated with γ-irradiation to suppress clonogenic survival of PaTuII pancreatic carcinoma cells compared to empty vector controls (Figure 2f). Together, these results demonstrate that targeting XIAP by shRNA-mediated knockdown of XIAP or overexpression of Smac sensitized PaTuII pancreatic carcinoma cells for γ-irradiation-induced apoptosis and loss of clonogenic survival.

XIAP targeting and γ-irradiation cooperate to trigger the mitochondrial pathway

To gain insight into the molecular mechanisms mediating the observed sensitization of PaTuII pancreatic carcinoma cells for γ-irradiation provided by XIAP knockdown or Smac overexpression, we first asked whether XIAP targeting has an effect on the mitochondrial pathway. To address this question, we determined mitochondrial membrane potential by flow cytometry in cells exposed to γ-irradiation. Downregulation of XIAP or overexpression of Smac significantly enhanced breakdown of the mitochondrial membrane potential following γ-irradiation compared to nonsense shRNA (Figure 3a) or empty vector control cells (Figure 3b). These findings indicate that targeting XIAP by shRNA-mediated knockdown of XIAP or overexpression of Smac cooperated with γ-irradiation to trigger the mitochondrial pathway in PaTuII pancreatic carcinoma cells.

XIAP targeting and γ-irradiation cooperate to activate caspases

To further explore the molecular mechanisms of the cooperative interaction of XIAP targeting and γ-irradiation, we analysed activation of the caspase cascade by enzymatic caspase assay using fluorogenic caspase substrates and flow cytometry. Notably, activity of caspase-3, -8, -9 or -2 following γ-irradiation was increased in PaTuII pancreatic carcinoma cells with shRNA-mediated knockdown of XIAP compared to nonsense shRNA in a time-dependent manner (Figure 4a). Also, overexpression of Smac significantly enhanced γ-irradiation-induced activity of caspase-3, -8, -9 or -2 compared to vector control cells in a time-dependent fashion (Figure 4b). Increased activity of caspase-3 and -9 in cells that overexpress Smac is likely the result of enhanced release of ectopically expressed Smac from mitochondria into the cytosol (data not shown), where Smac neutralizes XIAP and thus promotes caspase activation. The increase in caspase-8 or -2 activity in cells with XIAP knockdown or Smac overexpression may reflect the elevated caspase-3 activity in these cells, since caspase-3 has been reported to promote

Figure 2 XIAP targeting and γ-irradiation cooperate to induce apoptosis and inhibit clonogenic growth of pancreatic carcinoma cells. PaTuII pancreatic carcinoma cells transduced with nonsense or XIAP shRNA (**a**, **c** and **e**) or with empty vector or Smac cDNA (**b**, **d** and **f**) were treated with 10 or 20 Gy γ-irradiation and apoptosis and clonogenic survival was assessed in distinct clones. Apoptosis was determined 96 h after γ-irradiation by FACS analysis of DNA fragmentation of propidium-iodide-stained nuclei (**a** and **b**) or by annexin-V staining (**c** and **d**). In (**e** and **f**) clonogenic survival was determined by clonogenic assay as described in Materials and methods; colony formation is expressed as percentage of colonies of treated compared to untreated cells. In (**a**–**f**) mean $\pm$ s.e.m. of three independent experiments are shown; in (**e** and **f**) representative experiments are shown in upper panels. For statistical analysis, *t*-test was performed comparing nonsense versus XIAP shRNA or empty versus Smac-containing vectors (#$P<0.001$). Abbreviations: cDNA, complementary DNA; FACS, fluorescence-activated cell sorting; shRNA, short hairpin RNA; Smac, second mitochondria-derived activator of caspase; XIAP, X-linked inhibitor of apoptosis.

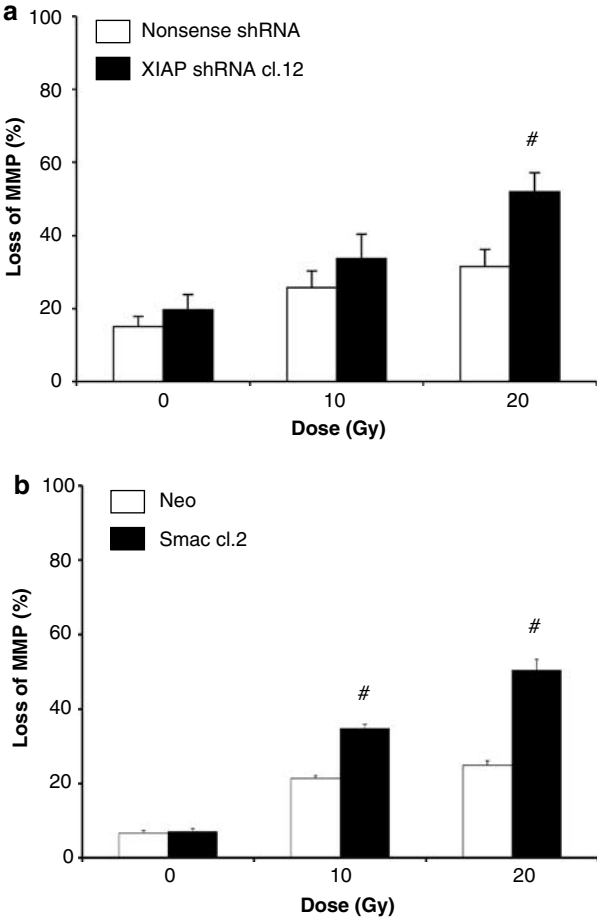


Figure 3 XIAP targeting and γ-irradiation cooperate to trigger loss of mitochondrial membrane potential. PaTuII pancreatic carcinoma cells transduced with nonsense or XIAP shRNA, clone 12 (**a**) or with empty vector or Smac cDNA, clone 2 (**b**) were treated with 10 or 20 Gy γ-irradiation. Mitochondrial transmembrane potential was determined 96 h after γ-irradiation by $DiOC_6(3)$ staining and FACS analysis. Mean $\pm$ s.e.m. of three independent experiments performed in triplicate are shown. For statistical analysis, *t*-test was performed comparing nonsense versus XIAP shRNA or empty versus Smac-containing vectors ($\#P<0.001$). Abbreviations: cDNA, complementary DNA; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanide iodide; FACS, fluorescence-activated cell sorting; shRNA, short hairpin RNA; Smac, second mitochondria-derived activator of caspase; XIAP, X-linked inhibitor of apoptosis.

activation of the initiator caspases-8 and -2 in a positive feedback loop (Slee *et al*., 1999, 2000; Sohn *et al*., 2005).

To investigate whether apoptosis was the result of caspase activation, we used the broad-range caspase inhibitor zVAD.fmk as well as relatively selective chemical inhibitors of individual caspases, that is, caspase-2 (zVDVAD.fmk), caspase-3 (zDEVD.fmk) and caspase-8 (zIETD.fmk). Sensitization of PaTuII pancreatic carcinoma cells for γ-irradiation-induced apoptosis by XIAP downregulation or Smac overexpression was substantially reduced in the presence of zVAD.fmk, zVDVAD.fmk, zDEVD.fmk or zIETD.fmk (Figure 4c and d). These findings demonstrate that sensitization of PaTuII pancreatic carcinoma cells for γ-irradiation-induced apoptosis provided by knockdown of XIAP or overexpression of Smac occurred in a caspase-dependent manner.

XIAP targeting promotes a caspase-dependent mitochondrial amplification loop upon γ-irradiation

We then wished to dissect the sequence of events leading to caspase activation and mitochondrial perturbations after γ-irradiation in PaTuII pancreatic carcinoma cells in which XIAP was antagonized. To address this question, we examined the effect of caspase inhibitors on mitochondrial membrane perturbations after γ-irradiation. γ-Irradiation-induced drop of mitochondrial membrane potential was inhibited by the wide-spectrum caspase inhibitor zVAD.fmk and the relatively specific inhibitors of caspase-2 (zVDVAD.fmk), caspase-3 (zDEVD.fmk) and caspase-8 (zIETD.fmk) in PaTuII pancreatic carcinoma cells with XIAP downregulation (Figure 5a). Similarly, loss of mitochondrial membrane potential following γ-irradiation was prevented in the presence of zVAD.fmk, zVDVAD.fmk, zDEVD.fmk or zIETD.fmk in cells overexpressing Smac (Figure 5b). These findings suggest that targeting XIAP enhanced mitochondrial perturbations of PaTuII pancreatic carcinoma cells in response to γ-irradiation in a caspase-dependent manner by increasing caspase activity, which in turn initiated a loop acting back on the mitochondria to cause further disruption of the mitochondrial membrane potential.

XIAP targeting by small molecule XIAP inhibitors potentiates γ-irradiation-induced apoptosis in various pancreatic carcinoma cell lines

Having established that neutralizing XIAP sensitized PaTuII pancreatic carcinoma cells for γ-irradiation-induced apoptosis, we then wished to extend our studies to additional pancreatic carcinoma cell lines to exclude that the sensitization effect was restricted to a particular cell line. Importantly, overexpression of Smac also sensitized ASPC1 pancreatic carcinoma cells for γ-irradiation-induced apoptosis and significantly reduced clonogenic growth after γ-irradiation (Figure 6a and b). In addition, overexpression of Smac significantly enhanced loss of mitochondrial potential upon γ-irradiation (Figure 6c).

Finally, to test whether our approach could be translated into a therapeutic setting, we antagonized XIAP by two distinct small molecule XIAP inhibitors that bind to the BIR3 domain of XIAP or a close structural analogue that only very weakly binds to the BIR3 domain of XIAP as control (Oost *et al*., 2004). Treatment with XIAP antagonists alone did not induce apoptosis in pancreatic carcinoma cells (Figure 7a–d). Importantly, XIAP inhibitors strongly sensitized different pancreatic carcinoma cell lines for γ-irradiation-induced apoptosis (Figure 7a–d). In contrast, the control compound had no effect on γ-irradiation-induced apoptosis compared to solvent demonstrating the specificity of the sensitizing effect (Figure 7a–d). Furthermore, we investigated whether or not XIAP inhibitors have an effect on normal cells. Notably, XIAP antagonists did not alter γ-irradiation-induced apoptosis of non-malignant fibroblasts compared to the control compound (Figure 7e), although these cells express high levels of XIAP (data not shown). These data demonstrate that small molecule

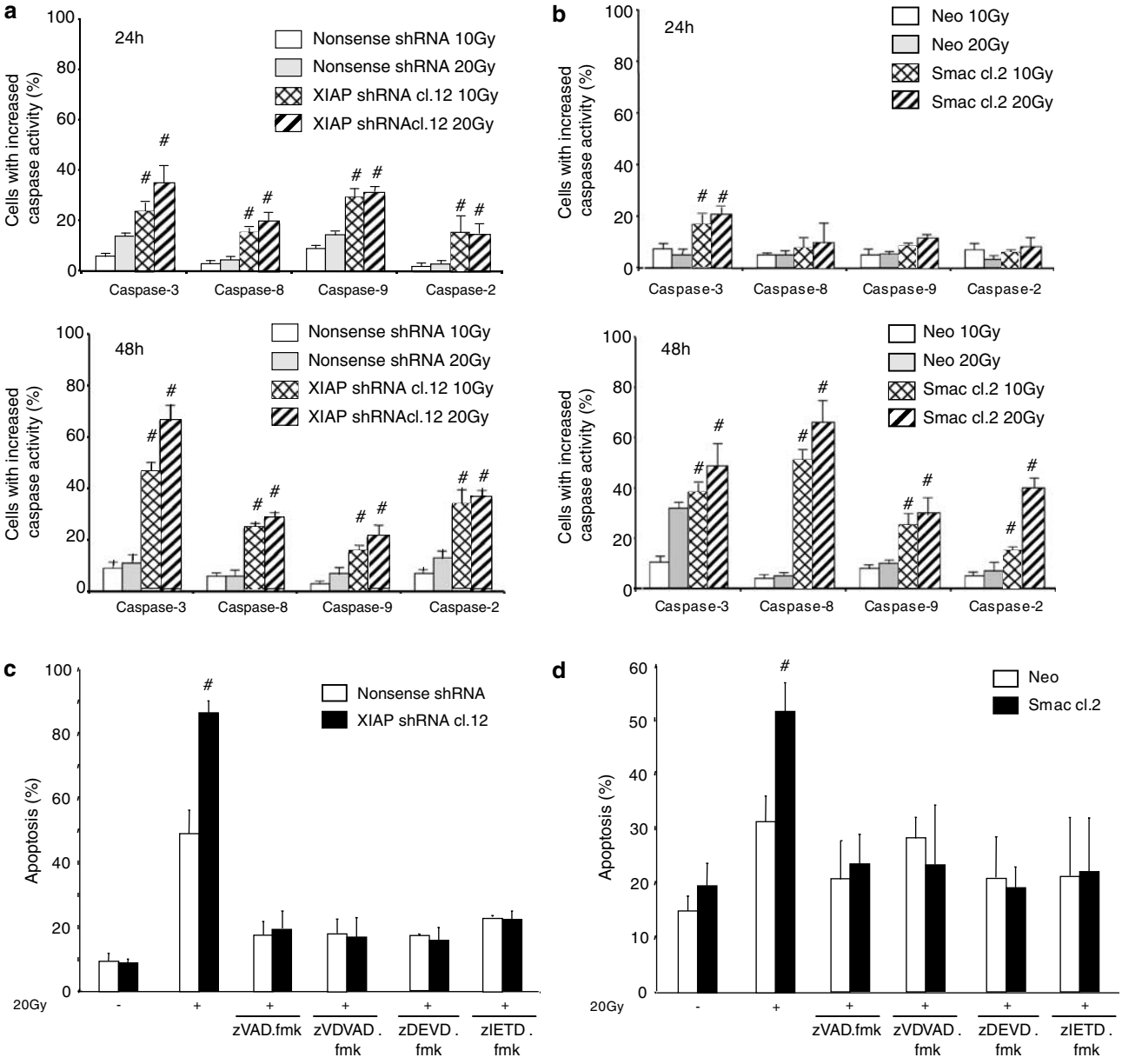


Figure 4 XIAP targeting and γ-irradiation cooperate to activate caspases. (**a** and **b**) Effect of XIAP targeting and γ-irradiation on caspase activity. PaTuII pancreatic carcinoma cells transduced with nonsense or XIAP shRNA, clone 12 (**a**) or with empty vector or Smac cDNA, clone 2 (**b**) were treated with 10 or 20 Gy γ-irradiation. Caspase activity of caspase-3, -8, -9 and -2 was determined 24 h (upper two panels) and 48 h (lower two panels) after γ-irradiation by FACS analysis as described in Materials and methods and the percentage of cells with increased caspase activity compared to untreated cells is shown. Mean $\pm$ s.e.m. of three independent experiments performed in triplicate are shown. For statistical analysis, *t*-test was performed comparing nonsense versus XIAP shRNA or empty versus Smac-containing vectors (#$P<0.001$). (**c** and **d**) Effect of caspase inhibitors on apoptosis induced by XIAP targeting and γ-irradiation. PaTuII pancreatic carcinoma cells transduced with nonsense or XIAP shRNA, clone 12 (**c**) or with empty vector or Smac cDNA, clone 2 (**d**) were treated with 20 Gy γ-irradiation in the presence or absence of 50 μM zVAD.fmk, 25 μM zVDVAD.fmk, 50 μM zIETD.fmk or 50 μM zDEVD.fmk. Apoptosis was determined 96 h after γ-irradiation by FACS analysis of DNA fragmentation of propidium-iodide-stained nuclei. Mean $\pm$ s.e.m. of three independent experiments performed in triplicate are shown. For statistical analysis, *t*-test was performed comparing nonsense versus XIAP shRNA or empty versus Smac-containing vectors (#$P<0.001$). Abbreviations: cDNA, complementary DNA; FACS, fluorescence-activated cell sorting; shRNA, short hairpin RNA; Smac, second mitochondria-derived activator of caspase; XIAP, X-linked inhibitor of apoptosis.

XIAP inhibitors are potent sensitizers for γ-irradiation-induced apoptosis in a variety of pancreatic carcinoma cell lines, but not in normal fibroblasts pointing to some tumor specificity.

Discussion

The intrinsic resistance of pancreatic carcinoma to established therapies including radiotherapy calls

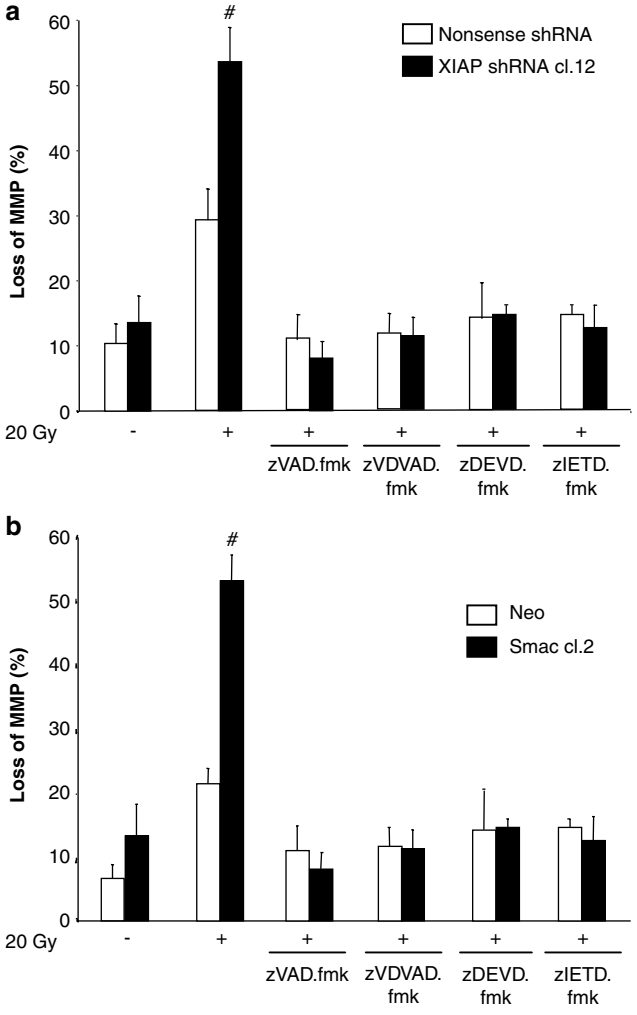


Figure 5 XIAP targeting promotes a caspase-dependent mitochondrial amplification loop upon γ-irradiation. PaTuII pancreatic carcinoma cells transduced with nonsense or XIAP shRNA, clone 12 (**a**) or with empty vector or Smac cDNA, clone 2 (**b**) were treated with 20 Gy γ-irradiation in the presence or absence of 50 μM zVAD.fmk, 25 μM zVDVAD.fmk, 50 μM zIETD.fmk or 50 μM zDEVD.fmk. Loss of mitochondrial transmembrane potential was determined 96 h after γ-irradiation by $DiOC_6(3)$ staining and FACS analysis. Mean $\pm$ s.e.m. of three independent experiments performed in triplicate are shown. For statistical analysis, *t*-test was performed comparing nonsense versus XIAP shRNA or empty versus Smac-containing vectors ($\#P<0.001$). Abbreviations: cDNA, complementary DNA; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanide iodide; FACS, fluorescence-activated cell sorting; shRNA, short hairpin RNA; Smac, second mitochondria-derived activator of caspase; XIAP, X-linked inhibitor of apoptosis.

for novel strategies to target tumor cell resistance (Schneider *et al*., 2005). Since IAPs block apoptosis at the core of the apoptotic machinery, therapeutic modulation of IAPs could addresses a key control point in deciding cell fate (Fulda and Debatin, 2006c). Here, we provide for the first time evidence that targeting XIAP by RNAi-mediated knockdown of XIAP or by small molecule XIAP inhibitors significantly enhanced γ-irradiation-induced apoptosis. Also, downregulation of XIAP markedly reduced clonogenic growth of pancreatic carcinoma cells upon γ-irradiation, demonstrating that it impaired long-term tumor cell survival. Similarly, overexpression of Smac, an inhibitor of XIAP that is released from mitochondria into the cytosol upon γ-irradiation, sensitized pancreatic carcinoma cells for γ-irradiation-induced apoptosis and loss of clonogenicity. These data provide convincing evidence that blocking XIAP, for example, by small molecule inhibitors, is a promising novel strategy to restore radiosensitivity of pancreatic carcinoma, a prototype cancer that is notoriously resistant to apoptosis.

There is mounting evidence that IAPs are involved in determining sensitivity to radiotherapy in human cancers (Rodel *et al*., 2003; Cao *et al*., 2004; Lu *et al*., 2004). To this end, high levels of XIAP or survivin have been associated with resistance to radiotherapy in lung or colorectal carcinoma (Holcik *et al*., 2000; Rodel *et al*., 2003; Cao *et al*., 2004; Lu *et al*., 2004). Inhibition of survivin or XIAP by antisense oligonucleotides was shown to enhance the efficacy of radiotherapy of lung cancer cells by reducing survival and increasing apoptosis (Cao *et al*., 2004; Lu *et al*., 2004). However, the question whether or not direct targeting of XIAP is an effective approach to overcome radioresistance of pancreatic cancer has not yet been explored. Our present study is the first to demonstrate that neutralizing XIAP by RNAi-mediated knockdown or small molecule inhibitors potentiates the antitumor activity of γ-irradiation in pancreatic carcinoma. By significantly advancing the present knowledge on the role of XIAP in pancreatic cancer biology and therapy, our study has important implications for the development of novel approaches designed to overcome apoptosis resistance of pancreatic cancer. Of note, it is increasingly becoming clear that XIAP regulates apoptosis in a context-dependent manner, since XIAP inhibition has been reported to fail to sensitize cancer cells for apoptosis under certain conditions, for example in leukemia cells treated with etoposide (Wilkinson *et al*., 2004) or in breast carcinoma cells upon treatment with carboplatin or doxorubicin (McManus *et al*., 2004). This underlines the importance of studies that evaluate the impact of XIAP inhibition in response to distinct stimuli, that is, γ-irradiation, in specific cancer types in order to see which tumor type might benefit from the concomitant use of XIAP inhibitors as adjuvant therapy. Thus, by identifying pancreatic carcinoma as a cancer that is vulnerable to XIAP inhibition when combined with γ-irradiation, our study has considerable impact on the successful development of experimental therapeutics for the treatment of pancreatic cancer. Of note, XIAP antagonists promoted apoptosis upon γ-irradiation in pancreatic cancer cells, but not in non-malignant fibroblasts. Although these data point to some tumor specificity of the radiosensitizing effect of XIAP inhibitors, this issue remains to be further addressed in normal cells that are more radiosensitive than fibroblasts.

Analysis of signaling pathways mediating the cooperative effect of XIAP targeting and γ-irradiation in pancreatic carcinoma cells revealed that downregulation of XIAP or overexpression of Smac enhanced activation

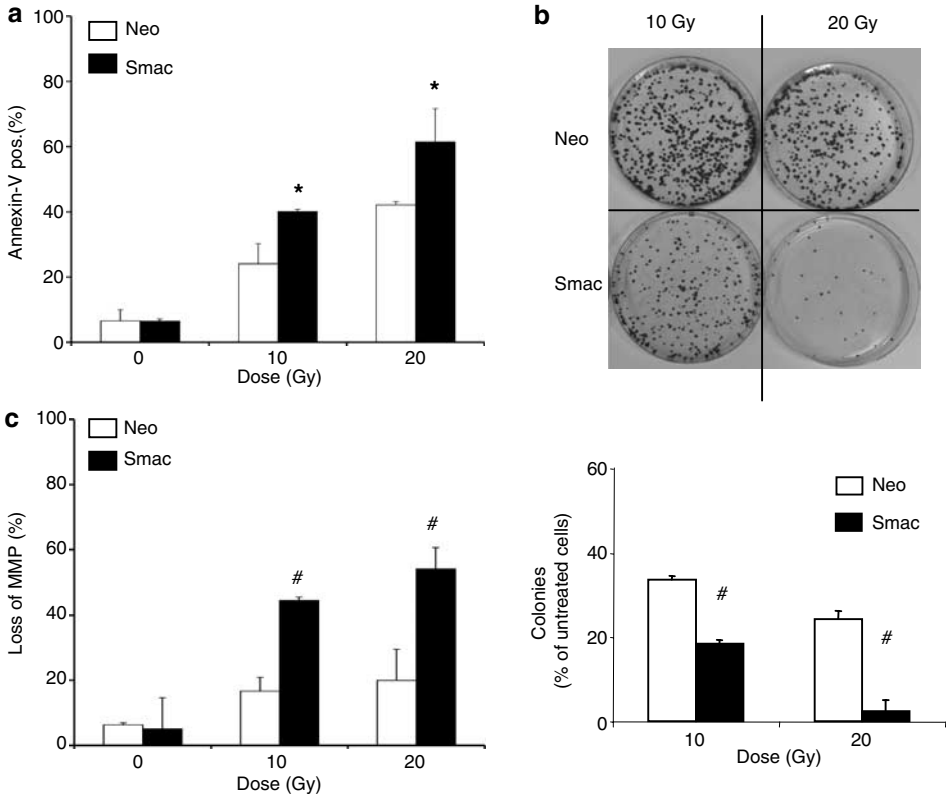


Figure 6 XIAP targeting by Smac overexpression cooperates with γ-irradiation to induce apoptosis in ASPC1 pancreatic carcinoma cells. ASPC1 pancreatic carcinoma cells stably transduced with empty vector or Smac cDNA were treated with 10 or 20 Gy γ-irradiation. Apoptosis was determined 96 h after γ-irradiation by annexin-V staining (**a**). In (**b**) clonogenic survival was determined by clonogenic assay as described in Materials and methods; colony formation is expressed as percentage of colonies of treated compared to untreated cells. In (**c**) mitochondrial transmembrane potential was assessed 96 h after γ-irradiation by $DiOC_6(3)$ staining and FACS analysis. In (**a**–**c**) mean $\pm$ s.e.m. of three independent experiments performed in triplicate are shown; in (**b**) a representative experiment is shown in the upper panel. For statistical analysis, *t*-test was performed comparing empty versus Smac-containing vectors (*$P<0.05$, #$P<0.001$). Abbreviations: cDNA, complementary DNA; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanide iodide; FACS, fluorescence-activated cell sorting; Smac, second mitochondria-derived activator of caspase.

of caspase-2, -3, -8 and -9 and promoted loss of mitochondrial membrane potential upon γ-irradiation in a caspase-dependent fashion. Thus, the increase in caspase activity in cells with XIAP downregulation or Smac overexpression initiated a feedback amplification loop acting back on the mitochondria to trigger further mitochondrial perturbations. Caspase-3 may contribute to this mitochondrial amplification loop by cleaving Bid, caspase-8 and/or caspase-2 (Slee *et al*., 1999, 2000; Sohn *et al*., 2005). Caspase-2 may also act upstream of mitochondria upon γ-irradiation, as suggested by our data showing that the caspase-2 inhibitor zVDVAD.fmk reduced loss of mitochondrial membrane potential upon γ-irradiation in the corresponding control cell lines, that is, cells infected with nonsense shRNA or empty vector. However, commercially available chemical inhibitors of caspases lack absolute specificity (Turk *et al*., 2002). Thus, the role of caspase-2 in controlling mitochondrial functions following γ-irradiation remains to be confirmed in future studies.

Clinically, resistance to apoptosis is a major cause of intrinsic or acquired resistance of cancers leading to treatment failure. By demonstrating that strategies to antagonize XIAP potentiate the antitumor activity of γ-irradiation in pancreatic carcinoma, a prototype tumor known to be notoriously resistant to current treatment options, our findings have important implications for the development of novel treatment concepts in radiotherapy. Thus, targeting XIAP, for example, by small molecule inhibitors, presents a promising approach to enhance the radiosensitivity of pancreatic cancer that warrants further investigation.

Materials and methods

Cell lines and chemicals

PaTuII, ASPC1, MiaPaCa2 and Colo357 pancreatic carcinoma cell lines and Reh B-precursor acute lymphocytic leukemia cells were cultured as described previously (Vogler *et al*., 2007). Generation of PaTuII or ASPC1 pancreatic carcinoma cells in which XIAP was stably downregulated by shRNA against XIAP at position 241 or full-length Smac was ectopically expressed were described previously (Vogler *et al*., 2005, 2007). Clones were selected by limiting dilutions. Nonsense shRNA directed against a sequence with no corresponding part in the human genome (Vogler *et al*., 2007) and shRNA against enhanced green fluorescence protein (data not shown) were

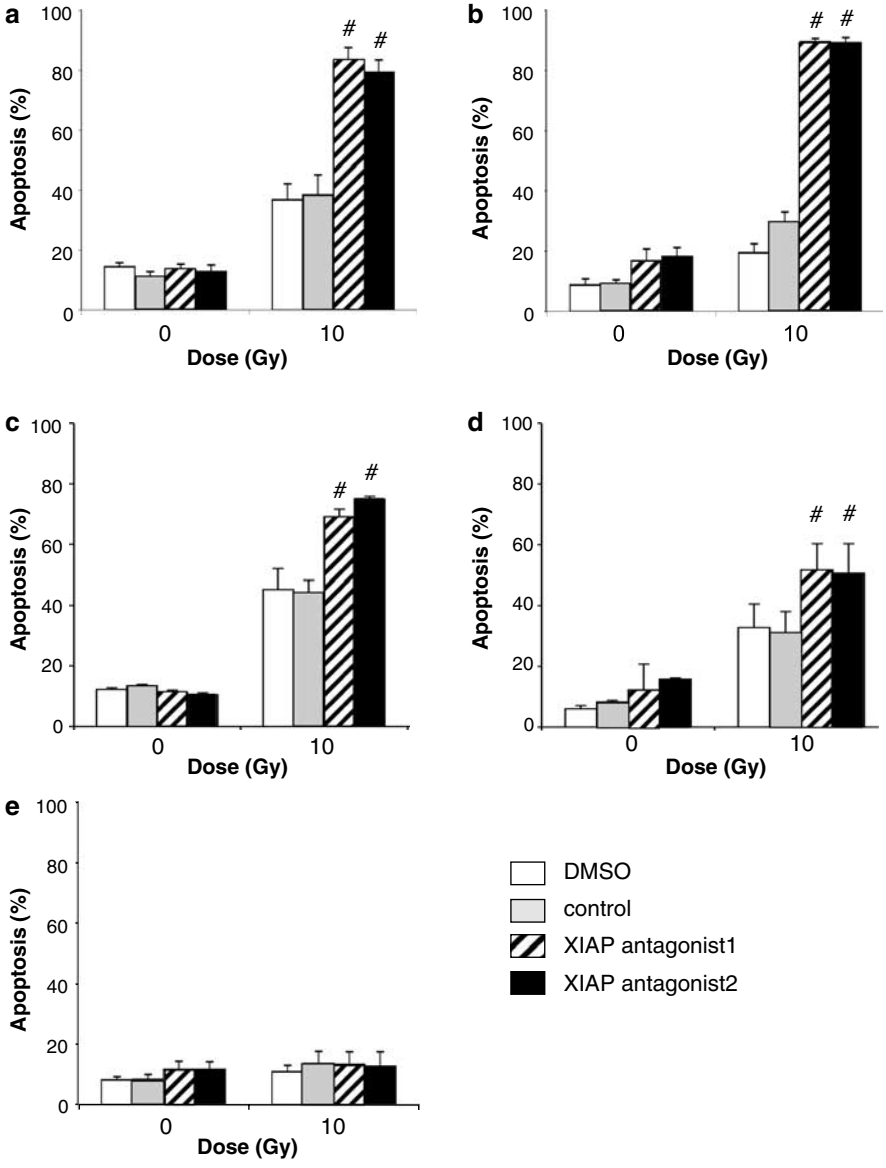


Figure 7 XIAP antagonists sensitize pancreatic carcinoma cells for γ-irradiation-induced apoptosis. PaTuII (**a**), ASPC1 (**b**), Colo357 (**c**), MiaPaCa2 (**d**) pancreatic carcinoma cells or NIH3T3 fibroblasts (**e**) were treated with 0 or 10 Gy γ-irradiation in the presence of 10 μM XIAP antagonists 1 or 2 (hatched or black bars), control compound (grey bars) or solvent (DMSO, white bars). Apoptosis was determined 96 h after γ-irradiation by FACS analysis of DNA fragmentation of propidium-iodide-stained nuclei. Mean $\pm$ s.e.m. of three independent experiments performed in triplicate are shown. For statistical analysis, *t*-test was performed comparing XIAP antagonists versus control compound (#$P<0.001$). Abbreviations: DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; XIAP, X-linked inhibitor of apoptosis.

used as negative controls. All chemicals were purchased by Sigma (Steinheim, Germany) unless indicated otherwise. XIAP antagonists 1 and 2 and control compound were kindly provided by Idun Pharmaceuticals now Pfizer Inc. (Groton, CN, USA) and correspond to compounds 2, 11 and 15 as described by Oost *et al*. (2004). They were dissolved in dimethyl sulfoxide and used at a final concentration of 10 μM.

Western blot analysis

Western blot analysis was performed as described previously (Fulda *et al*., 1997) using mouse anti-XIAP monoclonal antibody (1:1000; clone 28; BD Biosciences, Heidelberg, Germany), rabbit anti-cIAP2 polyclonal antibody (1:1000; R&D Systems Inc., Wiesbaden, Germany), rabbit anti-survivin polyclonal antibody (1:1000; R&D Systems Inc.), mouse anti-Smac monoclonal antibody (1:1000, BD Biosciences), mouse anti-Flag tag monoclonal antibody (M3, 1:5000; Sigma) or mouse anti-β-actin monoclonal antibody (1:5000; Sigma) followed by goat-antimouse IgG or goat-anti-rabbit IgG conjugated to horseradish peroxidase (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany). β-Actin was used as loading control. All western blots shown are representative of at least three independent experiments.

Determination of apoptosis and clonogenic assay

Cells were treated with γ-irradiation (Nuclear Data, CS-137, 44Tbq, 4 Gy/min, Frankfurt, Germany) at the indicated doses and times. For determination of apoptosis, cells were cultured

at 0.5×10^5 cells/cm^2. Apoptosis was determined by fluorescence-activated cell-sorting (FACS) analysis (FACScan, BD Biosciences) of DNA fragmentation of propidium-iodide-stained nuclei as described previously (Fulda *et al*., 1997) or by annexin-V staining (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Clonogenic growth was determined as described previously (Vogler *et al*., 2007). Clusters of >20 cells were counted as colonies.

Determination of mitochondrial membrane potential
3,3′-Dihexyloxacarbocyanide iodide (460 ng/ml, Molecular Probes, Karlsruhe, Germany) was used to measure the mitochondrial transmembrane potential. Cells were incubated for 15 min at 37°C in the presence of the fluorochrome, washed in phosphate-buffered saline (PBS)/1% fetal calf serum and immediately analysed by flow cytometry at fluorescence channel 1.

Determination of caspase activity
To determine caspase activity in living non-fixed, non-lysed cells, substrates conjugated to Rhodamine R110 were used according to the particular cleavage sequences: (*N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone-R110 (zDEVD-R110) for caspase-3, *N*-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone-R110 (zVDVAD-R110) for caspase-2, *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone-R110 (zIETD-R110) for caspase-8, *N*-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone-R110 (zLEHD-R110) for caspase-9, all purchased from Molecular Probes. Cells were trypsinized, collected by centrifugation at 1800 *g* at 4°C for 5 min, washed with PBS, incubated in 100 μM of caspase substrates at 37°C for 30 min, washed with PBS and measured immediately by FACS at fluorescence channel 1. For inhibition of caspases, the broad-range caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk, Bachem, Heidelberg, Germany), the relatively specific caspase-3 inhibitor zDEVD.fmk (Bachem), the relatively specific caspase-8 inhibitor zIETD.fmk (Bachem) or the relatively specific caspase-2 inhibitor zVDVAD.fmk (Sigma) were used.

Statistical analysis
Statistical significance was assessed by Student's *t*-test (two-tailed distribution, two-sample unequal variance).

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References

Cao C, Mu Y, Hallahan DE, Lu B. (2004). XIAP and survivin as therapeutic targets for radiation sensitization in preclinical models of lung cancer. *Oncogene* **23**: 7047–7052.

Cummins JM, Kohli M, Rago C, Kinzler KW, Vogelstein B, Bunz F. (2004). X-linked inhibitor of apoptosis protein (XIAP) is a nonredundant modulator of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human cancer cells. *Cancer Res* **64**: 3006–3008.

Fulda S, Debatin KM. (2006a). Therapeutic modulation of apoptosis in cancer therapy. In: Debatin, KM, Fulda, S (eds) *Apoptosis and Cancer Therapy*. Whiley-VCH: Weinheim, pp 849–861.

Fulda S, Debatin KM. (2006b). Extrinsic versus intrinsic apoptosis pathways in anti-cancer chemotherapy. *Oncogene* **25**: 4798–4811.

Fulda S, Debatin KM. (2006c). Targeting inhibitor of apoptosis proteins (IAPs) for diagnosis and treatment of human diseases. *Rec Patents Anti-Cancer Drug Disc* **1**: 81–90.

Fulda S, Sieverts H, Friesen C, Herr I, Debatin KM. (1997). The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. *Cancer Res* **57**: 3823–3829.

Fulda S, Wick W, Weller M, Debatin KM. (2002). Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma *in vivo*. *Nat Med* **8**: 808–815.

Giagkousiklidis S, Vogler M, Westhoff MA, Kasperczyk H, Debatin KM, Fulda S. (2005). Sensitization for γ-irradiation-induced apoptosis by Smac. *Cancer Res* **65**: 10502–10513.

Guo F, Nimmanapalli R, Paranawithana S, Wittman S, Griffin D, Bali P. (2002). Ectopic overexpression of second mitochondria-derived activator of caspases (Smac/DIABLO) or cotreatment with N-terminus of Smac/DIABLO peptide potentiates epothilone B derivative-(BMS 247550) and Apo-2L/TRAIL-induced apoptosis. *Blood* **99**: 3419–3426.

Holcik M, Yeh C, Korneluk RG, Chow T. (2000). Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. *Oncogene* **19**: 4174–4177.

Jendrossek V, Belka C. (2006). Radiation therapy and apoptosis. In: Debatin, KM, Fulda, S (eds) *Apoptosis and Cancer Therapy*. Whiley-VCH: Weinheim, pp 1049–1086.

Lu B, Mu Y, Cao C, Zeng F, Schneider S, Tan J *et al*. (2004). Survivin as a therapeutic target for radiation sensitization in lung cancer. *Cancer Res* **64**: 2840–2845.

McManus DC, Lefebvre CA, Cherton-Horvat G, St-Jean M, Kandimalla ER, Agrawal S *et al*. (2004). Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics. *Oncogene* **23**: 8105–8117.

Oost TK, Sun C, Armstrong RC, Al-Assaad AS, Betz SF, Deckwerth TL *et al*. (2004). Discovery of potent antagonists of the antiapoptotic protein XIAP for the treatment of cancer. *J Med Chem* **47**: 4417–4426.

Rodel C, Haas J, Groth A, Grabenbauer GG, Sauer R, Rodel F. (2003). Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor. *Int J Radiat Oncol Biol Phys* **55**: 1341–1347.

Saelens X, Festjens N, Walle LV, van Gurp M, van Loo G, Vandenabeele P. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene* **23**: 2861–2874.

Salvesen GS, Duckett CS. (2002). IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* **3**: 401–410.

Schmidt-Ullrich RK, Dent P, Grant S, Mikkelsen RB, Valerie K. (2000). Signal transduction and cellular radiation responses. *Radiat Res* **153**: 245–257.

Schneider G, Siveke JT, Eckel F, Schmid RM. (2005). Pancreatic cancer: basic and clinical aspects. *Gastroenterology* **128**: 1606–1625.

Slee EA, Adrain C, Martin SJ. (1999). Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ* **6**: 1067–1074.

Slee EA, Keogh SA, Martin SJ. (2000). Cleavage of BID during cytotoxic drug and UV radiation-induced apoptosis occurs downstream of the point of Bcl-2 action and is catalysed by caspase-3: a potential feedback loop for amplification of apoptosis-associated mitochondrial cytochrome *c* release. *Cell Death Differ* **7**: 556–565.

Sohn D, Schulze-Osthoff K, Janicke RU. (2005). Caspase-8 can be activated by interchain proteolysis without receptor-triggered dimerization during drug-induced apoptosis. *J Biol Chem* **280**: 5267–5273.

Turk B, Turk D, Salvesen GS. (2002). Regulating cysteine protease activity: essential role of protease inhibitors as guardians and regulators. *Curr Pharm Des* **8**: 1623–1637.

van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. (1998). Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **31**: 1–9.

Vogler M, Durr K, Jovanovic M, Debatin KM, Fulda S. (2007). Regulation of TRAIL-induced apoptosis by XIAP in pancreatic carcinoma cells. *Oncogene* **26**: 248–257.

Vogler M, Giagkousiklidis S, Genze F, Gschwend JE, Debatin KM, Fulda S. (2005). Inhibition of clonogenic tumor growth: a novel function of Smac contributing to its antitumor activity. *Oncogene* **24**: 7190–7202.

Westphal S, Kalthoff H. (2003). Apoptosis: targets in pancreatic cancer. *Mol Cancer* **2**: 6.

Wilkinson JC, Cepero E, Boise LH, Duckett CS. (2004). Upstream regulatory role for XIAP in receptor-mediated apoptosis. *Mol Cell Biol* **24**: 7003–7014.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).