Cell Death and Differentiation (2008) 15, 1745–1751
© 2008 Macmillan Publishers Limited All rights reserved 1350-9047/08 $32.00
www.nature.com/cdd

Zinc chelation induces rapid depletion of the X-linked inhibitor of apoptosis and sensitizes prostate cancer cells to TRAIL-mediated apoptosis

P Makhov[1,2], K Golovine[1,2], RG Uzzo[1], J Rothman[1], PL Crispen[1], T Shaw[1], BJ Scoll[1] and VM Kolenko*[,1]

The X-linked inhibitor of apoptosis (XIAP), the most potent member of the inhibitor of apoptosis protein (IAP) family of endogenous caspase inhibitors, blocks the initiation and execution phases of the apoptotic cascade. As such, XIAP represents an attractive target for treating apoptosis-resistant forms of cancer. Here, we demonstrate that treatment with the membrane-permeable zinc chelator, *N,N,N′,N′*-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) induces a rapid depletion of XIAP at the post-translational level in human PC-3 prostate cancer cells and several non-prostate cell lines. The depletion of XIAP is selective, as TPEN has no effect on the expression of other zinc-binding members of the IAP family, including cIAP1, cIAP2 and survivin. The downregulation of XIAP in TPEN-treated cells occurs via proteasome- and caspase-independent mechanisms and is completely prevented by the serine protease inhibitor, Pefabloc. Finally, our studies demonstrate that TPEN promotes activation of caspases-3 and -9 and sensitizes PC-3 prostate cancer cells to TRAIL-mediated apoptosis. Taken together, our findings indicate that zinc-chelating agents may be used to sensitize malignant cells to established cytotoxic agents via downregulation of XIAP.
Cell Death and Differentiation (2008) **15,** 1745–1751; doi:10.1038/cdd.2008.106; published online 11 July 2008

Inhibitor of apoptosis proteins (IAPs) are a family of caspase inhibitors that selectively bind and inhibit caspases at both the initiation phase and the execution phase of apoptosis.[1] All IAPs contain 1–3 baculoviral IAP repeat (BIR) motifs. Each BIR domain folds into a functionally independent structure that binds a zinc ion. Additionally, many IAPs contain another zinc-binding motif, the RING domain, which has E3 ubiquitin ligase activity.[2]

Of all the members of the IAP family, XIAP has received the most attention, possibly because it is the only member of this family that is able to directly inhibit both the initiation and execution phases of the caspase cascade.[3] The BIR2 domain of XIAP binds and inhibits caspases-3 and -7. Overexpression of cDNA corresponding to the BIR2 domain inhibits apoptosis from both death receptor and mitochondrial pathway stimuli, consistent with its ability to inhibit effector caspases.[4,5] The BIR3 domain of XIAP binds and inhibits caspase-9, an apical caspase in the mitochondrial arm of the apoptotic pathway.[5,6] Overexpression of cDNA corresponding to the BIR3 domain inhibits apoptosis in response to stimuli of the mitochondrial pathway of caspase activation, such as Bax, but not stimuli of the death receptor pathway.[4] XIAP levels are elevated in many cancer cell lines and suppression of XIAP protein levels sensitizes cancer cells to chemotherapeutic agents.[3]

Here, we demonstrate that treatment of PC-3 prostate cancer cells with the zinc-specific chelator *N,N,N′,N′*-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) induces rapid and selective depletion of XIAP at the post-translational level. XIAP depletion coincides with increased activation of caspases-3 and -9 and sensitization of PC-3 cells to apoptosis in response to subsequent treatment with TRAIL.

Results

Chelation of intracellular zinc induces rapid depletion of XIAP. The IAP family members suppress cell death by inhibiting caspase activity. The zinc-binding BIR domains are responsible for this inhibitory function. Therefore, we examined the impact of intracellular zinc chelation on the expression of the IAP family members. As seen in Figure 1a, treatment with TPEN induces a rapid decrease in XIAP protein levels in human PC-3 prostate cancer cells. Our experiments show that the inhibition of XIAP expression is selective, as TPEN had no effect on the levels of other zinc-containing members of the IAP family, namely cIAP1, cIAP2 and survivin (Figure 1a). Notably, a decrease in XIAP protein level was detectable at an extracellular TPEN concentration as low as 6 μM and was dose-dependent (Figure 1b).

To validate our observation that zinc chelation induces a rapid decrease of XIAP, we constructed an expression vector encoding a doubly tagged XIAP modified to incorporate both an amino-terminal HA tag and a carboxy-terminal FLAG tag in

[1]Department of Surgical Oncology, Division of Urology, Fox Chase Cancer Center, Philadelphia, PA 19111, USA
*Corresponding author: VM Kolenko, Department of Surgical Oncology, Division of Urology, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA. Tel: 215 728 5620; Fax: 215 728-4333; E-mail: Vladimir.Kolenko@fccc.edu
[2]These authors contributed equally to this work
Keywords: XIAP; zinc; cancer; prostate; apoptosis
Abbreviations: BIR, baculoviral IAP repeat; PARP, poly(ADP-ribose) polymerase; PSA, prostate specific antigen; RING, really interesting new gene; TPEN, *N,N,N′,N′*-tetrakis(2-pyridylmethyl) ethylenediamine; TRAIL, TNF-related apoptosis inducing ligand; XIAP, X-linked inhibitor of apoptosis protein
Received 20.3.08; revised 16.6.08; accepted 16.6.08; Edited by DL Vaux; published online 11.7.08

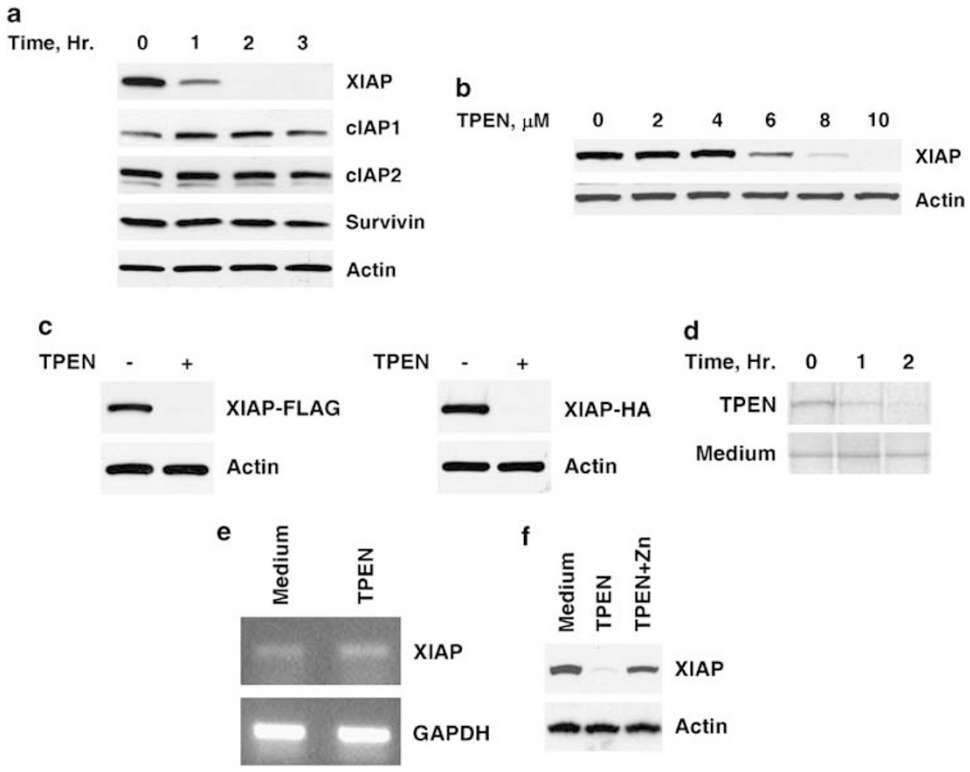

Figure 1 Zinc chelation induces rapid depletion of XIAP in PC-3 cells. (**a**) Cells were cultured for the indicated periods of time with TPEN (8 μM). The cell lysates were subjected to SDS-PAGE, blotted and probed with specific antibodies. (**b**) Cells were cultured in medium alone or treated with indicated concentrations of TPEN for 2 h. The cell lysates were subjected to SDS-PAGE followed by western blotting with anti-XIAP antibody. (**c**) Doubly tagged XIAP containing an amino-terminal HA tag and a carboxy-terminal FLAG tag was expressed in PC-3 cells. TPEN (8 μM) was added to the media for 2 h. XIAP was detected by immunoblotting using anti-FLAG or anti-HA antibodies. (**d**) [^{35}S]Methionine pulse-chase analysis of XIAP half-life in the presence or absence of TPEN. TPEN (8 μM) was added to the media for the indicated periods of time. Pulse-chase analysis was performed as described in Materials and Methods. (**e**) XIAP mRNA levels were not decreased in TPEN-treated PC-3 cells. TPEN (8 μM) was added to the media for 2 h. XIAP mRNA levels were detected by RT-PCR using specific primers. (**f**) The addition of zinc restores XIAP expression after TPEN-mediated depletion. PC-3 cells were pre-incubated with TPEN (8 μM) for 2 h followed by the addition of an equimolar concentration of $ZnSO_4$ to the cell culture medium for 4 h

the same protein. PC-3 cells were transfected with the construct followed by incubation with or without TPEN for 2 h. As demonstrated in Figure 1c, the presence of tagged XIAP protein was detectable only in untreated cells. To discriminate whether TPEN promotes XIAP degradation or prevents its synthesis, we examined the half-life of XIAP protein using [^{35}S]methionine pulse-chase analysis. Results of these experiments clearly demonstrate that exposure of PC-3 cells to TPEN markedly decreases the half-life of XIAP protein (Figure 1d). To test the possibility that TPEN might induce downregulation of XIAP at the mRNA level, we examined the levels of XIAP mRNA in PC-3 cells incubated with or without TPEN. As shown in Figure 1e, XIAP mRNA levels were not decreased in TPEN-treated cells. Therefore, zinc chelation induces depletion of XIAP specifically at the protein level. To determine whether downregulation of XIAP expression is a reversible process, we added equimolar concentration of $ZnSO_4$ to the cells pretreated with TPEN. The results presented in Figure 1f demonstrate that the addition of zinc restores XIAP expression in TPEN-treated cells.

To investigate whether the TPEN-induced XIAP down-regulation was mediated specifically via zinc depletion, PC-3 cells were exposed to TPEN (8 μM) with the addition of equimolar concentrations of physiologically relevant metal ions (Figure 2a). Addition of zinc and copper, but not other metals, completely blocked TPEN-induced XIAP degradation. Although TPEN has been used as a zinc-specific chelator in multiple studies,[7–9] it has a much higher affinity for copper ($K_d = 3 \times 10^{-20}$ M) than for zinc ($K_d = 2.6 \times 10^{-16}$ M).[10] This suggests that copper effectively competes with zinc for binding to TPEN and, therefore, 'neutralizes' its activity with respect to XIAP downregulation. In RPMI medium supplemented with 10% FBS, copper and zinc levels are 0.25 and 4.2 μM, respectively.[11] The results of our experiments demonstrating that a noticeable effect upon XIAP expression was detected only when TPEN was used at concentrations greater than 4 μM (Figure 1b) support the concept that TPEN-mediated reduction of XIAP expression was indeed mediated primarily by chelation of zinc and not other physiologically relevant metals.

Next, we tested the ability of TPEN to modulate XIAP expression in non-prostate cell lines. Figure 2b demonstrates that incubation with TPEN decreased XIAP levels in all tested cell lines, including cervical, colon, ovarian, leukemia and breast cancer cells.

Degradation of XIAP in TPEN-treated cells occurs via proteasome- and caspase-independent pathways and is prevented by the serine protease inhibitor Pefabloc. A potential mechanism explaining the TPEN-induced decrease

of XIAP at the protein level without a reduction in XIAP mRNA may be its increased degradation by the ubiquitin–proteasome or caspase-mediated pathways.[12,13] To address this hypothesis, we pre-incubated PC-3 cells with the proteasome inhibitor MG-132. The results presented in Figure 3a show that MG-132 did not block the ability of TPEN to reduce the XIAP protein levels, although MG-132 completely prevented TNF-α-induced degradation of IκBα. We have reported previously that caspases-3, -8 and -9 were rapidly activated in T lymphocytes treated with TPEN.[9] For this reason, we analyzed whether inhibition of caspase activity in TPEN-treated cells prevents XIAP degradation. The inhibition of caspase activity with the pan-caspase inhibitor Z-VAD-FMK failed to prevent TPEN-mediated degradation of XIAP, although the addition of Z-VAD-FMK completely blocked cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase (PARP) in PC-3 cells simultaneously treated with TRAIL and methylseleninic acid (Figure 3b). XIAP is a *bona fide* substrate of Omi/HtrA2 protease, which mediates XIAP cleavage under various stress conditions.[14,15] With this fact in mind, we treated cells with Omi's specific inhibitor, Ucf-101.[14] This also failed to prevent XIAP depletion (Figure 3c). Similar results were obtained with ALLM, an effective calpain and cathepsin inhibitor;[16] chloroquine, a lysosomal protease inhibitor;[17] and CA-074 Me, a cathepsin B inhibitor[18] (Figure 3d). To examine the potential contribution of other classes of proteases in TPEN-mediated XIAP degradation, PC-3 cells were pre-incubated with cell-permeable inhibitors specific for serine (Pefabloc), cysteine (E64d) and aspartic (pepstatin A) proteases. As demonstrated in Figure 3e, only the serine protease inhibitor Pefabloc was able to prevent XIAP depletion in TPEN-treated cells.

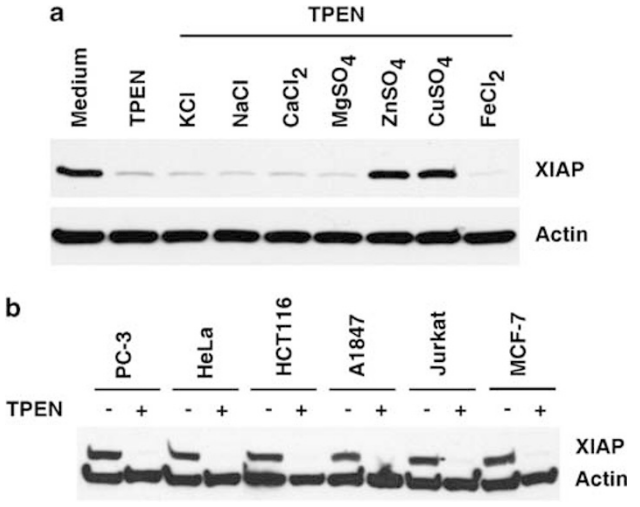

Figure 2 (**a**) Addition of equimolar concentrations of zinc and copper completely blocks TPEN-induced XIAP degradation. PC-3 cells were pre-incubated with 8 μM of KCl, NaCl, $CaCl_2$, $MgSO_4$, $ZnSO_4$, $CuSO_4$ and $FeCl_2$ for 1 h, and then incubated with an equimolar concentration of TPEN for 2 h. XIAP expression was detected by immunoblotting using an anti-XIAP antibody. (**b**) TPEN reduces XIAP expression in tumor cell lines of various origins. PC-3 (prostate cancer), HeLa (cervical cancer), HCT116 (colon cancer), A1847 (ovarian cancer), Jurkat (leukemia) and MCF-7 (breast cancer) cells were cultured in the presence of TPEN (8 μM) for 2 h. XIAP was detected by immunoblotting using an anti-XIAP antibody

XIAP domains possess variable sensitivity to intracellular zinc chelation. The potential sensitivity of various domains of XIAP to TPEN-mediated degradation was

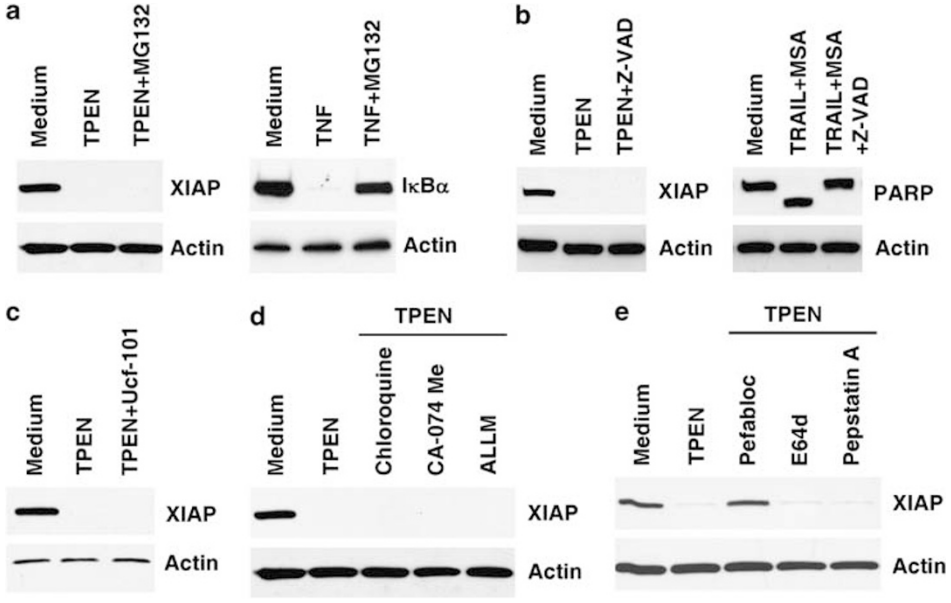

Figure 3 TPEN-mediated XIAP depletion is prevented by the serine protease-specific inhibitor Pefabloc. (**a**) PC-3 cells were pre-incubated with 10 μM of the proteasome inhibitor MG-132 for 30 min, followed by incubation with either 8 μM of TPEN for 2 h or 20 ng/ml of TNF-α for 15 min. (**b**) PC-3 cells were pre-incubated with 50 μM of the pan-caspase inhibitor Z-VAD-FMK for 30 min, followed by incubation with either 8 μM of TPEN for 2 h or 200 ng/ml of TRAIL and 5 μM methylseleninic acid (MSA) for 4 h. PC-3 cells were pre-incubated with (**c**) Ucf-101 (100 μM); (**d**) chloroquine (20 μM), CA-074 Me (20 μM) or ALLM (10 μM); (**e**) Pefabloc (200 μM), E64d (200 μM) or pepstatin A (200 μM) for 1 h, followed by incubation with 8 μM of TPEN for 2 h. Expression of XIAP, IκBα, PARP and actin was detected by immunoblotting with specific antibodies

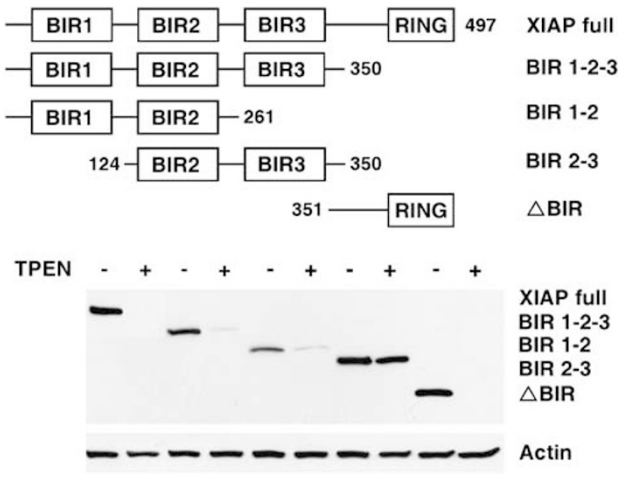

Figure 4 XIAP domains possess variable sensitivity to intracellular zinc chelation. The indicated XIAP mutants containing the HA tag were expressed in PC-3 cells. Cells were treated with TPEN (8 μM) for 2 h. Expression of XIAP and actin was detected by immunoblotting with anti-HA or anti-actin antibodies, respectively

evaluated by testing the ability of truncated versions of the protein to undergo the degradation after the addition of TPEN to cells transfected with appropriate constructs containing an amino-terminal HA tag. As shown in Figure 4, the amino-terminal portion of XIAP containing the three BIR domains and lacking the RING finger domain was degraded in the presence of TPEN, as were BIR1–2 and the full-length XIAP constructs. In addition, a truncation mutant lacking all BIR domains and containing the RING finger domain (ΔBIR) was also capable of undergoing the TPEN-induced degradation. In contrast, the BIR2–3 protein was resistant to TPEN-mediated cleavage. These results indicate that the BIR2–3 construct likely lacks the specific motif required for XIAP cleavage.

TPEN promotes activation of caspases-3 and -9 and sensitizes prostate cancer cells to TRAIL-mediated apoptosis. XIAP is probably the only *bona fide* inhibitor that selectively binds and inhibits caspases-3, -7 and -9.[1] With this understanding, we examined whether TPEN-mediated downregulation of XIAP coincides with increased proteolytic processing via caspases-3 and -9 and cleavage of intracellular caspase-3 substrate PARP in cells treated with TRAIL. When PC-3 cells were treated with TPEN alone, the level of proteolytic activation of caspases-3 and -9 remained consistently low throughout a 3 h incubation (Figure 5a). Although treatment of PC-3 cells with TRAIL alone was capable of inducing noticeable levels of caspases-3 and -9 processing, PARP cleavage was undetectable in cells treated with TRAIL alone (Figure 5a). Importantly, in mammals, XIAP binds exclusively to activated, processed forms of caspases-3, -7 and -9 and does not interact with their zymogenic forms.[19,20] These findings can explain the lack of PARP cleavage in the presence of processed caspases-3 and -9 in cells treated with TRAIL alone. In contrast, when PC-3 cells were treated with TPEN and TRAIL simultaneously, a clear effect on the processing of caspases-3 and -9 and PARP cleavage was detected (Figure 5a). To confirm that TPEN-mediated XIAP

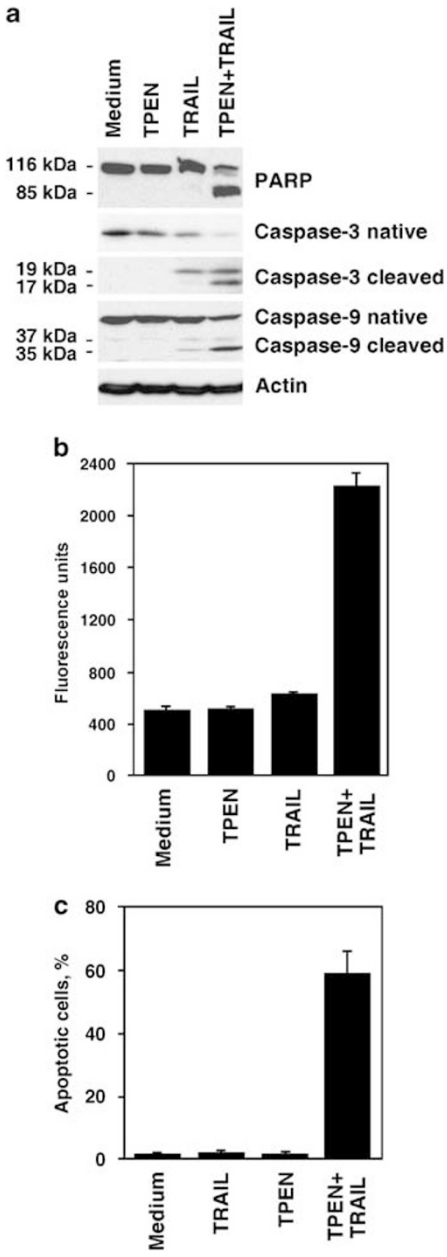

Figure 5 TPEN-mediated XIAP depletion coincides with increased activation of caspases-3 and -9 and sensitization of tumor cells to TRAIL-mediated apoptosis. (**a**) PC-3 cells were treated with TPEN (8 μM), TRAIL (200 ng/ml) or the TPEN/TRAIL combination for 3 h. Cell lysates were subjected to SDS-PAGE, blotted and probed with antibodies to full length and cleaved caspases-3 and -9, and PARP. (**b**) Caspase-3-like activity in PC-3 cells treated with TPEN (8 μM), TRAIL (200 ng/ml) or the TPEN/TRAIL combination for 3 h. Caspase-3-like activity was examined using the fluorogenic substrate DEVD-AMC, as described in Materials and Methods. Results are mean ± S.E.M. ($n = 3$). (**c**) Zinc chelation sensitizes prostate cancer cells to TRAIL-mediated apoptosis. PC-3 cells were cultured in medium alone or in the presence of TPEN (8 μM) with or without TRAIL (200 ng/ml) for 4.5 h. The percentage of apoptotic cells was determined by the APO-BRDU assay followed by flow cytometry analysis. Columns, means of four different experiments; bars, S.E.M.

deficiency contributes to increased activity of caspase family members in cells treated with TRAIL, caspase-3-like activity was examined in cell lysates from PC-3 cells treated with various combinations of TPEN and TRAIL using the

fluorogenic substrate DEVD-7-amino-4-methyl-coumarin (AMC). As expected, treatment with either TPEN or TRAIL alone failed to induce significant levels of caspase-3-like activity, whereas such an activity was easily detectable in PC-3 cells simultaneously treated with both TPEN and TRAIL (Figure 5b).

Given that suppression of XIAP activity/expression sensitizes cells to cytotoxic agents,[3,21] the pro-apoptotic effect of TPEN in combination with TRAIL was evaluated in PC-3 cells. Treatment with TRAIL alone had no significant effect on cell death in PC-3 cells, as prostate cancer cells are generally resistant to TRAIL-mediated killing. Treatment with TPEN alone also did not induce a significant degree of apoptosis. However, concomitant treatment with TRAIL and TPEN resulted in high levels of DNA fragmentation as early as 4.5 h after treatment initiation (Figure 5c). Furthermore, as demonstrated in Figure 6, direct small interfering RNA-mediated knockdown of XIAP resulted in the upregulation of caspase-3-like activity and TRAIL sensitization, suggesting that knockdown of XIAP by itself was sufficient to reverse resistance to TRAIL-mediated apoptosis in PC-3 cells. Yet, the results of our experiments presented in Figure 6c demonstrate that the level of apoptosis induced by TRAIL in the presence of TPEN was higher than the level of apoptosis induced by TRAIL in cells with XIAP knockdown. This may be due to the ability of TPEN to trigger more profound XIAP depletion compared with shRNA or due to a potential effect of TPEN on other critical components of the apoptotic pathway.

The present study provides the first evidence that intracellular zinc chelation induces rapid depletion of XIAP in tumor cells of various origins at the post-translational level. Figure 7 schematically illustrates the potential mechanism of tumor cell sensitization to TRAIL via TPEN-induced downregulation of XIAP.

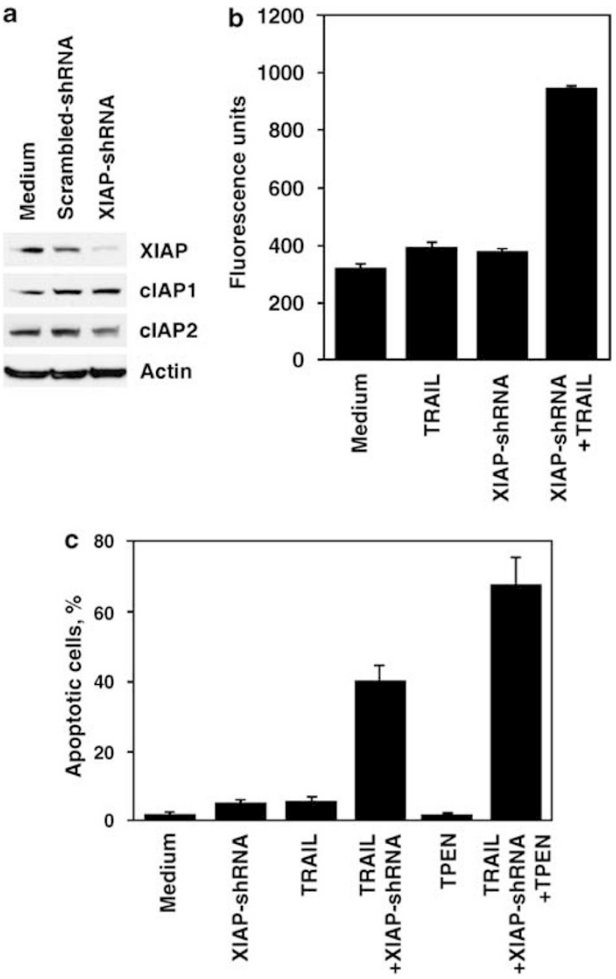

Figure 6 Small interfering RNA-mediated knockdown of XIAP sensitizes PC-3 cells to TRAIL-mediated apoptosis. (**a**) XIAP protein levels in native PC-3 cells and cells transfected with shRNA targeting XIAP. PC-3 cells were transfected with XIAP shRNA plasmid for 48 h, as described in Materials and Methods. XIAP, cIAP1, cIAP2 and actin levels were detected by immunoblotting using the appropriate antibodies. (**b**) Knockdown of XIAP causes upregulation of caspase-3-like activity in PC-3 cells treated with TRAIL. Cells were transfected with XIAP shRNA plasmid, as described above, followed by stimulation with TRAIL (200 ng/ml) for 6 h. Caspase-3-like activity was examined using the fluorogenic substrate DEVD-AMC, as described in Materials and Methods. Results are mean ± S.E.M. ($n = 3$). (**c**) Knockdown of XIAP sensitizes PC-3 cells to TRAIL-mediated apoptosis. Cells were transfected with XIAP shRNA plasmid, as described above, followed by stimulation with TRAIL (200 ng/ml), TPEN (8 μM) or a combination of both for 12 h. The percentage of apoptotic cells was determined by the APO-BRDU assay followed by flow cytometry analysis. Columns, means of three different experiments; bars, S.E.M.

Discussion

The therapeutic potential of XIAP suppression in cancer treatment has encouraged research to identify and characterize mechanisms that regulate XIAP expression as well as antagonists that block XIAP function. The inhibition or downregulation of XIAP in cancer cells lowers the apoptotic threshold, thereby inducing cell death and/or enhancing the cytotoxic action of chemotherapeutic agents. Indeed, XIAP antagonist 1396-34 sensitized PC-3 and DU-145 prostate cancer cells to chemotherapeutic agents and TRAIL.[22] Moreover, XIAP antisense enhanced pro-apoptotic TRAIL potency by 12- to 13-fold in DU-145 prostate cancer cells[23] demonstrating that knockdown of XIAP itself was sufficient to reverse TRAIL resistance.

Zinc, in addition to its catalytic role in more than 300 zinc metalloenzymes, is a known inhibitor of enzymes in general. Removal of zinc from an inhibitory zinc-specific enzymatic site results in a marked increase of enzyme activity.[24] For example, human tissue kallikreins, a subgroup of extracellular serine proteases, including prostate-specific antigen, are inactivated by the reversible binding of zinc (reviewed in the report by Pampalakis and Sotiropoulou).[25] Such findings provide a rationale for our observation that the addition of zinc chelators triggers serine protease-dependent depletion of XIAP. Nevertheless, the exact mechanism underlying the depletion of XIAP under zinc-deficient conditions remains to be elucidated. A possibility also exists that the TPEN-mediated pro-apoptotic effect is not exclusively due to XIAP depletion. Further studies are needed to fully explore the potential mechanisms of action of zinc-chelating agents.

Zinc is not the only metal ion influencing XIAP function. Elevated copper levels result in a conformational change in XIAP, which accelerates its degradation and significantly decreases its ability to inhibit caspase-3 in HEK 293 cells.[26] In contrast to our observation that zinc chelation induces specific

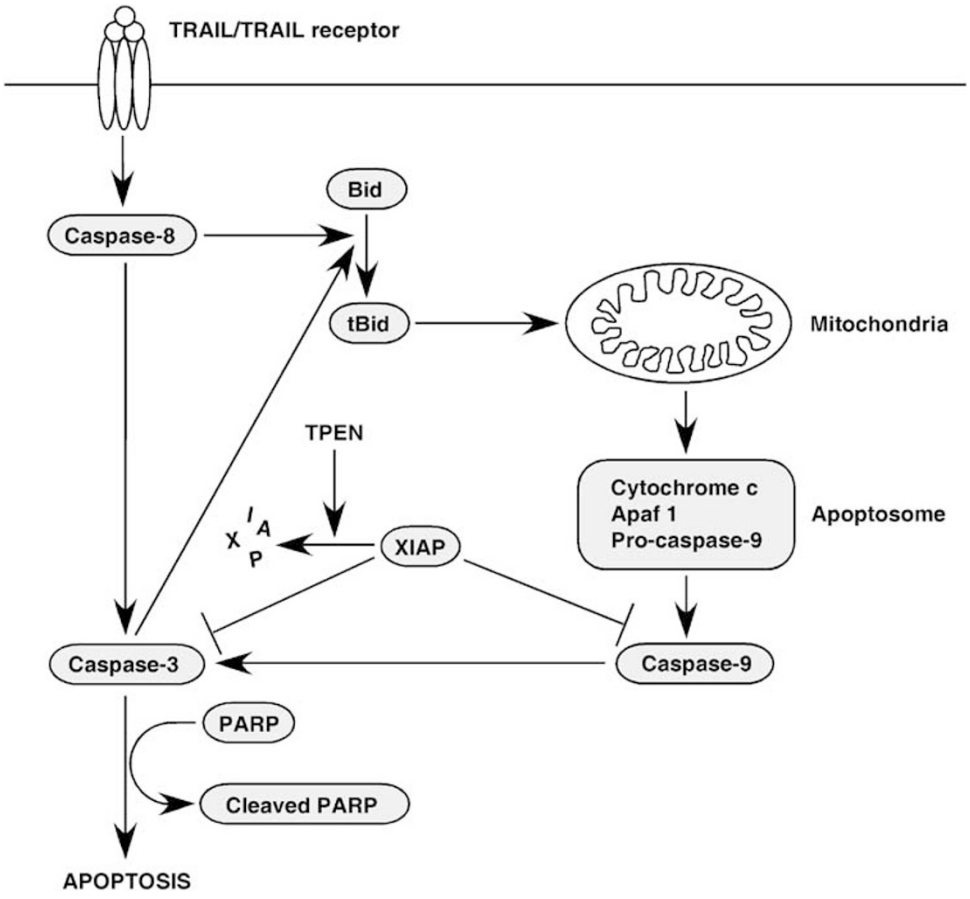

Figure 7 A proposed model for the enhanced susceptibility of prostate cancer cells to TRAIL-mediated apoptosis due to zinc chelation. The zinc-specific chelator TPEN induces rapid depletion of XIAP at the post-translational level, abrogating its apoptosis-inhibitory function, and thereby promoting amplification of an apoptotic signaling via activation of caspases-3 and -9

degradation of XIAP (Figure 1a), studies by Mufti *et al*. show that intracellular copper accumulation also affects other IAPs, such as Op-IAP and cIAP2. Mufti *et al*.[26] have also reported that copper induced no significant changes in XIAP mRNA expression. This finding is in agreement with our data that XIAP mRNA levels were not decreased in TPEN-treated cells (Figure 1e), indicating that both copper and TPEN can modulate XIAP expression at the post-transcriptional level. Furthermore, it has been suggested that direct binding of copper to cysteine residues within XIAP induces a conformational change that is associated with decreased stability.[26] An intriguing possibility exists that, in addition to direct binding of copper to XIAP, it can also substitute for zinc, and thereby promote the instability of XIAP. Indeed, the ability of various metals, including copper and cadmium, to substitute zinc in zinc finger domains is well established.[27,28]

Recent studies suggest that the presence or development of resistance may ultimately limit the use of small-molecule XIAP inhibitors.[3] It is unknown whether malignant cells acquire mutations in the BIR domains of XIAP and whether these mutations affect XIAP's function or its response to inhibitors. It is also unknown whether mutations in XIAP can develop after treatment with small-molecule XIAP inhibitors similar to the development of mutations in ABL kinase after treatment with the inhibitor Gleevec.[3,29] Therefore, agents capable of completely depleting XIAP in malignant cells might be superior to small-molecule XIAP inhibitors. Taken together, our findings indicate that zinc-chelating molecules capable of reducing XIAP expression, in combination with other treatment modalities, may be beneficial in treating apoptosis resistant tumors.

Materials and Methods

Cells and materials. Cell lines were obtained from ATCC (Rockville, MD, USA) and maintained in RPMI 1640 medium (Bio-Whittaker, Walkersville, MD, USA) supplemented with 10% FCS (Hyclone, Logan, UT, USA), gentamicin (50 mg/l), sodium pyruvate (1 mM) and non-essential amino acids (0.1 mM).

Antibodies and reagents. Antibodies to cIAP2, survivin, PARP, HA tag and IκBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to cIAP1, XIAP, caspase-3 and caspase-9 were obtained from Cell Signaling Technology (Beverly, MA, USA). Ucf-101, chloroquine, CA-074 ME and ALLM were purchased from Calbiochem (San Diego, CA, USA). Antibodies to FLAG tag, actin, TPEN and TNF-α were obtained from Sigma (St. Louis, MO, USA). Z-VAD-FMK, TRAIL, CD95 antibody, DEVD-AMC and MG132 were obtained from Biomol (Plymouth Meeting, PA, USA). TNF-α was obtained from Sigma. pSM2 vector encoding XIAP shRNAmir was obtained from Open Biosystems (Huntsville, AL, USA).

Western blot analysis. Cells were lysed in boiling SDS buffer (50 mM Tris (pH 7.6), 150 mM NaCl 2% SDS) for 10 min. SDS-PAGE and western blotting were performed as described previously.[9]

Plasmids and transfection. To create HA-XIAP-FLAG pEBB expression vector, the insert was obtained by PCR with 5′-atcttg ggatcc ATGACTT TTAACAGTTTTGAAGG-3′ (forward) and 5′-atcttg atcgat tta ctt atc gtc gtc atc ctt gta atcAGACATAAAAATTTTTTGCTT-3′ (reverse) specific primers (restriction sites underlined) using pEBB-HA-XIAP vector[30] as a template with subsequent cloning into BamH1/Cla1 sites of the pEBB-HA plasmid. The pEBB-HA-XIAP vector and XIAP truncation mutants were a kind gift from Dr. CS Duckett (University of Michigan Medical School, Ann Arbor, MI, USA). Transfections were performed using TransIT-Prostate transfection kit (Mirus Bio, Madison, WI, USA).

[^{35}S]Methionine pulse-chase analysis. Half-life of the XIAP protein was examined as described previously.[31]

Reverse transcription-PCR analysis. Total RNA was isolated from cells using MINI RNA isolation II Kit (Zymo-Research, Orange, CA, USA) and purified using DNA-free RNA Kit (Zymo-Research). Reverse transcription (RT) of 1 μg RNA was subsequently carried out using 200 U of SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and then amplified as follows: 94 °C for 3 min for one cycle; 94 °C for 20 s, 58 °C for 30 s and 72 °C for 1 min for 28 cycles. This was followed by a 5 min cycle at 72 °C using 1 U of HotTaq DNA polymerase (Eppendorf, Westbury, NY, USA); 125 μM each of dATP, dTTP, dCTP and dGTP; 50 pM of 5′-CGAAGTGAATCTGATGCTGTGAG-3′ (forward) and 5′-GTCTTCACTGGG CTTCCAATC-3′ (reverse) primers; 1 × HotMaster Taq buffer (Eppendorf) and 1 μl of cDNA from RT reaction. The GAPDH gene was used as a control for all RT reactions. The primer pair specific for GAPDH was 5′-ATGGGGAAGGTGAAGGT C-3′ (forward) and 5′-TCAGGCATTGCTGATGATCTT-3′ (reverse).

Measurement of caspase activity. Caspase-3-like activity was measured using the fluorometric tetrapeptide substrate DEVD-AMC. The assay was performed in 96-well plates by incubating 50 μg of cell lysates with 100 μl of reaction buffer (100 mmol/l HEPES, pH 7.5, 20% vol/vol glycerol, 5 mmol/l DTT and 0.5 mmol/l EDTA) containing 200 μM DEVD-AMC. Release of AMC was monitored after 15 min of incubation at 37 °C on a microplate fluorimeter with excitation and emission wavelengths of 380 and 460 nm, respectively.

Measurement of apoptosis. DNA fragmentation was detected using the APO-BRDU kit (The Phoenix Flow Systems Inc., San Diego, CA, USA).

Acknowledgements. This work was supported in part by the National Institutes of Health Grant RO1 CA108890 (to VMK).

1. Eckelman BP, Salvesen GS, Scott FL. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO R* 2006; **7**: 988–994.
2. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 2000; **288**: 874–877.
3. Schimmer AD, Dalili S, Batey RA, Riedl SJ. Targeting XIAP for the treatment of malignancy. *Cell Death Differ* 2006; **13**: 179–188.
4. Bratton SB, Lewis J, Butterworth M, Duckett CS, Cohen GM. XIAP inhibition of caspase-3 preserves its association with the Apaf-1 apoptosome and prevents CD95- and Bax-induced apoptosis. *Cell Death Differ* 2002; **9**: 881–892.
5. Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *EMBO J* 1999; **18**: 5242–5251.
6. Sun C, Cai M, Meadows RP, Xu N, Gunasekera AH, Herrmann J *et al.* NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP. *J Biol Chem* 2000; **275**: 33777–33781.
7. Hyun HJ, Sohn JH, Ha DW, Ahn YH, Koh JY, Yoon YH. Depletion of intracellular zinc and copper with TPEN results in apoptosis of cultured human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 2001; **42**: 460–465.
8. Kolenko V, Uzzo RG, Bukowski R, Bander NH, Novick AC, Hsi ED *et al.* Dead or dying: necrosis *versus* apoptosis in caspase-deficient human renal cell carcinoma. *Cancer Res* 1999; **59**: 2838–2842.
9. Kolenko VM, Uzzo RG, Dulin N, Hauzman E, Bukowski R, Finke JH. Mechanism of apoptosis induced by zinc deficiency in peripheral blood T lymphocytes. *Apoptosis* 2001; **6**: 419–429.
10. Armstrong C, Leong W, Lees GJ. Comparative effects of metal chelating agents on the neuronal cytotoxicity induced by copper (Cu^{+2}), iron (Fe^{+3}) and zinc in the hippocampus. *Brain Res* 2001; **892**: 51–62.
11. Glassman AB, Rydzewski RS, Bennett CE. Trace metal levels in commercially prepared tissue culture media. *Tissue Cell* 1980; **12**: 613–617.
12. Dan HC, Sun M, Kaneko S, Feldman RI, Nicosia SV, Wang HG *et al.* Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). *J Biol Chem* 2004; **279**: 5405–5412.
13. Johnson DE, Gastman BR, Wieckowski E, Wang GQ, Amoscato A, Delach SM *et al.* Inhibitor of apoptosis protein hILP undergoes caspase-mediated cleavage during T lymphocyte apoptosis. *Cancer Res* 2000; **60**: 1818–1823.
14. Althaus J, Siegelin MD, Dehghani F, Cilenti L, Zervos AS, Rami A. The serine protease Omi/HtrA2 is involved in XIAP cleavage and in neuronal cell death following focal cerebral ischemia/reperfusion. *Neurochem Int* 2007; **50**: 172–180.
15. Goffredo D, Rigamonti D, Zuccato C, Tartari M, Valenza M, Cattaneo E. Prevention of cytosolic IAPs degradation: a potential pharmacological target in Huntington's disease. *Pharmacol Res* 2005; **52**: 140–150.
16. Blagosklonny MV, An WG, Melillo G, Nguyen P, Trepel JB, Neckers LM. Regulation of BRCA1 by protein degradation. *Oncogene* 1999; **18**: 6460–6468.
17. Lee CM, Kim BY, Li L, Morgan ET. Nitric oxide-dependent proteasomal degradation of cytochrome P450 2B proteins. *J Biol Chem* 2008; **283**: 889–898.
18. Hentze H, Lin XY, Choi MS, Porter AG. Critical role for cathepsin B in mediating caspase-1-dependent interleukin-18 maturation and caspase-1-independent necrosis triggered by the microbial toxin nigericin. *Cell Death Differ* 2003; **10**: 956–968.
19. Foo RS, Mani K, Kitsis RN. Death begets failure in the heart. *J Clin Invest* 2005; **115**: 565–571.
20. Zachariou A, Tenev T, Goyal L, Agapite J, Steller H, Meier P. IAP-antagonists exhibit non-redundant modes of action through differential DIAP1 binding. *EMBO J* 2003; **22**: 6642–6652.
21. Vogler M, Durr K, Jovanovic M, Debatin KM, Fulda S. Regulation of TRAIL-induced apoptosis by XIAP in pancreatic carcinoma cells. *Oncogene* 2007; **26**: 248–257.
22. Schimmer AD, Welsh K, Pinilla C, Wang Z, Krajewska M, Bonneau MJ *et al.* Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity. *Cancer Cell* 2004; **5**: 25–35.
23. Amantana A, London CA, Iversen PL, Devi GR. X-linked inhibitor of apoptosis protein inhibition induces apoptosis and enhances chemotherapy sensitivity in human prostate cancer cells. *Mol Cancer Ther* 2004; **3**: 699–707.
24. Maret W, Jacob C, Vallee BL, Fischer EH. Inhibitory sites in enzymes: zinc removal and reactivation by thionein. *Proc Natl Acad Sci USA* 1999; **96**: 1936–1940.
25. Pampalakis G, Sotiropoulou G. Tissue kallikrein proteolytic cascade pathways in normal physiology and cancer. *Biochim Biophys Acta* 2007; **1776**: 22–31.
26. Mufti AR, Burstein E, Csomos RA, Graf PC, Wilkinson JC, Dick RD *et al.* XIAP is a copper binding protein deregulated in Wilson's disease and other copper toxicosis disorders. *Mol Cell* 2006; **21**: 775–785.
27. Meplan C, Mann K, Hainaut P. Cadmium induces conformational modifications of wild-type p53 and suppresses p53 response to DNA damage in cultured cells. *J Biol Chem* 1999; **274**: 31663–31670.
28. Predki PF, Sarkar B. Effect of replacement of 'zinc finger' zinc on estrogen receptor DNA interactions. *J Biol Chem* 1992; **267**: 5842–5846.
29. Soverini S, Martinelli G, Rosti G, Bassi S, Amabile M, Poerio A *et al.* ABL mutations in late chronic phase chronic myeloid leukemia patients with up-front cytogenetic resistance to imatinib are associated with a greater likelihood of progression to blast crisis and shorter survival: a study by the GIMEMA Working Party on Chronic Myeloid Leukemia. *J Clin Oncol* 2005; **23**: 4100–4109.
30. Lewis J, Burstein E, Reffey SB, Bratton SB, Roberts AB, Duckett CS. Uncoupling of the signaling and caspase-inhibitory properties of X-linked inhibitor of apoptosis. *J Biol Chem* 2004; **279**: 9023–9029.
31. Mabjeesh NJ, Post DE, Willard MT, Kaur B, Van Meir EG, Simons JW *et al.* Geldanamycin induces degradation of hypoxia-inducible factor 1alpha protein via the proteosome pathway in prostate cancer cells. *Cancer Res* 2002; **62**: 2478–2482.