

X-linked inhibitor of apoptosis protein inhibition induces apoptosis and enhances chemotherapy sensitivity in human prostate cancer cells

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apoptosis and enhanced chemotherapy sensitization in androgen-refractory prostate cancer cells. [Mol Cancer Ther 2004;3(6):699–707]

Abstract

Androgen-insensitive prostate cancer cells are highly resistant to several chemotherapeutic drugs and are characterized by the appearance of apoptosis-resistant cells. In this study, we identified the critical role of X-linked inhibitor of apoptosis protein (XIAP), a potent antiapoptotic factor, in conferring chemotherapy resistance in an androgen-insensitive DU145 human prostate cancer cell line. Results reveal that DU145 cells were highly resistant to cisplatin, but this resistance was overridden when the cells were treated for a prolonged time (>96 hours) with cisplatin ($IC_{50} = 27.5$ to $35.5 \mu\text{mol/L}$). A decrease in levels of XIAP and Akt/phospho-Akt and an increase in caspase-3 activity were identified to be key factors in cisplatin sensitivity (40% to 55% decrease in cell viability) at later time points. In contrast, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) treatment caused a 40% to 50% decrease in cell viability within 6 hours ($IC_{50} = 135$ to 145 ng/mL). However, increasing concentrations or prolonged treatment with TRAIL did not change drug potency. A significant increase in caspase-3 activity was observed with TRAIL treatment with no apparent change in XIAP levels. Specific inhibition of XIAP expression using an antisense XIAP phosphorodiamidate morpholino oligomer induced apoptosis and increased caspase-3 activity. Combination of cisplatin with XIAP antisense potentiated cisplatin sensitivity by decreasing the IC_{50} from $>200 \mu\text{mol/L}$ with cisplatin alone to 9 to $20 \mu\text{mol/L}$ and decreasing incubation time required for activity from 96 to 24 hours. Similarly, TRAIL in combination with XIAP antisense phosphorodiamidate morpholino oligomer enhanced TRAIL potency by 12- to 13-fold. In conclusion, abrogation of XIAP expression is essential for therapeutic

Introduction

The American Cancer Society in early 2003 predicted diagnosis of 220,900 new cases of prostate cancer, nearly 33% of overall new cancer cases, among American males (1). Current therapies like androgen ablation have been observed to precipitate changes in gene expression profile leading to an androgen-independent phenotype associated with chemotherapeutic resistance and increased mortality (2). Androgen-independent cells escape apoptosis induced by androgen ablation and cytotoxic drugs leading to drug resistance (3-5). Androgen unresponsiveness may be the result of stimulation of cell proliferation by alternative signaling pathways and/or up-regulation of antiapoptotic genes. It is evident that the apoptotic machinery of androgen-independent prostate cancer cells is in place and can be activated by apoptosis-inducing agents (6-10). Alternatively, the identification and reversal of antiapoptotic mechanisms responsible for drug resistance is very vital in the search of a successful treatment protocol.

Inhibitors of apoptosis proteins represent one set of potent endogenous modulators of apoptosis in mammalian cells. These include a family of intracellular antiapoptotic proteins consisting of five members: X-linked inhibitor of apoptosis protein (XIAP), human inhibitor of apoptosis protein-1, human inhibitor of apoptosis protein-2, neuronal apoptosis inhibitory protein, and survivin (11-15). These proteins mediate multiple biological functions that include binding and inhibiting caspases, regulating the cell cycle progression, and modulating receptor-mediated signal transduction.

XIAP has been identified as one of the most potent inhibitor of caspases and apoptosis. Unlike Bcl-2 protein, which blocks the mitochondrial branch of apoptosis, the antiapoptotic function of XIAP is due, at least in part, to its ability to inhibit both mitochondrial-dependent and mitochondrial-independent apoptotic pathways by directly binding to and inhibiting both initiator and effector caspases (16-19). XIAP overexpression in tumor cells has been shown to cause an inhibitory effect on cell death induced by a variety of apoptotic stimuli and induce resistance to chemotherapy (6, 20). We hypothesize herein that manipulation of XIAP expression using novel phosphorodiamidate morpholino oligomer (PMO) would induce therapeutic apoptosis and sensitize drug-resistant human prostate cancer cells to chemotherapy. We report that the observed cisplatin resistance and partial sensitivity

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to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in DU145 cells were associated with XIAP expression. XIAP inhibition using XIAP antisense PMO agent induced apoptosis and increased sensitivity of these cells to cisplatin and TRAIL.

Materials and Methods

Oligomers

XIAP antisense and scrambled PMOs were synthesized and purified at AVI BioPharma, Inc. (Corvallis, OR) with purity greater than 95% as determined by reverse-phase high-performance liquid chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy. The base compositions of the XIAP antisense and scrambled oligomers are 5'-CTG TTA AAA GTC ATC TTC TC-3' and 5'-CTT GAT AGA ATC TAC TCT CT-3', respectively. Lyophilized PMOs are water soluble and were dissolved in sterile distilled water for *in vitro* experiments.

Cell Culture

DU145 human prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA). RPMI 1640 was purchased from Hyclone Laboratories (Logan, UT). Penicillin and streptomycin were obtained from Life Technologies, Inc. (Grand Island, NY). Cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone Laboratories), 10 units/mL penicillin, and 10 $\mu\text{g}/\text{mL}$ streptomycin in a 5% CO_2 and 95% air humidified incubator at 37°C.

Cell Viability Assay

Cells were seeded at 4,000 cells per well in a 96-well plate (Becton Dickinson, Franklin Lakes, NJ) and allowed to reach at least 80% confluence. After treatment with agents, culture medium was aspirated and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) was added to each well at a concentration of 0.5 mg/mL medium from a 5 mg/mL stock. Cells were incubated at 37°C until the MTT reaction caused the formation of granulated purple coloration. Excess reagent was aspirated and replaced with 200 μL DMSO (Sigma Chemical) in each well. Absorbance was read at 540 nm in a Molecular Devices plate reader (Sunnyvale, CA). In some experiments, cell viability was also determined by trypan blue exclusion assay. Cells were trypsinized and resuspended in medium to achieve a homogenous mixture. An aliquot of cell suspension was mixed with an equal volume of 0.4% trypan blue solution (Sigma Chemical). Cell numbers were recorded in 10 μL of the resultant mixture using a hemocytometer.

Protein Expression

Cells were harvested and immediately lysed in solubilization buffer as described previously (21). Equal amounts of protein in cell lysates were subjected to SDS-PAGE under reducing conditions. Before loading onto the gel, all lysates, except those for XIAP immunodetection, were heated at 95°C to 100°C for 5 minutes and

immediately cooled on ice. The protein was transferred onto Immobilon-P transfer membranes (Millipore, Bedford, MA) previously soaked in methanol and transfer buffer. After the transfer process was complete, the membranes were allowed to dry, re-soaked in methanol, and incubated with blocking buffer (4% dry nonfat milk in 1 \times PBS-0.1% Tween 20) for 1 hour at room temperature. Membranes were incubated with primary antibody against XIAP (1:250 dilution; BD Transduction Laboratories, Lexington, KY) and Akt and phospho-Akt (1:1,000; Cell Signaling Technology, Beverly, MA) for 1 hour at room temperature and caspase-3 (1:100 dilution; Oncogene Research Products, San Diego, CA) and caspase-7 (1:1,000; PharMingen, San Diego, CA) overnight at 4°C. Membranes were washed three times with wash buffer (1 \times PBS-0.1% Tween 20) and subsequently incubated with appropriate secondary antibody conjugated with horseradish peroxidase (1:2,000 dilution) for 1 hour at room temperature. Membranes were washed three times, and immunoreactive bands were visualized using enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). Signals were developed after exposure to X-ray film (X-Omat films, Eastman Kodak Co., Rochester, NY). β -actin immunodetection was conducted to serve as loading control. This was done by stripping the same membrane in stripping buffer [100 mmol/L 2-mercaptoethanol-2% SDS-62.5 mmol/L Tris-HCl (pH 6.7)] at 50°C for 30 minutes followed by washing and blocking procedure as described previously (21). Densitometric analysis was done using Kodak 1D image analysis software (Eastman Kodak Co., New Haven, CT).

Treatment of Cells with Cisplatin and TRAIL

Cells were treated for 24 hours in serum-containing medium in a 96-well plate (Becton Dickinson) with cisplatin (0 to 200 $\mu\text{mol}/\text{L}$; Sigma Chemical) and recombinant TRAIL (0 to 250 ng/mL; BIOMOL Research Laboratories, Inc., Plymouth, PA). Cell viability was determined by MTT assay.

Plasmid-Based Test System for Screening PMO Antisense Activity

A fusion construct was generated by subcloning 29 bases of the 5' untranslated region, the AUG translation start site, and the first 16 bases of the protein coding sequence of the XIAP gene followed by the luciferase reporter gene into the pCiNeo expression vector (Promega, Madison, WI). The fusion construct was designated pCiNeoXIAP-luc Δ A. This plasmid features a T7 promoter capable of generating *in vitro* transcribed RNA from a cloned insert for use in the cell-free rabbit reticulocyte *in vitro* translation reactions and a cytomegalovirus promoter for constitutive expression in mammalian cells. Confluent HeLa cells were transiently transfected with pCiNeoXIAP-luc Δ A plasmid construct using Lipofectamine (Invitrogen Life Technologies, Carlsbad, CA) according to a previous study (22). Cells were trypsinized after 24 hours and plated in six-well plates (Becton Dickinson) at a density of 6×10^5 cells per well, allowed to adhere overnight, and scrape loaded with vehicle or PMOs at different concentrations. Cell lysates

were prepared 24 hours later and normalized for protein content, and luciferase activity was determined using the luminometer (Cardinal series 500, Cardinal Associates, Inc., Santa Fe, NM).

Delivery of Antisense PMO into Cells in Culture

Antisense and scrambled PMOs were delivered into the cells by scrape loading as described previously (21-23). Briefly, cells were plated at a density of 5×10^5 cells per well in a Falcon (San Jose, CA) six-well plate (Becton Dickinson) and allowed to reach at least 95% confluence. PMOs were diluted in 2 mL culture medium to yield the desired concentration. Cells were scraped with a sterile scraper (Sarstedt, Newton, NC) in the medium, mixed gently, and transferred into appropriately labeled fresh six-well plates.

Scheduled Treatment with Cisplatin, TRAIL, and XIAP Antisense PMO

Cells were treated with increasing concentrations of cisplatin and TRAIL. In one case, cisplatin treatment was conducted for a 24-hour period and was followed by XIAP antisense or scrambled PMOs by scrape loading for 24 hours. In the study involving treatment with TRAIL, cells were first treated with XIAP antisense or scrambled PMOs by scrape loading for 24 hours. This was followed by a 24-hour treatment with TRAIL. TRAIL, cisplatin, and vehicle controls were also sham scrape loaded. All treatments were carried out in six-well plates at 37°C in serum-containing culture medium.

Apoptosome ELISA-Based Assay

In this assay, the M30-Apoptosome ELISA kit (DiaPharma Group, Inc. West Chester, OH) was used to quantify apoptosis by detecting the levels of M30 antigen that is formed during apoptosis as a result of cleavage of cytokeratin 18 by active caspase-3. DU145 cells were treated with XIAP antisense and scrambled PMOs. Total protein extracts were prepared and 25 μ L of these were added to 75 μ L of diluted horseradish peroxidase conjugate solution per well of coated microstrips. This was allowed to react on a shaker at room temperature for 4 hours. Each well was washed three times with wash solution and 200 μ L of 3,3',5,5'-tetramethylbenzidine substrate solution were added to each well and incubated in darkness for 20 minutes. The reaction was stopped by adding 50 μ L stop solution and placed on a shaker for 5 to 10 seconds. An additional 5-minute period was allowed before absorbance readings were taken at 450 nm on a Molecular Devices plate reader.

Statistical Analysis

Statistical analyses were performed by using GraphPad Prism 4.0 Student's two-tailed *t* test. Differences were considered significant at $P < 0.05$. Data are expressed as means \pm SE.

Results

Cisplatin Resistance in DU145 Cells Correlated with XIAP Expression

Human androgen-independent DU145 cells were treated for 24 to 96 hours with various concentrations of cisplatin.

Data in Fig. 1A show that these cells are highly resistant to cisplatin up to 96 hours even at higher concentrations (200 μ mol/L). However, a 40% to 55% decrease in cell viability ($IC_{50} = 27.5$ to 35.5 μ mol/L) was observed when the cells were treated with cisplatin for 96 hours (Fig. 1A). To provide a mechanistic insight into the effect of cisplatin on DU145 cell viability, we examined the levels of XIAP, Akt, phospho-Akt, and caspases at different time points of postcisplatin treatment. No significant changes were observed in XIAP or active caspase-3 levels in lysates from cells treated up to 24 hours with cisplatin. In contrast, lysates from cells treated with cisplatin for 96 hours revealed a significant decrease in XIAP expression, a corresponding increase in active forms of caspase-3 and caspase-7, and a decrease in Akt and phospho-Akt levels in a dose-dependent manner, which correlated with decrease in cell viability (Fig. 1B and C). A significant decrease in Akt and phospho-Akt levels at the higher cisplatin concentration (200 μ mol/L) was also observed at the 24-hour time point (Fig. 1C) but with no measurable change in cell viability (Fig. 1A), suggesting the need for XIAP inhibition in inducing apoptosis in these cells.

TRAIL Activates Caspase-3 but Does Not Change XIAP Levels in DU145 Cells

TRAIL has been shown to induce apoptosis by binding to death receptors DR4 and/or DR5 followed by activation of caspase-8 and downstream effector caspases, particularly caspase-3. Treatment of DU145 cells with TRAIL caused a rapid (within 6 hours) increase in activated caspase-3 (Fig. 2B) and 40% to 50% cell death with IC_{50} of 135 to 145 ng/mL. However, there was no shift in IC_{50} or decreased cell viability with increasing concentration or duration of TRAIL treatment (Fig. 2A). In addition, TRAIL treatment had no effect on XIAP expression compared with untreated cells (Fig. 2B). Although TRAIL decreased Akt levels within 6 hours, no significant changes in phospho-Akt were observed compared with untreated cells as shown in Fig. 2C.

XIAP Antisense PMO Decreases XIAP Expression and Induces Apoptosis in DU145 Human Prostate Cancer Cells

To examine the role of XIAP in the regulation of apoptosis, a 20-mer antisense PMO agent was generated targeting the translational start site of XIAP mRNA. The effect of XIAP antisense PMO on endogenous XIAP levels was tested in DU145 cells, and the immunoblot analysis in Fig. 3A shows significant XIAP inhibition, which was accompanied by a modest dose-dependent decrease (20% to 30%) in cell viability compared with the controls as shown in Fig. 3B and C.

To further analyze antisense specificity and activity, a plasmid-based screening system was generated by subcloning 29 bases of the 5' untranslated region, the AUG translational start site, and the first 16 bases of the protein coding sequence of XIAP gene followed by the luciferase reporter gene. Confluent HeLa cells, which have been optimized for this screening system in earlier studies

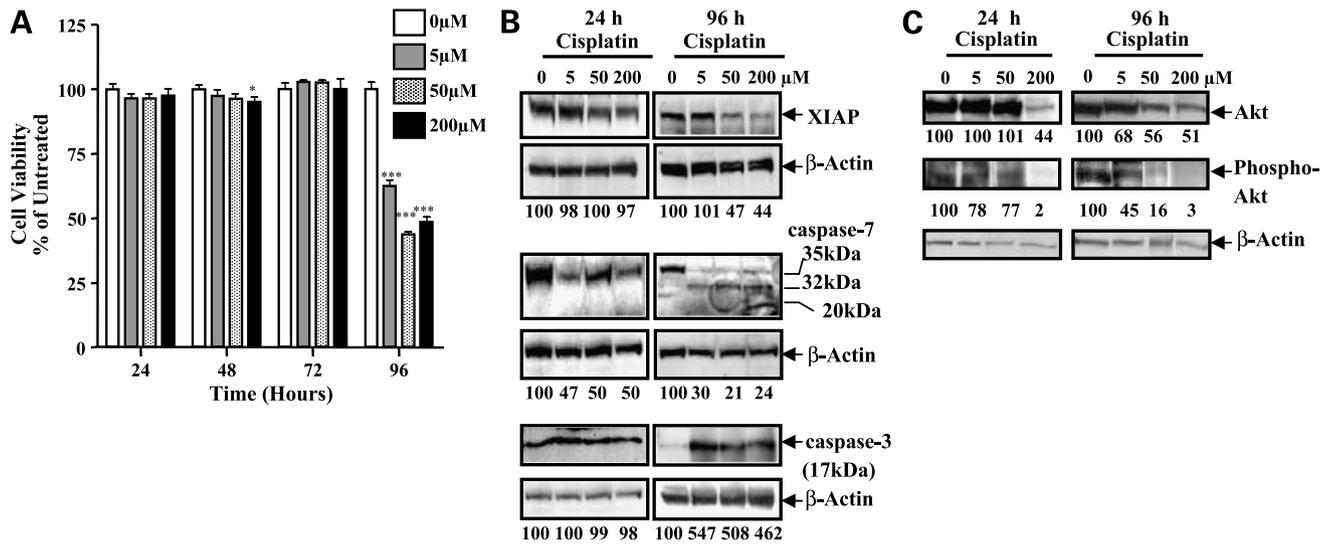


Figure 1. Effect of cisplatin on DU145 human prostate cancer cells. **A**, Cells were treated with indicated concentrations of cisplatin at four different time points. Cell viability was monitored by MTT assay after each time point. Columns, means of triplicate values ($n = 3$); bars, SE. *, $P < 0.05$, cisplatin versus untreated cells at 48 hours. ***, $P < 0.001$, cisplatin versus untreated cells at 96 hours. **B**, Immunoblot analysis of cell lysates treated with cisplatin at 24-hour and 96-hour time points with monoclonal antibodies against XIAP, caspase-7, and caspase-3. Respective membranes were stripped and probed for β -actin as loading control. Protein bands were quantified by densitometric analysis and expressed as percentage of control. Arrows, 57-kDa XIAP; 43-kDa β -actin; 35-, 32-, and 20-kDa forms of caspase-7; and 17-kDa active caspase-3 bands. **C**, Akt and phospho-Akt immunoblot analysis of lysates from cells treated with cisplatin at 24-hour and 96-hour time points. The phospho-Akt membrane was stripped and probed for Akt and β -actin as loading control. Protein levels were quantified by densitometric analysis and expressed as percentage of control. Arrows, 60-kDa Akt and phospho-Akt bands.

(21, 23), were transiently transfected with this plasmid construct in the presence of the delivery agent Lipofectamine. Controls included Lipofectamine and vector alone (pCiNeo-luc Δ A) transfected cells. After 24 hours, the

transfected cells were split into six-well plates and treated by scrape loading with scrambled PMO, unrelated PMO sequence, XIAP antisense PMO, or sham scrape loaded in the absence of any PMO. Data in Table 1 reveal

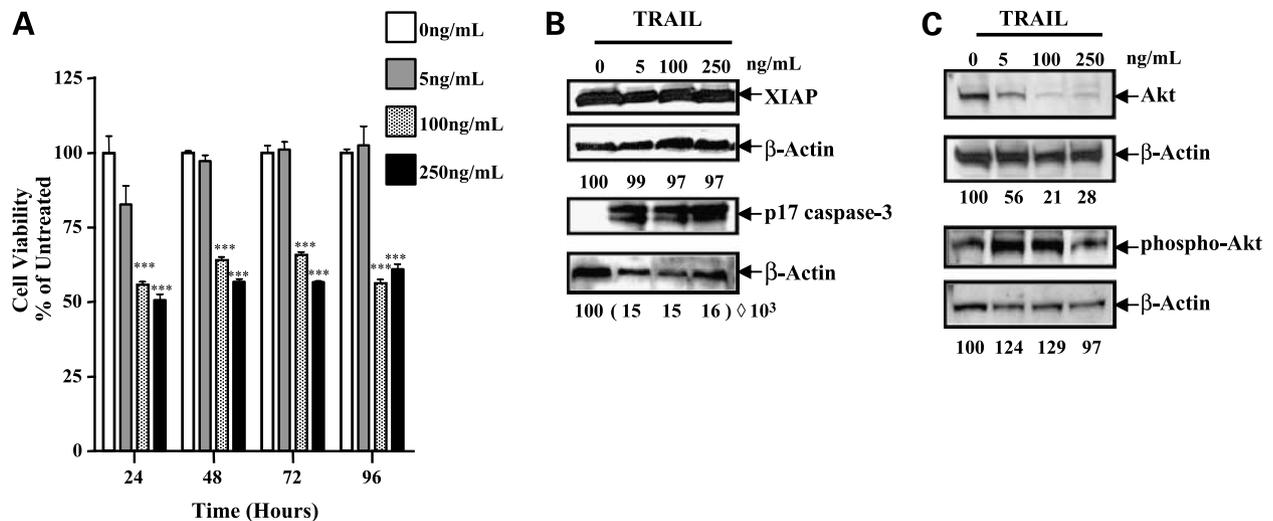


Figure 2. Effect of TRAIL on cell viability and protein expression in DU145 human prostate cancer cells. **A**, Cells were treated with indicated concentrations of TRAIL at four different time points. Cell viability was examined by MTT assay after each time point. Values obtained were compared with the respective controls. Columns, means of triplicate values; bars, SE. ***, $P < 0.001$, TRAIL treated versus untreated cells at each time point. **B**, Immunoblot analysis of lysates from DU145 cells treated with TRAIL for 6 hours. Blots were probed with XIAP and caspase-3 antibodies. Respective membranes were stripped and probed for 43-kDa β -actin as loading control. Numbers below each blot were obtained from densitometric analysis and expressed as percentage of control. Arrows, 57-kDa XIAP, 17-kDa caspase-3, and 43-kDa β -actin bands. **C**, Immunoblot analysis of lysates from DU145 cells treated with TRAIL for 6 hours. Phospho-Akt and Akt were probed with anti-Akt and phospho-Akt polyclonal antibodies. Respective membranes were stripped and probed for 43-kDa β -actin as loading control. Numbers below each blot were obtained from densitometric analysis and expressed as percentage of control. Arrows, 60-kDa Akt and phospho-Akt bands.

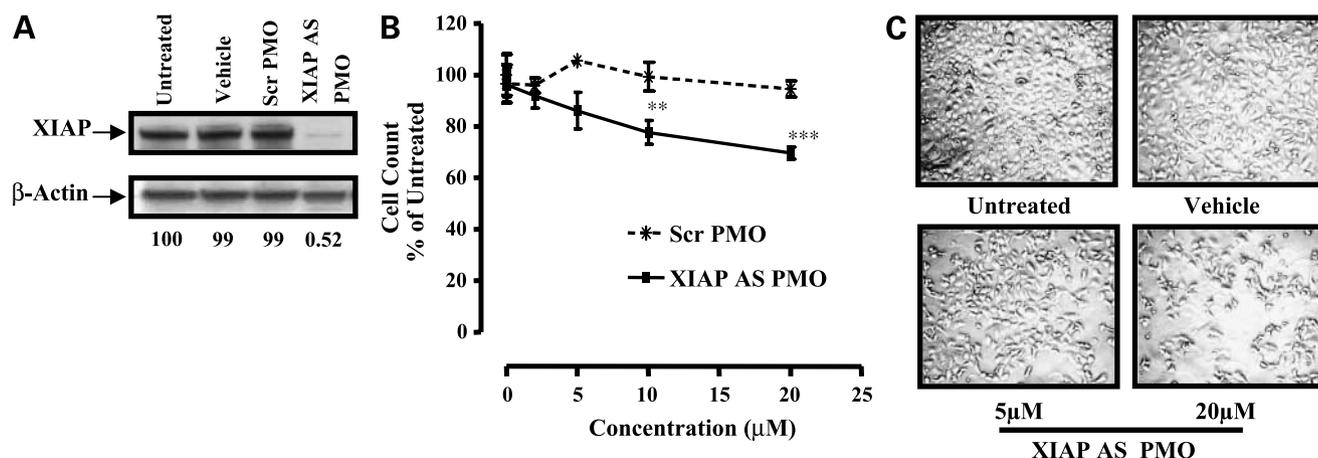


Figure 3. Effect of XIAP antisense PMO on XIAP expression and cell proliferation in DU145 cells. **A**, Immunoblot analysis of lysates from cells treated with XIAP antisense and scrambled PMOs by scrape loading for 24 hours. The same membrane was stripped and probed for β -actin as loading control. Protein levels were determined by densitometric analysis and expressed as percentage of control. *Arrows*, 57-kDa XIAP and 43-kDa β -actin bands. **B**, Effect of XIAP antisense PMO on viability of DU145 cells. The number of viable cells was determined by trypan blue exclusion using a hemocytometer. *Points*, means of triplicate values ($n = 3$); *bars*, SE. **, $P < 0.01$, ***, $P < 0.001$, XIAP antisense versus scrambled PMO and vehicle at the indicated PMO concentrations. **C**, Representative phase-contrast photomicrographs of DU145 cells scrape loaded from different treatments.

sequence-specific inhibition of luciferase activity in the presence of the antisense XIAP PMO compared with the scrambled or vehicle controls. In addition, immunoblot analysis (Fig. 4) of the cell lysates from the various treatment groups revealed specific inhibition of endogenous XIAP levels in the HeLa cells, which corresponded with about 30% decrease in cell viability similar to that seen in DU145 cells as discussed above.

To ascertain whether the observed cell death was mediated by apoptosis, immunoblot analysis was conducted to examine the status of caspase-3. Data in Fig. 5A showed that XIAP antisense PMO induced activation of caspase-3, which was further confirmed by the M30 ELISA-based method that detects the presence of M30 antigen formed during apoptosis due to cleavage of cytokeratin 18, a caspase-3 substrate. An increase in M30 antigen levels was observed in the XIAP antisense PMO treated cells compared with scrambled control (Fig. 5B).

XIAP Antisense PMO Potentiates Cisplatin and TRAIL in DU145 Human Prostate Cancer Cells

Our data suggest that cisplatin sensitivity in DU145 cells is inversely related to XIAP expression, and TRAIL-resistant DU145 cells continue to express high levels of XIAP. We therefore studied the effect of a combination treatment schedule consisting of cytotoxic agent and the XIAP antisense PMO. In one case, cells were treated with cisplatin for 24 hours followed by 24-hour XIAP antisense or scrambled PMO treatment. Controls included untreated cells (vehicle cells that were scraped in culture medium only). Cells that were treated with cytotoxic agent alone were also sham scrape loaded to account for any cell death due to the antisense delivery method.

A significant decrease ($P < 0.01$) in cell viability was observed in cells treated with a combination of XIAP antisense PMO + cisplatin ($\text{IC}_{50} = 9$ to $20 \mu\text{mol/L}$) compared with XIAP antisense alone (20% to 30% decrease

Table 1. Plasmid-based system for screening PMO sequence specificity and antisense activity

| Transfection | Treatments | % Luciferase Activity (Relative Luminescence Units/ μg Protein) | % Cell Viability |
|---------------------------------|----------------|--|--------------------|
| pCiNeoXIAP-luc ΔA | Vehicle | 100.00 \pm 1.93 | 100.00 \pm 2.72 |
| | Scrambled PMO | 115.00 \pm 4.23 | 93.78 \pm 4.15 |
| | XIAP antisense | 53.78 \pm 9.53 | 69.30 \pm 5.85 |
| Vector control | Vehicle | 100.00 \pm 8.35 | 100.00 \pm 15.38 |
| | Scrambled PMO | 97.21 \pm 3.91 | 84.89 \pm 9.50 |
| LipofectAMINE alone | Scrambled PMO | 0.006 \pm 0.01 | 83.00 \pm 7.57 |

NOTE: HeLa cells transiently transfected with pCiNeoXIAP-luc ΔA , vector alone (pCiNeo-luc ΔA), or delivery agent (Lipofectamine) were scrape loaded with different agents as indicated. Luciferase activity was measured 24 hours later. Data are expressed as luciferase activity in lysates normalized for protein content and represent means \pm SE of triplicate values ($n = 2$). Cell viability in each case was assessed by trypan blue staining and expressed as mean \pm SE of triplicate values. $P < 0.01$, vehicle versus XIAP antisense PMO treated cells. $P < 0.001$, scrambled PMO versus XIAP antisense PMO treated cells transiently transfected with pCiNeoXIAP-luc ΔA .

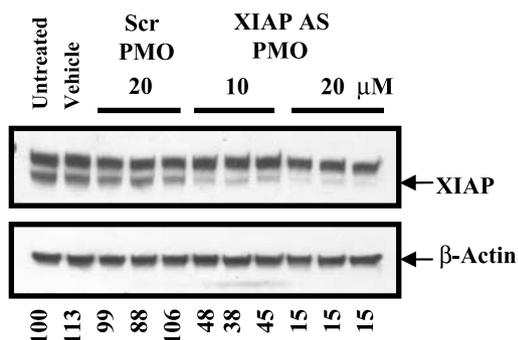


Figure 4. Lysates from the HeLa cells transiently transfected with pCiNeoXIAP-luc Δ A and scrape loaded with vehicle, antisense XIAP PMO, or scrambled PMO were also probed for endogenous XIAP expression. Protein bands were quantified by densitometric analysis and expressed as percentage of untreated.

in cell viability at 20 μ mol/L, cisplatin alone ($IC_{50} > 200$ μ mol/L), or combination of scrambled PMO + cisplatin ($IC_{50} > 200$ μ mol/L) treatment groups where IC_{50} was not reached within the range of cisplatin concentration tested (Fig. 6A). Immunoblot analysis (Fig. 6B) revealed a decrease in XIAP expression in the XIAP antisense PMO alone group and the XIAP antisense PMO + cisplatin combination treatment group. This effect was not seen in cisplatin alone or in cisplatin + scrambled PMO treatment groups.

Another combination treatment schedule with TRAIL and XIAP antisense PMO revealed that treatment of DU145 cells with XIAP antisense PMO for 24 hours followed by TRAIL treatment for another 24 hours enhanced TRAIL sensitivity as seen with increased cell death ($P < 0.05$) at 100 to 250 ng/mL concentrations in the XIAP antisense + TRAIL combination group ($IC_{50} = 3$ to 5 ng/mL) compared with TRAIL alone ($IC_{50} = 65$ to 68 ng/mL) or TRAIL + scrambled combination ($IC_{50} = 54$ to 65 ng/mL; Fig. 7A). Immunoblot analysis of the lysates revealed a marked decrease in XIAP levels in the XIAP antisense PMO alone and XIAP antisense + TRAIL combination treated cells. This decrease in XIAP levels was not observed in the TRAIL alone or TRAIL + scrambled PMO treated cells as shown in Fig. 7B.

Discussion

We report herein that XIAP, a potent caspase inhibitor, plays a critical role in modulating chemosensitivity in DU145 human androgen-unresponsive and invasive prostate cancer cell model. DU145 cells constitutively express XIAP, and time course experiments demonstrated that these cells are largely resistant to cisplatin and partially sensitive to TRAIL treatment. Although 24- to 72-hour cisplatin treatment caused decrease in Akt/phospho-Akt levels at higher concentrations along with some level of caspase-7 activation, XIAP levels and cell viability were comparable with untreated cells. However, prolonged incubation with cisplatin caused a dramatic increase in

sensitivity (70% decrease in cell viability at lower concentrations) and significant dose-dependent down-regulation of XIAP and activation of caspase-3 and caspase-7. In contrast, sensitivity to TRAIL was a rapid event, which occurred within 6 hours posttreatment and correlated with marked activation of caspase-3 and Akt cleavage. However, prolonged TRAIL treatment up to 96 hours had no effect on levels of XIAP and phospho-Akt and did not cause a time-dependent or dose-dependent increase in cell death compared with untreated cells.

Although chemotherapeutic agents such as cisplatin are widely used for the treatment of various forms of cancer, including prostate cancer (24), chemoresistance and toxicity present major hurdles. Multiple mechanisms have been implicated in the establishment of resistant phenotype including the role of XIAP. One of the well-characterized mechanisms of action of cisplatin involves DNA binding

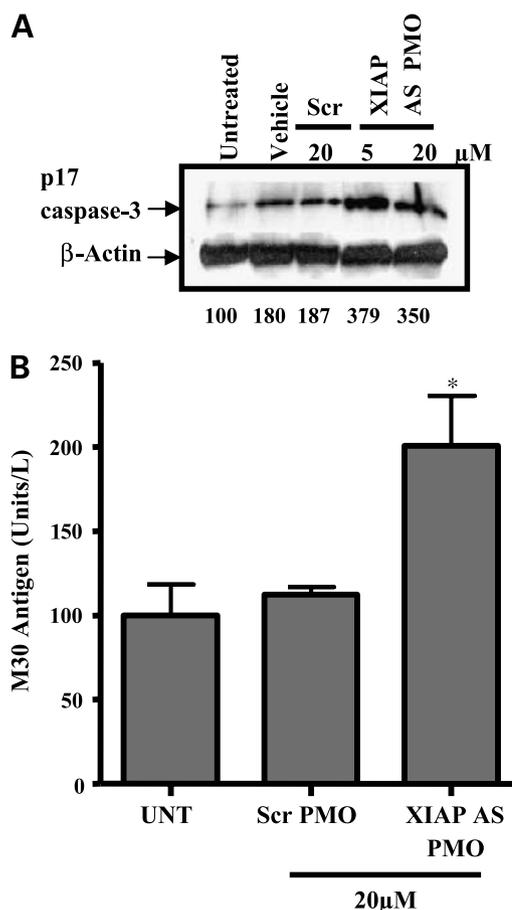


Figure 5. **A**, Caspase-3 immunoblot from cell lysates treated with XIAP antisense and scrambled PMOs by scrape loading for 24 hours. The same membrane was stripped and probed for β -actin as loading control. Numbers below each blot were obtained from densitometric analysis and expressed as percentage of control. Arrows, 17-kDa form of caspase-3 and 43-kDa β -actin bands. **B**, Levels of M30 antigen in cell lysates as determined by ELISA-based method. Levels of M30 antigen were normalized to total protein content in the cell extracts. Points, means of triplicate values from a single experiment; bars, SE. *, $P < 0.05$, XIAP antisense PMO versus untreated and scrambled PMO.

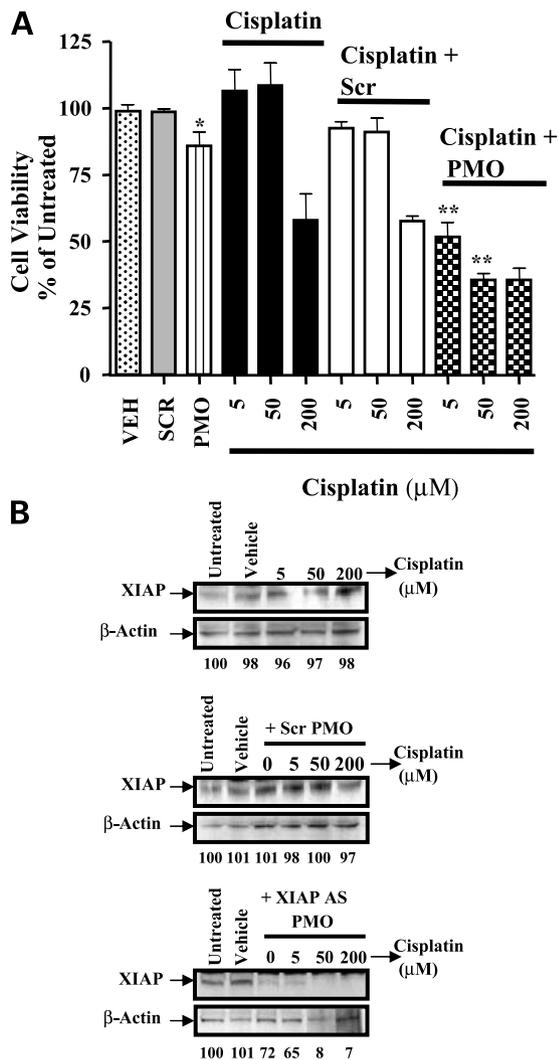


Figure 6. Role of combined XIAP antisense PMO and cisplatin treatment on protein expression and cell proliferation in DU145 human prostate cancer cells. Cells were treated with cisplatin for 24 hours followed by 24-hour XIAP antisense PMO and scrambled PMO treatments. **A**, Cell viability was monitored by MTT assay after 24 hours. *Columns*, means of triplicate values; *bars*, SE. *, $P < 0.05$, XIAP antisense PMO versus vehicle and scrambled PMO. **, $P < 0.01$, cisplatin + XIAP antisense PMO versus cisplatin and cisplatin + scrambled PMO. **B**, Immunoblot analysis of XIAP expression. Protein levels were quantified by densitometric analysis and expressed as percentage of control. *Arrows*, 57-kDa XIAP and 43-kDa β-actin bands.

and blocking of DNA replication (25). Recent studies have shown that cisplatin is capable of down-regulating XIAP expression with the induction of apoptosis in cisplatin-sensitive but not in resistant ovarian cancer cells (26). In addition, cisplatin has been observed to induce caspase-3 activation and apoptosis by up-regulating the expression of Fas (a 45-kDa cysteine-rich transmembrane glycoprotein) and its ligand FasL, which results in initiation of the apoptotic pathway by activation of caspase-8 and downstream effector caspase-3 and caspase-7 (27, 28).

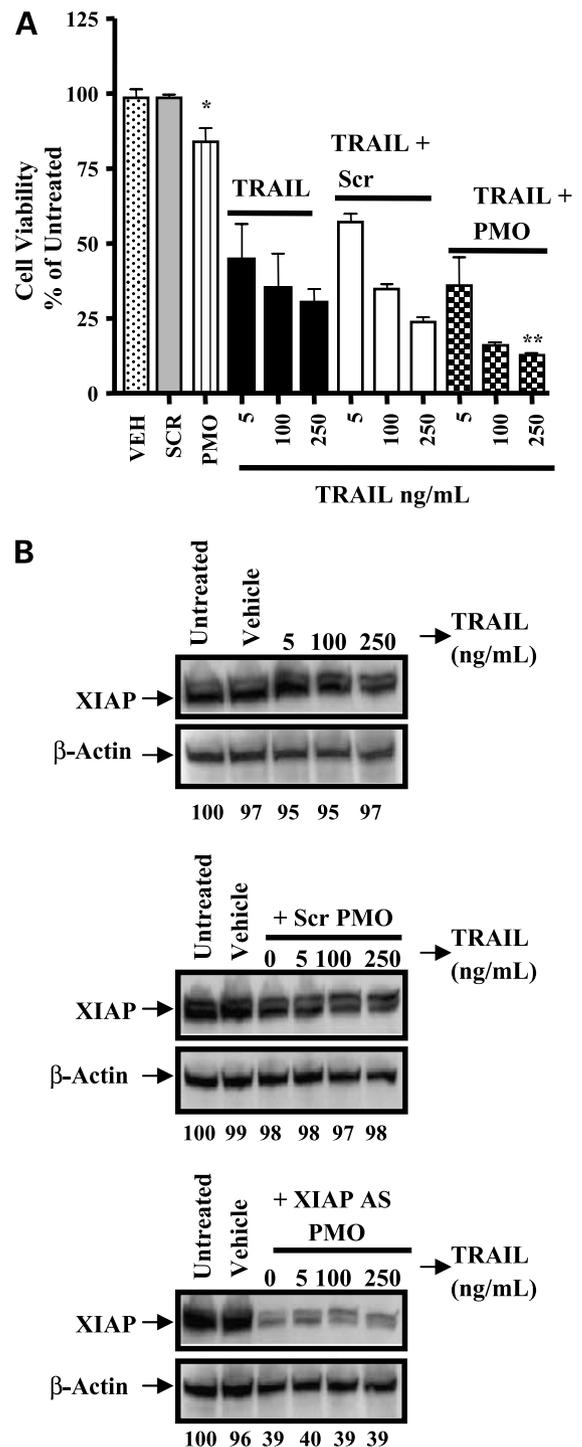


Figure 7. Effect of combined TRAIL and XIAP antisense PMO on cell proliferation and protein expression in DU145 cells. Cells were treated with XIAP antisense and scrambled PMOs for 24 hours followed by a 24-hour TRAIL treatment. **A**, Cell viability was monitored by MTT assay after 24 hours. *Columns*, means of triplicate values; *bars*, SE. *, $P < 0.05$, XIAP antisense PMO versus vehicle and scrambled PMO. **, $P < 0.05$, TRAIL + XIAP antisense PMO versus TRAIL and TRAIL + scrambled PMO. **B**, Immunoblot analysis of XIAP expression. Protein levels were quantified by densitometric analysis and expressed as percentage of control. *Arrows*, 57-kDa XIAP and 43-kDa β-actin bands.

These studies are in support of our observations, as a significant increase in active caspase-3 and caspase-7 levels correlated with XIAP inhibition and apoptosis after prolonged cisplatin treatment. This effect of cisplatin on XIAP down-regulation in the DU145 cells could be caspase-mediated following Fas activation because XIAP has also been shown to be a substrate of caspase-8, caspase-3, and caspase-7 leading to degradation of XIAP into inactive fragments that do not retain the ability to inhibit caspase activity (29).

The results from the TRAIL treatment caused a dramatic increase in active caspase-3 levels, but the continued presence of XIAP (potent caspase inhibitor) seemed to protect 50% to 60% of these cells from undergoing apoptosis even after prolonged TRAIL treatment. An earlier study showed that the levels of the active form of caspase-3 induced by TRAIL treatment were similar in both TRAIL-resistant and TRAIL-sensitive melanoma cancer cells, but the activated caspase-3 did not have a functional downstream effect on its substrates in the TRAIL-resistant cancer cells (30). Furthermore, the activated caspase-3 in the resistant cells was identified to be bound by high levels of XIAP and hence inactive, whereas the XIAP levels were decreased in the TRAIL-sensitive cells. Interestingly, TRAIL treatment caused decrease in Akt levels but no change in the amount of phospho-Akt, suggesting the presence of a survival signaling even in the presence of TRAIL. Asselin et al. (28, 31) have shown increased Akt activity in the presence of XIAP in cancer and developmental models. Antiapoptotic characteristics of TRAIL, particularly by Akt activation, have also been observed in primary human vascular endothelial (32), synovial (33), and neuronal (34) cells. Continued expression of XIAP combined with activated Akt could potentially explain the saturation effect seen in the present study, where the DU145 prostate cancer cells seemed to be only partially sensitive to TRAIL as there was no shift in IC_{50} or increased cell death with increasing concentration or duration of TRAIL treatment.

To correlate the role of XIAP in the inhibition of apoptosis, DU145 cells were treated with XIAP antisense PMO agent, which had the ability to specifically down-regulate XIAP expression. A dose-dependent decrease in cancer cell viability, albeit modest, and an increase in caspase-3 activity were observed 24 hours post-XIAP antisense PMO treatment. Current cytotoxic chemotherapy has limited antitumor activity in hormone-refractory prostate cancer (24). Therefore, the development of novel therapeutic strategies that target the molecular changes contributing to the etiology and progression of the disease is of immediate need. Presently, various studies have shown that XIAP down-regulation following adenoviral antisense expression induces apoptosis in chemoresistant human ovarian cancer (35, 36) and sensitizes lung cancer cells to low-dose γ -radiation (37). The clinical application of adenoviral vectors as a means of delivering the antisense agent is however limited by host immune response (38), whereas antisense oligonucleotides appear to be relatively

safer (39). The antiproliferative effect of a 19-mer XIAP phosphorothioate antisense oligonucleotide (targeting the baculovirus inhibitors of apoptosis protein repeat domain) in human lung cancer cell model has been reported (40). In the present study, a combination treatment strategy involving XIAP antisense PMO with cisplatin potentiated the activity of cisplatin in the DU145 cells by decreasing the IC_{50} from $>200 \mu\text{mol/L}$ with cisplatin alone to 9 to $20 \mu\text{mol/L}$ and decreasing incubation time required for activity from 96 to 24 hours. Similarly, TRAIL in combination with XIAP antisense PMO enhanced TRAIL potency by 12- to 13-fold. These results provide evidence that the XIAP antisense PMO can enhance the effect of cisplatin and TRAIL through the combined effect of decreasing XIAP and Akt levels coupled with caspase-3 activation.

In conclusion, abrogation of XIAP expression is essential for therapeutic apoptosis and enhanced chemotherapy sensitization in androgen-refractory prostate cancer cells. Unlike the phosphorothioate antisense oligonucleotides, PMOs represent a novel antisense oligomer chemistry in which the backbone is neutral at physiologic pH and consists of a six-membered morpholine ring. The absence of internucleoside charge allows the PMOs to avoid nonspecific effects associated with commonly used phosphorothioate oligonucleotides. The safety of the PMO antisense agents has been extensively established, and no serious adverse effects or mortality have been reported in animal models or human clinical trials (4, 41, 42). This novel PMO-based antisense strategy provides not only a nontoxic therapeutic approach to cancer but also an insight into the biochemical role of XIAP in cancer chemoresistance.

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