

Mithramycin A sensitizes cancer cells to TRAIL-mediated apoptosis by down-regulation of *XIAP* gene promoter through Sp1 sites

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Abstract

Mithramycin A is a DNA-binding antitumor agent, which has been clinically used in the therapies of several types of cancer and Paget's disease. In this study, we investigated the combined effect of mithramycin A and tumor necrosis factor- α -related apoptosis-inducing ligand (TRAIL) on apoptosis of cancer cells. In Caki renal cancer cells, which are resistant to TRAIL, cotreatment with subtoxic doses of mithramycin A and TRAIL resulted in a marked increase in apoptosis. This combined treatment was also cytotoxic to Caki cells overexpressing Bcl-2 but not to normal mesengial cells. Moreover, apoptosis by the combined treatment with mithramycin A and TRAIL was dramatically induced in various cancer cell types, thus offering an attractive strategy for safely treating malignant tumors. Mithramycin A-stimulated TRAIL-induced apoptosis was blocked by pretreatment with the broad caspase inhibitor zVAD-fmk or Crm-A overexpression, showing its dependence on caspases. We found that mithramycin A selectively down-regulated XIAP protein levels in various cancer cells. Luciferase reporter assay and the chromatin immunoprecipitation assay using the XIAP promoter constructs show that mithramycin A down-regulates the transcription of *XIAP* gene through inhibition of Sp1 binding to its promoter. Although XIAP overexpression

significantly attenuated apoptosis induced by mithramycin A plus TRAIL, suppression of XIAP expression by transfection with its small interfering RNA prominently enhanced TRAIL-induced apoptosis. We present here for the first time that mithramycin A-induced suppression of XIAP transcription plays a critical role in the recovery of TRAIL sensitivity in various cancer cells. [Mol Cancer Ther 2006;5(11):2737–46]

Introduction

The aggressive cancer cell phenotype is the result of a variety of genetic and epigenetic alterations leading to deregulation of intracellular signaling pathways (1). Despite aggressive therapies, resistance of many tumors to current treatment protocols still constitutes a major problem in cancer therapy (2). Targeting death receptors, especially DR4 or DR5, to trigger apoptosis in tumor cells is an attractive concept for cancer therapy because tumor necrosis factor- α -related apoptosis-inducing ligand (TRAIL) has been shown to induce apoptosis in a wide variety of cancer cells, including renal cancer cells, whereas most normal human cell types are resistant to TRAIL-induced cell death, which is supported by the presence of large numbers of decoy receptors on normal cells (3, 4). However, recent studies have shown that some cancer cells are resistant to the apoptotic effects of TRAIL (5, 6). TRAIL-resistant cancer cells can be sensitized by chemotherapeutic drugs *in vitro*, indicating that combination therapy may be a possibility. Therefore, understanding the molecular mechanisms of TRAIL resistance and ways to sensitize these cells to undergo apoptosis by TRAIL are important issues for effective cancer therapy.

The anticancer antibiotic mithramycin A, also called Plicamycin, was originally isolated from *Streptomyces griseus*. It has been used in cancer therapy in combination with hydroxurea or α -IFN (7, 8). Its mechanism of action has been proposed to interact with GC-rich domains of DNA contained in genes promoters (9), leading to gene transcription modulation, such as *multidrug resistance* gene (10), *c-myc*, or *h-ras* (11). It has recently been shown that mithramycin A can sensitize tumor cells to apoptosis induced by tumor necrosis factor- α and anti-Fas antibody (12, 13). However, the underlying mechanisms of mithramycin A-induced sensitization are not well understood. In this study, we show that mithramycin A can significantly enhance TRAIL-mediated apoptosis in various cancer cells, offering new possibilities for the treatment of malignant tumors. Furthermore, we show here for the first time that down-regulation of XIAP is critical for the sensitizing effect of mithramycin A on TRAIL-mediated apoptosis.

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Note: T.-J. Lee and E. Mi Jung contributed equally to this work.

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Materials and Methods

Cells and Materials

Human renal carcinoma Caki cell, colon cancer HT29 cell, breast cancer MDA231, prostate cancer PC3, and human astrogloma U87 cells were obtained from the American Type Culture Collection (Manassas, VA). Primary culture of human mesangial cells (Cryo NHMC) and its corresponding growth medium (CC-3146 MsGM) were purchased from Clonetics (San Diego, CA). The culture medium used throughout these experiments was DMEM, containing 10% FCS, 20 mmol/L HEPES buffer, and 100 mg/mL gentamicin. Mithramycin A was directly added to cell cultures at the indicated concentrations. Anti-Hsc70, anti-XIAP, anti-Bcl-2, anti-phospholipase C- γ 1, anti-poly(ADP-ribose) polymerase, anti-caspase-3, anti-Crm-A, anti-Bax, anti-cIAP2, anti-Bcl-xL, and anti-cIAP1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Soluble recombinant TRAIL was purchased from Calbiochem (San Diego, CA). Mithramycin A was obtained from Sigma (St. Louis, MO).

Transfections and Luciferase Gene Assays

Cells were plated onto six-well plates at a density 5×10^5 per well and grown overnight. Cells were cotransfected with 2 μ g of various plasmid constructs and 1 μ g of the pCMV- β -galactosidase plasmid for 5 hours by the LipofectAMINE method. After transfection, cells were cultured in 10% FCS medium with vehicle (DMSO) or drugs for 24 hours. Luciferase and β -galactosidase activities were assayed according to the manufacturer's protocol (Promega, Madison, WI). Luciferase activity was normalized for β -galactosidase activity in cell lysate and expressed as an average of three independent experiments.

XIAP Promoter Construction

Chromosomal DNA was prepared from Caki cells using the DNAzol reagent (Life Technologies, Gaithersburg, MD). Human XIAP promoter was amplified from chromosomal DNA with the following synthetic primers: 5'-AAG-GCAAAAGAGCTCGCTAATTC-3' (−1504 to −1482, sense), 5'-GGCAGCAAGAGCTCAACTCC-3' (−1204 to −1183, sense), 5'-ATTTTTCCTGAGCTCCCTC-3' (−674 to −653, sense), 5'-TAATGTCGAGCTCAGTATTT-3' (−184 to −163, sense), 5'-TTCCTAATATAATGTTCT-3' (−58 to −41), 5'-GCAGGTACACAAGCTTTAGAT-3' (+24 to +44, antisense), and 5'-AATCCAA AAGCTTAAACACAA-3' (deletion for −57 to +45 antisense). The PCR product was digested with *SacI* and *HindIII* and cloned upstream of the firefly luciferase gene of pGL2-basic (Promega). PCR products were confirmed by their size, as determined by electrophoresis and DNA sequencing. Point mutations of the Sp1-binding sites to the XIAP (−184) promoter were generated by a two-step PCR method using the following primers: mSp1-1 (5'-GTTTCTTAGCGGTCTGTAGTA-3' to 5'-GTTTCTTAGCAATCATGTAGTA-3') and mSp1-2 (5'-CTTTTATAGAAAAGGTGGACAAGT-3' to 5'-CTTTTATAGAAAATGTTGACAAGT-3'). Double mutants (mSp1-1 and mSp1-2) were generated by a two-step PCR method using the same primer, but template DNAs were used as point mutated plasmids. The XIAP promoter plasmid was

transfected into Caki cells using the LipofectAMINE reagent (Life Technologies) according to the manufacturer's instructions. To assess XIAP promoter luciferase activity, cells were collected and disrupted by sonication in lysis buffer [25 mmol/L Tris-phosphate (pH 7.8), 2 mmol/L EDTA, 1% Triton X-100, and 10% glycerol]. After centrifugation, aliquots of supernatants were tested for luciferase activity using the luciferase assay system (Promega), as specified by the manufacturer. Point mutations of the Sp1 binding sites to the XIAP promoter were generated by a two-step PCR method using the following primers: Sp1 (5'-CTTTTATAGAAAAGGTGGACAAGT-3' to 5'-CTTTTATAGAAAATGTTGACAAGT-3'). Clones representing each point mutation were sequenced to ensure the accuracy of the PCR amplification procedure.

Western Blotting

Cellular lysates were prepared by suspending 1×10^6 cells in 100 μ L of lysis buffer (137 mmol/L NaCl, 15 mmol/L EGTA, 0.1 mmol/L sodium orthovanadate, 15 mmol/L MgCl₂, 0.1% Triton X-100, 25 mmol/L MOPS, 100 μ mol/L phenylmethylsulfonyl fluoride, and 20 μ mol/L leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 minutes. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Detection of specific proteins was carried out with an Enhanced Chemiluminescence Western blotting kit according to the manufacturer's instructions.

Cell Count and Flow Cytometry Analysis

Cell counts were done using a hemocytometer. Approximately 1×10^6 Caki cells were suspended in 100 μ L PBS, and 200 μ L of 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 hour, washed with PBS, and resuspended in 250 μ L of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μ g RNase. Incubation was continued at 37°C for 30 minutes. The cellular DNA was then stained by applying 250 μ L of propidium iodide (50 μ g/mL) for 30 minutes at room temperature. The stained cells were analyzed by fluorescence-activated cell sorting on a FACScan flow cytometer for relative DNA content based on red fluorescence.

Asp-Glu-Val-Asp-ase Activity Assay

To evaluate Asp-Glu-Val-Asp-ase (DEVDase) activity, cell lysates were prepared after their respective treatment with TRAIL or mithramycin A. Assays were done in 96-well microtiter plates by incubating 20 μ g of cell lysates in 100 μ L reaction buffer [1% NP40, 20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 10% glycerol] containing the caspases substrate [Asp-Glu-Val-Asp-chromophore-*p*-nitroanilide (DVAD-pNA)] at 5 μ mol/L. Lysates were incubated at 37°C for 2 hours. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

RNA Isolation and Reverse Transcription-PCR

XIAP mRNA expression was determined by reverse transcription-PCR. Total cellular RNA was extracted from cells using the TRIzol reagent (Life Technologies). A cDNA was synthesized from 2 μ g of total RNA using M-MLV reverse transcriptase (Life Technologies). The cDNA for

XIAP was amplified by PCR with specific primers. The sequences of the sense and antisense primer for XIAP were 5'-CTTGAGGAGTGTCTGGTAA-3' and 5'-GTGACTA-GATGTCCACAAGGC-3', respectively. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were done as followed. Briefly, asynchronously growing Caki cells were incubated with formaldehyde to cross-link protein-DNA complexes. The cross-linked chromatin was then extracted, diluted with lysis buffer, and sheared by sonication. After preclearing with 1:2 mix of protein A/protein G-agarose beads (Upstate, Lake Placid, NY), the chromatin was divided into equal samples for immunoprecipitation with either anti-Sp1 or anti-immunoglobulin G (negative control) polyclonal antibody (Santa Cruz Biotechnology). The immunoprecipitates were pelleted by centrifugation and incubated at 65°C to reverse the protein-DNA cross-linking. The DNA was extracted from the eluate by the phenol/chloroform method and then precipitated by ethanol. Purified DNA was subjected to PCR with primers specific for a region (-214 to +60) in the XIAP promoter spanning two putative Sp1-binding sites. The sequences of the PCR primers used are as follows: PF1 (-214 to -187), 5'-TTTACTTTATGACTTGAATGATGTGG-3'; PR1 (+39 to +60), 5'-TTCCTTATTGATGTCTGCAGGT-3'.

RNA Interference

Caki carcinoma cells were seeded at a density of 1×10^5 per well in six-well tissue culture plates the day before transfection to achieve 50% to 60% confluence. Transfections were done with 70 nmol/L of small interfering RNA (siRNA) duplex using LipofectAMINE Plus (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Subsequent transfections were carried out every 24 hours. The antisense oligonucleotide was designed to the region 5'-AATAGTGCCACGCAGTC-TACA-3' corresponding to residues 331 to 351 of human XIAP cDNA (U45880). Predesigned siRNA duplexes 5'-AAGACCCGCGCCGAG GUGAAG-3' for green fluorescent protein were used as a negative control. Both oligonucleotides were synthesized and purified by Bioneer (Daejeon, Korea). Three independent XIAP-silencing experiments were carried out to confirm the reproducibility of the findings.

Results

Mithramycin A Sensitizes TRAIL-Mediated Apoptosis in Various Malignant Cancer Cells

To investigate the effect of mithramycin A on TRAIL-mediated apoptosis, Caki human renal cancer cells were treated with mithramycin A alone (10–200 μ mol/L), TRAIL alone (100 ng/mL), or combination of mithramycin A and TRAIL. Three established criteria were subsequently used to assess apoptosis in our system. First, apoptosis in Caki cells was determined using flow cytometric analysis to detect hypodiploid cell populations. As shown in

Fig. 1A, cotreatment of Caki cells with mithramycin A and TRAIL resulted in a markedly increased accumulation of sub-G₁ phase cells, whereas treatment with mithramycin A alone or TRAIL alone did not increase accumulation of sub-G₁ phase cells. Second, we analyzed DNA fragmentation, which is another hallmark of apoptosis. Following agarose gel electrophoresis of DNAs from Caki cells treated with mithramycin A and TRAIL for 24 hours, a typical ladder pattern of internucleosomal fragmentation was observed. In contrast, DNA fragmentation in Caki cells treated with TRAIL alone or mithramycin A alone was barely detected (Fig. 1B). In addition, we analyzed whether cotreatment with mithramycin A and TRAIL gave rise to the activation of caspase-3, a key executioner of apoptosis. Cotreatment of Caki cells with mithramycin A and TRAIL strongly stimulated DEVDase activity and led to a reduction of the protein levels of 32-kDa precursor together with a concomitant cleavage of phospholipase C- γ 1, a substrate protein of caspases (Fig. 1C). Taken together, these results indicate that treatment with mithramycin A sensitizes Caki cells to TRAIL-mediated apoptosis. Next, we investigated whether the combined treatment with mithramycin A and TRAIL affects the induction of apoptosis in normal human mesangial cells. The apoptotic characteristics, such as cell shrinkage, apoptotic bodies, and detachment from the plate, were frequently observed in Caki cells treated with mithramycin A plus TRAIL (Fig. 1D). However, the mesangial cells were resistant to mithramycin A (200 nmol/L) or TRAIL (100 ng/mL) alone, and their morphologic changes were not significantly affected by the combined treatment with mithramycin A and TRAIL. Furthermore, mithramycin A did not enhance TRAIL-induced apoptosis in normal mesangial cells (Fig. 1E). These results suggest that the sensitizing regimens using mithramycin A and TRAIL may be preferentially toxic for renal carcinoma cells over normal mesangial cells.

Overexpression of Bcl-2 is known to inhibit chemotherapy-induced apoptosis (14). To determine the effect of cotreatment with mithramycin A and TRAIL on the viability of Bcl-2-overexpressing cells, we employed Caki renal carcinoma cells engineered for overexpression of Bcl-2 (Caki/Bcl-2) and vector-transfected control cells (Caki/Vector). As shown in Fig. 2A, overexpression of Bcl-2 could not significantly attenuate the apoptosis induced by the combined treatment with 200 nmol/L mithramycin A and 100 ng/mL TRAIL. These results suggest that the combined treatment with mithramycin A and TRAIL may effectively induce apoptotic cell death in Bcl-2-overexpressing cancer cells, which are relatively resistant to chemotherapy. We further investigated whether mithramycin A enhances TRAIL-induced apoptosis in a synergistic fashion in other human cancer cell types. As shown in Fig. 2B, combined treatment with mithramycin A and TRAIL strongly enhanced TRAIL-induced apoptosis in human colon cancer HT29 cells, breast cancer MDA231 cells, prostate cancer PC3 cells, and glioma U87MG cells. These results suggest that the mithramycin A can sensitize various malignant cancer cells to TRAIL-mediated apoptosis.

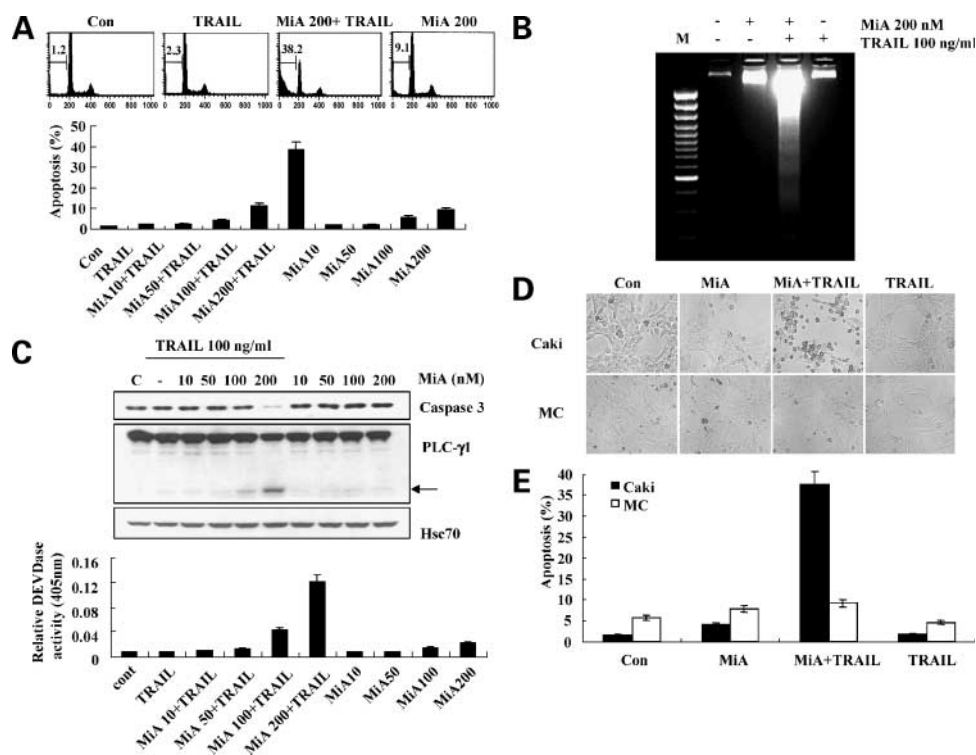


Figure 1. Mithramycin A sensitizes Caki cells but not normal mesangial cells to TRAIL-induced apoptosis. **A**, flow cytometric analysis of apoptotic cells. Caki cells were treated with TRAIL (100 ng/mL) in either the absence or the presence of mithramycin A (10–200 nmol/L) for 24 h. Apoptosis was analyzed as a sub-G₁ fraction by fluorescence-activated cell sorting. **B**, fragmentation of genomic DNAs in Caki cells treated for 24 h with the indicated concentrations of mithramycin A and TRAIL. Fragmented DNA was extracted and analyzed on 2% agarose gel. **C**, cells were treated with the indicated concentrations of mithramycin A and TRAIL. Equal amounts of cell lysates (40 µg) were subjected to electrophoresis and analyzed by Western blot for procaspase-3 and phospholipase C-γ1 (PLC-γ1). The proteolytic cleavage of phospholipase C-γ1 is indicated by an arrow. Representative result; two additional experiments yielded similar results. Enzymatic activities of DEVDase were determined by incubation of 20 µg of total protein with 200 µmol/L chromogenic substrate (DEVD-pNA) in a 100 µL assay buffer for 2 h at 37°C. The release of chromophore *p*-nitroanilide (*p*NA) was monitored spectrophotometrically (405 nm). **D**, effect of mithramycin A and/or TRAIL on normal mesangial cells. Caki and normal mesangial cells (MC) were treated with vehicle, 200 nmol/L mithramycin A alone, 100 ng/mL TRAIL, or mithramycin A plus TRAIL for 24 h. The morphologies of cells were determined by interference light microscopy. Magnification, ×200. **E**, quantitation of apoptosis by fluorescence-activated cell sorting analysis. Sub-G₁ fraction was measured in Caki or mesangial cells treated with vehicle, 200 nmol/L mithramycin A alone, 100 ng/mL TRAIL, or mithramycin A plus TRAIL for 24 h.

Sensitization to TRAIL-Induced Apoptosis by Mithramycin A Is Dependent on Caspases

To address the significance of caspase activation in mithramycin A-mediated sensitization for TRAIL-induced apoptosis, we used a general and potent inhibitor of caspases, z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone). As shown in Fig. 3A, increase in apoptotic population by the cotreatment with mithramycin A and TRAIL was significantly inhibited by pretreatment with z-VAD-fmk. The role of caspases in mithramycin A-stimulated TRAIL-induced apoptosis was further confirmed by overexpression of CrmA, a viral caspase-8 inhibitor, in U87MG cells. As shown in Fig. 3B, apoptosis induced by mithramycin A plus TRAIL was almost completely blocked in CrmA-overexpressing cells (U87MG/CrmA).

Mithramycin A Itself Down-Regulates XIAP Protein Levels

Next, we examined whether mithramycin A- and TRAIL-induced apoptosis is associated with the changes

in the expression levels of caspase inhibitory proteins, IAP (inhibitor of apoptosis protein) family proteins. As shown in Fig. 4A, combinatory treatment with mithramycin A and TRAIL led to reduction of the XIAP expression, but not cIAP1 and cIAP2, in dose-dependent manners. Because XIAP has been reported to be a substrate of caspases (15), we tested whether the decrease in the protein levels of XIAP induced by combined mithramycin A/TRAIL treatment was correlated with enhanced caspase activity. Although pretreatment with z-VAD-fmk completely blocked activation of caspase-3 induced by the combined treatment, it did not affect down-regulation of XIAP by the combined treatment with mithramycin A and TRAIL (Fig. 4B). Moreover, overexpression of CrmA, a viral inhibitor protein of caspase-8, showed a similar effect (Fig. 4C). These results suggest the possibility that mithramycin A itself may down-regulate XIAP protein levels. As expected, treatment of Caki cells with mithramycin A alone reduced the protein levels of XIAP dose and time dependently (Fig. 4D). Mithramycin-induced XIAP

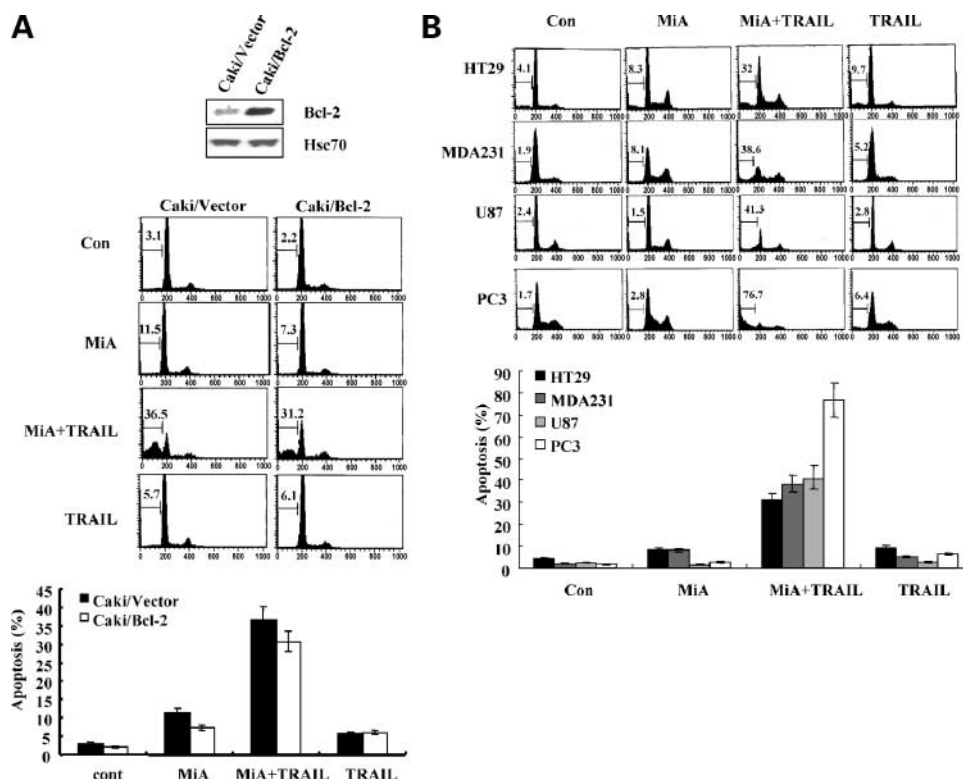
down-regulation was also observed in human colon HT29, breast MDA231, prostate PC3, and glioma U87MG cells, showing that mithramycin A treatment can reduce XIAP expression in various cancer cell types.

Mithramycin A Inhibits Transcription from the XIAP Gene Promoter Blocking DNA Binding of Sp1 Its Promoter

Next, we attempted to further clarify the underlying mechanisms of mithramycin A-induced XIAP down-regulation, employing the reverse transcription-PCR and the luciferase gene expression system. First, to further elucidate the mechanism responsible for the changes in amounts of XIAP protein, we determined levels of XIAP mRNA by reverse transcription-PCR. Treatment with mithramycin A resulted in marked decreases XIAP mRNA levels, an effect that was suppressed by mithramycin A in a dose-dependent and time-dependent manner (Fig. 5A). Second, to define the regulatory region of the XIAP promoter, a series of chimeric plasmids were constructed to contain progressive 5' end deletion of the XIAP 5' flanking sequence spliced to the firefly luciferase coding sequence in the promoterless plasmid vector pGL2-Basic (Fig. 5B). Plasmids were transiently transfected into Caki cells, and luciferase levels were measured to reflect promoter activity at 24 hours after transfection (Fig. 5B). The construct pXIAP/-1504 showed a ~2-fold increase in promoter activity compared with pXIAP/-58. The construct pXIAP/-184 produced the greatest activity (3.5-fold; Fig. 5B). These results show that potential

positive regulatory elements are located between -184 and +58 nucleotide and might be enough to regulate XIAP promoter activity. It was well known that mithramycin A inhibits Sp1 DNA binding activity to GC-rich promoter sequences (9). To characterize the mechanism of mithramycin A-induced down-regulation of XIAP gene expression, we treated mithramycin A to Caki cells and analyzed the promoter activities of several XIAP promoter constructs. As shown in Fig. 5C, mithramycin A inhibited XIAP promoter activity, suggesting that XIAP promoter was down-regulated by mithramycin A. To elucidate the involvement of Sp1 transcription factor in regulation of XIAP promoter activity, Sp1 expression vector was ectopically transfected, and promoter activities were analyzed. In all XIAP promoter constructs, Sp1 up-regulated XIAP promoter activity up to 4-fold (Fig. 5D). From a computer analysis, the human XIAP promoter region up to 184 bp contains sites for Sp1, nuclear factor- κ B, and activator protein (Fig. 6A). To decipher which Sp1 site plays a critical role in mithramycin A-mediated inhibition of pXIAP/-184 promoter, several XIAP promoter constructs, including Sp1 binding site mutants and deletion mutants, were made and tested in the transfection assay. As shown in Fig. 6B, deletion and mutation of Sp1-1 site (at -144 Sp1 site) or Sp1-2 site (at -25 Sp1 site) constructs decreased XIAP promoter activity, compared with the wild-type pXIAP/-184 construct but still induced by ectopic expression of Sp1. The promoter activity was significantly decreased by double mutation

Figure 2. Bcl-2 overexpression does not block apoptosis by mithramycin A plus TRAIL, and various cancer cells are also sensitized by mithramycin A to TRAIL-mediated apoptosis. **A**, effect of mithramycin A and/or TRAIL on the viabilities of Bcl-2-overexpressing Caki cells. Analysis of Bcl-2 expression levels in the stably transfected cell lines. Western blotting using an anti-Bcl-2 antibody was done to detect Bcl-2 expression levels in selected cell lines. Control cells (Caki/Vector) or Bcl-2-overexpressing cells (Caki/Bcl-2) were treated with TRAIL (100 ng/mL) in either the absence or the presence of mithramycin A (200 nmol/L) for 24 h. Apoptosis was analyzed as a sub-G₁ fraction by fluorescence-activated cell sorting. Columns, mean from three independent experiments; bars, SD. **B**, effect of mithramycin A and/or TRAIL on the viabilities of various cancer cells. HT29, MDA231, PC3, and U87MG cells were treated with TRAIL (100 ng/mL) in either the absence or the presence of mithramycin A (200 nmol/L) for 24 h. Apoptosis was analyzed as a sub-G₁ fraction by fluorescence-activated cell sorting.



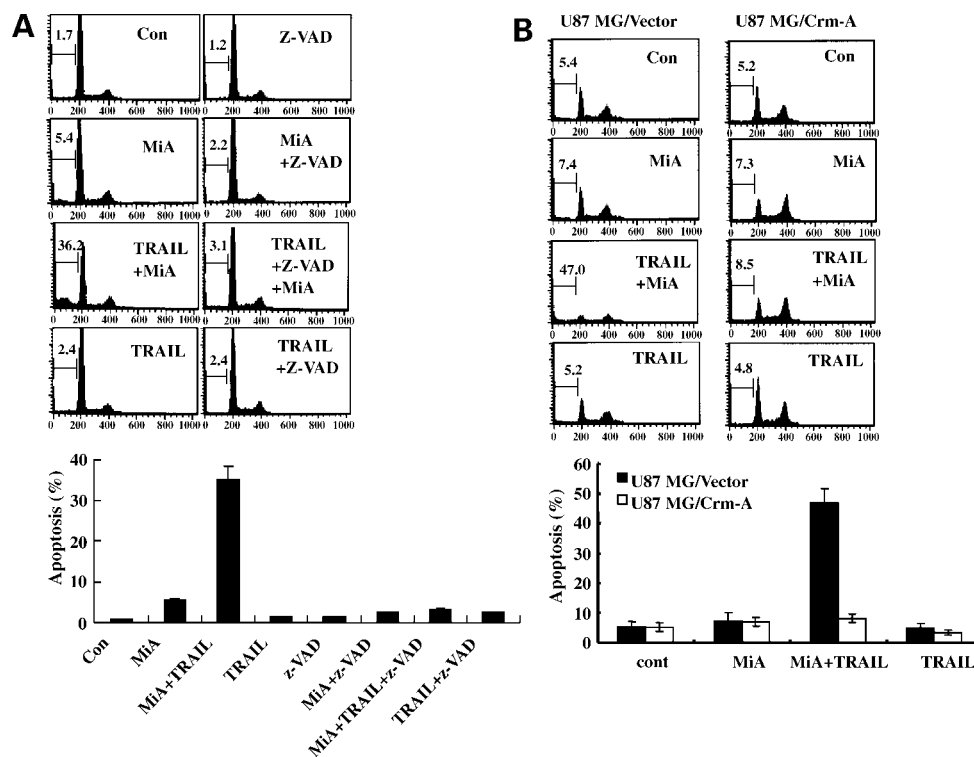


Figure 3. Inhibition of caspases by z-VAD-fmk or Crm-A overexpression significantly blocks apoptosis induced by mithramycin A plus TRAIL. **A**, effect of z-VAD-fmk on apoptosis induced by mithramycin A plus TRAIL. Caki cells were incubated with 50 μ mol/L z-VAD-fmk or solvent for 1 h before treatment with 200 nmol/L mithramycin A and/or 100 ng/mL TRAIL for 24 h. DNA contents of treated cells were evaluated after propidium iodide staining, and apoptosis was measured as a sub-G₁ fraction by fluorescence-activated cell sorting. *Columns*, mean from three independent experiments; *bars*, SD. **B**, effect of Crm-A overexpression on apoptosis induced by mithramycin A plus TRAIL. Control cells (U87 MG/Vector) or Crm-A-overexpressing stable cell lines (U87 MG/Crm-A) were treated with 200 nmol/L mithramycin A and/or 100 ng/mL TRAIL for 24 h, and then DNA contents were evaluated after propidium iodide staining. *Columns*, means ($n = 3$); *bars*, SD.

of two Sp1 sites. To confirm the involvement of Sp1 in the mithramycin A-induced response, chromatin immunoprecipitation assays were done with Sp1-specific antibody and PCR primers encompassing the two

putative Sp1-binding sequences present in XIAP (–184) construct (Fig. 6C). As shown in Fig. 6C, mithramycin A suppressed direct binding of Sp1 to XIAP promoter compared with the control, providing the evidence that

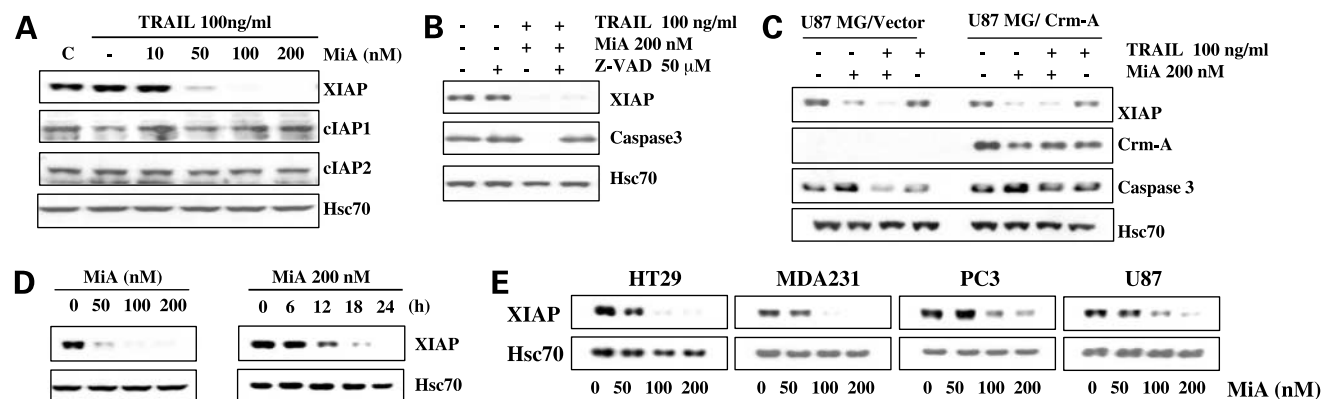


Figure 4. Mithramycin A itself down-regulates XIAP protein levels. **A**, changes in the protein levels of IAPs following cotreatment with mithramycin A and TRAIL. Caki cells were cotreated with mithramycin and TRAIL at the indicated concentrations for 24 h, and cell extracts were prepared. Equal amounts of cell lysates (40 μ g) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies (XIAP, cIAP1, and cIAP2) or with anti-Hsc70 antibody to serve as control for the loading of protein level. **B**, pretreatment with z-VAD-fmk does not block down-regulation of XIAP by the combined treatment with mithramycin and TRAIL. Caki cells were pretreated or not treated with 50 μ mol/L z-VAD-fmk for 30 min and further treated with 200 nmol/L mithramycin A and 100 ng/mL TRAIL for 24 h. Cell extracts were prepared for Western blotting of XIAP and caspase-3. Equal loading of the protein samples were confirmed by Western blotting of Hsc70. **C**, Crm-A overexpression does not block down-regulation of XIAP by the combined treatment with mithramycin A and TRAIL. Control cells (U87MG/Vector) and Crm-A-overexpressing cells were cotreated or not treated with 200 nmol/L mithramycin A and 100 ng/mL TRAIL for 24 h. Western blotting of Crm-A, XIAP, caspase-3, and Hsc70 was done. **D**, mithramycin A itself down-regulates XIAP protein levels dose and time dependently in Caki cells. Caki cells were treated with mithramycin A at the indicated concentrations for 24 h or 200 nmol/L mithramycin A for the indicated time points. Western blotting of XIAP was done. Hsc70 served as a protein loading control. **E**, mithramycin A itself down-regulates XIAP protein levels in various cancer cell types. HT29, MDA231, PC3, or U87MG cells were treated with mithramycin A at the indicated concentrations for 24 h. Western blotting of XIAP was done. Hsc70 served as a protein loading control.

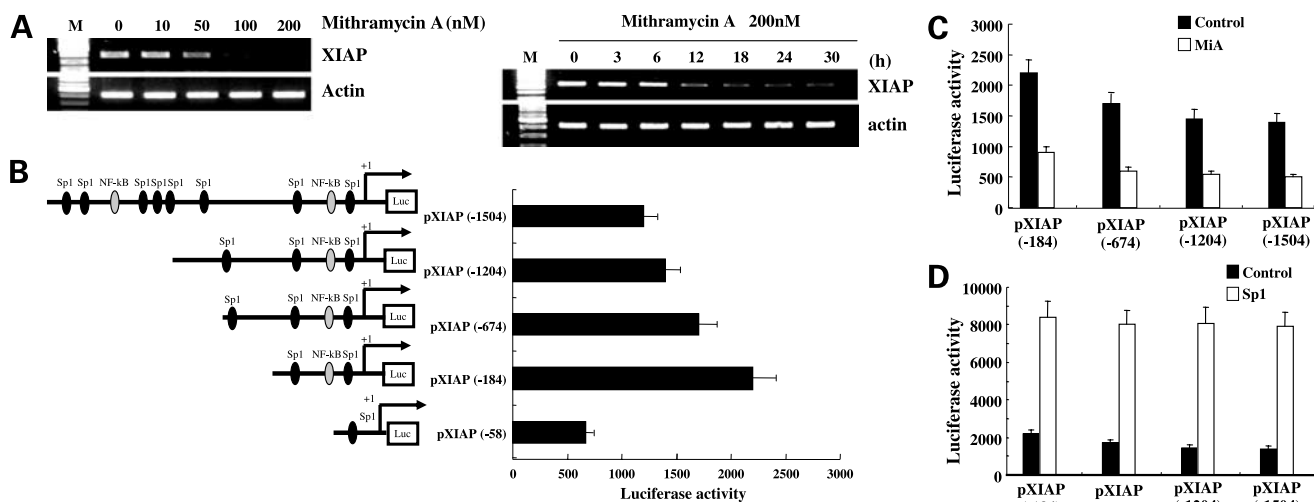


Figure 5. Effect of mithramycin A on the XIAP mRNA expression levels and the XIAP promoter activities. **A**, Caki cells were treated with the indicated concentrations or times of mithramycin A. Total RNA was isolated, and reverse transcription-PCR analysis was done as described in Materials and Methods. Representative study; two additional experiments yielded similar results. **B**, schematic structure of XIAP promoter constructs used for the analysis of luciferase activity. Basal activities of the constructed deletion mutants of XIAP promoter. Caki cells were transfected with each XIAP promoter vectors and luciferase activity measured. Columns, mean of at least three independent experiments; bars, SD. **C**, effects of mithramycin A on the XIAP promoter activities. The cells were treated with or without 200 nmol/L mithramycin A and luciferase activity measured. **D**, effects of ectopic expression of Sp1 on the XIAP promoter activities. Caki cells were cotransfected with Sp1 cDNA and XIAP promoter plasmids. The cells treated with mithramycin A and luciferase activity measured.

Sp1 directly binds the XIAP promoter and mithramycin A inhibits its binding. Taken together, XIAP promoter activity is regulated by Sp1 and down-regulated by mithramycin A through the inhibition of Sp1 binding to its promoter.

Although Overexpression of XIAP Inhibits Mithramycin A Plus TRAIL-Induced Apoptosis, siRNA-Mediated XIAP Silencing Increases Sensitivity to TRAIL

Next, we examined the functional significance of mithramycin A-induced XIAP down-regulation in apoptosis

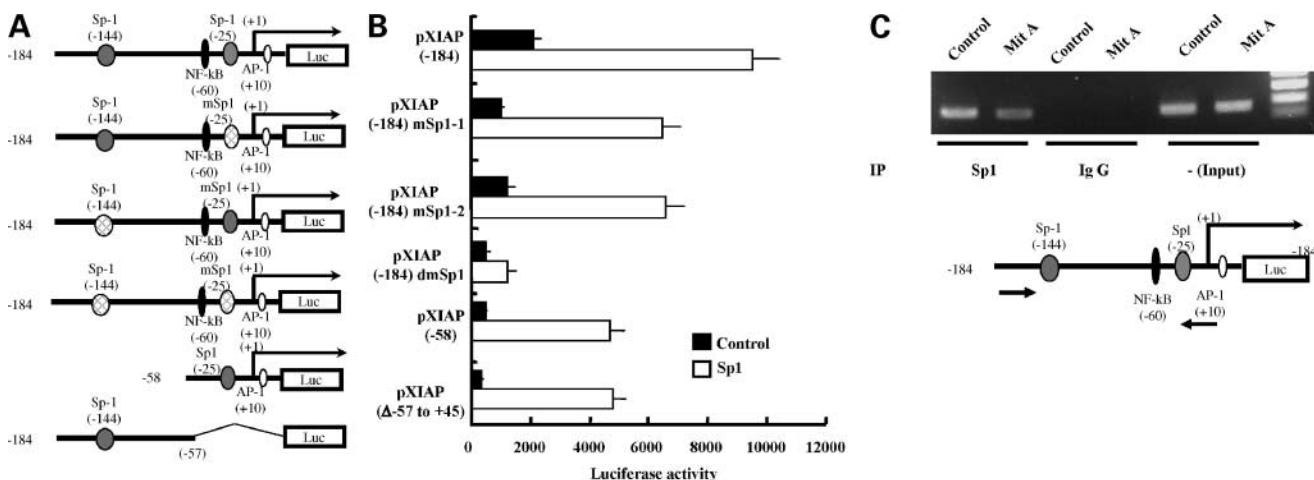


Figure 6. Mithramycin A down-regulates transcription of XIAP gene through inhibition of SP1 binding to its promoter. **A**, schematic structure of the mutant constructs of XIAP (-184) promoter used for analysis of luciferase activity. **B**, effects of ectopic expression of Sp1 on the XIAP (-184) promoter activities. Caki cells were cotransfected with Sp1 cDNA and XIAP promoter plasmids. The cells were treated with mithramycin A, and luciferase activity was measured. Nucleotides of the Sp1 consensus site in the promoter region were substituted as described in Materials and Methods. Caki cells were transfected with clones (left). **C**, Sp1 binds to the XIAP (-184) promoter region. Chromatin immunoprecipitation analyses were done with anti-Sp1 antibody, as described in Materials and Methods. The DNAs were extracted from total sonicated nuclei (Input), protein A without antibody (-), protein A with antibody (anti-Sp1), or with pre-immune serum (anti-IgG). Specific promoter regions of the XIAP genes were amplified by PCR, separated on 1.5% agarose gels, and stained with ethidium bromide. Also indicated is the relative position of the PCR product generated in chromatin immunoprecipitation assay.

induced by the combined treatment with mithramycin A and TRAIL. To test whether XIAP overexpression could block the cell death by the combined treatment, we employed Caki cells transiently transfected with XIAP (Caki/XIAP) and empty vector (Caki/Vector). Ectopic expression of XIAP significantly blocked not only the increase of sub-G₁ populations, DEVDase activity, and cleavage of poly(ADP-ribose) polymerase but also XIAP down-regulation, which were observed in Caki/vector cells cotreated with mithramycin A and TRAIL (Fig. 7A–C). These results indicate that restoration of XIAP expression, which was down-regulated by mithramycin A treatment, abolished mithramycin A- and TRAIL-mediated apoptosis.

We further examined whether down-regulation of XIAP using its siRNA could mimic the sensitizing effect of mithramycin on TRAIL-induced apoptosis. As shown in Fig. 7D, transfection of Caki cells with siRNA against XIAP resulted in a suppression of XIAP expression compared

with cells transfected with control green fluorescent protein siRNA. Immunoblot analysis of caspase-3 in Caki cells transfected with XIAP siRNA showed more caspase-3 degradation with formation of the 17-kDa active subunit compared with those in control siRNA-transfected cells, after TRAIL stimulation (Fig. 7E). When we analyzed the susceptibility of Caki cell to TRAIL-induced apoptosis, down-regulation of XIAP protein expression by siRNA augmented the sensitivity to TRAIL compared with control siRNA-transfected cells (Fig. 7E). DEVDase activity was also significantly enhanced in cells transfected with XIAP siRNA compared with cells transfected with green fluorescent protein siRNA following exposure to TRAIL (Fig. 7F). Taken together, these results suggest that XIAP may confer cancer cells resistance to TRAIL via inhibition of caspases. However, mithramycin A-induced XIAP down-regulation may provide an effective strategy to recover TRAIL sensitivity in various cancer cells.

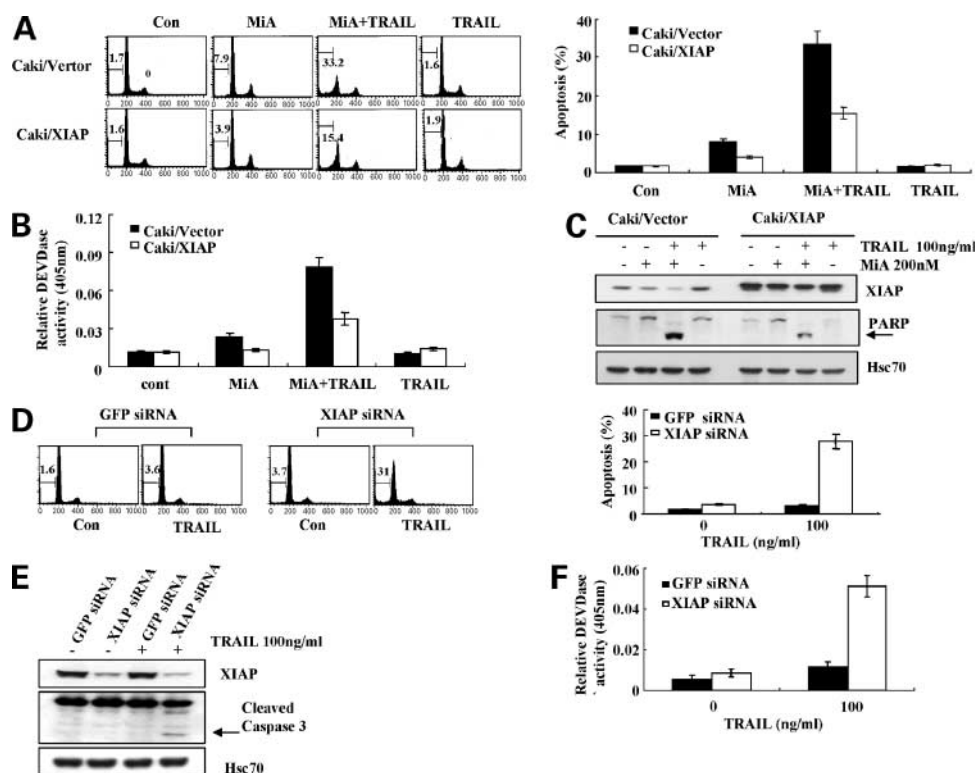


Figure 7. XIAP down-regulation by siRNA recovers TRAIL sensitivity in Caki cells, whereas XIAP overexpression attenuates mithramycin A plus TRAIL-induced apoptotic cell death. **A**, effect of mithramycin A and/or TRAIL on the viabilities of XIAP-overexpressing cells. Caki/Vector and Caki/XIAP cells were treated for 24 h with mithramycin A alone (200 nmol/L), TRAIL alone (100 ng/mL), or combination of mithramycin A and TRAIL. Apoptosis was analyzed as a sub-G₁ fraction by fluorescence-activated cell sorting. Columns, mean from three independent experiments; bars, SD. **B**, effect of XIAP overexpression on DEVDase activity induced by the combined treatment with mithramycin A and TRAIL. DEVDase activity was determined as described in Fig. 1C. Columns, means ($n = 3$); bars, SD. **C**, XIAP overexpression in Caki cells attenuates the activation of caspase-3 induced by the combined treatment with mithramycin A and TRAIL. After treatments of Caki/Vector or Caki/XIAP cells, cell extracts were prepared for Western blotting. The proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) is indicated by an arrow. **D**, down-regulation of XIAP by its siRNA increases TRAIL-induced apoptosis. Caki cells were transfected with green fluorescent protein (GFP) siRNA or XIAP siRNA and then treated with 100 ng/mL TRAIL for 24 h. Apoptosis was analyzed as sub-G₁ fraction by fluorescence-activated cell sorting. Columns, mean from three independent experiments; bars, SD. **E**, effect of down-regulation of XIAP by its siRNA on TRAIL-induced proteolytic processing of caspase-3. Equal amount of cell lysates (40 μ g) were subjected to electrophoresis and analyzed by Western blot for XIAP and caspase-3. Hsc70 served as a protein loading control. **F**, effect of down-regulation of XIAP by its siRNA on TRAIL-induced DEVDase activity. DEVDase activity was determined as described in Fig. 1C. Columns, means ($n = 3$); bars, SD.

Discussion

TRAIL, a recently identified member of the tumor necrosis factor family, is capable of inducing apoptosis in various tumor cells (16, 17). The anti-neoplastic specificity of TRAIL warrants interest in the use of this compound in the clinic; however, *in vitro* resistance of some cancer cell lines might predict a limited role for TRAIL as a single agent. Hence, an active search for novel therapeutics directed against diverse molecular targets to overcome resistance of tumors to TRAIL is ongoing. A burgeoning literature shows that combining TRAIL with chemotherapeutic or certain signaling inhibitors results in robust enhancement of apoptosis, albeit via different mechanisms. Synergism of the combination was induced through enhanced induction of intrinsic apoptotic pathway, inhibition of pro-survival signaling via AKT and/or nuclear factor- κ B, and down-regulation of inhibitor of apoptosis protein (XIAP). In the present study, we show for the first time that treatment of various cancer cells with mithramycin A in combination with TRAIL synergistically induced apoptosis. The mechanism of this synergy involves mithramycin A-induced down-regulation of XIAP expression. Functional significance of XIAP down-regulation in apoptosis induced by cotreatment with mithramycin A and TRAIL was confirmed by the fact that XIAP overexpression significantly attenuated the cell death by the combined treatment. In addition, XIAP siRNA transfection potentiated the apoptotic effect of TRAIL, mimicking the effect of mithramycin A.

XIAP is a member of the IAP family and plays a key role in cell survival by modulating death-signaling pathways at the post-mitochondrial level. XIAP is the most potent inhibitor of caspases and apoptosis among IAPs. It has been shown that XIAP is a direct inhibitor of caspase-3 and caspase-9 and modulates the Bax/cytochrome *c* pathway by inhibiting caspase-9 (18). Down-regulation of XIAP is an important mechanism for caspase activation in response to various apoptotic stimuli (19). Furthermore, chemotherapeutic agent-induced programmed cell death is accompanied by a decrease in XIAP protein content (20, 21). Several studies have addressed that the down-regulation of XIAP was involved in synergism of the combination drug treatment, such as indole-3-carbinol, sodium butyrate, and 17-allylamino-17-demethoxygeldanamycin, in various cancer cell types (22–24). In our study, we found that reduction of XIAP protein levels during mithramycin A-stimulated TRAIL-induced apoptosis was not mediated by caspase-dependent pathways. Neither pretreatment with z-VAD-fmk nor Crm-A overexpression did block down-regulation of XIAP protein levels in mithramycin A plus TRAIL treated-Caki cells.

It has been reported that mithramycin A inhibits transcription of many genes through suppression of Sp1 binding to their promoters. For example, *melanocortin-4 receptor* (MC4-R) gene (25), *myeloid Elf-1 like factor* (MEF) gene, *MUC6 mucin* gene, *DNA methyltransferase 3A* (DNMT3A), and *DNMT3B* genes promoter involves Sp1 site proximal to transcriptional start site (26–28). As shown in Fig. 5, XIAP (–184) basal promoter construct activity was

still high compared with other constructs, indicating that the 184-bp fragment of the XIAP promoter contains important elements to regulate XIAP transcription. Additionally, we found that mithramycin A-induced inhibition of XIAP expression involves two putative Sp1 sites (–144 and –25 bp) within the 5'-untranslated region using combination of chromatin immunoprecipitation assay and luciferase reporter assay. In addition, based on the chromatin immunoprecipitation assay results, we found that the Sp1 may be a genuine transcription factor, which binds to the GC-box site of the XIAP promoter.

In our study, treatment with mithramycin A and TRAIL did not enhance in apoptosis in normal human mesangial cells, whereas the same treatment significantly induced apoptosis in various types of cancer cells. Moreover, overexpression of Bcl-2 did not block apoptosis induced by combined mithramycin A/TRAIL treatment. Therefore, cotreatment with mithramycin A and TRAIL is a potentially safe and attractive treatment strategy against intractable human malignant cancer cells, particularly in cases where resistance to apoptosis induced by anticancer drugs is due to overexpression of Bcl-2. In addition, the ability of mithramycin A to reduce the apoptotic threshold targeting XIAP suggests its potential applicability as a chemosensitizer or radiosensitizer in the treatment of various human malignant cancer cells, although extensive *in vitro* and *in vivo* studies will be required.

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