

Andrographolide sensitizes cancer cells to TRAIL-induced apoptosis via p53-mediated death receptor 4 up-regulation

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is an important member of the tumor necrosis factor subfamily with great potential in cancer therapy. Andrographolide (Andro), a diterpenoid lactone isolated from a traditional herbal medicine *Andrographis paniculata*, is known to possess potent anti-inflammatory and anticancer activities. Here, we showed that pretreatment with Andro significantly enhances TRAIL-induced apoptosis in various human cancer cell lines, including those TRAIL-resistant cells. Such sensitization is achieved through transcriptional up-regulation of death receptor 4 (DR4), a death receptor of TRAIL. In search of the molecular mechanisms responsible for DR4 up-regulation, we found that the tumor suppressor p53 plays an essential role in DR4 transcriptional activation. Andro is capable of activating p53 via increased p53 phosphorylation and protein stabilization, a process mediated by enhanced reactive oxygen species production and subsequent c-Jun NH₂-terminal kinase activation. Pretreatment with an antioxidant (*N*-acetylcysteine) or a c-Jun NH₂-terminal kinase inhibitor (SP600125) effectively prevented Andro-induced p53 activation and DR4 up-regulation and eventually blocked the Andro-induced sensitization on TRAIL-induced apoptosis. Taken together, these results present a novel anticancer effect of Andro and support its potential application in cancer therapy to overcome TRAIL resistance. [Mol Cancer Ther 2008; 7(7):2170–80]

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Introduction

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor subfamily. TRAIL is a promising anticancer agent for its ability to selectively induce apoptosis in tumor cells but not in normal cells (1, 2). TRAIL-based therapies are now in phase I and II clinical trials (3). TRAIL induces apoptosis through recognizing and binding to its cognate death receptors, DR4 and DR5 (also named as TRAIL-R1 and TRAIL-R2), on the cell surface (2, 3). This ligand binding initiates receptor conformational changes and formation of a death-inducing signaling complex by further recruiting an adaptor molecule Fas-associated death domain and caspase-8/10, which consequently results in the autoactivation of caspase-8/10 to trigger the caspase cascade and eventually leads to apoptosis. However, the anticancer application of TRAIL is unfortunately shadowed by the fact that some types of cancer cells are resistant to TRAIL-induced cell death (4, 5). This resistance is generally conferred by a few molecular changes, such as lower expression of cell surface death receptors, and/or higher expression of antiapoptotic molecules [e.g., decoy receptors, FLICE-like inhibitory protein (FLIP) and X-linked inhibitors of apoptosis proteins (XIAP); refs. 6, 7]. Thus, identification of sensitizing agents capable of overcoming such resistance and establishment of TRAIL-based combination regimens may facilitate an improved treatment of TRAIL-resistant cancers. Several sensitizing agents have been reported, including histone deacetylase inhibitors (8), cyclin-dependent kinase inhibitors (9), proteasome inhibitors (10), and Myc oncoprotein and the Raf kinase inhibitor (11). Previous work in our laboratory has also identified several phytochemicals to be effective in overcoming TRAIL resistance, such as luteolin by degradation of XIAP (12) and 3,3'-diindolylmethane by down-regulation of FLIP (13).

Andrographolide (Andro) is a diterpenoid lactone isolated from a traditional herbal medicine *Andrographis paniculata*. This compound is known to possess strong anti-inflammatory activity mainly via its inhibitory effect on nuclear transcription factor nuclear factor- κ B (14). We have reported previously that Andro is capable of using the death receptor–mediated apoptotic pathway to induce apoptosis in human cancer cells (15). In the present study, we show that Andro sensitizes TRAIL-induced apoptosis in TRAIL-resistant human cancer cells. Such sensitization effect is achieved through p53-dependent transcriptional up-regulation of DR4, a process mediated by several sequential events including reactive oxygen species (ROS) production, c-Jun NH₂-terminal kinase (JNK) activation, p53 phosphorylation, and stabilization. Results from this study thus present a novel function of Andro as a potent sensitizer for TRAIL-induced apoptotic cell death.

Materials and Methods

Chemicals, Reagents, and Antibodies

Andro, 4',6-diamidino-2-phenylindole (DAPI), and other common chemicals were all purchased from Sigma-Aldrich. Human recombinant TRAIL (carrier free) was from R&D Systems. Pan-caspase inhibitor Z-VAD-FMK, caspase-8 inhibitor Z-IETD-CHO, and caspase-3 inhibitor Z-DEVD-CHO were from Biomol. JNK inhibitor SP600125 was from Calbiochem. Trizol RNA extraction kit and LipofectAMINE 2000 transfection reagent were from Invitrogen. Anti-p53, anti-phosphorylated p53 (Thr^{S1} and Ser²⁰), anti-caspase-3, anti-caspase-8, anti-survivin, anti-Bcl-2, anti-Bcl-xL, and anti-Mcl-1 antibodies were from Cell Signaling. The anti-XIAP and anti-poly(ADP-ribose) polymerase (PARP) antibody were from BD Pharmingen. Anti-DR4, anti-Dcr1, anti-Dcr2, anti-c-IAP1, anti-c-IAP2, and anti- α -tubulin antibodies were from Santa Cruz Biotechnology. Anti-DR5 antibody was from Chemicon. Anti-FLIP antibody, anti-DR4 and anti-DR5 antibodies used for receptor blockage were purchased from Alexis. Antibodies against DR4 and DR5 used for flow cytometry were from eBioscience. The secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG and rabbit anti-goat IgG) and the enhanced chemiluminescence substrate were from Pierce.

Cell Culture and Treatments

Human liver cancer cells HepG2 and Hep3B, human cervical cancer cells HeLa, and human colorectal cancer cells HCT116 were all obtained from the American Type Culture Collection. An isogenic pair of HCT116 colon cancer cell lines was kindly provided by Dr. Bert Vogelstein (Johns Hopkins University; ref. 16). All cell lines were maintained in DMEM (Sigma-Aldrich) supplemented with 100 units/mL penicillin, 100 μ g/mL streptomycin, and 10% fetal bovine serum (Hyclone) under standard incubator condition (37°C, 5% CO₂). Andro was dissolved in DMSO at 100 mmol/L as stock solution, and human recombinant TRAIL was prepared in PBS containing 0.1% bovine serum albumin at 50 mg/mL.

Detection of Apoptosis

Cells undergoing apoptosis were evaluated by DAPI staining for morphologic changes including chromatin condensation and nuclear shrinkage as reported previously (12). Briefly, after various designated treatments, medium was removed, and cells were fixed with 70% ethanol at room temperature for 10 min. This was followed by staining the fixed cells with 0.3 μ g/mL DAPI (in PBS) at room temperature for another 10 min and visualized under an inverted fluorescence microscope. The cell death was quantified by counting the percentage of cell with condensed nuclear among 200 randomly selected cells.

RNA Interference

For the RNA interference study, synthetic small interfering RNA (siRNA; scrambled siRNA and p53 siRNA) were from Qiagen. The cellular delivery of siRNA was carried out by using LipofectAMINE 2000 and optimized with various doses and post-transfection time and evaluated by Western blot experiment.

Immunoblot Analysis

For Western blot, equal amount of protein was fractionated on SDS-polyacrylamide gel in the Mini-PROTEAN II system (Bio-Rad) and blotted onto polyvinylidene difluoride membrane (Millipore). After blocking with 5% nonfat milk in TBST [10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween 20], the membrane was probed with various antibodies and developed with enhanced chemiluminescence (Pierce) using a Kodak Image Station (Kodak).

Measurement of Cell Surface Expression of Death Receptors

The DR4 and DR5 cell surface expression was determined by flow cytometry as described previously with minor modification (17). Briefly, after designated treatments, cells in six-well plates were collected with non-enzyme dissociation buffer (Sigma-Aldrich) and washed with PBS. Cells (5×10^5) were incubated with 100 μ L staining buffer containing saturating amounts of anti-DR4 or anti-DR5 antibody at room temperature for 30 min. After incubation, cells were washed twice with staining buffer and analyzed with flow cytometer (BD Biosciences).

Measurement of Intracellular ROS

Production of intracellular ROS was examined by CM-H₂DCFDA (Molecular Probe) as described previously (18). Cells were loaded with 10 μ mol/L CM-H₂DCFDA for 30 min in culture medium at 37°C followed with the treatments as indicated. The cells were collected, and the fluorescence intensity was analyzed using the flow cytometer.

RNA Extraction and Reverse Transcription-PCR

RNA extraction was carried out using a total RNA extraction kit (Trizol) following the instructions from the manufacturer. Total RNA (5 μ g) from each sample was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Promega). For PCR, the amplification reaction was carried out with 200 pmol of each primer, 200 μ mol/L of each deoxynucleotide triphosphates, and 0.5 units Taq DNA polymerase II (Promega). The reverse transcription-PCR primers used for glyceraldehyde-3-phosphate dehydrogenase, p53, DR4, and DR5 were based on our previous report (13). PCR products were size fractionated using 1.0% agarose gel and visualized by ethidium bromide staining.

Results

Andro Sensitizes TRAIL-Induced Apoptosis

We first tested the cytotoxicity of TRAIL in human cancer cells originated from various tissues, including human hepatoma cells HepG2, human cervical cancer cells HeLa, and colorectal cancer cells HCT116. As shown in Fig. 1A, these three cell lines show different sensitivity to TRAIL-induced cell death. HeLa and HCT116 cells are sensitive to as low as 2.5 ng/mL TRAIL. In contrast, HepG2 cells are rather resistant to TRAIL-induced apoptosis even at a much higher concentration (20 ng/mL). In contrast, Andro alone exhibits a similar dose-dependent cytotoxicity in both TRAIL-resistant cells and TRAIL-sensitive cells (IC₅₀, ~60 μ mol/L; Fig. 1B). To evaluate whether Andro

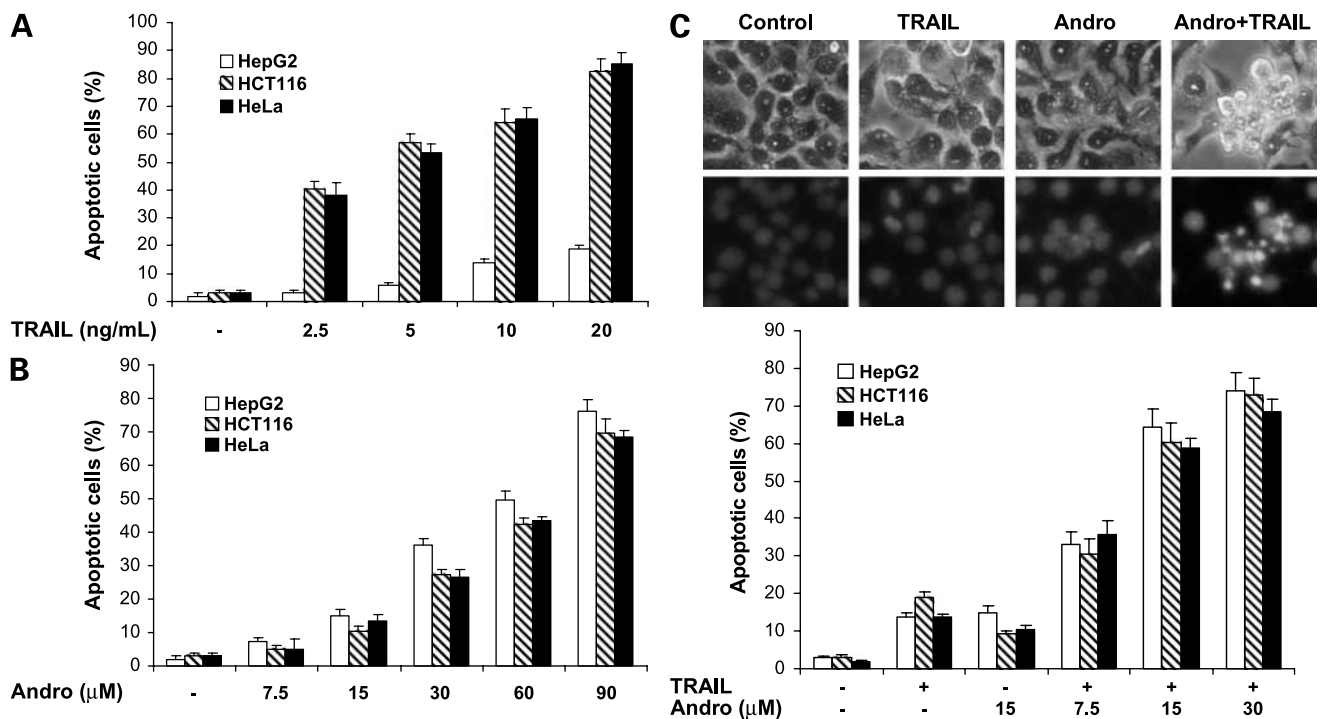


Figure 1. Andro sensitizes human cancer cells to TRAIL-induced apoptosis. **A**, TRAIL-induced apoptosis in human cancer cells. HepG2, HCT116, and HeLa cells were treated with indicated concentrations of TRAIL for 24 h. **B**, Andro-induced apoptosis in human cancer cells. Cancer cells were treated with indicated doses of Andro for 24 h. **C**, sensitization effect of Andro on TRAIL-induced apoptosis. HepG2, HCT116, and HeLa were pretreated with the indicated concentration of Andro for 2 h followed by treatment with a subtoxic concentration of TRAIL (10 ng/mL for HepG2 and 1 ng/mL for HCT116 and HeLa) for another 12 h. The percentage of apoptosis was determined using DAPI staining. Mean \pm SD of three independent experiments. Representative images of HepG2 cells with various treatments were photographed using a normal light microscope and an inverted fluorescence microscope. Magnification, $\times 200$.

could sensitize TRAIL-induced cell death, we treated three cell lines with the combination of Andro and TRAIL. As shown in Fig. 1C, when the cells were pretreated with Andro (7.5–30 μ mol/L) for 2 h followed by a low cytotoxic concentration of TRAIL for 12 h, all three cell lines tested underwent dramatic apoptotic cell death as evidenced by the typical apoptotic morphologic changes detected by DAPI staining. Similar results were obtained using other methods such as MTT assay (Supplementary Fig. S1A).³ In addition, we observed similar synergistic effects of Andro with lower concentrations of TRAIL on HepG2 cells (Supplementary Fig. S1B).³

Andro Promotes TRAIL-Induced Caspase Activation

It is well known that TRAIL-induced apoptosis is mainly executed by the extrinsic cell death receptor pathway, involving caspase-8 as the initiator and caspase-3 as the executor caspase (6). Here, we first examined the effect of Andro on TRAIL-initiated caspase cascade. As shown in Fig. 2A, subtoxic dose of Andro or TRAIL alone has no evident effect on the cleavage of caspase-8 and caspase-3. Notably, pretreatment with Andro significantly augmented TRAIL-induced cleavage/activation of caspase-8 and

caspase-3 as well as the cleavage of one of the caspase-3 substrates PARP (Fig. 2A). We next used various caspase inhibitors to confirm the role of caspase cascade in apoptotic cell death induced by Andro and TRAIL. As shown in Fig. 2B and C, the general caspase inhibitor (Z-VAD-FMK), caspase-8 inhibitor (Z-IETD-CHO), or caspase-3 inhibitor (Z-DEVD-CHO) are able to prevent PARP cleavage and apoptosis induced by the combined treatment of Andro and TRAIL. To further confirm the initiating function of caspase-8 activation in the apoptosis sensitized by Andro, we overexpressed a specific caspase-8 inhibitor protein (CrmA) in HepG2 cells. The overexpression of CrmA protein almost completely protected the cells from apoptotic cell death caused by Andro and TRAIL (data not shown). Therefore, it is believed that Andro enhances TRAIL-induced apoptosis mainly via promoting caspase-8 activation.

Andro Promotes FLIP-L Cleavage and XIAP Down-regulation as a Result of Enhanced Caspase Activation

To understand the underlying mechanism responsible for the sensitization effect of Andro on TRAIL-induced apoptosis, we systematically examined the involvement of various apoptosis regulatory proteins. Reestablishment of TRAIL-induced cell death in TRAIL-resistant cells is usually conveyed by the down-regulation of antiapoptotic molecules, including FLIP, inhibitor of apoptosis proteins

³ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

(XIAP, c-IAP1, and c-IAP2), Bcl-2, Bcl-xL, and Mcl-1 (11–13, 19, 20). As shown in Supplementary Fig. S2A,³ Andro significantly enhances TRAIL-induced cleavage of FLIP-L and reduction of XIAP protein level in HepG2 cells, whereas no evident changes are found for other apoptosis regulatory proteins. Moreover, ectopic expression of either FLIP-L or XIAP protected Andro-sensitized apoptosis (Supplementary Fig. S2B),³ indicating that FLIP-L or XIAP plays an important role in the sensitization effect of Andro on TRAIL-induced apoptosis. Interestingly, the pan-caspase inhibitor Z-VAD-FMK almost completely reversed the effect of Andro on FLIP-L cleavage and XIAP down-regulation (Supplementary Fig. S2C).³ It is thus believed that the enhanced FLIP-L cleavage and XIAP protein down-regulation lie downstream of the increased caspase activation as the result, but not the cause, of Andro-induced sensitization on TRAIL-induced apoptosis.

Andro Sensitizes TRAIL-Induced Apoptosis via DR4 Up-regulation

It has been well established that the decreased expression of TRAIL receptors DR4 and DR5 and/or up-regulation of the decoy receptors DcR1 and DcR2 account for TRAIL resistance in certain cancer cell lines (2, 6, 21). To determine the possible role of TRAIL receptors in Andro-sensitized apoptosis, we first detected the protein levels of various TRAIL receptors in cells treated with Andro and TRAIL. As shown in Fig. 3A, Andro treatment markedly increases the

expression of DR4 in HepG2 cells from 6 h onwards as detected by Western blot, whereas no evident changes were found for DR5, DcR1, and DcR2. To confirm the effect of Andro on DR4, we further detected the DR4 expression level at cell surface using flow cytometry. Consistently, Andro markedly increased the cell surface expression of DR4 but not DR5 (Fig. 3B). Similar to a previous report (22), it is noted that the basal level of DR5 is much higher than that of DR4 detected by either Western blot or flow cytometry (Fig. 3A and B).

To determine whether the increased DR4 protein level is due to enhanced gene transcription, we next measured the mRNA level of both DR4 and DR5 using RT-PCR (Fig. 3C). Consistent with the changes of the protein level, significant increase of DR4 mRNA level was observed from 3 h onwards, preceding the increase of DR4 protein level. Taken together, it is clear that Andro treatment selectively up-regulates DR4 expression at transcriptional level.

DR4 Up-regulation Is Critical for the Sensitization Effect of Andro

To further elucidate the critical role of DR4 in Andro-induced sensitization effects, we neutralized the function of TRAIL receptors by using DR4 or DR5 blocking antibody as reported previously (23). It is evident that DR4 blocking antibody is able to suppress caspase-8 cleavage (Fig. 3D) and protect against apoptotic cell death induced by the combined treatment of Andro and TRAIL (Fig. 3E). On

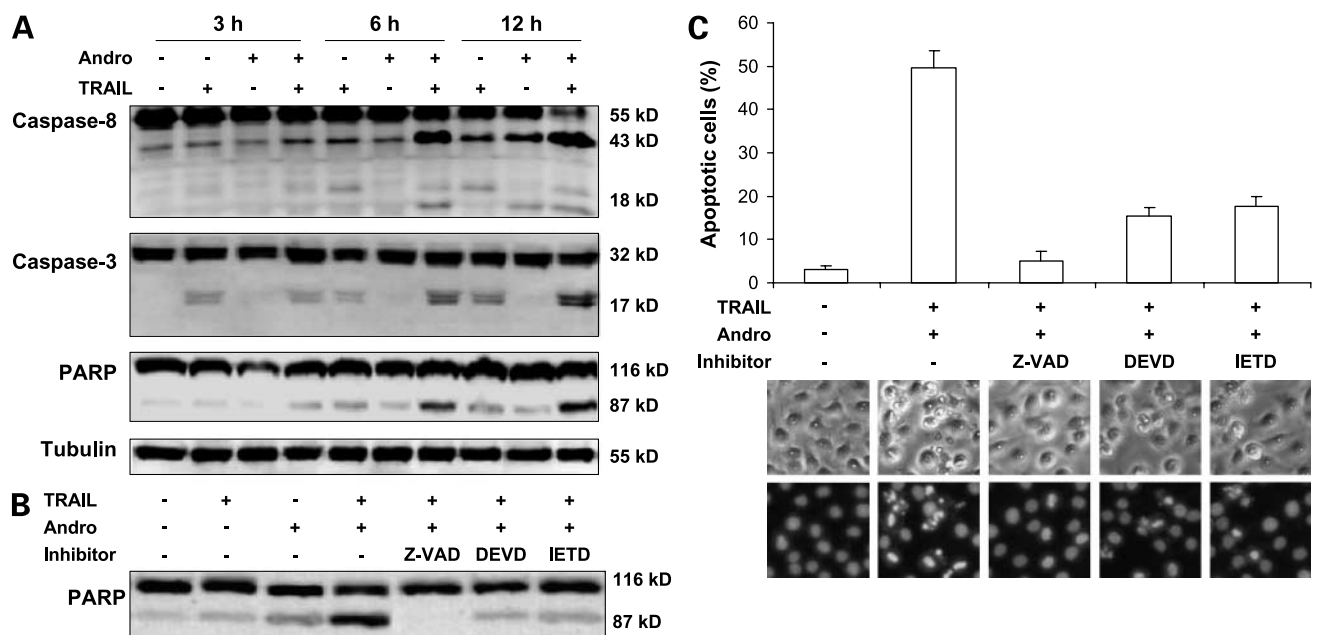


Figure 2. Andro enhances the caspase cascade triggered by TRAIL. **A**, Andro promotes TRAIL-induced caspase activation. HepG2 cells were pretreated with 15 $\mu\text{mol/L}$ Andro for 2 h followed by treatment with TRAIL (10 ng/mL) for indicated periods. Cell lysates were collected and subjected to Western blot for detecting the cleavage of caspase-8, caspase-3, and PARP. **B**, caspase inhibitors block PARP cleavage induced by Andro and TRAIL. **C**, caspase inhibitors block apoptosis induced by Andro and TRAIL. **B** and **C**, HepG2 cells were pretreated with Z-IETD-CHO (25 $\mu\text{mol/L}$), Z-DEVD-CHO (25 $\mu\text{mol/L}$), or Z-VAD-FMK (25 $\mu\text{mol/L}$) for 30 min followed by combined treatment of Andro (15 $\mu\text{mol/L}$) for 2 h and then TRAIL (10 ng/mL) for another 12 h. Cells were collected for measurement of PARP cleavage by Western blot (**B**) or determination of cell death using DAPI staining (**C**). Mean \pm SD of three independent experiments. Magnification, $\times 200$.

the contrary, these effects could not be detected after neutralization with the DR5 blocking antibody. These results thus strengthen our argument that DR4 but not DR5 plays a key role in the sensitization effect of Andro on TRAIL-induced apoptotic cell death.

p53 Is Required for the DR4 Up-regulation and Enhanced Apoptosis Induced by Andro

It has been well documented that p53 tumor suppressor is one of the main transcription factors regulating the

expression of both DR4 and DR5 (24–26). Here, we tested whether Andro-mediated DR4 transcriptional up-regulation is p53 dependent. We first showed that Andro treatment increases p53 protein level, which is coincident with the temporal change of DR4 protein level (Fig. 4A). Next, we manipulated the p53 expression by using specific siRNA against p53. As expected, knockdown of p53 resulted in the suppression of Andro-induced DR4 up-regulation (Fig. 4B) and consequently prevented

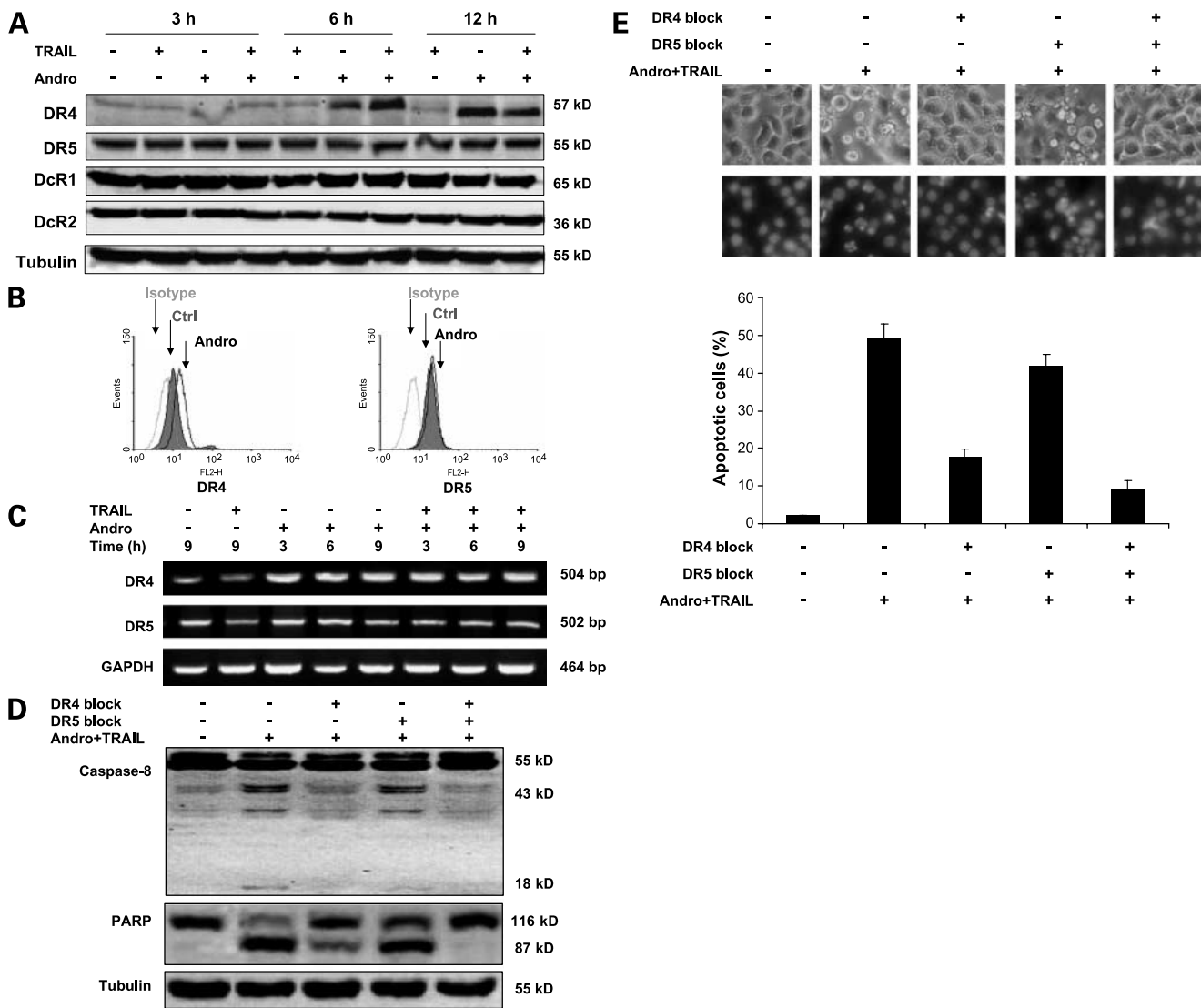


Figure 3. Andro up-regulates DR4 transcription. **A**, time-dependent up-regulation of DR4 protein level by Andro. HepG2 cells were treated with Andro (15 μmol/L) for 2 h followed by TRAIL (10 ng/mL) for indicated periods. Cell lysates were collected for Western blot to detect protein expression level of various TRAIL death receptors. **B**, effects of Andro on the cell surface expression of DR4 and DR5. HepG2 cells were incubated with Andro (15 μmol/L) for 6 h and the cell surface expression of DR4 and DR5 proteins was analyzed by flow cytometry. X axis, fluorescence intensity; Y axis, relative number of cells. Representative of three independent experiments. **C**, effects of Andro and TRAIL on DR4 and DR5 mRNA level. HepG2 cells were treated with Andro (15 μmol/L) for 2 h followed by TRAIL (10 ng/mL) for indicated periods. The mRNA levels of DR4 and DR5 were measured by reverse transcription-PCR. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. **D**, DR4 blocking antibody suppresses the cleavage of caspase-8 and PARP induced by Andro and TRAIL. HepG2 cells were pretreated with DR4 or DR5 blocking antibodies (10 μg/mL) for 30 min followed by pretreatment with Andro (15 μmol/L × 2 h) and then TRAIL (10 ng/mL) for another 12 h. At the end of treatment, cell lysates were collected for Western blot. **E**, DR4 blocking antibody prevents Andro-sensitized apoptosis. HepG2 cells were treated as indicated in **D**. At the end of treatment, cell death was determined using DAPI staining. Magnification, ×200.

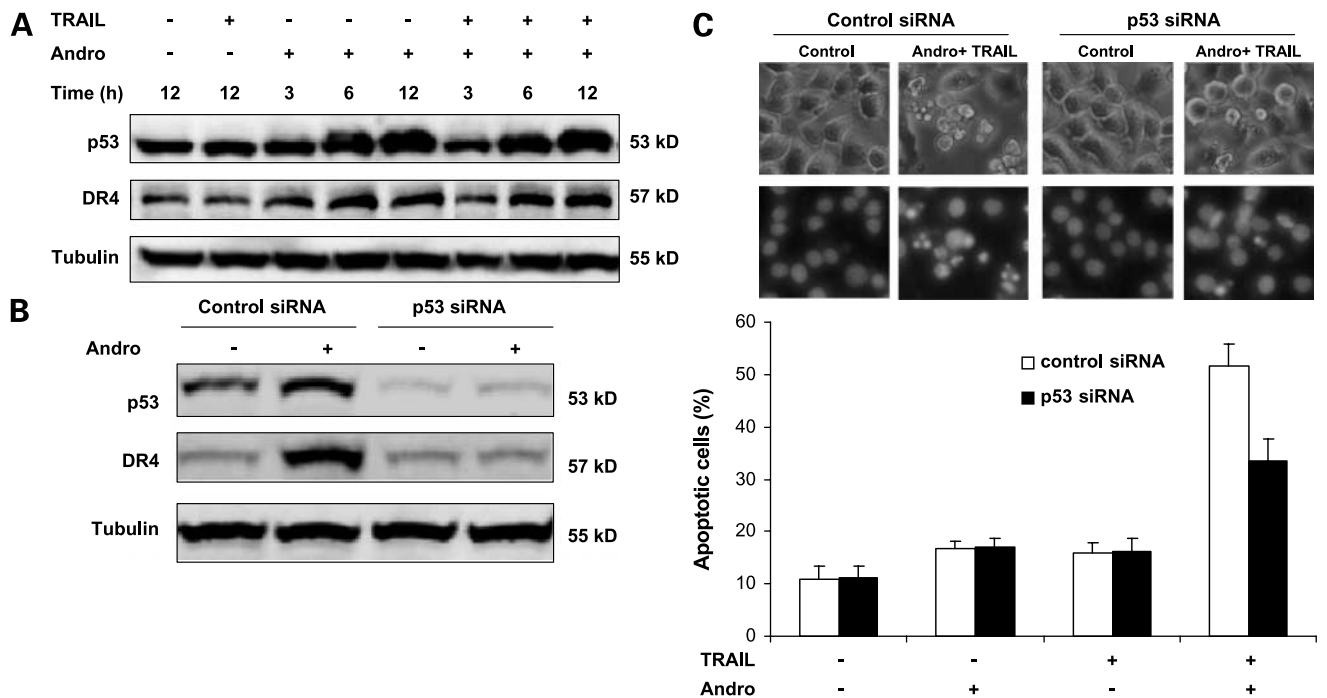


Figure 4. p53 is required for DR4 up-regulation and enhanced apoptosis by Andro. **A**, Andro promotes p53 protein accumulation. HepG2 cells were treated with Andro (15 $\mu\text{mol/L}$) for 2 h followed by TRAIL (10 ng/mL) for indicated periods. p53 and DR4 protein levels were detected by Western blot. **B**, p53 knockdown prevents Andro-induced DR4 up-regulation. HepG2 cells were transfected with scrambled siRNA or p53 siRNA for 24 h. Cells were treated with Andro (15 $\mu\text{mol/L}$) for 6 h. At the end of treatment, cells were collected for detection of p53 and DR4 by Western blot. **C**, p53 knockdown suppresses apoptosis induced by Andro and TRAIL. After p53 knockdown, HepG2 cells were treated with Andro (15 $\mu\text{mol/L}$) for 2 h followed by TRAIL (10 ng/mL) for 12 h. Cells were assessed using DAPI staining. Representative of three independent experiments. Magnification, $\times 200$.

Andro-sensitized apoptosis (Fig. 4C). These observations thus establish that p53 is required for Andro-induced DR4 up-regulation and sensitization to TRAIL-induced apoptosis in HepG2 cells.

To further confirm the critical role of p53 in Andro-sensitized apoptosis in TRAIL-treated cells, we selected two pairs of cancer cell lines with different genetic features of p53: p53 wild-type HepG2 versus p53 mutant Hep3B cells (27) and p53 wild-type HCT116 versus p53 knockout HCT116 cells (16). As shown in Fig. 5A, Andro plus TRAIL induce significant apoptosis in HepG2 cells but not in Hep3B cells. Similarly, the combined treatment causes obvious apoptotic death in HCT116 p53 wild-type cells but shows no effect in HCT116 p53 knockout cells (Fig. 5B). We next examined the DR4 and p21 expression levels in HCT116 cells treated with Andro. As expected, on Andro treatment, the dramatic enhancement of DR4 could be detected in HCT116 p53 wild-type but only slight increase in HCT116 p53 knockout cells (Fig. 5C), which is consistent to the change of p21, a well-established p53 target protein. Although p53 is a well-known transcriptional regulator of DR4 (24, 25), nuclear factor- κB (28), c-myc (29), and JAK/STAT pathway (30) also have been reported to be able to regulate the expression of DR4. Therefore, it is possible that other regulatory mechanisms may contribute to the slight up-regulation of DR4 in HCT116

p53 knockout cells. However, this slight up-regulation of DR4 is not sufficient to enhance the sensitivity of HCT116 p53 knockdown cells to TRAIL- and Andro-induced apoptosis (Fig. 5B). Therefore, it is believed that up-regulation of DR4 and sensitization of TRAIL-induced apoptosis by Andro are dependent on the presence of functional p53.

Andro Elevates p53 Expression through JNK-Mediated p53 Thr⁸¹ Phosphorylation

Activation of p53 may result from either the increased transcriptional level or post-translational modification and stabilization (31). In this study, Andro did not affect the mRNA level of p53 (Supplementary Fig. S3A).³ Consistently, it was found that, in the presence of cycloheximide, a protein synthesis inhibitor, Andro significantly prolongs the half-life of p53 from 30 min to ≥ 2 h (Supplementary Fig. S3B),³ suggesting that accumulation of p53 protein by Andro is most probably due to its post-transcriptional regulation.

It has been well established that p53 protein level is mainly regulated via the ubiquitination and proteasome pathway, a process closely associated with the phosphorylation status of p53 (32, 33). We next examined the pattern of p53 phosphorylation in response to Andro treatment. Notably, dramatic p53 phosphorylation at Thr⁸¹ site was detected on Andro treatment, with concurrent increase

of p53 and DR4 protein levels (Supplementary Fig. S3C).³ On the contrary, we failed to detect any significant changes at the other phosphorylation sites, such as Ser²⁰, in Andro-treated cells, in contrast to the effect of etoposide, a DNA-damaging agent (Supplementary Fig. S3C).³ It has been documented that p53 Thr⁸¹ is specifically phosphorylated by JNK in response to DNA damage and stress-inducing agents (34). More importantly, JNK phosphorylation of p53 on Thr⁸¹ is important for p53 stabilization and transcriptional activities as a stress response (35). To assess the involvement of JNK in the activation of p53 by Andro, we determined the activation of JNK in Andro-treated cells. As shown in Fig. 6A, Andro promotes JNK phosphorylation in a temporal pattern consistent with that of p53 phosphorylation and stabilization and subsequent DR4 up-regulation.

JNK is readily activated by ROS (36) and separate reports showed that Andro triggers cellular accumulation of ROS as well as JNK activation (37, 38). We thus

examined whether ROS plays a role in Andro-induced JNK activation. The DCF assay revealed that the intracellular ROS level increased significantly following the treatment with Andro in both time-dependent and dose-dependent patterns (Supplementary Fig. S4A),³ a process that could be completely blocked by an antioxidant *N*-acetylcysteine (NAC; Supplementary Fig. S4B).³ Next, we attempted to determine the role of Andro-induced ROS in the sequential events of JNK activation, p53 protein phosphorylation/stabilization, and DR4 up-regulation. Pretreatment with NAC or JNK inhibitor (SP600125) led to significant inhibition of JNK activation and p53 phosphorylation (Fig. 6B). Moreover, pretreatments with NAC or SP600125 also completely blocked the up-regulation of DR4 mRNA and protein level (Fig. 6C and D). As a result, both reagents effectively abolished the sensitization effect of Andro on TRAIL-induced apoptosis in HepG2 cells (Fig. 6E). Taken together, the above results indicate that ROS-mediated

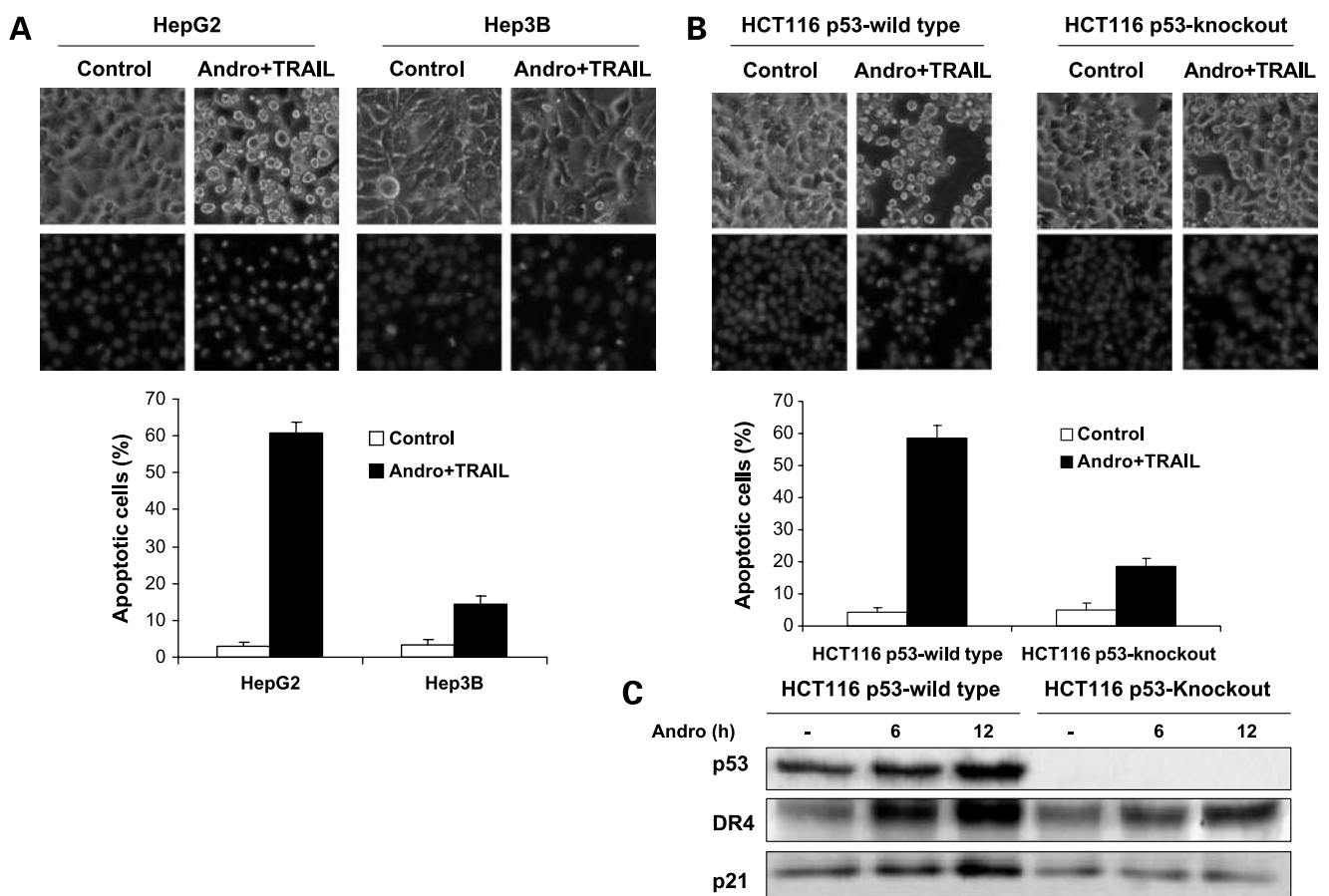


Figure 5. Andro sensitizes TRAIL-induced apoptosis in p53 wild-type cancer cells but not in p53-deficient cancer cells. **A**, HepG2 and Hep3B cells were pretreated with Andro (15 μ mol/L) for 2 h followed by TRAIL (10 ng/mL) for another 12 h. The percentage of apoptosis was determined using DAPI staining. Mean \pm SD of three independent experiments. Magnification, $\times 200$. **B**, HCT116 p53 wild-type cells and HCT116 p53 knockout cells were pretreated with Andro (15 μ mol/L) for 2 h followed by TRAIL (1 ng/mL) for another 12 h. The percentage of apoptosis was determined using DAPI staining. Mean \pm SD of three independent experiments. Magnification, $\times 200$. **C**, Andro fails to enhance DR4 level in HCT116 p53 knockout cells. HCT116 p53 wild-type cells and HCT116 p53 knockout cells were treated with Andro (15 μ mol/L) for 6 h or 12 h. At the end of treatment, cells were collected for detection of p53, DR4, and p21 using Western blot.

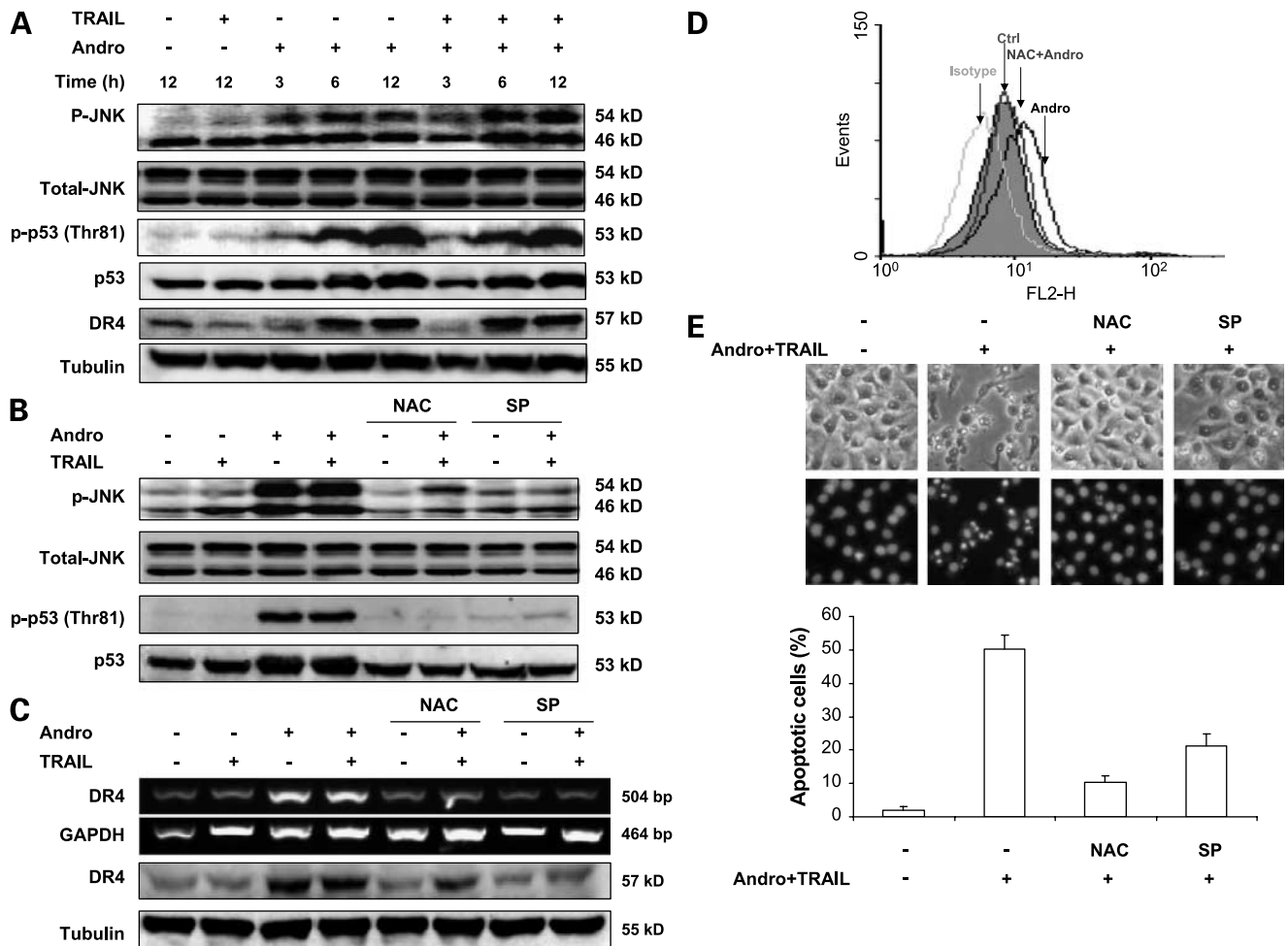


Figure 6. Andro promotes JNK activation, p53 stabilization, and DR4 up-regulation in ROS-dependent manner. **A**, Andro induces JNK activation. HepG2 cells were treated with Andro (15 $\mu\text{mol/L}$) for 2 h followed by TRAIL (10 ng/mL) for indicated periods. At the end of treatment, cell lysates were collected for the detection of protein level of phospho-JNK, total JNK, phospho-p53 Thr⁸¹, p53, DR4, and α -tubulin using Western blot. **B**, NAC and SP600125 abrogate Andro-induced JNK activation, p53 phosphorylation, and stabilization. HepG2 cells were pretreated with NAC (2.5 mmol/L for 30 min) or SP600125 (20 $\mu\text{mol/L}$ for 30 min) followed by the treatment of Andro (15 $\mu\text{mol/L}$ \times 2 h) and then TRAIL (10 ng/mL) for another 6 h. **C**, NAC and SP600125 prevent Andro-induced DR4 up-regulation. Cells were first pretreated with NAC and SP600125 as described in **B** followed by Andro (15 $\mu\text{mol/L}$ \times 2 h) and then TRAIL (10 ng/mL) for another 3 h (for detection of DR4 mRNA level) and 6 h (for detection of DR4 protein level). **D**, NAC prevents Andro-induced cell surface expression of DR4. HepG2 cells were pretreated with NAC as described above followed by Andro (15 $\mu\text{mol/L}$) for 6 h. The cell surface expression of DR4 was measured using flow cytometry as described in **B**. Representative of three independent experiments. **E**, NAC and SP600125 inhibit apoptosis induced by Andro and TRAIL. HepG2 cells were treated with Andro (15 $\mu\text{mol/L}$ \times 2 h) followed by TRAIL (10 ng/mL) for another 12 h. The percentage of apoptosis was measured with DAPI staining. Mean \pm SD of three independent experiments. Magnification, \times 200.

JNK activation by Andro accounts for the sensitization effect of Andro on TRAIL-induced apoptosis via sequential events including p53 phosphorylation/stabilization and DR4 up-regulation.

Discussion

The unique property of triggering apoptosis in a variety of human cancer cells while sparing normal cells makes TRAIL a highly promising cancer therapeutic agent. However, TRAIL resistance is a major limitation in its clinical application as a cancer therapeutic agent. One effective strategy to overcome this obstacle is to combine TRAIL with other anticancer agents (39). Several chemical

compounds have been identified as effective sensitizing agents to TRAIL-induced apoptosis, including some natural products (8–10, 12, 13). Results from this study provide substantial evidence that Andro, a diterpenoid lactone isolated from a traditional medicinal herb, is capable of sensitizing TRAIL-induced apoptosis in TRAIL-resistant cancer cells. Thus, this study presents a novel anticancer effect of Andro and supports its potential application in cancer therapy.

Andro Sensitizes TRAIL-Induced Apoptosis by Up-regulation of DR4

The expression level of death receptors (DR4 and DR5) plays a critical role in determining the cell fate in response

to TRAIL (2, 6, 21). Numerous literatures have shown convincing data that the up-regulation of DR4 and DR5 could sensitize TRAIL-resistant cells to TRAIL-induced cell death (22, 40). In this study, it is believed that Andro-induced sensitization on TRAIL-induced apoptosis is mainly executed via modulation of DR4, but not DR5, based on the following observations. First, Andro selectively enhanced the expression level of DR4 in HepG2 cells (Fig. 3A and B). Second, the administration of blocking antibody of DR4, but not DR5, effectively protected the cell death induced by combination of Andro and TRAIL (Fig. 3D and E), further confirming that up-regulation of DR4 is functionally important in Andro-sensitized apoptosis.

At present, there are inconsistent reports whether DR4 or DR5 is the main mediator for TRAIL-mediated apoptosis in cancer cells. It has been well reported that, in chronic lymphocytic leukemia cells, DR4 seems to be the principal receptor in response to TRAIL in the presence of histone deacetylase inhibitors (41–43). Suppression of DR4 expression or function by homozygous deletion or epigenetic silencing led to TRAIL resistance (44, 45). Recently, Kurbanov et al. used an established cell culture model for TRAIL resistance and regained TRAIL sensitivity and indicated that significant down-regulation of initiator caspase and DR4 was characteristic for TRAIL-resistant melanoma cells (46). Additionally, another recent report suggested that reactivation of DR4 expression contributed to restore sensitivity of melanoma cells to IFN- β or Apo2L/TRAIL (47). Moreover, Aza-Blanc et al. reported that a siRNA targeting DR4 was effective in blocking TRAIL-induced apoptosis, whereas knockdown of DR5 failed to confer any protection (48). In this regard, DR4 represents the major determinant of TRAIL sensitivity in certain cancer cells. On the other hand, there is evidence that DR5 may play a more prominent role than DR4 in mediating apoptotic signals emanating from TRAIL in cells expressing both death receptors (49) and TRAIL binds with a higher affinity to DR5 than to DR4 in some cells (50). One possible explanation for such inconsistent results might be the different expression levels of these two receptors in different types of cancer cells. In line with some previous studies (22), we found that in HepG2 cells DR5 is expressed in a much higher basal level but is more insensitive to regulatory mechanisms. In contrast, DR4 with a very low basal level is susceptible of being up-regulated by p53. Such a difference may well explain the fact that it is DR4 that is responsible for the sensitization effect of Andro to TRAIL-induced apoptosis.

Andro Up-regulates DR4 via p53-Dependent Transcriptional Regulation

The tumor suppressor gene p53 is a key apoptosis regulator in cancer cells and one of its proapoptosis functions is achieved via transcriptional activation of death receptors (24, 25). In the present study, we found that p53 played a critical role in Andro-sensitized apoptosis through up-regulation of DR4, owing to the following findings. First, RNA interference of p53 could effectively inhibit Andro-induced DR4 up-regulation (Fig. 4B) and abolish the

apoptotic cell death induced by the combined treatment of Andro and TRAIL (Fig. 4C). Second, evident apoptotic cell death and DR4 up-regulation were only observed in p53 wild-type cancer cells but not in p53-deficient cells (Fig. 5A and B). It is thus believed that Andro is capable of enhancing TRAIL-mediated cancer cell apoptosis via activating p53-dependent DR4 transcription.

The involvement of p53 in control of DR4 and DR5 transcription remains contradictory. Original work mainly by El-Deiry's group identified DR5 as the main target of p53 as the molecular link between the apoptotic pathways mediated by DNA damage and cell death receptors (51, 52). On the other hand, there is convincing evidence showing that DNA damage-induced p53 activation also leads to DR4 up-regulation (24, 25). It is likely that p53 is important for both DR4 and DR5 depending on the context including the nature of stimuli and the cell type. It remains to be further tested whether p53-mediated DR4 up-regulation is also applicable to other TRAIL-resistant cancer cells. One implication of such findings is that the therapeutic value of TRAIL is at least partly dependent on functional p53 in cancer cells and the sensitization effect of Andro may only be achievable in p53 wild-type cancer cells.

Andro Stabilizes p53 Protein via Oxidative Stress and JNK Activation

It has been well established that p53 activity is mainly regulated through complex networks of post-translational modifications, including phosphorylation, ubiquitination, and proteasome degradation (32, 33). The phosphorylation status of p53 directly affects MDM2 binding and subsequent ubiquitination and proteasome degradation. At present, more than a dozen phosphorylation sites have been mapped on p53, but only a few have been characterized with respect to the phosphorylating kinase and the relevance to p53 activities (32). Among them, activation of JNK has been linked with p53 Thr⁸¹ phosphorylation in response to DNA damage and stress-inducing agents (34). Consistently, we observed p53 phosphorylation at Thr⁸¹, but not Ser²⁰ in response to Andro treatment, in contrast to the effect of etoposide, a DNA-damaging agent. Meanwhile, it is intriguing to note that no up-regulation of DR4 was found in cells treated with etoposide despite the equally enhanced total p53 protein level (Supplementary Fig. S3C).³ The mechanism underlying such a discrepancy remains to be further investigated. More importantly, pretreatment of SP600125, a specific JNK inhibitor, suppressed Andro-induced JNK activation and thus abrogated p53 phosphorylation, total p53 protein accumulation, DR4 up-regulation, and subsequent cell death induced by combined treatment of Andro and TRAIL (Fig. 6). Collectively, our data reveal a sequence of events elicited by Andro, from ROS production to JNK activation, p53 phosphorylation, and stabilization to DR4 up-regulation and eventually enhanced cell death.

In conclusion, this study shows a novel anticancer effect of Andro: Andro is capable of activating p53 function via ROS-dependent JNK activation, leading to DR4 up-regulation and sensitization to TRAIL-induced apoptosis in

TRAIL-resistant cancer cells. Data from this study support the further development of Andro as a chemosensitizer in combined therapy with TRAIL to overcome TRAIL resistance in p53 wild-type cancers.

Disclosure of Potential Conflicts of Interest

The other authors disclosed no potential conflicts of interest.

References

- Hall MA, Cleveland JL. Clearing the TRAIL for cancer therapy. *Cancer Cell* 2007;12:4–6.
- Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* 2003;22:8628–33.
- Huang Y, Sheikh MS. TRAIL death receptors and cancer therapeutics. *Toxicol Appl Pharmacol* 2007;224:284–9.
- Duiker EW, Mom CH, de Jong S, et al. The clinical trail of TRAIL. *Eur J Cancer* 2006;42:2233–40.
- Dyer MJ, MacFarlane M, Cohen GM. Barriers to effective TRAIL-targeted therapy of malignancy. *J Clin Oncol* 2007;25:4505–6.
- LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* 2003;10:66–75.
- Shankar S, Srivastava RK. Enhancement of therapeutic potential of TRAIL by cancer chemotherapy and irradiation: mechanisms and clinical implications. *Drug Resist Updat* 2004;7:139–56.
- Inoue S, MacFarlane M, Harper N, Wheat LM, Dyer MJ, Cohen GM. Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies. *Cell Death Differ* 2004;11 Suppl 2:S193–206.
- Palacios C, Yerbes R, Lopez-Rivas A. Flavopiridol induces cellular FLICE-inhibitory protein degradation by the proteasome and promotes TRAIL-induced early signaling and apoptosis in breast tumor cells. *Cancer Res* 2006;66:8858–69.
- Ganten TM, Koschny R, Haas TL, et al. Proteasome inhibition sensitizes hepatocellular carcinoma cells, but not human hepatocytes, to TRAIL. *Hepatology* 2005;42:588–97.
- Ricci MS, Kim SH, Ogi K, et al. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell* 2007;12:66–80.
- Shi RX, Ong CN, Shen HM. Protein kinase C inhibition and X-linked inhibitor of apoptosis protein degradation contribute to the sensitization effect of luteolin on tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in cancer cells. *Cancer Res* 2005;65:7815–23.
- Zhang S, Shen HM, Ong CN. Down-regulation of c-FLIP contributes to the sensitization effect of 3,3'-diindolylmethane on TRAIL-induced apoptosis in cancer cells. *Mol Cancer Ther* 2005;4:1972–81.
- Xia YF, Ye BQ, Li YD, et al. Andrographolide attenuates inflammation by inhibition of NF- κ B activation through covalent modification of reduced cysteine 62 of p50. *J Immunol* 2004;173:4207–17.
- Zhou J, Zhang S, Ong CN, Shen HM. Critical role of pro-apoptotic Bcl-2 family members in andrographolide-induced apoptosis in human cancer cells. *Biochem Pharmacol* 2006;72:132–44.
- Bunz F, Dutriaux A, Lengauer C, et al. Requirement for p53 and p21 to sustain G₂ arrest after DNA damage. *Science* 1998;282:1497–501.
- Earell JK, Jr., VanOosten RL, Griffith TS. Histone deacetylase inhibitors modulate the sensitivity of tumor necrosis factor-related apoptosis-inducing ligand-resistant bladder tumor cells. *Cancer Res* 2006;66:499–507.
- Lu GD, Shen HM, Chung MC, Ong CN. Critical role of oxidative stress and sustained JNK activation in aloe-emodin-mediated apoptotic cell death in human hepatoma cells. *Carcinogenesis* 2007;28:1937–45.
- Mirandola P, Sponzilli I, Gobbi G, et al. Anticancer agents sensitize osteosarcoma cells to TNF-related apoptosis-inducing ligand downmodulating IAP family proteins. *Int J Oncol* 2006;28:127–33.
- Taniai M, Grambihler A, Higuchi H, et al. Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. *Cancer Res* 2004;64:3517–24.
- Takeda K, Stagg J, Yagita H, Okumura K, Smyth MJ. Targeting death-inducing receptors in cancer therapy. *Oncogene* 2007;26:3745–57.
- Galligan L, Longley DB, McEwan M, Wilson TR, McLaughlin K, Johnston PG. Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. *Mol Cancer Ther* 2005;4:2026–36.
- Sprick MR, Rieser E, Stahl H, Grosse-Wilde A, Weigand MA, Walczak H. Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. *EMBO J* 2002;21:4520–30.
- Liu X, Yue P, Khuri FR, Sun SY. p53 upregulates death receptor 4 expression through an intronic p53 binding site. *Cancer Res* 2004;64:5078–83.
- Guan B, Yue P, Clayman GL, Sun SY. Evidence that the death receptor DR4 is a DNA damage-inducible, p53-regulated gene. *J Cell Physiol* 2001;188:98–105.
- El-Deiry WS. Insights into cancer therapeutic design based on p53 and TRAIL receptor signaling. *Cell Death Differ* 2001;8:1066–75.
- Bressac B, Galvin KM, Liang TJ, Isselbacher KJ, Wands JR, Ozturk M. Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1990;87:1973–7.
- Jin F, Liu X, Zhou Z, et al. Activation of nuclear factor- κ B contributes to induction of death receptors and apoptosis by the synthetic retinoid CD437 in DU145 human prostate cancer cells. *Cancer Res* 2005;65:6354–63.
- Sussman RT, Ricci MS, Hart LS, Sun SY, El-Deiry WS. Chemotherapy-resistant side-population of colon cancer cells has a higher sensitivity to TRAIL than the non-SP, a higher expression of c-Myc and TRAIL-receptor DR4. *Cancer Biol Ther* 2007;6:1490–5.
- Tanaka F, Kawakami A, Tamai M, et al. IFN- γ /JAK/STAT pathway-induced inhibition of DR4 and DR5 expression on endothelial cells is cancelled by cycloheximide-sensitive mechanism: novel finding of cycloheximide-regulating death receptor expression. *Int J Mol Med* 2005;15:833–9.
- Ashcroft M, Taya Y, Vousden KH. Stress signals utilize multiple pathways to stabilize p53. *Mol Cell Biol* 2000;20:3224–33.
- Brooks CL, Gu W. p53 ubiquitination: Mdm2 and beyond. *Mol Cell* 2006;21:307–15.
- Ashcroft M, Vousden KH. Regulation of p53 stability. *Oncogene* 1999;18:7637–43.
- Wu GS. The functional interactions between the p53 and MAPK signaling pathways. *Cancer Biol Ther* 2004;3:156–61.
- Buschmann T, Potapova O, Bar-Shira A, et al. Jun NH₂-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress. *Mol Cell Biol* 2001;21:2743–54.
- Shen HM, Liu ZG. JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. *Free Radic Biol Med* 2006;40:928–39.
- Li J, Cheung HY, Zhang Z, Chan GK, Fong WF. Andrographolide induces cell cycle arrest at G₂/M phase and cell death in HepG2 cells via alteration of reactive oxygen species. *Eur J Pharmacol* 2007;568:31–44.
- Ji L, Liu T, Liu J, Chen Y, Wang Z. Andrographolide inhibits human hepatoma-derived Hep3B cell growth through the activation of c-Jun N-terminal kinase. *Planta Med* 2007;73:1397–401.
- Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. *Cancer Gene Ther* 2005;12:228–37.
- Jin Z, McDonald ER III, Dicker DT, El-Deiry WS. Deficient tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor transport to the cell surface in human colon cancer cells selected for resistance to TRAIL-induced apoptosis. *J Biol Chem* 2004;279:35829–39.
- MacFarlane M, Inoue S, Kohlhaas SL, et al. Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. *Cell Death Differ* 2005;12:773–82.
- Natoni A, Macfarlane M, Inoue S, et al. TRAIL signals to apoptosis in

chronic lymphocytic leukaemia cells primarily through TRAIL-R1 whereas cross-linked agonistic TRAIL-R2 antibodies facilitate signalling via TRAIL-R2. *Br J Haematol* 2007;139:568–77.

43. MacFarlane M, Kohlhaas SL, Sutcliffe MJ, Dyer MJ, Cohen GM. TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. *Cancer Res* 2005;65:11265–70.

44. Ozoren N, Fisher MJ, Kim K, et al. Homozygous deletion of the death receptor DR4 gene in a nasopharyngeal cancer cell line is associated with TRAIL resistance. *Int J Oncol* 2000;16:917–25.

45. Horak P, Pils D, Haller G, et al. Contribution of epigenetic silencing of tumor necrosis factor-related apoptosis inducing ligand receptor 1 (DR4) to TRAIL resistance and ovarian cancer. *Mol Cancer Res* 2005;3:335–43.

46. Kurbanov BM, Fecker LF, Geilen CC, Sterry W, Eberle J. Resistance of melanoma cells to TRAIL does not result from upregulation of antiapoptotic proteins by NF- κ B but is related to downregulation of initiator caspases and DR4. *Oncogene* 2007;26:3364–77.

47. Bae SI, Cheriya V, Jacobs BS, Reu FJ, Borden EC. Reversal of methylation silencing of Apo2L/TRAIL receptor 1 (DR4) expression

overcomes resistance of SK-MEL-3 and SK-MEL-28 melanoma cells to interferons (IFNs) or Apo2L/TRAIL. *Oncogene* 2007;27:490–8.

48. Aza-Blanc P, Cooper CL, Wagner K, Batalov S, Devereaux QL, Cooke MP. Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. *Mol Cell* 2003;12:627–37.

49. Kelley RF, Totpal K, Lindstrom SH, et al. Receptor-selective mutants of apoptosis-inducing ligand 2/tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR) 5 than DR4 to apoptosis signaling. *J Biol Chem* 2005;280:2205–12.

50. Truneh A, Sharma S, Silverman C, et al. Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. *J Biol Chem* 2000;275:23319–25.

51. Wu GS, Kim K, El-Deiry WS. KILLER/DR5, a novel DNA-damage inducible death receptor gene, links the p53-tumor suppressor to caspase activation and apoptotic death. *Adv Exp Med Biol* 2000;465:143–51.

52. Takimoto R, El-Deiry WS. Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. *Oncogene* 2000;19:1735–43.