Ursolic Acid, a Pentacyclin Triterpene, Potentiates TRAIL-induced Apoptosis through p53-independent Up-regulation of Death Receptors

Evidence for the Role of Reactive Oxygen Species and JNK

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Discovery of the molecular targets of traditional medicine and its chemical fingerprints can validate the use of such medicine. In the present report, we investigated the effect of ursolic acid (UA), a pentacyclic triterpenoid found in rosemary and holy basil, on apoptosis induced by TRAIL. We found that UA potentiated TRAIL-induced apoptosis in cancer cells. In addition, UA also sensitized TRAIL-resistant cancer cells to the cytokine. When we investigated the mechanism, we found that UA down-regulated cell survival proteins and induced the cell surface expression of both TRAIL receptors, death receptors 4 and 5 (DR4 and DR5). Induction of receptors by UA occurred independently of cell type. Gene silencing of either receptor by small interference RNA significantly decreased the expression of DcR1. Induction of both receptors by UA was p53-independent because UA induced DR4 and DR5 in p53-null cells. Down-regulation of survival proteins and up-regulation of the receptors required reactive oxygen species (ROS) and JNK-mediated up-regulation of the receptors. The down-regulation of survival proteins and DRs, however, was dependent on JNK because UA induced by UA decreased the expression of decoy receptor 2 (DcR2) but not DcR1. Induction of DRs was independent of p53 because UA induced by UA and the effect of TRAIL. In addition, UA also sensitized TRAIL-resistant cancer cells to UA potentiated TRAIL-induced apoptosis in cancer cells. In summary, our results showed that UA potentiates TRAIL-induced apoptosis in cancer cells in a nude mouse model. Overall, our results to induce the expression of DRs, down-regulate cell survival quenchers and JNK inhibitor. In addition, UA was also found to induce the expression of DRs, down-regulate cell survival proteins, and activate JNK in orthotopically implanted human colorectal cancer in a nude mouse model. Overall, our results showed that UA potentiates TRAIL-induced apoptosis through activation of ROS and JNK-mediated up-regulation of DRs and down-regulation of DcR2 and cell survival proteins.

More than 80% of people around the world, for their day-to-day medicinal needs, rely on traditional medicine, which has been around for centuries. Even modern medicine in most instances relies on natural products, and 70% of anticancer drugs have their roots in products derived from nature (1). The search for signature genes of different cancers has shown that most cancers are due to dysregulation of multiple genes and multiple cell signaling pathways; thus, drugs that are multi-targeted (once called "dirty drugs") are needed. Compounds from natural sources have an advantage in that they are usu-
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have cytoplasmic death domains that activate the apoptotic machinery upon TRAIL binding (3). Other receptors, such as decoy receptor 1 (DcR1) and DcR2, although membrane-bound, exhibit dominant negative effects by sequestering the ligand (24). Osteoprotegerin, although it binds TRAIL, lacks a transmembrane domain and thus is a soluble receptor.

Numerous reports have shown that various types of cancer cells are resistant to the apoptotic effects of TRAIL (25–27). The exact mechanism of resistance to TRAIL is still not fully understood; however, it can occur at different points in TRAIL-induced apoptotic signaling pathways. For instance, dysfunction of DR4 and DR5, overexpression of antiapoptotic proteins, and loss of proapoptotic proteins has all been linked with TRAIL resistance. Activation of different subunits of mitogen-activated protein kinases and nuclear factor-κB are also reported to develop TRAIL resistance in certain types of cancer cells (28). Therefore, modulation of TRAIL-induced apoptotic signaling molecules is an important strategy to sensitize cancer cells for effective cancer therapy.

In the current report, we tested whether UA can potentiate TRAIL-induced apoptosis and sensitize resistant cancer cells to TRAIL. We found that this triterpene can indeed enhance TRAIL-induced apoptosis through up-regulation of death receptors and down-regulation of antiapoptotic proteins via production of reactive oxygen species (ROS) and activation of JNK.

EXPERIMENTAL PROCEDURES

Reagents—A 50 mM solution of UA (from Sigma), with purity greater than 90%, was prepared in DMSO stock aliquots at −20 °C, and then diluted in culture medium as needed. Further fractionation of UA revealed that 15% fetal bovine serum 100 units/ml penicillin, and 100 mg/ml streptomycin. Iscove's modified Dulbecco's medium was supplemented with 15% human colon cancer HCT116 variant cell lines were cultured in Iscove’s modified Dulbecco’s medium. DMEM and RPMI were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Iscove’s modified Dulbecco’s medium was supplemented with 15% fetal bovine serum 100 units/ml penicillin, and 100 mg/ml streptomycin.

Live/Dead Assay—To measure apoptosis, we used the Live/ Dead® assay (Invitrogen), which assesses intracellular esterase activity and plasma membrane integrity. This assay was performed as described previously (29).

Cytotoxicity Assay—The effects of DBA on TRAIL-induced cytotoxicity were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method (29).

Propidium Iodide Staining for Apoptosis—To determine the effect of DBA on the cell cycle, treated and untreated cells were stained with PI as described earlier (18). A total of 10,000 events were analyzed by flow cytometry using an excitation wavelength of 488 nm and emission wavelength of 610 nm.

Analysis of Cell Surface Expression of DR4 and DR5—Treated and untreated cells were stained with phycoerythrin-conjugated mouse monoclonal anti-human DR5 or DR4 (R&D Systems) for 45 min at 4 °C according to the manufacturer’s instructions. Cells were then suspended, and analyzed by flow cytometry with a FACSCalibur (BD Biosciences).

Annexin V Staining for Apoptosis—A transient early indicator of apoptosis was determined with an annexin V/PI binding kit (Santa Cruz Biotechnology, Inc.) and then analyzed with a flow cytometer (FACSCalibur, BD Biosciences).

Intracellular ROS of cells were detected by using a flow cytometer (FACSCalibur, BD Biosciences).

Transfection with siRNA—HCT116 cells were plated in a well of six-well plates and allowed to adhere for 12 h. On the day of transfection, 12 μl of HiPerFect transfection reagent (Qiagen) was added to 50 nmol/liter siRNA in a final volume of 100 μl of culture medium. After 48 h of transfection, cells were treated with 20 μM UA for 12 h and then exposed to TRAIL for 24 h.

Western Blot Analysis—To determine the levels of protein expression, whole-cell extracts were prepared in lysis buffer as described previously (13). In the in vivo case, colorectal tumor tissues (75–100 mg/mouse) were minced and incubated on ice for 30 min in 0.5 ml of ice-cold whole-cell lysate buffer (10% Nonidet P-40, 5 mol/liter NaCl, 1 mol/liter HEPES, 0.1 mol/liter EGTA, 0.5 mol/liter EDTA, 0.1 mol/liter PMSF, 0.2 mol/liter sodium orthovanadate, 1 mol/liter NaF, 2 μg/ml aprotinin, 2 μg/ml leupeptin). The minced tissue was homogenized with a Dounce homogenizer and centrifuged at 16,000 × g at 4 °C for 10 min. The extracted proteins were then resolved on a 10% SDS gel, and Western blotting was performed as described previously (13).

RNA Analysis and RT-PCR—DR5 mRNA was detected using RT-PCR as follows. Total RNA was isolated from cells using TRizol reagent (Invitrogen) as instructed by the manufacturer. One microgram of total RNA was converted to cDNA using Superscript reverse transcriptase and then amplified by platinum Taq polymerase using the Superscript One Step RT-PCR kit (Invitrogen). The total RNAs were then amplified by PCR using the following primers: DR5 sense (5’-AAGACCCCTGTGCTCGTGTC-3’), DR5 antisense (5’-
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GAACATTCGTGACTCCCA-3'), DR4 sense (5'-CTGAGCAACGACTCTGCTCCAC3'), DR4 antisense (5'-TCCAAGGACACGGCAGACCTTGCTGCAA-3'), GAPDH sense (5'-GGTCTCACCACCATGGAG-3'), and GAPDH antisense (5'-CACCTGTTGCTGTAC-3'). The reaction sequence consisted of 50 °C for 30 min, 94 °C for 2 min, and 94 °C for 35 cycles of 15 s each; 50 °C for 30 s; and 72 °C for 45 s with an extension at 72 °C for 10 min. PCR products were run on 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed.

Animal Protocol—The HCT116 cells were orthotopically implanted as described previously (30). One week after implantation, the mice were randomized into the following treatment groups (n = 6/group): (a) untreated control (corn oil, 100 µl daily) and (b) UA (250 mg/kg once daily orally). Therapy was continued for 4 weeks, and the animals were euthanized 1 week later. Primary tumors in the colon were excised, snap-frozen in liquid nitrogen, and stored at −80 °C. Our experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at M. D. Anderson Cancer Center.

Statistical Analysis—All data are expressed as mean ± S.E. of three independent experiments. Statistical significance was determined using unpaired Student’s t test, and a p value of less than 0.001 was considered statistically significant.

RESULTS

The aim of this study was to investigate whether UA (Fig. 1A) can enhance the sensitivity of tumor cells to TRAIL, if so, through what mechanism. We used HCT116 cells for most of these studies, but other cell types were also used to determine the specificity of this effect.

UA Enhances TRAIL-induced Apoptosis—First, we studied the effect of UA on TRAIL-induced apoptosis by using the MTT method, which detects mitochondrial activity. The HCT116 cells were moderately sensitive to either UA or TRAIL alone. However, pretreatment with UA significantly enhanced TRAIL-induced cytotoxicity (Fig. 1B, left).

We also determined whether UA also enhances TRAIL-induced apoptosis of colon cancer cells. We found that UA and TRAIL treatments alone induced 21 and 16% apoptosis, respectively, in HCT116 cells. Interestingly, combination treatment with UA and TRAIL enhanced apoptosis to 76% (Fig. 1B, right).

To confirm the effect of UA on TRAIL-induced apoptosis, we measured apoptosis by FACS analysis of the sub-G1 fraction. The results indicated that the UA and TRAIL treatment alone induced 12 and 20% apoptosis, respectively. Combination treatment with both UA and TRAIL enhanced apoptosis to 50% (Fig. 1C, top). When apoptosis was examined using annexin V/PI staining, we found that apoptosis was induced at 13% by UA, 7% by TRAIL, and 47% by the combination of the two (Fig. 1C, bottom).

Next, we examined the effect of UA, TRAIL, and their combination on the activation of caspase-8, caspase-3, and PARP cleavage. We found that although UA and TRAIL alone had little effect on the activation of caspases and on PARP cleavage, the two together were highly effective (Fig. 1D). Together, our results indicate that UA can enhance TRAIL-induced apoptosis.

UA Induces the Expression of TRAIL Receptors DR4 and DR5 in Cancer Cell Lines—To explore the underlying mechanism that may be responsible for enhancement of TRAIL-induced apoptosis by UA, we examined the effect of UA on the expression of death receptors. UA induced both DR4 and DR5 in a dose-dependent manner (Fig. 2A, left). Whether this induction of the DRs was dependent on time was also examined. UA induced both DR4 and DR5 in a time-dependent manner as well (Fig. 2A, right).

We also investigated whether UA induces cell surface expression of TRAIL receptors. We found that UA increased the cell surface expression of both DR5 and DR4 in colon cancer HCT116 cells (Fig. 2B). The level of DR4 cell surface expression induced by UA was almost equal to that for DR5.

To determine whether up-regulation of TRAIL receptors by UA was specific to HCT116 cells or also occurs in other cell types, we exposed the following cells to 20 µM UA for 24 h: MCF-7, MDA-MB-231, PC3, SCC4, A293, HT29, Caco2, and KBM-5. UA induced expression of both DR5 and DR4 in almost all of these cell lines (Fig. 2C).

UA Down-regulates Decoy Receptor Gene Silencing of DRs Abolishes the Effect of UA on TRAIL-induced Apoptosis—To determine whether up-regulation of DR5 or DR4 is needed in TRAIL-induced apoptosis, we used a gene-silencing approach to abolish UA-induced expression of these receptors. We found that transfection of cells with DR5 siRNA but not with the control scrambled siRNA reduced the UA-induced up-regulation of DR5. Similarly, transfection of cells with siRNA for DR4 reduced the UA-induced DR4 expression (Fig. 3A).

We next examined whether the suppression of DR5 or DR4 by siRNA could abolish the effects of UA on TRAIL-induced apoptosis using the Live/Dead assay. We found that the silencing of DR5 reduced the UA-induced apoptosis from 22 to 12%, whereas silencing of DR4 reduced apoptosis to 14%. Silencing of both receptors reduced UA-induced apoptosis to 8%, whereas control scrambled siRNA had no effect. These results indicate that DRs contribute to UA-induced apoptosis as well.

The results indicate that DRs contribute to UA-induced apoptosis as well.
The TRAIL-induced apoptosis was reduced from 17 to 8% by silencing of DR5, but silencing of DR4 had no significant effect on TRAIL-induced apoptosis. When we examined the effect of UA and TRAIL in combination, we found that apoptosis was reduced from 62 to 34% by DR5 siRNA, to 48% by DR4 siRNA, and to 28% by the two siRNAs together, whereas with control scrambled siRNA, change in apoptosis was not significant (Fig. 3B). Silencing of DR5 had a more dramatic effect on TRAIL-induced apoptosis than silencing of DR4. The silencing of both receptors abolished the apoptosis as much as silencing of DR5 alone. Overall, these results suggest that DR5 plays a major role in TRAIL-induced apoptosis and that enhancement of apoptosis by UA is linked to up-regulation of the receptors.
UA Down-regulates the Expression of Antiapoptotic Proteins—Various antiapoptotic proteins, including survivin (31), Bcl-xL (32), XIAP (33), and cFLIP (34), have been shown to induce resistance to TRAIL-induced apoptosis. We examined whether UA sensitized the cells to TRAIL through down-regulation of the expression of these cell survival proteins. Results of Western blot showed that UA inhibited expression of the antiapoptotic proteins survivin, XIAP, cFLIP, and Bcl-2 (Fig. 4A, left). These results indicate that down-regulation of antiapoptotic proteins by UA could be another mechanism of potentiation of TRAIL-induced apoptosis.

UA Enhances the Expression of Proapoptotic Proteins—Whether UA can modulate the expression of proapoptotic proteins was also examined. We found that UA cleaved the proapoptotic protein Bid and induced the expression of Bax (Fig. 4A, right). Up-regulation of Bax by UA suggests that these proteins may disrupt mitochondrial homeostasis, which further leads to apoptosis.

UA-induced Up-regulation of TRAIL Receptors is p53-independent—Because p53 has been reported to induce death receptors (35), we investigated whether UA up-regulates DRs through up-regulation of p53. We found that UA did not up-regulate p53; if anything, at a higher dose (30 μM), it down-regulated p53 (Fig. 4B, left). We also determined whether UA-induced induction of TRAIL receptors is mediated through p53 in HCT116 cell lines that lack p53. We found that UA induced DR5 and DR4 in p53 parental as well as p53 knock-out HCT116 cells in a dose-dependent manner (Fig. 4B, right). These results indicate that induction of TRAIL receptors by UA is p53-independent.

![FIGURE 2. UA induces DR5 and DR4 expression.](image-url)
UA-induced Up-regulation of TRAIL Receptors Is Bax-independent—We found that UA induced Bax. Whether UA up-regulates DRs through up-regulation of Bax was investigated. For this, we used Bax knock-out HCT116 colon cancer cells. UA induced expression of DR5 and DR4 in both Bax parental and Bax knock-out HCT116 cells (supplemental Fig. 1A), indicating that induction of TRAIL receptors are independent of Bax expression.

UA-induced Up-regulation of Death Receptor Is Not Mediated through Activation of ERK1/2 and GSK-3β—Activation of ERK1/2 and GSK-3β has been linked with induction of TRAIL-induced apoptosis (36, 37). We therefore investigated whether UA activates ERK1/2 and GSK-3β. Results showed that UA activated neither ERK1/2 nor GSK-3β and had no effect on the expression levels of these proteins (Fig. 4C, left).

UA-induced Up-regulation of DRs Is Not Mediated through Activation of CHOP and PPARγ—Next we determined whether UA modulates CHOP and PPARγ. We found that DBA did not modulate either PPARγ or CHOP (supplemental Fig. 1B), indicating that induction of DRs is not mediated through PPARγ or CHOP.

UA-induced Up-regulation of DRs Requires JNK Activation—Activation of the TRAIL receptor by H2O2 (38) and by CDDO (39) requires activation of JNK. We investigated whether UA can activate JNK by exposing the cells to different concentrations of UA for 24 h and then examined the cells for activation of JNK. Western blotting results showed that UA induced JNK activation in a dose-dependent manner (Fig. 4C, right), but under the same conditions it had no effect on total JNK protein level.

Next, we investigated whether activation of JNK is needed for UA-induced up-regulation of death receptors. For this we used SP600125, a specific pharmacologic inhibitor of JNK. As shown in Fig. 4D (left), pretreatment of cells with this JNK inhibitor significantly suppressed the UA-induced up-regulation of DR5 and DR4 expression. These results suggest that JNK is involved in UA-induced up-regulation of DRs. Suppression of JNK also leads to inhibition of UA-induced cleavage of PARP (Fig. 4D, left).
Furthermore, we examined whether the suppression of JNK activation by its inhibitor could abrogate the apoptosis induced by UA, TRAIL, and the combination of UA and TRAIL. We found that apoptosis induced by UA, TRAIL, and the combination was reduced from 18 to 10%, from 24 to 13%, and from 64 to 42%, respectively (Fig. 4D, right). Thus, suppression of JNK activation substantially reduced the apoptosis, although not completely. 

**UA-induced Up-regulation of DR5 Requires ROS**—That ROS is needed for induction of death receptors by certain agents has been demonstrated (29). To determine whether UA has the ability to generate ROS, we treated HCT116 cells with UA and used dichlorodihydrofluorescein diacetate as a probe to measure the increase in ROS levels in the cells. We found that UA induced the production of ROS in a dose-dependent manner (Fig. 5A).
Next we determined whether ROS production is needed for up-regulation of expression of DR5 and DR4 by UA. We found that pretreatment of cells with the ROS scavenger NAC blocked UA-induced up-regulation of DR5 and DR4 protein expressions in a dose-dependent manner (Fig. 5B, top), indicating that ROS generation is critical for the effect of UA on TRAIL receptors.

NAC Abrogates the Effect of UA in Suppression of Antiapoptotic Proteins—Next we examined whether NAC abrogates UA-induced inhibition of antiapoptotic proteins. The results revealed that pretreatment of NAC effectively abolished the effect of UA in suppression of XIAP, cFLIP, and Bcl-2 but not survivin (Fig. 5B, bottom). The effect of NAC in inhibiting the effect of UA on XIAP is more prominent than others.

UA-induced Potentiation of Apoptosis Induced by TRAIL Is Reversed by Quenchers of ROS—We next examined whether ROS is needed for potentiation of TRAIL-induced apoptosis by UA. As shown in Fig. 5C, UA enhanced TRAIL-induced...
apoptosis in HCT116 cells, and pretreatment of cells with NAC markedly reduced this UA-induced enhancement from 68 to 34% (Fig. 5C).

We also found that NAC reversed the effect of UA on TRAIL-induced cleavage of procaspases and PARP (Fig. 5D), again suggesting the critical role of ROS in UA effects on TRAIL.

**UA Sensitizes TRAIL-resistant Colon Cancer Cells**—It has been shown that colon cancer HT-29 cells are completely resistant to TRAIL (40). We therefore investigated whether UA affects TRAIL-resistant HT29 cancer cells. We found that HT29 cells were moderately sensitive to UA but resistant to TRAIL. However, pretreatment with UA enhanced TRAIL-induced apoptosis from 2% to 42% (Fig. 6A).

Furthermore, we examined apoptosis by FACS analysis by PI staining and found that UA alone induced 8.2% apoptosis, whereas TRAIL showed no cell death in HT29 cells. Interestingly, the pretreatment with UA sensitized the cells to TRAIL and induced apoptosis of 24% (Fig. 6B). Results of the cytotoxicity assay by MTT uptake also showed that HT29 cells were moderately sensitive to UA but resistant to TRAIL. However, pretreatment of UA sensitized the HT29 cells to TRAIL and induced apoptosis (Fig. 6C).
We then determined whether UA up-regulates the expression of death receptors (DRs) in HT29 cells. UA induced DR5 and DR4 (Fig. 6D), suggesting that UA sensitized the HT29 cells to TRAIL-induced apoptosis.

UA Up-regulates DR Expression, Down-regulates Cell Survival Proteins, and Activates JNK in Colorectal Tumors in Vivo—Whether UA up-regulates the expression of DR4 and DR5 in vivo was examined in orthotopically implanted human colorectal tumor from nude mice treated with UA. The Western blot analysis revealed that tumor tissues from UA-treated animals had up-regulated expression of both of the death receptors, DR4 and DR5 (Fig. 7A), had down-regulated expression of cell survival proteins (survivin and Bcl-2) (Fig. 7B), and had activated JNK (Fig. 7C). These results in vivo are in agreement with those in vitro.

DISCUSSION

TRAIL has recently been considered a highly promising candidate as an anti-cancer drug, because it induces apoptosis specifically in malignant or transformed cells without any cytotoxicity toward a variety of normal cells (41–43). A considerable number of cancer cells, however, are resistant to apoptosis induced by TRAIL (28). TRAIL induces apoptosis by interacting with two different death-inducing receptors, DR4 and DR5. Therefore, targeting death receptors and their signaling molecules to trigger apoptosis in tumor cells is an attractive concept for cancer therapy.

Several reports demonstrated that chemotherapeutic agents and ionizing radiation can sensitize cells to TRAIL-induced apoptosis (44, 45). In the present study, we demonstrated that UA, a pentacyclic triterpene, can sensitize cells to TRAIL-induced apoptosis (Fig. 8).

To elucidate the mechanism, we found that UA induction of death receptors and down-regulation of antiapoptotic proteins. In our study, UA treatment induced dose- and time-dependent increases in the protein levels of DR5 and DR4. We also demonstrated the up-regulation of expression of cell surface death receptors by UA. Silencing of DR5, however, was more effective than silencing of DR4 in inhibiting UA and TRAIL-induced apoptosis.

The induction of death receptors by UA was not cell type-specific because induction was observed in a wide variety of cancer cell types, including breast, prostate, head and neck, kidney, leukemic, and colon cancer cells. Induction of TRAIL receptors in some cells, however, was much more pronounced than in other cell types. Thus, UA is likely to potentiate the anticancer effect of TRAIL in a wide variety of cells.

Resistance to TRAIL-induced apoptosis has been reported to be associated with overexpression of antiapoptotic proteins. Among Bcl-2 family proteins, Bcl-2 has been linked with suppression of apoptosis by TRAIL (46). In our study, Bcl-2 was suppressed by UA treatment. In addition, UA treatment also decreased the expression of Bcl-xl, survivin, and XIAP proteins but had no effect on c-FLIP expression, which is also linked to TRAIL resistance (31, 32). When we looked for other potential mechanisms, we found that UA significantly up-regulated the expression of Bax and cleavage of Bid proteins. The former has been shown to be critical for TRAIL-induced apoptosis (47). However, our result also
showed that UA-induced apoptosis mediated through expression of DR is independent of Bax expression.

It has been suggested that oxidative stress plays a major role as a mediator of cell death (48). ROS generation has been proposed to be involved in the up-regulation of DR5 by numerous cancer chemopreventive agents, including curcumin and sulforaphane (49, 50), zerumbone (51), and garcinol (29).

In the current study, our data showed that UA induces up-regulation of DRs through production of ROS. The antioxidant NAC abolished the up-regulation of DR induced by UA. Therefore, ROS generation is critical for UA-induced DR-mediated apoptosis in cancer cells.

The transcriptional regulation of DR5 is complex, and multiple potential binding sites of various transcription factors, including CHOP and p53, are present in the upstream region of DR5 (52, 53). However, we found that the induction of DR5 by UA occurs independently of p53 and CHOP. These results are consistent with those previously reported for the protosome inhibitor MG132 (54) and for HDAC inhibitors (55).

In addition to DR4 and DR5, other death receptors called decoy receptors (DcRs) are also involved in the TRAIL-induced apoptotic signaling pathway. TRAIL can also bind DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4). These latter receptors fail to induce apoptosis, because DcR1 lacks an intracellular death domain and DcR2 has a truncated cytoplasmic death domain. In addition, DcR1 and DcR2 inhibit DR4- and DR5-mediated apoptosis by TRAIL. While DcR1 prevents the assembly of the death-inducing signaling complex by sequestering TRAIL within lipid rafts, DcR2 is co-recruited with DR5 within the death-inducing signaling complex and inhibits initiator caspase activation (56, 57). However, UA reduced the expression of DcR1 and DcR2 in a ROS-dependent manner (39, 62). In contrast, no change in DcR1 occurred, yet expression of DcR2 has been shown to be regulated by p53. Therefore, we did find down-regulation of p53 at higher doses, it is possible that this suppression of p53 mediates decrease of DcR2.

Activation of stress-activated proteins such as JNK is known to enhance TRAIL-induced apoptosis (58). Our findings provide evidence that activation of JNK by UA up-regulates DRs, which may further lead to an increase in TRAIL-induced apoptosis. UA was found to be ineffective in activating ERK1/2 MAPK. Although ROS can lead to induction of MAPK (59), in our study, UA induced TRAIL receptors independently of MAPK. In another study, quercetin augmented TRAIL-induced apoptosis through the ERK-mediated down-regulation of the survivin signal transduction pathway (60). In our study, however, UA induced apoptosis through down-regulation of survivin but independently of ERK activation.

We observed that UA sensitized the tumor cells that are resistant to TRAIL. Although the mechanisms underlying sensitization of TRAIL-resistant cells are not clear, some important components, such as down-regulation of antiapoptotic proteins in signaling pathways, may be involved in this process (28). Because Bcl-2, XIAP (inhibitor of caspase), and survivin (46, 61) are involved in TRAIL resistance, down-regulation of these proteins by UA is the probable reason for the sensitizing of TRAIL-resistant cells. In addition, induction of death receptors could further contribute to the sensitivity.

UA is a pentacyclic triterpene isolated from various traditional medicinal plants. Interestingly, CDDO, which is a synthetic analog designed based on ursolic acid and betulinic acid and which is also a pentacyclic triterpene, has been shown to induce death receptors (39, 62). CDDO sensitized tumor cells to TRAIL through up-regulation of death receptors and down-regulation of cFLIP (62). In addition, Zou et al. (39) found that CDDO-Me induces DR5 up-regulation through induction of CHOP, and Hyer et al. (62) found that the cell surface expressions of DR4 and DR5 were significantly up-regulated by CDDO or CDDO-Im but not by CDDO-Me. Why Zou et al. found up-regulation of DRs by CDDO-Me and Hyer et al. did not is not clear. We showed, however, that UA-induced up-regulation of DR is independent of CHOP. All of these groups did show that activation of JNK is needed for up-regulation of the receptors.

Whether our in vitro results have relevance to those in vivo was also investigated. We found that UA up-regulated DRs expression, down-regulated cell survival proteins, and activated JNK in vitro. These effects are consistent with the effect of UA on the expression and surface expression of DRs. While CDDO sensitizes tumor cells to TRAIL-induced apoptosis through up-regulation of DR4 and DR5 and down-regulation of antiapoptotic proteins (Fig. 8), thus rendering cancer cells more sensitive to the cytotoxic activities of TRAIL. Considering that UA by itself is highly safe and exhibits anticancer activities against a wide variety of tumors in vitro (12, 13, 18) and in vivo (22, 63), its potential use in combination with TRAIL should be explored. Further studies in animals are needed to investigate the anticancer potential of UA in combination with TRAIL.

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SUPPLEMENTARY FIGURE 1: Upregulation of DRs is independent of bax, CHOP and PPARγ expression. (A) To determine whether bax were involved in the induction of DR5/DR4, bax parental and bax knockout cells were treated separately with UA for 24 h, and whole-cell extracts were prepared for Western blotting for DR5 and DR4. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. (B) HCT116 cells were pretreated with the indicated doses of UA for 24 h. Whole-cell extracts were prepared and subjected to Western blotting with the CHOP and PPARγ antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading.
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