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ORIGINAL ARTICLE

AW00179 potentiates TRAIL-mediated death of human lung cancer H1299 cells through ROS-JNK-c-Jun-mediated up-regulation of DR5 and down-regulation of anti-apoptotic molecules

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Abstract Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apoptosis in tumor cells, but when used alone, it is not effective at treating TRAIL-resistant tumors. This resistance is challenging for TRAIL-based anti-cancer therapies. In this study, we found that 1-(4-trifluoromethoxy-phenyl)-3-[4-(5-trifluoromethyl-2,5-dihydro-pyrazol-1-yl)-phenyl]-urea (AW00179) sensitized human lung cancer H1299 cells to TRAIL-mediated apoptosis. Even in the absence of TRAIL, AW00179 strongly induced DR5 expression and decreased the expression of anti-apoptotic proteins, suggesting that the sensitizing effect of AW00179 on TRAIL-mediated apoptosis is due to increased levels of DR5 protein and decreased anti-apoptotic molecules. AW00179 also induced the activation of c-Jun and ERK; however, a pharmacologic inhibition study revealed that JNK-c-Jun signaling is involved in the induction of DR5 expression. In addition, reactive oxygen species (ROS) appear to be involved in AW00179 activity. In conclusion, AW00179 has the potential to sensitize H1299 cells to TRAIL-mediated apoptosis through two distinct mechanisms: ROS-JNK-c-Jun-mediated up-regulation of DR5, and down-regulation of anti-apoptotic molecules.

Keywords TRAIL · Sensitization · DR5 · ROS · c-Jun

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) preferentially triggers apoptosis in a variety of cancer cells compared with normal cells, making it an excellent therapeutic candidate for the treatment of cancers (Walczak et al. 1999; Wang 2010; McGrath 2011; Voelkel-Johnson 2011). Apparently, TRAIL has induced the regression of cancer xenografts without detrimental effects on normal cells (Roth et al. 1999). However, after reporting resistance to TRAIL in several cancer cell lines (Braeuer et al. 2006), TRAIL resistance has been considered a challenging issue for effective TRAIL-based anticancer therapeutic strategies. Therefore, several approaches have been recently employed to overcome resistance to TRAIL (Amm et al. 2011). Most notably, combination therapy with TRAIL and chemotherapeutic agents or radiotherapy has been investigated as a method of sensitizing cancer cells to TRAIL-mediated apoptosis (Wissink et al. 2006; Chen et al. 2010).

TRAIL triggers apoptosis signaling by binding to its receptors, death receptor 4 (DR4) and DR5 (Pan et al. 1997; Walczak et al. 1997; Abdulghani and El-Deiry 2010; Yang et al. 2010). Next, the adaptor protein Fas-associated death domain (FADD) and procaspase-8 are recruited to the cytoplasmic death domain of the receptors to form the death-inducing signaling complex (DISC; Pennarun et al. 2010). Procaspase-8 subsequently undergoes autocatalytic activation in the DISC, leading to the activation of caspases, including caspase-3 (Cohen 1997). DISC also contains functional inhibitors, such as cellular FADD-like interleukin-1β-converting enzyme inhibitory protein (FLIP). Two forms of FLIP are known: $FLIP_L$, which has two death effector domains (DEDs) and a caspase-like domain, and $FLIP_s$, which lacks the caspase-like domain. Because FLIP is structurally similar to procaspase-8 (Krueger et al. 2001), it can inhibit caspase-8 activation by binding to either FADD or caspase-8, and consequently inhibits apoptosis. Caspase activation can also be inhibited

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by the inhibitor of apoptosis protein (IAP) family members (Schimmer 2004).

A better understanding of the molecular components or mechanisms involved in resistance to TRAIL-induced apoptosis would be helpful to develop sensitizers that can aid in overcoming TRAIL resistance. Apparently, dysfunction of DRs, defects in DISC components, and/or overexpression of anti-apoptotic molecules can lead to TRAIL resistance (Zhang and Fang 2005). Therefore, resistance to TRAIL-mediated apoptosis can be overcome via up-regulation of DRs or DISC components for bursting TRAIL-induced extrinsic cell death signaling and/or down-regulation of anti-apoptotic molecules, such as FLIP and IAP family members, to accelerate functional DISC and caspase-dependent cell death signaling activities (Zhang and Fang 2005).

In this study, to identify small molecules that sensitize cancer cells to TRAIL-induced apoptosis, we screened chemicals by comparing their effects on cell viability between human lung fibroblast WI38 cells and human lung cancer H1299 cells, which are relatively resistant to TRAIL-induced apoptosis as a normal cells and TRAIL-resistant cancer cells, respectively (Kim et al. 2000; Chen et al. 2008). We found that 1-(4-trifluoromethoxy-phenyl)-3-[4-(5-trifluoromethyl-2,5-dihydro-pyrazol-1-yl)-phenyl]-urea (AW00179, Fig. 1a) enhances TRAIL-mediated apoptosis in H1299 cells.

Materials and methods

Materials

AW00179 was purchased from Maybridge (Thermo Fisher Scientific Inc. Waltham, MA, USA). Soluble recombinant human TRAIL and anti-DR5 antibody were purchased from Chemicon International Inc. (Temecula, AC, USA). Primary antibodies against FLIP, survivin and XIAP were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Primary antibodies (against DR4, Bcl-2, Bcl-xL, cIAP-1, cIAP-2, p-c-Jun, c-Jun, p-ERK, ERK, and actin) and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-human DR5-PE was purchased from BioLegend, Inc. (San Diego, CA, USA). CM-H2DCFDA was purchased from Molecular Probes, Inc (Carlsbad, CA, USA). The JNK inhibitor SP600125 and the MEK-ERK inhibitor PD98059 were purchased from Calbiochem (EMD Biosciences, Inc., La Jolla, CA, USA). Caspase inhibitor Z-VAD-FMK was purchased from Promega (Madison, WI, USA). N-acetyl cysteine (NAC) was purchased from Sigma (St. Louis, MO, USA).

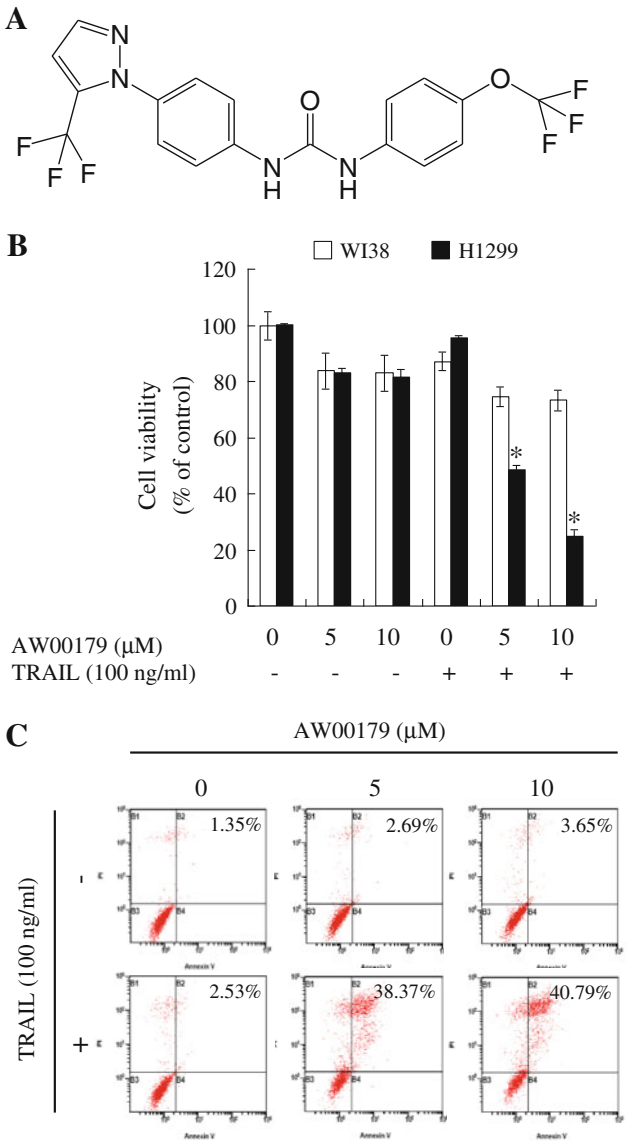


Fig. 1 AW00179 potentiates TRAIL-mediated apoptosis in H1299 cells. **a** Structure of AW00179. **b** Effects of AW00179 alone or in combination with TRAIL on the viability of H1299 and WI38 cells were evaluated. Briefly, WI38 cells and H1299 cells were seeded in a 96-well plate at 8 and 1.4 × 10^3 cells/well, respectively. After 24 h, cells were incubated with AW00179 alone or its combination with TRAIL (100 ng/ml) for 2 days and then the cell viability was evaluated by CCK-8 assay $*P < 0.001$. **c** Sensitization effect of AW00179 on TRAIL-mediated apoptosis was evaluated by flow cytometry analysis. Cells (2.8 × 10^5 cells/well) were plated in a 6-well plate. After 24 h, cells were incubated with AW00179 in the absence or presence of TRAIL (100 ng/ml) for 48 h and then the apoptotic cells were analyzed with flow cytometry. The number of apoptotic cells was presented to % of total cell number analyzed with flow cytometry

Cell culture

Human lung cancer H1299 cells and lung WI38 cells (American Type Culture Collection, Rockville, MD, USA)

were cultured in RPMI 1640 or MEM supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin in humidified atmosphere of 5% CO_2 at 37°C. The culture medium was changed every 3 days.

Apoptosis analysis

Apoptosis was determined using annexin V and propidium iodide (PI) staining-based ApoAlert annexin V apoptosis detection kit (Clontech, Palo Alto, CA, USA). H1299 cells (2.8×10^5 cells/well) were plated in a 6-well plate. After 24 h, cells were incubated with AW00179 in the absence or presence of TRAIL (100 ng/ml) for 48 h and then cells were harvested and analyzed by flow cytometry (Cytomics FC500; Beckman Coulter Inc., Fullerton, CA, USA). Data were analyzed using the Beckman Coulter Cytomic RXP software.

Cell viability assay

WI38 cells and H1299 cells were plated in a 96-well plate at 8 and 1.4×10^3 cells/well, respectively. After 24 h, cells were treated with AW00179 in the absence or presence of TRAIL (100 ng/ml) for 48 h. Then, cell viability was measured in triplicates by the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD, USA) according to the manufacturer's protocol. Absorbance was measured by using Wallac EnVision microplate reader (PerkinElmer, Finland). In order to evaluate the involvement of caspase cascade, MAP kinase signaling or oxidative stress in the sensitizing effect of AW00179 on the TRAIL-induced apoptosis, cells were pre-incubated with the caspase inhibitor, Z-VAD-FMK (10 μM), the JNK inhibitor SP600125 (10 μM), the MEK-ERK inhibitor PD98059 (20 μM) or the antioxidant NAC (10 mM) for 1 h and then incubated with AW00179 in the absence or presence of TRAIL (100 ng/ml) for 48 h. The cell viability was then measured by CCK-8.

Caspase-8 activity assay

Caspase-8 activity was measured by Caspase-8 Fluorometric Assay kit (R&D systems, Inc. Minneapolis, MN, USA). Assay was performed in triplicates in a 96-well plate based on the manufacturer's protocol.

Western blot analysis

Western blot was performed as described in a previous study (Hwang et al. 2009). Briefly, cells were homogenized and centrifuged at $10{,}000 \times g$ for 15 min. The supernatant was used as the cytoplasmic protein fraction. Denatured proteins were separated on the gels and transferred onto PVDF (Millipore, Temecula, CA, USA). After incubation with antibody, membranes were developed with Pierce SuperSignal West Femto Maximum Sensitivity Substrate (Fisher Scientific, Pittsburgh, PA, USA) using the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Japan).

Reverse transcription-PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and chloroform extraction. The RNA yield and purity were evaluated by measuring A260/A280. First-strand cDNA was synthesized with 1 μg of total RNA and 1 μM of oligo (dT_{18}) primer using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA). Using Taq PCR Master Mix kit (Qiagen), subsequent PCR was performed with 0.5 μl of first-strand cDNA and 20 pmol of primers. The PCR consisted of initial denaturation at 94°C for 3 min, 3-step cycling (30 cycles) at 94°C for 40 s, 53°C for 40 s, and 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were run on 1.2% agarose gel, stained with ethidium bromide and photographed using the Doc-Print imaging system (Vilber Lourmat, Germany). GAPDH was used as an internal control. The primers used for PCR were as follows: DR5, 5′-AGA GGG ATT GTG TCC ACC TG-3′ (forward) and 5′-AAT CAC CGA CCT TGA CCA TC-3′ (reverse); GAPDH, 5′-GTC AGT GGT GGA CCT GAC CT-3′ (forward) and 5′-AGG GGT CTA CAT GGC AAC TG-3′ (reverse).

Analysis of DR5-expressing cells

H1299 cells (2.8×10^5 cells/well) were pre-treated with SP600125 or PD98059 for 1 h and then treated with AW00179. Cells were harvested, washed with PBS with 0.5% FBS, stained with phycoerythrin (PE)-conjugated anti-human DR5 for 1 h at 4°C according to the manufacturer's protocol and then analyzed using flow cytometry. PE-conjugated IgG was used as an isotype control.

Flow cytometric analysis of H_2O_2

Intracellular concentration of H_2O_2 was determined by staining with the redox-sensitive dye CM-H_2DCFDA. Cells were (2.8×10^5 cells/well) plated in a 6-well plate. After 24 h, cells were incubated with AW00179 for 3 h, stained with 5 μM CM-H_2DCFDA for 15 min and evaluated by flow cytometry.

Statistical analysis

Significance was determined using the Student's t test and differences were considered significant when $P < 0.05$.

Results

AW00179 sensitizes caspase-dependent TRAIL-mediated apoptosis in H1299 cells

Because toxicity to normal cells is a major concern in the development of TRAIL sensitizers, we primarily screened chemicals by comparing their effects on cell viability between WI38 cells and H1299 cells and identified AW00179 as one of the TRAIL sensitizers with a new scaffold. TRAIL alone (100 ng/ml) was not cytotoxic to either cell line. However, its combination with AW00179 significantly decreased the viability of H1299 cells, but not WI38 cells (Fig. 1b). In addition, the effect of TRAIL plus AW00179 on the induction of apoptosis in H1299 cells was evaluated by annexin V/PI-based flow cytometric analysis. The respective percentages of apoptotic cells were 2.69 and 3.65% when treated with 5 μM and 10 μM AW00179 (Fig. 1c). However, in the presence of TRAIL (100 ng/ml), the percentage of apoptotic cells was dramatically increased from 2.53% (TRAIL alone) to 38.37% (5 μM AW00179) and 40.79% (10 μM AW00179).

Next, we examined the effects of AW00179, TRAIL, and their combination on the activation of caspase-8, which mainly mediates TRAIL-induced extrinsic cell death (LeBlanc and Ashkenazi 2003). AW00179 alone and TRAIL alone had no effect on the activation of caspase-8; however, their combination significantly induced caspase-8 activation (Fig. 2a). The involvement of caspase-dependent signaling in the effect of AW00179 on TRAIL-mediated cell death was confirmed by the use of the caspase inhibitor Z-VAD-FMK; in the presence of TRAIL (100 ng/ml), Z-VAD-FMK significantly inhibited the AW00179-mediated decrease of H1299 cell viability (Fig. 2b).

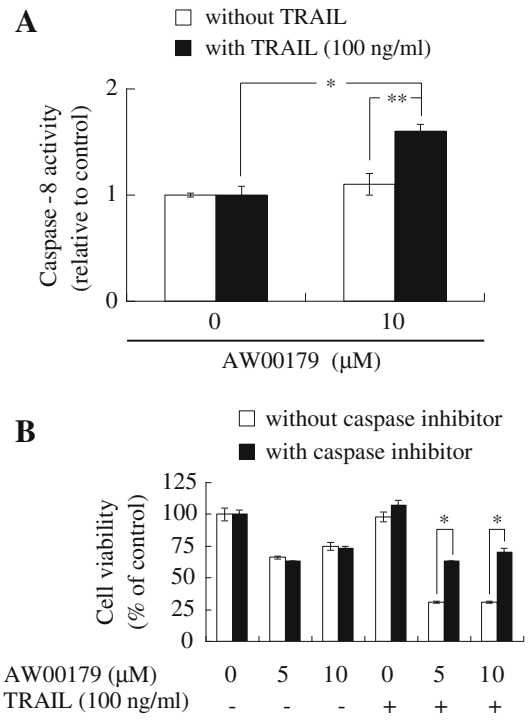


Fig. 2 Caspase signaling is involved in sensitization effect of AW00179 on TRAIL-mediated death of H1299 cells. **a** Effect of AW00179 alone or in combination with TRAIL on capase-8 activity was evaluated. Cells (1.4×10^3 cells/well) were plated in a 96-well plate. After 24 h, cells were incubated with AW00179 in the absence or presence of TRAIL (100 ng/ml) for 4 h and then caspase-8 activity was measured. $^*P < 0.01$, $^{**}P < 0.001$. **b** Effect of caspase inhibitor on cell death induced by AW00179 plus TRAIL was evaluated. Cells (1.4×10^3 cells/well) were plated in a 96-well plate. After 24 h, cells were pre-incubated with the caspase inhibitor, Z-VAD-FMK (10 μM) for 1 h, incubated with AW00179 alone or its combination with TRAIL (100 ng/ml) for 2 days and then the cell viability was evaluated by CCK-8 assay. $^*P < 0.001$

AW00179 up-regulates DR5 expression

To elucidate the underlying mechanism that may be responsible for AW00179-mediated sensitization to TRAIL-mediated apoptosis, we examined the expression levels of DRs that are directly involved in the TRAIL-induced extrinsic cell death cascade. AW00179 increased the protein expression level of DR5 but not DR4 in the absence and presence of TRAIL (Fig. 3a). AW00179 also up-regulated the mRNA level of DR5 in the absence and presence of TRAIL (Fig. 3b).

AW00179 induces phosphorylation of c-Jun and ERK

The up-regulation of DRs via activation of c-Jun N-terminal kinase (JNK) and/or extracellular signal-regulated kinase (ERK) has been reported in several studies (Gupta et al. 2011; Prasad et al. 2011a). Therefore, we further evaluated the effect of AW00179 on the phosphorylation of c-Jun (a target of JNK) and ERK. AW00179 induced the phosphorylation of both kinases in the absence or presence of TRAIL (Fig. 3c).

AW00179 down-regulates the expression of anti-apoptotic molecules

Next, we evaluated the effect of AW00179 on the expression of anti-apoptotic molecules, such as FLIPs, Bcl-2, and IAP family members because the down-regulation of anti-apoptotic molecules has been shown to potentiate TRAIL-mediated apoptosis (Chawla-Sarkar et al. 2004; Kim et al. 2004; Hetschko et al. 2008; Lin et al. 2008). As shown in Fig. 3d, the expression of $FLIP_L$ was not affected

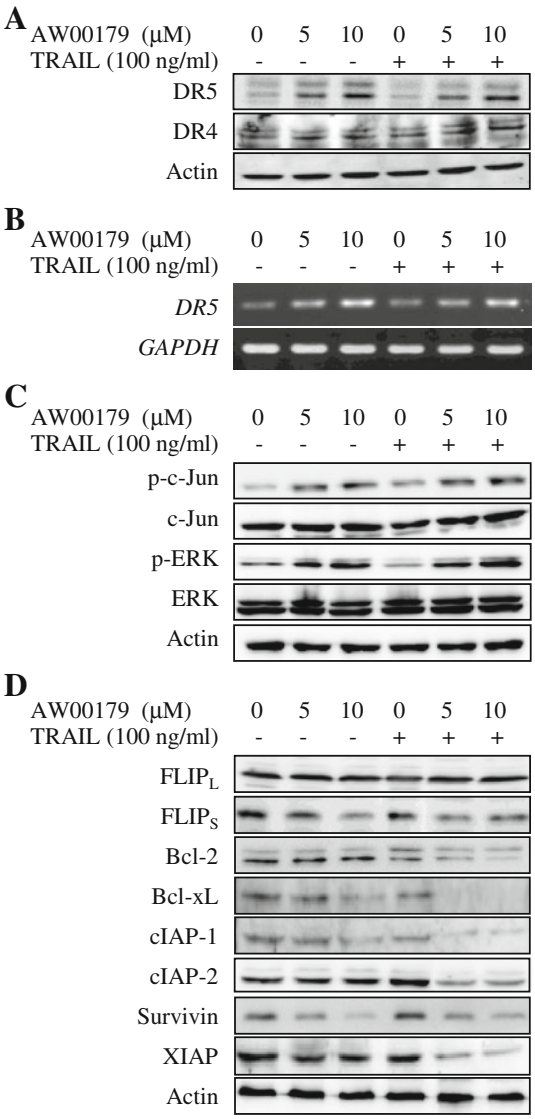


Fig. 3 AW00179 induces DR5 expression and MAP kinase activation, but down-regulates anti-apoptotic molecules. Cells (2.8×10^5 cells/well) were plated in a 6-well plate. After 24 h, cells were incubated with AW00179 in the absence or presence of TRAIL (100 ng/ml) for 24 h (for DRs **a** and anti-apoptotic molecules **d**) or 30 min (for signaling molecules **c**) and then western blot analysis was carried out as described in "Materials and methods". **b** The mRNA level of DR5 was evaluated in cells treated with AW00179 in the absence or presence of TRAIL (100 ng/ml) for 3 h by RT-PCR. *GAPDH* was used as an internal control

by AW00179 or its combination with TRAIL. However, the expression levels of Bcl-2 and cIAP-2 were decreased by their combination, but not by AW00179 alone. AW00179 also decreased the expression levels of $FLIP_S$, Bcl-xL, cIAP-1, survivin, and XIAP in the absence of TRAIL, and its combination with TRAIL synergistically decreased the expression levels of Bcl-xL, cIAP-1, and XIAP.

AW00179-induced activation of the JNK-c-Jun signaling pathway mainly potentiates TRAIL-mediated cell death via up-regulation of DR5

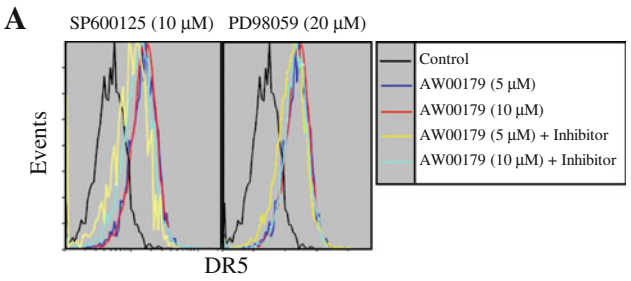
The possibility that AW00179 triggers DR5 up-regulation in H1299 cells via JNK-c-Jun and/or MEK-ERK activation was confirmed in a pharmacologic inhibition study. Flow cytometry analysis revealed that AW00179 induced the cell surface expression of DR5; however, the inhibition of JNK-c-Jun signaling by SP600125 strongly prevented this induction more than the inhibition of MEK-ERK signaling by PD98059 (Fig. 4a). The prevention of AW00179-induced DR5 expression by the pharmacologic blockade of the JNK-c-Jun or MEK-ERK signaling pathways was also confirmed by Western blot analysis (Fig. 4b). Consistent with the flow cytometry analysis, cell death induced by AW00179 plus TRAIL was strongly inhibited by SP600125 rather than PD98059, suggesting that the JNK-c-Jun signaling pathway was functional during the sensitization effect of AW00179 on cells for TRAIL-mediated cell death (Fig. 4c).

AW00179-generated reactive oxygen species sensitize TRAIL-mediated cell death via c-Jun phosphorylation and DR5 up-regulation

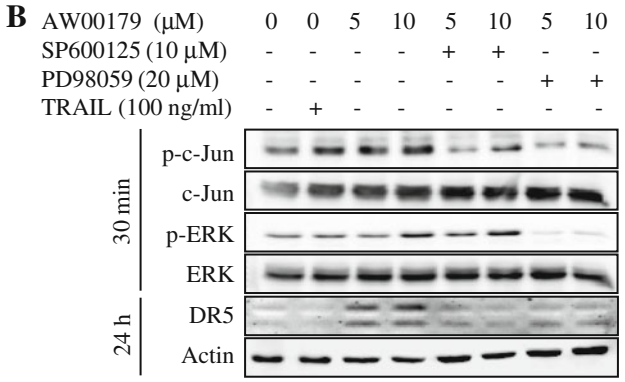
Because the generation of reactive oxygen species (ROS) has been shown to be needed for the induction of DR5 expression via activating JNK-c-Jun signaling (Prasad et al. 2011a, b), we evaluated the ability of AW00179 to generate ROS by using the redox-sensitive dye CM-H_2DCFDA, which becomes fluorescent when oxidized by H_2O_2 and its free radical products. As shown in Fig. 5a, AW00179 dose-dependently increased the number of CM-H_2DCFDA-positive cells. Next, we evaluated whether AW00179-induced ROS generation is needed for the activation of c-Jun and the induction of DR5 expression. The anti-oxidant NAC prevented AW00179-induced phosphorylation of c-Jun and the induction of DR5 protein expression (Fig. 5b). In addition, NAC significantly blocked the AW00179-induced death of H1299 cells in the presence of TRAIL (Fig. 5c).

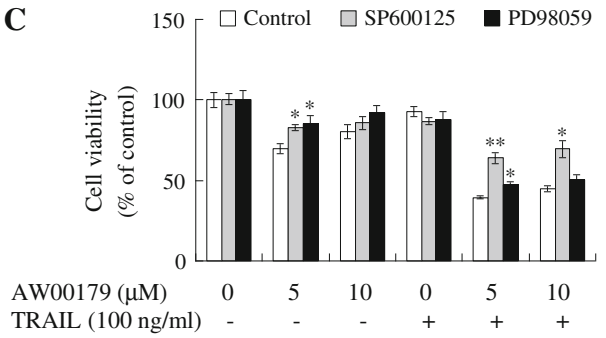
Discussion

We identified AW00179 as a sensitizer of human lung cancer H1299 cells to TRAIL-mediated apoptosis. Notably, the combination of AW00179 and TRAIL was less toxic to human lung fibroblast WI38 cells than to H1299 cells. WI38 cells have been used to evaluate the selectivity of apoptosis induction of cancer cells by combination treatment with TRAIL and its sensitizing compound (Xie et al.



AW00179 (μM)	% of DR5-positive cells		
	-	SP600125 (10 μM)	PD98059 (20 μM)
0	1.31	2.47	1.39
5	50.70	20.30	35.70
10	52.70	35.10	48.00



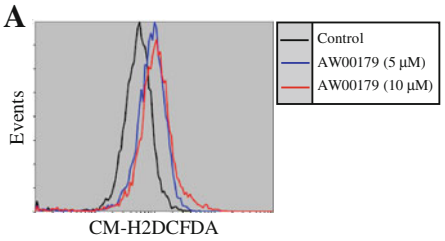


2011). The sensitizing action of AW00179 could be due to its potential to induce DR5 expression via the ROS-JNK-c-Jun signaling pathway; AW00179-induced c-Jun phosphorylation and DR5 expression were prevented by a JNK-specific inhibitor, SP600125, or a ROS scavenger, NAC, in H1299 cells. In addition, the AW00179-induced death of H1299 cells was attenuated by pre-treatment with either SP600125 or NAC. In WI38 cells, TRAIL, AW00179 alone or their combination did not induce the DR5 expression (data not shown).

◀ **Fig. 4** AW00179-induced activation of JNK-c-Jun signaling pathway potentiates TRAIL-mediated cell death via DR5 up-regulation. The effect of JNK inhibitor (SP600125) or MEK-ERK inhibitor (PD98059) on DR5 expression and viability was evaluated in H1299 cells. Cells (2.8×10^5 cells/well) were plated in a 6-well plate. After 24 h, cells were pre-incubated with 10 μM SP600125 or 20 μM PD98059 for 1 h and incubated with AW00179 for 16 h. Then, DR5-positive cells were counted by flow cytometry (**a**) and the protein level of DR5 was evaluated by western blot analysis (**b**). **c** Effect of SP600125 or PD98059 on cell death induced by AW00179 plus TRAIL was evaluated. Cells (1.4×10^3 cells/well) were plated in a 96-well plate. After 24 h, cells were pre-incubated with 10 μM SP600125 or 20 μM PD98059 for 1 h and incubated with AW00179 alone or its combination with TRAIL (100 ng/ml) for 2 days. Then, cell viability was evaluated by CCK-8 assay. control *vs.* inhibitor-treated, $*P < 0.01$; $**P < 0.001$

As mentioned above, among several strategies to overcome the resistance to TRAIL, the up-regulation of molecules involved in TRAIL-mediated extrinsic cell death signaling (such as DRs) could be an attractive approach to potentiate apoptosis in cancer cells. While in TRAIL signaling, DR5 expression can be induced in several ways, this study has demonstrated that AW00179 induces DR5 expression by activating the ROS-JNK-c-Jun signaling axis, which consequently increased the sensitivity of H1299 cells to TRAIL.

In fact, the involvement of ROS generation and/or JNK-c-Jun signaling pathways in the sensitization of cancer cells to TRAIL-mediated apoptosis has been reported in several studies (Nakshatri et al. 2004; Prasad et al. 2011a, b; Yang et al. 2011). The ROS generation induced by chemicals acting as TRAIL sensitizers (such as the inactive analogue of PI3 K inhibitor LY303511, the synthetic triterpenoid CDDO-Me, perifosine, and ursolic acid) has been considered important for the activation of MAP kinases and the up-regulation of DR5, leading to apoptosis. LY303511-induced TRAIL sensitization was mediated by the induction of intracellular H_2O_2 production, which subsequently induces the activation of MAP kinases and the up-regulation of DR expression (Shenoy et al. 2009). The results showing that CDDO-Me-induced activation of JNK and up-regulation of DR5 expression were prevented by NAC also suggest the involvement of ROS generation and JNK signaling in TRAIL sensitization (Zou et al. 2008). In addition, perifosine (an alkylphospholipid tested in phase II clinical trials) increased the levels of phosphorylated JNK and c-Jun with the induction of DRs; however, perifosine-induced up-regulation of DR5 was abrogated by either SP600125 or JNK-specific siRNA (Fu et al. 2010). Furthermore, anti-oxidants (including NAC) inhibited the perifosine-induced elevation of p-c-Jun and DRs. Similarly, ursolic acid (a pentacyclic triterpenoid found in rosemary and holy basil) also potentiated TRAIL-induced apoptosis through the activation of ROS and JNK-mediated up-regulation of DRs (Prasad et al. 2011a).



AW00179 (μM)	% of CM-H2DCFDA -positive cells
5	10.34
10	12.04

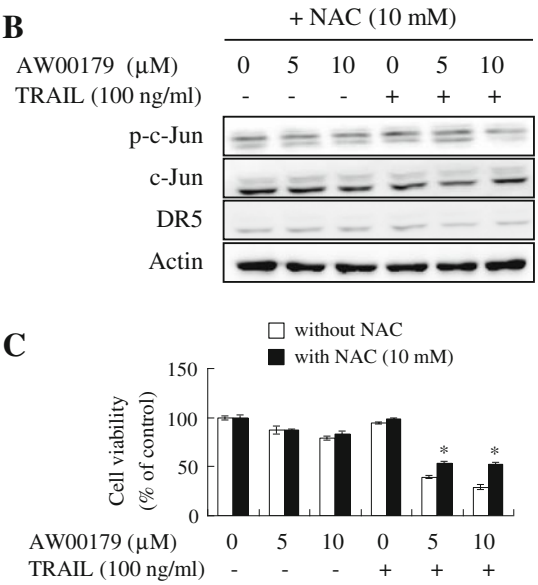


Fig. 5 AW00179-generated ROS sensitizes TRAIL-mediated cell death via c-Jun phosphorylation and DR5 up-regulation. **a** ROS generation induced by AW00179 was evaluated in H1299 cells. Cells (2.8×10^5 cells/well) were plated in a 6-well plate. After 24 h, cells were incubated with AW00179 for 3 h, stained with 5 μM CM-H2DCFDA for 15 min and evaluated by flow cytometry. **b** Effect of NAC on AW00179-induced phosphorylation of c-Jun and up-regulation of DR5 was evaluated in H1299 cells. Cells (2.8×10^5 cells/well) were plated in a 6-well plate. After 24 h, cells were pre-incubated with 10 mM NAC for 1 h and incubated with AW00179 alone or its combination with TRAIL (100 ng/ml) for 30 min (for c-Jun and p-c-Jun) or 16 h (for DR5 and actin) and then western blot analysis was carried out. **c** Effect of NAC on AW00179-induced death of H1299 cells was evaluated. Cells (1.4×10^3 cells/well) were plated in a 96-well plate. After 24 h, cells were pre-incubated with 10 mM NAC for 1 h and incubated with AW00179 alone or its combination with TRAIL (100 ng/ml) for 2 days. Then, cell viability was evaluated by CCK-8 assay. $^*P < 0.001$

In addition, in this study, AW00179-induced DR5 expression was likely to be prevented by the MEK-ERK signaling inhibitor PD98059; however, it could not strongly inhibit the cell death induced by AW00179 plus TRAIL, suggesting that ERK signaling may not be required for sensitizing H1299 cells to TRAIL-mediated cell death. However, in contrast to our results, several studies have reported the involvement of MEK-ERK activation in the expression of DR5 in TRAIL-mediated apoptotic signaling (Frese et al. 2003; Kannappan et al. 2010; Moon et al. 2010; Sung et al. 2010; Gupta et al. 2011).

The down-regulation of anti-apoptotic molecules could be another approach to overcome TRAIL resistance. With the exception of $FLIP_L$, anti-apoptotic molecules tested in this study were decreased by AW00179 or its combination with TRAIL, suggesting their involvement in the sensitization effect of AW00179 on TRAIL-mediated apoptosis. Although we could not fully elucidate whether the decrease of anti-apoptotic molecules by the combination of AW00179 with TRAIL potentiates or simply indicates TRAIL-mediated apoptosis, several studies have suggested that the down-regulation of anti-apoptotic molecules could sensitize cancer cells to TRAIL-mediated apoptosis.

FLIP is preferentially recruited to DISC, where it binds FADD and thwarts the activation of caspase-8 and caspase-10 (Irmler et al. 1997). Several studies have reported the involvement of $FLIP_S$ down-regulation in the sensitization of cells to TRAIL-mediated apoptosis (Lin et al. 2008; Kim et al. 2008a, b). Therefore, the down-regulation of $FLIP_S$ by AW00179 may account for its sensitization effect on TRAIL-mediated apoptosis.

The fundamental roles of Bcl-2 and Bcl-xL in TRAIL-mediated apoptosis have been proven by their specific inhibitors (Hetschko et al. 2008); the specific Bcl-2 inhibitor and the Bcl-2/Bcl-$_X$L inhibitor potently enhance apoptosis. However, in contrast, the ectopic expression of Bcl-2 and Bcl-xL completely abolishes TRAIL-induced cytotoxic effects (Lamothe and Aggarwal 2002). These results indicate the involvement of Bcl-2 family members in the sensitization of cells to TRAIL-mediated apoptosis.

IAP family members have also been linked with the sensitization of cells to TRAIL-mediated apoptosis. RNA interference-based knockdown of IAP family members, such as XIAP and survivin, has been shown to increase apoptosis in cancer cells when used with TRAIL (Chawla-Sarkar et al. 2004; Kim et al. 2004). In contrast, increased or sustained IAP protein levels in cancer cells confer resistance to TRAIL-induced apoptosis (Ng and Bonavida 2002; Ng et al. 2002; Lee et al. 2006).

In summary, AW00179 has been demonstrated to sensitize human lung cancer H1299 cells to TRAIL-mediated apoptosis through two distinct mechanisms: the ROS-JNK-c-Jun-mediated up-regulation of DR5, and the down-regulation of anti-apoptotic molecules. The identification of a TRAIL sensitizer with a new chemical scaffold, such as AW00179, might be an initial step toward developing efficient therapeutics for overcoming TRAIL resistance in future clinical applications. Further studies, including

animal experiments, are needed to identify a stronger sensitizer through the structure-activity relationship and determine the optimal ratio between TRAIL and its sensitizer(s) to improve the clinical efficacy of TRAIL.

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