Andrographolide sensitizes human renal carcinoma cells to TRAIL-induced apoptosis through upregulation of death receptor 4

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in cancer cells, with minimal toxicity to normal tissues. However, accumulating evidence suggests that certain cancer types are insensitive to TRAIL signaling. The aim of this study was to identify an effective combination regimen, which can overcome TRAIL resistance in renal cancer cell. Herein, we found that human renal carcinoma cells (RCCs) are widely resistant to TRAIL-mediated growth inhibition and subsequently identified that andrographolide (Andro), a major constituent of *Andrographis paniculate,* an annual herbaceous plant in the family *Acanthaceae*, counteracts TRAIL resistance in RCCs. Combined treatment with TRAIL and Andro suppressed cell viability as determined by MTS and proliferation as determined by EdU in a dose-dependent manner and inactivated the clonogenic and migration ability of RCCs. Andro significantly enhances TRAIL-mediated cell cycle arrest at the G2/M phase as determined by flow cytometry and senescence. Moreover, Andro restored TRAIL signaling, which in turns activated pro-apoptosis caspases as determined by immunoblot assay. The TRAIL receptor, death receptor (DR)4, but not DR5, was found to be significantly upregulated in Andro-treated RCC cells, which contributed to the role of Andro as a TRAIL sensitizer. The present study demonstrated that the combined treatment of Andro and TRAIL has potential therapeutic value against renal cancer.

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Introduction

Renal cell carcinoma (RCC) is one of the most commonly diagnosed human malignant neoplasms with more than 300,000 new patients diagnosed worldwide each year (1). The major type of kidney tumor (80-90%) originates from the epithelial lining of the proximal convoluted tubules and exhibits highly vascularized and metastatic characteristics (2). To date, the primary therapy for localized RCC is surgery (radical nephrectomy and nephron-sparing surgery), while for unresectable and metastatic RCC, the therapeutic options remain limited (3-5). RCC is sensitive to neither traditional chemotherapy nor radiation therapy (6). However, the existing therapies remain ineffective against metastatic and unresectable RCC. Therefore, exploring effective and safe strategies for the treatment of RCCs is crucial.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) family, is an optimal anticancer agent (7). The ability of TRAIL to induce apoptosis depends on the interaction of TRAIL and its membrane receptors death receptor (DR)4 and DR5 (named as TRAIL-R1 and TRAIL-R2) (8). Upon ligand stimulation, DR4 and DR5 bind Fas-associated death domain protein (FADD) through the death domain, which results in the formation of the death-inducing signaling complex (DISC). Caspase 8 is then recruited to DISC where it initiates the downstream apoptotic cascade. Activation of caspase 8 induces apoptosis via two well-elucidated apoptotic pathways: The extrinsic pathway (stimulating the effector caspases 3, 6, and 7) and the intrinsic-mitochondrial pathway [stimulating Bax and Bak, and releasing mitochondrial cytochrome *c* and mitochondrial-derived activator of caspase (Smac)] (9-11). As death receptors, DR4 and DR5 are normally upregulated in tumor cells, thus the TRAIL signaling pathway can be an optimal target for cancer therapy (12-14). Accumulating evidence

from basic and clinical studies indicates that various cancer types are not sensitized to TRAIL-induced apoptosis (15,16). TRAIL-based drug development has attracted significant interest to identify an effective combination regimen, which can overcome TRAIL resistance in cancer cells.

In renal cancer, a cancer type highly resistant to chemotherapy, the identification of specific agents that are able to sensitize TRAIL-induced apoptosis of unresponsive renal carcinoma cells holds the utmost importance for the targeted treatment of renal cancer. In the present study, our data showed that andrographolide (Andro), a major constituent of *Andrographis paniculate*, an annual herbaceous plant in the family *Acanthaceae*, remarkably improved the sensitivity of RCC cells to TRAIL-induced growth inhibition. The combined treatment stimulated caspase-dependent apoptosis, and enhanced DR4 expression. Our study provides proof-of-concept evidence for the clinical application of this traditional anti-inflammatory medical agent, andrographolide, in the treatment of renal cancers.

Materials and methods

Cell culture and treatments. The RCC cell lines 786-0, OS-RC-2, and ACHN were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). 786-0 cells were cultured in RPMI-1640 medium (HyClone; Cytiva). OS-RC-2 and ACHN cells were cultured in the DMEM medium (HyClone; Cytiva). All media were supplemented with 10% fetal bovine serum (FBS) (Biological Industries, USA) and penicillin/streptomycin solution. All cells were cultured under standard incubator conditions (37˚C, 5% CO_2).

Chemicals, reagents, and antibodies. Andrographolide (MedChemExpress, MCE) was dissolved in DMSO at 10 mmol/l as a stock solution, and recombinant human TRAIL (R&D Systems, Inc.) was prepared in PBS containing 0.1% bovine serum albumin at 20 μg/ml. Z-VAD (HY-16658) and Necrostatin-1 (HY-15760) were purchased from MCE. Antibodies used in this study were as follows: Phospho-HistoneH2A.X (product #9718), PARP1 (product #9532), DR4 (product #42533), caspase 9 (product #9502), caspase 8 (product #4790), GAPDH (product #51332) (from Cell Signaling Technology, Inc.), Bax (cat. #633601, BioLegend), DR5 (LM11912, Novus, USA), β-actin (ab8227, Abcam), anti-rabbit IgG (product #7054) and anti-mouse IgG (product #7056) (from Cell Signaling Technology, Inc.).

Cell viability assay. Cell viability was assessed by measuring the formazan production following the addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphop-henyl)-2H-tetrazolium, inner salt (MTS) (Promega Corp.). Approximately 5,000 cells/well were seeded in a 96-well plate and incubated at 37˚C in a 5% CO_2 incubator for 24 h. The cells were incubated in a medium containing 20 μl MTS for 3 h at 37˚C post treatment under different conditions for 24 h. Absorbance was detected using a BioTek ELISA reader (BioTek Instruments, Inc.) at a wavelength of 490 nm.

Cell proliferation assay. We used EdU (5-ethynyl-2'-deoxyuridine) and colony formation assays to evaluate the effect of Andro and/or TRAIL on cell proliferation. For the EdU Assay, $2x10^5$ cells/well were seeded in a 12-well plate, treated under Andro and/or TRAIL for 24 h, and then, cell proliferation was determined using BeyoClick EdU cell proliferation kit (Beyotime Biotech Inc.) according to the manufacturer's instructions. Images (x400 magnification) of the cells were acquired on a confocal microscope using OLYMPUS cellSens Standard software (Olympus).

For the colony formation assays, 200 cells/well were seeded in a 6-well plate in 2 ml of medium, treated under different conditions, and subjected to growth for 12 days. After 12 days of incubation, the cells were washed once with cold phosphate-buffered saline (PBS). Then, 4% paraformaldehyde was used to fix the cells for 20 min. Cells were then stained with 0.1% crystal violet solution for 15 min at 25˚C, and then washed with water thrice and air-dried for counting using an inverted microscope (x100 magnification), where cell colonies (>50 cells) were counted. All experiments were repeated thrice.

Cell migration assay. For cell migration, $2x10^5$ cells/well were seeded in a 6-well plate and incubated in an incubator. When the cells reached 90% confluence, straight scratches were made by using a sterile 200-μl pipette tip and the cells were then washed thrice with PBS. Then the cells were incubated in an incubator with serum-free medium containing Andro or/and TRAIL or DMSO for 24 h. An inverted microscope (x100 magnification) was used to monitor cell migration at 0, 6, 12, 18 and 24 h post scratching. Images of cells were acquired on a confocal microscope using OLYMPUS cellSens Standard software. Data were analyzed with Image J software (version 1.8.0, National Institutes of Health, Bethesda, MD, USA).

Flow cytometric analysis. For cell cycle analysis, cells treated under different conditions for 24 h were detached from the 6-well culture plates, washed twice with ice-cold PBS, and pelleted by centrifugation at 1,000 x g. The cells were then suspended in 75% ethanol overnight at -20˚C. Following an overnight suspension, cells were centrifuged at 1,000 x g for 5 min and washed twice with ice-cold PBS. The cell pellets were resuspended in buffer containing PI (propidium iodide) and RNase for 1 h in the dark at 37˚C, and the cell cycle distribution was examined by flow cytometry (BD Bioscience) after filtration.

Apoptotic cells were identified and quantified by using the Annexin V-FITC apoptosis detection kit (KeyGENBioTECH). After treatment under different conditions for 24 h, the cells were digested and collected with trypsin solution without EDTA, which were then washed twice with PBS and then centrifuged at 1,200 x g for 5 min to collect the cells. In the next step, the cells were re-suspended in binding buffer and incubated with Annexin V-FITC and PI for 15 min in the dark at 37˚C. A fluorescence-activated cell sorting (FACS) flow cytometer (BD Bioscience) was used to analyze cell apoptosis.

Cell senescence assay. Senescent cells were identified and quantified by Senescence β-Galactosidase staining kit (Cell Signaling Technology, Inc.). Following treatment under different conditions for 24 h, the cells were washed with PBS, fixed by the fixative solution for 15 min at 25˚C, and determined using Senescence β-Galactosidase (pH=6.0) staining

for 24 h. An inverted microscope (x400 magnification) was used to monitor senescent cells and to count them. Images of the cells were acquired on a confocal microscope using OLYMPUS cellSens Standard software.

Immunoblot assay. Whole-cell extracts, which were treated under different conditions for the corresponding times, were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (EMD Millipore), and 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (EMD Millipore) were used to visualize the protein bands. Images of the western blotting were acquired on a scanner (Epson Perfection V330 Photo) using Scan-n-Stitch Deluxe software (version 1.1.9, Arcsoft).

siRNAs for the construction of knockdown cells. Synthetic siRNA [negative-control siRNA, DR4 siRNA, and DR5 siRNA] which can specifically knock down the *TNFRSF10A* (DR4) gene and *TNFRSF10B* (DR5) gene, were obtained from GenePharma (Shanghai, China). The cellular delivery of siRNA was performed using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.), optimized using various siRNA concentrations, and evaluated by immunoblot assay. The siRNA sequences are listed in Table SI.

Data collection and bioinformatics analysis. We downloaded fragments per kilobase million (FPKM) values of RNA-sequencing profiles of RCC patients including 414 RCC tissues and 19 normal tissues from The Cancer Genome Atlas (TCGA) databse's official website (https://portal.gdc.cancer.gov/). RNA expression datasets were processed using the R software version 3.6.6 (https://www.r-project.org/).

Statistical analysis. Differences among test groups were analyzed by GraphPad Prism software (version 8.0; GraphPad Software Inc.). Data are expressed as the mean ± standard deviations (SD). An unpaired two-tailed Student's t test was performed to detect statistical difference between two individual experimental groups. For multiple comparisons, statistical analyses were performed using one-way analysis of variance (ANOVA) and two-way ANOVA with Dunnett and Tukey post-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Andro sensitizes TRAIL-induced survival and proliferation inhibition in renal cancer cells. As DR4 and DR5 are canonical TRAIL receptors involved in its antitumor effects, we analyzed mRNA expression data of RCC patients from the TCGA database. We found that the mean DR5 mRNA expression in renal cancer tissues exceeded that in normal tissues, whereas a mild was found in DR4 between tumor and normal tissues (Fig. 1A). These data hinted that TRAIL signaling could be a potential target for renal cancer therapy. However, our experiments indicated that renal cancer 786-0, OS-RC-2, and ACHN cells were resistant to the TRAIL-mediated suppression even at an extremely high concentration (200 ng/ml), while our previous study demonstrated that the 50% inhibitory concentration IC_{50} value of TRAIL in bladder cancer T24 cells was 38.35 ng/ml (Fig. 1C-E) (17). As noted, andrographolide (Andro), a diterpene lactone ($C_{20}H_{30}O$) (Fig. 1B), represents a potential agonist for TRAIL therapy. The IC_{50} of Andro was 50.19 μM in 786-0 cells, 45.32 μM in OS-RC-2 cells, and 45.55 μM in ACHN cells (Fig. 1C-E). Interestingly, cell viability of the RCC cell lines treated with the combination of Andro and TRAIL for 24 h was significantly decreased as compared with that of the cells treated with TRAIL or Andro alone (Fig. 1C-E).

Next, we evaluated the ability of Andro to sensitize TRAIL-mediated proliferation inhibition in RCC cells. As shown in Fig. 2B, TRAIL (50 ng/ml) or Andro (5 μM or 10 μM) alone mildly inhibited the growth rate of renal cancer cells. In contrast, Andro significantly sensitized 786-0 cells to TRAIL-mediated proliferation inhibition at a concentration of 5 μM. In agreement with this result, the morphological changes in treated RCC cells further supported that the combined treatment with TRAIL and Andro inhibited the survival and proliferation of 786-0 (Fig. 2A), OS-RC-2 (Fig. S1A) and ACHN cells (Fig. S2A) more potently than single-drug treatment. Furthermore, EdU cell proliferation assay showed that 786-0 cells treated with the combination of TRAIL and Andro proliferated much more slower than the cells exposed to single-drug treatment (Fig. 2C).

Andro promotes TRAIL-dependent inhibition of the clone formation and migration of renal cancer cells. Subsequently, we conducted clonogenic assays to determine the long-term anti-proliferative effects of Andro and TRAIL in invasive renal cancer cells. Our data indicated that the colony formation in case of cells treated with the combination of Andro and TRAIL was significantly (75%) inhibited compared to that in cells treated with only Andro or TRAIL in 786-0 (Fig. 2D), OS-RC-2 (Fig. S1B) and ACHN cells (Fig. S2B) cells.

To determine whether Andro increases the ability of TRAIL to suppress RCC migration, we applied wound healing measurements as functional readings. The results indicated that TRAIL or Andro alone modestly (<25%) decreased the migration of RCC 786-0 (Fig. 2E), OS-RC-2 (Fig. S1C) and ACHN cells (Fig. S2C) cells. However, there was an approximately 95% decrease in RCC migration induced by the combined treatment of Andro and TRAIL. These findings demonstrated that Andro effectively enhanced the suppression of the growth and migration of renal cancer cells mediated by TRAIL.

Andro enhances TRAIL-induced G2 cell cycle arrest and senescence in renal cancer cells. To understand the mechanism through which the combined treatment of Andro and TRAIL inhibited cell proliferation, we investigated the effects of the indicated drugs on the cell cycle distribution of RCC cells, and demonstrated that TRAIL (50 ng/ml) or Andro (5 μM) alone did not have a significant effect on cell cycle distribution. Yet, in the case of 786-0 cells treated with the same amounts of TRAIL and Andro significant cell cycle arrest at the G2 phase was triggered (Fig. 3A and B).

Moreover, RCC cells treated with a combination of Andro and TRAIL appeared larger, and flat morphological changes with time were exacerbated, which indicated that cell senescence was exacerbated. This was confirmed by the β-galactosidase staining assay (Fig. 3C and D).

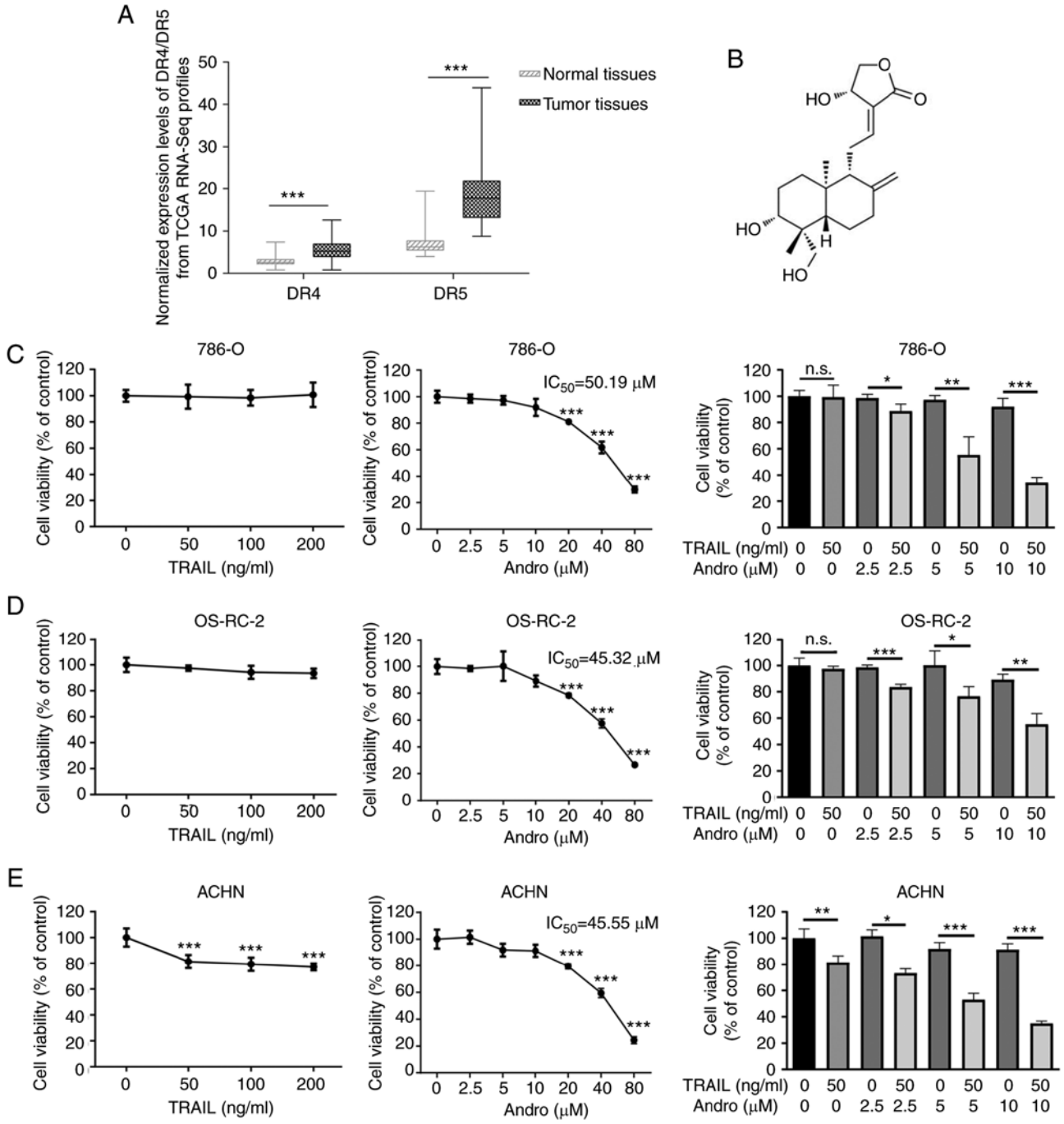

Figure 1. TRAIL combined with Andro inhibits RCC cell viability. (A) Normalized mRNA expression levels of DR4/DR5 in normal renal tissues and RCC tissues from TCGA RNA-Seq profiles. (B) Chemical structure of Andro. (C-E) Effects of TRAIL and Andro on the cell viability of 786-0 (C), OS-RC-2 (D) and ACHN (E) cells. Statistical analysis was carried out by one-way ANOVA and Dunnett's multiple comparisons test. Data are shown as mean ± SD; n.s. (not significant), $P>0.05$; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; n=3). Andro, andrographolide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TCGA, The Cancer Genome Atlas.

Combination of Andro and TRAIL triggers apoptosis in renal cancer cells. In RCC 786-0 cells treated with the combination of Andro and TRAIL, we observed apoptotic features, such as cell contraction, rounding, and floating. We then evaluated the roles of Andro in apoptosis progression using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI)-labeled flow cytometry. Compared with groups that were solely treated with TRAIL (4.07±0.29%) or Andro (6.33±0.24%), the groups treated with their combination for 24 h exhibited 39.26±1.17% apoptosis (Fig. 4A). Immunoblot assays were used to analyze changes in protein content in 786-0 cells treated with TRAIL and/or Andro. The results indicated that the combined treatment enhanced levels of cleaved-poly(ADP ribose) polymerase 1 (PARP1; 89 kDa), cleaved caspase 8 (17 kDa), and cleaved caspase 9 (35 kDa). It also decreased full-length caspase 8 (60 kDa) and caspase 9 (45 kDa) expression while increasing levels of apoptosis regulator Bax (21 kDa), indicating caspase 8 activation and initiation of the apoptotic signal (Fig. 4B). We also noted that the levels of the phosphorylated form of H2AX

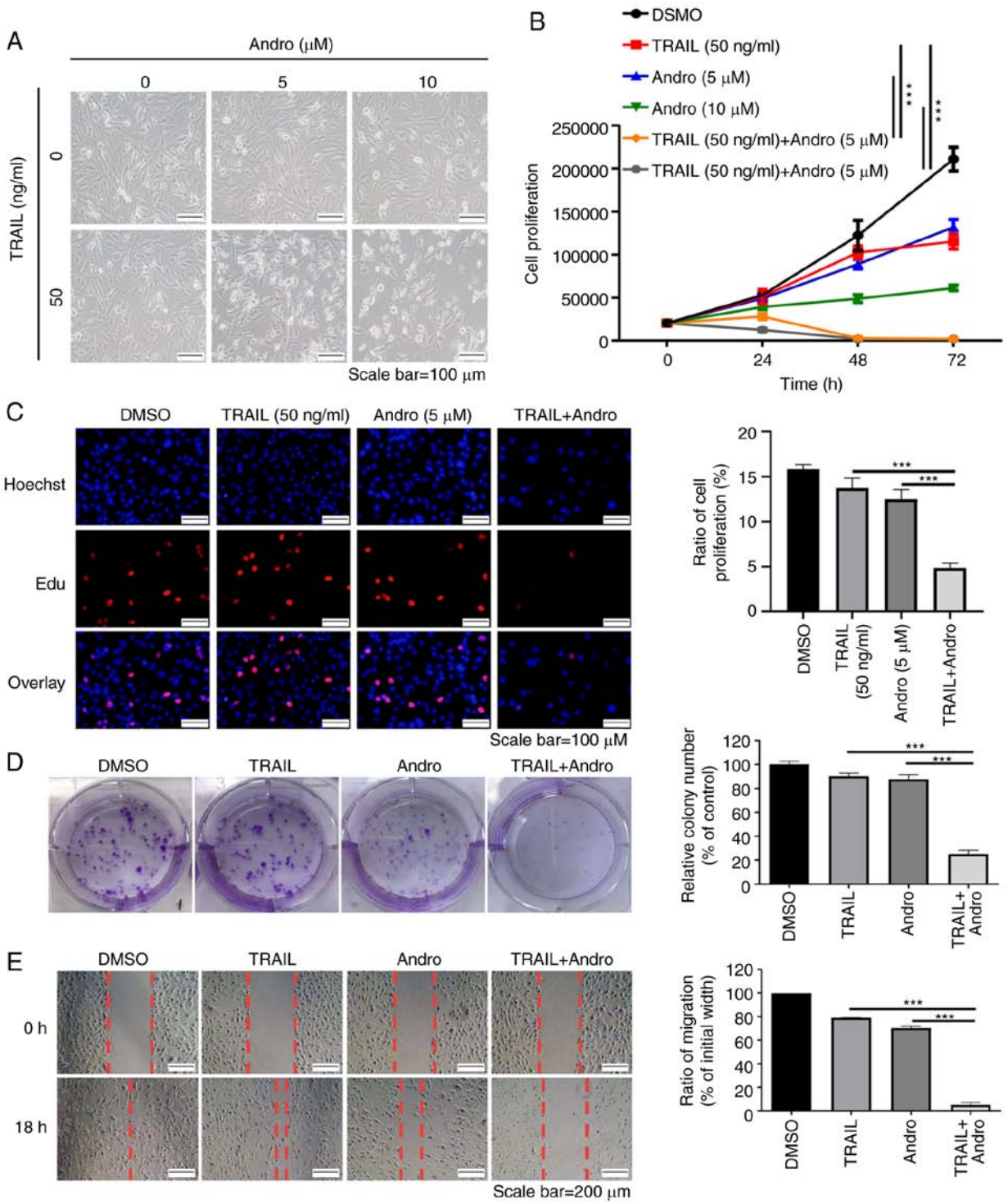

Figure 2. TRAIL combined with Andro inhibits the cell proliferation, colony formation, and migration of 786-0 cells. (A) Images (x200 magnification) show 786-0 cell morphology after treatment with various concentrations of TRAIL and/or Andro for 24 h. (B) Cell proliferation of 786-0 cells after treated with various concentrations of TRAIL and/or Andro for 24, 48 and 72 h (two-way ANOVA, Tukey). (C) Images (x200 magnification) show cells that were treated with TRAIL (50 ng/ml) and/or Andro (5 μM) for 24 h, and then proliferation was determined by BeyoClick EdU cell proliferation kit. Hoechst staining shows the entire nucleus, and EdU shows the nucleus which was proliferating. The histogram (right) shows the ratio of proliferation (one-way ANOVA, Tukey). (D) Effects of TRAIL (50 ng/ml) and Andro (0.5 μM) on the clonogenic formation of 786-0 cells. The histogram indicates the percentage of each group's clony number compared to the control group (one-way ANOVA, Tukey). (E) Images (x100 magnification) show the effects of TRAIL (50 ng/ml) and Andro (5 μM) on the migratory ability of 786-0 cells. The histogram indicates the percentage of migration compared to the initial width (one-way ANOVA, Tukey). Data are shown as mean ± SD; ***P<0.001; n=3). Andro, andrographolide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

(γ-H2AX) were increased in Andro and TRAIL combined treated groups (Fig. 4B). We also showed that the combination therapy also potently induced apoptosis in other RCC cell lines (OSR-C and ACHN cells); supporting that Andro enhanced TRAIL-induced apoptosis independent of RCC cell type (Fig. S3).

Additionally, we found that RCC apoptosis induced by combined treatment was initiated by caspase-specific

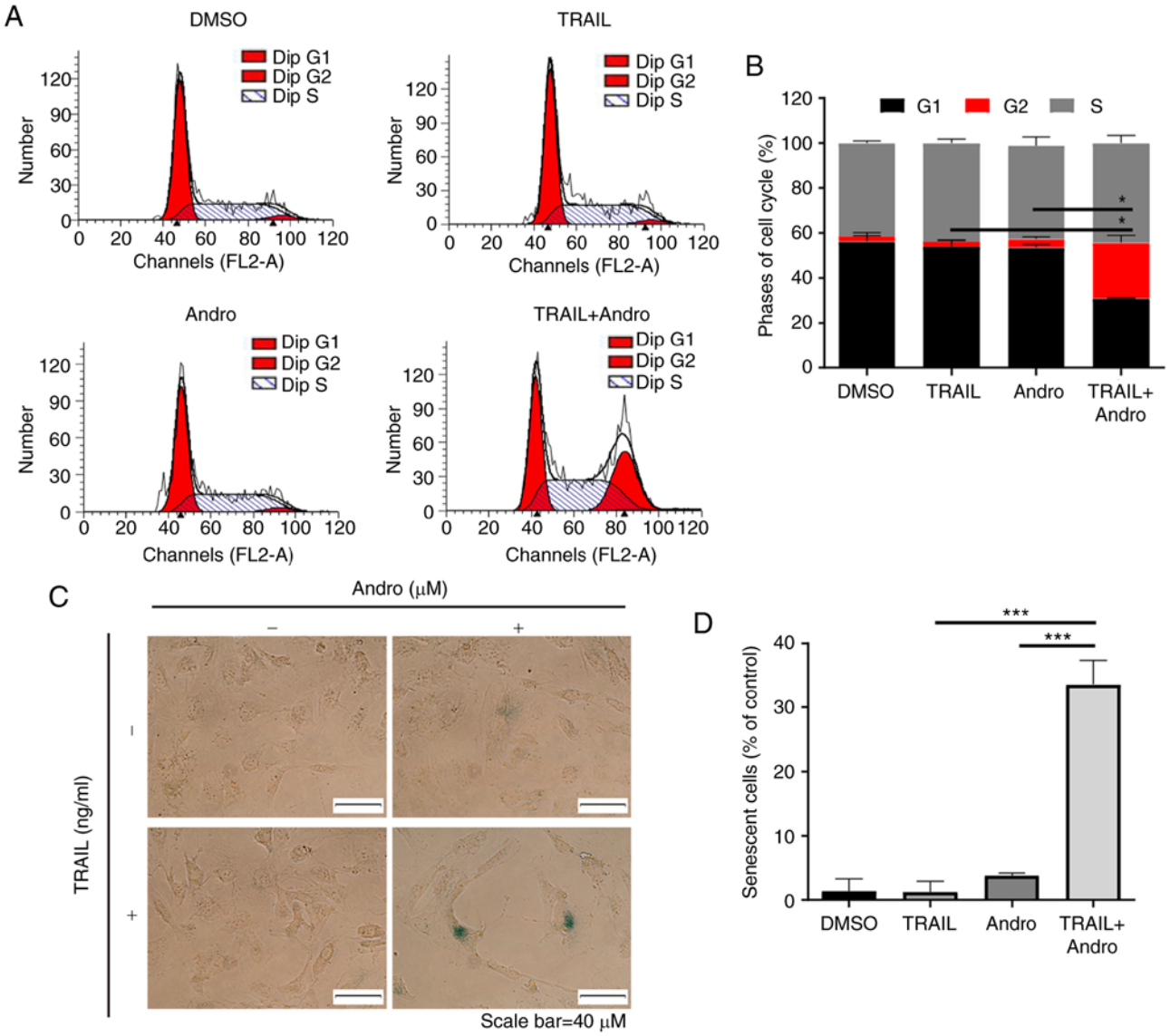

Figure 3. TRAIL combined with Andro induces G2 cell cycle arrest and senescence in 786-0 cells. (A) Cell cycle arrest by combined treatment with TRAIL (50 ng/ml) and Andro (5 μM) for 24 h. (B) The histogram shows the percentage of cells in each cell cycle phase (two-way ANOVA, Tukey). (C) Cells were treated with TRAIL (50 ng/ml) and/or Andro (5 μM) for 24 h before being subjected to Senescence β-Galactosidase staining. Images (magnification, x400) show the senescence of 786-0 cells after the treatments. (D) The histogram indicates the percentage of senescent cells of all cells in a view (one-way ANOVA, Tukey). Data are shown as mean ± SD; ***P<0.001, n=3). Andro, andrographolide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

activation that did not involve cell necrosis. The antitumor effects of the Andro and TRAIL combined treatment was almost blocked by a pan-caspase inhibitor Z-VAD (0.05 μM, 91.72±4.21%), but not by cell-necrosis inhibitor necrostatin-1 (0.05 μM, 45.67±3.29%). This further confirms that Andro enhanced TRAIL-mediated caspase-dependent apoptotic cell death in RCC cells (Fig. 4C). Cell morphology was also consistent with the MTS assay results (Fig. 4D).

Andro sensitizes TRAIL-induced apoptosis via upregulation of DR4. Our immunoblot assays demonstrated that Andro treatment selectively upregulated protein levels of DR4 (Fig. 5A), but not of DR5 (Fig. 5B). To determine whether one or both receptors are responsible for the pro-apoptotic effect of TRAIL in RCC cells, we used small RNA interference to block endogenous DR4/DR5 translation according to their knockdown efficiency determined by immunoblotting (Fig. 5E). The results demonstrated that cell viability was slightly restored in the DR5-knockdown cells (53.10±2.71%) and restored to a higher degree in the DR4-knockdown cells (80.07±3.71%) following combination treatment with TRAIL and Andro (Fig. 5D). Cell apoptosis assays and clonogenic assays further supported the important roles of DR4 in the effects of the combined treatment of TRAIL and Andro (Fig. 5F and G).

Discussion

Renal cell carcinoma (RCC) is the third most prevalent urinary tumor and claims more than 100,000 lives each year worldwide (18). At present, the primary treatment for RCC, either localized RCC or locally advanced RCC, is surgery. However, for patients with metastatic RCC, surgery does not significantly improve the prognosis or quality of life (4). Moreover, RCC is neither sensitive to radiotherapy nor chemotherapy

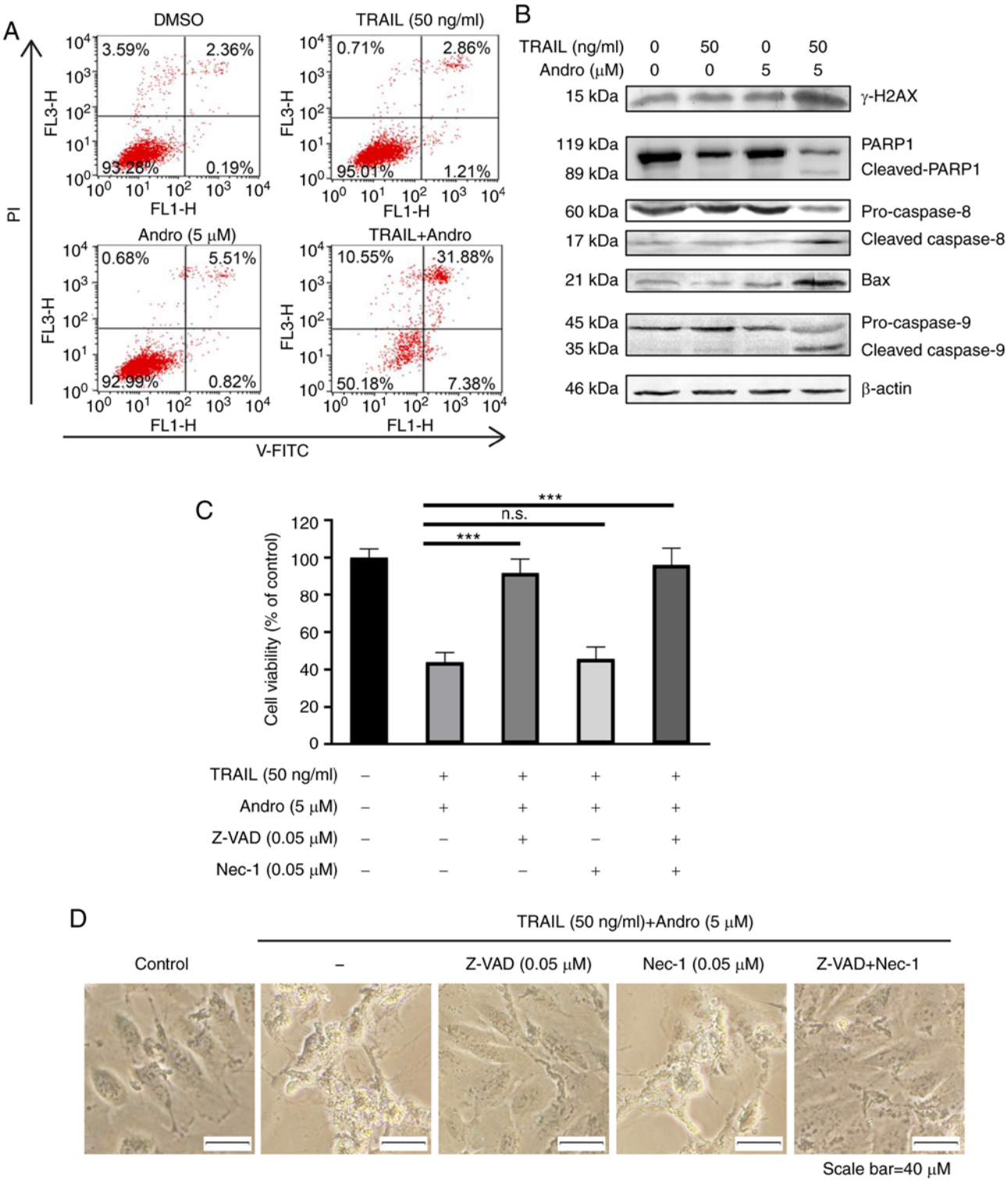

Figure 4. Combined treatment of Andro and TRAIL induces caspase-dependent apoptosis in 786-0 cells. (A) Cell apoptosis was determined by Annexin V-FITC after DMSO, TRAIL (50 ng/ml), and/or Andro (5 μM) treatment for 24 h. (B) Indicated protein levels in 786-0 cells treated with TRAIL (50 ng/ml) and/or Andro (5 μM) for 24 h as detected by immunoblotting. (C) Cells were treated with DMSO, TRAIL (50 ng/ml), and Andro (5 μM), pan-caspase inhibitor Z-VAD (0.05 μM), and cell-necrosis inhibitor necrostatin-1 (Nec-1) (0.05 μM). Then cell viability was determined by MTS assay (one-way ANOVA, Tukey). (D) Images (magnification, x400) show the apoptotic cells following treatment under different conditions. Data are shown as mean ± SD; n.s. (not significant), P>0.05, ***P<0.001, n=3). Andro, andrographolide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PI, propidium iodide; PARP1, poly(ADP ribose) polymerase 1; Bax, Bcl-2 associated X, apoptosis regulator, DR, death receptor.

and has a low response to cytokine therapy (19). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising agent for anticancer therapy due to its ability to selectively trigger cancer cell death (20). Moreover, in contrast to other members of the TNF superfamily, TRAIL administration *in vivo* is harmless (21-23). However, the resistance of cancer cells to TRAIL-mediated apoptosis is a major limitation to its clinical application (24,25). In the present study, we

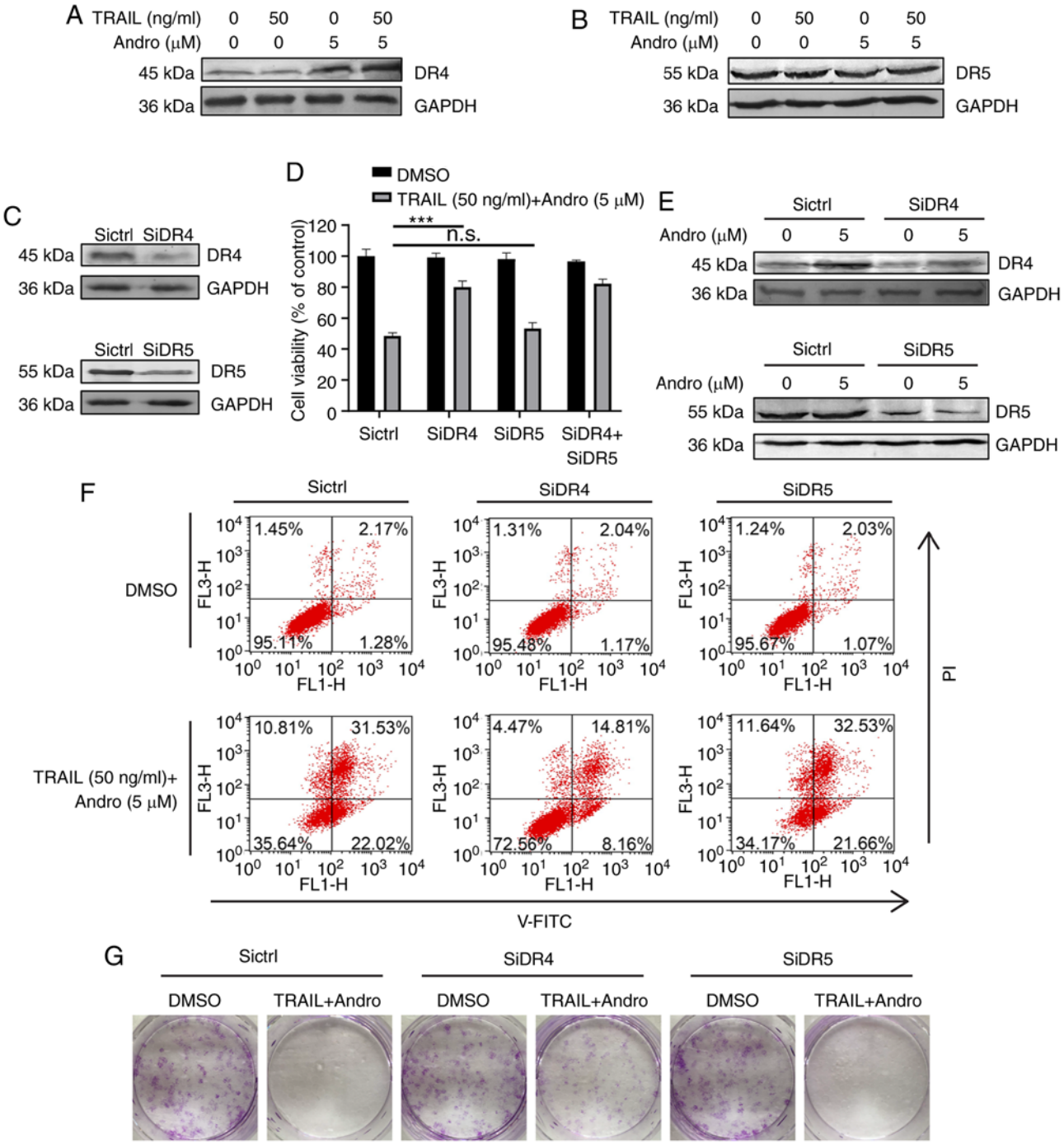

Figure 5. DR4 is critical for Andro-mediated sensitization of 786-0 cells to TRAIL. (A and B) DR4 and DR5 protein levels in 786-0 cells treated with TRAIL (50 ng/ml) and/or Andro (5 μM) for 24 h. (C) DR4 and DR5 protein levels in siRNA-targeted DR4 or DR5-transfected (siDR5 and siDR4) cells. (D) Cell viability of 786-0 cells transfected with siDR4 and/or siDR5 and treated with the combination of TRAIL (50 ng/ml) and Andro (5 μM) for 24 h (two-way ANOVA, Tukey). (E) DR4 and DR5 protein levels in siRNA-targeted DR4 or DR5-transfected cells and treated with Andro (5 μM) for 24 h. (F) Cell apoptosis was determined by Annexin V-FITC after transfection with siDR4 or siDR5 and treated with the combination of TRAIL (50 ng/ml) and Andro (5 μM) for 24 h. (G) Effects of TRAIL (50 ng/ml) and Andro (0.5 μM) on the clonogenic formation of 786-0 cells after transfection with siDR4 or siDR5. Data are shown as mean ± SD; n.s. (not significant), $P>0.05$, $^{***}P<0.001$, n=3). Andro, andrographolide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR, death receptor; PI, propidium iodide.

confirmed that human RCC cell lines were widely unresponsive to TRAIL-mediated cytotoxicity, which was primarily due to low expression levels of its receptor in these RCC cells. Hence, it is necessary to assess and find novel TRAIL sensitive agents with high efficacy and low toxicity.

In this preclinical study, we showed that andrographolide (Andro), a major constituent of *Andrographis paniculate*, an annual herbaceous plant in the family *Acanthaceae*, a natural compound, restored the sensitivity of RCC cell lines to TRAIL-mediated apoptosis. The findings provide critical insight into a novel therapeutic strategy for RCC patients. Andro administration enhanced TRAIL-mediated inhibition of cell viability, proliferation, migration, and colony formation of RCC cell lines. Moreover, our data revealed that

combination therapy also potently inhibited the proliferation of diverse RCC cell lines, suggesting that Andro enhanced the anticancer activity of TRAIL independent of RCC cell type.

Most cancer cells resist apoptosis (26,27). The combined treatment with TRAIL and Andro potently triggered cell cycle arrest, senescence, and apoptosis in RCCs, which largely relied on its ability to specifically increase death receptor (DR)4 expression. Elevation of membrane associated DR4 expression by Andro treatment amplified TRAIL-mediated initiation of apoptosis, cleavage of PARP1, and caspase activation. A pan-caspase inhibitor (Z-VAD-FMK), but not the necrosis inhibitor, Necrostatin-1, almost fully restored cell viability in RCC cells treated with both TRAIL and Andro, further supporting an Andro-specific increase in the cytotoxicity of TRAIL in RCC cells through its induction of caspase-dependent apoptosis. All of these results revealed that Andro treatment acts synergistically with TRAIL treatment on RCC cells.

In-depth understanding of the causes of TRAIL resistance in renal cancer may help to better develop drugs that are more effective. TRAIL binding to its receptors (DR4 and DR5) to initiate DISC assembly subsequently activates the caspase cascades and triggers apoptosis (28). Accumulating evidence suggests that an increase in TRAIL receptors is an effective strategy for enhancing the sensitivity of cancer cells to TRAIL-mediated effects (29-31). The tumor suppressor p53 is a key apoptosis regulator limiting cancer development via its proapoptotic function (11,32). Transcriptional activation of death receptors by p53 is essential for its tumor-suppressing functions. Recently, our findings and that of other authors have demonstrated that Andro activates p53 signaling which results in DR4 or DR5 upregulation in other cancer cell types (17,33-35). It has also been known that p53 signaling stimulates the *DR5* gene through an intronic sequence-specific DNA-binding site (36). In addition, previous findings that DNA damage-induced p53 activation leads to DR4 upregulation further supports the essential role of p53 signaling in the regulation of death receptors expression (37).

Interestingly, unlike our previous report that elevation of DR5 but not DR4 expression is one of the determinant factors for Andro-mediated sensitization of bladder cancer cells to TRAIL, we found that the expression levels of DR4 but not DR5 are critical for counteracting TRAIL-resistance in RCCs by Andro (17). These data hint that TRAIL signaling in diverse cancer types is selectively initiated by a certain TRAIL receptor, DR4 or DR5. Future studies need to clarify the detailed strategies of cancer cells to evade suppression by TRAIL. Furthermore, clinical database analysis revealed that a modest increase in mRNA expression levels of DR4 was noted in RCC patients which was in contrast to the dramatic elevation of DR5 mRNA levels. These results imply that the low expression of DR4 is one determinant strategy for the evasion of TRAIL proapoptosis signaling by renal cancer cells.

The potential application of andrographolide in clinical cancer treatment has several advantages (38,39). Andro is widely distributed in various plants of the genus *Andrographis* and has been used for centuries in Asia (40). Andro possesses therapeutic effects against various conditions, such as carcinoma, arthritis, ischemia, pyrogenesis, and oxidative stress (41-44). Due to its short half-life, Andro can be excreted from the body at a high rate with almost no toxic effects to normal cells (45). Considering these features, our results indicated that Andro counteracts TRAIL resistance in RCC cells providing proof-of-concept evidence for the clinical investigation of combined treatment of TRAIL and the traditional anti-inflammatory agent, andrographolide, in renal carcinoma therapy.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

WW, CW, and YD conceived and designed the experiments. RB, YD, CT, LX, BX performed the experiments and collected and analyzed the data. WW with the help of CW and BR wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the First Hospital of Jilin University (Changchun, Jilin, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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