# Overcoming resistance to TRAIL-induced apoptosis in solid tumor cells by simultaneously targeting death receptors, c-FLIP and IAPs

YING HUANG[1,2*], XIANG YANG[2,3*], TIANRUI XU[2], QINGHONG KONG[2], YAPING ZHANG[2], YUEHAI SHEN[2], YUNLIN WEI[2], GUANLIN WANG[2] and KWEN-JEN CHANG[2]

[1]Faculty of Environmental Science and Engineering, [2]Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, Yunnan 650500; [3]Institute of Translational Medicine, Zhejiang University, Hangzhou 310058, P.R. China

Received March 18, 2016; Accepted April 15, 2016

DOI: 10.3892/ijo.2016.3525

**Abstract.** The discovery of the TRAIL protein and its death receptors DR4/5 changed the horizon of cancer research because TRAIL specifically kills cancer cells. However, the validity of TRAIL-based cancer therapies has yet to be established, as most cancer cells are TRAIL-resistant. In this report, we demonstrate that TRAIL-resistance of many cancer cell lines can be overcome after siRNA- or rocaglamide-mediated downregulation of c-FLIP expression and simultaneous inhibition of IAPs activity using AT406, a pan-antagonist of IAPs. Combined triple actions of the TRAIL, the IAPs inhibitor, AT406, and the c-FLIP expression inhibitor, rocaglamide (ART), markedly improve TRAIL-induced apoptotic effects in most solid cancer cell lines through the activation of an extrinsic apoptosis pathway. Furthermore, this ART combination does not harm normal cells. Among the 18 TRAIL-resistant cancer cell lines used, 15 cell lines become sensitive or highly sensitive to ART, and two out of three glioma cell lines exhibit high resistance to ART treatment due to very low levels of procaspase-8. This study provides a rationale for the development of TRAIL-induced apoptosis-based cancer therapies.

## Introduction

Traditional chemotherapy and radiotherapy of cancer treatments suffer from severe side effects, development of drug resistance and cross-resistance, cancer migration and recurrence (1). In light of recent breakthroughs in molecular oncology, targeted therapies using monoclonal antibodies to mutated cell surface receptors and small molecule agents inhibiting tyrosine kinases, serine/threonine kinases, small GTP-binding proteins and other oncogenic proteins in the proliferation-driving signaling pathways have become standard in the current treatment of cancer (2). However, due to the genetic heterogeneous nature of cancer cells, particularly for solid tumors, resistance to these targeted agents can develop rapidly, and thus limit the overall efficacy (3). Novel cancer therapies are needed. One potential immuno-surveillance mechanism for therapy is apoptosis induced by cytokines produced by immune cells such as T and natural killer (NK) cells.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), discovered two decades ago by Wiley *et al* (4) and Pitti *et al* (5), attracted enthusiastic attention worldwide as a potential cancer therapy because of its capacity to specifically induce cancer cell death, but not the death of normal and healthy cells (6). TRAIL produced from immune NK cells (7), can induce apoptosis of cancer cells upon binding to the cell surface death receptors (DR, TRAIL receptor), DR4 (or TRAIL R1) and/or DR5 (or TRAIL R2). In addition, TRAIL recruits the adaptor Fas-associated death domain (FADD) and procaspase-8 to form death-inducing signaling complexes (DISC), which results in the activation of the initiator caspase-8, leading to the activation of extrinsic and intrinsic apoptotic signaling downstream of caspase-3 (4,8). Recently, several phase 2 clinical studies based on the use of recombinant human TRAIL or agonistic monoclonal antibodies against DR4/5 have failed to show clinical efficacy, even when combined with traditional chemotherapy (9,10). Thus, enthusiasm has greatly dampened for cancer therapies based on TRAIL-induced apoptosis. Moreover, in the past decade, studies have demonstrated that only a small portion of cancer cells are sensitive to TRAIL, while most tumors were TRAIL-resistant (11,12). This property limits the potential of TRAIL-based cancer therapy.

Currently, inhibitors of the apoptosis proteins, cellular FLICE-like inhibitory protein (c-FLIP) and inhibitors of

*Correspondence to:* Professor Kwen-Jen Chang or Dr Guanlin Wang, Faculty of Life Science and Technology, Kunming University of Science and Technology, 727 South Jingming Rd., Chenggong, Kunming, Yunnan 650500, P.R. China
E-mail: kwenjenchang@yahoo.com
E-mail: glwang83@foxmail.com

*Contributed equally

*Key words:* TRAIL, c-FLIP, IAPs, cancer therapy, apoptosis, drug combination

apoptosis protein (IAPs, including XIAP) are considered to be responsible for cellular TRAIL resistance. The utility of TRAIL-based therapy is dependent on mitigating this TRAIL resistance. IAPs bind to downstream executor caspases-3/6/7/9 to inhibit their activities and prevent the execution of apoptosis (13,14). To overcome this obstacle, IAPs antagonists with excellent activity *in vivo* have been developed, and several of these antagonist (e.g., AT406) are currently under clinical investigation (15-18). These IAP antagonists are second mitochondria-derived activator of caspase (Smac) mimetics. c-FLIP, a procaspase-8 homologue, can compete with procaspase-8 to bind to the death effective domain (DED) of FADD and block the apoptotic signal from upstream of the apoptosis pathway (19). *In vitro* studies with some cytotoxic anticancer agents revealed that the downregulation of c-FLIP induced by these agents was partly responsible for their pro-apoptotic effects (20).

However, there is no specific antagonist available for c-FLIP (21). Downregulating the expression of c-FLIP through specific siRNA sensitized resistant melanoma cells to TRAIL-induced apoptosis (22). Rocaglamide, a natural product isolated from *Aglaia* species, is a translational inhibitor of *de novo* c-FLIP synthesis (23,24). Previous studies showed that a c-FLIP inhibitor and a XIAP inhibitor cooperatively sensitized TRAIL-mediated apoptosis in Hodgkin's lymphoma cells (25). However, no studies have shown that a triple combination can be effective in other solid tumors. Recent genetic analysis for various tumor cells revealed the extremely heterogeneous nature of cancers (1). The results in a single cancer cell line cannot be generalized to other types of cancer cells without empirical evidence. Furthermore, there is no safety testing on normal cells for this combination treatment. In our investigation, a combination of AT406 (A) a pan-antagonist of IAPs, rocaglamide (R) or c-FLIP-siRNA and TRAIL (T) (ART triple combination) was used to evaluate its possible broad spectrum activities on selected 17 solid cancer cell lines (from different tissues or organs), three glioma cell lines and two normal cells (pulp cells and MRC5). In addition, various combination effects were assessed. Our study showed that the ART-triple combination may be applied as a broad-spectrum antitumor therapeutic approach for cancer treatment. We also confirmed that our triple combination treatment had no harmful effects on normal cells tested, similar to TRAIL-only treatment. These features provide a theoretical and experimental basis for the TRAIL-induced apoptosis pathway as a potential target for cancer treatment.

## Materials and methods

*Cell lines and culture conditions*. The cancer cell lines U87, SW480, U251 and U373 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HCT116, HT29, LOVO, H460, SK-OV-3, MDA-MB-231, A549, MCF7, SK-BR-3, T-47D, BT474, U2OS, HeLa, HepG2, MDA-MB-468, Vcap, and MRC5 were purchased from ATCC (MD, USA). HCT116, HT29, LOVO, H460, SK-OV-3, MDA-MB-231, A549, U87, MCF7, SK-BR-3, T-47D, BT474 and SW480 were maintained in RPMI-1640 (Hyclone, USA). U2OS, HeLa, HepG2, MDA-MB-468, Vcap, U251 and U373 were cultured in Dulbecco's modified minimal essential medium (DMEM) growth medium (Hyclone). MRC5 cells (human embryonic lung cells) were maintained in MEM growth medium (Hyclone). All culture media were supplemented with 10% fetal bovine serum (Hyclone). All cancer cells were maintained in a humidified incubator at 37˚C with 5% $CO_2$, and passaged with 0.25% trypsin-EDTA when ~80% confluence was reached. The pulp cells were isolated and cultured according to a previously described method (26).

*Antibodies and chemicals*. The antibodies for immunoblotting were from the following sources: mouse anti-caspase-8 p55/53/43/41/18 (#9746), rabbit anti-PARP p118/89 (#9532) and mouse anti-caspase-3 p35/19/17 (#9668) were from Cell Signaling Technology (Beverly, MA, USA); mouse anti-FADD (#F8053) was from Sigma (St. Louis, MO, USA); rabbit anti-DR5 was from Abcam (Cambridge, MA, USA); mouse anti-c-FLIP (clone 7F10, #ALX-804-961-0100) was from Enzo Life (New York, NY, USA) and mouse anti-GAPDH (sc-365062) was from Santa Cruz Biotechnology (Dallas, TX, USA). Recombinant hTRAIL (#310-04) was from R&D Systems (Minneapolis, MN, USA), rocaglamide (#350-121-C100) was purchased from Enzo Life, AT406 (#S2754) was from Selleckchem (Houston, TX, USA) and valproic acid was from J&K Scientific.

*Cell viability assay*. Cells were seeded in 96-well cell culture plates and treated the next day with the given agents for the indicated times. The cells were then incubated with 100 $\mu$g/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37˚C for 4 h. Finally, the medium was discarded carefully and 150 $\mu$l of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The absorbance was measured using a Microplate Reader (Perkin-Elmer 2030) at a wavelength of 490 nm. The experiments were performed in triplicate.

*Caspase activity*. Cells were seeded in 96-well cell culture plates (white/black walled) and treated the next day with the given agents for the indicated times, then manipulated according to the technical bulletin of Caspase-Glo 3/7 assay (Promega).

*Western blotting*. For each sample, $1x10^6$ cells were lysed using a solubilizing solution [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM PMSF, 0.02% $NaN_3$, protease inhibitor cocktail tablet; Roche, Mannheim, Germany]. Protein concentration was determined using a Bio-Rad Protein assay kit (Hercules, CA, USA). An equal quantity (10-30 $\mu$g) of proteins was separated by 10-15% SDS-PAGE and transferred onto a PVDF membrane (Millipore Corp., Billerica, MA, USA). The membrane was blocked in 10% skim milk (in TBS, pH 7.2, containing 0.1% Tween-20) overnight at 4˚C, then incubated with primary antibodies followed by peroxidase-conjugated anti-mouse or anti-rabbit IgG (Thermo Fisher, Inc., Rockford, IL, USA). The epitope was detected using an ECL western blot detection kit (Millipore Corp.). GADPH was used as an internal control for all western blots.

*Data analysis*. Data were expressed as the means ± SD values for western blots or mean ± SEM for cell viability MTT assays. Statistical analysis was performed by using Graphpad Prism 5 software. The $IC_{50}$ values were evaluated by SPSS version 19.

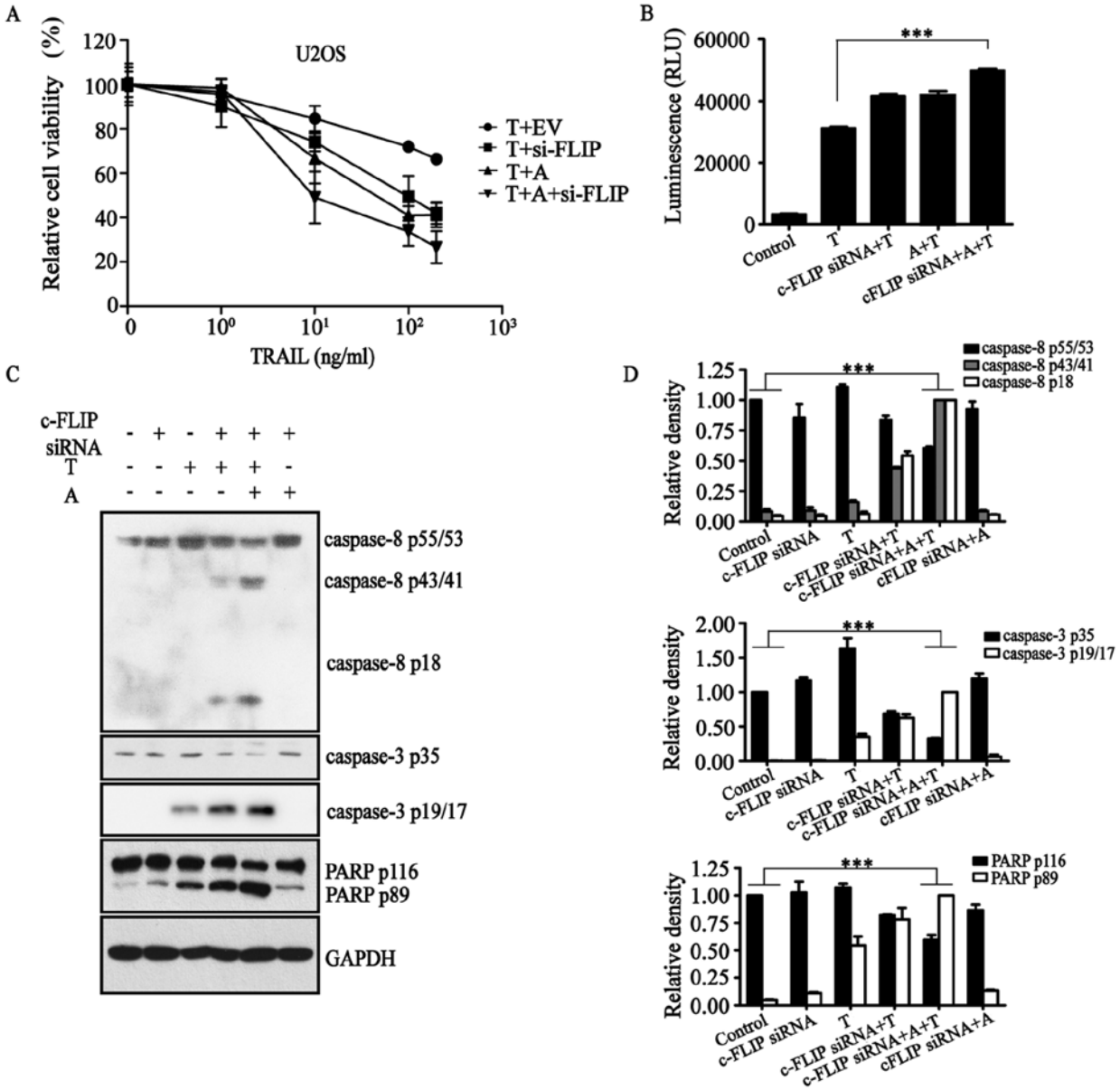


Figure 1. The combination of AT406 and downregulation of c-FLIP$_{L/S}$ by c-FLIP-siRNA sensitizes the osteosarcoma cells U2OS to TRAIL-induced apoptosis. (A) The osteosarcoma cells U2OS were treated with different concentrations of TRAIL for 24 h. In three other concentration-response experiments, cells were pre-treated with AT406 (A) for 1 h and with or without transient transfection with c-FLIP-siRNA for 24 h. The cell viabilities were determined by an MTT assay. The results are shown as the mean ± SEM of three separate experiments. (B) Caspase activity of U2OS cells in response to TRAIL with or without pre-treatments of A and c-FLIP-siRNA as indicated in the graph was measured by a Caspase-Glo 3/7 assay. (C) The U2OS cells were treated with TRAIL for 5 h, and with or without pre-treatment of A for 1 h, and transient transfection with c-FLIP-siRNA for 24 h as indicated in the graph. The extrinsic apoptosis related proteins were determined by western blots and statistical analyses and are shown in (D). In all experiments if not specified: A, AT406 at 1 $\mu$g/ml; EV, control empty vector; c-FLIP-siRNA, pSuper-puro-siRNA-c-FLIP vector; T, TRAIL at 100 ng/ml. The results are shown as the means ± SEM in all bar graphs of western blots of three independent experiments (NS>0.05, $^{***}$p<0.001).

The one-way ANOVA followed by a post hoc multiple comparison test was used to compare control (positive control) and treated groups. A p-value <0.05 was considered statistically significant. The cell viability and blot experiments were performed in triplicate. Densitometry analysis was performed by using ImageJ software.

## Results

*Inhibition of c-FLIP$_{S/L}$ and IAPs can increase the sensitivity of U2OS to TRAIL-induced apoptosis*. By evaluating TRAIL promotion of tumor cell apoptosis, we found that U2OS is resistant to TRAIL-induced apoptosis. Because c-FLIP is a crucial apoptotic resistance factor, we used the c-FLIP-siRNA overexpression plasmid which was reported in our previous study to inhibit the expression of c-FLIP (Fig. 1A) (27). Furthermore, we determined the effective concentrations of AT406 (SM406), a synthetic Smac mimetic that is a pan-antagonist of IAPs, including XIAP (Fig. 2A, left). The results of an MTT assay showed that in combination with TRAIL with or without c-FLIP-siRNA, AT406 could increase the cell death rate of U2OS (Fig. 1A).

Results of the Caspase-Glo 3/7 assay (Fig. 1B) confirmed that c-FLIP-siRNA in the presence of AT406 activated caspase

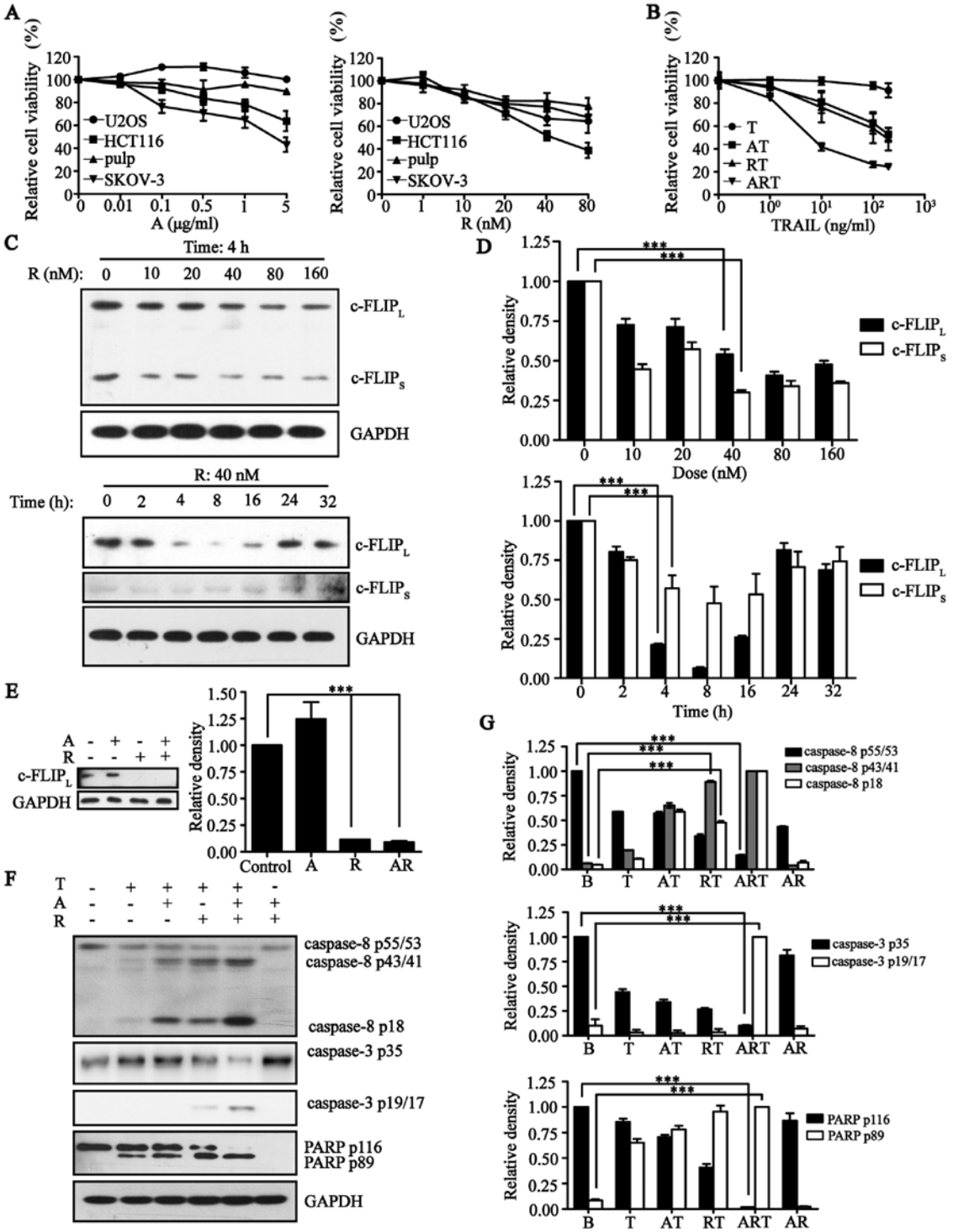


Figure 2. Rocaglamide behaves similarly to c-FLIP-siRNA to inhibit the expression of c-FLIP$_{L/S}$ and, in combination with AT406, sensitized U2OS cells for TRAIL-induced apoptosis. (A) Different cells (cancer cells and normal cells) were treated by different doses of AT406 (A) and rocaglamide (R). Cell viabilities were measured by an MTT assay. (B) Cell viabilities of U2OS in response to TRAIL with or without pre-treatments of A and R as indicated in the graph. Cell viability determined by an MTT assay in A and B are presented as the mean ± SEM of three separate experiments. (C) The osteosarcoma cells U2OS were treated with different concentrations of rocaglamide for 4 h or treated with 40 nM rocaglamide for different durations. The c-FLIP$_{L/S}$ expression was determined using western blots. (D) Statistical analysis of western blots in (C). (E) The U2OS cells were treated with rocaglamide and with or without AT406 for 8 h. The c-FLIP$_{L/S}$ expressions were determined using western blots. (F) The osteosarcoma cells U2OS were treated with TRAIL at 100 ng/ml for 5 h, and with or without pre-treatment of A at 1 $\mu$g/ml for 1 h, R at 40 nM for 4 h as indicated in the graph. The extrinsic apoptosis related proteins were determined by western blots. (G) Statistical analysis of western blots in F for caspase-3 and PARP related proteins. The bar graphs represent the mean ± SD. The results are representative of three independent experiments (NS>0.05, $^{***}p<0.001$).

proteins in the TRAIL-activated apoptotic pathway. That is, apoptosis initiator protein caspase-8, apoptosis executor protein caspase-3 and apoptosis substrate poly(ADP-ribose) polymerase (PARP) were all in their cleaved states to various degrees (Fig. 1C and D). All of these results demonstrated that c-FLIP-siRNA and AT406 together activated the extrinsic

Table I. The sensitivity to TRAIL-induced apoptosis of 20 cancer cell lines with or without pre-treatment of AT406 (A) or rocaglamide (R) or A + R combination.

| Entry | Cell line | $IC_{50}$ (ng/ml) | | | | $I_{max}$ (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | T | AT | RT | ART | T | ART-24 h | ART-72 h |
| 1 | LOVO | 4.2±1.4 | 0.3±0.9 | 7.4±7.9 | 0.5±0.3 | 22±7 | 33±8 | 6±0.3 |
| 2 | HCT116 | 54.2±19 | 12±2.9 | 5.4±5.1 | 2.7±3.3 | 22±8 | 17±6 | 2±0.1 |
| 3 | HT29 | >200 | 48.7±21 | 106.8±56 | 3.4±1.1 | 91±3 | 14±3 | 7±0.5 |
| 4 | H460 | >200 | 158±40.7 | 9±2.9 | 4.3±0.9 | 51±7 | 34±6 | 6±0.6 |
| 5 | BT474 | >200 | >200 | 89.3±26 | 26.2±7.8 | 97±3 | 20±1 | 3±0,7 |
| 6 | SK-BR-3 | >200 | >200 | 122.3±119 | 21.9±2.1 | 97±6 | 29±1 | 5±0.2 |
| 7 | U2OS | >200 | >200 | >200 | 11.3±12 | 91±8 | 24±2 | 3±0.5 |
| 8 | T-47D | >200 | >200 | >200 | 35.8±13.2 | 102±1 | 32±3 | 7±0.3 |
| 9 | MDA-MB-231 | >200 | >200 | >200 | 17.8±7.3 | 81±6 | 33±2 | 7±0.4 |
| 10 | SK-OV-3 | >200 | >200 | >200 | 56.6±32.8 | 83±7 | 41±2 | 13±1.8 |
| 11 | Vcap | >200 | >200 | >200 | 52.5±20 | 100±2 | 43±3 | 15±1.2 |
| 12 | SW480 | >200 | >200 | >200 | >200 | 88±10 | 62±8 | 14±5.3 |
| 13 | MDA-MB-468 | >200 | >200 | >200 | >200 | 93±4 | 71±2 | 7±2.4 |
| 14 | HeLa | >200 | >200 | >200 | >200 | 101±3 | 57±4 | 27±0.4 |
| 15 | Hep G2 | >200 | >200 | >200 | >200 | 70±6 | 76±7 | 17±7.9 |
| 16 | A549 | >200 | >200 | >200 | >200 | 81±8 | 68±12 | 32±10.9 |
| 17 | U87 | >200 | >200 | >200 | >200 | 85±9 | 73±8 | 36±2.7 |
| 18 | MCF7 | >200 | >200 | >200 | >200 | 104±9 | 93±7 | 48±13.2 |
| 19 | U251 | >200 | >200 | >200 | >200 | 108±7 | 101±12 | 81±3.4 |
| 20 | U373 | >200 | >200 | >200 | >200 | 94±4 | 102±4 | 93±4.4 |

Twenty different kinds of cancer cell lines were treated with different concentrations of TRAIL for 24 or 72 h, or pretreated with A for 1 h and/or R for 4 h. The cell viability was determined by an MTT assay. The $IC_{50}$ and $I_{max}$ (%) were obtained from Fig. 3 as well as the concentration-cell viability inhibition curves (not shown) for the AT and RT conditions. The concentrations which cause 50% reduction of cell viability ($IC_{50}$ values) were calculated using GraphPad Prism5 software. $I_{max}$ (%) represents the percentage (%) of cells that remain alive at a maximum dose of 200 ng/ml of TRAIL. The results are shown as the means ± SEM of three independent experiments.

apoptotic pathway in the presence of TRAIL to induce apoptosis.

*Triple combination of TRAIL/AT406/rocaglamide functions via an extrinsic apoptosis pathway*. The c-FLIP-siRNA, mentioned above, cannot be transfected successfully to most cell lines. Rocaglamide is a natural product isolated from *Aglaia* species and has been previously shown to downregulate the expression of c-FLIP in leukemic T cells (23) and Hodgkin's lymphoma cells (25). Therefore, we tested whether rocaglamide could replace the siRNA. After determining the scope of drug safety of rocaglamide (Fig. 2A), we found that rocaglamide could also inhibit the expression of c-FLIP effectively (Fig. 2C and D). Because AT406 did not interfere with rocaglamide (Fig. 2E), we combined AT406 and rocaglamide with TRAIL. This combination could also increase the cell death rate of U2OS cells (Fig. 2B) and similarly activated caspase-8, caspase-3 and PARP by increasing the cleaved and active forms of these caspases (Fig. 2F and G). This result showed that, rocaglamide could inhibit c-FLIP as well as c-FLIP-siRNA, and further activated the extrinsic apoptotic pathway in combination with AT406 in the presence of TRAIL.

*The actions of the triple combination overcame the resistance of most solid tumor cell lines to TRAIL-induced apoptosis*. We collected 20 tumor cell lines that originated from colorectal cancers (HCT116, HT29, LOVO, SW480), lung cancers (H460, A549), ovarian cancer (SK-OV-3), osteosarcoma (U2OS), breast cancers (MDA-MB-231, SK-BR-3, T-47D, BT474, MDA-MB-468, MCF7), cervical cancer (HeLa), liver cancer (HepG2), prostate cancer (Vcap) and gliomas (U87, U251, U373) to test for apoptotic effects induced by TRAIL alone (Table I). Among these twenty tumor cell lines, only two cell lines, HCT116 and LOVO, responded to TRAIL; the remaining 18 tumor cell lines were highly resistant.

The addition of AT406 to HCT116 (a TRAIL sensitive cell line) or HT29 (a TRAIL-resistant cell line) to antagonize IAPs significantly improved the apoptotic effects of TRAIL (Fig. 3A and B). Pre-treating these two cell lines with rocaglamide to downregulate the expression of c-FLIP showed similar effects (Fig. 3A and B). Furthermore, after a combined pre-treatment

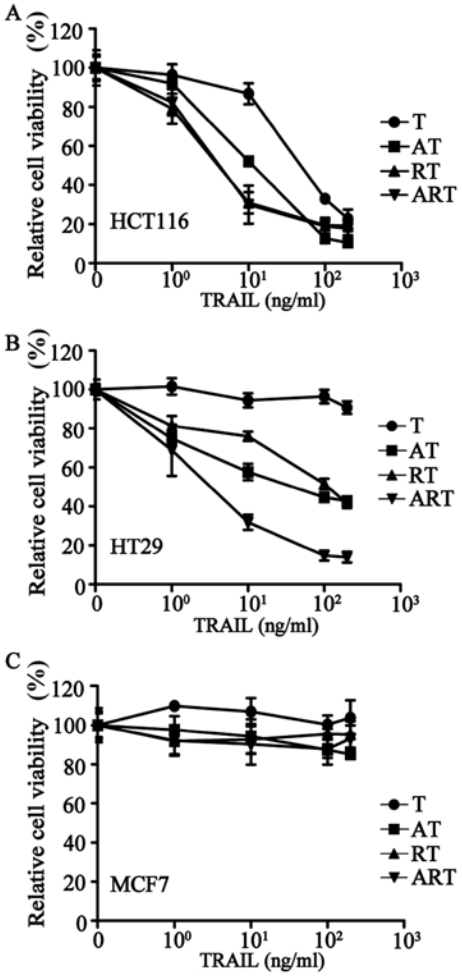


Figure 3. Concentration-cell viability inhibition curves of TRAIL in the absence and presence of AT406 and rocaglamide, and the time course of ART triple combination treatments in cancer cell lines. (A-C) Cell viabilities of example cell lines HCT116 (A), HT29 (B) and MCF7 (C) in response to TRAIL with or without pre-treatment of A and R as indicated in the graph (A at 1 $\mu$g/ml; R at 40 nM. In each TRAIL concentration-response experiments, TRAIL treating time is 24 h). The results are shown as the mean ± SEM of three independent experiments.

of rocaglamide and AT406, TRAIL-resistant HT29 cells became highly sensitive to TRAIL (Fig. 3B).

Next, we treated the 20 cancer cell lines with a combination of AT406 (1 $\mu$g/ml), rocaglamide (40 nM) and TRAIL at various concentrations (ART combination) (Table I) for 24 h of treatment. Our results showed that these 20 tumor cell lines could be divided into the following three groups: group 1, highly sensitive (<50% relative cell viability), 11 cell lines (e.g., HCT116) (Fig. 3A); group 2, sensitive (50-80% relative cell viability), 6 cell lines (e.g., HT29) (Fig. 3B); and group 3, resistant (>80% relative cell viability), 3 cell lines (e.g., MCF7) (Fig. 3C). This ART-induced cell death can be further improved over time by extending the treatment to a 72-h incubation. Only three cancer cell lines were resistant to ART even after 72-h treatment (Table I), i.e., the breast cancer cell line, MCF7, and brain cancer cell lines, U373 and U251.

*Inhibition of IAPs or c-FLIP alone is not sufficient to achieve the remarkable cell death induced with an ART triple combination*. To confirm that an ART triple combination is necessary for inducing profound cell death, we determined the cell viability inhibition concentration ($IC_{50}$ values) of TRAIL on cancer cells with or without AT406 or rocaglamide pre-treatment (Table I). When cells were treated with TRAIL alone, $IC_{50}$ values could be obtained only on TRAIL-sensitive LOVO and HCT116 cells. After combination with either AT406 or rocaglamide pre-treatment (AT or RT combination), the potency of TRAIL increased >4-fold (lower $IC_{50}$ values) in these two cell lines (entries 1 and 2), and $IC_{50}$ values could be obtained on other cancer cells (8 cell lines for AT, 11 cell lines for RT). ART triple combination further enhanced the apoptosis-inducing effects of TRAIL. For the 11 highly sensitive cancer cell lines, >50% apoptosis was observed after 24-h treatment (entries 1-11). $IC_{50}$ values using TRAIL on these cells were markedly lower than those values using AT or RT combinations, and 7 of them were <10 ng/ml. When ART exposure was extended to 72 h, the relative cell viability ($I_{max}$ values) further decreased to <10% for 10 cell lines, and 10-40% for 7 cell lines (column of ART-72 h). Only three cells remained resistant to ART (entries 18-20).

On a protein level, cleaved caspases and PARP were barely detected when U2OS cells were treated with TRAIL alone. However, ART completely activated the extrinsic apoptotic pathway and resulted in cleavage of all related proteins (Fig. 2F and G).

*Triple combination is safe for normal cells*. Mounting evidence has demonstrated that TRAIL does not hurt normal cells. To assess the safety of ART, cell viability assays were carried out using pulp and MRC5 cells. Our results showed that both ART and its equivalent AT406/c-FLIP-siRNA/TRAIL combination could not induce cell death in these normal cells (Fig. 4A-C). Both of these normal cells were resistant to ART even after a 72-h treatment (Fig. 4D). Next, we evaluated the variation of protein expression levels during TRAIL-induced extrinsic apoptosis. Pulp expression of procaspase-3 and procaspase-8 were much lower than expression in the ovarian cancer cell line SK-OV-3. After stimulation by A/R/T in different combination patterns, we did not observe cleavage of the apoptotic proteins procaspase-3/8, but rather, only a slight upregulation of these two apoptotic proteins (Fig. 4E and F).

*ART-resistance may attribute to the low expression of procaspases*. To investigate the molecular mechanism of TRAIL- and ART-resistance in normal and cancer cells, western blot analysis was carried out for the key proteins involved in the TRAIL apoptotic pathway, i.e., DR5, FADD, procaspase-3/-8 and c-$FLIP_{L/S}$ (Fig. 5A and B). DR5 was expressed in all of the cell lines, but levels in TRAIL-resistant cells were lower. FADD was expressed in all cells at levels unrelated to TRAIL- or ART-resistance. The expression of c-$FLIP_L$ was found in all ten cell lines, although it was relatively higher in U87, U251 and pulp cells. c-FLIPS could be clearly observed in HT29, U87 and pulp cells, and it was difficult to detect in other cells. Notably, procaspase-8 was expressed at relatively high levels in ART-sensitive cells (LOVO, HCT116, HT29, U2OS and SK-OV-3 cells) and at significantly lower levels in ART-resistant cells (U251, U373 and pulp cells). In contrast, procaspase-3 in ART-resistant MCF7 cells was nearly invisible. Valproic acid (VA) is an antiepileptic drug with histone

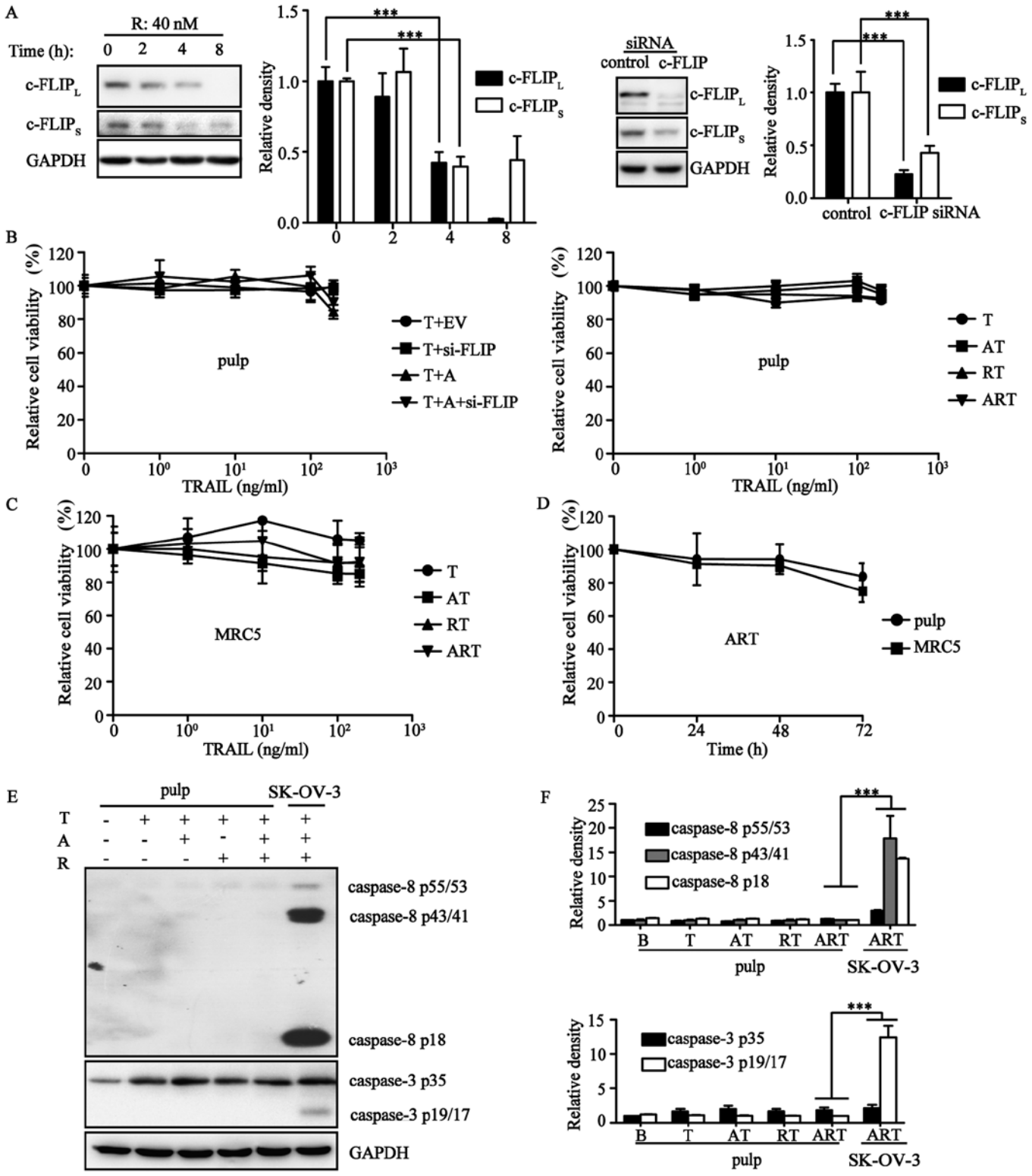


Figure 4. Normal cell pulp and MRC5 are resistant to ART combination treatment. (A) The pulp cells were treated with 40 nM rocaglamide for different durations or with/without c-FLIP-siRNA transfection for 24 h. The c-FLIP$_{L/S}$ expression was determined using western blots. (B) Left, the normal cell pulp was treated with different concentrations of TRAIL for 24 h in combination with c-FLIP-siRNA (si-FLIP, transient transfection with c-FLIP-siRNA for 24 h), AT406 (A) or empty vector (EV) as indicated in the graph. (B) Right, pulp cells were treated with various concentrations of TRAIL for 24 h with or without the combination of A and R as indicated in the graph. (C) MRC5 cells were treated with various concentrations of TRAIL for 24 h with or without the combination of A and R as indicated in the graph. In experiments of B and C, cells were pretreated by R at 40 nM for 4 h, and A at 1 $\mu$g/ml for 1-h pretreatment. The cell viability was determined by an MTT assay. (D) The time course of cell viability of pulp and MRC5 cells after treatment with the ART combination. In cell viability assays, results are shown as the means ± SEM of three independent experiments. (E) The ART triple combination did not activate the extrinsic apoptosis pathway. The normal cell pulp was treated with TRAIL at 100 ng/ml for 5 h, with or without the pretreatment of A at 1 $\mu$g/ml for 1 h and R at 40 nM for 4 h as indicated in the graph. The ovarian cancer cell SK-OV-3 is shown as a positive control after ART combination treatments. (F) Statistical analysis of western blots in (E). Means ± SD are shown in all bar graphs of western blots on the right side. The results are representative of three independent experiments.

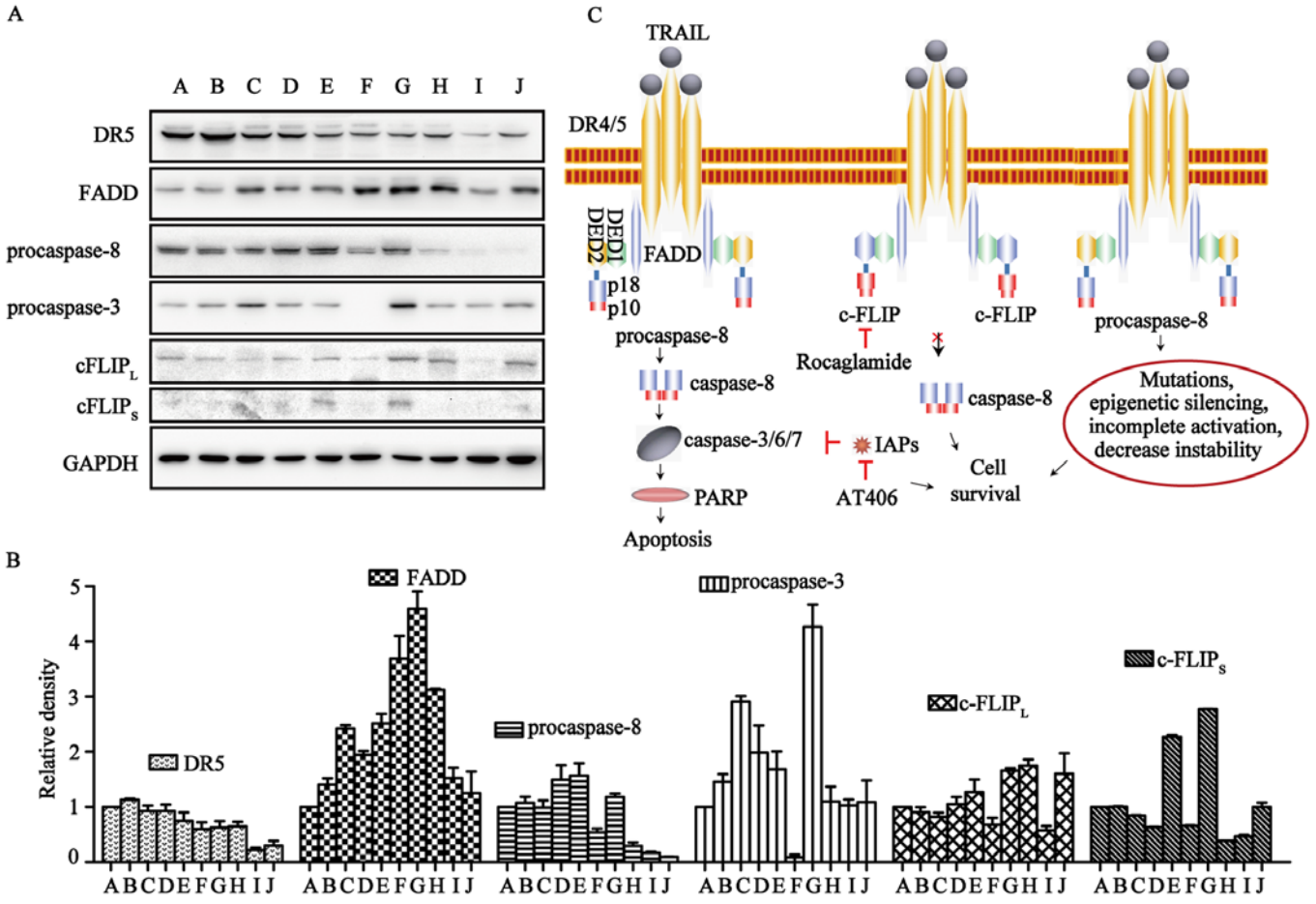


Figure 5. The resistance to synergistic actions of triple combination may be attributed to the low expression of procaspases. (A) TRAIL related apoptosis proteins of the 9 selected cell lines and normal cell pulp were detected by western blots without any drug treatment. (B) All of the TRAIL-related apoptosis proteins in these 9 cancer cell lines and pulp cells were compared with LOVO. (A) LOVO; (B) HCT116; (C) U2OS; (D) SK-OV-3; (E) HT29; (F) MCF7; (G) U87; (H) U251; (I) U373; (J) pulp. The results are presented in the bar graphs as the means ± SD for western blots from three independent experiments. (C) Model showing the mechanisms of sensitization of TRAIL-induced apoptosis by rocaglamide as a c-FLIP synthetic inhibitor, and AT406 as a pan-antagonist of IAPs. Abnormal expression of procaspase-8 prevents the progress of apoptosis pathway in cancer cells and cells develop resistance to both TRAIL- and ART triple combination-induced apoptosis.

deacetylase inhibitory activity (28). We used VA at an appropriate concentration to increase procaspase-8 expression (Fig. 6). The improvement of procaspase-8 expression resulted in the enhancement of apoptotic rate (Fig. 6B-D). Overall, levels of procaspase-3/-8 correlated with ART-sensitivity.

## Discussion

Recombinant human TRAIL and several agonistic monoclonal antibodies of DR4/5 have been developed and used in clinical trials (28-30). However, their development as a cancer therapy is hampered by the resistance observed in most cancer cells (31,32). Although synergy has been described for combinations of TRAIL with a variety of cytotoxic agents, including etoposide, 5-FU, oxamflatin, sorbitol, staurosporine, MG132, bortezomib, doxorubicin, azacitidine and sorafenibin, these synergistic effects were apparent in only TRAIL-sensitive tumor cells (33). However, in resistant tumor cells, a combination of TRAIL or DR4/5 agonist with chemotherapy showed no profound effect on cell death.

In this study, we hypothesized that TRAIL resistance could be overcome by simultaneously attacking three apoptosis inhibiting factors. First, we demonstrated that a higher concentration of TRAIL (200 ng/ml) would be necessary for competitive binding of DcR1/2 (34). Second, we showed that the apoptosis-inhibiting function of IAPs can be blocked by adding AT406, a small molecule IAPs inhibitor in clinical trials. Third, we showed that the cytosolic apoptotic inhibitor c-FLIP$_{L/S}$ can be downregulated by transient transfection of a plasmid containing c-FLIP-siRNA or by adding rocaglamide, a known c-FLIP expression suppressor (Fig. 5C). We firmly believe that only with the elimination of the main inhibitory factors of an extrinsic apoptotic pathway, can a drug combination based on TRAIL achieve the strongest and broadest therapeutic effects. This may also be the reason for failure of clinical trials of TRAIL or agonist monoclonal antibodies of death receptors and cytotoxic agent combinations on different cancer types. Additionally, *in vitro* culture systems are limited in making *in vivo* predictions because TRAIL is quickly degraded *in vivo* (35,36).

A double combination of either AT or RT only slightly improved the TRAIL-induced apoptosis on cancer cells. In contrast, powerful combined effects were observed for ART on most of the resistant cancer cells. Among the 18 TRAIL-

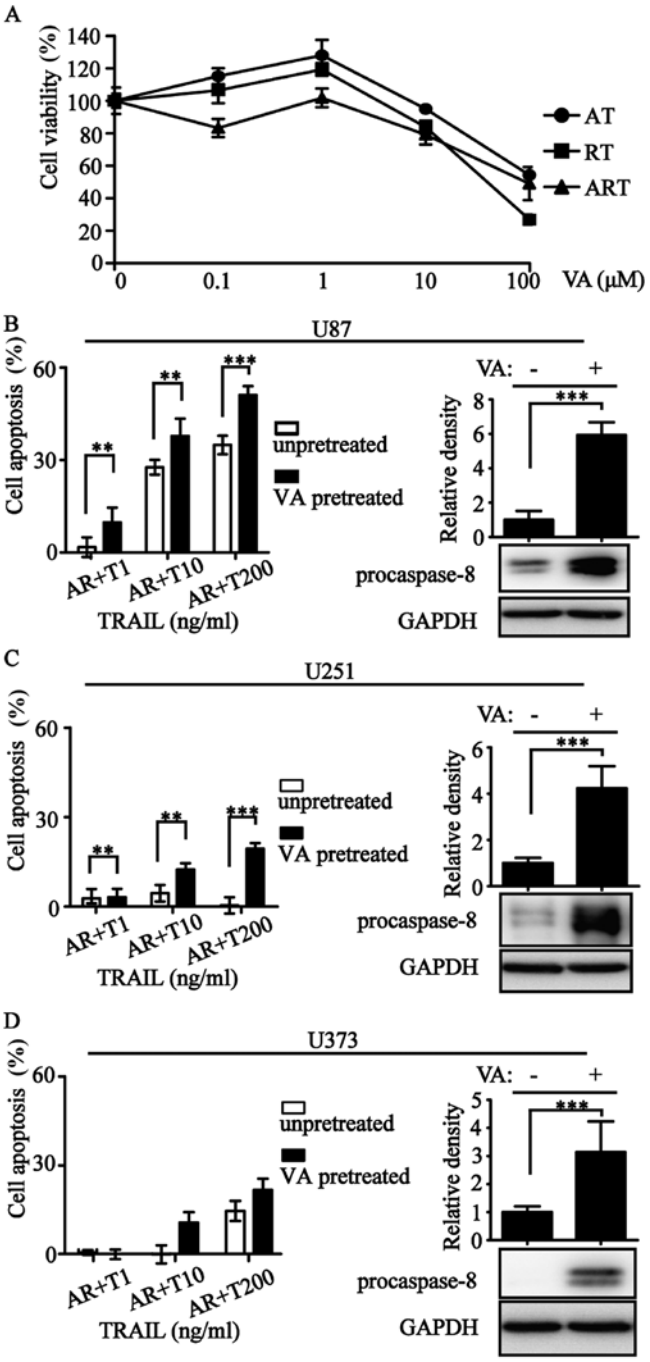


Figure 6. The procaspase-8 expression positively correlates with TRAIL inducing apoptosis. (A) Different cells (U87, U251, U373) were treated by different concentrations of valproic acid (VA) for 24 h. Cell viabilities were measured by MTT assay. (B-D) Cell viabilities of U87, U251 and U373 in response to ART with or without pre-treatments of VA (1 $\mu$M, 24 h) as indicated in the graph. Procaspase-8 in U87/U251/U373 were tested by western blots with or without VA (1 $\mu$M) pretreated for 24 h. Statistical analyses of each western blots are on the top. Cell viability determined by MTT assays in A/B/C/D were presented as mean ± SEM of three separate experiments performed in triplicate. The results are shown as the means ± SEM in all bar graphs of western blots of three independent experiments (NS>0.05, $^{**}p<0.01$, $^{***}p<0.001$).

resistant cancer cells, after a 72-h triple combination treatment, 8 became highly sensitive (<10% relative cell viability), and 7 became sensitive (10-50% relative cell viability). The normal pulp and MRC5 cells remained highly resistant to an ART triple combination treatment (Fig. 3 and Table I). These results clearly demonstrate that the ART triple combination generates effects that specifically activates an extrinsic apoptotic pathway in solid tumor cells. The profound apoptotic effects of ART triple combination on cell lines originally from solid tumors also suggests that c-FLIPs and IAPs contribute to the high resistance of these cancer cells to TRAIL-induced apoptosis. Therefore, a combination of DR 4/5 agonist, IAPs antagonist and a c-FLIP antagonist, i.e., the ART combination and its equivalents, is likely a better cancer therapy than TRAIL or agonistic monoclonal antibodies of the death receptors alone. These results also suggest that simultaneous activation of death receptors, inhibition of c-FLIP and antagonization of IAPs are minimally required for induction of profound apoptosis of solid tumor cells.

It is worth noting that ART triple combination does not impair normal cells. Previous research suggests that TRAIL-resistant cancer cells are resistant due to the expression of only one anti-apoptotic protein and that these cells have lost the redundancy in resistance mechanisms observed in non-transformed cells (37). In contrast, our study suggests that most of these highly resistant cancer cells rely on multiple tolerance mechanisms rather than only one resistance mechanism. We suggest that TRAIL-resistance mechanisms of normal cells are far more complicated than cancer cells and at the same time, confirm that ART triple combination is safe for normal primary culture cells from adults or normal passage cells from the human embryo.

Among the 20 cancer cell lines used in this study, the breast cancer cell line MCF7 and two brain cancer cell lines remained highly resistant to ART triple combination, even after a 72-h exposure (Table I and Fig. 3). Western blot analysis revealed that c-FLIP is expressed in these cell lines; however, the levels of procaspase-8 in these cell lines are lower than in the other cell lines. In addition, MCF7 showed extremely low, nearly undetectable, levels of procaspase-3 (Fig. 5). These results suggest that low expression or loss of procaspase-8 and -3 is a likely mechanism for resistance to the ART combination. MCF-7 is deficient of caspase-3 and is relatively insensitive to many traditional chemotherapeutic agents (38-40). Because of the indispensable role of the extrinsic apoptotic pathway, deficiency of caspase-3 in MCF-7 results in high TRAIL-resistance during exposure to the ART combination.

Eggert *et al* (41) reported that only one out of 18 neuroblastoma cell lines showed sensitivity to TRAIL-induced apoptosis. This number of TRAIL sensitive cell lines increased to five by adding cycloheximide (CHX, a protein synthesis inhibitor) to cultured cells to inhibit the synthesis of c-FLIP. The remaining 13 TRAIL-resistant neuroblastoma cell lines (70%) showed a loss of procaspase-8 expression, correlating with resistance to TRAIL-induced apoptosis. These results suggest that in addition to c-FLIP, the loss of procaspase-8 plays a major role in TRAIL resistance in brain cancer cells that originated from a neuroblastoma. In our present study, similarly, all three glioma cell lines and normal pulp cells showed high resistance to TRAIL, as well as ART triple combination treatment (Fig. 3 and Table I). Two (U373 and U251) of these glioma cell lines and pulp cells also expressed at very low levels of procaspase-8 and variable levels of c-FLIP$_{L/S}$, suggesting that the resistance of these cells to TRAIL and ART are the

results of low level expression of procaspase-8 and the presence of anti-apoptosis factors, such as c-FLIP and/or IAPs. The underlying mechanisms of TRAIL resistance caused by decreased expression of the caspase-8 initiator have been attributed to epigenetic silencing, such as DNA methylation, histone acetylation modification (42-45). Indeed, when histone deacetylase inhibitor was used on three glioma cell lines, the procaspase-8 expression increased. There is more cell apoptosis under ART treatment (Fig. 6). Decreased caspase-8 activities in some cancer cells are related to procaspase-8 gene mutation (41,46-48), decrease in stability, and incomplete activation (42-46,49,50) (Fig. 5C). The loss of procaspase-8 expression is particularly prevalent in both neuroblastoma (39) and glioma brain cancer cells. These results may explain the poor prognosis of patients suffering from malignant brain cancers regardless of the therapy.

Recently reported unsuccessful clinical phase 2 studies of rhTRAIL or agonistic monoclonal antibodies to death receptors using a randomly unselected patient population, with and without traditional chemotherapy (10,11), suggest that future clinical studies should consider the aforementioned mechanisms of TRAIL resistance in tumors. For peripheral solid tumors, it appears that a majority of tumor cells are sensitive to ART triple combination therapy; however, a small number of these tumors may have mutations in the procaspase-8 gene rendering caspase-8 inactive (50). Future clinical protocol designs and prognosis analysis based on death receptors should exclude patients with abnormal expression of procaspase-8 (51).

Indeed, the TRAIL resistance of cancer cell lines was successfully reversed with ART in the majority of peripheral solid tumor cells with the exception of brain cancer cells. While being used alone, AT406 has only a limited effect on cancer cell death (Fig. 2A). For rocaglamide, certain degrees of cytotoxicity were observed on both cancer cell lines and normal cells (Fig. 2A). The fact that rocaglamide is cytotoxic to normal cells could raise concerns for the future application of ART combination therapy. To study the cause of this cytotoxicity, we carried out additional experiments in which rocaglamide was replaced with c-FLIP-siRNA. However, similar results were obtained using c-FLIP-siRNA alone or in combination with TRAIL and AT406, confirming that this cytotoxicity is the result of c-FLIP downregulation and the suppression of the anti-apoptotic functions of c-FLIP (52,53). Therefore, it is highly desirable to develop a specific disruptor or antagonist of c-FLIP-FADD interactions to avoid the cytotoxicity caused by changes in the cellular levels of c-FLIP in normal cells.

## Acknowledgements

This study was supported by Chinese National Natural Science Foundation, grant no. 81360162, 81260351 and U1302225.

## References

1. Tannock IF: Cancer: Resistance through repopulation. Nature 517: 152-153, 2015.
2. Santarpia L, Lippman SM and El-Naggar AK: Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. Expert Opin Ther Targets 16: 103-119, 2012.
3. Sriraman SK, Aryasomayajula B and Torchilin VP: Barriers to drug delivery in solid tumors. Tissue Barriers 2: e29528, 2014.
4. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA, *et al*: Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3: 673-682, 1995.
5. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A and Ashkenazi A: Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem 271: 12687-12690, 1996.
6. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, *et al*: Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 104: 155-162, 1999.
7. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, Iwakura Y, Yagita H and Okumura K: Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med 7: 94-100, 2001.
8. Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ and Ashkenazi A: Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity 12: 611-620, 2000.
9. Lemke J, von Karstedt S, Zinngrebe J and Walczak H: Getting TRAIL back on track for cancer therapy. Cell Death Differ 21: 1350-1364, 2014.
10. Holland PM: Death receptor agonist therapies for cancer, which is the right TRAIL? Cytokine Growth Factor Rev 25: 185-193, 2014.
11. Dyer MJ, MacFarlane M and Cohen GM: Barriers to effective TRAIL-targeted therapy of malignancy. J Clin Oncol 25: 4505-4506, 2007.
12. Koschny R, Walczak H and Ganten TM: The promise of TRAIL - potential and risks of a novel anticancer therapy. J Mol Med Berl 85: 923-935, 2007.
13. Salvesen GS and Duckett CS: IAP proteins: Blocking the road to death's door. Nat Rev Mol Cell Biol 3: 401-410, 2002.
14. Fulda S: Inhibitor of apoptosis proteins as targets for anticancer therapy. Expert Rev Anticancer Ther 7: 1255-1264, 2007.
15. Cai Q, Sun H, Peng Y, Lu J, Nikolovska-Coleska Z, McEachern D, Liu L, Qiu S, Yang CY, Miller R, *et al*: A potent and orally active antagonist (SM-406/AT-406) of multiple inhibitor of apoptosis proteins (IAPs) in clinical development for cancer treatment. J Med Chem 54: 2714-2726, 2011.
16. Fulda S, Wick W, Weller M and Debatin KM: Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. Nat Med 8: 808-815, 2002.
17. Fakler M, Loeder S, Vogler M, Schneider K, Jeremias I, Debatin KM and Fulda S: Small molecule XIAP inhibitors cooperate with TRAIL to induce apoptosis in childhood acute leukemia cells and overcome Bcl-2-mediated resistance. Blood 113: 1710-1722, 2009.
18. Vogler M, Walczak H, Stadel D, Haas TL, Genze F, Jovanovic M, Bhanot U, Hasel C, Möller P, Gschwend JE, *et al*: Small molecule XIAP inhibitors enhance TRAIL-induced apoptosis and antitumor activity in preclinical models of pancreatic carcinoma. Cancer Res 69: 2425-2434, 2009.
19. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schröter M, Burns K, Mattmann C, *et al*: Inhibition of death receptor signals by cellular FLIP. Nature 388: 190-195, 1997.
20. Aoudjit F and Vuori K: Integrin signaling in cancer cell survival and chemoresistance. Chemother Res Pract 2012: 283181, 2012.
21. Safa AR and Pollok KE: Targeting the anti-apoptotic protein c-FLIP for cancer therapy. Cancers (Basel) 3: 1639-1671, 2011.
22. Chawla-Sarkar M, Bae SI, Reu FJ, Jacobs BS, Lindner DJ and Borden EC: Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. Cell Death Differ 11: 915-923, 2004.
23. Bleumink M, Köhler R, Giaisi M, Proksch P, Krammer PH and Li-Weber M: Rocaglamide breaks TRAIL resistance in HTLV-1-associated adult T-cell leukemia/lymphoma by translational suppression of c-FLIP expression. Cell Death Differ 18: 362-370, 2011.
24. Zhu JY, Giaisi M, Köhler R, Müller WW, Mühleisen A, Proksch P, Krammer PH and Li-Weber M: Rocaglamide sensitizes leukemic T cells to activation-induced cell death by differential regulation of CD95L and c-FLIP expression. Cell Death Differ 16: 1289-1299, 2009.

25. Giaisi M, Kohler R, Fulda S, Krammer PH and Li-Weber M: Rocaglamide and a XIAP inhibitor cooperatively sensitize TRAIL-mediated apoptosis in Hodgkin's lymphomas. Int J Cancer 131: 1003-1008, 2012.
26. Gronthos S, Mankani M, Brahim J, Robey PG and Shi S: Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA 97: 13625-13630, 2000.
27. Zhang YP, Kong QH, Huang Y, Wang GL and Chang KJ: Inhibition of c-FLIP by RNAi enhances sensitivity of the human osteogenic sarcoma cell line U2OS to TRAIL-induced apoptosis. Asian Pac J Cancer Prev 16: 2251-2256, 2015.
28. Ziauddin MF, Yeow WS, Maxhimer JB, Baras A, Chua A, Reddy RM, Tsai W, Cole GW Jr, Schrump DS and Nguyen DM: Valproic acid, an antiepileptic drug with histone deacetylase inhibitory activity, potentiates the cytotoxic effect of Apo2L/TRAIL on cultured thoracic cancer cells through mitochondria-dependent caspase activation. Neoplasia 8: 446-457, 2006.
29. Micheau O, Shirley S and Dufour F: Death receptors as targets in cancer. Br J Pharmacol 169: 1723-1744, 2013.
30. Wiezorek J, Holland P and Graves J: Death receptor agonists as a targeted therapy for cancer. Clin Cancer Res 16: 1701-1708, 2010.
31. Graves JD, Kordich JJ, Huang TH, Piasecki J, Bush TL, Sullivan T, Foltz IN, Chang W, Douangpanya H, Dang T, *et al*: Apo2L/TRAIL and the death receptor 5 agonist antibody AMG 655 cooperate to promote receptor clustering and antitumor activity. Cancer Cell 26: 177-189, 2014.
32. Fulda S: Safety and tolerability of TRAIL receptor agonists in cancer treatment. Eur J Clin Pharmacol 71: 525-527, 2015.
33. Sayers TJ: Targeting the extrinsic apoptosis signaling pathway for cancer therapy. Cancer Immunol Immunother 60: 1173-1180, 2011.
34. Menke C, Bin L, Thorburn J, Behbakht K, Ford HL and Thorburn A: Distinct TRAIL resistance mechanisms can be overcome by proteasome inhibition but not generally by synergizing agents. Cancer Res 71: 1883-1892, 2011.
35. Daniel PT, Wieder T, Sturm I and Schulze-Osthoff K: The kiss of death: Promises and failures of death receptors and ligands in cancer therapy. Leukemia 15: 1022-1032, 2001.
36. Schneider P, Olson D, Tardivel A, Browning B, Lugovskoy A, Gong D, Dobles M, Hertig S, Hofmann K, Van Vlijmen H, *et al*: Identification of a new murine tumor necrosis factor receptor locus that contains two novel murine receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). J Biol Chem 278: 5444-5454, 2003.
37. Wu GS, Burns TF, Zhan Y, Alnemri ES and El-Deiry WS: Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor. Cancer Res 59: 2770-2775, 1999.
38. van Dijk M, Halpin-McCormick A, Sessler T, Samali A and Szegezdi E: Resistance to TRAIL in non-transformed cells is due to multiple redundant pathways. Cell Death Dis 4: e702, 2013.
39. Jänicke RU, Sprengart ML, Wati MR and Porter AG: Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 273: 9357-9360, 1998.
40. Yang XH, Sladek TL, Liu X, Butler BR, Froelich CJ and Thor AD: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin- and etoposide-induced apoptosis. Cancer Res 61: 348-354, 2001.
41. Eggert A, Grotzer MA, Zuzak TJ, Wiewrodt BR, Ho R, Ikegaki N and Brodeur GM: Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. Cancer Res 61: 1314-1319, 2001.
42. Hopkins-Donaldson S, Ziegler A, Kurtz S, Bigosch C, Kandioler D, Ludwig C, Zangemeister-Wittke U and Stahel R: Silencing of death receptor and caspase-8 expression in small cell lung carcinoma cell lines and tumors by DNA methylation. Cell Death Differ 10: 356-364, 2003.
43. Duiker EW, Meijer A, van der Bilt ARM, Meersma GJ, Kooi N, van der Zee AG, de Vries EG and de Jong S: Drug-induced caspase 8 upregulation sensitises cisplatin-resistant ovarian carcinoma cells to rhTRAIL-induced apoptosis. Br J Cancer 104: 1278-1287, 2011.
44. Jia SH, Parodo J, Kapus A, Rotstein OD and Marshall JC: Dynamic regulation of neutrophil survival through tyrosine phosphorylation or dephosphorylation of caspase-8. J Biol Chem 283: 5402-5413, 2008.
45. Jin Z, Li Y, Pitti R, Lawrence D, Pham VC, Lill JR and Ashkenazi A: Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. Cell 137: 721-735, 2009.
46. Bellail AC, Olson JJ, Yang X, Chen ZJ and Hao C: A20 ubiquitin ligase-mediated polyubiquitination of RIP1 inhibits caspase-8 cleavage and TRAIL-induced apoptosis in glioblastoma. Cancer Discov 2: 140-155, 2012.
47. Fulda S, Küfer MU, Meyer E, van Valen F, Dockhorn-Dworniczak B and Debatin KM: Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. Oncogene 20: 5865-5877, 2001.
48. Casciano I, De Ambrosis A, Croce M, Pagnan G, Di Vinci A, Allemanni G, Banelli B, Ponzoni M, Romani M and Ferrini S: Expression of the caspase-8 gene in neuroblastoma cells is regulated through an essential interferon-sensitive response element (ISRE). Cell Death Differ 11: 131-134, 2004.
49. Fulda S and Debatin KM: IFNgamma sensitizes for apoptosis by upregulating caspase-8 expression through the Stat1 pathway. Oncogene 21: 2295-2308, 2002.
50. Crowder RN and El-Deiry WS: Caspase-8 regulation of TRAIL-mediated cell death. Exp Oncol 34: 160-164, 2012.
51. Soung YH, Lee JW, Kim SY, Sung YJ, Park WS, Nam SW, Kim SH, Lee JY, Yoo NJ and Lee SH: Caspase-8 gene is frequently inactivated by the frameshift somatic mutation 1225_1226delTG in hepatocellular carcinomas. Oncogene 24: 141-147, 2005.
52. Shirley S and Micheau O: Targeting c-FLIP in cancer. Cancer Lett 332: 141-150, 2013.
53. Wajant H, Haas E, Schwenzer R, Muhlenbeck F, Kreuz S, Schubert G, Grell M, Smith C and Scheurich P: Inhibition of death receptor-mediated gene induction by a cycloheximide-sensitive factor occurs at the level of or upstream of Fas-associated death domain protein (FADD). J Biol Chem 275: 24357-24366, 2000.