

Plumbagin enhances TRAIL-induced apoptosis of human leukemic Kasumi-1 cells through upregulation of TRAIL death receptor expression, activation of caspase-8 and inhibition of cFLIP

XIAOYANG KONG¹, JING LUO¹, TONGPENG XU¹, YUNJIAO ZHOU², ZENGKAI PAN¹,
YINGHUA XIE¹, LIMIN ZHAO¹, YINGTING LU¹, XIYAO HAN¹, ZHIXIONG LI⁴ and LIGEN LIU^{1,3}

¹Department of Hematology, ²Central Laboratory, Shanghai No. 5 Hospital, Fudan University, Shanghai 200240;

³Department of Hematology, Shanghai Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200336, P.R. China; ⁴Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School, D-30625 Hannover, Germany

Received November 10, 2016; Accepted April 24, 2017

DOI: 10.3892/or.2017.5627

Abstract. Although the patients with t(8;21) acute myeloid leukemia (AML) have a favorable prognosis compared with other non-acute promyelocytic leukemia AML patients, only ~50% patients with this relatively favorable subtype can survive for 5 years and refractory/relapse is common in clinical practice. So it is necessary to find novel agents to treat this type of AML. In this study, the effects and the mechanisms of plumbagin and recombinant soluble tumor necrosis factor- α -related apoptosis-inducing ligand (rsTRAIL) on leukemic Kasumi-1 cells were primarily investigated. Plumbagin and/or rsTRAIL could significantly inhibit the growth of Kasumi-1 cells and induce apoptosis *in vitro* and *in vivo*. Plumbagin enhanced TRAIL-induced apoptosis of Kasumi-1 cells in association with mitochondria damage, caspase activation, upregulation of death receptors (DRs) and decreased cFLIP expression. The effects of plumbagin on the expression of DR5, Bax and cFLIP could be partially abolished by the reactive oxygen species (ROS) scavenger NAC. Glutathione (GSH) depletion by plumbagin increased the production of ROS. *In vivo*, there was no obvious toxic pathologic change in the heart, liver and kidney tissues in any of the groups. Comparing with the control mice, a significantly increased number of apoptotic cells were observed in the combined treated mice by flow cytometry. Plumbagin also increased the expression of DR4

and DR5 in cells of xenograft tumors. Collectively, our results suggest that both plumbagin and rsTRAIL could be used as a single agent or synergistical agents to induce apoptosis of leukemic Kasumi-1 cells *in vitro* and *in vivo*.

Introduction

Tumor necrosis factor- α -related apoptosis-inducing ligand (TRAIL/Apo2L), a member of the tumor necrosis factor (TNF) family of apoptosis triggering proteins, is believed to selectively induce apoptosis in cancer cells but not the normal cells (1,2). Recombinant soluble TRAIL (rsTRAIL) initiates extrinsic apoptosis through binding to death receptors (DRs) 4 and/or 5 expressed on the cell surface in a variety of tumor cells (3). Preclinical and clinical studies indicate that TRAIL is safe for potential therapeutic use (4,5). However, TRAIL resistance has been widely reported, and this resistance appears to be mediated through the loss of TRAIL receptors, inhibitors such as cFLIP, X-linked inhibitor of apoptosis protein (XIAP), cIAP, and survivin alternations in expression of the Bcl-2 family proteins (6-9). Combinational therapy enhancing TRAIL-induced apoptosis through differential regulation of pro- and anti-apoptotic proteins have been reported, including combination with chemo-radiotherapy/biological agents. These kinds of strategies have been shown to have favorable development prospects (2).

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), which is isolated from the roots of the medicinal plant *Plumbago zeylanica* L., has been safely used for centuries in Indian Ayurvedic and Oriental medicine for the treatment of various ailments (10,11). Plumbagin and its analogs exhibit a variety of potent pharmacological and biological activities including anti-microbial (12,13), anti-malarial (14), anti-inflammatory (15), anti-atherosclerotic (16), anti-diabetic (17) and neuroprotective (18) effects. The promising antineoplastic effect of plumbagin has also been demonstrated in various cancer models both *in vivo* and *in vitro*. It effectively

Correspondence to: Dr Ligen Liu, Department of Hematology, Shanghai No. 5 Hospital, Fudan University, 801 Heqing Road, Shanghai 200240, P.R. China
E-mail: 2512034560@qq.com

Key words: plumbagin, tumor necrosis factor- α -related apoptosis-inducing ligand, death receptor, t(8;21), reactive oxygen species, xenograft

induces apoptosis and exerts anti-proliferation activity in diverse cancer cell lines, such as leukemia (19,20), lung cancer (21,22), prostate cancer (23), breast cancer (24-26), ovarian cancer (27), cervical cancer (28) and melanoma (29). The mechanisms of its antitumor effect are still not fully unveiled.

The balanced translocation between chromosome eight and twenty-one [t(8;21)] is one of the most frequent chromosomal abnormalities observed in acute myeloid leukemia (AML) and only ~50% of patients with this relatively favorable subtype can survive for 5 years (30). The Kasumi-1 cell line with the expression of t(8;21) is widely used as a model for the study of this AML subtype (31). Although TRAIL and/or plumbagin were investigated in some leukemic cell lines, there is no previous report on plumbagin, TRAIL and their combination on Kasumi-1 cell line.

In this study, we determined the cell proliferation inhibition activity of both agents alone and/or combination by using *in vitro* and *in vivo* experimental models on Kasumi-1 cells and also explored their possible mechanisms. Collectively, our results suggest that combination treatment with plumbagin and TRAIL might be an effective therapeutic strategy for TRAIL resistant AML with t(8;21).

Materials and methods

Reagents. Plumbagin and NAC were purchased from Sigma-Aldrich (USA). Plumbagin was dissolved in dimethyl sulfoxide (DMSO). The recombinant human zinc ion positioned trimeric form of TRAIL/Apo-2L was a gift from Shanghai Qiaer Co. Revert Aid First strand cDNA synthesis kit was from Fermentas (EU). SYBR Green PCR master mix was from AB Applied Biosystems (UK). For western blot analysis, the antibodies used were: anti-DR5 (Abcam), anti-caspase-3, anti-caspase-8, anti-caspase-9 (R&D), anti-Bid (Abcam), anti-bcl-2 (R&D), anti-bax (R&D), anti-GAPDH (Cell Signaling Technology), anti-cFLIP (Stressgen) and peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies (Chemicon Co., USA and Canada). For flow cytometry antibodies used were: anti-DR4 (LifeSpan BioSciences, Inc., USA), anti-DR5 (LifeSpan BioSciences, Inc.), anti-TRAIL (Biolegend, USA), anti-DcR1 (LifeSpan BioSciences, Inc.), anti-DcR1 (LifeSpan BioSciences, Inc.). Neutralizing anti-TRAIL-R2 (DR5) was purchased from Diaclone (French).

Cell cultures, cell morphology and TUNEL assay. Human leukemic Kasumi-1 cell lines were kindly provided by Professor J.X. Wang (Institute of Hematology and Blood Diseases Hospital, CAMS and PUMC) or J. Zhu (Shanghai Institute of Hematology) and confirmed by morphology, cytogenetics, immunophenotype and/or molecular analysis. Kasumi-1 cells were cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS). Cultures were maintained in a humidified atmosphere of 95% air/5% CO₂ at 37°C. Cell morphology was evaluated by Wright's staining of cells prepared by cytospin centrifugation. Cells were assessed by imaging the TUNEL-positive cells under a light microscope after incubating for 12 h using TUNEL kit (Chemicon Co.).

Cell proliferation assay. Cell proliferation was analyzed using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan).

In brief, cells were seeded on a 96-well plate (Corning Inc., Corning, NY, USA) at a concentration of 5x10⁵ cells/well in a volume of 200 µl. The cells were incubated with plumbagin, rsTRAIL or the combination of plumbagin and rsTRAIL at different concentrations for 12, 24 or 48 h and the absorbance was measured at 490 nm by spectrophotometry.

Flow cytometry analysis of Annexin V/PI, cell cycle, mitochondrial membrane potential, and death receptors. A total of 2x10⁴ cells were evaluated by Annexin V/PI kit (BD Pharmingen, USA) according to FACSCanto™ flow cytometer. To assess the cell cycle, cells were collected, washed in PBS and fixed overnight in 75% ethanol at -20°C, incubated with PI/RNase staining buffer (BD Pharmingen, USA) for 15 min at room temperature. For the detection of mitochondrial membrane potential, 1x10⁶ cells were washed twice with PBS, incubated with JC-1 kit (BD Pharmingen) for 15 min at 37°C in the dark. Cell surface expression of DR4 and DR5 was detected by incubation of Kasumi-1 cells with primary antibodies (anti-DR4, anti-DR5) with FITC (fluorescein isothiocyanate) for 15 min at room temperature. Fluorescence intensity was monitored using a Beckman flow cytometry. All experiments were performed in triplicate and data were analyzed by software.

Quantitative real-time RT-PCR. Total RNA was isolated using TRIzol (Invitrogen Co., Carlsbad, CA, USA). Total RNA template (2 µg) was used per 1 µl of reverse transcriptase reaction by AMV reverse transcriptase (Fermentas, EU) using oligo(dT) primers at 42°C for 1 h according to the manufacturer's instructions. For semi-quantitative real-time RT-PCR, duplicate 1 µl samples of each cDNA using SYBR Green mix were amplified as follows: 95°C for 10 min, 40 cycles at 95°C for 15 sec, and 60°C for 60 sec. The relative amount of gene expression was calculated using the expression of β-actin as an internal standard. DR4: sense, 5'-GGCTGAGGACAATGCTCACA-3'; antisense, 5'-TTGCTGCTCAGAGACGAAAGTG-3'. DR5: sense, 5'-CCAGCCCTCCCTCAGATGTAC-3'; antisense, 5'-TGTAAGGTTGTCCAGTTCAAAGACT-3'. β-actin: sense, 5'-CCAGCTCACCATGGATGATG-3'; antisense, 5'-ATGCCGAGCCGTTGTC-3'. Data were normalized to the level of β-actin expression in each individual sample according to $\Delta\Delta Ct$ ($Ct_{DR4/DR5} - Ct_{\beta-actin}$) value.

Western blot analysis. Cells treated with plumbagin alone, rsTRAIL alone, or their combinations were harvested, washed with PBS, and treated with RIPA lysis buffer (Merck Millipore, Darmstadt, Germany). The lysates were centrifuged at 10,000 x g for 15 min at 4°C and the concentration of protein in each lysate was determined using BCA protein assay reagent (Biyuntian) following the manufacturer's protocol. After being mixed with loading buffer, 30 µg of protein sample in each group was loaded for SDS-polyacrylamide gel electrophoresis and subsequently transferred to a PVDF membrane. Then, PVDF membrane was blocked with appropriate blocking solution for 2 h at room temperature (RT), incubated with primary antibody overnight at 4°C, and followed with HRP conjugated secondary antibody for 2 h at RT. After being incubated with electrochemiluminescence substrate (Immobilon Western Chemiluminescent HRP substrate), PVDF membrane

was visualized and scanned via FluorChem E system (Bio-Techne, San Jose, CA, USA)

Glutathione determination. Kasumi-1 cells were treated with diverse concentration of plumbagin. The cells were centrifuged with 800 x g for 10 min. The concentrations of total glutathione (T-GSH), reduced glutathione (GSH) and oxidized disulfide (GSSG) in different group cells were measured by an enzymatic method according to the commercial assay kit procedure (Beyotime Institute of Biotechnology, Jiangsu, China).

In vivo tumor xenograft study. NOD/SCID male mice (4-6-week-old) (Beijing HFK Bioscience Co. Ltd., Beijing, China) were housed under pathogen-free conditions with a 12-h light/12-h dark schedule and fed with an autoclaved diet and water *ad libitum*. All animal procedures adhered to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals with all efforts to minimize animal number and suffering. Kasumi-1 cells were injected s.c. into the flanks of the mice (2.5×10^6 in 2 ml) and tumors were allowed to develop for 18-30 days until they reached 50-100 mm³, then treatment was initiated. Mice (n=25) were randomly assigned to one of the following treatment groups: i) mice (n=5) was treated daily with vehicle (0.4 ml physiological saline) as a normal saline group; ii) Ara-C group (n=5) was treated daily with Ara-C at doses of 50 mg/kg body weight in 0.4 ml PBS; iii) mice (n=5) had an injection of rsTRAIL alone (10 mg/kg body weight in 0.1 ml PBS); while iv) plumbagin alone group (n=5) were treated with plumbagin (6 mg/kg of body weight) in a volume of PBS 0.4 ml every day; v) mice (n=5) were treated daily with rsTRAIL (10 mg/kg) and plumbagin (6 mg/kg) in a final volume of 0.6 ml for two weeks. Tumor volumes and weights were measured twice a week. Tumor size was based on the formula: tumor volume (TV, mm³) = 1/2 (long diameter) x (short diameter)², measured by calipers. After 3 weeks of treatment, tumor-bearing mice were sacrificed. Xenograft tumors, as well as other vital organs of the treated and control mice were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 3-5- μ m sections. The pathological slices of tumor and organs of mouse were stained with H&E method and observed under a microscope. The apoptotic rate and the expression of TRAIL and TRAIL receptors on the surface of the cells were detected by flow cytometry.

Statistical analysis. Data are expressed as mean values \pm standard deviation (SD). Analyses were carried out using Student's t-tests. Values of $P < 0.05$ were considered statistically significant.

Results

Combined treatment with plumbagin and rsTRAIL decreased the cell viability and induced cell apoptosis in human leukemic Kasumi-1 cells. Using CCK-8 assay, we first treated Kasumi-1 cells with different concentrations of rsTRAIL or plumbagin alone for different incubation times to determine cell viability. rsTRAIL alone from 10 to 1,000 ng/ml could inhibit cell proliferation in a dose- and time-dependent

manner after 12-72 h (Fig. 1A). Plumbagin inhibited cell proliferation in Kasumi-1 cells with a dosage of 2-12 μ M (Fig. 1B). Then, we tested their combinational effects. As shown in Fig. 1C, rsTRAIL-inhibited cell viability of Kasumi-1 cells were dosage and time-dependently enhanced by 2 and 4 μ M plumbagin, which is slightly inhibited in Kasumi-1 cells.

To confirm whether the cell viability decrease was caused by apoptosis, cell morphology and TUNEL staining assay were performed. We exposed Kasumi-1 cells to various concentrations of rsTRAIL alone or in combination with 2 μ M plumbagin for 12 h. Many more cells showed characteristic changes with plumbagin plus rsTRAIL, such as cell shrinkage, nuclear condensation, and formation of apoptotic bodies (Fig. 1D). As shown in Fig. 1D, more cells with brown precipitate were toward the combination group than rsTRAIL or plumbagin alone. Cell cycle analysis also revealed a markedly increased sub-G1 population in the combined treatment with plumbagin and rsTRAIL, compared with the results of plumbagin or rsTRAIL alone (Fig. 1E).

Plumbagin upregulated expression of DR5 and contributed to the enhancement of TRAIL-induced cell death. Death receptors (DRs) play key roles in TRAIL-induced apoptosis (32,33). It has been reported that sensitivity to TRAIL-induced apoptosis may result from the differential expression of death receptors, and a positive correlation of the sensitivity to TRAIL with the expression of DR4 and DR5 is documented (1,34). We, therefore, investigated whether these receptors are similarly regulated by plumbagin in Kasumi-1 cells. We examined the effect of plumbagin on the expression of death receptors at both mRNA and protein levels by using real-time PCR, flow cytometry and western blot analyses. We found that treatment of Kasumi-1 cells with 1 and 2 μ M plumbagin for 4, 8 and 12 h resulted in an increased mRNA level expression of DR5 but not DR4 in a dose- and time-dependent manner (Fig. 2A). The surface levels of DR4 and DR5 were also detected by flow cytometry analysis, and upregulation of DR5 was further confirmed as shown in Fig. 2B. Then we extracted protein of Kasumi-1 cells treated by 1 and 2 μ M plumbagin for 4 and 8 h. Western blot analysis was performed and clearly showed the upregulated expression of DR5 (Fig. 2C). To observe the upregulation of DR5 in both agents combination-induced apoptosis, Kasumi-1 cells were further treated by plumbagin alone, rsTRAIL alone, and their combination for 4, 8 and 12 h. As shown in Fig. 2D, upregulation of DR5 expression was observed in plumbagin alone and combination with rsTRAIL group but not in rsTRAIL group.

To investigate the functional upregulation of DR5 induced by plumbagin in Kasumi-1 cells, we used an anti-DR5 neutralizing antibody to inhibit DR5 activity by flow cytometry analysis. We demonstrated that the apoptosis induced by rsTRAIL alone and the combination of plumbagin and rsTRAIL were significantly abolished in the presence of anti-DR5 neutralizing antibody but not plumbagin alone (Fig. 2E). The results demonstrated that upregulation of DR5 by plumbagin contribute to apoptosis of Kasumi-1 cells induced by TRAIL.

Caspase activation, mitochondrial damage and decreased cFLIP expression were involved in the enhancement effect of

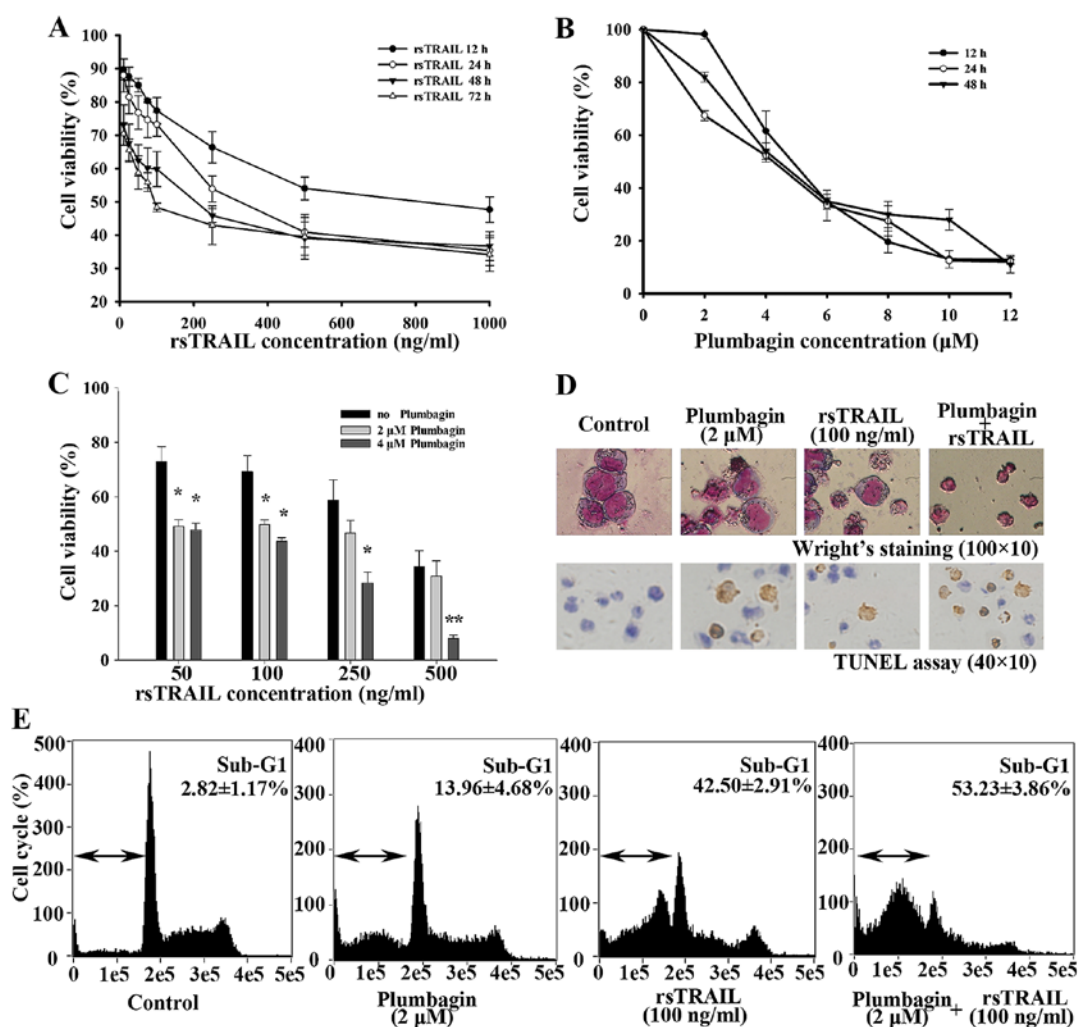


Figure 1. The growth inhibitory effect and apoptosis of plumbagin, rsTRAIL, or both in Kasumi-1 cells. (A) Various concentration (10-1,000 ng/ml) of rsTRAIL effects on cell viability at 12, 24, 48 or 72 h. (B) Different concentration (2-12 μ M) of plumbagin effects on cell viability at 12, 24 or 48 h. (C) rsTRAIL combines with 2 or 4 μ M plumbagin effect on cell viability at 24 h. * P <0.05, ** P <0.01 comparing with the no plumbagin cells. (D) Characteristic apoptotic cells treated with plumbagin, rsTRAIL or combination plumbagin with rsTRAIL for 12 h by Wright's staining (100x10) on top. Apoptotic cells underneath detected by TUNEL assays were treated for 12 h. The content of TUNEL-positive cells was the number of brown precipitate in each photograph (40x10). (E) Contribution of nuclear DNA content treated with plumbagin, rsTRAIL or plumbagin plus rsTRAIL for 12 h. Data are one representative from three independent experiments.

plumbagin on rsTRAIL-induced Kasumi-1 cell apoptosis. Both the death receptor (extrinsic) pathway and the mitochondria (intrinsic) pathway are involved in TRAIL-induced apoptosis, and TRAIL apoptosis signaling pathway finally results in the activation of caspases (35). Cellular FLICE-inhibitory protein (c-FLIP) is major inhibitor of TRAIL-mediated apoptosis and is involved in the resistance to TRAIL-induced apoptosis (2). We then analyzed the caspases, Bcl-2, Bid, Bax and cFLIP by western blot analysis. As shown in Fig. 3A, combined treatment led to more significantly increasing caspase-8, -3 and -9 activities than treatment with rsTRAIL alone, indicating that combined treatment induces apoptosis of Kasumi-1 cells through a caspases-dependent pathway. Upregulation of Bax and activation of Bid were also observed in the combined group, suggesting intrinsic pathway is involved (Fig. 3B). In parallel to the western blot analysis, combined treatment with plumbagin and rsTRAIL also significantly decreased the MMP (Fig. 3C). As shown in Fig. 3B, the expressions of c-FLIP was decreased in plumbagin group and the combined

group, but not in rsTRAIL alone group, suggesting that down-regulation of c-FLIP might be involved in plumbagin-induced apoptosis of Kasumi-1 cells and might be used as the sensitizer of TRAIL resistance cells.

The effects of plumbagin on the expression of DR5, Bax and cFLIP is partially abolished by ROS scavenger NAC in Kasumi-1 cells. Plumbagin contains a quinone nucleus which could transfer one electron in aerobic metabolism under oxidative stress, and the semiquinone radical participates in redox cycling to generate reactive oxygen species (ROS) like superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (36). Published data reported that plumbagin effectively induced hematological malignancies, in which ROS levels play an important role in mediating apoptosis (19,20,37). We postulated that the mechanism might also underlie plumbagin-induced apoptosis in Kasumi-1 cells. Then, ROS scavenger NAC was combined with plumbagin to induce apoptosis of Kasumi-1 cells. Apoptosis of Kasumi-1 cells induced by

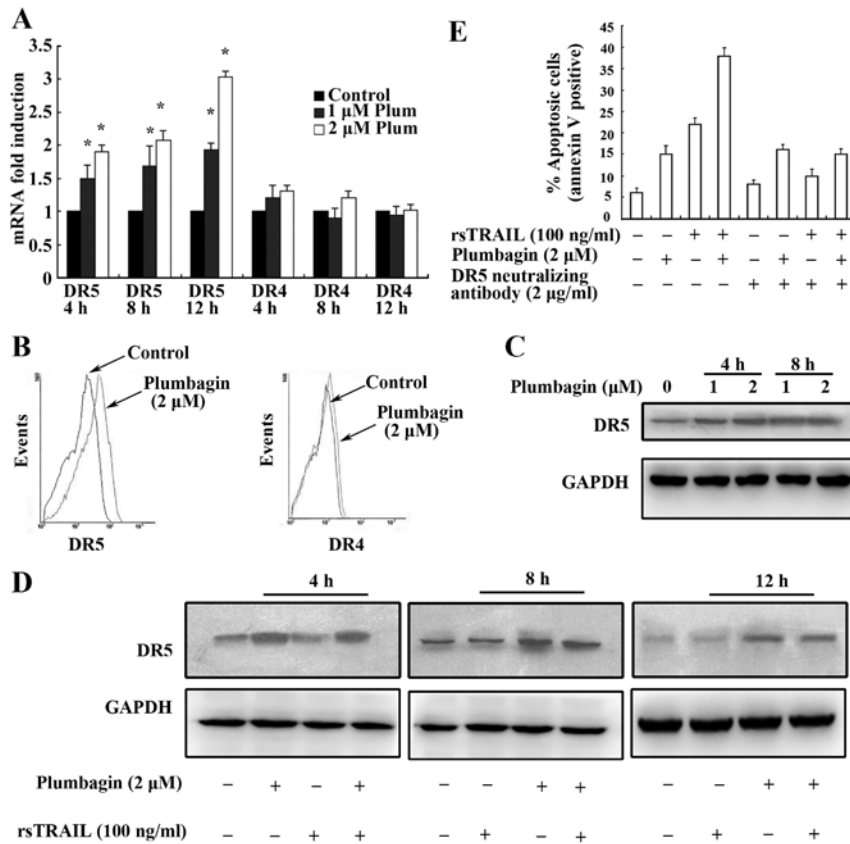


Figure 2. Analysis of TRAIL receptor expression of DR4 and DR5. (A) Analysis of DR4 and DR5 on mRNA levels from Kasumi-1 cells 4, 8 or 12 h after treatment with 1 and 2 μ M of plumbagin. Results are reported as average fold induction normalized to the untreated sample. All values are normalized to the β -actin internal controls. * P <0.05, significantly higher than control. (B) Analysis of TRAIL receptors DR4 and DR5 by flow cytometry. Percentage of cells expressing the receptor is reported for Kasumi-1 cells after plumbagin (2 μ M) treatment for 8 h. Data are one representative from three independent experiments. (C) Western blot analysis of DR5 in Kasumi-1 cells treated with plumbagin at 1 and 2 μ M for 4 and 8 h. (D) DR5 expression was increased in Kasumi-1 cells treated with plumbagin or combination with rsTRAIL at the indicated concentrations for 4, 8 and 12 h. (E) Kasumi-1 cells were cultured in control conditions (-), in the presence of rsTRAIL (+) with (+) or without (-) plumbagin and anti-DR5 neutralizing antibodies for 12 h and apoptosis rate was assessed by Annexin V/PI staining. The graph represents the mean values \pm SE of Annexin V-positive cell of three independent experiments.

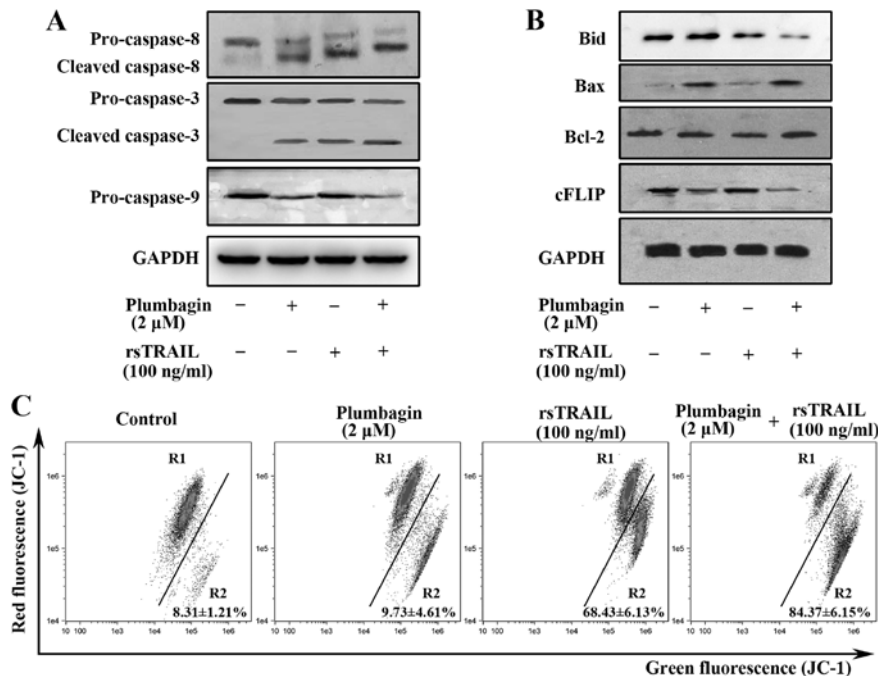


Figure 3. Plumbagin and rsTRAIL induces caspase activation, bax upregulation, cFLIP downregulation and MMP loss. (A) Western blot analysis of caspase-8, -3, and -9 in Kasumi-1 cells treated with plumbagin (2 μ M), rsTRAIL (100 ng/ml), or both reagents for 8 h. (B) Western blot analysis for Bid, Bax, Bcl-2, and cFLIP. Kasumi-1 cells were treated with plumbagin (2 μ M), rsTRAIL (100 ng/ml), or both reagents for 8 h. (C) The cells stained with JC-1 were exposed to the indicated concentrations of plumbagin and rsTRAIL for 12 h and then were analyzed by flow cytometry. The data are representative of three separate experiments.

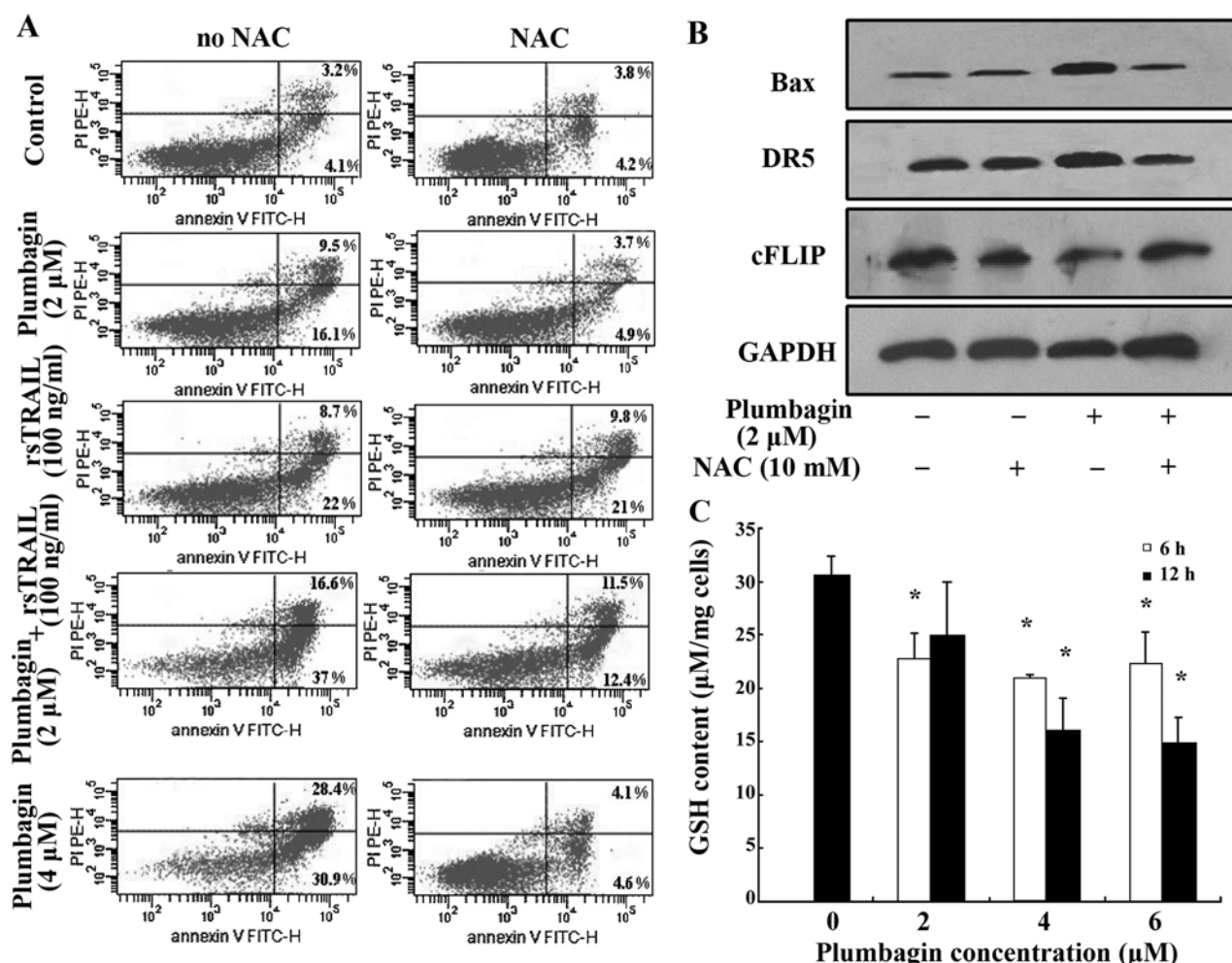


Figure 4. The effects of ROS scavengers on plumbagin-induced apoptosis, DR5 upregulation, Bax expression, and cFLIP degradation. (A) Kasumi-1 cells pretreated with 10 mM NAC for 1 h were incubated with 4 μ M plumbagin alone, or 2 μ M plumbagin plus 100 ng/ml rsTRAIL for 12 h. Cells were stained with Annexin V/PI and analyzed by FACS. (B) Cells pretreated with 10 mM NAC for 1 h were incubated with 2 μ M plumbagin for 12 h. The expression of DR5, Bax and cFLIP were determined by western blot analysis. (C) Kasumi-1 cells were treated with different concentration of plumbagin (2-6 μ M). Contents of GSH in Kasumi-1 cells were measured by an enzymatic method. Values are mean \pm SD. *Significant difference from control ($P < 0.05$).

plumbagin (4 μ M) alone or plumbagin (2 μ M) plus rsTRAIL (100 ng/ml) were inhibited by NAC from 59.3 to 8.7 and 53.6 to 23.9%, respectively (Fig. 4A). However, NAC did not show a significant effect on rsTRAIL (100 ng/ml) alone induced apoptosis of Kasumi-1 cells (Fig. 4A). Cell lysates containing equal amounts of total protein from cells treated by plumbagin with or without NAC were assayed for western blot analysis, and the expression of DR5, Bax and cFLIP were detected. Plumbagin-induced DR5 upregulation was attenuated by the ROS scavenger NAC, the expression of Bax and cFLIP was also influenced by the ROS scavenger NAC (Fig. 4B).

GSH depletion by plumbagin increases the production of ROS. It has been shown that the decrease of GSH content is a common feature in apoptotic cell death (38). Previous studies reported that plumbagin regulates the cellular redox state by modulation of GSH (36,39). In this study, we observed the content of GSH in Kasumi-1 cells treated by plumbagin for 6 and 12 h. The content of GSH was significantly decreased in groups of plumbagin treatment. The decrease was negatively correlated with plumbagin concentrations (Fig. 4C).

Combined treatment with plumbagin and rsTRAIL inhibits tumor growth *in vivo*. To determine whether combined treatment with plumbagin and rsTRAIL can inhibit tumor growth *in vivo*, we injected human leukemic Kasumi-1 cells s.c. into NOD/SCID male mice. Combined treatment with plumbagin and rsTRAIL had a synergistic effect and was found to markedly inhibit tumor growth compared with the control group, plumbagin alone, or TRAIL alone ($P < 0.05$) (Fig. 5A). The tumor volume was 1783.12 ± 891.28 mm³ in the control group and 293.51 ± 86.06 mm³ in combined group ($P < 0.01$) (Fig. 5B). Moreover, the average volume of the tumors in combined group was reduced by 19.44% when compared with the control group ($P < 0.01$) (Fig. 5B). As shown in Fig. 5C, the decrease of tumor weight is obvious in Ara-C group, plumbagin alone group and combined group. Apart from the infiltration of tumor cells in spleen and bone marrow, there was no obvious toxic pathologic change in the heart, liver or kidney tissues in any of the groups (Fig. 5D).

To gain insight into the mechanism of combined treatment with plumbagin and rsTRAIL inhibition of tumor growth *in vivo*, we harvested the Kasumi-1 tumor xenografts from

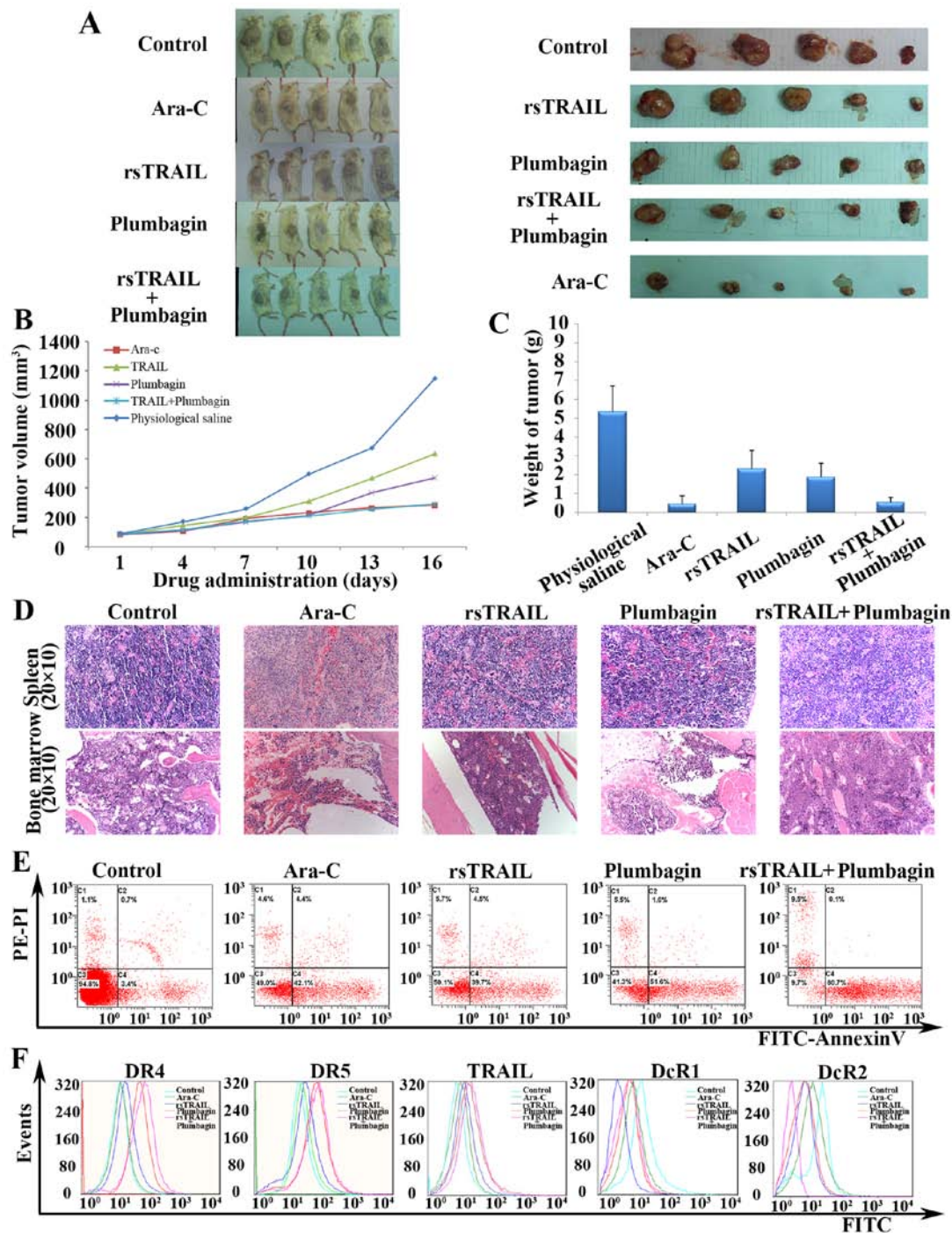


Figure 5. Effect of combined treatment with plumbagin and rsTRAIL on Kasumi-1 xenograft in NOD/SCID mice. From days 18-30, mice with established tumors (50-100 mm³) were dosed s.c. with plumbagin (6 mg/kg), rsTRAIL (10 mg/kg) or Ara-C (50 mg/kg), and the control mice were treated with vehicle every day. Data were presented as means \pm SD. (A) Tumor-bearing mice and tumors from the control and drug-treated groups. (B) Tumor volume measured at the days of drug administration. (C) Tumor weight of NOD/SCID mice in course of treatment. Data are presented as means \pm SD. (D) Tissue sections of spleen and bone marrow from experimental mice discerned by H&E staining (20x10). The histopathologic examination of organs was made after mice were sacrificed. (E) Apoptotic cells were evaluated by Annexin V/PI staining. (F) The expression of TRAIL and TRAIL receptor on the surface of the cells were detected by flow cytometry in each group.

the treated mice. Comparing with the control mice, a significantly increased number of apoptotic cells was observed in the combined treated mice ($P < 0.01$) (Fig. 5E). As shown in Fig. 5F, combined treatment with plumbagin and rsTRAIL could increase the expression of both DR4 and DR5 receptors and decrease the DcR2 receptors expression on the surface of the cells. In addition to this, plumbagin not only increased

the expression of DR5 in cells of xenograft tumors, but also increased the expression of DR4, which was different with the expression of DRs *in vitro*. There were no obvious difference on the expression of both TRAIL and DcR1 receptors between combined groups and control groups. These data indicated that the administration of plumbagin plus rsTRAIL induces tumor regression associated with apoptosis *in vivo*.

Discussion

AML with t(8;21) accounts for ~15% of AML. It is generally considered as a subtype of better outcomes, but resistance to drugs is still a tough problem in clinic. So the approach of finding new agents and/or methods is important to conquer this problem. In this study, we used Kasumi-1 cells, which is a classic cell line derived from t(8;21) positive acute leukemia patient, as model to study the effect of two agents, the rsTRAIL and plumbagin. Although rsTRAIL could significantly induce apoptosis of Kasumi-1 cells, resistance to TRAIL apoptotic pathway is a common phenomenon in refractory/relapse AML patients (35). Here we report for the first time, that plumbagin could enhance TRAIL-induced apoptosis on acute myeloid leukemia cells with t(8;21). This was observed not only in well-established cell lines *in vitro*, but also in a murine xenograft tumor model. Combined treatment with plumbagin and rsTRAIL decreased the cell viability mainly due to the induction of apoptosis, which has been demonstrated by morphological assay and increase of apoptotic cells both *in vitro* and *in vivo*. The mechanisms by which plumbagin mediates its effects on TRAIL-induced apoptosis appears to involve the induction of TRAIL receptor upregulation, activation of caspase-8 and inhibition of cFLIP.

Firstly, we found upregulation of DR5 expression was closely involved in the enhancement effect of plumbagin on TRAIL-induced apoptosis of Kasumi-1 cells both *in vitro* and *in vivo*. This further supports the notion that TRAIL receptor expression is related to TRAIL sensitivity in tumor cells (35). As shown on Fig. 1B, the concentration of plumbagin $<2\ \mu\text{M}$ could not induce significant apoptosis of Kasumi-1 cells within 12 h. Then, the concentrations of plumbagin at 1 and $2\ \mu\text{M}$ were applied to test the effect on TRAIL-induced apoptosis of Kasumi-1 cells and their influence of TRAIL receptors as well as death signaling pathway. The results of real-time PCR showed that upregulation of DR5 mRNA level, but not DR4, by plumbagin was significant in plumbagin-induced apoptosis of Kasumi-1 cells. Flow cytometry and western blot analysis further confirmed the findings at protein levels. Similar results were seen by Li *et al* previously finding that plumbagin upregulated death receptor mRNA and protein expression of human melanoma A375 cells (40).

Although expressed on the cell surface, DR4 and DR5 may not be functional, and both functional and non-functional TRAIL receptors are detected by FCM and western blot analysis (2). Then, we used DR5 neutralizing antibody to observe its effect on Kasumi-1 cells. The enhancement effect of plumbagin on TRAIL-induced apoptosis of Kasumi-1 cells was significantly decreased after the neutralization of DR5. It means upregulation of DR5 by plumbagin is functionally taken effect among TRAIL-induced apoptosis of Kasumi-1 cells. Then we asked whether these findings could be translated into *in vivo* situations. Interestingly, our study found that plumbagin could increase the expression of both DR4 and DR5 in murine xenograft tumors, which was different from the results that only the DR5 expression on the cell surface *in vitro*. Recently, von Karstedt *et al* (41) found that high TRAIL-R2 expression correlates with invasion of pancreatic ductal adenocarcinoma into lymph vessels. Upregulation of DR4 not only DR5 might imply more clinical importance and need to be further investigated.

Secondly, the augmented effect of plumbagin on TRAIL-induced apoptosis of Kasumi-1 cell is ROS-dependent, and ROS levels are mainly negatively regulated by content of GSH in the cell has been reported. Plumbagin can induce ROS-mediated apoptosis in various leukemic cells *in vitro* (20,37). In this study, plumbagin was also confirmed to induce significant apoptosis of leukemic Kasumi-1 cells *in vitro*, which was completely abolished by NAC treatment.

There are two main mechanisms with the therapeutic activity of quinones including the production of the semiquinone radical under aerobic conditions which participates in redox cycling to generate ROS, like superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) and as a potent electrophile reacting with the thiol groups of proteins and GSH (36). Plumbagin is a bicycling naphthoquinone, its cytotoxic properties is related to its quinone core and contribute to its therapeutic activity.

Castro *et al* (36) reported the toxicity of plumbagin acts mainly as a potent electrophile in *Saccharomyces cerevisiae*, and then decrease the concentration of GSH. The GSH level is an important factor in the protection against several stress conditions, and low GSH levels can be associated with decrease in cell proliferation, protection against apoptosis and ROS elimination. The contents of GSH in Kasumi-1 cells after treated with plumbagin were negatively correlated with the concentration of plumbagin from 2 to $6\ \mu\text{M}$. Therefore, we postulate that plumbagin-induced ROS-mediated apoptosis of leukemic Kasumi-1 cells are mainly through reacting with thiol group of GSH. Glutathione-S-transferases (GST), which is related to the contents of GSH in cells and is important in quinone detoxification, may also be inactivated by plumbagin. This modulation is paralleled by ROS formation (42).

Thirdly, we found the activation of both extrinsic and intrinsic caspase pathway is involved in plumbagin-induced apoptosis of Kasumi-1 cells, which in turn may potentially promote TRAIL-induced apoptosis in Kasumi-1 cells. As shown in Fig. 3A, both intrinsic and extrinsic caspases could be activated in plumbagin alone and the combination with rsTRAIL-induced apoptosis of Kasumi-1 cells. It was interesting that caspase-8 (the substrate of extrinsic pathway) could be activated by plumbagin alone at the concentration $<2\ \mu\text{M}$ in our study, and the result was consistent with the finding of Xu and Lu that caspase-8 could be activated after caspase-9 activation in NB4 cells (19). McKallip *et al* (43) also suggested that the observed effects on caspase-8 activity following plumbagin exposure were mediated through the production of ROS. Wieder *et al* (44) reported that drug-induced caspase-8 activation in B-lymphoma cells is independent of death receptor signaling and is mediated by postmitochondrial caspase-3 activation. Plumbagin is generally considered as an intrinsic apoptosis inducer, our results supported that there is cross-talk between the extrinsic and intrinsic apoptotic pathways in plumbagin-induced apoptosis of tumor cells. Therefore, we think plumbagin-induced caspase-8 activation might also involve in its enhancement effect on TRAIL-induced apoptosis of Kasumi-1 cells.

In conclusion, we found that plumbagin could enhance TRAIL-induced apoptosis in Kasumi-1 cells, and the mechanisms include ROS-mediated upregulation of DR5 expression, caspase-8 activation and inhibition of cFLIP expression. Although overcoming TRAIL resistance still remains a

challenge, our *in vitro* and *in vivo* data suggest that rational combination of plumbagin and TRAIL is a regimen that would optimize the anti-leukemic activity in refractory/relapse AML with t(8;21) in future.

Acknowledgements

This study was supported by a grant from National Natural Science Foundation of China (NSFC, no. 30672415), Science and Technology Commission of Shanghai Municipality (STCSM, no. 054119528) and Japanese Society for the Promotion of Science Ronpaku program (2004-CSC-2).

References

- LeBlanc HN and Ashkenazi A: Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* 10: 66-75, 2003.
- Mahalingam D, Szegezdi E, Keane M, de Jong S and Samali A: TRAIL receptor signalling and modulation: Are we on the right TRAIL? *Cancer Treat Rev* 35: 280-288, 2009.
- Sessler T, Healy S, Samali A and Szegezdi E: Structural determinants of DISC function: New insights into death receptor-mediated apoptosis signalling. *Pharmacol Ther* 140: 186-199, 2013.
- Ashkenazi A, Holland P and Eckhardt SG: Ligand-based targeting of apoptosis in cancer: The potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). *J Clin Oncol* 26: 3621-3630, 2008.
- 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.
- Zhang L and Fang B: Mechanisms of resistance to TRAIL-induced apoptosis in cancer. *Cancer Gene Ther* 12: 228-237, 2005.
- Van Geelen CMM, de Vries EGE and de Jong S: Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: Paving the road to patient-tailored therapy. *Drug Resist Updat* 7: 345-358, 2004.
- O'Kane HF, Watson CJ, Johnston SR, Petak I, Watson RW and Williamson KE; O Kane HF: Targeting death receptors in bladder, prostate and renal cancer. *J Urol* 175: 432-438, 2006.
- Cheng J, Hylander BL, Baer MR, Chen X and Repasky EA: Multiple mechanisms underlie resistance of leukemia cells to Apo2 Ligand/TRAIL. *Mol Cancer Ther* 5: 1844-1853, 2006.
- Padhye S, Dandawate P, Yusufi M, Ahmad A and Sarkar FH: Perspectives on medicinal properties of plumbagin and its analogs. *Med Res Rev* 32: 1131-1158, 2012.
- Krishnaswamy M and Purushothaman KK: Plumbagin: A study of its anticancer, antibacterial & antifungal properties. *Indian J Exp Biol* 18: 876-877, 1980.
- Mishra BB, Singh DD, Kishore N, Tiwari VK and Tripathi V: Antifungal constituents isolated from the seeds of *Aegle marmelos*. *Phytochemistry* 71: 230-234, 2010.
- Abdul KM and Ramchender RP: Modulatory effect of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) on macrophage functions in BALB/c mice. I. Potentiation of macrophage bactericidal activity. *Immunopharmacology* 30: 231-236, 1995.
- Pradeepa V, Sathish-Narayanan S, Kirubakaran SA and Senthil-Nathan S: Antimalarial efficacy of dynamic compound of plumbagin chemical constituent from *Plumbago zeylanica* Linn (Plumbaginaceae) against the malarial vector *Anopheles stephensi* Liston (Diptera: Culicidae). *Parasitol Res* 113: 3105-3109, 2014.
- Checker R, Patwardhan RS, Sharma D, Menon J, Thoh M, Sandur SK, Sainis KB and Poduval TB: Plumbagin, a vitamin K3 analogue, abrogates lipopolysaccharide-induced oxidative stress, inflammation and endotoxic shock via NF- κ B suppression. *Inflammation* 37: 542-554, 2014.
- Sharma I, Gusain D and Dixit VP: Hypolipidaemic and anti-atherosclerotic effects of plumbagin in rabbits. *Indian J Physiol Pharmacol* 35: 10-14, 1991.
- Sunil C, Duraipandian V, Agastian P and Ignacimuthu S: Antidiabetic effect of plumbagin isolated from *Plumbago zeylanica* L. root and its effect on GLUT4 translocation in streptozotocin-induced diabetic rats. *Food Chem Toxicol* 50: 4356-4363, 2012.
- Son TG, Camandola S, Arumugam TV, Cutler RG, Telljohann RS, Mughal MR, Moore TA, Luo W, Yu QS, Johnson DA, *et al*: Plumbagin, a novel Nrf2/ARE activator, protects against cerebral ischemia. *J Neurochem* 112: 1316-1326, 2010.
- Xu KH and Lu DP: Plumbagin induces ROS-mediated apoptosis in human promyelocytic leukemia cells in vivo. *Leuk Res* 34: 658-665, 2010.
- Gaascht F, Teiten MH, Cerella C, Dicato M, Bagrel D and Diederich M: Plumbagin modulates leukemia cell redox status. *Molecules* 19: 10011-10032, 2014.
- Hsu YL, Cho CY, Kuo PL, Huang YT and Lin CC: Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53 accumulation via c-Jun NH2-terminal kinase-mediated phosphorylation at serine 15 in vitro and in vivo. *J Pharmacol Exp Ther* 318: 484-494, 2006.
- Shieh JM, Chiang TA, Chang WT, Chao CH, Lee YC, Huang GY, Shih YX and Shih YW: Plumbagin inhibits TPA-induced MMP-2 and u-PA expressions by reducing binding activities of NF-kappaB and AP-1 via ERK signaling pathway in A549 human lung cancer cells. *Mol Cell Biochem* 335: 181-193, 2010.
- Aziz MH, Dreckschmidt NE and Verma AK: Plumbagin, a medicinal plant-derived naphthoquinone, is a novel inhibitor of the growth and invasion of hormone-refractory prostate cancer. *Cancer Res* 68: 9024-9032, 2008.
- Kawiak A, Zawacka-Pankau J and Lojkowska E: Plumbagin induces apoptosis in Her2-overexpressing breast cancer cells through the mitochondrial-mediated pathway. *J Nat Prod* 75: 747-751, 2012.
- Kuo PL, Hsu YL and Cho CY: Plumbagin induces G2-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells. *Mol Cancer Ther* 5: 3209-3221, 2006.
- Lee JH, Yeon JH, Kim H, Roh W, Chae J, Park HO and Kim DM: The natural anticancer agent plumbagin induces potent cytotoxicity in MCF-7 human breast cancer cells by inhibiting a PI-3 kinase for ROS generation. *PLoS One* 7: e45023, 2012.
- Sinha S, Pal K, Elkhanany A, Dutta S, Cao Y, Mondal G, Iyer S, Somasundaram V, Couch FJ, Shridhar V, *et al*: Plumbagin inhibits tumorigenesis and angiogenesis of ovarian cancer cells in vivo. *Int J Cancer* 132: 1201-1212, 2013.
- Nair S, Nair RRR, Srinivas P, Srinivas G and Pillai MR: Radiosensitizing effects of plumbagin in cervical cancer cells is through modulation of apoptotic pathway. *Mol Carcinog* 47: 22-33, 2008.
- Wang CCC, Chiang YM, Sung SC, Hsu YL, Chang JK and Kuo PL: Plumbagin induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human melanoma A375.S2 cells. *Cancer Lett* 259: 82-98, 2008.
- Marcucci G, Mrózek K, Ruppert AS, Maharry K, Kolitz JE, Moore JO, Mayer RJ, Pettinati MJ, Powell BL, Edwards CG, *et al*: Prognostic factors and outcome of core binding factor acute myeloid leukemia patients with t(8;21) differ from those of patients with inv(16): A Cancer and Leukemia Group B study. *J Clin Oncol* 23: 5705-5717, 2005.
- Asou H, Tashiro S, Hamamoto K, Otsuji A, Kita K and Kamada N: Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation. *Blood* 77: 2031-2036, 1991.
- Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J and Dixit VM: The receptor for the cytotoxic ligand TRAIL. *Science* 276: 111-113, 1997.
- Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, Timour MS, Gerhart MJ, Schooley KA, Smith CA, *et al*: TRAIL-R2: A novel apoptosis-mediating receptor for TRAIL. *EMBO J* 16: 5386-5397, 1997.
- Ashkenazi A: Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2: 420-430, 2002.
- Fulda S: Cell death in hematological tumors. *Apoptosis* 14: 409-423, 2009.
- Castro FA, Mariani D, Panek AD, Eleutherio EC and Pereira MD: Cytotoxicity mechanism of two naphthoquinones (menadione and plumbagin) in *Saccharomyces cerevisiae*. *PLoS One* 3: e3999, 2008.
- Sun J and McKallip RJ: Plumbagin treatment leads to apoptosis in human K562 leukemia cells through increased ROS and elevated TRAIL receptor expression. *Leuk Res* 35: 1402-1408, 2011.
- Franco R and Cidrowski JA: Glutathione efflux and cell death. *Antioxid Redox Signal* 17: 1694-1713, 2012.

39. Inbaraj JJ and Chignell CF: Cytotoxic action of juglone and plumbagin: A mechanistic study using HaCaT keratinocytes. *Chem Res Toxicol* 17: 55-62, 2004.
40. Li J, Shen Q, Peng R, Chen R, Jiang P, Li Y, Zhang L and Lu J: Plumbagin enhances TRAIL-mediated apoptosis through up-regulation of death receptor in human melanoma A375 cells. *J Huazhong Univ Sci Technol* 30: 458-463, 2010.
41. von Karstedt S, Conti A, Nobis M, Montinaro A, Hartwig T, Lemke J, Legler K, Annewanter F, Campbell AD, Taraborrelli L, *et al*: Cancer cell-autonomous TRAIL-R signaling promotes KRAS-driven cancer progression, invasion, and metastasis. *Cancer Cell* 27: 561-573, 2015.
42. Turella P, Cerella C, Filomeni G, Bullo A, De Maria F, Ghibelli L, Ciriolo MR, Cianfriglia M, Mattei M, Federici G, *et al*: Proapoptotic activity of new glutathione S-transferase inhibitors. *Cancer Res* 65: 3751-3761, 2005.
43. McKallip RJ, Lombard C, Sun J and Ramakrishnan R: Plumbagin-induced apoptosis in lymphocytes is mediated through increased reactive oxygen species production, upregulation of Fas, and activation of the caspase cascade. *Toxicol Appl Pharmacol* 247: 41-52, 2010.
44. Wieder T, Essmann F, Prokop A, Schmelz K, Schulze-Osthoff K, Beyaert R, Dörken B and Daniel PT: Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3. *Blood* 97: 1378-1387, 2001.