BIOMOLECULES & THERAPEUTICS

The Histone Deacetylase Inhibitor Trichostatin A Sensitizes Human Renal Carcinoma Cells to TRAIL-Induced Apoptosis through Down-Regulation of c-FLIP$_L$

Min Ho Han[1], Cheol Park[2], Taek Kyu Kwon[3], Gi-Young Kim[4], Wun-Jae Kim[5], Sang Hoon Hong[6], Young Hyun Yoo[7],* and Yung Hyun Choi[1,8,*]

[1]Department of Biochemistry, College of Oriental Medicine, Dong-Eui University, Busan 614-851,
[2]Department of Molecular Biology, College of Natural Sciences, Dongeui University, Busan 614-714,
[3]Department of Immunology, School of Medicine, Keimyung University, Daegu 704-701,
[4]Laboratory of Immunobiology, Department of Marine Life Sciences, Jeju National University, Jeju 690-756,
[5]Department of Urology, College of Medicine, Chungbuk National University, Cheongju 362-763,
[6]Department of Internal Medicine, College of Oriental Medicine, Dong-Eui University, Busan 614-851,
[7]Department of Anatomy and Cell Biology, Dong-A University College of Medicine and Mitochondria Hub Regulation Center, Busan 602-714,
[8]Anti-Aging Research Center & Blue-BioIndustry RIC, Dongeui University, Busan 614-714, Republic of Korea

Abstract

Histone acetylation plays a critical role in the regulation of transcription by altering the structure of chromatin, and it may influence the resistance of some tumor cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) by regulating the gene expression of components of the TRAIL signaling pathway. In this study, we investigated the effects and molecular mechanisms of trichostatin A (TSA), a histone deacetylase inhibitor, in sensitizing TRAIL-induced apoptosis in Caki human renal carcinoma cells. Our results indicate that nontoxic concentrations of TSA substantially enhance TRAIL-induced apoptosis compared with treatment with either agent alone. Cotreatment with TSA and TRAIL effectively induced cleavage of Bid and loss of mitochondrial membrane potential (MMP), which was associated with the activation of caspases (-3, -8, and -9) and degradation of poly (ADP-ribose) polymerase (PARP), contributing toward the sensitization to TRAIL. Combined treatment with TSA and TRAIL significantly reduced the levels of the cellular Fas-associated death domain (FADD)-like interleukin-1β-converting enzyme (FLICE) inhibitory protein (c-FLIP), whereas those of death receptor (DR) 4, DR5, and FADD remained unchanged. The synergistic effect of TAS and TRAIL was perfectly attenuated in c-FLIP$_L$-overexpressing Caki cells. Taken together, the present study demonstrates that down-regulation of c-FLIP contributes to TSA-facilitated TRAIL-induced apoptosis, amplifying the death receptor, as well as mitochondria-mediated apoptotic signaling pathways.

Key Words: Trichostatin A, TRAIL, Apoptosis, c-FLIP$_L$

INTRODUCTION

Renal cell carcinoma (RCC) derived from renal tubular cells is a common urologic neoplasm. Among urologic cancers, the incidence of RCC is the third highest after prostate and bladder cancers, and up to half of RCC patients eventually develop metastatic RCC (Brugarolas, 2007; Ather *et al*., 2010). In contrast to many other malignancies, RCC is highly aggressive and generally resistant to chemotherapy, radiotherapy, and hormone therapy (Staehler *et al*., 2005; Inman *et al*., 2013). Therefore, new therapeutic alternatives are needed for more effective treatment of this malignancy.

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is considered a

promising anticancer agent due to its unique ability to induce cancer cell death, with only negligible effects on normal cells. TRAIL induces apoptosis by interacting with transmembrane type-I receptors, death receptor 4 (DR4, TRAIL-R1), and DR5 (TRAIL-R2), leading to the formation of a death-inducing signaling complex (DISC) consisting of Fas-associated death domain protein (FADD) and procaspase-8 (Srivastava, 2001; Thomas *et al*., 2004). Once the DISC has been assembled, self-cleaved caspase-8 activates effector caspases (-3 and -7). This results in DR-mediated extrinsic apoptotic cell death. The FADD-like apoptosis regulator (c-FLIP), a well-known anti-apoptotic gene, efficiently regulates apoptotic cell death (Rae *et al*., 2007; Benayoun *et al*., 2008; Hwang *et al*., 2014). Activated caspase-8 can cleave Bid to truncated Bid (tBid), which is responsible for translocation of apoptotic signals to mitochondria, resulting in the sequential induction of mitochondrial cytochrome *c* release, activation of caspase-9/-3 and cleavage of cellular proteins (intrinsic or mitochondria pathway). This process culminates in the promotion of apoptosis. Caspase-8 can also directly promote the proteolytic activation of effector caspases (Billen *et al*., 2008; Kantari and Walczak, 2011).

Despite the valuable selective tumoricidal activity of TRAIL, many cancer cells, including RCC cells (O'Kane *et al*., 2006; Voelkel-Johnson, 2011), are resistant to its apoptotic effects, possibly due to intrinsic or acquired resistance. The underlying mechanism of TRAIL resistance is not clear. However, several studies reported that it is intimately associated with loss of expression of caspase-8, DRs, and Fas/FasL and overexpression of antiapoptotic proteins, including c-FLIP, antiapoptotic Bcl-2 family proteins (e.g., Bcl-2 and Bcl-xL), and inhibitor of apoptosis proteins (IAPs) family members (Jönsson *et al*., 2003; Van Geelen *et al*., 2004; Zhang *et al*., 2004; Giménez-Bonafé *et al*., 2009; Safa and Pollok KE, 2011; Crowder and El-Deiry, 2012). The aforementioned indicates that TRAIL alone may not be sufficient for the treatment of various malignant tumor cells. Recent studies demonstrated that chemotherapeutic drugs and biochemical inhibitors can sensitize TRAIL-resistant tumor cells by suppressing survival signals and gene expression, as well as elevating apoptosis-associated gene expression (Kelly *et al*., 2002; Teraishi *et al*., 2005; Mühlethaler-Mottet *et al*., 2006; Jin *et al*., 2007; 2011; Crowder and El-Deiry, 2012; Park *et al*., 2012; Seo *et al*., 2012). These observations suggest that combination therapy may provide a possible strategy to overcome TRAIL resistance and that developing ways to make these cells vulnerable to apoptosis by TRAIL are important strategies for effective cancer therapy.

Recently, accumulating evidence has suggested that inhibition of histone deacetylases (HDACs) is a promising new target in cancer therapy. Histones are typically catalyzed by two opposing enzymes: histone acetyltransferases (HATs) and HDACs. Histone acetylation and deacetylation of lysine residues in histones are essential in the initiation of gene regulation and in the modulation of chromatin structure (Kouraklis and Theocharis, 2002; Yang and Seto, 2007; Barneda-Zahonero and Parra, 2012). Previous studies reported that aberrant gene expression and subsequent functional inactivation of HAT activity or dysregulation of HDAC activity can contribute to cancer initiation and mediate tumor cell proliferation (Monneret, 2005; Barneda-Zahonero and Parra, 2012). As a result, HDAC inhibitors are now considered attractive anticancer drugs. Moreover, recent work demonstrated that several

HDAC inhibitors can synergize with TRAIL in a variety of human cancers but not in normal cells, although the different synergy mechanisms may be dependent on the cell type or the particular HDAC inhibitors used (Sonnemann *et al*., 2005; Carew *et al*., 2008; Fulda, 2012). These observations indicate that HDAC inhibitors represent promising new agents for the restoration of TRAIL-resistant cancer cells. However, molecular mechanisms underlying the enhancement of TRAIL-induced renal cancer cell apoptosis by HDAC inhibitors are not fully defined.

In the present study, we assumed that treatment with HDAC inhibitors would increase the sensitivity of human renal cancer cells to TRAIL-mediated apoptosis. To test our hypothesis, we investigated whether trichostatin A (TSA), a pan-HDAC inhibitor originally isolated from *Streptomyces hygroscopius* as an antifungal antibiotic (Yoshida *et al*., 2003), could sensitize TRAIL-resistant renal cancer Caki cells (Mizutani *et al*., 2002) to TRAIL-induced apoptosis, and we explored the underlying mechanisms involved in TSA- and TRAIL-induced apoptosis.

MATERIALS AND METHODS

Materials

TSA and recombinant human TRAIL were purchased from Calbiochem (San Diego, CA, USA) and KOMA Biotech Inc. (Seoul, Republic of Korea), dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemicals, St. Louis, MO, USA), and then diluted with the medium to the desired concentration prior to use. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, penicillin, and streptomycin were purchased from GIBCO-BRL (Gaithersburg, MD). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT), 4,6-diamidino-2-phenylindole (DAPI), and propidium iodide (PI) were obtained from Sigma-Aldrich. Annexin V-fluorescein isothiocyanate (FITC) was obtained from Calbiochem. 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and caspase activity assay kits were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Chemicon (Temecula, CA, USA), PharMingen (San Diego, CA, USA) and Sigma-Aldrich. Peroxidase-labelled donkey antirabbit, sheep antimouse immunoglobulin, and enhanced chemiluminescence (ECL) kits were purchased from Amersham (Arlington Heights, IL, USA). All other chemicals were purchased from Sigma-Aldrich.

Cell culture and cell viability assay

The human RCC Caki cell line was purchased from the American Type Culture Collection (Manassas, MD, USA), and maintained at 37°C in a humidified 95% air and 5% CO_2 atmosphere in DMEM supplemented with 10% heat-inactivated FBS, 2 mM of glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. c-FLIP$_L$-overexpressing Caki cells were a generous gift from Dr. T. K. Kwon (Department of Immunology, Keimyung University School of Medicine, Daegu, Republic of Korea) and were maintained in a medium containing 0.7 μg/ml of geneticin (G418 sulfate, Calbiochem). Cells were treated with TRAIL (50 ng/ml) in the presence or absence of various concentrations of TSA for 24 h. Control cells were supplemented with complete media containing 0.05% DMSO (vehicle control). Following treatment, cell viability was determined with an

MTT assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. The inhibitory effect of cell growth was assessed as the percentage of cell viability, where vehicle-treated cells were considered 100% viable.

Nuclear staining with DAPI

For DAPI staining, the cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with 2.5 μg/ml of DAPI solution for 10 min at room tem-

Fig. 1. Effect of TSA on inhibition of cell viability in TRAIL-treated Caki cells. The cells were seeded at 1×10⁵ cells/ml and then treated with the indicated concentrations of TRAIL, TSA, or TRAIL plus TSA for 24 h. The viability of the cells was determined by an MTT assay. Each point represents the mean ± SD of three independent experiments. The significance was determined using the Student's *t*-test (*$p<0.05$ vs. untreated control; #$p<0.05$ vs. TRAIL-treated cells).

perature. The cells were then washed twice with PBS and analyzed by fluorescence microscopy (Carl Zeiss, Oberkochen, Germany).

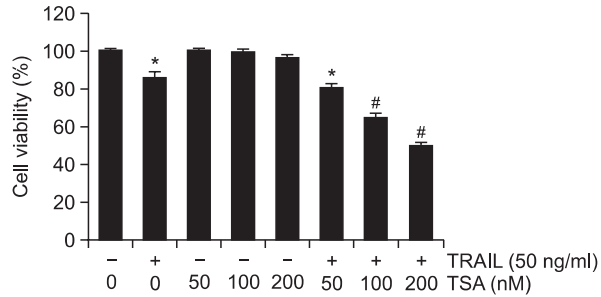## DNA flow cytometric detection of apoptosis

The cells were stained with annexin V-FITC and PI in each sample. After incubation for 15 min at room temperature in the dark, the degree of apoptosis was quantified as a percentage of the annexin V-positive and PI-negative (annexin V⁺/PI⁻ cells) cells by a flow cytometer (Li and Gao, 2013).

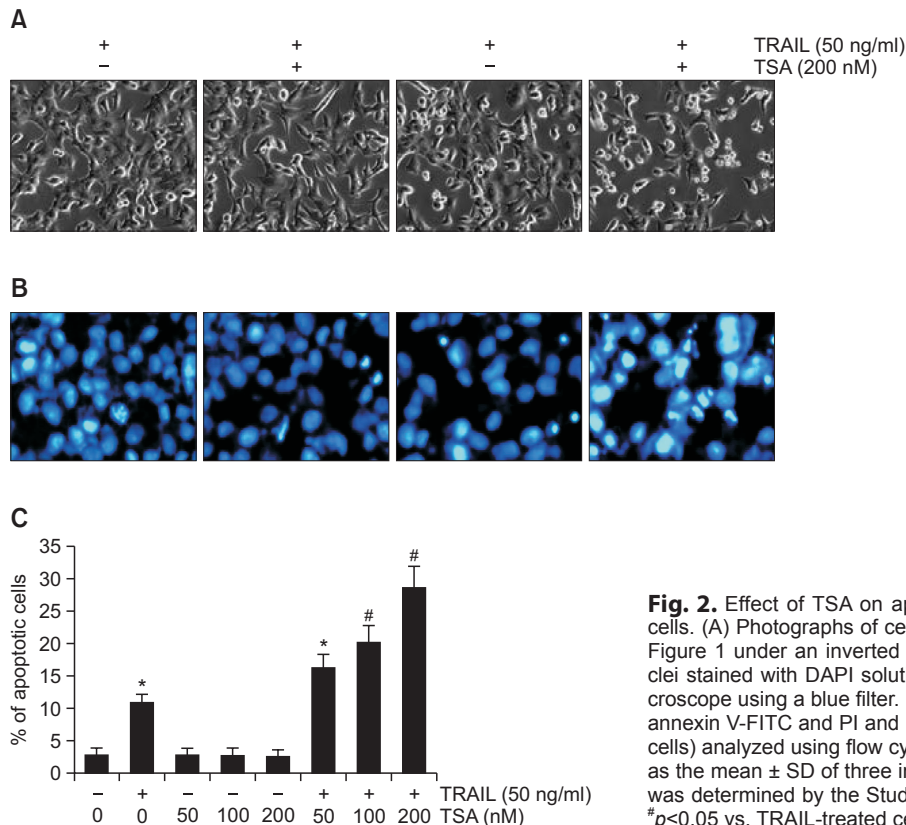## Protein extraction and Western blot analysis

Cellular lysates were prepared by suspending cells in lysis buffer (25 mM Tris-Cl [pH 7.5], 250 mM of NaCl, 5 mM of ethylenediaminetetra acetic acid, 1% nonidet of P40, 1 mM of phenymethylsulfonyl fluoride, and 5 mM of dithiothreitol) for 30 min. The protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). For Western blot analysis, the proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then electrotransferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The blots were probed with the desired antibodies for 1 h and incubated with the diluted enzyme-linked secondary antibody. Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

Measurement of mitochondrial membrane potential (MMP, $\Delta\psi m$)

Retention of JC-1, a dual-emission fluorescent dye, was

Fig. 2. Effect of TSA on apoptotic cell death in TRAIL-treated Caki cells. (A) Photographs of cells grown under the same conditions as in Figure 1 under an inverted microscope. Magnification, ×200. (B) Nuclei stained with DAPI solution photographed with a fluorescence microscope using a blue filter. Magnification, ×400. (C) Cells stained with annexin V-FITC and PI and percentages of apoptotic cells (annexin V⁺ cells) analyzed using flow cytometric analysis. The data are expressed as the mean ± SD of three independent experiments. The significance was determined by the Student's *t*-test (*$p<0.05$ vs. untreated control; #$p<0.05$ vs. TRAIL-treated cells).
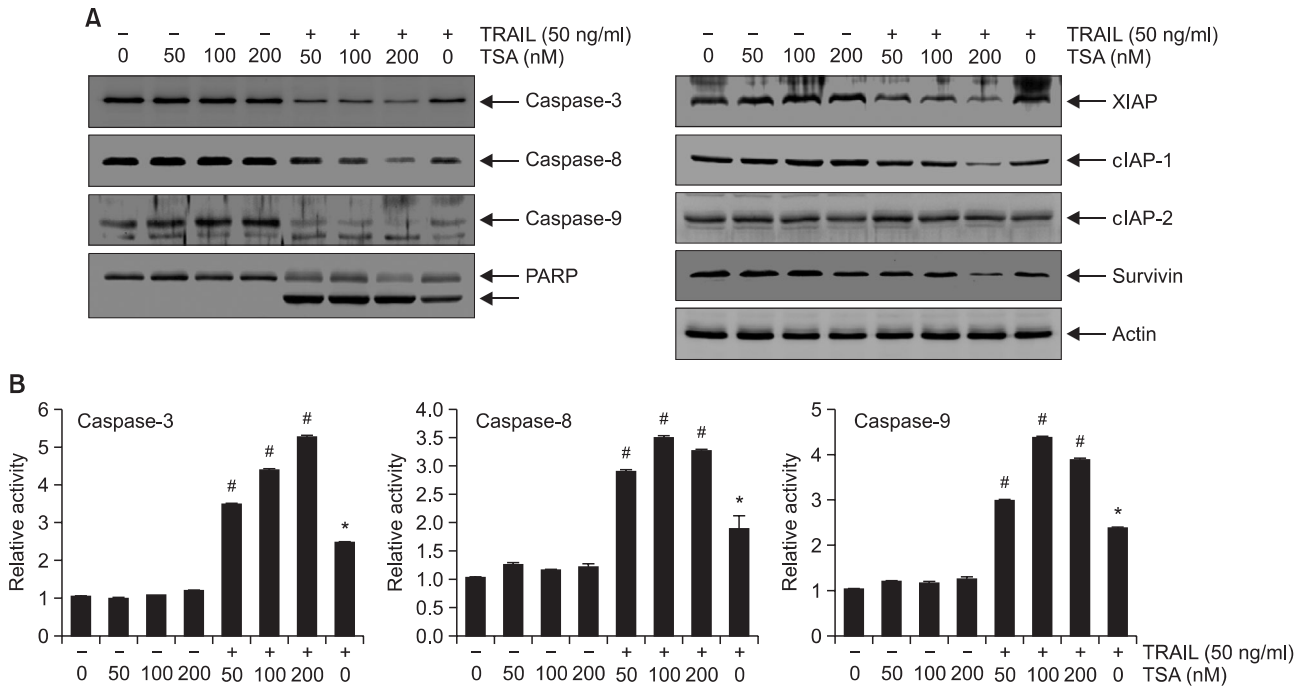
Fig. 3. Effects of TSA and TRAIL on the levels of caspases and IAP family proteins in Caki cells. (A) The cells were treated with TSA and TRAIL alone or combination treatment for 24 h. Cells were lysed, and equal amounts of cell lysates (30-50 μg) were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with the indicated antibodies. An ECL detection system was used for visualization of proteins. Actin was used as an internal control. (B) The cells were treated as in (A), and caspase (-3, -8, and -9) activity was determined using caspase assay kits obtained from R&D Systems and used according to the protocol of the manufacturer. Data are expressed as the mean ± SD of three independent experiments. The significance was determined using the Student's t-test (*$p<0.05$ vs. untreated control; #$p<0.05$ vs. TRAIL-treated cells).

used as a measure of loss of MMP. Briefly, the cells were treated with TSA in the presence or absence of TRAIL and incubated for 24 h. JC-1 (40 nM) was added during the last 30 min of treatment. The cells were washed twice with PBS to remove unbound dye. The concentration of retained JC-1 dye was determined by a flow cytometer.

Determination of caspase activity

The enzymatic activity of the caspases was assayed using colorimetric assay kits according to the manufacturer's protocol. In brief, equal amounts of protein (100 μg per 50 μl) were incubated with 50 μl of a reaction buffer and 5 μl of colorimetric tetrapeptides (Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (pNA) for caspase-3, Ile-Glu-Thr-Asp (IETD)-pNA for caspase-8, and Leu-Glu-His-Asp (LEHD)-pNA for caspase-9) at 37°C for 2 h in the dark. Caspase activity was determined by measuring proteolytic cleavage of the substrates at 405 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Data analysis and statistics

The data are presented as means ± standard deviation (SD) of at least three separate experiments using the unpaired Student's t-test. A value of $p<0.05$ was accepted as statistically significant. All the figures shown represent results from at least two independent experiments.

RESULTS

## TSA increases TRAIL-induced apoptotic cell death in Caki cells

We first examined whether TSA would increase TRAIL-induced apoptosis in Caki cells. To determine a suitable concentration of TSA, the dose response to this agent was investigated using an MTT assay (data not shown) and various concentrations of TSA (50, 100, and 200 nM) were then combined with TRAIL. As shown in Fig. 1, treatment with 50 ng/ml of TRAIL alone for 24 h only slightly decreased the cell viability (86.2%). However, when Caki cells were treated with both TSA and TRAIL, TSA inhibited the growth of the cells in a dose-dependent manner compared to treatment with TRAIL alone. To assess apoptosis, we analyzed chromatin condensation and apoptotic bodies. Treatment with TRAIL (50 ng/ml) or TSA (200 nM) alone did not induce any morphological changes or apoptotic bodies indicative of cell death (Fig. 2A and B). However, the cotreatment with TSA and TRAIL significantly increased membrane shrinkage, cell rounding, and the appearance of apoptotic bodies in Caki cells. In addition, the results of flow cytometric analysis demonstrated that cotreatment markedly increased the accumulation of annexin V⁺ cells compared to treatment with TRAIL alone, whereas treatment with TSA alone did not (Fig. 2C). These results indicate that a nontoxic dose of TSA significantly enhances TRAIL-induced apoptosis in Caki cells.

A

B
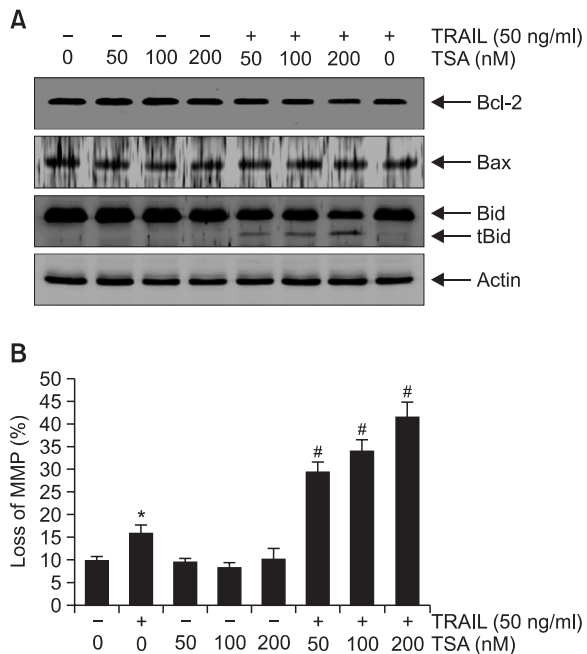
Fig. 4. Effects of TSA and TRAIL on the levels of Bcl-2 family proteins and MMP values in Caki cells. (A) The cells were treated with the indicated concentrations of agents for 24 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with the indicated antibodies. Actin was used as an internal control. (B) The cells were exposed to the indicated concentrations of TSA and TRAIL for 24 h, after which the values of MMP were evaluated by flow cytometric analysis. Data are expressed as the mean ± SD of three independent experiments. The significance was determined using the Student's t-test ($*p<0.05$ vs. untreated control; $\#p<0.05$ vs. TRAIL-treated cells).

TSA elevates TRAIL-induced activation of caspases in Caki cells

As caspases are known to act as important mediators of apoptosis, we investigated the expression and activation of caspases (-3, -8, and -9) in Caki cells treated with TSA and TRAIL. Although we did not observe active forms of caspase-8 and -9, Western blot analyses revealed that treatment with TRAIL alone slightly decreased the levels of procaspase-8 and -9, which are initiator caspases of the extrinsic and intrinsic apoptotic pathways, respectively (Fig. 3A left panel). In conjunction with these results, treatment of Caki cells with TRAIL alone down-regulated procaspase-3. However, combined treatment with TSA and TRAIL significantly decreased procaspase-3 levels levels and markedly increased the cleavage of poly(ADP-ribose) polymerase (PARP) from an 116 kDa band to an 89 kDa fragment. PARP is a substrate of active caspase-3 and also serves as a marker of cells undergoing apoptosis (Lazebnik *et al*., 1994). Analysis of the enzyme activity using the specific synthetic substrates for each caspase indicated that TSA significantly increased the TRAIL-induced activation of these caspases in a concentration-dependent manner (Fig. 3B). Activation was associated with down-regulation of members of the IAP family, including XIAP, cIAP-1, and survivin but not cIAP-2 (Fig. 3A right panel).
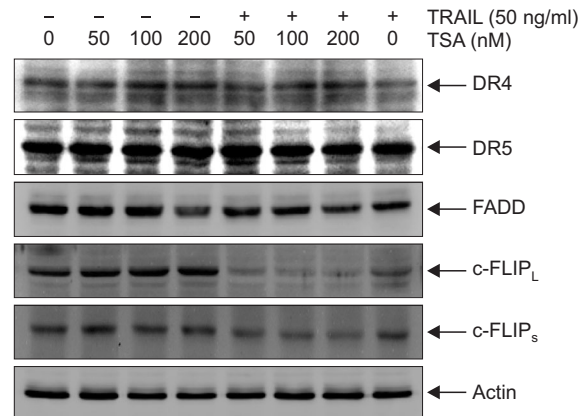

Fig. 5. Effects of TSA and TRAIL on the levels of DR-related proteins in Caki cells. Cells were treated with the indicated concentrations of TSA and TRAIL for 24 h and harvested. Western blotting was performed using the indicated antibodies. Anti-actin antibody served as a control for the loading of protein level.

TSA enhances down-regulation of Bcl-2 and truncation of Bid in TRAIL-treated Caki cells

To identify the involvement of Bcl-2 family proteins in synergistic interactions between TSA and TRAIL, expression patterns of antiapoptotic Bcl-2 and proapoptotic Bax, as well as those of the BH3 domain-only protein Bid, which is cleaved by active caspase-8 (Billen *et al*., 2008; Kantari and Walczak, 2011), were investigated. As shown in Figure 4A, combined exposure of Caki cells to TSA and TRAIL did not change total levels of Bax but moderately reduced those of Bcl-2. Moreover, the combination of TSA and TRAIL significantly decreased the level of full-length Bid proteins, accompanied by increased amounts of its cleavage product, tBid (Fig. 4A). We also examined the values of MMP using JC-1 fluorescent cationic dye to determine whether mitochondrial signaling is involved in the synergic effects of TSA in TRAIL-induced apoptotic cell death. Treatment of cells with TRAIL alone slightly reduced the MMP of Caki cells. In contrast, combined treatment with TSA and TRAIL significantly decreased the MMP in a dose-dependent manner (Fig. 4B).

Down-regulation of c-FLIP contributes to TSA-sensitized TRAIL-induced apoptosis in Caki cells

TRAIL triggers apoptotic signals via two types of death receptors, DR4 and DR5, which have cytoplasmic death domains. These domains facilitate the recruitment of the adaptor FADD to the receptor complex and the incorporation of the initiator procaspase-8 prodomain into the DISC. The antiapoptotic protein c-FLIP can interrupt cytotoxic signaling by the TRAIL receptor complex (Srivastava, 2001; Crowder and El-Deiry, 2012). Therefore, we examined whether TSA-enhanced TRAIL-induced apoptosis modulates these regulators. TSA and TRAIL did not alter protein levels of DR4 and DR5 or those of FADD (Fig. 5). Furthermore, TSA only treatment of Caki cells did not change the expression of the two main forms of c-FLIP, c-FLIP$_S$ and c-FLIP$_L$. However, cotreatment with TSA and TRAIL significantly reduced both c-FLIP$_S$ and c-FLIP$_L$ protein levels. Next, we examined whether down-regulation of c-FLIP is critical to stimulate TRAIL-induced apoptosis by TSA using a stable cell line overexpressing c-FLIP$_L$. As shown
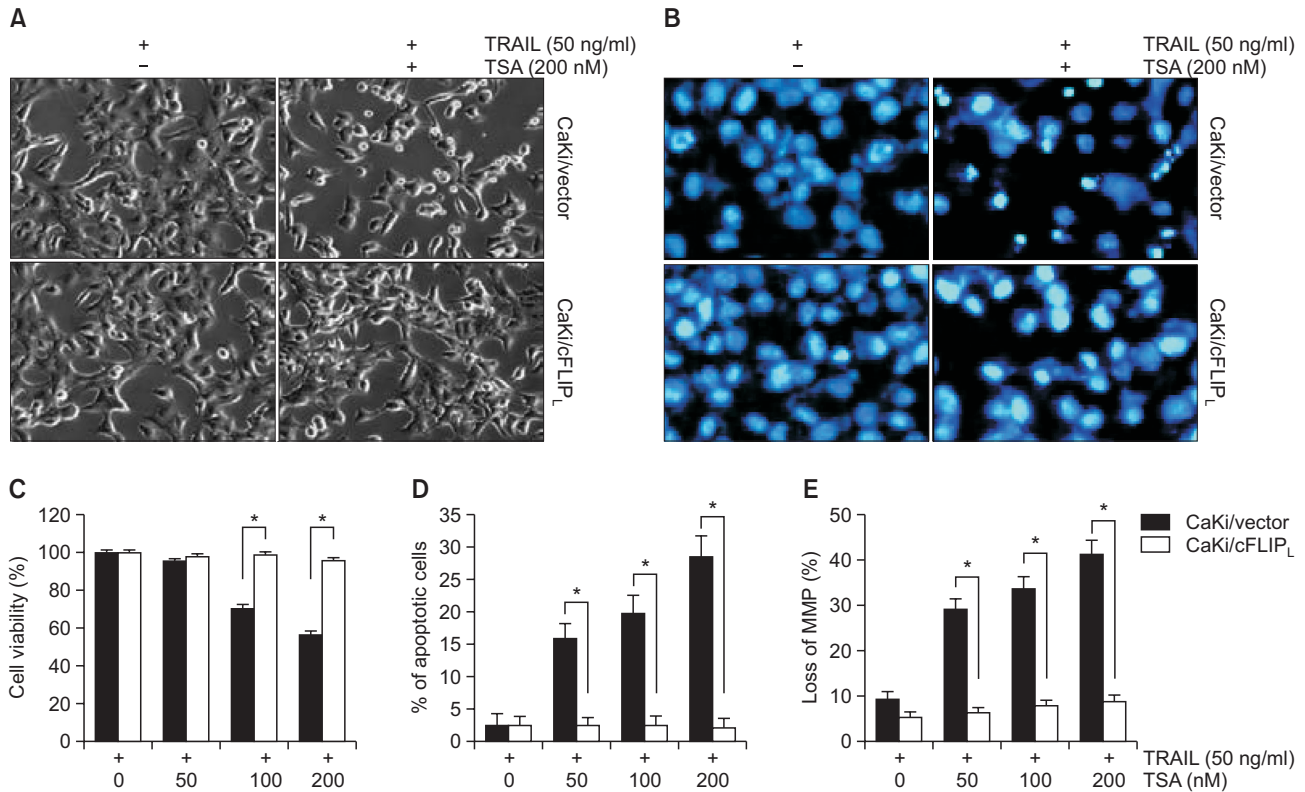
Fig. 6. Influence of down-regulation of c-FLIP on TSA-stimulated TRAIL-induced apoptosis in Caki cells. Caki/vector and Caki/c-FLIP$_L$ cells were treated for 24 h with TSA and TRAIL alone or a combination of TSA and TRAIL. (A and B) Cellular morphological changes (A) and nuclei stained with DAPI (C), photographed using an inverted microscope (Magnification, ×200.) and a fluorescence microscope (Magnification, ×400). (C) Cell viability determined with an MTT assay. (D and E) Percentages of apoptotic cells (annexin V$^+$ cells, D) and loss of MMP (E) analyzed by flow cytometry. The significance was determined using the Student's *t*-test (*$p<0.05$ vs. untreated control).

in Fig. 6 A and B, combined treatment with TSA and TRAIL significant altered cellular morphology and induced chromatin condensation in Caki/vector cells, whereas these changes were markedly blocked in Caki/c-FLIP$_L$ cells. Overexpression of c-FLIP$_L$ also significantly attenuated TSA-facilitated TRAIL-induced growth inhibition and apoptosis, and cotreatment with the two agents markedly inhibited cell viability and induced apoptosis in Caki/vector cells (Fig. 6C and D). Furthermore, the loss of MMP by the combination treatment with TSA and TRAIL was also inhibited in Caki/c-FLIP$_L$ cells (Fig. 6E). Taken together, the results indicate that TSA may recover TRAIL-sensitivity in Caki cells through down-regulation of c-FLIP and amplification of the death receptor and mitochondria-mediated apoptotic signaling pathways.

DISCUSSION

The death ligand TRAIL has recently emerged as a particularly promising candidate for cancer therapy because it preferentially induces apoptosis in transformed or malignant cells while sparing most normal cells. Nevertheless, many cancer cells are refractory to TRAIL due to the dominance of antiapoptotic signals, suggesting that treatment with TRAIL alone may be insufficient for cancer therapy. Combination therapy may be a useful strategy to enhance the therapeutic effects of TRAIL (i.e., the addition of other treatment modalities that escape TRAIL resistance or sensitize cancer cells to TRAIL-induced apoptosis).

Epidemiological investigations have pointed to the prognostic value of TRAIL and TRAIL receptors in RCC (Macher-Goeppinger *et al.*, 2009). However, the clinical application of TRAIL is hampered due to its short half-life and intrinsic resistance. A growing body of recent evidence supports the feasibility of combined therapy, such as TRAIL combined with HDAC inhibitors. Several reports have demonstrated that HDAC inhibitors can increase TRAIL-induced apoptosis in many types of cancer. In the present study, we investigated the effect of combined treatment with TSA and TRAIL on apoptosis of RCC Caki cells resistant to TRAIL (Mizutani *et al.*, 2002) and found that TSA sensitized TRAIL-resistant Caki cell to TRAIL-induced apoptosis.

There are two major pathways by which apoptosis can proceed: the extrinsic pathway via death receptors and the intrinsic pathway via mitochondrial dysfunction. TRAIL binds to death receptors on the cell surface, activating caspase-8 and triggering the extrinsic apoptotic pathway by activating caspase-3. The activation of caspase-8 amplifies the intrinsic pathway by Bid cleavage, causing mitochondrial dysfunction and activation of caspase-9 (Billen *et al.*, 2008; Kantari and Walczak, 2011). Generally, these processes are augmented by overexpression of DRs but are negatively regulated by antiapoptotic proteins (Abdulghani J, El-Deiry, 2010). Our data indicated that cotreament with TSA and TRAIL significantly

activated two initiator caspases (caspase-8 and -9) of extrinsic and intrinsic pathways and downstream effector caspase-3 (Fig. 3). In addition, cotreatment with TSA increased TRAIL-induced PARP cleavage in Caki cells. Although we did not observe up-regulation of proapoptotic Bax proteins in the present study, combined treatment with TSA and TRAIL led to Bcl-2 down-regulation, Bid truncation, and MMP loss, suggesting the involvement of both extrinsic and intrinsic apoptotic pathways in overcoming TRAIL resistance caused by TSA.

In addition to enhancing apoptosis at the level of mitochondria, other DISC components have been shown to play a role in sensitization to TRAIL. For example, modulation of the expression of c-FLIP, which can interrupt cytotoxic signaling by the TRAIL receptor complex, is another mechanism potentially involved in the regulation of TRAIL sensitivity (Rae *et al*., 2007; Benayoun *et al*., 2008). Previous studies indicated that several anticancer agents can induce down-regulation of c-FLIP through a protease-dependent mechanism and subsequent sensitization to TRAIL-induced apoptosis in some cancer cell lines (Kelly *et al*., 2002; Zhang *et al*., 2004; Palacios *et al*., 2006; Son *et al*., 2007; Jang *et al*., 2010; Lee *et al*., 2011; Woo *et al*., 2012; Yoon *et al*., 2013; Min *et al*., 2014). In the present study, treatment with TSA alone markedly down-regulated two major isoforms of c-FLIP, c-FLIP$_S$ and c-FLIP$_L$, in the presence of TRAIL, whereas cotreatment with TSA and TRAIL did not alter protein levels of DR4, DR5, and FADD (Fig. 5). Among both isoforms of c-FLIP, the long isoform c-FLIP$_L$ has an additional carboxyterminal caspase-like domain and structurally resembles procaspase-8, whereas the short isoform c-FLIP$_S$ only has the amino-terminal death effector domain (Matsuda *et al*., 2008). Moreover, c-FLIP$_L$ can heterodimerize with procaspase-8, thereby enhancing caspase-8 activation at the receptor complex, suggesting that c-FLIP$_L$ may inhibit TRAIL-mediated apoptosis via other molecular mechanisms (Benayoun *et al*., 2008; Murtaza *et al*., 2009). Consistent with this concept, we found that overexpression of c-FLIP$_L$ significantly prevented Caki cell apoptosis and attenuated the growth inhibition induced by combined treatment with TSA and TRAIL by blocking the loss of the MMP (Fig. 6). Therefore, this study provides evidence that down-regulation of c-FLIP$_L$ sensitizes cells to TRAIL-induced apoptosis following TSA treatment in Caki cells.

In summary, the present study demonstrated that TSA enhances TRAIL-induced apoptosis in renal cancer Caki cells by triggering extrinsic and intrinsic pathways. Moreover, overexpression of c-FLIP$_L$ overcame the TSA- plus TRAIL-induced apoptosis, indicating that down-regulation of the antiapoptotic protein may critically contribute to the sensitizing effect of TSA on TRAIL-mediated apoptosis. Accordingly, we recommend that this combination should be further evaluated in animal models of renal cancer and that it should be considered for clinical trials.

ACKNOWLEDGMENTS

REFERENCES

Abdulghani, J. and El-Deiry, W. S. (2010) TRAIL receptor signaling and therapeutics. *Expert Opin. Ther. Targets* **14**, 1091-1108.

Ather, M. H., Masood, N. and Siddiqui, T. (2010) Current management of advanced and metastatic renal cell carcinoma. *Urol. J.* **7**, 1-9.

Barneda-Zahonero, B. and Parra, M. (2012) Histone deacetylases and cancer. *Mol. Oncol.* **6**, 579-589.

Benayoun, B., Baghdiguian, S., Lajmanovich, A., Bartoli, M., Daniele, N., Gicquel, E., Bourg, N., Raynaud, F., Pasquier, M. A., Suel, L., Lochmuller, H., Lefranc, G. and Richard, I. (2008) NF-kappaB-dependent expression of the antiapoptotic factor c-FLIP is regulated by calpain 3, the protein involved in limb-girdle muscular dystrophy type 2A. *FASEB J.* **22**, 1521-1529.

Billen, L. P., Shamas-Din, A. and Andrews, D. W. (2008) Bid: a Bax-like BH3 protein. *Oncogene* **27**, S93-104.

Brugarolas, J. (2007) Renal-cell carcinoma--molecular pathways and therapies. *N. Engl. J. Med.* **356**, 185-187.

Carew, J. S., Giles, F. J. and Nawrocki, S. T. (2008) Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. *Cancer Lett.* **269**, 7-17.

Crowder, R. N. and El-Deiry, W. S. (2012) Caspase-8 regulation of TRAIL-mediated cell death. *Exp. Oncol.* **34**, 160-164.

Fulda, S. (2012) Histone deacetylase (HDAC) inhibitors and regulation of TRAIL-induced apoptosis. *Exp. Cell Res.* **318**, 1208-1212.

Giménez-Bonafé, P., Tortosa, A. and Pérez-Tomás, R. (2009) Overcoming drug resistance by enhancing apoptosis of tumor cells. *Curr. Cancer Drug Targets* **9**, 320-340.

Inman, B. A., Harrison, M. R. and George, D. J. (2013) Novel immunotherapeutic strategies in development for renal cell carcinoma. *Eur. Urol.* **63**, 881-889.

Hwang, E. Y., Jeong, M. S., Park, S. Y. and Jang, S. B. (2014) Evidence of complex formation between FADD and c-FLIP death effector domains for the death inducing signaling complex. *BMB Rep.* **47**, 488-493.

Jang, J. H., Lee, T. J., Yang, E. S., Min, do. S., Kim, Y. H., Kim, S. H., Choi, Y. H., Park, J. W., Choi, K. S. and Kwon, T. K. (2010) Compound C sensitizes Caki renal cancer cells to TRAIL-induced apoptosis through reactive oxygen species-mediated down-regulation of c-FLIPL and Mcl-1. *Exp. Cell Res.* **316**, 2194-2203.

Jin, C. Y., Park, C., Cheong, J., Choi, B. T., Lee, T. H., Lee, J. D., Lee, W. H., Kim, G. Y., Ryu, C. H. and Choi, Y. H. (2007) Genistein sensitizes TRAIL-resistant human gastric adenocarcinoma AGS cells through activation of caspase-3. *Cancer Lett.* **257**, 56-64.

Jin, C. Y., Park, C., Hwang, H. J., Kim, G. Y., Choi, B. T., Kim, W. J. and Choi, Y. H. (2011) Naringenin up-regulates the expression of death receptor 5 and enhances TRAIL-induced apoptosis in human lung cancer A549 cells. *Mol. Nutr. Food Res.* **55**, 300-309.

Jönsson, G., Paulie, S. and Grandien, A. (2003) High level of cFLIP correlates with resistance to death receptor-induced apoptosis in bladder carcinoma cells. *Anticancer Res.* **23**, 1213-1218.

Kantari, C. and Walczak, H. (2011) Caspase-8 and bid: caught in the act between death receptors and mitochondria. *Biochim. Biophys. Acta* **1813**, 558-563.

Kelly, M. M., Hoel, B. D. and Voelkel-Johnson, C. (2002) Doxorubicin pretreatment sensitizes prostate cancer cell lines to TRAIL induced apoptosis which correlates with the loss of c-FLIP expression. *Cancer Biol. Ther.* **1**, 520-527.

Kouraklis, G. and Theocharis, S. (2002) Histone deacetylase inhibitors and anticancer therapy. *Curr. Med. Chem. Anticancer Agents* **2**, 477-484.

Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. and Earnshaw, W. C. (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371**, 346-347.

Lee, S. J., Noh, H. J., Sung, E. G., Song, I. H., Kim, J. Y., Kwon, T. K. and Lee, T. J. (2011) Berberine sensitizes TRAIL-induced apoptosis through proteasome-mediated downregulation of c-FLIP and Mcl-1 proteins. *Int. J. Oncol.* **38**, 485-492.

Li, Z. and Gao, Q. (2013) Induction of apoptosis in HT-29 cells by quercetin through mitochondria-mediated apoptotic pathway. *Animal Cells Syst.* **17**, 147-153

Macher-Goeppinger, S., Aulmann, S., Tagscherer, K. E., Wagener, N.,

Haferkamp, A., Penzel, R., Brauckhoff, A., Hohenfellner, M., Sykora, J., Walczak, H., The, B. T., Autschbach, F., Herpel, E., Schirmacher, P. and Roth, W. (2009) Prognostic value of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors in renal cell cancer. *Clin. Cancer Res*. **15**, 650-659.

Matsuda, F., Inoue, N., Goto, Y., Maeda, A., Cheng, Y., Sakamaki, K. and Manabe, N. (2008) cFLIP regulates death receptor-mediated apoptosis in an ovarian granulosa cell line by inhibiting procaspase-8 cleavage. *J. Reprod. Dev*. **54**, 314-320.

Min, K. J., Seo, B. R., Bae, Y. C., Yoo, Y. H. and Kwon, T. K. (2014) Antipsychotic agent thioridazine sensitizes renal carcinoma Caki cells to TRAIL-induced apoptosis through reactive oxygen species-mediated inhibition of Akt signaling and downregulation of Mcl-1 and c-FLIP(L). *Cell Death Dis*. **5**, e1063.

Mizutani, Y., Nakanishi, H., Yoshida, O., Fukushima, M., Bonavida, B. and Miki, T. (2002) Potentiation of the sensitivity of renal cell carcinoma cells to TRAIL-mediated apoptosis by subtoxic concentrations of 5-fluorouracil. *Eur. J. Cancer* **38**, 167-176.

Monneret, C. (2005) Histone deacetylase inhibitors. *Eur. J. Med. Chem*. **40**, 1-13.

Mühlethaler-Mottet, A., Flahaut, M., Bourloud, K. B., Auderset, K., Meier, R., Joseph, J. M. and Gross, N. (2006) Histone deacetylase inhibitors strongly sensitise neuroblastoma cells to TRAIL-induced apoptosis by a caspases-dependent increase of the pro- to anti-apoptotic proteins ratio. *BMC Cancer* **6**, 214.

Murtaza, I., Saleem, M., Adhami, V. M., Hafeez, B. B. and Mukhtar, H. (2009) Suppression of cFLIP by lupeol, a dietary triterpene, is sufficient to overcome resistance to TRAIL-mediated apoptosis in chemoresistant human pancreatic cancer cells. *Cancer Res*. **69**, 1156-1165.

O'Kane, H. F., Watson, C. J., Johnston, S. R., Petak, I., Watson, R. W. and Williamson, K. E. (2006) Targeting death receptors in bladder, prostate and renal cancer. *J. Urol*. **175**, 432-438.

Palacios, C., Yerbes, R. and López-Rivas, A. (2006) Flavopiridol induces cellular FLICE-inhibitory protein degradation by the proteasome and promotes TRAIL-induced early signaling and apoptosis in breast tumor cells. *Cancer Res*. **66**, 8858-8869.

Park, C., Jin, C. Y., Hwang, H. J., Kim, G. Y., Jung, J. H., Kim, W. J., Yoo, Y. H. and Choi, Y. H. (2012) J7, a methyl jasmonate derivative, enhances TRAIL-mediated apoptosis through up-regulation of reactive oxygen species generation in human hepatoma HepG2 cells. *Toxicol. In Vitro* **26**, 86-93.

Rae, C., Langa, S., Tucker, S. J. and MacEwan, D. J. (2007) Elevated NF-kappaB responses and FLIP levels in leukemic but not normal lymphocytes: reduction by salicylate allows TNF-induced apoptosis. *Proc. Natl. Acad. Sci. U.S.A*. **104**, 12790-12795.

Safa, A. R. and Pollok, K. E. (2011) Targeting the anti-apoptotic protein c-FLIP for cancer therapy. *Cancers (Basel)* **3**, 1639-1671.

Seo, O. W., Kim, J. H., Lee, K. S., Lee, K. S., Kim, J. H., Won, M. H., Ha, K. S., Kwon, Y. G. and Kim, Y. M. (2012) Kurarinone promotes TRAIL-induced apoptosis by inhibiting NF-κB-dependent cFLIP expression in HeLa cells. *Exp. Mol. Med*. **44**, 653-664.

Son, Y. G., Kim, E. H., Kim, J. Y., Kim, S. U., Kwon, T. K., Yoon, A. R., Yun, C. O. and Choi, K. S. (2007) Silibinin sensitizes human glioma cells to TRAIL-mediated apoptosis via DR5 up-regulation and down-regulation of c-FLIP and survivin. *Cancer Res*. **67**, 8274-8284.

Sonnemann, J., Gänge, J., Kumar, K. S., Müller, C., Bader, P. and Beck, J. F. (2005) Histone deacetylase inhibitors interact synergistically with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to induce apoptosis in carcinoma cell lines. *Invest. New Drugs* **23**, 99-109.

Srivastava, R. K. (2001) TRAIL/Apo-2L: mechanisms and clinical applications in cancer. *Neoplasia* **3**, 535-546.

Staehler, M., Rohrmann, K., Haseke, N., Stief, C. G. and Siebels, M. (2005) Targeted agents for the treatment of advanced renal cell carcinoma. *Curr. Drug Targets* **6**, 835-846.

Teraishi, F,, Kagawa, S., Watanabe, T., Tango, Y., Kawashima, T., Umeoka, T., Nisizaki, M., Tanaka, N. and Fujiwara, T. (2005) ZD1839 (Gefitinib, 'Iressa'), an epidermal growth factor receptor-tyrosine kinase inhibitor, enhances the anti-cancer effects of TRAIL in human esophageal squamous cell carcinoma. *FEBS Lett*. **579**, 4069-4075.

Thomas, L. R., Johnson, R. L., Reed, J. C. and Thorburn, A. (2004) The C-terminal tails of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas receptors have opposing functions in Fas-associated death domain (FADD) recruitment and can regulate agonist-specific mechanisms of receptor activation. *J. Biol. Chem*. **279**, 52479-52486.

Van Geelen, C. M., de Vries, E. G. and de Jong, S. (2004) Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. *Drug Resist. Updat*. **7**, 345-358.

Voelkel-Johnson, C. (2011) TRAIL-mediated signaling in prostate, bladder and renal cancer. *Nat. Rev. Urol*. **8**, 417-427.

Woo, S. M., Min, K. J. and Kwon, T. K. (2012) Calyculin A causes sensitization to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by ROS-mediated down-regulation of cellular FLICE-inhibiting protein (c-FLIP) and by enhancing death receptor 4 mRNA stabilization. *Apoptosis* **17**, 1223-1234.

Yang, X. J. and Seto, E. (2007) HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene* **26**, 5310-5318.

Yoon, M. J., Kang, Y. J., Kim, I. Y., Kim, E. H., Lee, J. A., Lim, J. H., Kwon, T. K. and Choi, K. S. (2013) Monensin, a polyether ionophore antibiotic, overcomes TRAIL resistance in glioma cells via endoplasmic reticulum stress, DR5 upregulation and c-FLIP down-regulation. *Carcinogenesis* **34**, 1918-1928.

Yoshida, M., Matsuyama, A., Komatsu, Y. and Nishino, N. (2003) From discovery to the coming generation of histone deacetylase inhibitors. *Curr. Med. Chem*. **10**, 2351-2358.

Zhang, X., Jin, T. G., Yang, H., DeWolf, W. C., Khosravi-Far, R. and Olumi, A. F. (2004) Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. *Cancer Res*. **64**, 7086-7091.