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 histone deacetylase inhibitor (HDACi), 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-FLIPL-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

THE HISTONE DEACYTYLASE INHIBITOR TRICHOSTATIN A SENSITIZES HUMAN RENAL CARCINOMA CELLS TO TRAIL-INDUCED APOPTOSIS THROUGH DOWN-REGULATION OF c-FLIP_L

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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-FLIPL-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.
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 (innisitric or mitochondria pathway). This process culminates in the promotion of apoptosis. Caspase-8 can also directly promote the proteolytic activation of effector caspases (Billem 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; Voeikel-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 Theocaris, 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 hygroscopicus 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% CO2 atmosphere in DMEM supplemented with 10% heat-inactivated FBS, 2 mM of glucose, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. c-FLIP, 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 temperature. 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 phenylmethylsulfonyl 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, ΔΨm)**

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

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, we investigated whether down-regulation of c-FLIP (Bcl-2-like protein-I) is critical to stimulate TRAIL-induced apoptosis by TSA. As shown in Figure 4A, cotreatment with TSA did not alter the expression of c-FLIP (c-FLIPs and c-FLIPL) but slightly increased the cleavage of Bid, which is a substrate of caspase-8 and serves as an activator of the death domain (Doherty et al., 2003). Moreover, cotreatment with TSA only treatment of Caki cells did not change the expression of the two main forms of c-FLIP, c-FLIPs and c-FLIPL. However, cotreatment with TSA and TRAIL significantly reduced both c-FLIPs and c-FLIPL 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-FLIPL. As shown

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 anti-apoptotic 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-FLIPs and c-FLIPL. However, cotreatment with TSA and TRAIL significantly reduced both c-FLIPs and c-FLIPL 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-FLIPL.
in Fig. 6A 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<sub>L</sub> cells. Overexpression of c-FLIP<sub>L</sub> 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<sub>L</sub> 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 anti-apoptotic 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 cotreatment 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 L, and c-FLIP S, 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 procapase-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 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.

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