Triptolide Sensitizes Resistant Cholangiocarcinoma Cells to TRAIL-induced Apoptosis

TASANEE PANICHAKUL[1,5], PAKAMAS INTACHOTE[1], ADISAK WONGKAJORSILP[2], BANCHOB SRIPA[3] and STITAYA SIRISINHA[4]

[1]Laboratory of Immunology, Chulabhorn Research Institute, Bangkok;
[2]Department of Pharmacology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok;
[3]Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen;
Departments of [4]Microbiology and [5]Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand

Abstract. *Background: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) promotes apoptosis by binding to transmembrane receptors. It is known to induce apoptosis in a wide variety of cancer cells, but TRAIL-resistant cancers have also been documented. In this study, the relative resistance of human cholangiocarcinoma (CCA) cell lines against TRAIL-induced apoptosis is reported and the possible potential synergistic effect with triptolide, a diterpene triepoxide extracted from the Chinese herb Tripterygium wilfordii, in killing TRAIL-resistant CCA cells is investigated. Materials and Methods: Six human CCA cell lines were treated with various concentrations of TRAIL and the resistant cells were identified and subsequently tested for their sensitivity to a combination of TRAIL and triptolide. The susceptibility and resistance of the cells were based on analysis of cytotoxic and apoptotic induction and expression of anti-apoptotic factors (Mcl-1 and cFLIP). Results: The treatment of TRAIL induced a dose-dependent decrease in cell viability in 4 out of the 6 cell lines. A combination of TRAIL and triptolide enhanced cytotoxicity and apoptosis in these 2 resistant cell lines. The combined treatment enhanced activation of caspase-8 and its downstream signaling processes compared with the treatment with either one alone. Conclusion: The results presented show that human CCA cells were heterogeneous with respect to susceptibility to TRAIL-induced apoptosis. The combination of TRAIL and triptolide could enhance susceptibility to TRAIL-induced apoptotic killing in these TRAIL-resistant CCA cells, thus offering an alternative approach for the treatment of TRAIL-resistant cholangiocarcinoma.*

Correspondence to: Tasanee Panichakul, Department of Pathobiology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand. Tel: (662) 201-5555, Fax: (662) 354-7158, e-mail: tasanee_p@yahoo.com

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The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L), a member of tumor necrosis factor family, is a promising candidate for anti-cancer treatment for a number of cancer cell lines (1, 2). At the concentration used, it is not toxic for normal cells. A recent *in vivo* study confirmed its selective tumor cytotoxicity (3-5). The signaling pathway underlying TRAIL sensitivity is induced by the initial interaction of TRAIL with distinct trans-membrane receptors including 2 pro-apoptotic death receptors (DR4 and DR5) and 3 anti-apoptotic (decoy) receptors (DcR1, DcR2 and osteoprotegerin) (6-8).

Cholangiocarcinoma (CCA), a highly fatal malignant tumor of the bile duct system, is often associated with chronic biliary tract inflammation, resulting from either liver fluke infestation or primary sclerosing cholangitis. This cancer is predominantly found in several Asian countries where liver fluke is endemic (9). However, the incidence has also been reported to be increasing in Western countries (10-12). CCA is known to be resistant to virtually all chemotherapeutic agents that have been tested (13, 14), but recently some CCA cell lines were found to be susceptible to TRAIL-induced apoptosis (15).

TRAIL resistance is known to be common in many cancers, possibly by interfering with apoptosis *via* the cellular FLICE-inhibitory protein (cFLIP) and anti-apoptotic factors in the Bcl-2 family (16-18). High levels of cFLIP correlate with resistance to TRAIL-induced apoptosis in human bladder cancer and gastric cancer (19, 20). cFLIP has multiple splice variants, of which cFLIP long (cFLIPL) and cFLIP short (cFLIPS) are the 2 dominating forms (21). The prominent Mcl-1 (myeloid cell leukemia-1) is a member of the Bcl-2 family that has 2 splicing variants: Mcl-1 short (Mcl-1S) and Mcl-1 long

(Mcl-1L). Mcl-1S has pro-apoptotic activity that is antagonized by the anti-apoptotic Mcl-1L (22). Mcl-1 expression has been investigated in different types of carcinomas, *e.g.*, ovarian, breast and colorectal cancers (23-25). A recent study with 3 CCA cell lines showed that Mcl-1 mediated TRAIL resistance by blocking a mitochondrial cell death pathway (16). On the other hand, there are studies demonstrating that TRAIL resistance could also be reduced in the presence of chemotherapeutic agents or other chemicals, including the triptolide found in the Chinese herb, *Tripterygium wilfordii* (26-28). The latter has been shown to complement the apoptosis-inducing effect of tumor necrosis factor–alpha (TNF-α), chemotherapeutic drugs and TRAIL in killing a number of cancer cells *via* the inhibition of NF-κB activation or activation of ERK2 (27, 29, 30). In this study, the sensitivity of CCA cell lines to TRAIL-induced apoptosis was analyzed and the possible synergistic effect with triptolide in killing TRAIL-resistant CCA was explored.

Materials and Methods

Cell cultures. Five cholangiocarcinoma cell lines, HuCCA-1, KKU-100, KKU-M139, KKU-M156, and KKU-M214 available for study, were established from tumor mass obtained during routine surgical resection. The sixth cell line, HubCCA-1, was prepared from sediments of the biliary fluid from a patient receiving routine palliative endoscopic retrograde cholecystopancreatomy. The epithelial nature of HuCCA-1, KKU-100, KKU-M156, KKU-M214, and HubCCA-1 were confirmed by microscopic examination of cell monolayers stained with specific anti-cytokeratin and exhibited columnar appearance with Mucicarmine reagent for mucin production (31). The KKU-M139 exhibited a squamous cell-like morphology. All cell lines were cultured at 37˚C with 5% CO_2 atmosphere in Hams'F12 culture medium (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 100 U/ml penicillin and 100 μg/ml streptomycin.

Reagents and antibodies. Lyophilized recombinant human TRAIL (amino acids 114-281, R&D Systems, Minneapolis, MN, USA) was reconstituted in phosphate buffered saline (PBS pH 7.2) containing 0.1% sterile bovine serum albumin and kept in small aliquots at –70˚C. Triptolide (99% purity, Sigma-Aldrich, St. Louis, MO, USA) was solubilized in 0.01% dimethylsulfoxide (DMSO) in PBS, filtered through a 0.2 μm Millipore filter and kept at –70˚C. Antibodies for caspases 3 (H277), 8 (C20), and 9 (H170), BID (Bcl-2 interacting death domain) (C20), Bax (Bcl-2-associated X protein) (N20), cytochrome c (C20), poly(ADP-ribose)polymerase (PARP, H250), cFLIP (G11), Mcl-1 (22) and actin (C11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated rabbit anti-mouse and anti-goat and swine anti-rabbit antibodies were from Dako A/S (Glostrup, Denmark).

Cytotoxic activity. Confluent cell monolayers were harvested and then plated at density of $2x10^4$ per 100 μl of medium in 96-well tissue culture plates. After an overnight incubation for 24 h, the cells were treated for 24 h with various concentrations of TRAIL or different combinations of TRAIL and triptolide. Cell viability was determined by staining with crystal violet (32). Briefly, after 24 h of treatment, the medium was removed and the cell monolayer was washed, fixed with 95% ethanol and then stained with 0.5% crystal violet. The stained cells were subsequently lysed with 100 mM HCl in absolute methanol and the optical density (OD) was determined with a microtiter plate reader set to read at a wavelength of 540 nm.

Detection of apoptosis. Apoptosis was determined using a nuclear binding dye, 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI) (Roche Diagnostics Gmbh, Roche Molecular Biochemicals, Mannheim, Germany), as described previously (32). The confluent cell cultures were harvested and plated in 6-well tissue culture plates at a density of $1x10^6$ per 4 ml of medium. After 24 h of treatment, the cells were harvested, washed with PBS and stained with 2 μg/ml DAPI at room temperature for 15 min. The characteristic nuclear change typical of apoptosis (*i.e.*, chromatin condensation and nuclear fragmentation) was examined under a fluorescence microscope fitted with a 340/380 nm excitation filter. The percentage of apoptotic cells was calculated from a total of 1,000 cells counted.

Immunoblot analysis. Whole-cell lysate was prepared by lysing $1x10^6$ cells in 100 μl of lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM PMSF and 1 mM TPCK) and followed by sonication on ice for 20 seconds (29, 33). The total cell lysate was subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Schleicher & Schuell, Dassel, Germany). After blocking nonspecific binding sites with 10% nonfat milk in PBST (PBS with 1% Tween 20), the membrane was incubated overnight at 4˚C with specific antibodies. The membrane was then washed with PBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. Bound antibody on the membrane was detected with chemiluminescent substrate as described by the manufacturer (Roche Diagnostics Gmbh, Roche Molecular Biochemicals) using Hyperfilm film (Amershampharmacia biotech, Amersham Pharmacia Biotech UK Limited., England). For analysis of cytochrome c, a cytosolic fraction was prepared and used instead of the whole-cell lysate (26, 34).

Detection of TRAIL receptors. The expression of TRAIL receptor mRNA was assessed by reverse-transcription reaction and polymerase chain reaction (RT-PCR) (15). The following primers were used: DR4 (5'-CAGAACGTCCTGGAGCCTGTAAC-3', 5'-ATGTCCATTGCCTGATTCTTTGTG-3'), DR5 (5'-CCTTGGA GACGCTGGGAGAGA-3', 5'-TGGGTGATGTTGGATGGGAG AGT-3'), DcR1(5'-CGTTAGG GAACTCT GGGGACAG-3',5'-GGAAGCGTTGGTGTAATCCACA-3'), and DcR2(5'-AATTT GCCTTCTTGCCTGCTATGTA-3',5'-CTCCTCCGCTGCTGGG GTTTTC-3'). The expression of glyceraldehyde-3-phosphate dehydrogenase mRNA was included as reference for gel loading. The amplification cycles used were 94˚C for 45 sec, 55˚C for 30 sec, and 72˚C for 45 sec and carried on for 30 cycles. The PCR-amplified products were run on a 1.5% agarose gel containing ethidium bromide and were visualized under ultraviolet light.

Results and Discussion

TRAIL has been reported to induce apoptosis in a variety of cancer cell types. In the present study, 6 human CCA cell lines that exhibited a pronounced difference in TRAIL sensitivity (Figure 1) were described. These cell lines were treated with various concentrations of TRAIL (0.1-100 ng/ml) for 24 h before the cytotoxic assay was performed. The results indicate that of these 6 cell lines, 4 were sensitive and 2 were resistant to TRAIL treatment. The 4 sensitive cell lines (KKU-100, KKU-M156, KKU-M214 and HubCCA-1) exhibited TRAIL-induced cytotoxicity in a dose-dependent manner. In contrast, the treatment of the 2 resistant cell lines (KKU-M139 and HuCCA-1) with TRAIL did not result in significant cytolysis. At a concentration as high as 800 ng/ml, more than 80% of these 2 cell lines survived (data not shown), compared with the 4 sensitive cells which were almost completely killed (more than 90%) by a concentration of 100 ng/ml of TRAIL (Figure 1). Microscopic examination of the TRAIL-treated cells (10 ng/ml, for 24 h) presented in Figure 2A is in agreement with the cytotoxic assays shown in Figure 1. DAPI-staining of these 6 cell lines revealed that only the TRAIL sensitive cells exhibited a nuclear morphology typical of apoptotic death (Figure 2B) similar to those reported previously by other groups of investigators (15, 16). In addition, the TRAIL receptor expression of our TRAIL-sensitive and resistant CCA cell lines was also examined and the results showed that there was no difference in the receptor expression among these 6 cell lines (data not shown), thus confirming the results reported previously by other investigators (16, 19, 27).

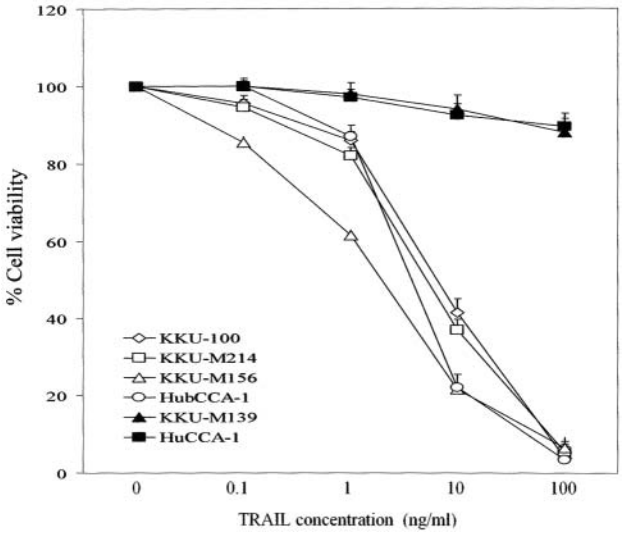

Figure 1. *Sensitivity of human CCA cell lines to TRAIL treatment. Cytotoxic effects of TRAIL were tested on a panel of 6 human CCA cell lines: HuCCA-1, HubCCA-1, KKU-100, KKU-M139, KKU-M156 and KKU-M214. Viability was determined after the cells were exposed to different concentrations of TRAIL for 24 h. The sensitivity of 2 cell lines (KKU-M139 and HuCCA-1) was distinct from that of the remaining 4 cell lines. The results shown are the mean with S.D. of 3 independent experiments.*

We previously demonstrated that triptolide could sensitize CCA cells to TNF-α -induced apoptosis (29). In the present study, whether or not the triptolide could also synergistically kill the TRAIL-resistant KKU-M139 and HuCCA-1 cells was investigated. In this experiment, the 2 cell lines were simultaneously exposed to 10 ng/ml TRAIL and optimal concentrations of triptolide (30 ng/ml for KKU-M139 and 50 ng/ml for HuCCA-1) and the cytotoxic assays were analyzed after 24 h. As shown in Figure 3A, less than 20% of the cells were killed when treated with either compound alone. However, with the combined treatment, apoptosis as high as 70-75% was noted (Figure 3B), indicating that the two agents had synergistic cytotoxic activity in the TRAIL-resistant CCA cells similar to that reported for lung cancer cells (27, 30).

In an attempt to elucidate the possible mechanism of killing, both the treated and untreated KKU-M139 and HuCCA-1 cells were subjected to immunoblot analysis (Figure 4). The results indicate that a combination of triptolide and TRAIL could enhance the activation of procaspase-8 (55kDa), resulting in the proteolytic cleavage to a 20 kDa fragment. The results presented further indicate that the combined treatment also induced the cleavage of Bid with a release of cytochrome c from mitochondria. It is possible that the latter activated caspase-9 and caspase-3 and induced PARP cleavage. Altogether, these observations suggest that the combined treatment selectively utilized a mitochondria-dependent pathway. It should be mentioned that the TRAIL receptors in the cells treated with combined triptolide and TRAIL were not noticeably different from untreated cells (data not shown), suggesting that the synergy did not involve the regulation of receptor expression.

The intracellular levels of anti-apoptotic factors (Mcl-1, cFLIP and Bax) were analyzed in our CCA cell lines. As mentioned above, the combined triptolide and TRAIL could render the KKU-M139 and HuCCA-1 cells to TRAIL-sensitive cells. The results presented in Figure 5A show that down-regulation of Mcl-1 protein occurred in the KKU-M139 cells treated with the combined triptolide and TRAIL, while no Mcl-1 down-regulation was detected in the HuCCA-1 cells treated. In addition, no down-regulation of the cFLIP and Bax proteins was detected in either of these two cell lines after combination treatment of triptolide and TRAIL. These observations suggest that Mcl-1 is involved in TRAIL resistance only in KKU-M139 cells but not in the HuCCA-1 cells. The intracellular levels of Mcl-1, cFLIP and Bax varied considerably among the 6 CCA cell lines (Figure

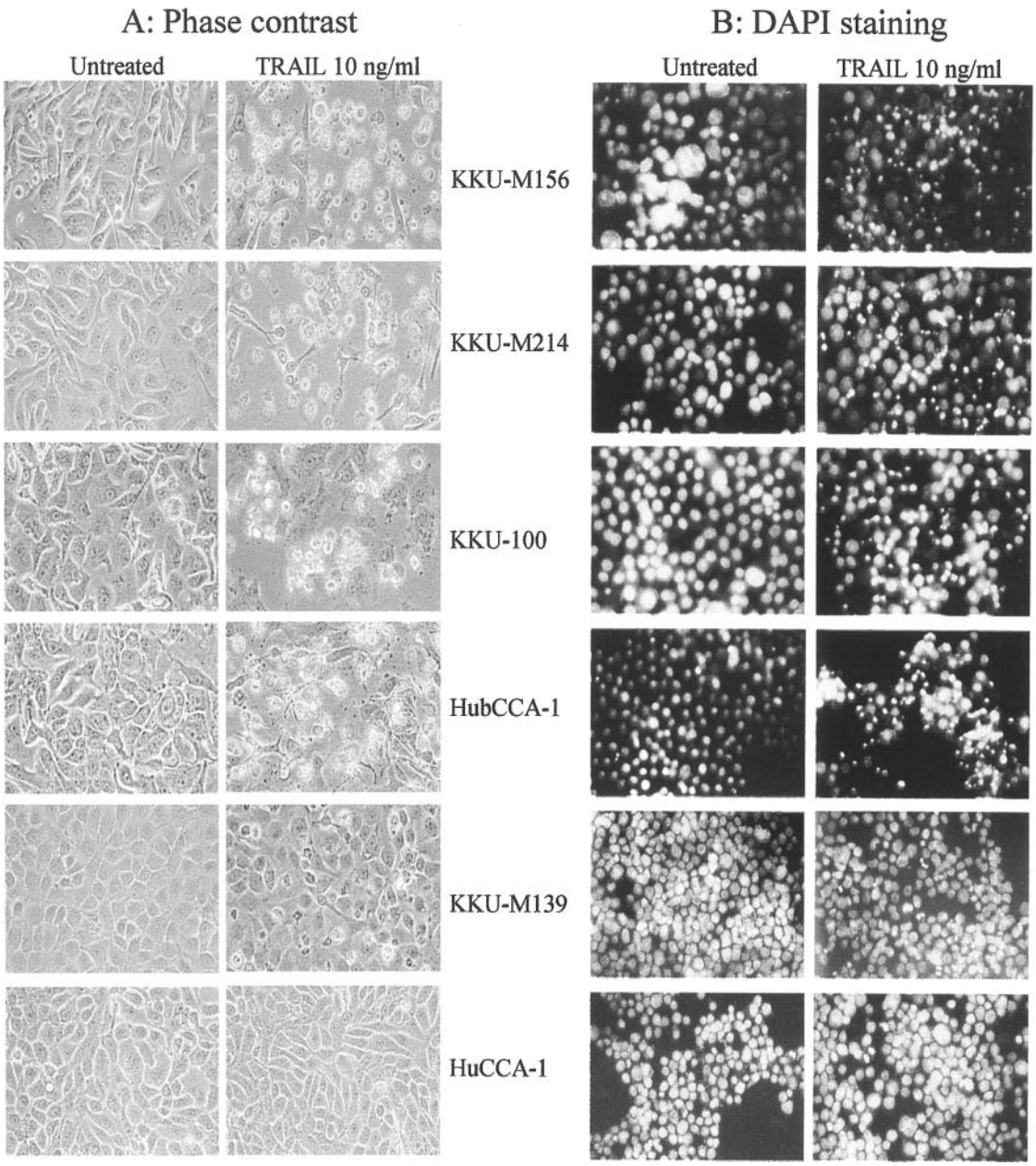

Figure 2. *Microscopic appearance of TRAIL-treated CCA cells. HuCCA-1, HubCCA-1, KKU-100, KKU-M139, KKU-M156 and KKU-M214 were treated with 10 ng/ml of TRAIL for 24 h and were observed under phase contrast microscope for morphological changes (A) or fluorescence microscope of DAPI-staining for fragmented nuclei (B). These microscopic appearances are consistent the results of cell viability observed in Figure 1, i.e., KKU-M139 and HuCCA-1 are more resistant to TRAIL cytotoxicity. Magnification, 200x.*

5B). The expression of the Mcl-1L protein, an anti-apoptotic factor, was high in only KKU-M139, supporting the Mcl-1 involvement in TRAIL resistance previously reported (16). The expression of the cFLIPS protein was high in the other resistant cell line (HuCCA-1) and 2 out of the 4 sensitive cell lines (KKU-M156 and KKU-M214). On the other hand, there was no difference in the expression of Bax among these cells. These data indicate that cFLIP and Bax are not related with TRAIL resistance in CCA cells, in contrast to other TRAIL-resistant cancer cells, such as breast, colon and bladder cancer cells (17-19). The expression of procaspase-8 was low in the 2 resistant cell lines (KKU-M139 and HuCCA-1) and high in the 4 sensitive cell lines (Figure 5C), suggesting that low expression of procaspase-8 might involve TRAIL resistance in CCA cells. It is interesting to note that TRAIL could

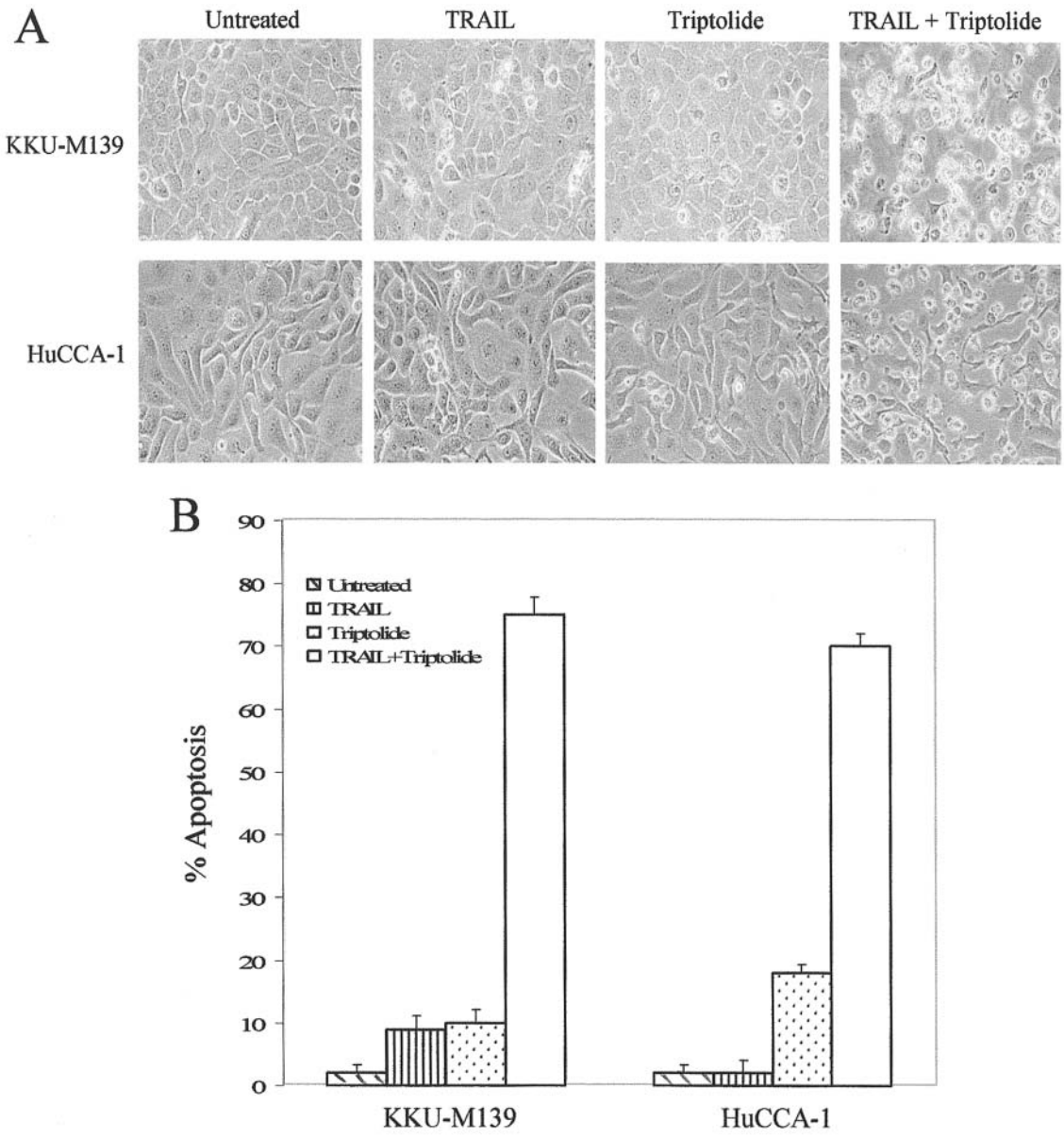

Figure 3. *Augmentation of cell death induced by a combination of TRAIL and triptolide in the KKU-M139 and HuCCA-1 cells. The cells were seeded into 6-well plates at a density of 1x10^6 cells/well and treated for 24 h with a combination of triptolide (30 ng/ml for KKU-M139 and 50 ng/ml for HuCCA-1 cells) and a 10 ng/ml of TRAIL, or with either triptolide or TRAIL alone. (A) Phase contrast micrographs showed massive cell death in the combined treatment groups. Magnification, 200X. (B) After 24 h of treatment, the cells were harvested, stained with DAPI and the percentage of apoptotic cells was determined. Data are mean values with S.D. of 3 independent experiments.*

induce apoptosis in 4 sensitive cell lines which expressed high levels of procaspase-8 but could not in 2 resistant cell lines that had low procaspase-8 expression (data not shown). As shown in Figure 4, combined TRAIL and triptolide could induce apoptosis in these two resistant cell lines. The intracellular mechanism analysis showed that procaspase-8 in the 2 resistant cell lines was activated by this combined treatment. However, high expression of Mcl-1 was one factor involved in the TRAIL-resistance but other anti-apoptotic factors were also involved. The expression of caspase-8 may be one factor that correlated with TRAIL resistance in CCA cells.

In the present study, our results indicated that triptolide could convert TRAIL-resistant CCA cells to be susceptible to TRAIL-induced apoptosis, most likely by enhancing the activation of caspase-8 (Figure 4) and down-regulating

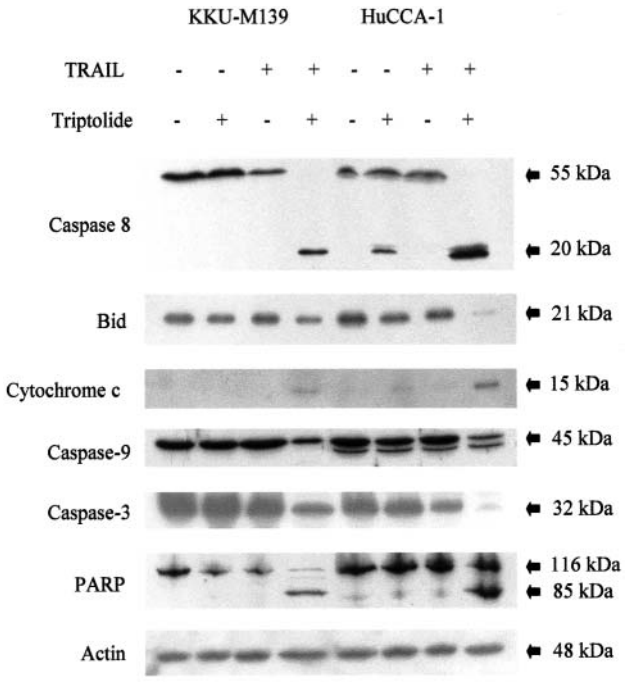

Figure 4. *Possible mechanism of a combined triptolide and TRAIL treatment in inducing apoptosis in KKU-M139 and HuCCA-1 cells. The treated cells were harvested after 24 h, lysed with lysis buffer and whole cell lysates or cytosolic fractions were subjected to immunoblot as described in the Materials and Methods (caspase-3, 8 and 9, Bid, cytochrome c, PARP). Actin served as loading control. Representative results of at least 3 different experiments are depicted.*

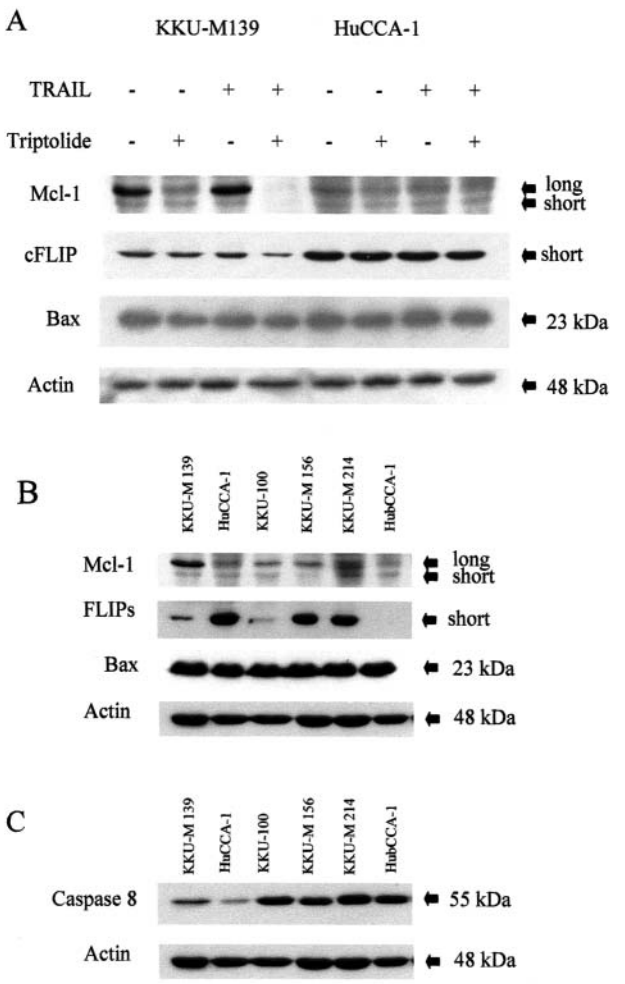

Figure 5. *Intracellular levels of anti-apoptotic factors in human CCA cells. (A) Two resistant CCA cell lines exposed to a combination of triptolide and TRAIL and (B) and (C) 6 untreated cell lines were subjected to electrophoresis and immunoblot reaction, as described in the Materials and Methods (cFLIP, Mcl-1, Bax and caspase-8). It should be noted that the 2 TRAIL-resistant cell lines (KKU-M139 and HuCCA-1) exhibited remarkably lower procaspase-8 levels than the TRAIL-sensitive cell lines. Actin served as the loading control. Representative results of at least 3 different experiments are depicted.*

Mcl-1 expression (Figure 5A). Triptolide was also previously reported to enhance the activity of TRAIL-induced apoptosis by inhibiting NF-κB activation or by activating ERK2 (27, 30). The data presented suggest that the combination of triptolide and TRAIL represents an alternative approach for the management of patients with TRAIL-resistant cholangiocarcinoma.

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References

1 Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C and Smith CA: Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity *3*: 673-682, 1995.

2 Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A and Ashkenazi A: Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem *271*: 12687-12690, 1996.

3 Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC and Lynch DH: Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat Med *5*: 157-163, 1999.

4 Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR and Strom SC: Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat Med *6*: 564-567, 2000.

5 Almasan A and Ashkenazi A: Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. Cytokine Growth Factor Rev *14*: 337-348, 2003.

6 Sheridan JP, Marsters S A, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P and Ashkenazi A: Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science *277*: 818-821, 1997.

7 Pan G, Ni J, Yu G, Wei YF and Dixit VM: TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signaling. FEBS Lett *424*: 41-45, 1998.

8 Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dul E, Appelbaum ER, Eichman C, DiPrinzio R, Dodds RA, James IE, Rosenberg M, Lee JC and Young PR: Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem *273*: 14363-14367, 1998.

9 Watanapa P and Watanapa WB: Liver fluke-associated cholangiocarcinoma. Brit J Surg *89*: 962-970, 2002.

10 Gores GJ: Cholangiocarcinoma: current concepts and insights. Hepatology *37*: 961-969, 2003.

11 Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P and Thomas HC: Changing international trends in mortality rates for liver, biliary and pancreatic tumours. J Heptol *37*: 806-813, 2002.

12 Davila JA and El-Serag HB: Cholangiocarcinoma: the "other" liver cancer on the rise. Am J Gastroenterol *97*: 3199-3200, 2002.

13 Shimada M, Takenaka K, Kawahara N, Yamamoto K, Shirabe K, Maehara Y and Sugimachi K: Chemosensitivity in primary liver cancers: evaluation of the correlation between chemosensitivity and clinicopathological factors. Hepatogastroenterology *43*: 1159-1164, 1996.

14 Moradpour D and Wands JR: Hepatic oncogenesis. Tumors of the liver. *In*: Hepatology: A Texbook of Liver Disease Zakim D, Boyer ID (eds). Philadelphia, Saunders, pp. 1490-1512, 1996.

15 Tanaka S, Sugimachi K, Shirabe K, Shimada M, Wands JR and Sugimachi K: Expression and antitumor effects of TRAIL in human cholangiocarcinoma. Hepatology *32*: 523-527, 2000.

16 Taniai M, Grambihler A, Higuchi H, Werneburg N, Bronk SF, Farrugia DJ, Kaufmann SH and Gores GJ: Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. Cancer Res *64*: 3517-3524, 2004.

17 Panka DJ, Mano T, Suhara T, Walsh K and Mier JW: Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. J Biol Chem *276*: 6893-6896, 2001.

18 Kreuz S, Siegmund D, Scheurich P and Wajant H: NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol *21*: 3964-3973, 2001.

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

20 Nam SY, Jung GA, Hur GC, Chung HY, Kim WH, Seol DW and Lee BL: Upregulation of FLIPs by Akt, a possible inhibition mechanism of TRAIL-induced apoptosis in human gastric cancers. Cancer Sci *94*: 1066-1073, 2003.

21 Dierbi M, Darreh-Shori T, Zhivotovsky B and Grandien A: Characterization of the human FLICE-inhibitory protein locus and comparison of the anti-apoptotic activity of four different flip isoforms. Scand J Immunol *54*: 180-189, 2001.

22 Bae J, Leo CP, Hsu SY and Hsueh AJW: Mcl-1S, a splicing variant of the antiapoptotic Bcl-2 family member Mcl-1, encodes a proapoptotic protein possessing only the BH3 domain. J Biol Chem *33*: 25255-25261, 2000.

23 Baekelandt M, Holm R, Nesland JM, Trope CG and Kristensen GB: Expression of apoptosis-related proteins is an independent determinant of patient prognosis in advanced ovarian cancer. J Clin Oncol *18*: 3775-3781, 2000.

24 Backus HHJ, van Riel MGH, van Groeningen, Vos W, Dukers DF, Bloemena E, Wouters D, Pinedo HM and Peters GJ: Rb, Mcl-1 and p53 expression correlate with clinical outcome in patients with liver metastases from colorectal cancer. Ann Oncol *12*: 779-785, 2001.

25 O'Driscoll L, Cronin D, Kennedy SM, Purcell R, Linehan R, Glynn S, Larkin A, Scanlon K, Mcdermott EW, Hill AD, O'Higgins NJ, Parkinson M and Clynes M: Expression and prognostic relevance of Mcl-1 in breast cancer. Anticancer Res *24*: 473-482, 2004.

26 Lacour S, Micheau O, Hammann A, Drouineaud V, Tschopp J, Solary E and Dimanche-Boitrel MT: Chemotherapy enhances TNF-related apoptosis-inducing ligand DISC assembly in HT29 human colon cancer cells. Oncogene *22*: 1807-1816, 2003.

27 Frese S, Pirnia F, Miescher D, Krajewski S, Borner MM, Reed JC and Schmid RA: PG490-mediated sensitization of lung cancer cells to Apo2L/TRAIL-induced apoptosis requires activation of ERK2. Oncogene *22*: 5427-5435, 2003.

28 Rohn TA, Wagenknecht B, Roth W, Naumann U, Gulbins E, Krammer PH, Walczak H and Weller M: CCNU-dependent potentiation of TRAIL/Apo2L-indued apoptosis in human glioma cells is p53-independent but may involve enhanced cytochrome c. Oncogene *20*: 4128-4137, 2001.

29 Panichakul T, Wanun T, Reutrakul V and Sirisinha S: Synergistic cytotoxicity and apoptosis induced in human cholangiocarcinoma cell lines by a combined treatment with tumor necrosis factor-alpha (TNF-α) and triptolide. Asian Pacific J Allergy Immunol *20*: 167-173, 2002.

30 Lee KY, Park JS, Jee YK and Rosen GD: Triptolide sensitizes lung cancer cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by inhibition of NF-κB activation. Exp Mol Med *34*: 462-468, 2002.

31 Sirisinha S, Tengchaisri T, Boonpucknavig S, Prempracha N, Ratanarapee S and Pausawasdi A: Establishment and characterization of a cholangiocarcinoma cell line from a Thai patient with intrahepatic bile duct cancer. Asian Pacific J Allergy Immunol *9*: 153-157, 1991.

32 Tengchaisri T, Chawengkirttikul R, Rachaphaew N, Reutrakul V, Sangsuwan R and Sirisinha S: Antitumor activity of triptolide against cholangiocarcinoma growth *in vitro* and in hamster. Cancer Lett *133*: 169-175, 1998.

33 Catlett IM, Xie P, Hostager BS and Bishop G: Signaling through MHC class II molecules blocks CD95-induced apoptosis. J Immunol *166*: 6019-6024, 2001.

34 Ruiz-Ruiz C and Lopez-Rivas A: Mitochondria-dependent and -independent mechanisms in tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis are both regulated by interferon-γ in human breast tumour cells. Biochem J *365*: 825-832, 2002.

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