Int J Clin Exp Pathol 2017;10(6):6435-6443
www.ijcep.com /ISSN:1936-2625/IJCEP0050559

Original Article

Bio-available selective IAP inhibitor dual-activated TRAIL-induced apoptosis via caspase-mediated pathway in human colorectal cancer

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Received February 10, 2017; Accepted April 6, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: TRAIL is a new orientation of anti-tumor research ligand due to its tumor selectivity and its non-toxicity to normal cells. Nevertheless, the potential of TRAIL as a chemotherapeutic agent is limited, because of the emergence of TRAIL resistance. So, this research aimed to explore the ability of representative XIAP inhibitor to overcome TRAIL resistance by increasing apoptosis in colorectal cancer models, as well as to investigate the potential mechanisms of regulating inflammatory cytokines TNF-α, IL-1β by TRAIL-induced cascade pathway. Cell viability was assessed by MTT assay for incubating 24 h and 48 h, with the expression level of apoptotic and anti-apoptotic proteins was measured by Western-blot and analyzed by ImageJ software. The mRNA expression level of inflammatory markers, TNF-α, IL- 1β, was determined by qRT-PCR method. The results shown combined treatment with AT406 and TRAIL augmented the activation of caspase-dependent manner as compared with treating them alone. Moreover, the data indicated that after TRAIL treatments, the expression level of inflammation-related genes (TNF-α, IL-1β) was higher up-regulation ($P<0.05$) as compared with placebo. Therefore, we demonstrated here for the first time that co-treatment in vitro with TRAIL and AT-406 in two human colorectal cancer models resulted in significantly greater tumor growth inhibition compared to single treatments, and the combination effects were synergistic and significantly enhanced apoptosis in colorectal cancer cells, but have no cytotoxicity to normal cell. Additionally, combination therapy on targeting TRAIL-induced apoptosis not only play significant for cell death through cancer apoptosis signaling, but could be shed light on the future research directions.

Keywords: Apoptosis, colorectal cancer, TRAIL, caspase, inflammatory cytokines

Introduction

Cancer is one of the most serious diseases endangering human life and health according to WHO (WHO) 2013 Statistics (http://www.who.int/research/en/). In 2008, there are 12.4 million people were newly diagnosed with cancer worldwide, and 7.6 million people died of cancer. Worst of that, 15 million new cases are projected to occur by the end of 2020 [1]. Among all cancers, colorectal cancer (CRC) is the second most commonly diagnosed cancer with over 1.2 million new cases and 608,700 deaths in 2008 [2, 3]. In addition, the incidence rate of CRC is rapidly increasing in a number of countries within Eastern Asia, such as China. World Health Organization estimated that 220,000 new CRC cases and 109,000 deaths occurred in China in 2008. Besides, the development and progression of CRC involves unregulated epithelial cell proliferation due to a series of accumulated genetic alteration [4]. Evidence has shown that prolonged survival of such genetically unstable colorectal epithelial cells, leading eventually to their ultimate malignant transformation, is associated with progressive inhibition of apoptosis.

As far as our knowledge, utilization of apoptosis for therapy is a hallmark of human cancers [5, 6], and targeting key apoptosis regulators to overcome apoptosis resistance of tumor cells is

being pursued as a new cancer therapeutic strategy [7, 8]. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor superfamily, is considered a promising anticancer agent due to its tumor selectivity. Moreover, the cytotoxic activity of TRAIL is selective for human tumor cells and leaves normal cells unharmed [9, 10]. Thus, TRAIL is a promising anticancer cytokine [11]. TRAIL induces apoptosis in various types of tumor cells through the death receptor pathway [12-15]. Besides, TRAIL reacts with the death receptor DR4 or DR5, leading to the aggregation of the receptors, recruitment of the adaptor molecule Fas-associated death domain protein, and activation of initiator caspase-8. Activated caspase-8 is released into the cytoplasm and initiates a protease cascade that activates effector caspases such as caspase-3 [16]. Nevertheless, the only disadvantage for treatment of TRAIL is its resistance to some kind of cancer cells.

The current understanding of TRAIL resistant can divide into two main mechanisms: 1), c-FLIP can inhibit the interaction of procaspase-8 to the DED (death effective domain) of FADD, then block the signal from upstream. 2), the high level of IAPs expression, such as XIAP is a critical inhibitor of TRAIL resistance. Its apoptosis prevention depends on binding to and inhibiting activated caspase-dependent pathway. Moreover, Smac-mimetic IAP-antagonists sensitize TRAIL-induced apoptosis by blocking XIAP function in multiple tumor models, including Pancreatic cancer, ovarian cancer, breast cancer , and multiple myeloma. These findings provide a strong rationale for using Smac-mimetics to achieve TRAIL-sensitization by functional inhibition of the over-expressed XIAP.

In this sense, we investigated the molecular mechanisms of AT-406 (formerly known as SM-406) which is a bioavailable potent IAP (inhibitor of apoptosis protein) of XIAP, sensitizes colon cancer cells to the therapeutic potential of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Notably, combined treatment with AT406 and TRAIL were also more effective in inducing caspase activities, and apoptosis than treatment with either agent alone. While, the viability of normal cell was not affected by TRAIL incubating alone and combination actions. So, these data suggest that AT406 is a useful agent for TRAIL-based cancer treatments. Moreover, we try to test the expression level of inflammation-related genes (TNF-α, IL-1β,) after TRAIL treatments, the data showed the expression level was significantly changed ($P<0.05$). Then, it is rational to use the network immunotherapy for stimulated immune system of anti-tumor with TRAIL treatment modalities to eradicate the neoplasm disease.

Materials and methods

Reagents and antibodies

AT406 was purchased from Selleckchem (Selleckchem, USA). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (Sigma Chemical, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma. Antibodies against caspase-8, capase -3, PARP were purchased from Cell Signaling (Cell Signaling Technology, Inc, USA). The antibody against GAPDH was purchased from Sigma (Sigma Chemical, USA). Peroxidase-labeled goat anti-mouse was purchased from Thermo Scientific (ThermoFisher Scientific, USA), goat anti-rabbit was purchased from Cwbiotech (C-Wbio, China). Recombinant Human TRAIL/TNFSF10 was purchased from R&D Systems (R&D Systems, Inc., USA). Flag-tagged TRAIL was purchased from Alexis Biochemicals (Alexis Biochemicals, USA).

Cell lines and cell culture

Human colon cancer cell line HCT116, lovo was obtained from the Chinese Academy of Sciences, Shanghai Type Culture Collection. Pulp was obtained from The Affiliated Hospital of Kunming University of Science and Technology. H-glucose/DMEM culture medium, RPMI 1640 culture medium were purchased from Thermo Scientific (ThermoFisher Scientific, USA), IMDM culture medium and fetal bovine serum were purchased from Gibco (Gibco, Carlsbad CA, USA). The 1% penicillin-streptomycin was purchased from Sigma (Sigma Chemical, USA). The cells were seeded (5.0 × 104 cells/mL), grown at 37°C in a humidified atmosphere with 5% carbon dioxide for 24 h, and then incubated for up to 24 h with TRAIL and/or AT406.

Table 1. Primer sequences for real-time PCR

Gene	Forward primer	Reverse primer
GAPDH	CCCCTTCATTGACCTCAACTAC	GATGACAAGCTTCCCGTTCTC
TNF-α	CCCAGGGACCTCTCTCTAAT	AGCTGCCCCTCAGCTTGAG
IL-1β	GCACGATGCACCTGTACGAT	CACCAAGCTTTTTTGCTGTG

Viability assay

Cell viability was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. In brief, the cells were cultured in 96-well microtiter plates under 50 ml/L CO_2 in a humidified atmosphere at 37°C. Each well was then incubated for 4 h with MTT solution (final concentration 0.45 g/L, Sigma Chemical, USA) under the same condition. Culture medium in each well was discarded and replaced with 100 ml of dissolving solution (DMSO). The absorbance of each well was determined by a spectrophotometer (VICTOR X5, PerkinElmer Inc, USA) with a 490 nm wavelength. Controls were treated with DMSO, and experiments were repeated in triplicate. Cell viability is expressed as a percentage of the absorbance value determined for the control cultures.

Cytotoxicity assays and calculation of combination indices

The IC_{50} concentrations of TRAIL and AT406 in colon cancer cell lines were established in MTT assays after continuous incubation with drugs. Results were quantified as described in [17], with the method of Chou and Tremblay [18] used to determine combination indices at IC_{50}. CI's values above 0.9 but less than 1.1 represent additive effect and ones less than 0.9 or above 1.1 represent synergism or antagonism, respectively.

Cell morphology and apoptosis by fluorescence microscopy

The HCT-116 cells (5.0×10^5/mL) were seeded 24 h before the experiments in a 6-well plate. TRAIL (100 ng/mL) and/or AT406 (1 μg/ml) were added to cancer cells, and 12 h later the cells were washed with PBS and detached from cell culture wells by trypsin. Next, the cells were centrifuged to discard supernatant, washed with PBS and resuspended in binding buffer (100 μL/sample). The stained cells were observed under a fluorescence inverted microscope CTR6000 (Leica, Germany).

Western blot analysis

For isolation of total protein, treated HCT-116 and lovo cells were centrifuged at 300 g for 3 min, and cell pellets were lysed in 2 ml of ice-cold lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.4, 20 mM EGTA, 1 mM dithiothreitol, 1 mM Na3V04, and protease inhibitor cocktail tablets) for 10 min. Lysates were centrifuged at 12,000 g for 20 min, and supernatant containing 30 μg proteins was boiled in SDS sample loading buffer for 10 min before undergoing electrophoresis on 12% NuPAGE Bris-Tris gels (Invitrogen). After electrophoresis for 2 h, proteins in gel were transferred to a poly-vinylidened ifluoride membrane (Pall Inc., East Hills, NY), and the blots were blocked with 5% nonfat dry milk-phosphate-buffered saline containing 0.1% Tween 20 (PBST) for 60 min at room temperature. The membranes were incubated overnight at 4°C with primary antibodies. The primary antibodies used were anti-caspase-3, anti-caspase-8, anti-Parp, and anti-GAPDH. The membranes were detected by the appropriate secondary antibodies and washed with TBST three times. Reaction products were visualized by using ECL Western Blotting Detection Reagent (Thermo scientific, USA) and GAPDH was used as the loading control. The membranes were incubated overnight at 4°C.

Quantitative RT-PCR

Cells (1.0×10^6) were lysed and RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized with 5 μg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Subsequently, RT-PCR was performed and GAPDH was used as a control. The design of TNF-α, IL-1β Primer were followed by Genbank Data and obtained from GeneRay Biotechnology. cDNA was produced from 2 μg of total RNA samples using Oligo(dT)20 and SuperScript II (Invitrogen). The primers for real-time PCR as seen in (**Table 1**). Light-Cycler PCR was performed with DNA SYBR Green I according to the manufacturer's instructions (Roche Diagnostics). Expression levels of target gene were normalized to the housekeeping gene GAPDH (ΔCt). Gene expression values were then calcu-

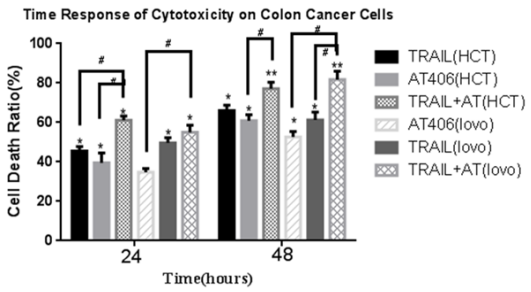

Figure 1. Time Respond of Cytotoxicity for TRAIL with AT406 on Colon cells. The cell viability was determined by MTT for 24 h and for 48 h, respectively. (AT406, 1 μg/mL, TRAIL, 100 ng/mL). And the ability of the apoptosis caused time-dependent growth inhibition in HCT-116 and lovo cells. The values represent mean ± SD of three independent experiments performed in quadruplicate (n = 3). **P<0.01, *P<0.05, compared with the control (both 24 h and 48 h), respectively. #P<0.05 compared with cells treated with AT406 or TRAIL alone.

lated based on the ΔΔCt method using the equation: RQ = 2-ΔΔCt. Each assay was performed in triplicate and repeated three times.

Analysis of western blot band density

Analysis of band density was performed with ImageJ software available at http://rsb.info.nih.gov/ij/. Each band was examined for particle analysis. All bands were compared with their respective GAPDH control. The percent expression of the housekeeping gene GAPDH was assumed to be 100% and the level of protein band density for each experimental antibody was compared with this. The data was eventually normalized.

Statistical analysis

Each experiment was repeated independently three times. Statistical analysis was performed with SPSS 19.0 statistical software package. Continuous variables were presented as mean ± standard deviation (SD) and categorical variables as frequencies and group percentages. The value of *P*<0.05 was considered statistically significant.

Results

AT406 sensitize colon cancer cells to TRAIL-induced cytotoxicity

Results showed that synergistic actions of network combination can significantly improve the death effect in each of colon cancer cell lines (P<0.05). Synergism of TRAIL and AT406 was also evident in cytotoxicity assays (**Figures 1, 3**), and both two colon cells of CI's values less than 0.9, where the combination caused a significant increase in cell death compared to that with each drug alone. The cell death rate of separate treatments on HCT-116 with 100 ng/ml TRAIL was (66.10±2.80)% and with AT406 was 1 μg/mL (60.90±3.08)% for 48 hour, respectively, and the cell death rate on lovo with that was (61.32±3.92)% and was (52.70±2.68)% for 48 hour, respectively. However, their combined use markedly decreased cell viability in above-mentioned cell lines (P<0.05), and the death rate (100 ng/ml TRAIL with 1 μg/mL AT406) was (77.15±2.97)% on HCT-116 and was (81.80±3.97)% on lovo for 48hour, respectively (**Figure 1**). What's more, AT406 increased TRAIL-induced cytotoxicity toward the two colon carcinoma cell lines, as well as this potentiation was strengthened over time from 24 hours to 48 hours.

Observation of the morphology of apoptotic cells

The morphological changing of tumor cells was directly observed by inverted microscopy to examine characteristic of early apoptosis. The cancer cells were incubated for 24 h with TRAIL at the concentrations of 100 ng/mL and AT406 at the concentration of 1 μg/mL. A cover slide was placed in the 6-well plate with HCT-116 cells seeded, fixed for 10 min, Apoptotic cells were determined by fluorescent staining and were observed under Leica CTR6000 systems. (**Figure 2**).

Combination of AT406 and TRAIL does not induce apoptosis in normal human Pulp cells

We have also confirmed that this death effect was caused by the activation of the TRAIL inducing apoptotic pathway. Previous studies have focused on XIAP as the primary molecular target for Smac mimetics in combination with TRAIL. While, the viability of normal cell was not affected by TRAIL alone and combination actions. At concentrations ranging from 10 ng/ml to 200 ng/ml for TRAIL alone or with AT406 1 μg/mL, the result demonstrated that a very limited cytotoxic effect on the tested cell line (**Figure 3**). Moreover, TRAIL-induced cytotoxicity was not significantly enhanced by co-treatment

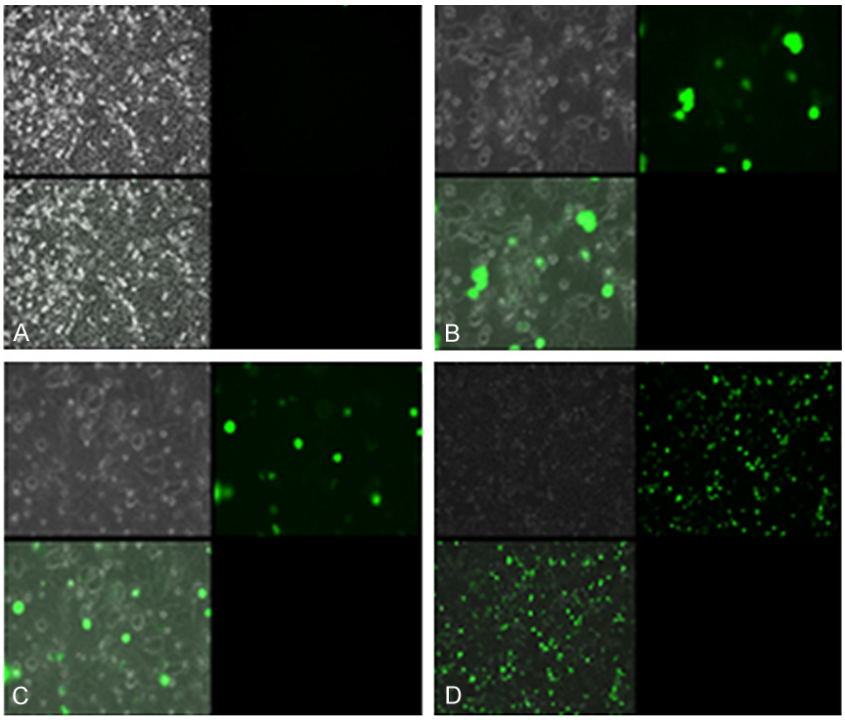

Figure 2. AT406 Enhanced TRAIL-induced Apoptosis in HCT-116 by Cell Morphology. A: control cells; B: cells incubated with AT406 (1 μg/mL); C: cells incubated with TRAIL (100 ng/mL); D: cells incubated with TRAIL (100 ng/mL) and A T406 (1 μg/mL).

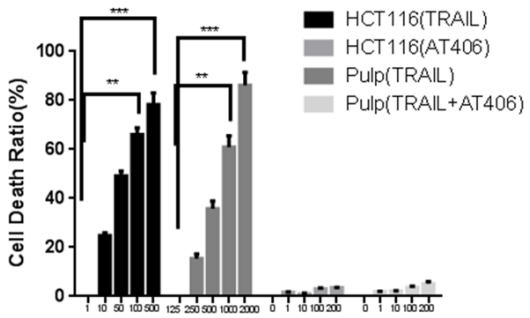

Figure 3. AT406 sensitizes HCT116 cells to TRAIL-induced apoptosis but does not induce apoptosis in normal human Pulp cells. HCT116 cells and normal Pulp cells were treated with TRAIL, AT406 and combination. The cells viability was determined by MTT. (AT406, 1 μg/mL; TRAIL1 ng/mL, 10 ng/mL, 100 ng/mL, 200 ng/mL, respectively). The values represent mean ± SD of three independent experiments performed in quadruplicate (n = 3). The viability of normal cell were not affected by synergistic actions of combination.

with low concentrations of AT406 ($P>0.05$). So, the viability of Pulp cell was not affected by synergistic actions of combination.

Combination Treatment induces Caspase-dependent activation

After detecting the combination effects of AT-406 and TRAIL, we test the proteins expression of caspase level, the initiator and effector-caspases of the extrinsic apoptotic pathways. The data illustrated that cytotoxic activity of TRAIL was found to be related to its ability to trigger apoptotic cell death. The specificity of TRAIL-induced apoptosis was demonstrated in HCT116 cells by showing its activation in the presence of the *Caspase*3/-8. Furthermore, HCT-1116 cells were treated with AT406, TRAIL or the combination of both as indicated. The results showed that combining AT 406 with TRAIL led to a significant increase in active PARP, and the appearance of caspase-8 cleaved forms, p41/43, and p18 could be detected, as counterpart with decrease in Procaspase-8, Procapase-3 (**Figure 4A**). Both PARP, and the appearance of caspase-8 cleaved forms, p41/43, and p18 were significant increase with co-treating in HCT-116 cells relative to GAPDH. While, Procaspase-8 and Procapase-3 protein level were markedly reduced with co-treating in HCT-116 cells relative to GAPDH. The bars display the level of expression of each band relative to GAPDH (**Figure 4C**). Besides, we also confirmed that both AT406 and TRAIL modulating the pathway of exogenous apoptosis for cancer cells by stimulating DR5 expression (**Figure 4B**). These data also suggest that synergistic actions of combination activated the extrinsic apoptosis pathway and by activating through amplification of the caspase-8 mediated extrinsic apoptosis pathway.

Taken together, these *in vitro* apoptosis data reveal that AT406 enhanced TRAIL-induced

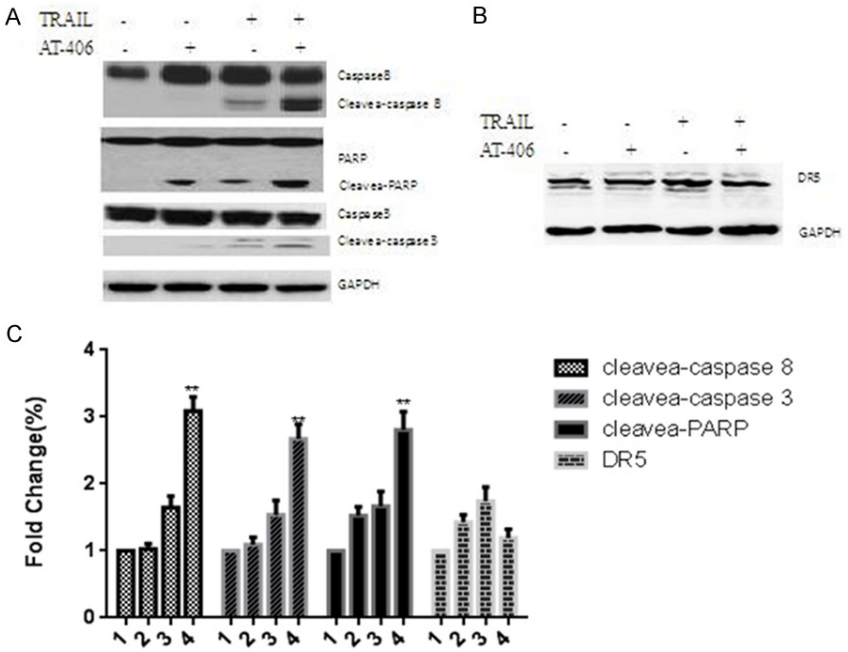

Figure 4. AT406 potentiated TRAIL-induced apoptosis by activating caspase-dependent pathway. A: HCT-116 cells were treated with 100 ng/ml of TRAIL, in the presence or absence of 1 μg/ml of AT406, incubating 24 h, respectively. Whole cell lysates (30 μg) were subjected to Western blot analysis. Membranes were probed with antibodies against caspase-8, caspase-3, and PARP. GAPDH was shown as a loading control. B: The effect of TRAIL and AT-406 on DR5 by Western blotting. C: The expression protein level of PARP, and caspase-8, caspase-3 cleaved forms, and DR5 indicated on HCT-116 cells relative to GAPDH as a loading control (column 1: control, column 2: AT406, column 3: TRAIL, column 4: combination. The fold change of apoptosis proteins was analyzed by Image J software.

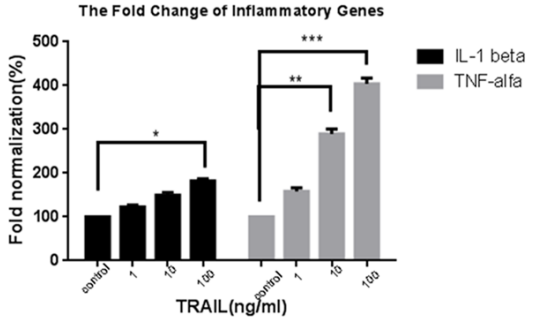

Figure 5. Regulating of inflammatory cytokines TNF-α and IL-1β with TRAIL. After treatment ranging from 1 ng/ml, 10 ng/ml to 100 ng/ml of TRAIL, cells were harvested for quantitative RT-PCR detect target genes. Fold increase of gene expression was calculated by dividing the normalized gene expression activity by that of the untreated control. Representative results of three independent experiments. Columns, mean; bars, SD (n = 3). Placebo, DMSO vehicle control. Student's t-test (n = 3). Expression levels of target gene were normalized to the housekeeping gene GAPDH (ΔCt). Gene expression values were then calculated based on the ΔΔCt method. The data indicated that after TRAIL treatments, the expression level of inflammation-related genes (TNF-α, IL-1β) was higher up-regulation ($P<0.05$).

apoptosis by activating both the extrinsic death receptor pathway and the typically through activation of the cascade-dependent manner. More importantly, AT406 combined with TRAIL induced a longer- lasting apoptosis than TRAIL alone, showing that AT406 enhanced the effect of TRAIL-induced apoptosis.

Regulation of inflammatory cytokines TNF-α and IL- 1β with TRAIL

Tumor Necrosis Factor alfa (TNF-α), Interleukin 1 beta (IL-1β), which were key pro-inflammatory cytokine gene, have been associated with chronic inflammation and play an important role in cancer. Elevated levels of TNF-α and IL-1β, in particular IL-1β greatly enhance the intensity of the inflammatory response. According to (**Figure 5**), increased mRNA expression of TNF-α and IL-1β were observed in TRAIL 1 ng/ml, TRAIL10 ng/ml, and TRAIL 100 ng/ml group as compared to the control, by qRT-PCR analysis. The values represent mean ± SD of three independent experiments performed in quadruplicate (n = 3). What's more, the expression level of TNF-α and IL-1β was correlated with TRAIL dose-response manner. So, our work has indicated that the molecular status of TNF-α and IL-1β may play a significant role in the pathogenesis of colon carcinoma. Moreover, these findings imply that continued research into IL-1β mRNA expression and polymorphisms will be an important source of information in understanding the role of inflammation in the pathogenesis of colon cancer.

Discussion

Cancer, which has the features of heterogeneity, is a systematic disease. This is because tumor cell growth, proliferation, differentiation

and metastasis were controlled by multi-factor, multi-way modulation of protein network systems. And differences in the biological role of each molecule are likely to explain unique, clinical features of agents that target each pathway. So, the strategy of focusing on a single target-spot in cancer therapy is limited in their effect due to the ability of cancer cells to escape antibody efficacy by down regulating the respective pathway, up regulating of alternative pathways. And, the recent review confirm that more potent agents, which can clearly deliver superior clinical efficacy, may lead to more diverse mechanisms of escape. Then these treatments in clinics were generally well tolerated, but not all patients respond to these therapies, and evaluation of biomarkers predictive of network-response is ongoing. These findings reinforce the importance of network therapy to achieve long term clinical curing and prevention of cancer. More importantly, this network therapy will be mandatory not only for detection of early signs of immune-mediated adverse events for patients, but also for judicious use of network therapy to maximize clinical benefits. Therefore, it would be a promising direction to add a layer of complexity to the design of combination regimens, and to purse new network model for cancer therapy.

In contrast to traditional therapies triggering the mitochondrial apoptotic pathway, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) activates the extrinsic apoptotic pathway upon binding to the surface death receptors, DR4 and/or DR5, leading to the recruitment of Fas-associated death domain (FADD) and the formation of the death inducing signaling complex (DISC), resulting in activation of the initiator caspase-8 activates extrinsic apoptotic signaling. TRAIL has attracted public attention for its potential as an anticancer agent based on evidence of its beneficial therapeutic effects for a range of advanced cancers of hematologic and solid tumor origin [19]. Unfortunately, in the post researches, proved that nearly half of cancer cells are resistant with TRAIL. This property limits its potentiality in cancer curing [20]. So, a TRAIL-sensitizing agent is believed to be required in chemotherapy for TRAIL- resistant cancers. There is great speculation about the mechanisms that mediate TRAIL sensitivity/resistance of tumor cells. Sensitivity is dependent on TRAIL-receptor expression of the pro-apoptotic receptors, low levels of IAP, as well as a level of pro-apoptotic proteins that favor apoptosis as opposed to cell survival. The main draw of TRAIL as a potential anticancer therapy is its ability to induce apoptosis only in cancerous and not in non-transformed cells, and it was therefore of importance for us to test the TRAIL-sensitizing ability of the IAP inhibitors in normal cells. Importantly, normal human fibroblasts were not sensitive to the combination of high concentrations of TRAIL and IAP antagonists that had resulted in profound killing of cancer cells. Furthermore, both normal mammary fibroblasts and normal mammary endothelial cells were refractory to TRAIL-induced apoptosis with or without the IAP antagonists, and no cytotoxicity was observed in these cells when the IAP inhibitors were applied as single agents [21].

So far as we know, previous studies have demonstrated that embelin and TRAIL combination treatment synergistically induce apoptosis in pancreatic cancer cells and malignant glioma cells, as well as to enhance the effects of treating TRAIL-resistant A549 NSCLC cells with TRAILR2 mAb in combination with embelin. And the synergistic effect of the combination treatment was due to modulation of multiple components in the TRAIL receptor-mediated apoptotic signaling pathway, including TRAILR2, XIAP, survivin, Bcl-2 and c-FLIP [22]. These finding suggest that the combination of TRAIL and XIAP inhibitor might be used as a new strategy not only for the treatment of NSCLC cells cancer, but for other kind of cancer cells. Similar with that, our results showed that synergistic actions of TRAIL and antagonist of IAPs combination can significantly improve the death effect in CRC cell lines. We have also confirmed that this death effect was caused by the activation of the TRAIL inducing apoptotic pathway. Then caspase-8 activates its downstream effector caspase-3 thus functionalizing extrinsic pathway [23, 24]. Comparatively, intrinsic pathway is also activated via Caspase-8 mediated processing of Bid into truncated Bid. Bid moves into mitochondrion to promote release of cytochrome c, SMAC/DIABLO, Omi/Htra. Decrease in cytosolic levels of Smac/Diablo released from the mitochondria and considerably higher expression of inhibitor of apoptosis (IAP) proteins are some of the mechanisms, which induce TRAIL resistant phenotype [25]. This study

furthers our understanding on the underlying molecular mechanism of the strong synergy between AT406 and TRAIL and provides strong support that the combination of IAP inhibition and TRAIL should be evaluated in the clinic as a new cancer therapeutic strategy for the treatment of a variety of human cancers.

Our results also demonstrated that TNF-α and IL-1β, two of inflammatory biomarkers, which have been closed to NF-κB pathway, were involved in regulating sensitivity of colon cancer cells to TRAIL. What's more, elevated levels of TNF-α and IL-1β, in particular interleukin 1 beta greatly enhance the intensity of the inflammatory response. Then these work have indicated that the molecular status of TNF-α and IL-1β may play a significant role in the pathogenesis of colon carcinoma. Moreover, these findings imply that continued research into IL-1β mRNA expression and polymorphisms will be an important source of information in understanding the role of inflammation in the pathogenesis of colon cancer.

It is known that inflammatory biomarkers, such as TNF-α and IL-1β can remove anti-tumor immunity as well as be used to monitor the progression of the cancer [26]. TNF-a has been found to be involved in all the stages of carcinogenesis such as cellular transformation, promotion, survival, proliferation, angiogenesis and metastasis. It acts primarily through the induction of genes encoding nuclear factor-kB (NF-kB)-dependent anti-apoptotic molecules [27]. The link between inflammation and cancer could be the activation of NF-kB, a hallmark of inflammatory response, which is involved in regulating sensitivity of cancer cells to TRAIL, is frequently detected in malignant tumors [28, 29]. Besides, it has also been reported that constitutively active NF-κB signaling leads to TRAIL-resistance by up regulating XIAP in multiple human cancer cells, and in certain tumor cell types, NF-κB is the primary cause for TRAIL resistance [30]. Previous research reported that IAPs are valid molecular targets for modulating TRAIL sensitivity in prostate cancer cells, and show that blocking IAPs achieves improved efficacy and overcomes resistance to TRAIL. The research team of Yadav VR also [31] examined the potential role of the NF-kB pathway activation in TRAIL signaling, they genetically "knocked down" NF-kB2 in MDA-MB-231 cells and pretreated the cells with an IAP antagonist. They found that NF-kB2 ablation resulted in increased TRAIL-induced loss of cell viability. Also, a recently described chemical inhibitor of NF-kB signaling, 3-FC, results in sensitization to TRAIL and induces even greater sensitization with IAP inhibition. Thus, these preliminary results suggest that modulation of NF-kB pathway signaling may be another important intervention strategy in TRAIL-resistant cancers. Therefore, it is reasonable to postulate that a Smac-mimetic can augment TRAIL-induced apoptosis by blocking both IAPs and NF-κB. And blockade of IAPs by a small molecule Smac-mimetic promotes TRAIL-induced apoptosis in colon cancer cells via modulating both the apoptosis pathway and NF-κB pathway. Consequently , small molecule Smac-mimetics that specifically target IAPs may yield a potential therapeutic benefit with TRAIL- based therapy.

In summary, as IAPs are key molecular targets for the development of cancer cell-selective therapeutics, our findings approved a potential mechanism for a Smac-mimetic IAP-antagonist AT406 on TRAIL-mediated signaling, and suggested that inhibiting IAPs may contribute to enhanced TRAIL efficacy. Besides, our result also indicated that in two colon cell lines which are sensitive to both Smac and TNFα, IL-1β related to TRAIL potential therapeutic pathway of method. Anyhow, combination therapy on targeting TRAIL-induced apoptosis not only play significant for cell death through cancer apoptosis signaling, but could be shed light on the future research directions.

Acknowledgements

This work was financially supported by Scientific Research Fund of Yunnan Provincial Education Department (KKSY201326121), and the Translational Tumor for Science and Technology Project of Yunnan Province (2011DH011).

Disclosure of conflict of interest

None.

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