Paclitaxel enhances tumoricidal potential of TRAIL via inhibition of MAPK in resistant gastric cancer cells

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) holds promise for cancer therapy due to its unique capacity to selectively trigger apoptosis in cancer cells. However, TRAIL therapy is greatly hampered by its resistance. A preclinical successful strategy is to identify combination treatments that sensitize resistant cancers to TRAIL. In the present study, we fully assessed TRAIL sensitivity in 9 gastric cancer cell lines. We found combined administration of paclitaxel (PTX) markedly enhanced TRAIL-induced apoptosis in resistant cancer cells both in vitro and in vivo. The sensitization to TRAIL was accompanied by activation of mitochondrial apoptotic pathway, upregulation of TRAIL receptors and downregulation of anti-apoptotic proteins including C-IAP1, C-IAP2, Livin and Mcl-1. Noticeably, we found PTX could suppress the activation of mitogen-activated protein kinases (MAPKs). Inhibition of MAPKs using specific inhibitors (ERK inhibitor U0126, JNK inhibitor SP600125 and P38 inhibitor SB202190) facilitated TRAIL-mediated apoptosis and cytotoxicity. Additionally, SP600125 upregulated TRAIL receptors as well as downregulated C-IAP2 and Mcl-1 suggesting the anti-apoptotic role of JNK. Thus, PTX-induced suppression of MAPKs may contribute to restoring TRAIL sensitivity. Collectively, our comprehensive analyses gave new insight into the role of PTX on enhancing TRAIL sensitivity, and provided theoretical references on the development of combination treatment in TRAIL-resistant gastric cancer.

Introduction

Gastric cancer (GC) is the fourth most frequent malignancy and the second leading cause of cancer mortality worldwide (1). In China, most of the patients are identified at the advanced stage which leads to dismal prognosis. Radical surgery is the only curative therapy and chemotherapy serves as common strategy for late stage patients. Patients with GC, however, are not particularly sensitive to conventional chemotherapeutic drugs. Thus, it is of great interest to find new approaches, and targeted manipulation of apoptosis is a research hot spot (2). TRAIL, a member of tumor necrosis factor (TNF) superfamily, selectively triggers cell death in transformed cells while causing virtually no toxic towards normal cells (3). TRAIL initiates apoptotic signals via binding to two cell surface death receptors (DRs), DR4 (also known as TRAIL-R1) and DR5 (also known as TRAIL-R2), leading to receptor aggregation and recruitment of Fas-associated death domain (FADD) followed by procaspase-8 (4). The apoptotic process then follows two signaling pathways. In type I cells, activated caspase-8 enables the autocatalytic cleavage of caspase cascade triggers apoptosis. While in type II cells, caspase-8 additionally triggers mitochondrial apoptosis pathway by activating cleavage of Bid, which induces the release of cytochrome c from mitochondria and in turn activates caspase-9 to finally execute cell death (5). Drugs targeting TRAIL pathway, including recombinant TRAIL and agonistic antibodies have been demonstrated with robust anticancer activity in a number of preclinical studies (6-8).

Despite the attractive tumoricidal potential, TRAIL-based therapy is greatly hampered by its resistance, a major obstacle to clinical application (8-10). The most basic cause is dysfunction of death receptors due to hyper-methylation (11), mutation (12) and loss of cell surface expression (13). The defects in caspase protein (14) and overexpression of pro-survival proteins, such as IAP family and anti-apoptotic Bcl-2 family members (15-18), are also linked to TRAIL sensitivity. In addition, more recent findings suggested that cell survival signals, including mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase/Akt pathway, and transcription factor NF-xB played pivotal roles in regulation of TRAIL signaling (19-22). Previous studies...
demonstrated that TRAIL resistance could be alleviated or even reversed by combination therapy (23,24). PTX, one of the cytoskeletal drugs targeting tubulin, is commonly used in advanced GC treatment. It inhibits tumor cell proliferation through stabilizing microtubule network and inhibiting microtubule dynamics (25). PTX has been explored as sensitizing agent towards TRAIL in some cancer types (26-28), yet, the molecular mechanisms were not fully elucidated.

In the present study, we demonstrate that combined treatment of PTX and TRAIL significantly boosted apoptosis of TRAIL-resistant GC cells both in vitro and in vivo. The molecular mechanisms underlying the synergism involved enhanced activation of caspases, upregulation of DRs and downregulation of anti-apoptotic proteins. Moreover, we present herein the inhibitory effect of MAPKs in TRAIL-induced apoptosis in GC and reveal for the first time that TRAIL-augmenting effect of PTX was partly due to suppression of the MAPK pathway.

Materials and methods

Cell culture and reagents. GC cells lines AGS, NCI-N87, SNU-1 and SNU-16 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). MKN28 and NUGC3 were purchased from Health Science Research Resources Bank (Tokyo, Japan), and SGC-7901, BGC-823 and MGC-803 were obtained from Cell Research Institute (Shanghai, China). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C with 5% CO2. Recombinant human TRAIL was purchased from PeproTech EC Ltd. (London, UK). The small molecule inhibitors U0126 (#S1102), SP600125 (#S1460) and SB202190 (#S1077) were obtained from Selleck Chemicals (Houston, TX, USA). The chemotherapeutic drug PTX was from Beijing Union Pharmaceutical Factory (Beijing, China).

Cell viability assay. Cell viability was assessed using Cell Counting kit-8 assay (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, cells were seeded onto 96-well plates at a density of 3-5×103 cells/well and absorbance was measured at 450 nm using multiscan spectrum.

Flow cytometry for apoptosis analysis and expression of cell surface DRs. For cell apoptosis assay, cells (3×10⁴) were seeded onto 35-mm² culture dishes and treated with TRAIL and/or PTX for 24 h as indicated in the figure legends. Then, the cells were stained using an Annexin V/PI double staining kit (Dojindo Molecular Technologies) according to the manufacturer's protocol.

For cell surface expression analysis, cells (1×10⁶) treated with PTX or vehicle for 24 h were incubated with allopurinol (APC)-conjugated anti-DR4 and anti-DR5 antibody, or isotype control (BioLegend, Inc., San Diego, CA, USA) for 30 min at 4°C, and analyzed by flow cytometry (BD Accuri C6; BD Biosciences, San Jose, CA, USA). Positive cells were identified and measured by subtracting relative isotype control values.

Western blot assay. Total proteins were extracted using RIPA lysis buffer (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) with protease inhibitors and phosphatase inhibitors (Beijing Solarbio Science and Technology). Protein concentration was quantified using the Bradford method (Pierce, Rockford, IL, USA). Equivalent amount of protein (20 µg) was separated by 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk in TBS-T for 1 h and incubated with primary antibodies overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies. Signals were detected using chemiluminescent agents (Pierce). Primary antibodies of DR4 and GAPDH were from Abcam (Cambridge, MA, USA), and DR5 was from Sigma-Aldrich (St. Louis, MO, USA). Other primary antibodies purchased from Cell Signaling Technology (Danvers, MA, USA) were as follows: caspase-3 (#9662), cleaved-caspase-3 (#9664), caspase-7 (#9492), cleaved-caspase-7 (#8438), caspase-8 (#9746), caspase-9 (#9502), cleaved-caspase-9 (#7237), PARP (#9542), cleaved-PARP (#5625), Bid (#2002), C-IAP1 (#7065), C-IAP2 (#3130), XIAP (#2045), Livin (#5471), Bcl-xL (#2764), Bcl-2 (#2870), Mcl-1 (#5453), ERK (#4695), phosphorylated-ERK (#4370), JNK (#9258), phosphorylated-JNK (#4668), p38 (#8690), phosphorylated-p38 (#4511).

siRNA transfection. C-IAP1 siRNA and scrambled siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells (1×10⁵) in 6-well plates were incubated for 24 h and then transfected with siRNA (100 nmol/l) and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in Opti-MEM without serum, according to the manufacturer's specifications. After 6 h, the medium was replaced with fresh medium. Forty-eight hours after transfection, C-IAP1 knockdown effect was verified by western blotting.

In vivo xenograft study. Animal studies were carried out in strict adherence with institutional guidelines. SGC-7901 cells (2×10⁷/200 µl per mouse) were subcutaneously injected into the right hind legs of 6-8 week-old female nude mice. When tumors volume reached 50-100 mm³, the mice were randomized to 4 groups and dosing was initiated. They were: i) control (vehicle only); ii) TRAIL (100 µg/kg intratumoral injection); iii) PTX (10 mg/kg i.p.); and iv) the sequential administration of PTX (10 mg/kg i.p.) followed by TRAIL (100 µg/kg intratumoral injection) after 24 h. All groups were treated once every 3 days for 18 days. The tumor size and weight were monitored three times a week. Tumor volume (V) was calculated as \( V = 0.5 \times x \times y \times z \). Tumor growth inhibition (TGI) was assessed in accordance with the formula \( 1 - \frac{V_T}{V_T}\times \left(\frac{C-C_0}{C_0}\right) \times 100 \), where \( T \) and \( T_0 \) were the mean tumor volumes at the end of the drug administration and day 0, respectively for treated group, and \( C-C_0 \) were those for vehicle control group.

Statistical analysis. All the values are presented as mean ± SD of three independent experiments. The statistical significance among experimental groups was assessed by two-sided Student's t-test. P<0.05 were considered to be significant. All
statistical analyses were performed using GraphPad Prism 5.0 software.

**Results**

**Response of GC cells to TRAIL and expression of TRAIL receptors.** First, we analyzed the effects of TRAIL on cell viability in 9 GC cell lines using CCK-8 assay. As shown in Fig. 1A, GC cells had varying degree of sensitivity to TRAIL. SNU-16, NUGC3, NCI-N87 and SNU-1 were defined as sensitive cells, in which >50% growth inhibition was achieved when treated with TRAIL (200 ng/ml) for 24 h. By contrast, the growth inhibition rates of TRAIL-resistant cells (BGC-823, MKN28, MGC-803, SGC-7901 and AGS) were <50% even under condition of elevated concentration or prolonged exposure time indicating an intrinsic resistance mechanisms in these cells. TRAIL-induced apoptosis was also confirmed by Annexin V/PI double staining in the same cell lines (Fig. 1B). To determine whether TRAIL pathway was intact, we measured cell surface expression of DR4 and DR5 (Fig. 1C and D). Flow cytometric analysis revealed a predominant expression of DR5 in all these cells except SNU-16. DR4, however, exhibited diverse levels in 9 cell lines and the top three sensitive cells (SNU-16, NUGC3 and NCI-N87) expressed the highest levels.

**PTX and TRAIL act synergistically to inhibit GC cell growth.** PTX exhibited growth inhibiting effect in TRAIL-resistant cell lines (Fig. 2A). We attempted to investigate whether PTX augments TRAIL-inducing apoptosis and found a significant decrease on cell viability in combination group compared with TRAIL or PTX alone in all 5 resistant cell lines (P<0.05) (Fig. 2B). To confirm this synergism, SGC-7901 and MGC-803 cells were cultured with different combinations of TRAIL (100 and 200 ng/ml) and PTX (5 and 10 nM). The results indicated that PTX promoted TRAIL-mediated cytotoxic effect in a dose-dependent manner (Fig. 2C). Considering changes in the order of drug exposure could enhance cell death (29) and sequential application of PTX followed by other anticancer chemicals is common in GC treatment (30), we tested the possibility of sequential administration of PTX and TRAIL. Cells were pre-incubated with PTX alone for 12 h and subsequently treated with TRAIL for an additional 12 h. Reduction of cell viability was more distinct in the sequential exposure group compared with simultaneous administration group (Fig. 2D), indicating that pre-treatment of PTX rendered cancer cell more vulnerable to TRAIL.

**PTX sensitizes GC cells to TRAIL-induced apoptosis by activating caspase-dependent mitochondrial apoptotic pathway.**

Figure 1. The sensitivity of GC cells to TRAIL and expression of death receptors. (A) CCK-8 assay for 9 GC cell lines after incubation with TRAIL at indicated doses for 24 h. (B) Apoptotic analysis for 9 GC cell lines after incubation with TRAIL (200 ng/ml) for 24 h. (C) Cell surface expression of DR4 and DR5 assessed by flow cytometric method. Blue, black and red lines indicate isotype control, DR4 and DR5, respectively. (D) Quantified DR4 and DR5 expression on cell surface.
To ascertain whether cell growth inhibition was due to apoptosis, Annexin V/PI double staining was performed. A significant increase in apoptosis was observed in the combination treatment group compared with either agent alone.
Taking SGC-7901 cell line as an example, the mean apoptotic rate was 2.8 and 8% treated with PTX and TRAIL alone, respectively, but as much as 22.4% after combination treatment. The expression of full length and cleaved caspase-3, -7, -8 and -9 and PARP was measured by western blotting (Fig. 3C). Combination of TRAIL and PTX markedly enhanced cleavage of all the caspase proteins compared with single agent alone. Noteworthy, cleavage of caspase-9, but not caspase-8, was induced by PTX, indicating the effect of PTX on activation of mitochondrial pathway. We further tested expression of Bid, another key component in mitochondrial pathway, which was significantly decreased in combination group.

PTX upregulated TRAIL receptors and downregulated anti-apoptotic proteins. Given the therapeutic potential of combined regimen, we focused the following studies on unraveling the molecular mechanism of the synergism. As shown in Fig. 4A, PTX induced DR4 and DR5 expression in both SGC-7901 and MGC-803 cells, albeit the trend was not distinct in MGC-803 cell line as an example. (A) DR4 and DR5 expression was assessed by western blotting in SGC-7901 and MGC-803 cells after 24-h incubation with PTX at indicated doses. (B) Cell surface expression of DR4 and DR5 was assessed by flow cytometric method in SGC-7901 and MGC-803 cells after incubation with 100 nM PTX for 24 h. The blue lines indicate staining with isotype control, black lines and red lines indicate absence and presence of PTX, respectively. (C) SGC-7901 and MGC-803 cells were treated with TRAIL (100 ng/ml) and/or PTX (1 µM) for 24 h. Anti-apoptotic proteins were analyzed by western blotting. (D) SGC-7901 and MGC-803 cells were treated with indicated doses of PTX for 24 h or 1 µM PTX for indicated time-points. Anti-apoptotic proteins were analyzed by western blotting. (E) Effect of C-IAP1 silencing on cell viability induced by TRAIL plus PTX. SGC-7901 and MGC-803 cells were transiently transfected with C-IAP1 specific siRNA for 48 h and then incubated with TRAIL (100 ng/ml) and/or PTX (10 nM) for 24 h. Cell viability was analyzed by the CCK-8 assay. *P<0.05, **P<0.01.
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Additionally, enhanced cell surface expression of DR4, not DR5, was also examined after PTX treatment by flow cytometric analysis (Fig. 4B). To further uncover the role of PTX on modulating cell death, apoptosis-related proteins were investigated. Notably, combination treatment significantly inhibited expression of anti-apoptotic regulators including C-IAP1, C-IAP2, Livin and Mcl-1 (Fig. 4C). Consistent with these findings, decreased expression of these proteins was also observed in SGC-7901 and MGC-803 cells treated with PTX alone in a dose- and time-dependent manner (Fig. 4D). Moreover, a representative protein C-IAP1 was chosen for functional verification. Silencing of C-IAP1 by siRNA enhanced TRAIL-mediated cytotoxicity and amplified the TRAIL-sensitizing effect of PTX (Fig. 4E). All these results suggested that PTX sensitized TRAIL-induced apoptosis via upregulating DRs and downregulating anti-apoptotic proteins.

**PTX induced inactivation of the MAPK pathway.** Considering MAPKs have been implicated in regulation of TRAIL-resistance (19,31) in conjunction with the reports that PTX could modulate activation of MAPKs (32), we hypothesized that PTX-mediated MAPK activity may contribute to TRAIL sensitivity in GC cells. To this end, we first evaluated the effect of PTX on MAPK activity. Results depicted in Fig. 5A demonstrated ERK, JNK and p38 were strongly suppressed after treatment of PTX alone as well as in combination with TRAIL. Next, we investigated the possible role of MAPK deprivation on the sensitivity to TRAIL. Treatment of U0126 (ERK inhibitor), SP600125 (JNK inhibitor) and SB202190 (p38 inhibitor) respectively, significantly enhanced TRAIL-induced apoptosis and cytotoxicity, as did PTX (Fig. 5B and C). Among the three inhibitors, SP600125 showed the strongest sensitization effect. To further assess the effect of MAPKs on TRAIL signalling, expression of DRs and anti-apoptotic proteins were evaluated after pretreatment with specific inhibitors. Results showed SP600125 increased DR4 and DR5 expression while U0126 suppressed them indicating MAPKs exhibited opposite effect on DRs (Fig. 5D). Moreover, C-IAP2
was suppressed by SP600125 and Mcl-1 was decreased by all three inhibitors (Fig. 5E). Collectively, these results suggested that PTX-mediated inhibition of MAPKs might contribute to facilitate TRAIL potential in GC cells.

**PTX enhances the antitumor effect of TRAIL in a tumor xenograft model.** To assess the therapeutic effect of PTX and TRAIL, we established nude mouse models bearing SGC-7901 tumor xenografts. The results revealed that TRAIL or PTX alone only slightly suppressed growth of tumors (TGI was 14.8 and 56.0%, respectively), whereas in the combination group, TGI was 85.1% at the end of drug administration (Fig. 6). The data further confirmed that PTX enhanced the tumor-suppressing capacity of TRAIL in resistant GC cells.

**Discussion**

Identification of new drugs causing tumor specific apoptosis has roused enormous interest. TRAIL legend and its receptors are attractive targets for the selective eradication of tumor cells. Recombinant TRAIL protein and TRAIL receptor agonistic antibodies have been tested in clinical trials, displaying encouraging antitumor activities with mild side-effects. Nevertheless, resistance to TRAIL limits their clinical application. In the present study, we found not all the cell lines underwent significant apoptosis when treated with TRAIL. Numerous chemotherapeutic agents can sensitize tumor cells to TRAIL-mediated apoptosis (26-28). However, there are scarce data elucidating the synergistic interaction between PTX and TRAIL in GC cells. In the present study, we assessed the tumoricidal potential of TRAIL combined with PTX both in vitro and in vivo, and analyzed the mechanism by which PTX sensitized TRAIL-resistant GC cells.

One possible mechanism is synergistic activation of caspase. Apoptosis can be induced by two pathways. TRAIL and PTX share two complementary characteristics for performing apoptosis: one is high efficacy of cell death triggered by death receptor-mediated pathway, and the other is activation of mitochondria-controlled signaling. In the present study, we observed that TRAIL alone only slightly activated caspase-3, -8 and PARP in SGC-7901 and MGC-803 cells. In contrast, simultaneous administration of PTX and TRAIL resulted in a dramatic increase in cleavage of all caspase proteins. Notably, the combined administration markedly activated caspase-9 and induced cleavage of Bid, which are key events in mitochondrial apoptosis signaling, suggesting that PTX facilitated sensitivity to TRAIL by reinforcing mitochondria-mediated apoptosis. This observation is supported by others reporting that PTX induces apoptosis via a death receptor-independent, caspase-3/-8-driven mitochondrial amplification loop (33).

Apoptosis signals are initiated when TRAIL binds to DR4 and DR5. Upregulation of DRs can enhance the responsiveness of cancer cells to TRAIL-mediated cell death (26,34). According to our data, high DR5 expression was prevalent and almost undifferentiated in GC cells. Intriguingly, GC cells shared diverse surface expression of DR4 with the tendency that TRAIL-resistant cell lines exhibited relatively low levels. These findings provided novel evidence that susceptibility of GC cells to TRAIL might be mainly ascribed to the surface expression of DR4. As we found PTX could increase DR4 expression, it was plausible that induction of DR4 would be an efficient way to potentiate tumoricidal potential of TRAIL in GC. Another crucial point for increasing cell susceptibility to TRAIL is inhibition of the anti-apoptotic protein. Previous studies supported that TRAIL-resistant cells were re-sensitized by Bcl-2 or IAP antagonist (17,18). Here we revealed that PTX itself markedly inhibited expression of C-IAP1, C-IAP2, Livin and Mcl-1 in both SGC-7901 and MGC-803 cells, and combined application of PTX and TRAIL exhibited similar effects. In addition, knockdown of C-IAP1 by
siRNA augmented cytotoxic potential of TRAIL indicating PTX-mediated suppression of anti-apoptotic proteins contributed to the sensitizing effect.

Noteworthy, we found MAPKs were involved in the PTX-mediated sensitization to TRAIL. MAPK pathway, regulating cell proliferation, differentiation, mitosis and apoptosis, mainly consists of ERK, JNK1/2 and p38 MAPK members. They are frequently activated in GC (35). In this study, we found MAPKs (including ERK, JNK and p38) acted as negative regulators in TRAIL-induced apoptosis. PTX, however, suppressed MAPK activation, abolished the inhibition effect, and therefore partly restored TRAIL sensitivity. We also revealed suppression of JNK by the specific inhibitor SP600125, which showed the most effective sensitization effect. Consistent with the results of Mucha et al (19), JNK inhibition sensitised hepatocellular carcinoma cells to TRAIL. Similarly, ERK abrogated TRAIL-induced apoptosis by phosphorylating pro-caspase-8 and inhibited the cleavage of Bid (36,37). However, other groups reported that enforced activation of ERK and p38 conferred sensitization of tumor cells to TRAIL by suppressing expression of FLIP and increasing expression of DRs (34,38). These inconsistent results indicate that MAPK signaling may play various roles in the way the diverse cancer cells react to TRAIL. Moreover, our results showed that MAPKs played opposing roles on modulating DRs and only JNK inhibitor could induce both DR4 and DR5 expression. In agreement with our results, Kim et al (39) have shown SP600125 upregulated DRs surface expression on hepatocellular cancer cells. We also found MAPK inhibitors could suppress expression of anti-apoptotic protein C-IAP2 and Mcl-1. All these results suggested that inhibition of MAPKs was involved in TRAIL-sensitizing effect of PTX.

In summary, the present study suggested that PTX sensitized resistant GC cells to TRAIL-mediated tumoricidal effect both in vitro and in vivo. Potentiation of mitochondrial apoptotic signals, upregulation of death receptors, downregulation of anti-apoptotic proteins and inactivation of MAPKs were all involved in the synergistic interaction. Our findings strongly suggested that the combination of PTX with TRAIL could serve as a new therapeutic strategy for GC. Further study on the potential application in this direction is warranted.

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