

Emetine enhances the tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of pancreatic cancer cells by downregulation of myeloid cell leukemia sequence-1 protein

YUJEONG HAN¹, SOJUNG PARK¹, ANN W. KINYUA³, LADISLAV ANDERA⁴, KI WOO KIM³ and INKI KIM^{1,2}

¹Asan Institute for Life Sciences, Asan Medical Center, Seoul 138-736; ²Department of Medicine, University of Ulsan College of Medicine, Seoul 138-736; ³Department of Pharmacology, Institute of Lifestyle Medicine and Nuclear Receptor Research Consortium, Yonsei University Wonju College of Medicine, Wonju 220-701, Republic of Korea;

⁴Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 14220 Prague 4, Czech Republic

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Abstract. Although the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapeutic agent, it shows limited efficacy in human pancreatic cancer cells. Protein synthesis inhibition has been reported to sensitize cancer cells to apoptosis-inducing agents, but the detailed mechanism by which protein synthesis inhibition sensitize cells to TRAIL has not been determined. To investigate the mechanism underlying pancreatic cancer cell resistance to TRAIL, we performed a small scale high-throughput compound screening in AsPC-1 pancreatic cancer cells using a bioactive small molecule library. We identified 8 compounds that reproducibly sensitize AsPC-1 cells to TRAIL-induced apoptosis. One of these compounds, emetine hydrochloride, when combined with subtoxic concentrations of TRAIL, induced massive apoptosis in AsPC-1 and BxPC-3 pancreatic cancer cells. Cell death analysis revealed that the sensitizing effects of emetine were specific to TRAIL. Emetine downregulated the expression of the TRAIL-related anti-apoptotic protein Mcl-1 in a dose- and time-dependent manner. Furthermore, specific knockdown of Mcl-1 using small interfering RNA without emetine treatment sensitized pancreatic cancer cells to TRAIL. Emetine sensitization of pancreatic cancer cells

to TRAIL via Mcl-1 was confirmed under hypoxic conditions. Taken together, these findings strongly suggest that Mcl-1 is involved in pancreatic cancer cell resistance to TRAIL, and emetine facilitates the apoptosis of TRAIL-tolerant pancreatic cancer cells by specifically inhibiting Mcl-1 function.

Introduction

Pancreatic cancer is one of the most aggressive types of cancer. Although accounting for only 3% of all cancers, pancreatic cancer is the fourth most frequent cause of cancer-related mortality, with a median survival of 6 months and a 5-year survival rate under 5% (1). Many clinical trials have tested new therapeutic agents in patients with pancreatic cancer, but the median survival has remained virtually unchanged (2,3). New agents and combination agents are, therefore, needed to treat patients with pancreatic cancer.

Apoptosis is a cellular process that maintains the normal homeostasis of eukaryotic cells, with abnormally controlled apoptosis being one of the primary causes of cancer development and progression (4). Most anticancer agents induce apoptosis by activating the intrinsic or extrinsic cell death pathway. Activation of the extrinsic pathway is triggered by the ligation of a death ligand to its receptor on plasma membranes, leading to the formation of the death-inducing signaling complex (DISC) and the activation of apoptosis proteases (5).

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces cell death by signaling through TRAIL receptors on the plasma membrane, including TRAIL-receptor 1 (DR4) and TRAIL-receptor 2 (DR5) (6,7). TRAIL is a cytokine with selective cell death-inducing activity in malignant cancer cells, but with minimal toxicity towards normal cells (8). Due to its cancer-specific cell death-inducing activity, several clinical trials have been initiated to test the anticancer activities of recombinant human TRAIL protein and antibodies that bind to the TRAIL receptor, but most

Correspondence to: Professor Inki Kim, Asan Institute for Life Sciences, Asan Medical Center, Seoul 138-736, Republic of Korea
E-mail: ik.kim@amc.seoul.kr

Professor Ki Woo Kim, Department of Pharmacology, Institute of Lifestyle Medicine and Nuclear Receptor Research Consortium, Yonsei University Wonju College of Medicine, Wonju 220-701, Republic of Korea
E-mail: kiwoo@yonsei.ac.kr

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primary tumors and cancer cell lines were found to be resistant to TRAIL-induced apoptosis (9). These include various human pancreatic cancer cell lines, highlighting the need to understand their mechanisms of resistance (10-14).

Among the various mechanisms by which cells are resistant to TRAIL are abnormalities in TRAIL receptors (15-18), cell death signaling (19-22) and the assembly of death-inducing signaling complexes (23,24). Many chemical agents have been reported to enhance TRAIL toxicity in cancer cells. In particular, protein synthesis inhibitors, including cycloheximide and emetine, are thought to sensitize cancer cells to TRAIL (25-28). These protein synthesis inhibitors are thought to sensitize cells to TRAIL by targeting certain cellular pathways, including the c-Jun N-terminal kinase in prostate cancer cells (27). However, these effects are only partly understood.

In this study, we show that emetine hydrochloride strongly sensitizes pancreatic cancer cells to TRAIL-induced apoptosis by downregulating myeloid cell leukemia sequence-1 (Mcl-1) protein. We also found that Mcl-1 activity was required during hypoxia-induced resistance of pancreatic cancer cells to TRAIL, suggesting that Mcl-1 is the main regulator of TRAIL-induced apoptosis under both normoxic and hypoxic conditions.

Materials and methods

Cell culture and materials. Human pancreatic AsPC-1 and BxPC-3 cancer cells were maintained in RPMI-1640 medium, and PANC-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM). All media were supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA), 2 mM L-glutamine and penicillin-streptomycin (100 U/ml). Emetine hydrochloride was from Sigma (St. Louis, MO, USA). Recombinant human TRAIL, Lipofectamine 2000 and Lipofectamine RNAiMAX reagents were purchased from Life Technologies and CellTiter-Glo ATP measuring luminescence assay solution was from Promega (Madison, WI, USA).

Compound screening. AsPC-1 cells were recovered from culture plates and seeded in 96-well plates at a density of 5×10^3 cells/well in 50 μ l medium. The plates were incubated for 20 h at 37°C in a cell culture incubator. Using an automatic liquid handler (Perkin-Elmer model AJM8M01), 25 μ l of culture medium containing the test compound was added to each well in columns 2 to 11 to achieve final concentrations of 5 μ M, while 25 μ l of the culture medium was added to each well in columns 1 and 12. After 2 h, 25 μ l of TRAIL-containing medium was added to each well, yielding a final TRAIL concentration of 50 ng/ml, except for the wells in column 1, to which 25 μ l of culture medium was added. After 24 h, the ATP reactive luminescence value of each well was measured using a cellular ATP content assay (CellTiter-Glo) and an EnVision Multilabel reader (Perkin-Elmer). Raw values were transferred to Prism Statistics software to calculate relative cell survival. Using this system, we screened a library of 1,200 bioactive drugs (Prestwick-1200™).

Immunoblotting. Cell lysates were prepared by adding lysis buffer (50 mM Tris-Cl, pH 7.4, 1% Igepal, 300 mM NaCl,

2 mM $MgCl_2$, 2 mM Na_3VO_4 , 5 mM β -glycerophosphate, protease inhibitor mixture and phosphatase inhibitor mixture) to collected cells. After determining the sample protein concentration by bicinchoninic acid assay, 40 μ g of the cell extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes, which were incubated with specific antibodies diluted at 1:1,000. Primary antibodies for immunoblotting included antibodies to DR5 (CS-8074), caspase-3 (CS-9665), Bid (CS-2002), PARP (CS-9532), Bcl-2 (CS-2872), Bcl-X_L (CS-2764) and Mcl-1 (CS-5453), (all from Cell Signaling Technology, Danvers, MA, USA); and antibodies to DR4 (cat #7863; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α -tubulin (T9026; Sigma). After thorough washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) and the enhanced chemiluminescent substrate (SuperSignal West Pico-Pierce).

Cell viability analysis. Cell viability was evaluated using a cellular ATP content-based luminescence assay (CellTiter-Glo assay; Promega), flow cytometric analysis or trypan blue dye exclusion assay. For the ATP assays, cells were seeded in 96-well plates at a density of 5×10^3 cells/well in 50 μ l of culture medium. The cells were pretreated for 1 h with 25 μ l of the emetine-containing medium, at the indicated concentrations, followed by incubation for 24 h with the indicated concentrations of recombinant human TRAIL in 25 μ l of medium. To each well, 10-20 μ l assay solution was added and luminescence was evaluated. Raw values were analyzed using GraphPad Prism software (Graphpad Software Inc., La Jolla, CA, USA) to calculate relative cell survival rate.

For the flow cytometric analysis, 2×10^5 cells were placed in each well of a 12-well plate, pre-incubated with 2.5 μ M emetine chloride and treated with TRAIL, as described above. After 24 h, the recovered cells were washed with cold phosphate-buffered saline (PBS) and resuspended in 500 μ l Annexin V reaction buffer containing Annexin V-FITC (50 μ g/ml). The percentage of apoptotic cells was evaluated by flow cytometry, using a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA).

siRNA transfection and cell viability assay after Mcl-1 knock-down. Mcl-1 siRNA (5'-aagauacacagacguucuctt-3') and non-silencing siRNA (5'-acgugacacguucggagaauu-3') were synthesized by Genolution Pharmaceuticals, Inc. (Seoul, Korea) and transfected into pancreatic cancer cells using RNAiMax Reagent™ (Life Technologies). In brief, 30 pmol of siRNA in 5 μ l of transfection reagent was added to cells in 0.5 ml culture medium and incubated for 2 days. The cells were treated with TRAIL for 24 h, and cell viability was assessed using trypan blue exclusion assays. Gene knockdown was confirmed by western blotting.

Cell viability assay under hypoxic conditions. PANC-1 cells were seeded into 96-well plates in a normoxic incubator. The plates were transferred to an Xvivo hypoxia incubator (model Step Two; Biospherix Ltd., Lacona, NY, USA) and pre-subjected to hypoxia for 6 h. After treatment with emetine and TRAIL, as described above, the cells were further incubated

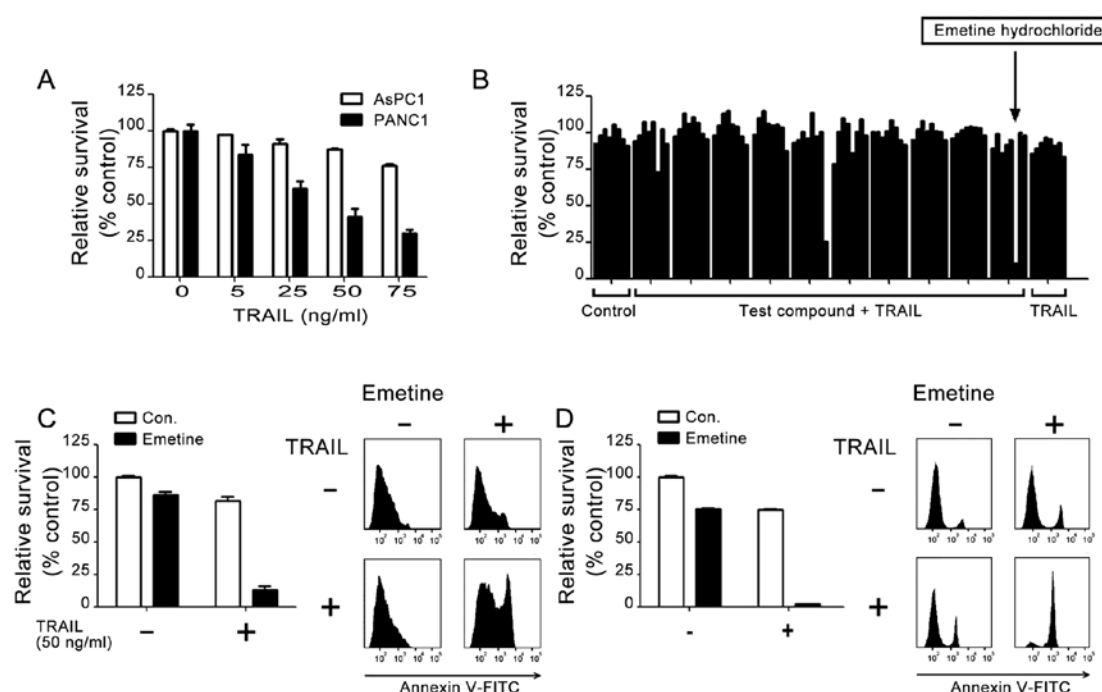


Figure 1. Response of pancreatic cancer cells to TRAIL and compound screening to isolate TRAIL-sensitizers. (A) Viability of pancreatic cancer cell lines treated with TRAIL. Cells were treated with the indicated concentrations of recombinant TRAIL for 24 h. Relative cell survival was evaluated by ATP content assays. Each bar represents the mean \pm standard deviation (SD) of triplicate samples. (B) Representative graph of an assay plate identifying emetine hydrochloride as sensitizing cells to TRAIL-induced apoptosis. Normalized relative survival (y-axis) was plotted against well number (x-axis). Wells in column 1 are controls (cells treated with DMSO only) and wells in column 12 are experimental controls (DMSO with TRAIL). Emetine-induced sensitization is indicated by the arrow. (C and D) Emetin-induced sensitization to TRAIL of (C) AsPC-1 and (D) BxPC-3 pancreatic cancer cell lines. Cells were incubated with emetine and TRAIL, and ATP content was assayed as described in A (left panels) or by flow cytometry after staining with Annexin V-FITC (right panels).

Table I. Sensitizers of TRAIL in AsPC-1 cells (% relative viability are shown).

Function		- TRAIL	+ TRAIL	Ref.
Control		100	82	
Daunorubicin	DNA intercalating agent	81	13	(46)
Doxorubicin	DNA intercalating agent	83	11	(47)
Vorinostat	Histone deacetylase inhibitor	89	21	(48)
Topotecan	Topoisomerase II inhibitor	82	11	(49)
Docetaxel	Microtubule stabilizer	89	47	(50)
CGP74514A	Cyclin-dependent kinase 1 inhibitor	94	9	
Emetine	Protein synthesis inhibitor	82	7	
Brefeldin A	Golgi complex transport inhibitor	80	39	

for 24 h under hypoxic conditions. Cell viability was measured using the ATP contents assay (CellTiter-Glo).

Results

Screening of compounds sensitizing AsPC-1 cells to TRAIL.

To assess the sensitivity of human pancreatic cancer cells to TRAIL, we first analyzed the viability of the cells after exposure to recombinant TRAIL protein. PANC-1 cells showed dose-dependent cell death when treated with TRAIL (Fig. 1A), whereas AsPC-1 cells were resistant to TRAIL. At a concentration of 75 ng/ml, TRAIL induced the death of 71% of PANC-1 cells relative to the control (Fig. 1A), while >70% of AsPC-1 cells remained viable.

To characterize the mechanism by which AsPC-1 cells are resistant to TRAIL, we screened a library of bioactive small molecules and marketed drugs (Prestwick-1200™) (Fig. 1B). In brief, these cells were pretreated with 5 μ M compound for 1 h, followed by treatment with a subtoxic dose of TRAIL (50 ng/ml). A primary 'hit' was defined as a compound that, when combined with TRAIL, induced >50% of cell death, relative to the control treatment. This screening identified 8 compounds (Table I) that reproducibly sensitized cells to TRAIL and had minimal self cytotoxicity, including emetine hydrochloride (Fig. 1B).

We next compared the ability of emetine to sensitize AsPC-1 and BxPC-3 cells to TRAIL. ATP assays of relative cell viability and flow cytometric analysis showed that treat-

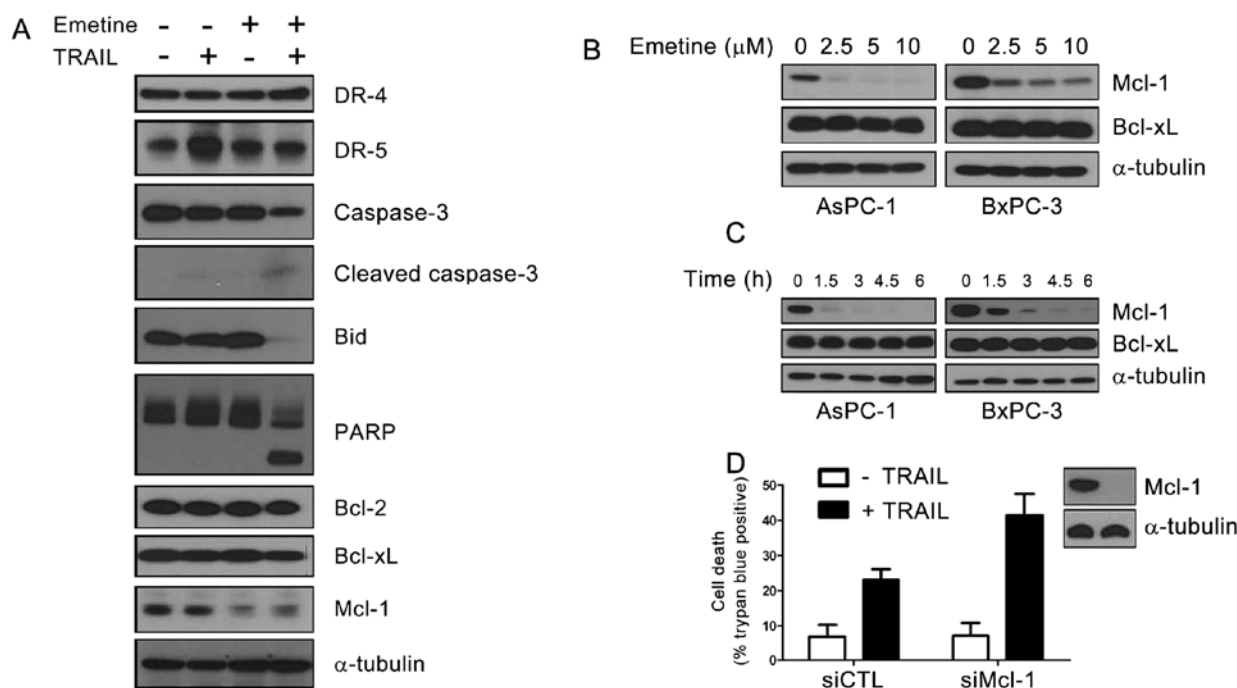


Figure 2. Downregulation of Mcl-1 by emetine sensitizes pancreatic cancer cells to TRAIL. (A) Western blot analysis of TRAIL-associated apoptotic proteins in AsPC-1 cells after treatment with TRAIL (50 ng/ml), emetine (2.5 μ M) or both. (B) Emetine downregulates Mcl-1 in a dose-dependent manner. Cells were incubated with the indicated concentrations of emetine for 6 h, and cell extracts were subjected to immunoblotting. (C) Emetine downregulates Mcl-1 in a time-dependent manner. Cells were incubated with 2.5 μ M emetine for the indicated times, and Mcl-1 expression levels were analyzed by western blotting. (D) Knockdown of Mcl-1 sensitizes AsPC-1 cells to TRAIL. Cells were transfected with siRNA of Mcl-1 and treated with TRAIL (50 ng/ml). After 24 h of incubation, the cell death rate was evaluated using trypan blue exclusion assay. Data represent means \pm SD (n=2).

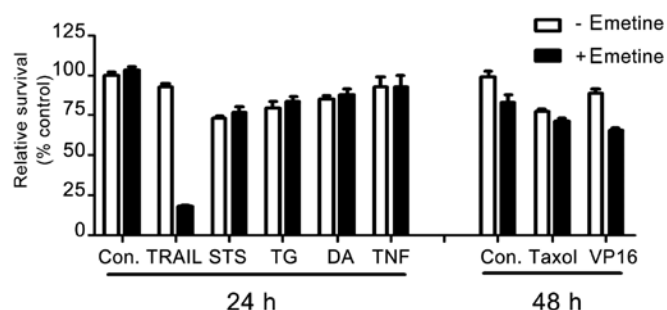


Figure 3. Emetine selectively sensitizes AsPC-1 cells to TRAIL-induced cell death. AsPC-1 cells were cultured overnight in 96-well plates, treated with emetine for 1 h, and treated with various cell death-inducing reagents, including 15 μ M thapsigargin (TG), 2.5 μ M staurosporine (STS), 5 μ M daunorubicin (DA), 30 ng/ml tumor necrosis factor α (TNF), 10 ng/ml paclitaxel (Taxol) and 50 μ M etoposide (VP16). Cellular ATP content was measured, with results normalized relative to cells treated with DMSO alone and presented as the percentage of the control (mean \pm SD; n=3).

ment of these cells with 2.5 μ M emetine hydrochloride and TRAIL (50 ng/ml) reduced the viability of both cell lines to <20% (Fig. 1C and D). By contrast, treatment with TRAIL or emetine alone showed minimal effects in both assays.

Emetine pretreatment downregulates Mcl-1. To characterize the mechanism by which emetine enhances TRAIL-induced cell death, we attempted to identify TRAIL-associated cell death proteins modulated by emetine in AsPC-1 cells. Western blot analysis showed that the levels of expression of DR4, Bcl-2 and Bcl-X_L were not altered by emetine or TRAIL (Fig. 2).

DR5 expression was upregulated by TRAIL, but this upregulation was abolished by emetine. However, the expression level of Mcl-1 protein was strongly reduced by emetine treatment, with or without TRAIL (Fig. 2A).

To determine whether Mcl-1 is downregulated in response to emetine in different pancreatic cancer cells, we examined the dose- and time-dependent effects of preincubation with emetine, followed by incubation with TRAIL, on Mcl-1 expression in AsPC-1 and BxPC-3 cells. We found that emetine downregulated Mcl-1 expression in both cancer cell lines in a dose- and time-dependent manner (Fig. 2B and C). In both cell lines, 2.5 μ M emetine reduced Mcl-1 expression to <50%. Moreover, after treatment with 2.5 μ M of emetine, 1.5 h was required for Mcl-1 expression in AsPC-1 and 3 h for BxPC-3 cells to reach a minimum level (Fig. 2C).

Sensitization effect of emetine is specific to TRAIL. We next assessed whether the ability of emetine to enhance cell death is specific to TRAIL signaling, or whether emetine can sensitize cells to a broad spectrum of cell death signaling, either intrinsic or extrinsic. Briefly, to test its involvement in intrinsic cell death signaling, AsPC-1 cells were pre-incubated in the presence or absence of 2.5 μ M emetine, followed by treatment with staurosporine (STS), a pan kinase inhibitor; thapsigargin (TG), a SERCA inhibitor that induces ER stress; daunorubicin (DA), a DNA damaging agent; taxol, a mitosis inhibitor; or VP16, a DNA damaging agent. To test its involvement in extrinsic cell death signaling, cells pre-incubated with emetine were incubated with tumor necrosis factor α (TNF- α) or with TRAIL as a control. We found that emetine sensitized AsPC-1

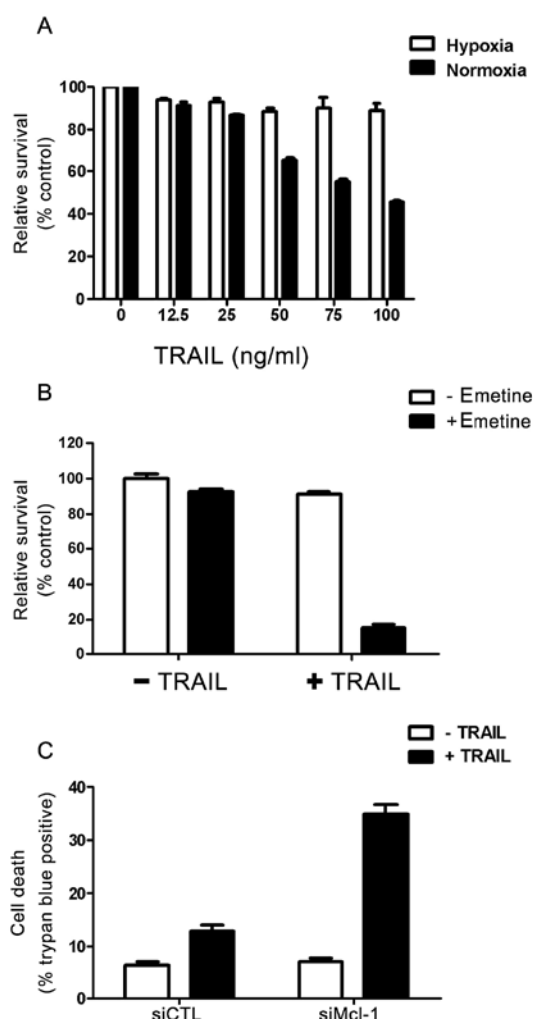


Figure 4. Emetine sensitizes PANC-1 cells to TRAIL under hypoxic conditions via Mcl-1 downregulation. (A) Viability of PANC-1 cells treated with TRAIL. Cells were seeded and incubated for 24 h under normoxic or hypoxic conditions and treated with the indicated concentrations of TRAIL for 24 h. Relative cell survival was evaluated using ATP content assays. Each value represents the mean \pm SD of triplicate samples. (B) Emetine sensitizes PANC-1 cells to TRAIL under hypoxic conditions. Cells were pretreated in the presence or absence of emetine for 1 h and then with TRAIL as in A. (C) PANC-1 cells transfected with scrambled (siCTL) or Mcl-1 (siMcl-1) siRNA were plated in 24-well plates and incubated in Xvivo culture chambers to induce hypoxia. Cells were treated with TRAIL for 24 h and cell death rates were evaluated by trypan blue exclusion. Each value represents the mean \pm SD of triplicate samples.

cells only to TRAIL, not to any of these other agents (Fig. 3) implying that the cell death sensitization effect of emetine is a specific event for TRAIL-induced apoptotic signaling.

Emetine-induced Mcl-1 downregulation sensitizes pancreatic cancer cells to TRAIL under hypoxic conditions. Tumor hypoxia is a situation in which primary tumor cells are deprived of their oxygen supply. Desmoplasia, a characteristic of pancreatic cancers, has been reported to reduce blood supply and induce hypoxia. In general, hypoxic tumor cells are resistant to radiotherapy and chemotherapy.

To assess the effect of a hypoxic environment on TRAIL-induced pancreatic cancer cell death, we tested whether hypoxia affects emetine-mediated sensitivity to TRAIL. We found that TRAIL induced PANC-1 cell death in a dose-

dependent manner, with 100 ng/ml of TRAIL reducing PANC-1 cell viability to 45%, as measured by the ATP content assay (Fig. 1A and 4A). Under hypoxic conditions, the same concentration of TRAIL had little effect on cell viability, suggesting that hypoxia is associated with the mechanism of pancreatic cancer cell resistance to TRAIL.

We subsequently assessed whether hypoxia-induced desensitization to TRAIL can be reversed by emetine. Importantly, pretreatment of emetine significantly increased the sensitivity of PANC-1 cells to TRAIL under a hypoxic condition (Fig. 4B). To determine whether this increased sensitivity was mediated by downregulation of Mcl-1, we knocked down Mcl-1 by treating PANC-1 cells with Mcl-1-specific siRNA and measured the cell viability following treatment with TRAIL (Fig. 4C). The cells showed marked sensitivity to TRAIL treatment, suggesting that Mcl-1 is a critical mediator of TRAIL-induced cell death in pancreatic cancer cells under hypoxic conditions.

Discussion

The ultimate goal of oncology research is the development of anticancer therapeutic regimes with little toxicity to normal cells. TRAIL has a potent therapeutic window as a tumor-specific agent (7,29,30), but many primary tumors have been found to be resistant to TRAIL (31,32). The development of TRAIL as a potent anticancer therapeutic agent requires understanding of the mechanism by which cells become resistant.

Inhibition of protein synthesis by cycloheximide and emetine has been found to effectively sensitize cancer cells to extrinsic cell death pathways. For example, TNF- α itself does not induce cell death in various cell lines, due to anti-apoptotic proteins that inhibit activation of the cell death pathway triggered by TNF- α . Chemical inhibition of the synthesis of these anti-apoptotic proteins has been found to sensitize these cells to extrinsic cell death inducers, including TNF- α Fas ligand and TRAIL. However, the main regulatory proteins targeted by cycloheximide and emetine in their inhibition of protein synthesis have not yet been clearly identified.

Emetine is a natural alkaloid derived from *Psychotria ipecacuanha* that strongly inhibits the synthesis of biomolecules. While cycloheximide is limited to *in vitro* research, emetine has been widely used as an anti-amoebiasis drug since the early 1900s (33). Although the severe side-effects of emetine led to its replacement by metronidazole (34), several studies suggest that emetine may have anticancer drug activity (35-38). Emetine was found to induce apoptosis in U937 lymphoma cells (36), A549-S lung cancer cells (37), Jurkat T cell leukemia cells (39), HL-60 promyelocytic leukemia cells (40) and rat hepatocytes (38). The cytotoxicity of emetine towards cancer cells is due to its ability to inhibit protein synthesis in eukaryotic cell ribosomes and to interact with DNA (35). In addition, recent reports have shown that emetine regulates the level of expression of apoptosis-associated genes.

Emetine has been found to control alternative splicing of Bcl-x in cancer cells (41), downregulating anti-apoptotic Bcl-X_L mRNA and upregulating pro-apoptotic Bcl-xS mRNA, and finally significantly reducing the Bcl-X_L/Bcl-xS ratio in MCF-7 breast cancer, PC-3 prostate cancer, C33A cervical

cancer and A549 lung cancer cell lines (41). Emetine has also been found to upregulate the pro-apoptotic genes caspase-9, death associated protein 6 (Daxx), granzyme B, caspase-8 and fas receptor (TNFRSF6) in leukemia cells and to down-regulate the anti-apoptotic genes Bcl-2 and EGFR in Jurkat cells (42,43).

Our findings indicate that emetine sensitizes pancreatic cancer cells to TRAIL-induced apoptosis by downregulating the expression of Mcl-1 protein (Figs. 1 and 2). The effect of emetine was highly specific to TRAIL since emetine did not have any effect on other cell death-inducing agents involved in intrinsic and extrinsic apoptosis signaling (Fig. 3). Our knockdown approaches involving the targeting of Mcl-1 also strongly support the specificity of emetine to TRAIL as Mcl-1 knockdown effectively sensitized AsPC-1 cells to TRAIL. These results also indicate that Mcl-1 is the main regulator of pancreatic cancer cell resistance to TRAIL-induced apoptosis.

Significantly, only Mcl-1, but not other apoptosis-related proteins, was downregulated in pancreatic cancer cells by short-term exposure to emetine (Fig. 2). This result can be explained by the fact that Mcl-1 is a fragile protein which is readily degraded by the ubiquitin proteasome system with a very short half life (44). Additionally, we tested whether emetine can enhance ubiquitination of Mcl-1. However, we found that emetine did not specifically increase the ubiquitination of Mcl-1 (data not shown).

One characteristic of pancreatic cancer is the desmoplastic reaction with strong hypoxia which limits cancer drug delivery (45). Therefore, we also assessed whether emetine-induced Mcl-1 downregulation effectively sensitizes pancreatic cancer cells to TRAIL under hypoxic conditions (Fig. 4). Although PANC-1 cells are normally TRAIL-sensitive, they became resistant to TRAIL-induced apoptosis under hypoxic conditions (Fig. 4A). We found, however, that emetine re-sensitized these PANC-1 cells to TRAIL under hypoxic conditions in an Mcl-1-dependent manner (Fig. 4B and C). These findings, therefore, suggest that: i) Mcl-1 is a critical regulator of TRAIL resistance in pancreatic cancer cells, under both normoxic and hypoxic conditions; and that ii) treatment with emetine is a promising method of downregulating Mcl-1 under hypoxic conditions and sensitizing cells to TRAIL. Emetine sensitization of cancer cells to TRAIL via Mcl-1 downregulation may be limited to pancreatic cancer cells, a possibility that requires further investigation.

In summary, we found that emetine sensitizes pancreatic cancer cells, which were initially resistant to TRAIL, to TRAIL-induced apoptosis. Although emetine was previously shown to sensitize cells by inhibiting protein synthesis, we demonstrated in the present study, for the first time, that the downregulation of the expression of Mcl-1 protein by emetine is a key factor in sensitizing pancreatic cancer cells to TRAIL. Our findings also indicate that Mcl-1 downregulation may be a promising target for sensitizing pancreatic cancers to TRAIL.

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