Anticancer effects of phenoxazine derivatives combined with tumor necrosis factor-related apoptosis-inducing ligand on pancreatic cancer cell lines, KLM-1 and MIA-PaCa-2

SEIKO KATO[1], KEN SHIRATO[2], KAZUHIKO IMAIZUMI[2], HIROKO TOYOTA[4], JUNICHIRO MIZUGUCHI[4], MASATO ODAWARA[1], XIAO-FANG CHE[3], SHINICHI AKIYAMA[3], AKIHISA ABE[5] and AKIO TOMODA[5]

[1]Third Department of Internal Medicine, Tokyo Medical University, Tokyo 160-0023; [2]Laboratory of Physiological Sciences, Graduate School of Human Sciences, Waseda University, Tokorozawa 359-1192; [3]Department of Oncology, Kagoshima University, Graduate School of Medicine, Kagoshima 890-8544; Departments of [4]Immunology and [5]Biochemistry and Research Institute of Immunological Treatment, Tokyo Medical University, Tokyo 160-8404, Japan

Received October 18, 2005; Accepted December 22, 2005

Abstract. The aim of this study was to investigate the anticancer effects of the phenoxazine derivatives, 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx-1), 3-amino-1,4α-dihydro-4α,8-dimethyl-2H-phenoxazine-2-one (Phx-2), and 2-aminophenoxazine-3-one (Phx-3) on human pancreatic cancer cell lines, KLM-1 and MIA-PaCa-2, in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor superfamily of cytokines. Of these three phenoxazines, Phx-1 and Phx-3 inhibited proliferation of KLM-1 dose-dependently, but Phx-2 did not. Phx-3 caused both apoptosis and necrosis in KLM-1 cells, as evidenced by the phosphatidylserine externalization and propidium iodide permeable cells detected by a flow cytometric method using annexin-V and propidium iodide. Down-regulation of Bcl-2 expression appeared to be involved in the Phx-3-induced cell death. TRAIL did not affect proliferation of KLM-1, and the inhibitory effects of Phx-1 and Phx-3 on the KLM-1 cell line were not augmented by the combination with TRAIL. On the other hand, proliferation of the MIA-PaCa-2 cell line was not affected by Phx-1, Phx-2 and Phx-3, although it was significantly inhibited by TRAIL in a dose-dependent manner. Inhibitory effects of TRAIL on MIA-PaCa-2 were synergistically augmented by the addition of Phx-1 and Phx-3, but not by Phx-2. These results suggest that both Phx-1 and Phx-3 exert anticancer effects against human pancreatic cancer cells, KLM-1 and MIA-PaCa-2, through distinct action modes. Phx-1 and Phx-3 may be effective for the treatment of pancreatic cancer.

Correspondence to: Dr Akio Tomoda, Department of Biochemistry and Research Institute of Immunological Treatment, Tokyo Medical University, 6-1-1 Shinjuku, Tokyo 160-8404, Japan
E-mail: tomoda@tokyo-med.ac.jp

Key words: phenoxazine, pancreatic cancer cells, proliferation inhibition, apoptosis, tumor necrosis factor-related apoptosis-inducing ligand

Introduction

Pancreatic cancer is resistant to chemotherapy, therefore making it a highly lethal malignancy that often occurs in developed countries (1,2). Gemcitabine (2',2'-difluorodeoxy-cytidine) is the only active chemotherapeutic agent and has an objective response rate of <20% (3). Thus, the development of drugs with anticancer effects on pancreatic cancer is urgently required.

On the other hand, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor superfamily of cytokines, has been demonstrated to induce apoptosis in a wide range of tumor cells, with little effect on normal tissues, both *in vitro* and *in vivo* (4-7). TRAIL-induced apoptosis has been shown to be enhanced in some cell types through the mitochondrial amplification pathway (8,9), which involves Bcl-2 family members. Bcl-2 family members are composed of pro-(Bax, Bak) and anti-apoptotic (Bcl-2, Bcl-xL and Mcl-1) members (10). Matsuzaki *et al* demonstrated that Aspc-1, a pancreatic cell line, became sensitive to TRAIL in combination with DNA-damaging agent actinomycin D, which induces mitochondrial depolarization (11). Since phenoxazine is a component of actinomycin D (12), and Hara *et al* found that the phenoxazine derivative, Phx-1, shows synergistic anticancer effects on Jurkat cells with TRAIL (13), it will be valuable to examine whether or not the phenoxazine derivatives Phx-1, Phx-2 and Phx-3, which were produced by the reaction of o-aminophenol and its derivatives with bovine hemoglobin (14,15), may exert anticancer effects on pancreatic cancer cell lines in combination with TRAIL.

In this study, we investigated whether the combination of TRAIL and Phx-1, Phx-2 or Phx-3 can suppress the viability of TRAIL-sensitive and -resistant human pancreatic cancer cells such as MIA-PaCa-2, often used for *in vitro* research on pancreatic cancer (11,16), and KLM-1, a highly metastatic

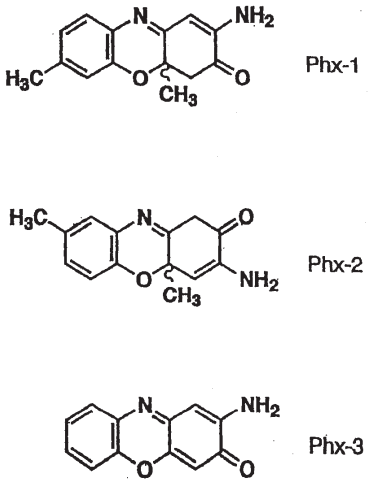

Figure 1. Chemical structure of phenoxazine derivatives, 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx-1), 3-amino-1,4α-dihydro-4α,8-dimethyl-2H-phenoxazine-2-one (Phx-2), and 2-aminophenoxazine-3-one (Phx-3).

variant (17), in addition to whether apoptosis and/or necrosis may be induced in KLM-1 cells by the phenoxazine.

Materials and methods

2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx-1), 3-amino-1,4α-dihydro-4α,8-dimethyl-2H-phenoxazine-2-one (Phx-2), and 2-amnophenoxazine-3-one (Phx-3). Phx-1, Phx-2 and Phx-3 were prepared according to the methods described by Tomoda *et al* (14) and Shimizu *et al* (15), respectively. The chemical structure of these compounds is illustrated in Fig. 1. Phx-1, Phx-2 and Phx-3 were dissolved in ethanol and dimethylsulfoxide (DMSO) (1:3) solution to make 20 mM.

Cell lines and cell culture. KLM-1 cells were cultured in RPMI-1640 medium (Daigo) (Wako Pure Chemicals, Tokyo) with kanamycin sulfate (70 mg/l) and 10% heat inactivated fetal bovine serum. MIA-PaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) with 70 mg/l kanamycin sulfate and 10% heat-inactivated fetal bovine serum.

Cell viability. The viability of cells was measured using a Cell Counting Kit-8 (Wako). Briefly, two types of tumor cells (KLM-1, $8x10^4$ cells/well; Mia-PaCa-2, $6x10^4$ cells/well) were precultured in a 24-well flat-bottomed plate for 24 h at 37˚C in a 5% CO_2 humidified chamber. The various concentrations of Phx-1, Phx-2 and Phx-3 and TRAIL (human recombinant TRAIL; Sigma) were then added and incubated for 24-72 h. After incubation, culture medium was discarded and 1 ml of fresh medium, including 40 μl of the Cell Counting Kit-8, was added to each well and incubation was continued for 1.5 h. After incubation, 40 μl of 0.2 N HCl was added to each well. The medium of each well was then analyzed using a multi-detection microplate reader (Powerscan HT; Dainippon Pharm. Co. Ltd., Osaka, Japan) at a wavelength of 450 nm. Cell viability was determined by referring to the absorbance of non-treated cells.

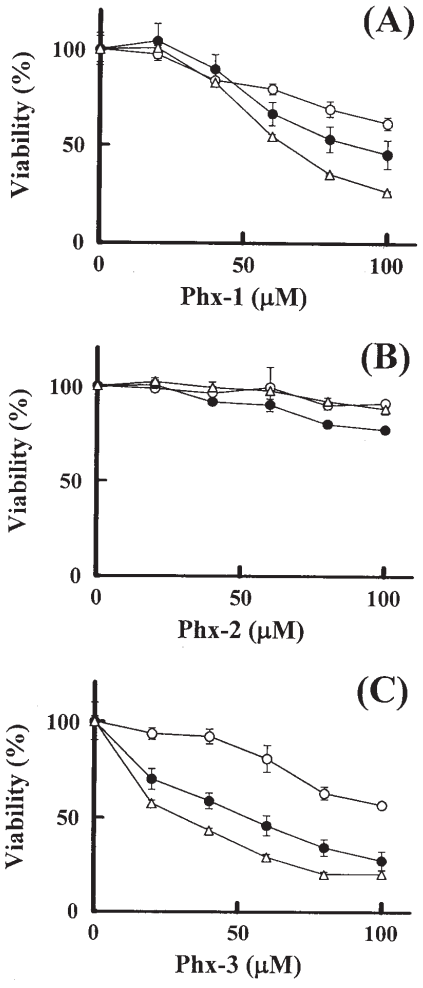

Figure 2. Viability of KLM-1 cells in the presence of different concentrations of Phx-1, Phx-2 or Phx-3. (A) Effects of Phx-1, (B) Phx-2; and (C) Phx-3 with 24 h (○), 48 h (●) and 72 h (Δ) treatment. n=4.

Analysis of apoptosis and necrosis by annexin V and propidium iodide staining. Apoptosis was determined using an Apoptosis Detection kit (R&D Systems). Briefly, after KLM-1 cells were incubated with or without 50 μM Phx-3 for 48 h, cells were collected, washed twice in cold PBS, then resuspended in binding buffer (HEPES-buffered saline solution containing 2.5 mM calcium chloride) at a density of $1x10^5$ cells/ml. Fluorescence-labeled annexin-V and propidium iodide (PI) were added to cells, and the samples were incubated for 15 min before analysis with a FACScan (Becton-Dickinson). Annexin-V-FITC-generated signals and PI signals were monitored using a FITC signal detector (FL1, 525 nm) and a detector reserved for phycoerythrin emission (FL2, 575 nm), respectively.

Western blotting. Western blotting was carried out as described previously (18). Briefly, the cells pretreated with 100 μM Phx-3 for 48 h were lysed with Triton-X 100 lysis buffer, and lysates were resolved on a 15% SDS-PAGE, followed by transfer to a PVDF membrane. The blots were blocked with 5% BSA, and incubated with the primary antibodies anti-Bcl-2, anti-Bax (MBL, Nagoya, Japan), and anti-Bcl-xL (BD Transduction Laboratories, San Jose, CA, USA), and control anti-actin antibodies (Sigma). After several washes, the blots were developed with horseradish peroxidase (HRP)-labeled

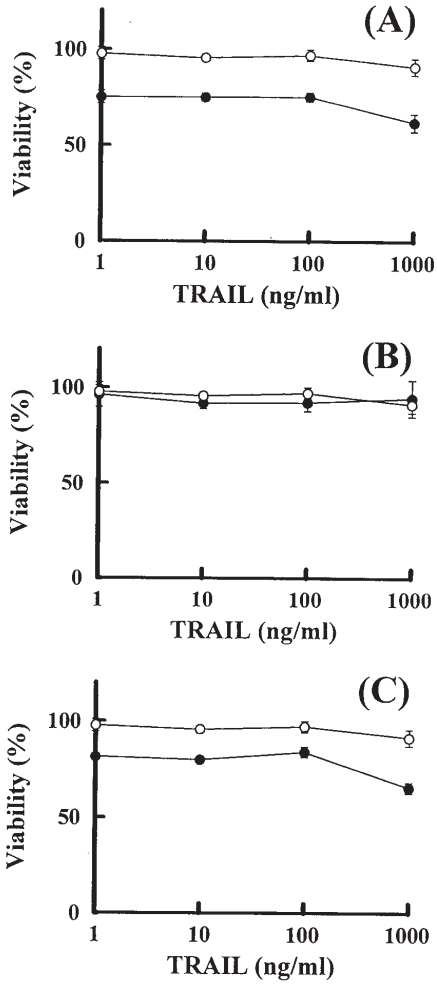

Figure 3. Effect of the combination of TRAIL with Phx-1, Phx-2 or Phx-3 on the viability of KLM-1 cells. KLM-1 cells were treated with different concentrations (1, 10, 100 and 1,000 ng/ml) of TRAIL with or without 50 μM Phx-1, Phx-2 or Phx-3 for 24 h. (A) Effect of the combination of TRAIL with Phx-1, (B) TRAIL with Phx-2, and (C) TRAIL with Phx-3. TRAIL without phenoxazines (○); TRAIL with 50 μM phenoxazines (●); n=4.

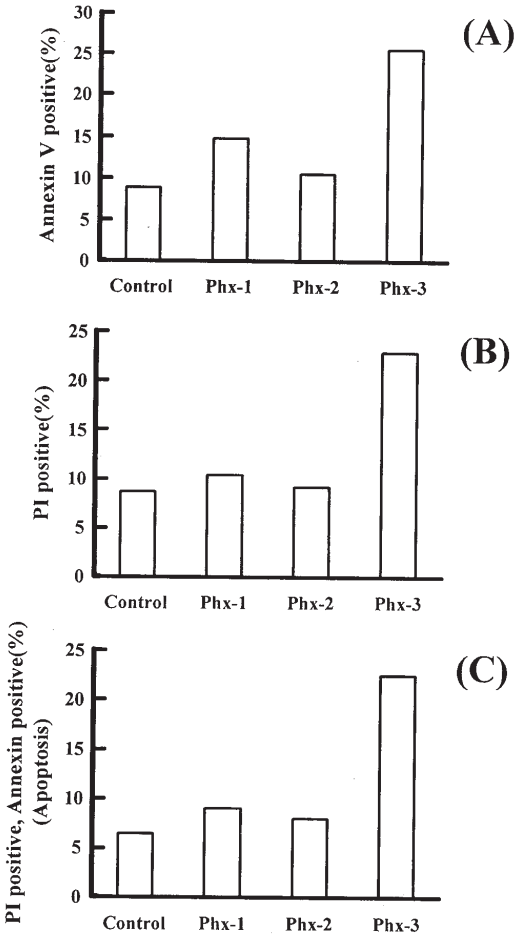

Figure 4. Analysis of the population of cells with early stage apoptosis and late stage apoptosis/necrosis detected by annexin-V and PI staining. KLM-1 cells were grown in the presence or absence of 50 μM Phx-3 for 48 h. The Phx-3-treated cells were stained with FITC-annexin-V and propidium iodide, then examined by flow cytometry. (A-C) The percentage of annexin-V+PI$^-$ in cells in early stage apoptosis (A), annexin-V-PI$^+$ in cells in necrosis (B), and annexin-V+PI$^+$ in cells in late stage apoptosis/necrosis (C) are shown.

goat anti-mouse immunoglobulin (IgG)(Fc) or HRP-labeled goat anti-rabbit IgG (Fc) antibodies, and an ECL detection kit (Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's recommendations.

Results

We studied the effects of different concentrations of Phx-1, Phx-2 and Phx-3 on the viability of pancreatic cell line, KLM-1 (Fig. 2A-C). Fig. 2A shows the effects of different concentrations of Phx-1 on the viability of KLM-1 cells at 24, 48 and 72 h. Phx-1 inhibited the proliferation of cells in a dose- and time-dependent manner. However, as shown in Fig. 2B, Phx-2 showed little inhibitory effect on proliferation at different concentrations and incubation times. Of the 3 phenoxazines, the inhibitory effects of Phx-3 was strongest at each concentration (Fig. 2C). The proliferation of KLM-1 was inhibited by 70% with 60 μM Phx-3 for 72 h.

The inhibitory effect of different concentrations of TRAIL on KLM-1 cells was studied in the presence or absence of Phx-1, Phx-2 or Phx-3 as shown in Fig. 3A-C. The proliferation of KLM-1 cells was not affected by TRAIL alone at different concentrations for 24 h (open circle in the figures). Furthermore, the inhibitory effects of 50 μM Phx-1 and Phx-3 on the KLM-1 cell line were indicated, but not augmented in the presence of different concentrations of TRAIL (closed circle in the figures), suggesting that no synergism between the phenoxazines and TRAIL against KLM-1 cells was present.

Since the viability of KLM-1 cells was greatly reduced by Phx-3 (Fig. 2C), the presence of apoptotic cells and necrotic cells was examined in KLM-1 treated with 50 μM Phx-3 for 48 h, using flow cytometry (Fig. 4A-C). In this case, the staining of cells with both PI and FITC-labeled annexin-V was examined; FITC-labeled annexin-V binds specifically to the phosphatidylserine exposed to the outer membrane in apoptotic cells (19), and PI can penetrate necrotic cells, but not viable or early apoptotic cells. As shown in Fig. 4A-C, annexin-V-positive cells, PI-positive cells, and PI- and annexin-V-positive cells increased to 25.0%, 22.5%, and 22.5%, respectively, in KLM-1 cells treated with 50 μM Phx-3 for 48 h compared with the control cells without Phx-3 (9%, 8% and 6%, respectively). The effects of Phx-1 on the

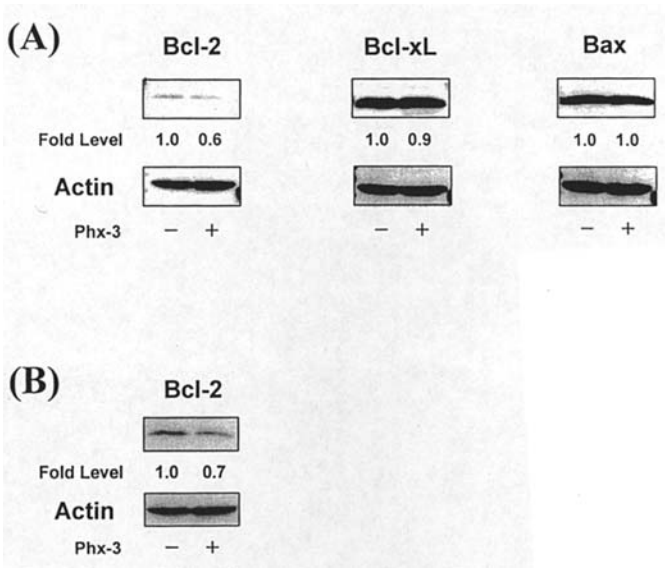

Figure 5. Down-regulation of Bcl-2 expression in KLM-1 cells by Phx-3. KLM-1 cells were cultured with 100 μM Phx-3 for 48 h, and cell lysates of 30 μg (A) or 100 μg (B) were used for Western blotting to assess the expression of Bcl-2 family members, Bcl-2, Bcl-xL and Bax. Expression of control actin was also determined. Levels of the Bcl-2 family members (Bcl-2, Bcl-xL, and Bax) were normalized to actin levels and expressed as a fold of their levels from unstimulated cells. Experiments were done twice with similar results.

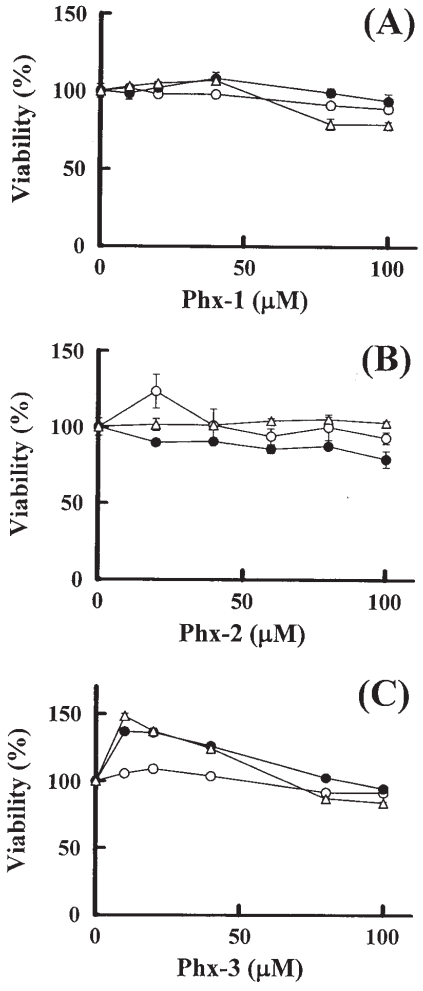

Figure 6. Viability of MIA-PaCa-2 cells in the presence of different concentrations of Phx-1, Phx-2 or Phx-3. (A) Effects of Phx-1, (B) Phx-2 and (C) Phx-3 with 24 h (○), 48 h (●) and 72 h (Δ) treatment. n=4.

induction of apoptosis and necrosis in KLM-1 cells appeared weaker than those of Phx-3, and there was little effect by Phx-2, which was consistent with results shown in Fig. 2A-C.

To assess whether Bcl-2 family proteins are involved in Phx-3-induced apoptotic cell death in KLM-1 cells, the cells cultured with 100 μM Phx-3 for 48 h were assayed for Western blotting (Fig. 5). The levels of Bcl-2 proteins decreased in response to Phx-3, while those of both Bcl-xL and Bax were unchanged. Control actin levels were unchanged. These results suggest that Phx-3 down-regulates expression of Bcl-2, an anti-apoptotic member, with unaltered expression of Bcl-xL, an anti-apoptotic member and Bax, a pro-apoptotic member, resulting in apoptosis.

We then studied the effect of different concentrations of Phx-1, Phx-2 and Phx-3 on the viability of pancreatic cell line, MIA-PaCa-2, for 24, 48, and 72 h (Fig. 6 A-C). Although approximately 20% inhibition of the proliferation of MIA-PaCa-2 cells was observed only at 80 and 100 μM Phx-1 at 72 h, it appears that Phx-1 has little antiproliferative effect on these cells at different concentrations for 24 and 48 h (Fig. 6A). There was no inhibition of proliferation of MIA-PaCa-2 cells in the presence of Phx-2 and Phx-3 at different concentrations for 72 h (Fig. 6B and C). Of note, cell proliferation was accelerated in the presence of lower concentrations of Phx-3 (10 and 20 μM) (Fig. 6C).

Fig. 7 shows the viability of MIA-PaCa-2 cells treated with TRAIL with or without 50 μM Phx-1, Phx-2 or Phx-3 for 24 h. MIA-PaCa-2 cells were sensitive to TRAIL alone at different concentrations (1, 10, 100 and 1,000 ng/ml) in a dose-dependent manner. When MIA-PaCa-2 cells were treated with both TRAIL and 50 μM Phx-1, Phx-2 or Phx-3, the viability of cells was synergistically inhibited, suggesting that the addition of Phx-1, Phx-2 or Phx-3 sensitized this cell line to TRAIL.

Discussion

In this study, we demonstrated that Phx-1 and Phx-3 suppress the viability of KLM-1 cells, a highly metastatic pancreatic variant reported by Kimura *et al* (17), in a dose-and time-dependent manner, but Phx-2 did not (Fig. 2 A-C). In particular, Phx-3 had stronger cytotoxic effects on KLM-1 cells than Phx-1 (Fig. 2A and C). The present results are consistent with a study by Shimizu *et al* who reported that the proliferation of human malignant melanoma, which is resistant to chemotherapeutic agent, was exclusively suppressed by Phx-3 (20).

As a newly discovered anticancer cytokine, TRAIL has been extensively investigated for its ability to induce apoptosis against a wide range of cancer cells (4-7). Matsuzaki *et al* reported that TRAIL induces apoptosis in several human pancreatic cancer cell lines, and the combination of TRAIL and actinomycin D induces apoptosis even in TRAIL-resistant human pancreatic cancer cells (11). Jazirehi *et al* also indicated

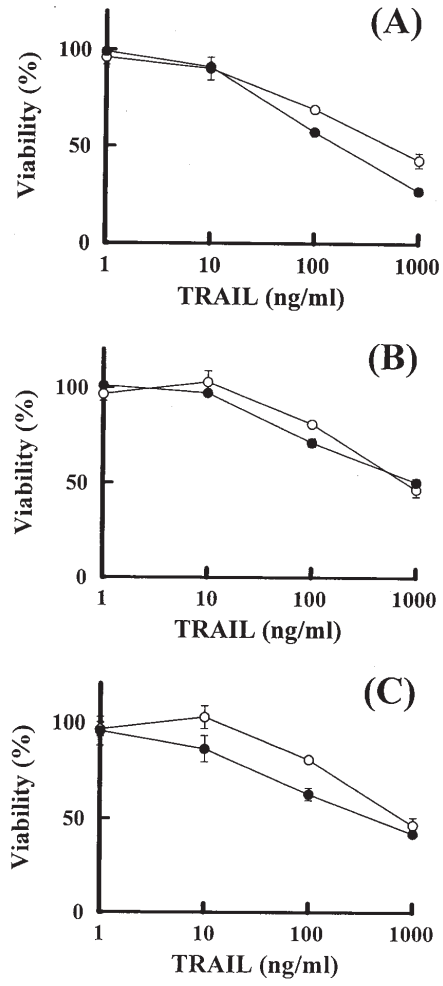

Figure 7. Effect of a combination of TRAIL with Phx-1, Phx-2 or Phx-3 on MIA-PaCA-2 cells. MIA-PaCa-2 cells were treated with different concentrations (1, 10, 100 and 1,000 ng/ml) of TRAIL with or without 50 μM Phx-1, Phx-2 or Phx-3 for 24 h. (A) Effect of the combination of TRAIL with Phx-1; (B) effect of the combination of TRAIL with Phx-2; (C) effect of the combination of TRAIL with Phx-3. TRAIL without phenoxazines (○); TRAIL with 50 μM phenoxazines (●); n=4.

that adriamycin sensitizes the adriamycin-resistant human multiple myeloma cells to TRAIL-mediated apoptosis (21). The human hepatocellular carcinoma cell lines, HepG2, Hep3B and SK-Hep1 cells, were resistant to TRAIL, but sensitized by actinomycin D (22). In the light of these reports, we studied the effects of TRAIL on human pancreatic cancer cell lines such as KLM-1 and MIA-PaCa-2 cells in the presence or absence of Phx-3. We first studied the effect of a single treatment of TRAIL on the viability of KLM-1 cells. As shown in Fig. 3A-C, TRAIL did not affect the viability of KLM-1 cells, showing their resistance to TRAIL-mediated cytotoxicity. Second, we studied the combined effect of TRAIL with Phx-1, Phx-2 or Phx-3 on the viability of KLM-1 cells. Though Phx-1 and Phx-3 suppressed the viability of cells in the presence of different concentrations of TRAIL, no synergistic effects with TRAIL were observed (Fig. 3A and C). Therefore, it is likely that Phx-1 and Phx-3 exert anticancer effects on KLM-1 cells without synergism with TRAIL.

Since apoptotic cells are engulfed by phagocytes whereas the membrane of necrotic cells is disrupted, and inflammation and release of toxic substances occur, the induction of apoptosis in cancer cells would reduce adverse effects (23,24). The population of annexin-V+PI$^-$ cells (cells in early stage apoptosis), annexin-V-PI$^+$ cells (cells in necrosis) and annexin-V+PI$^+$ cells (cells in late stage apoptosis/necrosis) increased significantly in KLM-1 cells treated with Phx-3 (Fig. 4), suggesting that Phx-3 induces a mix of apoptosis and necrosis in KLM-1 cells. As a mechanism of cellular apoptosis, the activation of caspases regulated by many intracellular factors has been indicated (25). Although we did not study the effects of Phx-3 on caspases in KLM-1 cells in the present study, the involvement of caspases in the apoptotic mechanism of these cells may be conceivable, as Abe *et al* showed that the apoptosis of human lung carcinoma cells by Phx-1 was reversed in part by the inhibition of caspase (26).

Since Bcl-2 family proteins have been shown to be involved in the regulation of caspase-9-related cellular apoptosis, and the down-regulation of Bcl-2 leads to the activation of caspase-9 and finally cellular apoptosis (26,27), we studied the levels of Bcl-2, Bcl-xL and Bax in KLM-1 cells with or without Phx-3 (Fig. 5). It was found that the levels of Bcl-2 decreased significantly in response to Phx-3, suggesting that caspase-9 activation due to the down-regulation of Bcl-2 might operate in KLM-1 cells treated with Phx-3, causing the apoptosis of cells.

The behavior of MIA-PaCa-2, another human pancreatic cancer cell line, was different from that of KLM-1. There were no inhibitory effects by Phx-1, Phx-2 and Phx-3 on MIA-PaCa-2 at different concentrations for 24, 48 and 72 h (Fig. 6A-C). When MIA-PaCa-2 was treated with TRAIL alone at various concentrations for 24 h, cell proliferation was suppressed in a concentration-dependent manner (Fig. 7A-C). The inhibition rate was 25% and 55% at 100 and 1,000 ng/ml of TRAIL, respectively. In addition, Phx-1 and Phx-3 sensitized MIA-PaCa-2 cells to TRAIL-mediated cytotoxicity, i.e. the viability of this cell line was extensively suppressed when TRAIL and Phx-1 or Phx-3 were treated in combination (Fig. 7A and C). The mechanism for such synergistic effects of TRAIL and phenoxazine is currently unclear, but the present results are consistent with those reported by Hara *et al* in which Phx-1 shows synergistic anticancer effects on Jurkat cells with TRAIL (13). Such synergism between TRAIL and chemotherapeutic agents has been reported in various cancer cell lines (4-7).

Although phenoxazine is an essential component of actinomycin D, having strong anticancer effects against Wilms's tumor (12), the anticancer effects of phenoxazines were considered negligible (28). Phx-1, Phx-2 and Phx-3 can be produced by the reactions of human or bovine hemoglobin with o-aminophenol and its derivatives (14,15), and has been demonstrated to exert various biological activities such as anticancer effects *in vivo* and *in vitro* (29-31), and antiviral (32), antimicrobial (33), and immunosuppressive effects (34). The present results demonstrated that the action mode of Phx-1 and Phx-3 against two human pancreatic cancer cell lines, KLM-1 and MIA-PaCa-2, was quite different in terms of combined effects with TRAIL. Since pancreatic cancer is extremely resistant to chemotherapeutic agents except gemcitabine with its objective response rate of less than 20% (3), Phx-1 and Phx-3 may be used in the treatment of pancreatic cancer in the future. Further investigation is needed to clarify the action mechanism of these compounds.

Acknowledgements

We would like to thank Professor J. Patrick Barron of the International Information Center, Tokyo Medical University, for reviewing the manuscript. Research was supported by funds from the High-Tech Research Project for Private Universities, and a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology, Japan (2003-2007) and the Ito Foundation.

References

1. Bardeesy N and Depinho RA: Pancreatic cancer biology and genetics. Nat Rev 2: 897-909, 2002.
2. Gansauge S, Gansage F and Berger HG: Molecular oncology in pancreatic cancer. J Mol Med 74: 313-320, 1996.
3. Burris HA III, Moore MJ, Andersen J, Breen MR, Modiano MR, Rothenberg ML, Cripps MC, Portenoy RK, Storniolo AM, Tarassofff P, Nelson R, Dorr FA, Stephens CD and Von Hoff DD: Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 15: 2403-2413, 1997.
4. Hernandwez A, Wang QD, Schwartz SA and Evers BM: Sensitization of human colon cancer cells to TRAIL-mediated apoptosis. Gastrointest Surg 5: 56-65, 2001.
5. Olsson A, Diaz T, Aquilar-Santelises M, Osterborg A, Celsing F, Jondal M and Osorio LM: Sensitization to TRAIL-induced apoptosis and modulation of FLICE-inhibitory protein in B chronic lymphocytic leukemia by actinomycin D. Leukemia 15: 1868-1877, 2001.
6. Yagita H, Takeda K, Hayakawa Y, Smyth MJ and Okumura K: TRAIL and its receptors as targets for cancer therapy. Cancer Sci 95: 777-783, 2004.
7. Mizutani Y, Nakao M, Ogawa O, Yoshida O, Bonavida B and Miki T: Enhanced sensitivity of bladder cancer cells to tumor necrosis factor released apoptosis inducing ligand mediated apoptosis by cisplatin and carboplatin. J Urol 165: 263-270, 2001.
8. Hotta T, Suzuki H, Nagai S, Yamamoto K, Imakiire A, Takada E, Itoh M and Mizuguchi J: Chemotherapeutic agents sensitize sarcoma cell lines to tumor necrosis factor-related apoptosis-inducing ligand-induced caspase-8 activation, apoptosis and loss of mitochondrial membrane potential. J Orthopaed Res 21: 949-957, 2003.
9. Walczak H, Bouchon A, Stahl H and Krammer PH: Tumor necrosis factor-related apoptosis-inducing ligand retains its apoptosis-inducing capacity on Bcl-2 or Bcl-xL-overexpressing chemotherapy-resistant tumor cells. Cancer Res 60: 3051-3057, 2000.
10. Reed JC: Bcl-2 family proteins. Oncogene 17: 3225-3236, 1998.
11. Matsuzaki H, Schmied BM, Ulrich A, Standop J, Schneider MB, Batra SK, Picha KS and Pour PM: Combination of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and actinomycin D induces apoptosis even in TRAIL-resistant human pancreatic cancer cells. Clin Cancer Res 7: 407-414, 2001.
12. Hollstein U: Actinomycin. Chemistry and mechanism of action. Chem Rev 74: 625-652, 1974.
13. Hara K, Okamoto M, Aki T, Yagita H, Tanaka H, Mizukami Y, Nakamura H, Tomoda A, Hamasaki N and Kang D: Synergistic enhancement of TRAIL- and tumor necrosis factor α-induced cell death by a phenoxazine derivative. Mol Cancer Ther 4: 1121-1127, 2005.
14. Tomoda A: An improved method from the rapid preparation of a novel antitumor agent. Biorg Med Chem Lett 11: 1057-1058, 2002.
15. Shimizu S, Suzuki M, Tomoda A, Arai S, Taguchi H, Hanawa T and Kamiya S: Phenoxazine compounds produced by the reactions with bovine hemoglobin show antimicrobial activity against non-tuberculosis mycobacteria. Tohoku J Exp Med 203: 47-52, 2004.
16. Schlieman MG, Fahy BN, Ramsamooj R, Beckett L and Bold RJ: Incidence, mechanism and prognostic value of activated AKT in pancreas cancer. Br J Cancer 89: 2110-2115, 2003.
17. Kimura Y, Kobari M, Yusa T, Sunamura M, Kimura M, Shimamura H and Matsuno S: Establishment of an experimental liver metastasis model by intraportal injection of a newly derived human pancreatic cancer cell line (KLM-1). Int J Pancreatol 20: 43-50, 1996.
18. Toyota H, Yanase N, Yoshimoto T, Moriyama M, Sudo T and Mizuguchi J: Calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax. Cancer Lett 189: 221-230, 2003.
19. Andree HAM, Reutelingsperger CPM, Hauptomann R, Hecker HC, Hermens WT and Willems GM: Binding of vascular anticoagulant a (VACa) to planar phospholipid bilayers. J Biol Chem 265: 4925-4928, 1990.
20. Shimizu S, Suzuki M, Miyazawa K, Yokoyama T, Ohyashiki K, Miyazaki K, Abe A and Tomoda: Differentiation and apoptosis in human malignant melanoma G-361 cells induced by 2-aminophenoxazine-3-one. Oncol Rep 14: 41-46, 2005.
21. Jazirehi AR, Ng C-P, Gan X-H, Schiller G and Bonavida B: Adriamycin sensitizes the adriamycin-resistant 8226/Dox-40 human multiple myeloma cells to Apo2L/tumor necrosis factor-related apoptosis-inducing ligand-mediated (TRAIL) apoptosis. Clin Cancer Res 7: 3874-3883, 2001.
22. Yamanaka T, Shiraki K, Sugimoto K, Ito T, Fujikawa K, Ito M, Takase K, Moriyama M, Nakano T and Suzuki A: Chemotherapeutic agents augment TRAIL-induced apoptosis in human hepatocellular carcinoma cell lines. Hepatology 32: 483-490, 2000.
23. Hawkins HK, Ericsson JL, Biberfield P and Trum BF: Lysosome and phagozome stability in lethal cell injury. Morphologic tracer studies in cell injury due to inhibition of energy metabolism, immune cytolysis and photosensitization. Am J Pathol 68: 255-258, 1972.
24. Mignotte B and Vayssiere JL: Mitochondria and apoptosis. Eur J Biochem 252: 1-15, 1998.
25. Kandel ES and Hay N: The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp Cell Res 253: 210-229, 1999.
26. Huang Z: Bcl-2 family proteins as targets for anticancer drug design. Oncogene 19: 6627-6631.
27. Manion MK and Hockenbery DM: Targeting BCL-2-related proteins in cancer therapy. Cancer Biol Ther 2: S105-S114, 2003.
28. Motohashi N, Mitscher LA and Myer R: Potential antitumor phenoxazines. Med Res Rev 11: 239-294, 1991.
29. Mori H, Honda K, Ishida R, Nohira T and Tomoda A: Antitumor activity of 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one against Meth A tumor transplanted into BALB/c mice. Anticancer Drugs 11: 653-657, 2000.
30. Shimamoto T, Tomoda A, Ishida R and Ohyashiki K: Antitumor effects of a novel phenoxazine derivative on human leukemia cell lines *in vitro* and *in vivo*. Clin Cancer Res 704: 704-708, 2001.
31. Nakada T, Isaka K, Nishi H, Osakabe Y, Shimamoto T, Ohyashiki K, Tomoda A and Takayama M: A novel phenoxazine derivative suppresses proliferation of human endometrial adenocarcinoma cell lines, inducing G2/M accumulation and apoptosis. Oncol Rep 10: 1171-1176, 2003.
32. Iwata A, Yamaguchi T, Sato K, Yoshitake N and Tomoda A: Suppression of proliferation of poliovirus and porcine parvovirus by novel phenoxazines, 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one and 3-amino-1,4α-dihydro-4α,8-dimethyl-2H-phenoxazine-2-one. Biol Pharm Bull 28: 905-907, 2005.
33. Uruma T, Yamaguchi H, Fukuda M, Kawakami H, Goto H, Kishimoto T, Yamamoto Y, Tomoda A and Kamiya S: Chlamydia pneumoniae growth inhibition in human monocyte cell line THP-1 cells and human epithelial HEp-2 cells by a novel phenoxazine derivative. J Med Microbiol (In press).
34. Gao S, Takano T, Sada K, He J, Noda C, Hori-Tamura N, Tomoda A and Yamamura H: A novel phenoxazine derivative suppresses surface IgM expression in DT40 B cell line. Br J Pharmacol 137: 749-755, 2002.