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

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Abstract. The aim of this study was to investigate the anticancer effects of the phenoxazine derivatives, 2-amino-4,4··,7-dimethyl-3H-phenoxazine-3-one (Phx-1), 3-amino-1,4··,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 MIA-PaCa-2, in combination with tumor necrosis factor superfamily of cytokines. Of these three phenoxazines, Phx-1 and Phx-3 inhibited proliferation of KLM-1 cell line, 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.

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'-difluorodeoxycytidine) 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...
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-4,4·-dihydro-4·,7-dimethyl-3H-phenoxazine-3-one (Phx-1), 3-amino-1,4·-dihydro-4·,8-dimethyl-2H-phenoxazine-2-one (Phx-2), and 2-aminophenoxazine-3-one (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, 8×10^4 cells/well; MIA-PaCa-2, 6×10^4 cells/well) were precultured in a 24-well flat-bottomed plate for 24 h at 37°C in a 5% CO₂ 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.

**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 1x10⁵ 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.
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 phenoazines, 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 phenoazines 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). The staining of cells with both PI and FITC-labeled annexin-V 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.
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...
in cancer cells would reduce adverse effects (23,24). The and release of toxic substances occur, the induction of apoptosis the membrane of necrotic cells is disrupted, and inflammation Therefore, it is likely that Phx-1 and Phx-3 exert anticancer synergistic effects with TRAIL were observed (Fig. 3A and C).

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.
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