Apoptosis Induction Preceded by Mitochondrial Depolarization in Multiple Myeloma Cell Line U266 by 2-Aminophenoxazine-3-one

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The aim of the present study was to investigate the mechanism of apoptosis in human multiple myeloma cell line, U266, caused by 2-aminophenoxazine-3-one (Phx-3). Flow-cytometrical and morphological analyses showed that Phx-3 increased the population of annexin V-positive cells including early stage apoptotic cells and late stage apoptotic cells and induced DNA fragmentation or apoptotic body formation in U266 cells, indicating that Phx-3 induced the apoptosis of U266 cells. Activity of caspase-3 was extensively increased in U266 cells treated with Phx-3 time-dependently within 24 h, but this Phx-3-stimulated activity of the enzyme in the cells was completely cancelled by the addition of N-benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), a pan-caspase inhibitor. The addition of z-VAD-fmk almost blocked the apoptotic effect of Phx-3 against U266 cells, indicating that Phx-3-induced apoptosis of U266 cells was dependent on a caspase signaling pathway. Moreover, the apoptosis of U266 cells occurred after the induction of cell cycle arrest of the cells in the S and G2/M phase, the loss of mitochondrial membrane potential, and activation of caspase-3 reached maximum, which were caused by Phx-3 within 24 h. These results support the views that the apoptosis of U266 cells caused by Phx-3 may be preceded by the cell cycle arrest, depolarization of mitochondria and activation of caspase-3. These results support the view that Phx-3 may be utilized in future as chemotherapeutic agent against multiple myeloma which is extremely refractory to chemotherapy.

Key words 2-aminophenoxazine-3-one; multiple myeloma; apoptosis; caspase-3; mitochondrial membrane potential

Multiple myeloma is a disseminated malignancy of monoclonal plasma cells in the bone marrow secreting monoclonal immunoglobulins in the serum and/or urine. Patients with this disease show a concomitant decrease in normal immunoglobulins and subsequent formation of lytic bone lesions, and are refractory to chemotherapy, which often is dependent, it has been recognized that loss of mitochondrial function may be essential to apoptosis. Therefore, it may be possible that the depolarization of mitochondria is pivotal to apoptosis-related events, and eventually leads to the apoptosis of cancer cells. In order to verify this hypothesis, we studied the apoptotic effects of Phx-3 against multiple myeloma cell line U266 cells under various conditions. The present study demonstrated that mitochondrial depolarization, the accompanying activation of caspase-3 and cell cycle arrest precedes apoptosis in U266 cells treated with Phx-3.

MATERIALS AND METHODS

2-Aminophenoxazine-3-one 2-Aminophenoxazine-3-one (Phx-3) was prepared according to the method described by Shimizu et al. The chemical structure of Phx-3 is illustrated in Fig. 1. Phx-3 was dissolved in a mixture of dimethylsulfoxide (DMSO) and ethyl alcohol (3:1) as a vehicle to make 20 mM solution, and then this solution was added in culture medium to reach final concentrations of 20—100 μM during experiments. The final volume of vehicle in culture medium was equivalent (0.2%) among Phx-3-free and Phx-3-treated cells in each experiment.

Fig. 1  Chemical Structure of 2-Aminophenoxazine-3-one (Phx-3)
Cell Line and Culture Condition The human multiple myeloma cell line, U266, was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, U.S.A.). The cells were cultured in RPMI-1640 medium (Wako Pure Chemical, Osaka, Japan) supplemented with 80 mg/l kanamycin sulfate (Wako) and 10% heat inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, U.S.A.) in a humidified incubator containing 5% CO2 and 95% air at 37 °C.

Apoptosis and Necrosis Detection The detection of apoptosis and necrosis was performed by flow-cytometry using Annexin V-FITC Apoptosis Detection Kit (Medical & Biological Laboratories (MBL), Nagoya, Japan). U266 cells (1×10^6 cells/10 ml) were treated with 0, 50 and 100 μM Phx-3 in 25 cm² cell culture flasks. After 24, 48, and 72 h of treatment, the cells were collected, once washed with phosphate buffered saline (PBS) (pH 7.4), and resuspended in 500 μl of binding buffer, into which 5 μl of fluorescein isothiocyanate (FITC)-labeled annexin V (Annexin V-FITC) and propidium iodide (PI) were added. These samples were incubated at room temperature for 5 min in the dark, and then analyzed with a flow-cytometer (Partec PAS, Partec, Münster, Germany). Annexin V-FITC binding and PI staining were monitored using FITC signal detector (FL1, 520 nm) and phycoerythrin emission signal detector (FL3, 590—650 nm), respectively.

Morphological Change Analysis For assessment of morphological changes in U266 cells in response to Phx-3, Shandon Cytospin 2 (Shandon Inc., Pittsburgh, PA, U.S.A.) was used for the cytospin preparations, and the prepared cells were stained with the May-Giemsa.17)

Caspase-3 Activity Measurement The activity of caspase-3 was measured using a fluorometrical substrate of caspase-3, Asp-Glu-Val-Asp-4-trifluoromethyl-coumarin (DEVD-AFC) (MBL). U266 cells (1×10^6 cells/10 ml) were treated with 0, 50 and 100 μM Phx-3 in 25 cm² cell culture flasks. After 24, 48 and 72 h of treatment, the cells were collected, washed once with PBS (pH 7.4), and treated with 200 μl of Cell Lysis Buffer (MBL), which were incubated on ice for 10 min. We then added 50 μl of 2X Reaction Buffer (MBL) and 5 μl of DEVD-AFC into 50 μl of aliquots, which were incubated at 37 °C for 1 h in the dark. These samples were analyzed using a multi-detection microplate reader (Powerscan HT, Dainippon Pharmaceutical) at a wavelength of 400 nm excitation/505 nm emission. The activity of caspase-3 was in evaluated triplicate, corrected by protein contents, and presented as relative ratio of the activity in Phx-3-treated cells to that in Phx-3-free cells at each time point.

Cell Cycle Analysis The analysis of cell cycle was performed by flow-cytometry using CyStain DNA 2 step (Partec). U266 cells (1×10^6 cells/10 ml) were treated with 0, 50 and 100 μM Phx-3 in 25 cm² cell culture flasks. After 24, 48 and 72 h of treatment, the cells were collected, once washed with PBS (pH 7.4), and treated with 1 ml of nuclei extraction buffer, which were incubated at room temperature for 10 min. The extracted nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) staining reagent and incubated at room temperature for 10 min in the dark. These samples were analyzed with a flow-cytometer (Partec PAS, Partec) using the cell cycle analysis program, MultiCycle AV (Phoenix Flow System, San Diego, CA, U.S.A.).

Mitochondrial Membrane Potential Analysis The analysis of mitochondrial membrane potential was performed by flow-cytometry using 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolyl-carboxyanine iodide (JC-1) (Wako).18—20 JC-1 can accumulate and aggregate in normal mitochondria, resulting in J-aggregate formation, although JC-1 is not capable of forming J-aggregate in membrane potential lost mitochondria, resulting in JC-1 monomer formation in cytosol. U266 cells (1×10^6 cells/10 ml) were treated with 0, 50 and 100 μM Phx-3 in 25 cm² cell culture flasks. After 1, 6, 12 and 24 h of treatment, the cells were collected, resuspended in 1 ml of fresh culture medium containing 10 μg/ml of JC-1, and incubated at 37 °C for 10 min in the dark. Then, the cells were twice washed with PBS (pH 7.4) and analyzed with a flow-cytometer (Partec PAS, Partec). J-aggregates in mitochondria and JC-1 monomer in cytosol were monitored using phycoerythrin emission signal detector (FL3, 590—650 nm) and FITC signal detector (FL1, 520 nm), respectively.

Caspase Inhibitor In the present study, as a pan-caspase inhibitor, N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl-ketone (z-VAD-fmk) (MBL) was used. z-VAD-fmk was dissolved in DMSO to make 20 mM solution, and then this solution was added in culture medium to reach a final concentration of 20 μM. z-VAD-fmk-free cells were treated with culture medium containing the equivalent volume of DMSO. U266 cells were treated with or without z-VAD-fmk for 1 h before treatment with or without 100 μM Phx-3 and for 24 h with or without 100 μM Phx-3. The measurement of caspase-3 activity, apoptosis and necrosis detection, and mitochondrial membrane potential analysis were performed as described above.

Effects of Phx-3 on DNA Replication in U266 Cells DNA replication in U266 cells was measured by using a Cell Proliferation ELISA, 5-bromo-2′-deoxyuridine (BrdU) kit (Roche Diagnostics, Indianapolis, IN, U.S.A.). U266 cells (2×10^4 cells/well) were cultured in 96 well plates in the presence of Phx-3 (0, 1, 2, 5, 10, 25 μM) at 37 °C for 24 h. Then, Brd-U (10 μM final concentration) was added and the cells were re-incubated for further 2 h. The cells were fixed and incubated with anti-Brd-U-peroxidase (10 μl/well) for 1 h at room temperature. Substrate solution was added for 10 min and the reaction was stopped by adding 1 M H₂SO₄ solution. The absorbance was measured within 5 min at 450 nm with a reference wavelength at 690 nm using an ELISA plate reader (TOSOH Co., Tokyo).

Statistical Analysis Experimental data were presented as mean±standard error of the mean (S.E.M.). A one-way analysis of variance (ANOVA) was performed to examine the differences of values between Phx-3-free cells and Phx-3-treated cells. The differences were considered significant when p<0.05.

RESULTS

Apoptosis and Necrosis Whether the apoptosis of U266 cells might be caused by Phx-3 was investigated by flow-cytometry using annexin V-FITC binding and PI staining methods (Fig. 2). Consequently, the population of both annexin V- and PI-negative cells (viable cells; plotted in bottom left quadrant) was dominantly observed in the control cells

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treated with vehicle alone during 24—72 h (Fig. 2). The population of annexin V-positive and PI-negative cells (early phase apoptotic cells: plotted in the bottom right quadrant), and that of both annexin V- and PI-positive cells (late phase apoptotic/necrotic cells, actually detected as buoyant dead cells in the culture well: plotted in the top right quadrant) increased in U266 cells treated with 50 and 100 μM Phx-3 (Fig. 2). The rate of increase in late phase apoptotic/necrotic cells was dose- and time-dependent. These results suggest that Phx-3 induces apoptosis of U266 cells.

**Morphological Change** To confirm the results in Fig. 2, we further assessed the morphological changes in U266 cells after 24 h-treatment with different concentrations of Phx-3. As compared with the untreated control cells, chromatin condensation became evident in U266 cells treated with 10 μM Phx-3, and some cells showed the fragmented nuclei. These findings were the typical features of the cells undergoing apoptosis (Fig. 3). The ratio of the cells with the fragmented nuclei increased at 50 μM. Finally, more than 90% of the cells became shrunken without apparent cytoplasm after exposure to 100 μM Phx-3. All these data are consistent with those shown in Fig. 2, and indicate the strong apoptosis inducing effect of Phx-3 in U266 cells.

**Caspase-3 Activity** We investigated the effect of Phx-3 on the activity of caspase-3, in order to investigate whether Phx-3-induced apoptosis of U266 cells was dependent or independent on the caspase family signaling cascade (Fig. 4A). In addition, it was examined whether U266 cells were affected by z-VAD-fmk, a pan-caspase inhibitor, in terms of the anti-caspase or apoptogenic effects of the reagent (Figs. 4B, C). As shown in Fig. 4A, caspase-3 in U266 cells was activated extensively in the presence of Phx-3, within 24 h, in a dose-dependent manner. When U266 cells were treated with z-VAD-fmk, Phx-3-induced activation of caspase-3 was completely inhibited (Fig. 4B). Phx-3-induced apoptotic cell death, which was illustrated in the third column from the left in Fig. 4C, was fully diminished by the addition of z-VAD-fmk (the fourth column from the left in Fig. 4C), indicating that the induction of apoptosis of U266 cells caused by Phx-3.

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**Fig. 2. Flow-Cytometrical Detection of Apoptosis and Necrosis of U266 Cells Treated with Phx-3**

U266 cells were treated with 0 (control), 50 and 100 μM Phx-3 for 24, 48 and 72 h. Both annexin V- and PI-negative (bottom-left quadrant), annexin V-positive and PI-negative (bottom-right quadrant), and both annexin V- and PI-positive (top-right quadrant) cells were considered as the viable, early-phase apoptotic, and late-phase apoptotic/necrotic (dead) cells, respectively. The percentages (%) of each cell were described in each quadrant except for viable cells.

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**Fig. 3. Morphological Changes of U266 Cells Treated with Phx-3**

U266 cells were cultured in the presence or absence of 10, 50 and 100 μM Phx-3 for 24 h in RPMI-1640 containing 10% FBS. (May-Giemsa stain, original magnification: ×1000).
Cell Cycle

Since it has been suggested that the apoptotic cellular death of cancer cells caused by anti-cancer drugs are linked to cell cycle arrest, we studied the induction of cell cycle arrest in U266 cells in the presence of Phx-3. As shown in Fig. 5, the cell cycle was arrested in U266 cells, within 24 h, dose-dependently, when Phx-3 was administered. In particular, 100 μM Phx-3 strongly induced S and G2/M phase arrest after 24 h.

DNA Replication

Progress of the cell cycle is closely associated with DNA replication in the cells. Thus, the effects of Phx-3 on DNA replication measured by Brd-U incorporation into DNA of U266 cells were examined at different concentrations of Phx-3. As shown in Fig. 6, DNA replication in U266 cells was suppressed by the increasing amounts of Phx-3, which occurred within 24 h, being consistent with the cell cycle arrest at S and G2/M phase, as shown in Fig. 5.

Mitochondrial Membrane Potential

Since it has been shown that the loss of mitochondrial membrane potential induces the apoptotic events, we studied whether depolarization of mitochondria in U266 cells might be caused by Phx-
3. Through the treatment of U266 cells with 50 \( \mu M \) or 100 \( \mu M \) Phx-3, the population of the cells with loss of mitochondrial membrane potentials (bottom right quadrant in Fig. 7) increased with time. After 6 h, the loss of mitochondrial membrane potentials was significantly observed, i.e., 9.3%, 37.4% and 67% in cell population, at 6 h, 12 h and 24 h, respectively.

**Time Course of Mitochondrial Depolarization and Apoptotic Events in U266 Cells Treated with Phx-3**

Figure 8A illustrates the changes in population of the cells with depolarized mitochondrial membrane and that of the apoptotic cells in U266 cells, and the changes in activity of caspase-3 during 72 h treatment of the cells with 100 \( \mu M \) Phx-3. The loss of mitochondrial membrane potentials occurred drastically after 12 h. The population of the cells with lost mitochondrial membrane potentials reached 70% after 24 h in U266 cells treated with Phx-3. The activation of caspase-3 in U266 cells occurred within 24 h, which is comparable to the loss of mitochondrial membrane potentials in the cells. Apoptotic events occurred thereafter. Namely, the population of apoptotic cells increased after 24 h in U266 cells treated with Phx-3. The activation of caspase-3 in U266 cells occurred within 24 h, which is comparable to the loss of mitochondrial membrane potentials in the cells.

**DISCUSSION**

In the present study, it was demonstrated that a phenoxazine derivative, Phx-3 induced cell apoptosis in a human multiple myeloma cell line, U266, in a dose- and time-dependent manner (Figs. 2, 3). Since caspase-3 has been shown to be the executing enzyme of apoptosis, we investigated whether this enzyme may be activated in U266 cells treated with Phx-3. It was found that the activation of caspase-3 was dependent on the dose of Phx-3, and its activity reached almost the maximum level within 24 h (Fig. 4A). Since the apoptosis of U266 cells initiated to occur 24 h after treatment of Phx-3 (Fig. 8A), it may be possible to say that the activation of caspase-3 may precede apoptosis in U266 cells. This view was further supported by the results obtained by the addition of z-V AD-fmk, a pan-caspase inhibitor. Namely, when U266 cells were co-treated with both 100 \( \mu M \) Phx-3 and 20 \( \mu M \) z-VAD-fmk, the activity of caspase-3 was completely inhibited (Fig. 4B) and the apoptosis of the cells was blocked (Fig. 4C). These results suggest that the apoptosis of U266 cells caused by Phx-3 was closely dependent on the activity of caspase-3.

It has recently been reported that Phx-3 caused apoptosis independent of the caspase signaling pathway in neuroblastoma cells,\(^7\) human glioblastoma cells,\(^8\) and gastric cancer cells,\(^15\) because Phx-3-induced apoptosis of these cancer cell lines was not prevented even in the presence of z-VAD-fmk. These results and the present results suggest that the contribution of the caspase signaling pathway to the induction of apoptotic cell death are different in the type of cancer cells, although Phx-3 ultimately induces apoptosis or necrotic-like cell death in various types of cancer cells.

Since the activation of caspase-3 in cancer cells has been recognized as a result of mitochondrial dysfunction, including the loss of mitochondrial membrane potential,\(^16\) we studied whether the depolarization of mitochondria might occur...
in U266 cells in the presence of Phx-3. Consequently, we found that Phx-3 induced mitochondrial membrane potential loss of U266 cells dose- and time-dependently at a relatively early phase of treatment, and that the mitochondrial membrane potential of almost all cells was lost by 24 h treatment with 50 μM Phx-3 (Fig. 7). This depolarization of mitochondrial membrane would cause the activation of caspase-3 (Fig. 4A), through release of cytochrome c from mitochondria, leading to the formation of the apoptosome constructed by apoptotic protease activating factor-1 (Apaf-1) and caspase-9 in cytosol.22) As shown in Fig. 8A, it was found that the loss of mitochondrial membrane potential precedes the apoptotic event in U266 cells, which is consistent with the activation of caspase-3, as a function of time. This suggests that there may be a schematic flow from the depolarization of mitochondrial membrane, release of cytochrome c from the mitochondria, activation of caspase-3, and finally to DNA fragmentation, causing apoptosis, in U266 cells treated with Phx-3.

It is still unclear how Phx-3 causes the depolarization of mitochondria. Hendrich et al.23) reported that phenoxazines cause the perturbation of phospholipids of the membrane, locating close to the polar/apolar interface of lipid bilayer and interacting weakly with the bilayer. Thus, a plausible explanation may be that Phx-3 may perturb the membrane of mitochondria, inducing the depolarization, which should be clarified by further examination. Concerning cell cycle arrest by Phx-3, weak intercalation of this compound to DNA in U266 cells may be possibly involved, because it was reported that Phx-3 (2-aminophenoxazine-3-one) interacts with DNA24) and binds the minor groove of DNA.25) Such intercalation of Phx-3 to DNA may cause the suppression of DNA replication in U266 cells by Phx-3 (Fig. 6). It is obscure at present how cell cycle arrest and suppressed DNA replication induced by Phx-3 in U266 cells may influence the cellular apoptosis that is induced thereafter, in cooperation with the mitochondrial depolarization and activation of caspase-3, though these events were seen almost at the same time within 24 h (Figs. 8A, B).

Azuine et al.26) reported that the simple tri- cyclic compound phenoxazine prevented the mouse skin tumor promotion caused by 12-O-tetradecanoylphorbol-13-acetate (TPA), without causing any toxicities. Miyano-Kurosaki et al.27) also observed that Phx-3 extensively inhibited the growth of mouse melanoma cells transplanted in nude mice, without evident adverse effects. It is likely that phenoxazines may prevent the proliferation of cancer cells through the inhibition of Akt-phosphorylation as suggested by Hara et al.27) and Thimmaiah et al.28) being consistent with the results of Enoki et al.29) that Phx-1 suppresses the antigen-induced degranulation in rat basophilic leukemia cells through inhibiting the Akt phosphorylation. Taken these reports and the present result that Phx-3 induces apoptosis in multiple myeloma cell line U266 cells, Phx-3 may be a useful candidate as chemotherapeutic agent against multiple myeloma which is extremely refractory to chemotherapy.

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