Phenoxazine Derivatives 2-Amino-4,4 α -dihydro-4 α -phenoxazine-3-one and 2-Aminophenoxazine-3-one-Induced Apoptosis through a Caspase-Independent Mechanism in Human Neuroblastoma Cell Line NB-1 Cells

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The aim of the present study was to determine whether phenoxazines such as 2-amino-4,4 α -dihydro-4 α -phenoxazine-3-one (Phx-1) and 2-aminophenoxazine-3-one (Phx-3) may suppress the proliferation of human neuroblastoma cell line, NB-1 that is refractory to chemotherapeutic agents, inducing apoptosis through the activation of caspase pathway or not. Phx-1 and Phx-3 suppressed the proliferation of NB-1 cells extensively dependent on dose and time. The IC₅₀ of Phx-1 and Phx-3 was about 20 μ M and 0.5 μ M, respectively, when the cells were treated with Phx-1 or Phx-3 for 72 h. Phx-1 and Phx-3 caused the mixed types of cell death—apoptosis and necrosis—in NB-1 cells, which was detected by flow cytometry. The induction of apoptosis/necrosis caused by these phenoxazines seemed to be correlated dominantly with the caspase independent pathway, because the increased activity of effector caspase 3/7 in NB-1 cells caused by 50 μ M Phx-1 or 20 μ M Phx-3 was completely cancelled by the addition of z-VAD-fmk, a pan-caspase inhibitor, but such phenoxazines-suppressed viability of NB-1 cells was not recovered to normal levels by this inhibitor. The results of this study demonstrate that Phx-1 and Phx-3 have antitumor activity against the neuroblastoma cell line, NB-1, though the IC₅₀ was extremely low for Phx-3, inducing the mixed types of cell death, apoptosis and necrosis, caspase-independently.

Key words phenoxazine; viability inhibition; apoptosis; neuroblastoma cell line; caspase 3/7

Neuroblastoma is a solid tumor with extracranial malignancy that mainly occurs in childhood. Since this carcinoma manifests high-risk biological and clinical characteristics such as advanced tumor stage,^{1–4)} the treatment of this carcinoma has been difficult. According to recent advances in the therapy for carcinoma, tumor-specific induction of apoptosis is a crucial strategy to overcome therapy-resistant and neoplastic progressive tumors.^{3,4)} It is, therefore, important to investigate the drugs that can induce both cellular apoptosis and suppression of proliferation, in order to treat high-risk tumors including neuroblastoma.

Phenoxazines are fundamental component of actinomycin D which exerts strong anticancer activity, but also has marked adverse effects such as myelosuppression and renal cytotoxicity,⁵⁾ it is conceivable that phenoxazines may possess anticancer activity. It has been reported recently that phenoxazines produced by the reaction of bovine hemoglobin with *o*-aminophenol and its derivatives^{6,7)} are relatively soluble in water and have various biological effects including anti-cancer effects,^{8,9)} immunosuppressive effects through the inhibition of Akt phosphorylation and activation of the proteasome^{10,11)} antimicrobial effects^{7,12)} and anti-viral effects.¹³⁾ In particular, Phx-1 showed antiproliferative effects against lung carcinoma cells,¹⁴⁾ leukemia cells⁹⁾ and Burkitt lymphoma cells,¹⁵⁾ but the anticancer effects of Phx-3 have only been evaluated with regard to a human malignant melanoma cell line¹⁶⁾ and pancreatic carcinoma cell line.¹⁷⁾

In the present work, we investigated apoptotic anticancer effects of Phx-1 and Phx-3 against neuroblastoma cell line NB-1, which is refractory to chemotherapeutic agents, and the contribution of caspases to the apoptotic cell death in the cells.

MATERIALS AND METHODS

Synthesis of Phx-1 and Phx-3 2-Amino-4,4 α -dihydro-4 α ,7-dimethyl-3*H*-phenoxazine-3-one (Phx-1) and 2-aminophenoxazine-3-one (Phx-3) were prepared according to the methods described previously.^{6,7)} The chemical structure of these compounds is illustrated in Fig. 1. Phx-1 and Phx-3 were dissolved in dimethylsulfoxide (DMSO) and ethyl alcohol (EtOH) (3 : 1) solution at a concentration of 20 mM.

Cell Line and Culture Condition The human neuroblastoma cell line, NB-1 was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (Kohjin Bio, Saitama, Japan) supplemented with 80 mg/l kanamycin sulfate (Wako Pure Chemicals, Tokyo, Japan) and 10% heat inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, U.S.A.) in a humidified incubator containing 5% CO₂/95% air at 37 °C.

Cell Viability Assay The viability of cells was assessed using a Cell Titer-Blue™ Cell Viability Assay (Promega, Madison, WI, U.S.A.). NB-1 cells were pre-cultured in a Nunclon 48-well flat-bottomed microtiter plate at

Fig. 1. Chemical Structure of Phx-1 and Phx-3

A: Phx-1; B: Phx-3.

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5×10^4 cells/well for 24 h. After the culture medium was discarded, the cells were treated with fresh medium containing various concentrations of Phx-1 (0–100 μM) and Phx-3 (0–4 μM). After 24, 48 and 72 h of treatment, culture medium was discarded, and fresh medium and 100 μl /well of the Cell Titer-Blue reagent (Resazurin, 7-hydroxy-3*H*-phenoxazine-3-one-10-oxide) was added, further incubation was continued for 1 h. Then, Resazurin reduced by living cells was analyzed using a multi-detection microplate reader (Powerscan HT, Dainippon Pharmaceutical, Osaka, Japan) at 560 nm excitation/570 nm emission. Cell viability was determined by referring to the fluorescence of cells treated without Phx-1 or Phx-3.

Apoptosis and Necrosis Detection The detection of apoptosis and necrosis was performed flow cytometrically using an Annexin V-FITC Apoptosis Detection Kit (BioVision Research Products, Mountain View, CA, U.S.A.). NB-1 cells (1×10^5 cells/ml) treated with or without 20 μM Phx-1 or 1 μM Phx-3 for 24, 48 and 72 h were collected and resuspended in 500 μl of binding buffer. Then, 5 μl of fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI) were added to cells and incubated at room temperature for 5 min in the dark. These samples were analyzed with 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.

Cell Cycle Analysis Cell cycle analysis was performed flow cytometrically using a nuclei extraction buffer (CyStain DNA 2 step, Partec). NB-1 cells (1×10^5 cells/ml) were treated with or without 50 μM Phx-1 or 20 μM Phx-3. After 24, 48 and 72 h, the culture medium was discarded, and cells were washed once with phosphate buffered saline (PBS, pH 7.4). Then, 1 ml of the nuclei extraction buffer was added and incubated at room temperature for 10 min. Extracted nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI) staining reagent and incubated at room temperature for 10 min in a 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.).

Measurement of Caspase Family Activities The activities of caspase 3/7, caspase 9 and caspase 8 were measured using Caspase-GloTM 3/7, 9 and 8 Assay (Promega), respectively. NB-1 cells were pre-treated in a Nunclon white-walled 96-well flat-bottomed microtiter plate at 5×10^3 cells/well for 24 h. Then, culture medium was discarded, and cells were treated with fresh medium containing 50 μM Phx-1 or 20 μM Phx-3. After 24, 48 and 72 h of treatment, culture medium was replaced, and 100 μl of Caspase-GloTM 3/7, 9 and 8 reagents were added to each well, and the cells were incubated at room temperature for 30 min. After incubation, a luminescent signal was detected using a multi-detection microplate reader (Powerscan HT, Dainippon Pharmaceutical). Under the same experimental condition, the viability of cells was concurrently assessed as described above. The activation of caspases 3/7, 9 and 8 was corrected by cell viability, and presented as relative ratio of the activity in cells with Phx-1 or Phx-3 to that in control cells with vehicle (DMSO/EtOH) at each time point.

Determination of Reversing Effect of Caspase Inhibitor

on the Antiproliferative Activity of Phx-1 or Phx-3 against NB-1 Cell NB-1 cells (1×10^4 cells/well) were pre-incubated in a Nunclon 96-well flat-bottomed microtiter plate for 24 h. Then, cells were divided in three groups as follows: 1) control cells treated with vehicle, 2) cells treated with indicated concentrations of Phx-1 or Phx-3, and 3) cells co-treated with indicated concentrations of Phx-1 or Phx-3 and 100 μM *N*-benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) (Kamiya Biochemical, Seattle, WA, U.S.A.), a pan-caspase inhibitor, which was dissolved in DMSO to make 20 mM solution before use. The third group cells and another group cells were pre-treated with 100 μM z-VAD-fmk and the same quantity of DMSO for 2 h, respectively, and then treated with or without indicated concentrations of Phx-1 or Phx-3 and 100 μM z-VAD-fmk for 48 h. The activity of caspase 3/7 and the viability of cells were examined, and the detection of apoptosis and necrosis was performed as described above.

Statistical Analysis Experimental data were presented as means $\pm$ S.E. Unpaired Student's *t*-test was performed to examine the differences between cells treated with Phx-1 or Phx-3 and control cells treated with vehicle alone, and between cells treated with and without z-VAD-fmk. The differences were considered significant when *p* was 0.05.

RESULTS

As shown in Fig. 2, Phx-1 and Phx-3 suppressed the proliferation of human neuroblastoma NB-1 cells time- and dose-dependently. The proliferation of the cells was almost completely suppressed at concentrations of more than 50 μM Phx-1 at 72 h, and the 50% inhibition concentration (IC_{50}) was about 20 μM . Phx-3 almost completely suppressed the viability of the cells at concentrations as low as 4 μM , and the IC_{50} was about 0.5 μM . These results show that Phx-1 and Phx-3 inhibit the proliferation of human neuroblastoma NB-1 cells *in vitro*, and that Phx-3, in particular, has stronger inhibitory activity against the proliferation of NB-1 cells.

We evaluated the population of viable cells and the cells in early stage apoptosis, late stage apoptosis/necrosis and necrosis among human neuroblastoma NB-1 cells treated with Phx-1 or Phx-3, by using flow cytometry (Fig. 3). In this case, binding and staining of the cells with both FITC-labeled annexin-V and PI were examined, because FITC-labeled annexin-V binds specifically to the phosphatidylserine exposed to the outer membrane in apoptotic cells¹⁸⁾ and be-

Fig. 2. Viability of Human Neuroblastoma Cell Line NB-1 in the Presence or Absence of Different Concentrations of Phx-1 or Phx-3

NB-1 cells were pre-cultured in a Nunclon 48-well flat-bottomed microtiter plate at 5×10^4 cells/well for 24 h. After the culture medium was discarded, the cells were treated with fresh medium containing various concentrations of Phx-1 (0–100 μM) and Phx-3 (0–4 μM), and were incubated for 72 h. (○) 24 h, (●) 48 h, (△) 72 h.

cause PI can penetrate into necrotic cells, but not viable or early apoptotic cells. As shown in Fig. 3, viable cells were mainly seen as the bottom-left population of cells which excluded PI and did not bind with annexin-V (control at 24, 48, 72 h) in non-treated human neuroblastoma NB-1 cells. In Phx-1 or Phx-3 treated NB-1 cells, annexin-V+ and PI+ cells (=late stage apoptotic/necrotic cells; top right) increased to after 24, 48 and 72 h but annexin-V+ and PI- cells (=early stage apoptotic cells; bottom right) tended to decrease (details are shown in Fig. 4). Annexin-V- and PI+ cells (=necrotic cells; top left) increased in NB cells treated with Phx-1 or Phx-3 for 72 h. These data were depicted as the changes in percentages (%) of the viable cells and the cells in early stage apoptosis, late stage apoptosis/necrosis and necrosis among NB-1 cells treated with Phx-1 or Phx-3 (Fig. 4). As a result, Phx-1 and Phx-3 induced mixed types of cell death including apoptosis and necrosis.

Cell cycle of cancer cells has been recognized to be often interrupted by the treatment of anticancer drugs such as

etoposide and 5-fluorouracil.¹⁹⁾ It has been indicated that G₁ arrest was observed in KB cells.²⁰⁾ human leukemia cell lines K562 and HL-60 cells⁹⁾ when the cells were treated with Phx-1, and in human malignant melanoma cell line G-361, when the cells were treated with Phx-3.¹⁶⁾ Thus, we studied the effects of 50 μ M Phx-1 and 20 μ M Phx-3 on cell cycle of NB-1 cells. As shown in the column of Fig. 5, the percentages of various cell cycle stages including G₀/G₁, S and G₂/M did not alter in the presence or absence of Phx-1 and Phx-3, showing that a block of cell cycle progression was not caused in NB-1 cells treated with these phenoxazines.

Since cellular apoptosis has been suggested to be caused by the activation of caspases,^{21,22)} we investigated whether the activity of caspase 3/7, caspase 9 and caspase 8 might be activated in NB-1 cells treated with Phx-1 or Phx-3 (Fig. 6). Consequently, caspase 3/7 activity was increased about 23 times at 72 h in NB-1 cells treated with 50 μ M Phx-1 compared with that in control cells without Phx-1, though its activation was twice at 48 h (top left column). The activity of caspase 3/7 was increased several times at 24, 48 and 72 h, in NB-1 cells treated with 20 μ M Phx-3, compared with that in control cells (top right column). The increase in caspase-9 and caspase-8 was moderate (about 2.5–3 fold increase) after 24 to 72 h in NB-1 cells treated with Phx-1 or Phx-3 (second and third columns). According to the increase in the activity of these caspases, the viability of the cells was greatly suppressed in NB-1 cells with phenoxazines, for 72 h (bottom columns).

In order to study how much these caspases may contribute to the viability and apoptosis/necrosis of NB-1 cells in the presence of Phx-1 or Phx-3, we studied the effects of 100 μ M

Fig. 3. Changes in Population of the Viable Cells and the Cells in Early Stage Apoptosis, Late Stage Apoptosis/Necrosis, and Necrosis among Human Neuroblastoma Cell Line NB-1, Detected by Annexin-V and PI Staining

Human neuroblastoma cell line NB-1 cells were grown in the presence or absence of 20 μ M Phx-1 or 1 μ M Phx-3, for 24, 48 and 72 h. These cells were stained with FITC-annexin-V and propidium iodide, and then examined by flow cytometry. In each dot plot, the percentages of annexin-V-PI- (viable cells, bottom-left quadrant), annexin-V+PI- (cells in early stage apoptosis, bottom-right quadrant), annexin-V+PI+ (cells in late stage apoptosis/necrosis, top-right quadrant), annexin-V-PI+ (cells in necrosis, top-left quadrant) are described.

Fig. 4. Changes in Percentages of Viable Cells and the Cells in Early Apoptosis Stage, Late Apoptosis/Necrosis Stage and Necrosis Stage in NB-1 Cells Treated with Phx-1 or Phx-3

The results in Fig. 3 were expressed by the changes in percentages (%) of cells in various apoptotic stages. (○) viable cells, (●) cells in early stage apoptosis, (△) cells in late stage apoptosis/necrosis, (▲) cells in necrosis.

Fig. 5. Effects of Phx-1 and Phx-3 on Cell Cycle in NB-1 Cells

Cell cycle analysis was performed flow-cytometrically using a CyStain DNA 2 step (Partec) for NB-1 cells (1×10^5 cells/ml) treated with or without 50 μ M Phx-1 or 20 μ M Phx-3 for 48 h. Percentages (%) of various cell cycle stages including G₁, S, and G₂/M were depicted in the figures.

z-VAD-fmk, a pan-caspase inhibitor on caspase 3/7 activity, viability of NB-1 cells and population of apoptosis/necrosis in the cells, which were treated with 50 μ M Phx-1 or 20 μ M Phx-3 for 48 h (Figs. 7A, B, Fig. 8). The activity of caspase 3/7 increased as much as 4 times in NB-1 cells treated with Phx-1 alone (Fig. 7A), but such activation of caspases 3/7 was completely cancelled when z-VAD-fmk was added. Concerning the viability of NB-1 cells treated with Phx-1 alone, it was decreased by 45% (Fig. 7B). However, the viability remained at 55%, when the cells were treated with both Phx-1 and z-VAD-fmk, indicating that cancellation of the augmented caspase 3/7 activity did not lead to the restoration of viability of NB-1 cells.

Such results were apparently indicated, when NB-1 cells were treated with Phx-3 alone or Phx-3 and z-VAD-fmk.

Fig. 6. Changes in the Activity of Caspase 3/7, Caspase 8 and Caspase 9 in NB-1 Cells and Cellular Viability during Incubation with 50 μ M Phx-1 or 20 μ M Phx-3 for 72 h

* $p < 0.05$ and ** $p < 0.01$ (vs. control cells).

Namely, when NB-1 cells were treated with Phx-3 alone, caspase 3/7 was extensively activated in the cells, and the cell viability was suppressed by 90%. When the cells were treated with both Phx-3 and z-VAD-fmk, the activated activity of caspase 3/7 was completely cancelled (Fig. 7A), but the suppressed viability of the cells did not recover to the levels of the control without Phx-3 (100%) (Fig. 7B).

We confirmed such behavior of NB-1 cells treated with Phx-1 or Phx-3 in combination of z-VAD-fmk, in terms of the population of the cells in late stage apoptosis/necrosis (Fig. 8). In the control cells without Phx-1, Phx-3 and z-VAD-fmk, the viable cells occupied dominantly. When the cells were treated with Phx-1, the population of the cells in the late stage apoptosis/necrosis increased up to 39.1%. Even when the cells were treated with Phx-1 and z-VAD-fmk, the population of the cells in the late stage apoptosis/necrosis (32.8%) did not alter compared with the case with Phx-1 alone, and the population of the viable cells did not increase. Such tendency was also observed when NB-1 cells were

Fig. 7. Effects of z-VAD-fmk on the Activation of Caspase 3/7 and the Suppression of Viability of NB-1 Cells Induced by 50 μ M Phx-1 or 20 μ M Phx-3

(A) Effects of z-VAD-fmk on the activation of caspase 3/7 in NB-1 cells induced by Phx-1 or Phx-3. (B) Effects of z-VAD-fmk on the suppression of viability of NB-1 cells induced by Phx-1 or Phx-3. * $p < 0.05$ and *** $p < 0.001$ (vs. cells treated without z-VAD-fmk).

Fig. 8. Effects of z-VAD-fmk on the Apoptosis/Necrosis of NB-1 Cells Induced by Phx-1 or Phx-3

The population of viable cells and the cells in early stage apoptosis, late stage apoptosis/necrosis, and necrosis among human NB-1 cells was detected by a flow cytometric assay as described in the legends of Fig. 3. NB-1 cells were grown in the presence or absence of 20 μ M Phx-1 or 1 μ M Phx-3 with or without 100 μ M z-VAD-fmk, for 48 h. In each dot plot, the percentages of annexin-V-PI- (viable cells, bottom-left quadrant), annexin-V-PI- (cells in early stage apoptosis, bottom-right quadrant), annexin-V-PI+ (cells in late stage apoptosis/necrosis, top-right quadrant), annexin-V-PI+ (cells in necrosis, top-left quadrant) are described.

treated with Phx-3 or with Phx-3 and z-VAD-fmk. It should be noted that when the cells were treated with Phx-3 alone, the group of the cells in late stage apoptosis/necrosis closer to necrosis was eminently present in addition to the cells in the late stage apoptosis/necrosis. The cells in the late stage apoptosis/necrosis closer to necrosis disappeared when the cells were treated with both Phx-3 and z-VAD-fmk (the fourth and fifth columns from the left in Fig. 8), the reason for which remains unclear.

DISCUSSION

Neuroblastoma manifests many aberrant molecular processes such as deregulated oncogenes, chromosomal segregation defects, loss of cell-cell contacts and nutrient deprivation *in situ*, associated with high-risk clinical features.³⁾ Chemotherapeutic treatment of neuroblastoma is, therefore, extremely difficult.²⁾ In this manuscript, we showed that Phx-1 and Phx-3 demonstrate anticancer activity against human neuroblastoma cell line NB-1 cells, namely, these phenoxazines caused marked suppression of the proliferation of the cells at micromolar concentrations (Fig. 2), inducing the late stage apoptosis/necrosis (Figs. 3, 4). These results suggest that these phenoxazines may act as an anticancer drug against neuroblastoma.

It has been previously reported that Phx-3 exerted anticancer effects against human malignant melanoma cell line G-361¹⁶⁾ and human pancreatic carcinoma cell line KLM-1 cells¹⁷⁾ at concentrations of more than 10 μM . With regard to human neuroblastoma cell line NB-1 cells, Phx-3 exerted antiproliferative activity at concentrations of lower than 1 μM ($\text{IC}_{50}=0.5 \mu\text{M}$) (Fig. 2). Thus, the antiproliferative effect of Phx-3 against NB-1 cells may be particularly strong. The reason why NB-1 cells were exclusively sensitive to Phx-3 remains obscure at current, but may be associated with the fact that phenoxazine derivatives act on nervous system as depressants.^{23,24)} The difference in chemical structure between Phx-1 and Phx-3, *i.e.*, the presence or absence of the methyl group in the tricyclic chromophore as shown in Fig. 1 may affect the action mode of these phenoxazines against NB-1 cells, because Phx-3 which does not include the methyl groups shows stronger antiproliferative effects on NB-1 cells than Phx-1 having the methyl groups.

We found that Phx-1 and Phx-3 caused mixed type apoptosis, *i.e.*, they greatly increased the population of late stage apoptosis/necrosis in human neuroblastoma cell line NB-1 cells, with a moderate increase in necrotic cells, dependent on time, when the cells were treated with these phenoxazines for 72 h (Figs. 3, 4). Apoptosis has been recognized as a highly regulated cellular response to various external signals, and can be triggered by extrinsic pathways activated through cell surface death receptors and the intrinsic pathway involving changes in permeability of the mitochondrial membrane and release of apoptogenic substances such as cytochrome c leading to the activation of caspase 3, a major final executing caspase of apoptosis.⁴⁾ However, it has been recently recognized that caspase independent pathway associated with apoptosis inducing factor (AIF) and BNIP3 is operating in induction of apoptosis.^{25–29)} AIF, a mitochondrial flavoprotein that is released from the intermembrane space, and translocates to the nucleus, once liberated, induces apoptosis,

in a caspase-independent fashion.²⁸⁾ BNIP3-mediated cell death is independent of caspase activation, cytochrome c release and nuclear translocation of apoptosis-inducing factor, and is associated with mitochondrial dysfunction characterized by opening of the mitochondrial permeability transition pore, yielding a morphotype that is typical of necrosis.²⁷⁾

The results in Fig. 6 showed that caspase 3/7 were markedly activated and caspase 8 and caspase 9 were moderately activated in NB-1 cells treated with Phx-1 and Phx-3. Therefore, we expected that the apoptotic cell death in NB-1 cells caused by Phx-1 or Phx-3 might be induced by the activation of caspase family in the cells. However, our results in Figs. 7A and B threw the doubt on this idea. The addition of z-VAD-fmk, a pan-caspase inhibitor, to NB-1 cells treated with Phx-1 or Phx-3 induced complete cancellation of stimulated activity of caspases in the cells (Fig. 7A), but did not reverse the phenoxazines-suppressed viability of the cells to normal levels (Fig. 7B). In addition, we observed that when the cells were treated with Phx-1 or Phx-3, the population of the cells in the late stage apoptosis/necrosis was extensively increased, and the similar tendency was observed when the cells were treated with Phx-1 or Phx-3 in combination of z-VAD-fmk (Fig. 8). These results strongly suggest that Phx-1 or Phx-3 may cause apoptosis/necrosis through caspase-independent pathway, and the contribution of caspase 3/7 to the apoptosis/necrosis induction in NB-1 cells treated with Phx-1 or Phx-3 may be smaller than expected. The detailed mechanism should be clarified by further research in future.

A block of progression of the cell cycle is shown to cause suppression of cellular proliferation and cellular death.^{25,30)} However, the cellular arrest was not indicated in NB-1 cells treated with Phx-1 or Phx-3 (Fig. 4), suggesting that the suppression of proliferation of NB-1 caused by these phenoxazines was not related with a block of cell cycle progression.

The drugs to induce apoptosis of cancer cells have been recently recognized as the most expectable candidates for anticancer drugs,^{19,24)} because inflammation and release of toxic substance may be minimized in the apoptotic cell death. It is obscure that Phx-1 and Phx-3 may cause little adverse effects, when administered *in vivo*, because the present results showed that these phenoxazines caused mixed type apoptosis/necrosis accompanying with the suppressed proliferation, in NB-1 cells (Figs. 2, 3). However, it has been recently reported that little adverse effects were observed when Phx-1^{8,9)} or Phx-3³¹⁾ was administered to mice transplanted with some cancer cells. These results are also consistent with the findings of Eckert and Eyer³²⁾ that when *o*-aminophenol was administered to dogs, it was quickly converted to 2-aminophenoxazine-3-one in the blood, causing no adverse effects such as nephrotoxicity, while *m*-aminophenol or *p*-aminophenol, which could not be metabolized in the blood, caused severe nephrotoxicity in dogs. Therefore, Phx-1 and Phx-3 may be a possible candidate for the treatment of neuroblastoma, without causing adverse effects. In addition, phenoxazine derivatives and phenothiazines in use are shown to pass the blood brain barrier and show various pharmacological effects without any reported adverse effect.³³⁾ In the light of these facts, Phx-1 and Phx-3, in particular, may hold promise as agents to treat neuroblastoma which is extremely refractory to treatment.

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