Antitumor Effects of a Novel Phenoazine Derivative on Human Leukemia Cell Lines 

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ABSTRACT
2-Amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoazine-3-one (Phx) was synthesized by reacting 2-amino-5-methylphenol with bovine hemolysates. Because Phx is a phenoazine derivative like actinomycin D, we examined its effects on the proliferation of the human leukemia cell lines K562, HL-60, and HAL-01. Phx inhibited proliferation and induced apoptosis in all of the leukemia cell lines we tested, in a dose-dependent manner. We further investigated the antitumor effect of this compound on HAL-01-bearing nude mice. Treatment with Phx markedly reduced tumor growth rate in the experimental group, as compared with the control group. Moreover, Phx was found to have few adverse effects on weight loss and WBC count. In addition, we examined the effects of Phx on human normal hematopoietic progenitor cells by a clonogenic assay, and we observed less suppression of normal progenitor cells than of leukemic progenitors. These results suggest that Phx may be used to treat patients affected by different types of leukemia.

INTRODUCTION
Actinomycin D has a strong antitumor activity and is known to be a DNA intercalator (1). The basic structure of actinomycin D is similar to that of phenoazine compounds. Although phenoazine is the basic chemical structure of actinomycin D that exerts a strong antitumor effect by intercalating DNA, chemically synthesized phenoazines show little solubility in water and exert no antitumor effects (2). On the other hand, it has been demonstrated by Tomoda et al. that 2-amino-phenoazine, cinnabaric acid, and new kinds of phenoazines could be synthesized by reacting o-aminophenol or its derivatives with bovine hemoglobin and that these phenoazine compounds were relatively more soluble in water than those synthesized chemically (3–5). Tomoda et al. (5) also found that a relatively water-soluble phenoazine was produced when 2-amino-5-methylphenol was reacted with human or bovine hemoglobin. This compound was identified as Phx (2).

A cell line designated HAL-01 was established in our laboratory from the blood cells of a patient with acute lymphoblastic leukemia characterized by the chromosomal translocation t(17;19)(q21;p13) (6). Immunophenotyping of this cell line revealed that the cells expressed B-lineage antigens (CD10+, CD19+, CD20+, and CD22+). The HAL-01 cells were s.c. transplantable into untreated nude mice, in which they grew as a s.c. tumor (6). Therefore, HAL-01-bearing mice are useful to evaluate the effects of drugs developed to treat human leukemias.

Several lines of evidence have shown that Phx has some growth-inhibitory effects on the epidermoid carcinoma cell line KB (7). In this study, we investigated the effect of Phx on the proliferation and apoptosis of various human leukemia cell lines and its antitumor effect in HAL-01-bearing nude mice. We also evaluated the toxicity of this compound, especially on normal hematopoietic progenitors. The results indicated that Phx strongly inhibited cell proliferation and induced apoptosis of various human leukemia cells in vitro. In addition, Phx markedly reduced the growth of the tumor in mice that had received a leukemia cell transplant.

MATERIALS AND METHODS
Reagents.
Phx was synthesized and purified as described previously (5). Phx was dissolved in ethanol at a concentration of 25 ms and diluted with PBS before use.

Cell Lines and Cell Culture.
Human erythroleukemia cells (K562) and human myeloid leukemia cells (HL-60) were obtained from the American Type Culture Collection, whereas the human lymphoblastic leukemia HAL-01 cell line was established in our laboratory. These cells were maintained in RPMI 1640 supplemented with 10% FCS.

Cell Proliferation Assay.
A WST-1 Cell Counting Kit (Dojin East, Tokyo, Japan) was used to assess cell proliferation, and absorption was measured at 450 nm. The viability of cells was determined by the trypan blue dye exclusion test.

Detection of Apoptosis.
FITC-conjugated APO2.7 mAb (clone 2.7), which was raised against the M6,38,000 mitochondrial membrane protein (7A6 antigen) expressed by cells undergoing apoptosis, was purchased from Immunotech (Marseille, France). Cells were washed twice with PBS containing 5% FBS. Cells (1 × 10^6) suspended in 0.5 ml of PBS containing 5% FBS and 0.02% NaN3 were incubated with the mouse phycoerythrin cyanine 5-conjugated APO2.7 mAb for 30 min at 4°C (8). Immunofluorescence was analyzed using an EPICS XL2 flow...
RESULTS

Inhibitory Effect of Phx on the Proliferation of Human Leukemia Cell Lines. Phx is a phenoxazine derivative whose primary structure is shown in Fig. 1. We examined whether Phx inhibited the proliferation of various leukemia cell lines using the WST-1 assay. To examine the effect of Phx on cell proliferation, culture of K562, HL-60, or HAL-01 cells was added in medium with various doses of Phx. As shown in Fig. 2, exposure to increasing concentrations of Phx for 72 h resulted in a dose-dependent inhibition of cell proliferation of all leukemia cells used in this study. The IC50 was 73 μM for K562, 81 μM for HL-60, and 93 μM for HAL-01 (Fig. 3).

Phx Induces Apoptosis of Leukemia Cells. We used mAb APO2.7, which was raised against the M, 38,000 mitochondrial membrane protein 7A6 antigen, to detect apoptotic cells because 7A6 antigen is selectively expressed on the mitochondrial membrane in cells undergoing apoptosis (8, 9). There were no detectable apoptotic cells among untreated K562, HL-60, or HAL-01 cells. In contrast, Phx induced apoptosis of each leukemia cell line in a dose-dependent manner (Fig. 4). However, there were some differences in the degree of apoptosis among the cell lines examined. An additional confirmatory experiment, such as terminal deoxynucleotidyl transferase-mediated nick end labeling assay using the Apoptag kit, also demonstrated similar results (data not shown) and supported this conclusion.

Antitumor Effect in Vivo. We studied the antitumor effect of Phx in HAL-01-bearing nude mice. The tumor was observed 4–5 weeks after HAL-01 cells were injected into the back of 6-week-old female nude mice (Fig. 4). In this study, we started to treat these animals 6 weeks after tumor inoculation, once transplantability was confirmed (tumor weight was 50–100 mg). These animals were treated with Phx s.c. once a week at the 2.5 and 5 mg/kg dose levels until 11 weeks after tumor inoculation; therefore, there was a total of six injections, whereas control mice were injected with 0.9% NaCl solution on the same schedule. In the control group, tumors grew rapidly, although the tumor size varied greatly among animals (Fig. 5). In contrast, tumor growth was markedly inhibited in both experimental groups. The tumor disappeared completely in the 5 mg/kg treatment group (Fig. 5). However, no significant differences in the survival period were observed among these groups because all animals survived more than 4–5 months after treatment (data not shown).

![Chemical structure of Phx.](image)

**A**: 2-amino-5-methylphenol  
**B**: quinoimine  
**C**: Phx

cytometer (Coulter Japan, Tokyo, Japan). The terminal deoxyribonucleotidyl transferase-mediated nick end labeling assay was performed using the Apoptag kit-Fluorescein (Oncor, Gaithersburg, MD) according to the manufacturer’s protocol.

Mice. The animals (BALB/c nu/nu) used in this study were housed under special pathogen-free conditions at room temperature and 55% humidity with a circadian light rhythm of 12 h and given standard diet pellets and tap water. Ten million viable HAL-01 cells in 0.1 ml of were injected s.c. into the back of 6-week-old female nude mice.

**Antitumor Effects.** For chemotherapy experiments, each control or drug-treated group included five mice bearing HAL-01 cells. HAL-01 cells were implanted on day 0, and the s.c. tumor volume was recorded twice a week until the end of the treatment. The tumor volume (TV) was calculated for each individual mouse from the recorded caliper measurements of the longest (L) and shortest (W) diameters (expressed in mm) of the approximately ellipsoid tumor, according to the following formula: TV (mg) = (W2 × L)/2. Drug treatment started when mean tumor weight was 50–100 mg. Phx was administered s.c. 6 times/week. A single preparation was used for Phx as 0.5 mg/mL. Experimental mice were given different volumes/kilogram (2.5 and 5 mg/kg Phx), whereas control mice were injected with the 0.9% NaCl solutions.

**WBC Count.** Twenty μl of whole blood were obtained from the orbital sinus of adult mice and immediately diluted with 1.98 ml of diluent using the Unopette-5854 microcollection system (Becton Dickinson, Rutherford, NJ). The WBCs were counted manually in duplicate using a hemocytometer counting chamber.

**Methylcellulose Culture.** Clonogenic assays were performed as described elsewhere (9). Briefly, 10,000 (bone marrow) or 500 (K562, HL-60, or HAL-01) cells were treated with Phx for 24 h and seeded in quadruplicate in conditioned medium MethoCult GF H4434 (Stem Cell Technologies, Vancouver, Canada). The blast colonies (≥50 cells) of K562, HL-60, and HAL-01 were scored on day 7. Progenitor cell-derived colonies were scored on day 14 and classified as either BFU-Es, CFU-GMs, or CFU-GEMMs, according to standard criteria (10).

**Statistical Analysis.** Statistical tests were performed using the Statview (Abacus Concepts Inc., Calabasas, CA) software package for the Macintosh. Comparisons between the experimental group and control group were made using Student’s t test or repeated measure ANOVA test. Values of P < 0.05 were considered significant.
Toxicity Studies in Vivo. The possible toxicity of Phx was also evaluated. To evaluate general toxicity, we measured body weight loss in healthy mice (10-week-old female nude mice) treated with 5 mg/kg Phx or injected with 0.9% NaCl solution weekly until the 12 week time point and compared their body weight curve. No significant body weight change was observed in either group during the observation period (Fig. 6A).

To evaluate hematological toxicity, we counted WBCs in healthy mice (10-week-old female nude mice) treated with 5 mg/kg Phx or injected with 0.9% NaCl solution weekly until the 12 week time point. There was no difference between the two groups until 6 weeks after the start of treatment; after that period, a slight reduction of the WBC count was observed in the group treated with 5 mg/kg Phx (Fig. 6B). However, hematological toxicity seemed to be mild because the WBC count was 6820 ± 1285 in the control group and 4300 ± 412 in the group treated with 5 mg/kg Phx at the end of the treatment (Fig. 6B).

Phx Inhibits Proliferation of Various Leukemia Cell Lines but not of Normal Bone Marrow Progenitors. To compare the effects of Phx on leukemia cells and its effects on normal hematopoietic cells, we examined the plating efficiency of K562, HL-60, and HAL-01 cells and the standard progenitor colony assay of normal bone marrow treated with Phx. The effect of Phx on the plating efficiency of leukemia cells was dose dependent for all cell lines we tested. Treatment with 50 μM Phx suppressed the plating efficiency of K562 by 35%, suppressed the plating efficiency of HL-60 by 22%, and suppressed the plating efficiency of HAL-01 by 93% (Fig. 6A). Furthermore, treatment with 100 μM Phx completely suppressed the plating efficiency of all of the cell lines tested (Fig. 6A). In contrast, treatment with up to 100 μM Phx did not significantly reduce BFU-Es, CFU-GMs, or CFU-GEMMs of normal bone marrow cells (Fig. 7B).

**DISCUSSION**

Although the chemically synthesized phenoxazine compounds show no antitumor effects against various types of cancer cells, this novel phenoxazine derivative, which was synthesized by reacting 2-amino-5-methylphenol with bovine hemoglobin, inhibited the cell proliferation of KB cells (human epidermoid carcinoma cells; Ref. 7). In the present study, we demonstrated that this phenoxazine derivative showed similar inhibitory effects on the proliferation of HAL-01, HL-60, and K562 cells, suggesting that Phx may have antitumor effects on any lineage including lymphoid, myeloid, and erythroid lineages. Phx even inhibited the proliferation of K562 cells, which were established from a patient with blastic crisis of chronic myelogenous leukemia and usually show resistance to various anticancer drugs (11). Therefore, Phx may be expected to be useful for the treatment of patients with various types of leukemia, including blastic crisis of chronic myelogenous leukemia.

For the clinical application of this drug, a preliminary study in healthy mice was set up to assess the toxicity of Phx. Because weekly treatment with 2.5 or 5 mg/kg Phx markedly reduced tumor growth in leukemia-bearing mice, we treated healthy mice with 5 mg/kg Phx. No obvious adverse effect including myelosuppression...
was observed in the treated mice. Furthermore, we demonstrated that the suppressive effects of Phx on human hematopoietic cells were significantly less compared with those observed on leukemic cells, suggesting a selective elimination of leukemic cells by Phx. This might indicate that Phx can be used for in vitro purging in case of auto-stem cell transplantation.

We demonstrated that Phx had a potent apoptosis-inducing effect on all of the leukemic cell lines tested. The mechanism of induction of apoptosis by Phx is unclear. BCL-2 and BAX protein levels did not change, whereas the p53 level was markedly increased after treatment with Phx (data not shown), suggesting that Phx may be involved in p53-related apoptotic pathway not through BCL-2 or BAX, possibly yet to be defined. Furthermore, although the proliferation of HAL-01 cells was significantly inhibited by 50 μM Phx, the treated cells underwent only a little apoptosis. The cell cycle assay revealed G2-M accumulation of HAL-01 cells after treatment with 50 μM Phx. This might indicate that the mechanism by which Phx inhibits the growth of leukemic cells is not only apoptosis but also cell cycle arrest. The possible mechanism of therapeutic selectivity might be due to G2-M arrest because many leukemic cells enter G2-M as compared with normal hematopoietic cells.

![Fig. 4](image_url)  
Induction of apoptosis in various leukemia cell lines after treatment with Phx. K562, HL-60, and HAL-01 cells were incubated with the indicated concentrations of Phx for 24 h. Apoptosis was examined by the cell surface expression of APO2.7, as detected by flow cytometry. The percentages of apoptotic cells are shown at the top right of each panel.

![Fig. 5](image_url)  
Effect of Phx on tumor growth in the HAL-01-bearing nude mice. Tumor-bearing mice were treated with either 5 mg/kg/week Phx (△), 2.5 mg/kg/week Phx (△), or 0.9% NaCl (○; control group) as described in “Materials and Methods.” Tumor volume in individual animals was measured and plotted versus time. The compound induces a significant reduction in s.c. tumor growth rate.

![Fig. 6](image_url)  
A, body weight of nude mice treated with either 5 mg/kg/week Phx (△), 2.5 mg/kg/week Phx, or 0.9% NaCl (○; control group). B, WBC counts of nude mice treated with either 5 mg/kg/week Phx (△), 2.5 mg/kg/week Phx, or 0.9% NaCl (○; control group).
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also caused G₂-M accumulation, similar to actinomycin D. However, in contrast to actinomycin D, Phx did not intercalate in DNA. We also demonstrated that Phx caused an accumulation of cells in the S and G₂ phases in leukemic cell lines, suggesting that Phx inhibits DNA synthesis. To confirm this speculation, we have performed a [³H]thymidine incorporation experiment and demonstrated a >50% inhibition of DNA synthesis in HAL-01 and HL60 cells 8 h after treatment with 100 μM Phx (data not shown). Because Phx does not intercalate in DNA, the manner in which Phx inhibits DNA synthesis remains to be resolved.

Horton et al. (12) reported that N-substituted phenoxazines increased accumulation of vinblastine and vincristine. Moreover, 2-chloro-N10-substituted phenoxazine can inhibit P-glycoprotein-mediated efflux of vinblastine in multidrug-resistant cells (13). We will further investigate the antitumor effects of Phx administered in combination with other anticancer drugs and the effects of Phx on multidrug-resistant leukemic cells.

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REFERENCES

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