Anticancer Effects of Phenoxazine Derivatives Revealed by Inhibition of Cell Growth and Viability, Disregulation of Cell Cycle, and Apoptosis Induction in HTLV-1–Positive Leukemia Cells

Naoko Miyano-Kurosaki,† Kou Ikegami,† Kunihiko Kurosaki, Takahiko Endo, Hoshimi Aoyagi, Mari Hanami, Jun Yasumoto, and Akio Tomoda,*

1Department of Life and Environmental Sciences, Faculty of Engineering, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan
2Department of Legal Medicine, School of Medicine, Toho University, 5-21-16 Ohmori-Nishi, Ota-ku, Tokyo 143-8540, Japan
3Department of Forensic Medicine and 4Department of Biochemistry, Tokyo Medical University, Shinjuku 6-1-1, Shinjuku-ku, Tokyo 160-8402, Japan

Received December 27, 2008; Accepted March 15, 2009

Abstract. Adult T-cell leukemia (ATL) is a malignant tumor of human CD4+ T cells infected with a human retrovirus, T lymphotropic virus type 1 (HTLV-1). The aim of the present study was to investigate the apoptotic effects of phenoxazines, 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 a T cell leukemia cell line from ATL patients, MT-1 cells; HTLV-1 transformed T-cell lines, HUT-102 cells and MT-2 cells; and an HTLV-1–negative rat sarcoma cell line, XC cells. Among these phenoxazines, Phx-3 at concentrations of less than 10 μg/ml extensively inhibited growth and cell viability; arrested cell cycles at sub G0/G1 phase; and augmented apoptosis of MT-1, HUT-102, and MT-2 cells. However, these phenoxazines did not affect the cell viability of an HTLV-1–negative rat sarcoma cell line, XC cells, and phytohemaggutinin-activated human peripheral blood mononuclear cells, although they markedly inhibited the growth of these cells. The transmission of HTLV-1 from HTLV-1–positive cells (MT-2 cells) to HTLV-1–negative cells (XC cells) was considered to be prevented by Phx-1, Phx-2, or Phx-3 because the syncytium formation between these cells was inhibited markedly in the presence of these phenoxazines. The present results suggest that Phx-1, Phx-2, and, in particular, Phx-3 may be useful as therapeutic agents against ATL, which is extremely refractory to current therapies.

Keywords: adult T-cell leukemia, HTLV-1, phenoxazine, apoptosis, syncytium formation

Introduction

Adult T-cell leukemia (ATL) is a fatal malignancy of T lymphocytes associated with the infection of human T-cell lymphotropic virus type 1 (HTLV-1) (1, 2), which has been shown to be endemic in the southern part of Japan; the Caribbean; and parts of Africa, South America, Melanesia, and the Middle East (3, 4). ATL develops after a latency period of 30 to 40 years. It is recognized to precede by oligoclonal expansion of HTLV-1–infected T cells, where the virus expands via cell-to-cell transmission (5, 6). However, it is very difficult to cure patients with ATL by current therapeutic methods, in spite of various trials including chemotherapeutic treatment, allogeneic hematopoietic stem cell transplantation, and so on. In the light of recent findings on cellular apoptosis, it appears that it may be possible to prevent the propagation of viruses in virus-infected host cells by inducing cellular apoptosis. From this viewpoint, it is conceivable that the drugs causing apoptosis in cancer cells may prevent the cell-to-cell...
transmission of HTLV-1 in ATL patients, resulting in successful ATL treatment.

ATL cell lines can be classified into two categories. One type is the T cell lines of leukemic cell origin, which were established from ATL patients (7), and are characterized by the negative expression of the viral transcriptional transactivator (Tax) playing a pivotal role in viral replication, transformation, and gene regulation in the cells (8). MT-1 cells belong to this category with negative expression of Tax. The other category of cells is the HTLV-1-transformed T cell lines established by an in vitro coculture protocol (1, 9), and these cells are Tax-positive. MT-2 cells and HUT-102 cells belong to this category. The inhibitory effects of synthetic retinoid (10), epigallocatechin-3-gallate (11), a derivative compound of imidazoquinoline (12), and brown algae fucoxanthin (13) on these two different categories of cell lines have been reported. These reports described that these compounds induced apoptosis in these ATL cells, suggesting that the development of useful therapeutic agents against ATL may be feasible. It remains, however, unclear whether the expansion of HTLV-1 was inhibited or not in these cases.

On the other hand, Tomoda and the colleagues (14, 15) synthesized several phenoxazine compounds by reacting o-aminophenol and its derivatives with human or bovine hemoglobin solution. Among them, 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) have been shown to exert various biological activities, including anticancer effects (16–20), antiviral effects (21–23), and immunosuppressive effects (24–26). In particular, Phx-3 exerted strong anticancer activity against various cancer cells from human melanoma (16), pancreatic cancer (17), neuroblastoma (18), glioma (19), and multiple myeloma (20), exerting apoptotic activity against these cancer cell lines (16–20). Moreover, Phx-1 inhibited human leukemia cells (27) and HTLV-1–negative T cell lymphoblastoid cells (28). Taking these findings into account, it is possible that Phx-1, Phx-2, and Phx-3 may induce apoptosis of ATL-derived cells, which can lead to prevention of HTLV-1 expansion and transmission from the host cells to the target cells.

In the present study, we investigated whether Phx-1, Phx-2, and Phx-3 may induce apoptosis of three different HTLV-1–associated ATL cells: MT-1, HUT-102, and MT-2 cells, and inhibit the transmission of the virus from the infected cells to uninfected target cells in vitro, by examining the syncytium formation between MT-2 and an HTLV-1–negative rat sarcoma cell line, XC cells.

**Materials and Methods**

**Reagents**

Phenoxazines, Phx-1, Phx-2, and Phx-3, were prepared according to the method described by Tomoda et al. (14) and Shimizu et al. (15). The chemical structures of these compounds are shown in Fig. 1. Phx-1, Phx-2, or Phx-3 was dissolved in a mixture of dimethylsulfoxide (DMSO) and ethyl alcohol (3:1) as a vehicle to make a 5 mg/ml solution, and then these solutions were added in culture medium to obtain final concentrations of 0.1, 1, and 10 μg/ml during the experiments. The final volume of vehicle in the culture medium was equivalent (0.2%) among Phx-1–, Phx-2–, or Phx-3–free and Phx-1–, Phx-2–, or Phx-3–treated cells in each experiment.

**Cell lines and cell culture**

MT-1 cells are a T-cell line of leukemia cell origin established from ATL patients (7). MT-2 cells are a HTLV-1–transformed T-cell line and constitutively expresses viral genes including Tax (9). HUT-102 cells, which were kindly provided by Prof. Naoki Yamamoto, Tokyo Medical and Dental University, School of Medicine, are a T-cell line established from a patient with ATL and constitutively expresses viral genes (1). XC is a rat sarcoma cell line. HTLV-1–infected T-cell lines, MT-1 cells, HUT-102 cells, and MT-2 cells, and an HTLV-1–uninfected rat sarcoma cell line, XC cells, were cultured in RPMI 1640 medium (Wako Pure Chemical, Osaka) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), 2 mM L-glutamine, and 80 mg/l kanamycin.

**Fig. 1.** Chemical structure of 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).
sulfate (Wako), in a humidified incubator containing 5% CO₂ and 95% air at 37°C.

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and were isolated on a Ficoll-Isopaque gradient. The cells were previously grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 80 mg/l kanamycin and treated with 10 U/ml interleukin-2 (IL-2) (Shionogi Co., Ltd., Osaka) and 5 μg/ml phytohemagglutinin (PHA) (Seikagaku Co., Tokyo), for a week, in a humidified incubator containing 5% CO₂ and 95% air at 37°C.

Cell growth and cell viability assay

For cell growth studies, MT-1 cells, HUT-102 cells, and MT-2 cells (5 × 10⁵ cells/2 ml, each); XC cells (3 × 10⁵ cells/2 ml); and PBMC (1 × 10⁶ cells/2 ml) in RPMI-1640 medium were seeded in each well of 24-well plates, cultured for 72 h at 37°C, with or without various concentrations of Phx-1, Phx-2, and Phx-3 (0, 0.1, 1, and 10 μg/ml) in a humidified incubator containing 5% CO₂ and 95% air at 37°C. Samples were collected at 24, 48, and 72 h and were analyzed for the measurement of cell growth and viability.

Cell growth was estimated microscopically by counting the number of cells by using a hemocytometer. Experiments were performed in triplicate.

Cell viability was estimated microscopically by counting the number of viable cells treated with trypan blue. Briefly, after the collection of the cells at 0, 24, 48, and 72 h, the medium of each well was replaced with 1 ml fresh medium including 100 μl of trypan blue. Then, the number of viable cells was counted by microscopy. Experiments were performed in triplicate.

Apoptosis and necrosis detection

The detection of apoptosis and necrosis was performed by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit (Merck, Ltd., Calbiochem., CA, USA). MT-1 cells (5 × 10⁵ cells/2 ml) were treated with 0, 0.1, 1, and 10 μg/ml Phx-1, Phx-2, or Phx-3 in 6-well flat-bottomed plates. 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 1.25 μl of fluorescein isothiocyanate (FITC)-labeled annexin V (Annexin V-FITC) and 10 μl of 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 (FACSCalibur; Becton, Dickinson Co., Franklin Lakes, NJ, USA). Annexin V-FITC binding and PI staining were monitored using a FITC signal detector (FL1, 518 nm) and a phycoerythrin emission signal detector (FL2, 620 nm), respectively.

RNA purification and RT-PCR

MT-1 cells (5 × 10⁵ cells/2 ml) were treated with 0, 0.1, 1, and 10 μg/ml of Phx-1, Phx-2, or Phx-3. After 24 h, the cells were collected and total cellular RNA was extracted from the cells with the GenElute mammalian total RNA kit (Sigma Aldrich Co., St. Louis, MO, USA). Then, reverse transcription (RT)-PCR assays were carried out with the PrimeScript™ RT reagent kit (Takara Bio Inc., Shiga). The levels of mRNA of caspase family (caspase-3, -5, -8, and -9 and Apaf-1) were analyzed by PCR kit for human apoptotic genes set-5 (Maxim Biotech, Inc., CA, USA). To analyze the extent of the RNA expression in the cells, the products from the RT-PCR amplified RNAs were electrophoresed through a non-denaturing 2% agarose gel in TAE buffer and were stained with ethidium bromide (Merck Ltd., Darmstadt, Germany). The results of caspasas-3, -5, -8, and -9 and Apaf-1 were quantified and normalized with glyceraldehydes 3-phosphate dehydrogenase (GAPDH).

Caspase-3 assay procedure

The activity of caspase-3 in MT-1 cells was estimated by the reaction with the CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (BioVision Research Products, CA, USA). Briefly, MT-1 cells (5 × 10⁵ cells/2 ml) were suspended in medium and incubated in 6-well flat-bottomed plates in the presence or absence of 10 μg/ml Phx-1, Phx-2, or Phx-3 for 16 h in a humidified incubator containing 5% CO₂ and 95% air at 37°C. Then, the cells were collected, washed once with PBS (pH 7.4), treated with 1 μl aliquot of FITC-DEVD-FMK, and were incubated at 37°C for 1 h in the dark. Then, the cells were washed twice with washing buffer and were moved to a 96-well flat bottomed plate. These samples were analyzed using a multi-detection microplate reader (Spectra Max Gemiine XS; Molecular Devices Co., Tokyo) at a wavelength of 485 nm excitation / 535 nm emission. The activity of caspase-3 was evaluated in triplicate and corrected by protein contents.

Cell cycle analysis

MT-1 cells (1.5 × 10⁶ cells/ml) in RPMI-1640 medium supplemented with antibiotics were incubated in 12-well flat-bottomed plates at 37°C for 24 h in a humidified incubator containing 5% CO₂ and 95% air at 37°C. The supernatant was replaced with 2 ml fresh medium, and then a final concentration of 10 μg/ml Phx-1, Phx-2, or Phx-3 was added to each well. Two hours before collecting the cells at the indicated time (24, 48, and 72 h), the cells were labeled with bromodeoxyuridine (BrdU) and were incubated for a further 2 h. The BrdU-
pulsed cells were collected at the indicated time, fixed, and permeabilized with BD Cytofix/Cytoperm buffer (Becton, Dickinson Co.). Then, the cells were treated with DNase for 1 h at 37°C and stained with FITC-labeled anti-BrdU antibody. The cells were stood for 20 min at room temperature in the dark, washed with Perm/Wash buffer, and then treated with actinomycin D. Cell cycle in the cells was analyzed by a flow cytometer using the FITC BrdU Flow Kit (Becton, Dickinson Co.).

Syncytium formation assay
The syncytium formation assay consisted of a coculture of HTLV-1–bearing MT-2 cells and indicator XC cells (29–31). XC cells were collected after treatment with 0.25% trypsin in 1 mM EDTA, washed three times by PBS, and then resuspended in RPMI 1640 medium with antibiotics. The cells (3 × 10^5 cells/ml) were incubated in 24-well flat-bottomed plates for 24 h in a humidified incubator containing 5% CO₂ and 95% air at 37°C. Then the medium was removed and 3 × 10⁵ cells/ml MT-2 cells were added to each well. Each of the Phx’s was added to separate wells at the final concentrations of 0.1, 1.0, and 10 μg/ml at the start of cocultivation of XC cells and MC-2 cells. After 24, 48, and 72 h, the cells were washed once with PBS and then subjected to microscopic analysis, without or with staining by 0.5% methylene blue and 0.125% crystal violet (Wako Pure Chemical Co., Ltd., Osaka) in methanol. Syncytia were counted in five fields of vision at random by phase-contrast inverted microscopy (magnification, ×100). Syncytia containing more than five nuclei were counted under an inverted microscope. In this assay system, the HTLV-1–bearing cell line MT-2 routinely formed 500 to 800 syncytia per 10⁶ indicator XC cells. All experiments were performed in triplicate.

Results

Inhibition of growth and viability of MT-1, MT-2, HUT-102, and XC cells by Phx-1, Phx-2, or Phx-3
Figure 2A shows the effect of various concentrations of Phx-1, Phx-2, or Phx-3 (0, 0.1, 1, and 10 μg/ml) on the growth and viability of MT-1 cells, an HTLV-1–positive ATL cell line, during 72 h. At the dose of 10 μg/ml, these phenoxazines suppressed the growth (solid bars in the figure) and viability (broken lines and solid dots in the figure) of MT-1 cells, time- and dose-dependently. In particular, the growth was completely suppressed by Phx-1, Phx-2, or Phx-3; and the viability was suppressed by 60% for Phx-1, 80% for Phx-2, and 70% for Phx-3 at 72 h. At the dose of less than 1 μg/ml of Phx-1, Phx-2, or Phx-3, the viability of MT-1 cells was not affected at all, while the growth of the cells was significantly inhibited time- and dose-dependently by these phenoxazines during 72 h.

HUT-102 cells, another HTLV-1–infected T cell line derived from a ATL patient, were also sensitive to 10 μg/ml Phx-1, Phx-2, or Phx-3 (Fig. 2B), i.e., the cell growth (bars) and cell viability (dots and lines) were extensively suppressed by these phenoxazines, both time- and dose dependently.

At 72 h, the viability (solid dots and broken lines in Fig. 2C) of MT-2 cells was suppressed completely at the dose of 10 μg/ml Phx-3, but was not by Phx-1 and Phx-2 at this dose. The cell growth (bars in the figure) of MT-2 cells was suppressed by Phx-1, Phx-2, and Phx-3. Among these phenoxazines, Phx-3 exerted the strongest inhibitory effects to the cell growth and viability of MT-2 cells at the 10 μg/ml dose.

Although the growth of XC cells, a rat sarcoma cell line, at 72 h was extremely suppressed by 10 μg/ml Phx-1, Phx-2, or Phx-3, the cell viability was not affected by Phx-1 and Phx-2 (Fig. 2D). The decrease of viability of XC cells was slight (approximately 20% reduction at 72 h).

The viability of PHA-activated PBMC was not suppressed by 10 μg/ml Phx-1 and Phx-2 (Fig. 2E), but moderately suppressed by 10 μg/ml Phx-3 (approximately 38% at 72 h), while the growth of PBMC was significantly suppressed by these phenoxazines both time- and dose-dependently, indicating that PBMC is far more resistant to these phenoxazines than ATL cell lines, in terms of cell viability.

Apoptosis and necrosis
Since the growth and viability of MT-1 were significantly affected by Phx-1, Phx-2, or Phx-3 (Fig. 2A), we investigated whether the apoptosis of MT-1 cells might be caused by these phenoxazines, by flow cytometry using annexin V–FITC binding and PI staining methods (Fig. 3). 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 treated with vehicle alone during 24–72 h (the left columns) (Fig. 3). 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 MT-1 cells treated with 10 μg/ml Phx-1, Phx-2, or Phx-3 (Fig. 3). The rate of increase in late phase apoptotic/necrotic cells was time-dependent in each sample with Phx-1, Phx-2, or Phx-3. These results suggest that Phx-1, Phx-2, and Phx-3 induce apoptosis of MT-1 cells.
Fig. 2. Cell growth and viability of HTLV-1–positive T-cell cells, HTLV-1–uninfected rat sarcoma cells, and phytohemagglutinin (PHA)-activated human peripheral blood mononuclear cells (PBMC), in the presence of Phx-1, Phx-2, or Phx-3. Cell growth (bars in the figure) and viability (lines and circles) of HTLV-1–positive T-cell lines (MT-1 cells, HUT-102 cells, and MT-2 cells); HTLV-1–uninfected rat sarcoma cells (XC cells); and PHA-activated PBMC were examined in the presence of various concentrations (0, 0.1, 1, and 10 µg/ml) of Phx-1, Phx-2, or Phx-3 for 72 h. A: MT-1 cells, B: HUT-102 cells, C: MT-2 cells, D: XC cells, E: PHA-activated PBMC.
We investigated the effect of Phx-3 on the levels of transcripts of caspase family enzymes and Apaf-1 in MT-1 cells at 24 h (Fig. 4). It was found that the levels of the mRNAs of caspase-3, -8, and -9 and Apaf-1 were not altered even in the presence of 10 \( \mu g/ml \) Phx-3 after 24 h. In spite of these results, the activity of caspase-3 in MT-1 cells was enhanced extensively (Fig. 5) when the cells had been incubated with 10 \( \mu g/ml \) Phx-1, Phx-2, or Phx-3 for 16 h. These results suggest that the activation of caspase-3 in MT-1 caused by phenoxazines may be due to the post-translational events including the depolarization of mitochondria (32 – 34) and may be compatible with the apoptosis in MT-1 cells as shown in Fig. 3.

**Caspase-3 activity**

We investigated the effect of Phx-3 on the levels of transcripts of caspase family enzymes and Apaf-1 in MT-1 cells at 24 h (Fig. 4). It was found that the levels of the mRNAs of caspase-3, -8, and -9 and Apaf-1 were not altered even in the presence of 10 \( \mu g/ml \) Phx-3 after 24 h. In spite of these results, the activity of caspase-3 in MT-1 cells was enhanced extensively (Fig. 5) when the cells had been incubated with 10 \( \mu g/ml \) Phx-1, Phx-2, or Phx-3 for 16 h. These results suggest that the activation of caspase-3 in MT-1 caused by phenoxazines may be due to the post-translational events including the depolarization of mitochondria (32 – 34) and may be compatible with the apoptosis in MT-1 cells as shown in Fig. 3.

**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 (35), we studied the induction
Fig. 7. Microscopic aspects of the syncytium cell formation between MT-2 cells and XC cells without (control: A, B) and with Phx-1 (C, D), Phx-2 (E, F), or Phx-3 (G, H). The open circles in the figures show the syncytium formation between these cells (Fig. 7: A – H). The syncytium formation between MT-2 cells and XC cells, which were cocultivated in the presence or absence of phenoxazines for 72 h, was examined microscopically without staining (left columns: A, C, E, G) or after staining with 0.5% methylene blue and 0.125% crystal violet (the right columns: B, D, F, H) (magnification, ×100).
of cell cycle arrest in MT-1 cells in the presence of 10 µg/ml Phx-1, Phx-2, or Phx-3 for 72 h. As shown in Fig. 6, extensive changes in cell cycle arrest were indicated in MT-1 cells with Phx-1, Phx-2, or Phx-3 after 72 h. Namely, the percentage of the sub-G₀/G₁ phase arrest increased to 80%, 50%, and 70%, for MT-1 cells with Phx-1, Phx-2, and Phx-3, respectively, while that for MT-1 cells without these phenoxazines (control) was only 20%, after 72 h. These results suggest that Phx-1, Phx-2, and Phx-3 cause the apoptosis of MT-1, which is consistent with the results in Fig. 3. However, there were only small changes in population of the S phase after the treatment of the cells with these phenoxazines.

**Inhibition of Phx-3 against cell-to-cell transmission of HTLV-1**

It has been demonstrated that HTLV-1 infects in a cell-to-cell manner in patients with ATL (5, 6), and that syncytium formation is characteristic for the viral transmission between the host cells and the target cells (7, 9, 29). Figure 7, A – H, shows the microscopic analysis of the syncytium formation between HTLV-1–positive MT-2 cells and HTLV-1–negative XC cells in the control experiments without phenoxazines (Fig. 7: A and B) and in the experiments with 10 µg/ml Phx-1 (Fig. 7: C and D), Phx-2 (Fig. 7: E and F), or Phx-3 (Fig. 7: G and H). As shown in Fig. 7A, in the samples without staining (magnification: ×100), five syncytium formations between these cells were indicated. When the samples were stained with methylene blue and crystal violet, these syncytium formations were indicated more clearly (Fig. 7B). When XC cells and MT-2 cells were cocultivated with Phx-1 (Fig. 7: C and D), Phx-2 (Fig. 7: E and F), or Phx-3 (Fig. 7: G and H), the syncytium formations were extremely reduced.

We further studied the effects of various concentrations of phenoxazines on the syncytium formations between these cells for 72 h. As shown in the histograms of Fig. 8, the syncytium formations between MT-2 cells and XC cells were dose-dependently suppressed by these phenoxazines at 24, 48, and 72. In particular, the syncytium formations were significantly suppressed by 10 µg/ml Phx-1 and Phx-2 at each time. As for 10 µg/ml Phx-3, there were no syncytium formations at 72 h. The present results suggest that cell-to-cell HTLV-1 transmission would be inhibited by Phx-1, Phx-2, and Phx-3.

**Discussion**

We performed this study to examine the inhibitory effect of Phx-1, Phx-2, and Phx-3 on the growth and viability of HTLV-1–infected T-cell lines, MT-1 cells, HUT-102 cells, and MT-2 cells. The growth of MT-1 cells, HUT-102 cells, and MT-2 cells was extensively suppressed by Phx-1, Phx-2, and Phx-3 time- and dose-dependently (Fig. 2: A – C). However, inhibitory effects of these phenoxazines on the viability differed according to the cell line, that is, at 72 h, the viability of MT-1 cells was suppressed 60% by Phx-1, 80% by Phx-2, and 70% by Phx-3 (Fig. 2A). The viability of HUT-102 cells was decreased 20% by Phx-1, 25% by Phx-2, and 60% by Phx-3 (Fig. 2B), while that of MT-2 cells was not affected by Phx-1 or Phx-2 at all, but almost completely suppressed by Phx-3 at 72 h (Fig. 2C). These results indicate that HTLV-1–positive T-cells are sensitive to the phenoxazine compounds like Phx-1, Phx-2, and in particular, Phx-3, in terms of cell growth and viability.

On the other hand, the XC cells that were derived from rat sarcoma and HTLV-1–negative, were insensitive to phenoxazine treatment in terms of cell viability (Fig. 2D), although cell growth was extensively suppressed by Phx-1, Phx-2, or Phx-3 at the dose of 10 µg/ml. This result indicates that XC cells may be suitable target cells for studying syncytium formation between HTLV-1–positive cells (host cells) and HTLV-1–negative cells (target cells). The viability of PHA-activated PBMC was not affected by Phx-1 or Phx-2, but was moderately decreased by 10 µg/ml Phx-3, suggesting that PBMC, which includes normal T cells, is relatively resistant to these phenoxazines. This fact may be important because the selective induction of
apoptosis in HTLV-infected ATL cells may be feasible by the administration of Phx-1, Phx-2, or Phx-3, without causing any adverse effects on normal T cells. The cell growth of PBMC was greatly inhibited both time- and dose-dependently by Phx-1, Phx-2, or Phx-3 (Fig. 2E, bars in the figure). This result is consistent with the view that these phenoxazines may play an immuno-suppressive role (24–26).

We also observed that apoptosis of MT-1 cells was time-dependently induced by 10 μg/ml Phx-1, Phx-2, or Phx-3 (Fig. 3). Furthermore, the apoptosis of MT-1 cells was compatible with the activation of caspase-3 (Fig. 5), an executing enzyme of the apoptosis, in cells treated with Phx-3, although the mRNA levels of the caspase family enzymes, including caspase-3, -5, -8, and -9 and Araf-1, were not altered by this phenoxazine (Fig. 4), suggesting that posttranslational events including the activation of caspase-3 in the cells, are critical to apoptosis induction (32–34). We also observed that the cell cycle arrest at the sub-G0/G1 phase, which corresponds to apoptotic cells, was induced in MT-1 cells by 10 μg/ml Phx-1, Phx-2, or Phx-3 (Fig. 6). Thus the cell cycle arrest and induction of apoptosis may prevent the propagation of the provirus of HTLV-1, which is integrated in the genomic DNA of ATL cells, possibly by inhibiting the replication of HTLV-1 provirus on one hand and by eliminating selectively the HTLV-1 positive ATL cells on the other hand.

It has been recognized that HTLV-1 is transmitted through the cell-to-cell interaction between HTLV-1–bearing cells and target cells (5, 6), resulting in syncytium formation (29–31), where the viral envelop glycoprotein gp46-197 expressed on HTLV-1–bearing cells interacts with 71-kDa heat shock cognate (HSC) protein expressed on the target cell surface (36). Therefore, the examination of syncytium formation between HTLV-1–bearing cells and the target cells is important for evaluating HTLV-1 transmission and its inhibitors. Our results demonstrate that syncytium formation was indicated between HTLV-1–positive MT-2 cells and HTLV-1–negative XC cells (Fig. 7) and that it was significantly inhibited by the addition of Phx-1, Phx-2, or Phx-3, time- and dose-dependently, particularly by 10 μg/ml of these phenoxazines (Figs. 7 and 8). These results suggest that the transmission of HTLV-1 from the host cells to the target cells could be suppressed by Phx-1, Phx-2, or Phx-3. However, it is currently unclear whether the inhibition of syncytium formation is due to the decreased number of viable MT-2 cells or due to the direct inhibition of HTLV-1 in MT-2 cells in the presence of these phenoxazines.

Phx-1, Phx-2, and Phx-3 show a variety of biological activities such as apoptotic effects against cancer cells (16–20), antiviral effects (21–23), and immuno-suppressive effects (24–26), the mechanism for which are diverse and are not sufficiently clarified. Since actinomycin D is a phenoxazine compound and strongly intercalates DNA (37), Phx-1, Phx-2, and Phx-3 could be anticipated to intercalate DNA. However, Phx-1 does not intercalate DNA (38), but Phx-3 does intercalate DNA moderately (39). Such differences may be related to the differences in action mode among these phenoxazines; for example, the cytotoxic effects of Phx-3 on ATL derived T cells including MT-1 cells, Hut-102 cells, and MT-2 cells were strongest among Phx-1, Phx-2, and Phx-3 (Fig. 2: A–C). Phx-1, Phx-2, and Phx-3, which are produced by biological reactions with human hemoglobin (40), bovine hemoglobin (14), cytochrome c and cytochrome oxidase (41), and phenoxazone synthetase (42), are in the oxidized form as is the case for actinomycin D and are relatively soluble in water. On the other hand, the chemically synthesized phenoxazines are commonly in the reduced form and almost insoluble in water. The oxidized or reduced state of phenoxazines may be reflected to the differences in the strength of biological activities because Phx-1, Phx-2, and Phx-3 exert strong anticancer effects, but the chemically synthesized phenoxazines do not, with the exception of some compounds (43, 44). The detailed mechanisms for the proliferative suppression of ATL cells and inhibition of HTLV-1 propagation in the cells caused by these oxidized phenoxazines should be clarified by further investigation.

ATL is a peripheral T-cell lymphoma, which develops after a long latency period, and is classified into 4 clinical subtypes including the acute form, chronic form, smoldering form, and ATL lymphoma (45). These ATL subtypes have different prognoses, but are very difficult to treat. In the present study, we found that phenoxazines such as Phx-1, Phx-2, and Phx-3 cause suppression of cell growth and cell viability, arrest of cell cycles, induction of apoptosis, and decreased syncytium formation. In addition, these phenoxazines seem to exert few adverse effects in vivo, as shown by Mori et al. (46), Shimamoto et al. (27), Azuine et al. (47), and Miyano-Kuroiwa et al. (48). These results strongly support the view that Phx-1, Phx-2, and Phx-3 may applicable as a specific therapy for patients with ATL.

**Acknowledgments**

We are grateful to Professor J. Patrick Barron (International Medical Communications Center of Tokyo Medical University) for reviewing the English of this manuscript. The present research was supported by the fund of the Private University Strategic Research Based Support from the Ministry of Education, Culture, Sports, and Technology, Japan (2008–2012).
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