Suppression of the proliferation of cancer cell lines, KB-3-1 and K562 cells preceded by a decrease in intracellular pH caused by phenoxazine derivatives

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Abstract. The intracellular pH (pHi) of cancer cells such as the KB-3-1 (human epidermoid carcinoma cell line) and K562 cells (human chronic myeloid leukemia cell line) cultured in a medium (pH 7.4) was found to be much higher (pH 7.65 and 7.8, respectively) than that of normal cells (pHi is usually ≤7.2). When a phenoxazine derivative, 2-aminophenoxazine-3-one (Phx-3) or 2-amino-4,4α-dihydro-4α-7-dimethyl-3H-phenoxazine-3-one (Phx-1) was added to these cells, pHi rapidly decreased within 20 min, dose-dependently, though the extent of the decrease of pHi was significantly larger for Phx-3 (a decrease of 0.9 units) than for Phx-1 (a decrease of 0.4 units). Phx-3 and Phx-1 caused the proliferative suppression of these cells 24 h after the addition, dose-dependently. The anti-proliferative effects of Phx-3 on KB-3-1 and K562 cells were far greater than those of Phx-1. It was proposed that the proliferative suppression of KB-3-1 and K562 cells caused by Phx-3 and Phx-1, may be preceded by a rapid and extensive decrease in pHi, which possibly influenced the intracellular homeostasis, finally causing the suppressed proliferation and apoptosis of these cancer cells. The present results suggest that the anti-cancer effects of Phx-3 and Phx-1 may be strengthened by the intracellular acidi-fication of cancer cells by these compounds.

Introduction

The role of intracellular pH (pHi) has recently attracted much attention with regard to cellular proliferation, oncogenic transformation and apoptotic cell death. The increase in pHi augments cellular proliferation (1,2) and increases tumorigenesis (3). However, cytoplasmic acidification suppresses cellular proliferation and promotes apoptotic cell death through mitochondrial dysfunction and the activation of endonuclease, which is responsible for the digestion of the genomic DNA (4-6). Therefore, pHi possibly plays a critical role in maintaining homeostasis in normal and cancer cells. In cancer cells, the increase of pHi was indicated in several reports (7-10), although, systemic studies on pHi in cancer cells at steady-state are lacking. Furthermore, it was reported that apoptosis in cancer cells such as Jurkat (9) and leukemia cells (HL-60 and P388) (10,11), induced by anti-cancer drugs including cycloheximide, camptothecin and etoposide, was preceded by intracellular acidification caused by these drugs. This finding suggests that compounds, which are capable of decreasing pHi and exerting certain anti-proliferative or apoptogenic effects on the cancer cells with a higher pHi are promising as an anti-cancer drug.

Phenoxazine compounds such as 2-aminophenoxazine-3-one (Phx-3) and 2-amino-4,4α-dihydro-4α-7-dimethyl-3H-phenoxazine-3-one (Phx-1) have been reported to exert anti-cancer effects against various cancer cells, by inducing the suppression of cellular proliferation and apoptotic cell death (12-15). Previously, the suppressive effects of phenoxazine on the growth of skin cancer cells promoted by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) was found in mice (16). It is also reported that Akt-phosphorylation was inhibited by certain phenoxazine derivatives in cancer cells and that this may be possibly related to the anti-cancer mechanism of phenoxazines (17-19). However, the influence of these phenoxazine compounds on pHi and the relationship between pHi and phenoxazine-induced proliferative suppression and apoptosis has not been examined. In the present study, we investigated whether phenoxazine compounds, Phx-3 and Phx-1 induce changes in pHi, thereby suppressing the proliferation of cancer cells such as KB and K562 cells.

Materials and methods

Materials. Phx-3 and Phx-1 were prepared from the reactions of 2-amino-5-methylphenol or *o*-aminophenol with bovine hemoglobin solution, according to the method described by Tomoda *et al* (20) and Shimizu *et al* (21), respectively. The

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chemical structure of these phenoxazines is illustrated in Fig. 1. Phx-3 and Phx-1 were dissolved in a mixture of dimethylsulfoxide (DMSO) and ethyl alcohol (3:1) as a vehicle to make 20 mM solution. This solution was then added to a culture medium in order to reach final concentrations of 20-100 μM during experiments.

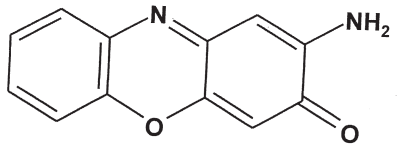
Cell line and culture condition. KB-3-1 (human chronic myeloid epidermoid carcinoma cell line) and K562 cells (human leukemia cell line) were a gift from Dr Michel M. Gottesman (NCI, Bethesda, MD) and Professor Yoshikazu Sugimoto (Kyoritsu Pharmaceutical University, Tokyo). The cells were cultured in MEM and 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) in a humidified incubator containing 5% CO_2 and 95% air at 37˚C.

Determination of pHi. Determination of the pHi of KB-3-1 and K562 cells was decided according to the method as described by Litman *et al* (8). Briefly, KB-3-1 or K562 cells ($4x10^7$/ml) were loaded with the pH-sensitive fluorescent probe BCECF-AM (3 μM) (Dojin Chemical Co. Ltd., Kumamoto, Japan) for 30 min at 37˚C in HEPES buffer (153 mM NaCl, 5 mM KCl, 5 mM glucose, 20 mM HEPES, pH 7.4). After being washed once with HEPES buffer, the cells were re-suspended in HEPES buffer. Cells ($3x10^6$) were treated with or without various concentrations of Phx-3 or Phx-1 for 0, 1, 2, 5, 10 and 20 min. Fluorescence was measured at an excitation wavelength of 500 nm and an emission wavelength of 530 nm, using a FP750 microplate fluorescence reader (Jasco, Tokyo). For the calibration of fluorescence, BCECF-AM-loaded cells ($3x10^6$) were suspended in pH 6.6, 7.2, 7.4, 7.8 and 8.0 calibration buffer (130 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, 10 mM Na-MOPS) and 10 μg/ml nigericin was added to equilibrate the external and internal pH. The relative fluorescence ratio values were plotted against corresponding pHi values, which allowed the determination of the unknown pHi. The calibration curve for pHi is shown in Fig. 2. With an increase of the pH, the optical density of the solution including BCECF-AM increased linearly. From this curve, it was possible to estimate the pHi of KB-3-1 and K562 cells loaded with BCECF-AM.

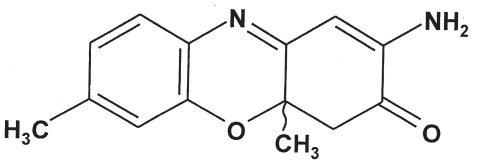
Cell growth assay. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed according to the manufacturer's instructions (Dojin). Briefly, KB-3-1 ($3x10^3$/well) or KB562 cells ($1x10^4$/well) were plated onto a 96-well plate and incubated with different concentrations of Phx-3 or Phx-1 for 24, 48, 72, 96 or 120 h. Ten microliters of 5 mg/ml MTT was added onto each well and the cells were incubated for another 4 h. For KB-3-1 cells, after removing the medium, the resulting insoluble dye was solubilized by adding 100 μl DMSO. For K562 cells, MTT was solubilized by adding 100 μl 0.04 N HCl/isopropanol. The absorbance was measured at 570-650 nm.

Results

Fig. 3A shows the time-course of changes in the pHi of human epidermoid carcinoma KB-3-1 cells caused by 100 μM Phx-3.



Phx-3



Phx-1

Figure 1. Chemical structure of Phx-3 and Phx-1.

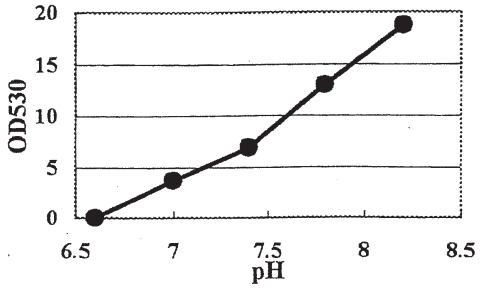


Figure 2. Calibration curve for pHi.

The pHi of KB-3-1 cells was 7.65 in the absence of Phx-3 or Phx-1 and decreased to 6.8, 1 min after the addition of 100 μM Phx-3. The decrease of pHi was maintained constantly in the presence of Phx-3, thereafter. When 100 μM Phx-1, another phenoxazine with two methyl groups at different positions in the phenoxazine ring as shown in Fig. 1, was administered to KB-3-1 cells, the pHi gradually decreased from 7.65 to 7.25 after 20 min, though the effects of Phx-1 were less than those of Phx-3 (Fig. 3B).

We studied the effects of Phx-3 and Phx-1 on the pHi change of human leukemia K562 cells. Fig. 3C and D shows the time-course of pHi change in K562 cells after treatment with 100 μM Phx-3 or Phx-1, respectively. Without these phenoxazines, the pHi of K562 cells was around 7.8. The pHi of K562 cells decreased within 1 min after the addition of Phx-3 (Fig. 3C) or Phx-1 (Fig. 3D), though the extent of the decrease was larger for Phx-3 than for Phx-1. Namely, the pHi of the K562 cells decreased from 7.8 to 6.8 and from 7.8 to 7.5, due to the addition of Phx-3 or Phx-1, respectively.

Fig. 4 shows the effects of various concentrations of Phx-3 on the pHi of KB-3-1 cells. Though the medium of the pH was maintained at 7.4, the pHi of the cells decreased from 7.65 to 7.25, 7.1 and 6.8, 20 min after the addition of 20, 50 and 100 μM Phx-3, respectively, showing that Phx-3 causes a significant decrease of pHi in KB-3-1 cells in a dose-dependent manner.

Since Phx-3 and Phx-1 have been demonstrated to exert anti-cancer effects on various types of cancer cells (12-15), we studied the effects of Phx-3 or Phx-1 on the proliferation of KB-3-1 and K562 cells. As shown in Fig. 5A, the proliferation

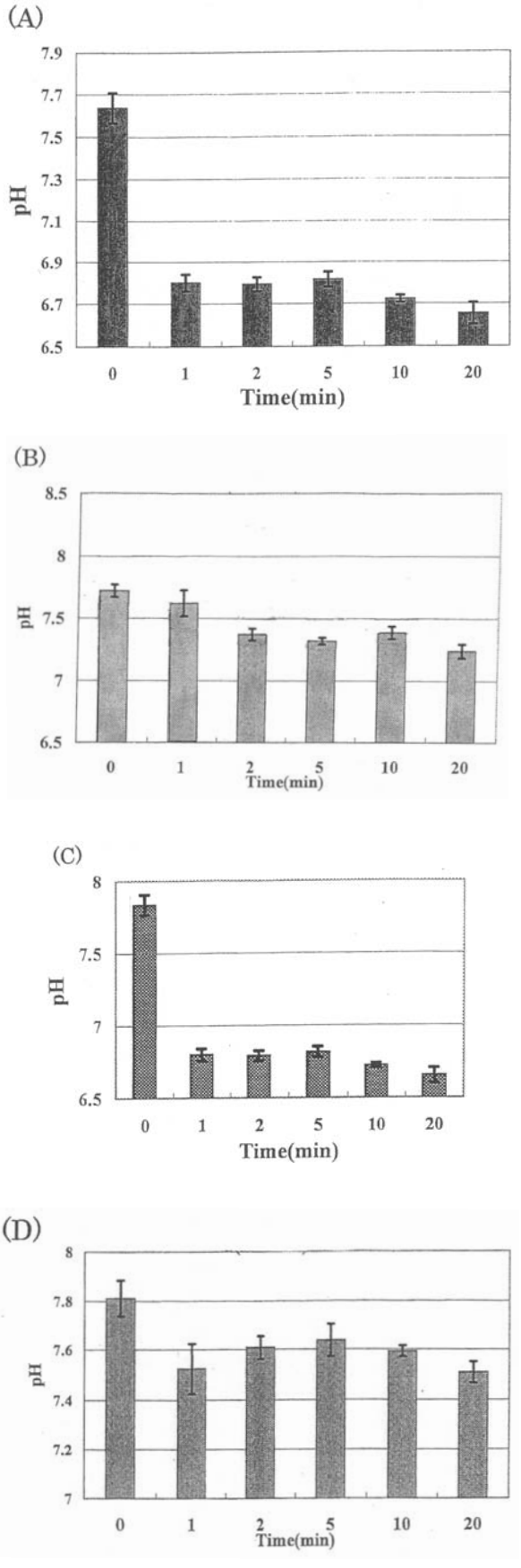


Figure 3. Time-course of pHi change in KB-3-1 and K562 cells caused by Phx-3 and Phx-1. (A) Changes in pHi in KB-3-1 cells after the addition of 100 μM Phx-3. (B) Changes in pHi in KB-3-1 cells after the addition of 100 μM Phx-1. (C) Changes in pHi in K562 cells after the addition of 100 μM Phx-3. (D) Changes in pHi in K562 cells after the addition of 100 μM Phx-1.

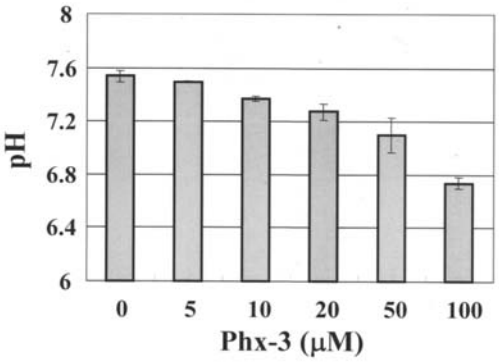


Figure 4. Effects of various concentrations (0, 5, 10, 20, 50 and 100 μM) of Phx-3 on pHi change in KB-3-1 cells.

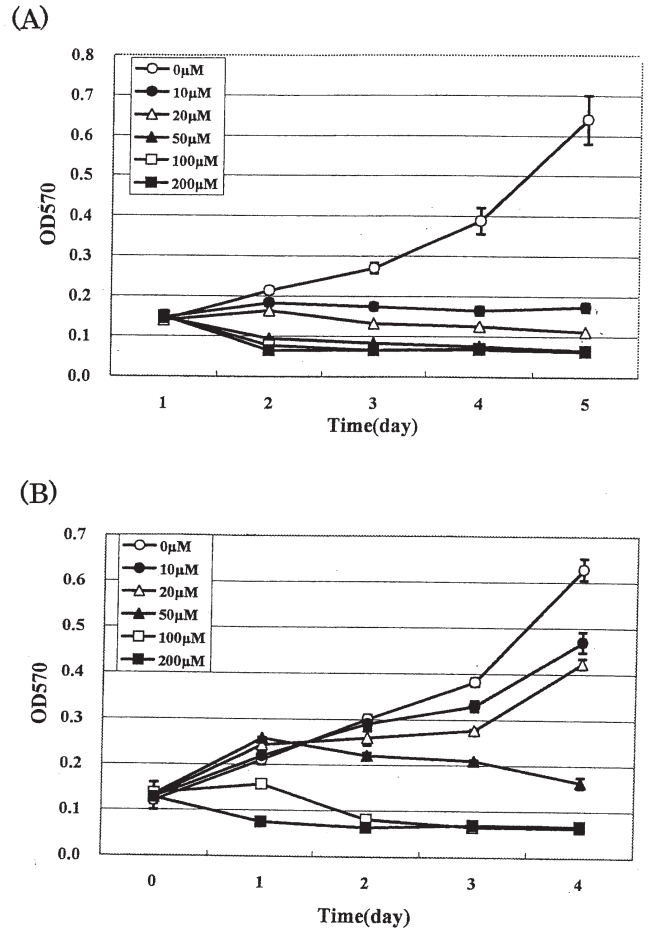


Figure 5. Effects of different concentrations (0, 5, 10, 20, 50, 100 and 200 μM) of Phx-3 and Phx-1 on the proliferation of KB-3-1 cells for 4 days. (A) Effects of Phx-3. (B) Effects of Phx-1.

of KB-3-1 cells was significantly suppressed according to the increase of the concentrations of Phx-3. When Phx-1 was administered to the KB-3-1 cells, the proliferation of the cells was suppressed by this phenoxazine, though the anti-proliferative effects of Phx-1 were less than those of Phx-3 (Fig. 5B).

From these results, the dose-response curves of cell growth of KB-3-1 cells in the presence of various concentrations of Phx-3 or Phx-1 were obtained (Fig. 6A and B). Phx-3 inhibited

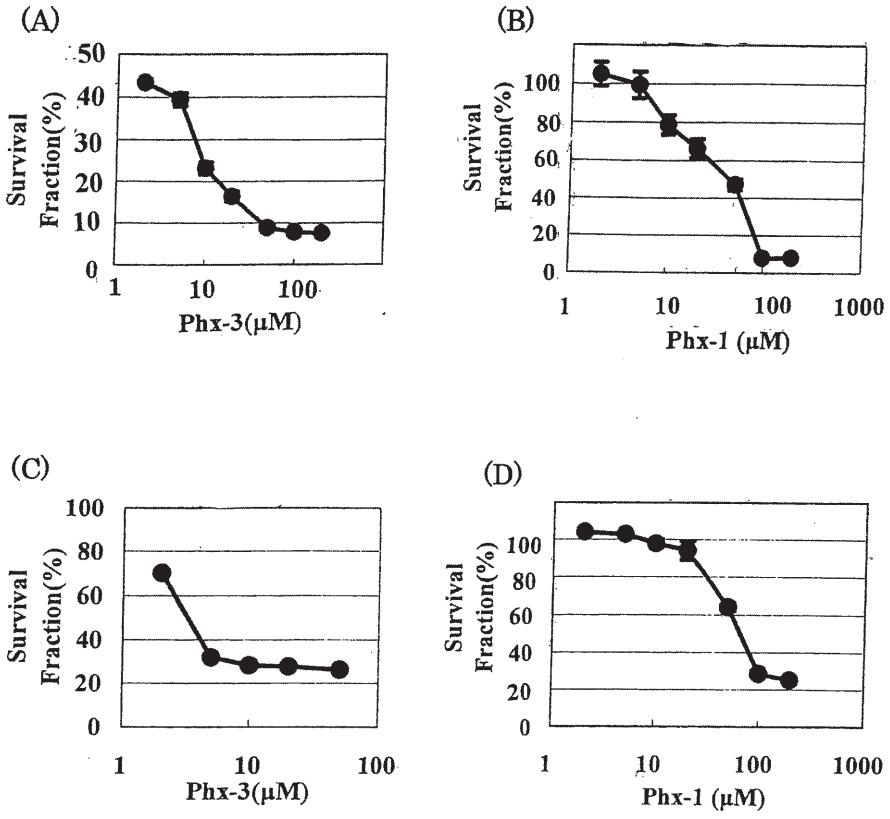


Figure 6. Effects of different concentrations of Phx-3 and Phx-1 on the proliferation of KB-3-1 and K562 cells. KB-3-1 or K562 cells were incubated with or without different concentrations of Phx-3 or Phx-1, for 24 h. The survival (%) of these cells was estimated from the data in Fig. 5 (for KB-3-1 cells) and from the data for K562 cells (not illustrated as a figure). (A) Effects of Phx-3 on KB-3-1 cells. (B) Effects of Phx-1 on KB-3-1 cells. (C) Effects of Phx-3 on K562 cells. (D) Effects of Phx-1 on K562 cells.

the proliferation of the KB-3-1 cells at lower concentrations (IC_{50}: <1 μM) (Fig. 6A). Phx-1 inhibited the proliferation of KB-3-1 cells, as well (IC_{50}: ~40 μM) (Fig. 6B). We obtained the dose-response curves of the proliferation of K562 cells in the presence of various concentrations of Phx-3 or Phx-1 (Fig. 6C and D). The inhibitory effects of Phx-3 (IC_{50}: ~3 μM) were greater than those of Phx-1 against K562 cells (IC_{50}: ~60 μM).

Discussion

We showed that the pHi of KB-3-1 and K562 cells was much higher (pHi: 7.65 for KB-3-1 cells, Fig. 3A; pHi: 7.8 for K562 cells, Fig. 3C) than that of normal cells (pHi, usually <7.2) (22,24). Our findings in the KB-3-1 and K562 cells are consistent with the general tendency towards the increased pHi in other cancer cells at steady-state, as reported by Litman *et al* (Ehrlich ascites tumor cells EHR2, pHi=7.3) (8) and by Goossens *et al* (P388 cells, pHi=7.6; HL60 cells, pHi=7.4) (10). The increased pHi in cancer cells may be consistent with the findings that increased pHi is commonly associated with proliferative stimuli and oncogene transformation (11) and that the glycolytic activity is enhanced in cancer cells, which has been known as the Warburg effects (23), since the glycolytic activity is shown to be profoundly enhanced at alkaline pH in many cells (22,25).

We found that the pHi of KB-3-1 and K562 cells was significantly decreased by phenoxazine compounds, Phx-3 and Phx-1, time- and dose-dependently (Figs. 3A-D and 4). In particular, Phx-3 and Phx-1 decreased the pHi of KB-3-1 cells within 1 and 20 min, respectively and the extent of the pHi decrease was larger for 100 μM Phx-3 (0.9 units) than for 100 μM Phx-1 (0.3 units) (Fig. 3A and B). Similar results were obtained for K562 cells with Phx-3 or Phx-1 (Fig. 3C and D). Such a rapid and extensive decrease in the pHi of KB-3-1 and K562 cells induced by these phenoxazines could cause changes in the intracellular homeostasis accompanying decreased intracellular metabolism, suppressing cellular proliferation (Fig. 6A-D), however the dysfunction of mitochondria and the activation of endonuclease II as suggested by Barry and Eastman (6), causing the apoptotic cell death. Therefore, it may be possible to say that suppressed proliferation of KB-3-1 and K562 cells caused by Phx-3 and Phx-1 may be preceded by the decrease of pHi.

Hendrich *et al* (26) indicated that phenoxazine molecules are located close to the polar/apolar interface of lipid bilayers and weakly interact with lipid bilayers, resulting in the alteration of lipid phase properties of the cell membrane. Furthermore, they suggested that such an interaction of phenoxazines with the lipid bilayers may be associated with the molecular mechanism underlying the biological activity of these compounds through changing the intramembrane conditions. Therefore, it is conceivable that the decrease of pHi in KB-3-1 and K562 cells may be taken for the consequence of perturbation of cell membranes caused by Phx-3 and Phx-1, though the detailed mechanism remains

currently unclear. Notably, the action of Phx-3 and Phx-1 seems to be opposite to that of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which interacts with phospholipid bilayers to increase pHi (1,3). Azuine *et al* (16) showed that the cancer-promoter action of TPA was cancelled by the phenoxazine compounds.

Authors Kim and Lee (27) demonstrated that amiloride, an inhibitor of the Na^+/H^+ antiporter, decreased the pHi without exerting cytotoxicity on human prostatic adenocarcinoma DU-145 cells. This result suggests that the decrease of pHi does not necessarily cause cytotoxic effects on cancer cells and that other additive mechanisms would be required to exert cytotoxic effects on cancer cells. Camptothecin is a typical drug capable of decreasing pHi and inhibiting topoisomerase II, resulting in extensive cytotoxic effects on cancer cells (10). In view of this, Phx-3 and Phx-1 seem analogous to camptothecin, since these phenoxazines are capable of decreasing pHi (Fig. 3A-D) and suppressing the proliferation of KB-3-1 and K562 cells in a dose-dependent manner (Fig. 6A-D).

In addition, Kim and Lee (27) found that when amyloride was added to DU-45 cancer cells, TRAIL-induced cytotoxicity in the cells was augmented, through the decrease of pHi. This result could be interpreted as that DU-145 cells became sensitive to TRAIL-induced cytotoxicity after the decrease of pHi. Hara *et al* (18) showed that in Jurkat lymphoblastoma cells, Phx-1 augmented the sensitivity to TRAIL as much as 100 times. Kato *et al* (13) observed that Phx-1 and Phx-3 extensively increased the sensitivity to TRAIL in human pancreatic cancer cells such as MIA-PaCa-1 and KLM-1 cells. These results suggest that the enhanced sensitivity of cancer cells to TRAIL caused by Phx-1 and Phx-3 might be associated with decreased pHi, as we found that pHi was significantly decreased in MIA-PaCa-1 and KLM-1 cells treated with these phenoxazines (our unpublished data).

In conclusion, it is likely that the proliferative suppression in KB and K562 cells caused by Phx-1 and Phx-3 might be preceded by a rapid and extensive decrease in pHi, which possibly influenced intracellular homeostasis including glycolytic metabolism, mitochondrial function, DNA transcription and genome-digestive activity of endonuclease etc., finally causing the apoptosis of these cancer cells. These results suggest that the anti-cancer effects of Phx-3 and Phx-1 may be strengthened by the intracellular acidification of cancer cells by these compounds.

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