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Report

Prevention of growth of human lung carcinoma cells and induction of apoptosis by a novel phenoxazinone, 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one

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Anti-tumor effects of a novel phenoxazinone, 2-amino-4,4-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx), which was synthesized by the reaction of 2-amino-5-methylphenol with bovine hemoglobin, were studied in terms of suppression of the proliferation of human lung carcinoma cells and apoptosis induction. When Phx was added to cultures of the human lung carcinoma cell lines A549 (adenocarcinoma) and H226 (squamous carcinoma), it caused the growth inhibition and the death of these cells. Phx also fragmented the DNA of these cells to oligonucleosomal-sized fragments, which is characteristic of the apoptosis, dependent on the dose and exposure time. The cellular death caused by the administration of Phx was partially reversed by the addition of Z-VAD-fmk, a caspase family inhibitor. Present results suggest that Phx demonstrates anti-cancer activity against human lung carcinoma cell lines A549 and H226, by inhibiting growth and inducing apoptosis. [© 2001 Lippincott Williams & Wilkins.]

Key words: Apoptosis, dose dependency, growth inhibition, lung carcinoma cell lines, phenoxazinone.

Introduction

Actinomycin D is known to inhibit the activity of DNA-dependent RNA polymerase,[1] to intercalate DNA,[2] and to have anti-cancer activity but with strong adverse effects such as immunosuppressive and anti-hematopoeitic toxicities. Tomoda *et al.* found that 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx) (see Figure 1) was produced by the reaction of 2-amino-5-methylphenol with human hemoglobin[3] or bovine hemoglobin[4] and reported that Phx was an elemental component of Actinomycin D, as a phenoxazinone. Thus, it is conceivable that Phx might cause the anti-cancer activity of Actionmycin D. Ishida and Tomoda *et al.*[5] reported that Phx suppressed the *in vitro* proliferation of KB cells, a human epidermoid carcinoma cell line, in a dose-dependent manner, probably by inhibiting the DNA synthesis of the cells. Mori and Tomoda *et al.*[6] also described that when Phx was administered to mice transplanted with Meth A carcinoma cells derived from BALB/c mice, it caused extensive suppression of the growth, without causing adverse effects such as immunological and hematopoeitic toxicities. On the other hand, adenocarcinoma and squamous carcinoma of the lung are reported to be resistant to chemotherapeutic drugs.[7] Specific anti-cancer drugs to treat these carcinomas are therefore essential. We set out to clarify whether Phx might exert cytotoxic activity against lung carcinoma-derived cells using the human lung cancer cell lines, A549 and H226, *in vitro*.

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Materials and methods

Drug

Phx was synthesized and purified as described previously.[4] The chemical structure of Phx is shown in Figure 1, in comparison with that of Actinomycin D. Phx was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical, Tokyo, Japan) at a concentration of 100 mM, prior to use.

Cell lines and culture

The human lung adenocarcinoma cell line, A549, was obtained from the Health Science Research Resources Bank (Osaka, Japan). The human lung squamous carcinoma cell line, H226, was obtained from ATCC (Rockville, MD). These cell lines were cultured in modified Eagle's medium (MEM) (Gibco/BRL, Tokyo, Japan) with non-essential amino acids, 110 μg/ml sodium pyruvate, 10% heat-inactivated fetal bovine serum and 50 μg/ml kanamycin sulfate at 37°C in a humidified incubator with 5% CO_2 in air.

Assay for cell growth and its inhibition by Phx

Exponentially growing cells were placed in quadruplicate at 3×10^4 cells/cell in the 24-well plates and cultivated in the presence of vehicle (DMSO) or vehicle and various concentrations of Phx, at 37°C. During cultivation for 1–3 days, the cell number was counted at constant intervals, by a hemocytometer.

Cell viability

The cell viability was assessed by the WST-8 reagent [2-(2-methoxy-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-tetrazolium, monosodium salt] method (Cell Counting Kit-8; Dojindo, Kumamoto, Japan).[8] Briefly, the tumor cells (5×10^3 cells/well) were precultured in a 96-well flat-bottomed microtiter plate for 24 h at 37°C in a 5% CO_2 humidified chamber. Then, various concentrations of Phx were added and incubated for 1–3 days. After incubation, culture medium was discarded and 100 μl of new medium including 10 μl of WST-8 reagent of Cell Counting Kit-8 was added to each well. Then, the cells were incubated for 90 min. After incubation, the microtiter plate was analyzed by a microplate reader (Model ELNX 96; TFB, Tokyo, Japan) at a test wavelength of 450 nm and a reference wavelength of 600 nm. Cell viability was determined by referring to the absorbance of non-treated cells.

Analysis of DNA fragmentation

A549 and H226 cell lines were treated with 100 μM Phx for 1–3 days. After treatment, detached cells were collected. The cells were centrifuged at 600 g for 5 min at 4°C and washed twice with phosphate buffer solution. Cells were lyzed in 100 μl of the lysis buffer containing 10 mM Tris–HCl buffer (pH 7.4), 10 mM EDTA and 0.5% Triton X-100, and were kept on ice for 15 min. Then, the samples were centrifuged at

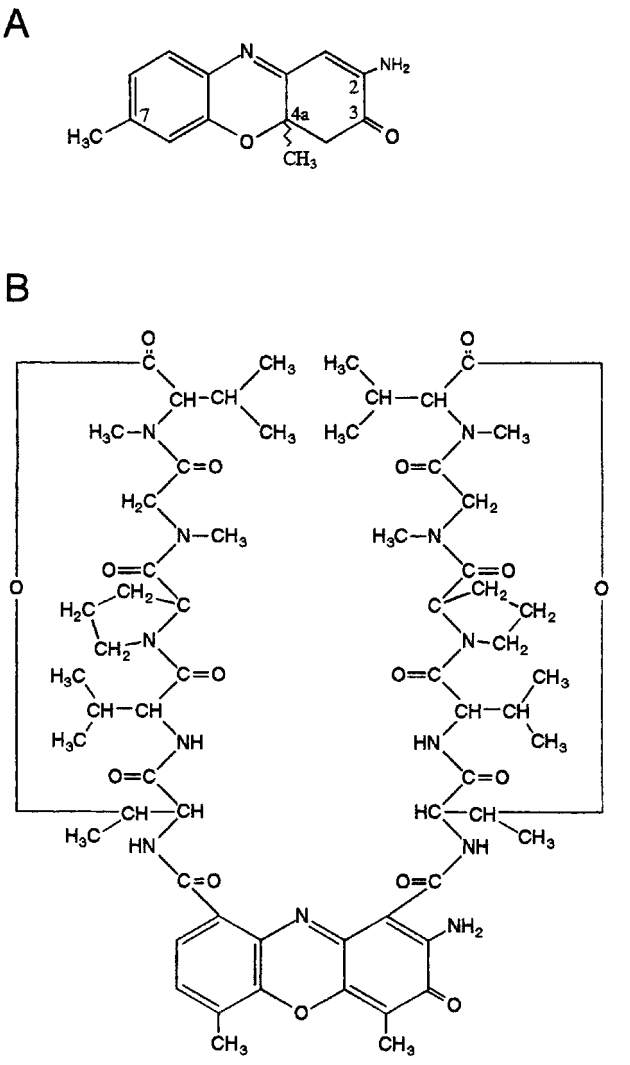

Figure 1. Chemical structure of Phx (A) and Actinomycin D (B).

12 000 *g* for 15 min at 4°C. After centrifugation, supernatant was incubated with 40 μg RNase A (Wako Pure Chemicals) at 37°C for 1 h, followed by a further 1 h incubation at 37°C with 40 μg Proteinase K (Wako Pure Chemicals). Then, DNA was precipitated with 20 μl of 5 M NaCl and 300 μl of 2-propanol, and kept overnight at −30°C. After centrifugation for 15 min, DNA was separated by electrophoresis using 1.5% agarose gel and visualized by UV illumination after SYBR gold (Molecular Probes, Eugene, OR) staining.

Measurement of the effects of Z-VAD-fmk, the caspase family inhibitor, on Phx-induced apoptosis of A549 and H226 cells

The tumor cells (4×10^4 cells/ well) were precultured in a 24-well flat-bottomed microtiter plate for 24 h at

37°C in a 5% CO_2 humidified chamber; further, these cells were pretreated with 75 μM Z-VAD-fmk (MBL, Nagoya, Japan) for 1 h, and then maintained for 2 days in a MEM medium containing 10% FCS, 100 μM Phx and 75 μM Z-VAD-fmk. The relative number of dead cells was estimated by the Cell Counting Kit-8 as described above.

Statistics

The results were analyzed by Student's *t*-test for comparison between the two groups. Values were expressed as means ± SEM. A p value of less than 0.05 was considered to indicate a significant difference in all statistical analysis.

Results

Growth inhibition and cytotoxic effects of Phx in human lung carcinoma cell line A549 and H226

Table 1 shows the effects of various concentrations of Phx on the growth of the human lung carcinoma cell lines, A549 (adenocarcinoma) and H226 (squamous cell carcinoma), which were incubated at 37°C for 3 days. Proliferation of both A549 and H226 cells was inhibited by Phx in a time- and dose-dependent manner. Namely, after day 1 of incubation, the growth of A549 and H226 cell lines was inhibited by 26.5 and 12.8%, respectively, in the presence of 50 μM Phx, and by 49.1 and 42.0%, respectively, in the presence of 100 μM Phx. After day 3 of incubation, the growth of A549 and H226 cell lines was inhibited by 42.9 and 23.4%, respectively, in the presence of 50 μM Phx, and by 100%, respectively, in both cell lines the presence of 100 μM Phx.

Table 1. The growth inhibition effect of a 3-day treatment with Phx on A549 and H226 cells

Cell line	Phx concentration	Inhibition (%)		
	(μM)	1 day	2 day	3 day
A549	12.5	4.5	7.2	4.2
	25	12.3	9.0	4.9
	50	26.5	40.6	42.9
	75	29.4	60.9	80.6
	100	49.1	74.4	100.0
H226	12.5	4.7	6.9	15.1
	25	5.9	8.6	17.7
	50	12.8	13.1	23.4
	75	22.2	49.5	71.2
	100	42.0	100.0	100.0

The values are means of quadruplicate determinations.

Figure 2(A and B) shows the effect of various concentrations of Phx on the viability of A549 and H226 cells during the 3-day study period. On day 1, cell death was slightly observed after treatment of A549 with 10–100 μM Phx, and was significantly accelerated on days 2 and 3 after treatment with 50–100 μM Phx (Figure 2A). After 3 days treatment, Phx had high cytotoxicity to A549 cells, at concentrations above 50 μM, i.e. the viability of A549 cells was 65, 25 and 8.8% at doses of 50, 75 and 100 μM, respectively, when the cells were treated with Phx for 3 days. Similar results were obtained for H226 cells (Figure 2B). Phx exerted high cytotoxicity to H226 cells at 75 and 100 μM Phx, when treated for 2 and 3 days. The viability of H226 cells after 3 days was 45 and 8.9%, at the doses of 75 and 100 μM Phx.

Effect of Phx on DNA fragmentation of the human lung carcinoma cell lines

DNA fragmentation is a characteristic feature of apoptosis. A typical experimental result of agarose gel electrophoresis is shown in Figure 3(A and B). Increased DNA fragmentation was observed in both A549 (Figure 3A) and H226 (Figure 3B) cells, after treatment of the cells with 100 μM Phx for 1–3 days. Fragmented DNA was especially detected on days 2 and 3 after the Phx treatment of both cell lines.

Effect of Z-VAD-fmk, the inhibitor of caspases, on Phx-induced apoptosis

The effects of Z-VAD-fmk, the inhibitor of caspases, on the number of dead cells in A549 and H226 cell lines treated with 100 μM Phx in the presence or absence of Z-VAD-fmk for 2 days was studied. The numbers of dead cells were about 50 and 70% when A549 and H226 cell lines were treated with Phx, in the absence of Z-VAD-fmk. The numbers of dead cells were decreased by 16.2 and 29.1%, respectively, in the presence of Z-VAD-fmk (Figure 4). These results show that the apoptosis of A549 and H226 induced by Phx was reversed, in part, by the inhibition of caspase family enzymes, because Z-VAD-fmk inhibits the caspases, and that the caspase system is involved in the apoptosis of A549 and H226 cell lines caused by Phx.

Discussion

We set out to clarify whether Phx plays a role in the growth inhibition and apoptosis of adenocarcinoma and squamous carcinoma of the lung, which are non-

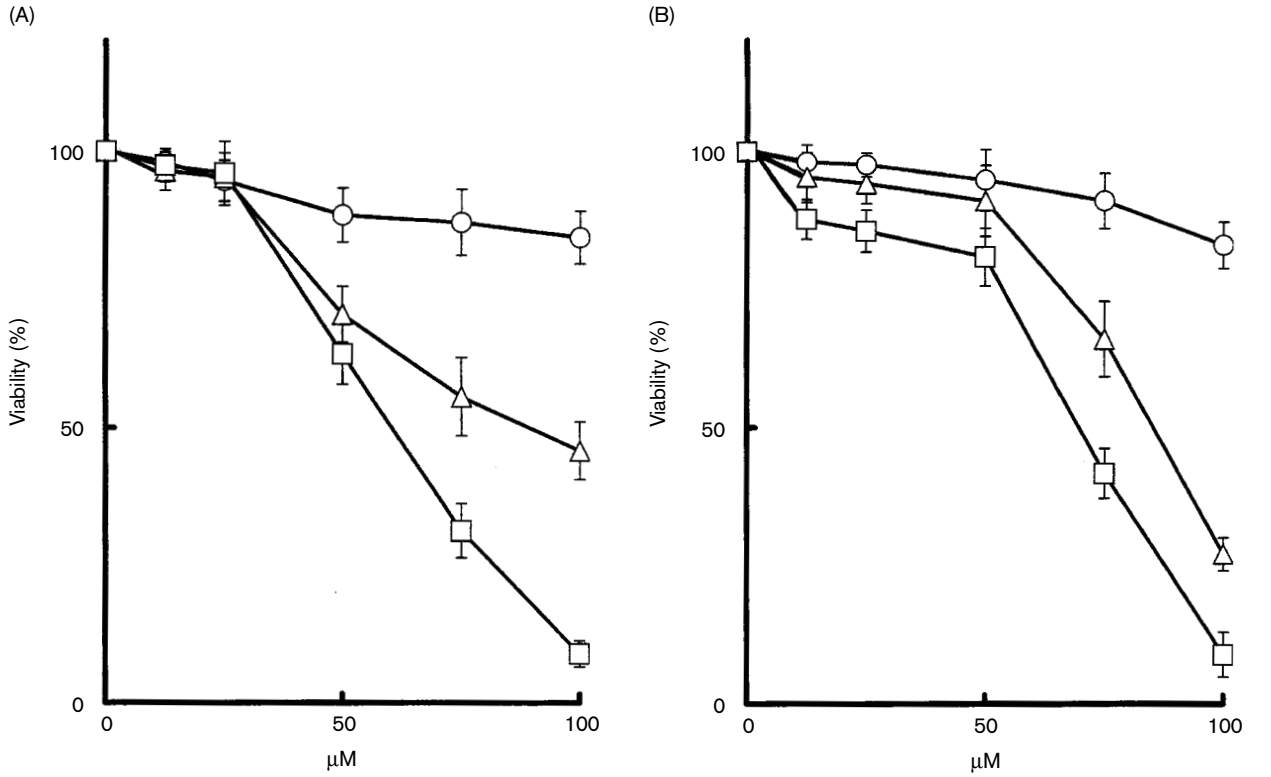

Figure 2. Effect of Phx treatment period and concentration in two lung carcinoma cell lines. (A) A549 cells. (B) H226 cells. Each treatment period is indicated by three different markers: (○), 1 day; (△), 2 days; (□), 3 days. The result of Phx treatment assay is shown as means ± SE from four independent experiments.

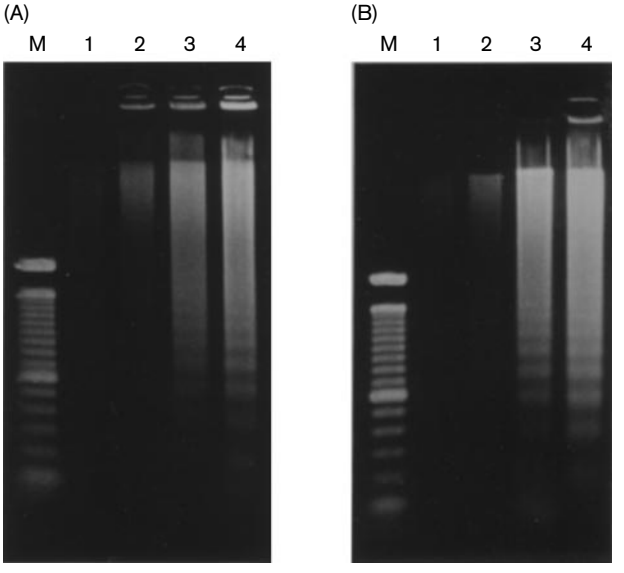

Figure 3. Electrophoretic analysis of DNA from A549 and H226 cells treated with 100 μM of Phx for 1–3 days. DNA from A549 cells (A) and H226 cells (B). M, DNA size markers, 100 bp ladder (100–3000 bp); lane 1, DNA from non-treated cells; lane 2, treatment with Phx for 1 day; lane 3, 2 days; lane 4, 3 days.

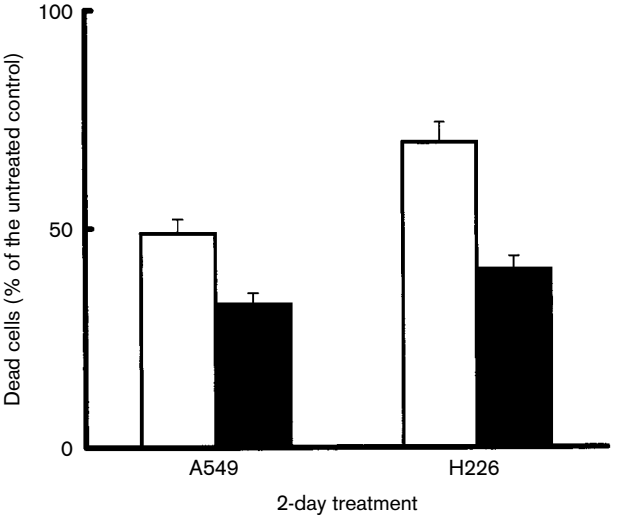

Figure 4. Effect of Z-VAD-fmk (caspase family inhibitor) on A549 and H226 cell lines which were treated with Phx.

small cell lung cancers and are difficult to treat, because they are generally resistant to chemotherapeutic drugs.[7] It is essential to discover an effective new therapy for these carcinomas. Recently, several therapeutic stimuli including hyperthermia,[9] toxins,[10] cytokines[11] and other chemicals[12] have been reported to induce apoptosis in cancer cells. Induction of apoptosis specifically in cancer cells without affecting the immune cell system might be of benefit to cancer therapy.

This study showed that Phx had strong anti-cancer effects against the human lung carcinoma cell lines A549 and H226 cells in a dose-dependent manner, because it inhibited the growth of these cell lines (Table 1) and induced cell death at doses above 50 μM (Figure 2A and B). The present results are consistent with the report that the growth of KB cells (human epidermoid carcinoma cells) was inhibited extensively by 100 μM Phx *in vitro*,[5] and that the growth of Meth A cells derived from BALB/c mice was suppressed in both *in vivo* and *in vitro* experiments.[6]

The mechanism for the induction of cell death of human lung carcinoma cell lines A549 and H226 (Figures 2A and B) by Phx would be correlated with the apoptosis. This view was supported by the results that the DNA ladder, a biochemical hallmark of apoptosis, was observed in both H226 cells and A549 cells treated with 100 μM Phx over 2 days (Figure 3A and B). Lokshin and Levitt[8] reported that DNA laddering was observed when NCI-H596 cells (adenosquamous lung cell carcinoma) were treated with suramin, an anti-cancer drug shown to be effective in the treatment of prostate cancer, but it was not observed when NCI-H226 cells (squamous lung cell carcinoma) were treated with the same drug. Since we observed that Phx induced DNA laddering of both H226 cells (squamous lung cell carcinoma) and A549 cells (adenocarcinoma cell of the lung), the mechanism of cell apoptosis caused by Phx might be different from that by suramin. Furthermore, we found that Z-VAD-fmk, an inhibitor of caspases, the activation of which induces the apoptosis, could partially inhibit the cell death process caused by Phx (Figure 4). This result suggests the involvement of caspase-dependent pathways in the mechanism of Phx-induced apoptosis on H226 and A549 cells, and that Phx is involved in the activation of the caspase pathway in these cells, inducing the apoptosis of these cells.

Motohashi reported that the chemically synthesized phenoxazinones showed little anti-tumor activity due to their poor solubility in water.[13] On the contrary, Phx, which is also a kind of phenoxazine and is produced by long-term reaction of 2-amino-5-methylphenol with human or bovine hemoglobin,[3,4] is relatively soluble in water. Therefore, the chemical structure of Phx might be different from that of the chemically synthesized phenoxazinones, the details of which should be investigated in further studies.

Mori and Tomoda *et al*. also observed that Phx exerts little adverse effects in mice, including hematopoeitic toxicity, decrease of body weight and deterioration of general appearance.[6] Thus, Phx can be expected to be available for the treatment of adenocarcinoma and squamous cell carcinoma of the human lung in the future.

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