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Experimental report

Antitumor activity of 2-amino-4,4α-dihydro-4α, 7-dimethyl-3H-phenoxazine-3-one, a novel phenoxazine derivative produced by the reaction of 2-amino-5-methylphenol with bovine hemolysate

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2-Amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx) was synthesized by the reaction of 2-amino-5-methyl-phenol with bovine hemolysates. Since Phx is a phenoxazine derivative like actinomycin D, which exerts a strong anti-tumor effect by intercalating DNA, we examined the effects of Phx on cell proliferation and cell cycle progression in human epidermoid carcinoma cells (KB cells). Phx inhibited the proliferation of KB cells in a dose-dependent manner. When KB cells were incubated for 9 h with medium containing 50 μM Phx, a transient accumulation of cells in S and G_2/M phase was observed and at 24 h many of cells had lower DNA content. Although Phx had antitumor activity, the drug did not intercalate DNA, showing a different mode of action from actinomycin D.

Key words: 2-Amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx), antitumor activity, flow cytometry, non-DNA intercalator.

Introduction

The phenoxaziones are present in insect pigments called ommochrones,[1] in some mold metabolites[2,3] and in the actinomycins synthesized by a number of *Streptomyces* species.[4] The chemically synthesized phenoxazines show little solubility in water and exert no anti-tumor effects.[5] On the other hand, it has been demonstrated by Tomoda *et al.*[6–8] that 2-amino-phenoxazine, cinnabarinic acid and novel kinds of phenoxazines could be synthesized by the reaction of *o*-aminophenol or its derivatives with human hemoglobin and that these phenoxazine compounds had relatively higher solubility in water than those chemically synthesized. Tomoda *et al.*[8] recently reported that 2-amino-5-methylphenol was converted to a reddish brown compound in the presence of hemoglobin, which was identified as 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx). We also found that Phx can be abundantly produced by the reactions of 2-amino-5-methylphenol with bovine hemoglobin and hemolysates. Since the phenoxazine compound is an essential chemical structure of actinomycin D which exerts a strong anti-tumor effect by intercalating DNA,[9] we investigated whether or not Phx is a DNA intercalator and if the drug has antitumor activity. In the present paper, we report that Phx produced by the reaction of 2-amino-5-methylphenol with bovine hemolysates inhibited the proliferation of human epidermoid carcinoma cells (KB cells).

Materials and methods

Drug

Phx was synthesized and purified as described previously,[11] except that bovine hemolysates were used instead of human hemolysates. Phx was dissolved in dimethyl sulfate at a concentration of 25 μM and was diluted with distilled water before use.

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Cell lines and culture

Human epidermoid carcinoma cells, KB, were cultured in Dulbecco's modified Eagle' medium (DME) supplemented with 10% calf serum, penicillin (10 units/ml) and streptomycin (100 μg/ml) under humidified atmosphere of 5% CO_2 in air.

Determination of growth inhibition and cell survival

Cells were seeded at 10^4 cells/60 mm dish with 4 ml of DME medium and grown in the presence or absence of Phx. Cells attached on the culture were trypsinized and the cell number was examined with a Coulter Industrial D cell counter (Coulter Electronics, Luton, UK). To determine cell survival, cells were seeded at 6×10^2 or 6×10^3 cells/60 mm dish. They were treated with various concentrations of Phx and cultured for about 8 days. After fixing with 10% formalin in 0.9% NaCl and staining with 0.1% crystal violet, colonies were counted. Percent survival is expressed as the ratio of the number of colonies in drug-treated cultures to that in control cultures.

Analysis of cell cycle phase by flow cytometry

KB cells were seeded at 10^6 cells/140 mm dish and cultured for 1 day. The cells were grown in the presence of 50 μM Phx for various times, and then trypsinized and collected by centrifugation. After cells were washed with phosphate-buffered saline (PBS), they were fixed with 70% ethanol for 30 min at 37°C, washed with PBS and treated with 1 mg/ml ribonuclease A in PBS for 20 min at 37°C. The cells were washed with PBS at once and stained with 50 μg/ml propidium iodide in 1% sodium citrate at 4°C.

Analysis of DNA content by Flow cytometry was carried out in a FACScan (Becton Dickinson) with the Lysis II program.

Assay for DNA intercalation

The reaction mixture contained 20 μl of 50 mM Tris–HCl buffer, pH 8.0, 100 mM NaCl, 0.6 μg bovine serum albumin, 0.5 mM dithiothreitol, 0.2 mM EDTA 10% glycerol, relaxed colicin E1 DNA (0.3 μg), 2 units topoisomerase I and various concentrations of drugs. The reaction was carried out for 15 min at 37°C and stopped by addition of 4 μl of 10% sodium dodecyl sulfate. After deproteinization with phenol:chloroform:isoamyl alcohol (50:49:1), 15 μl of supernatant was mixed with 3 μl of 0.1% bromphenol blue and 50% glycerol. An aliquot was run on 0.8% agarose gel with Tris–acetate buffer, pH 8.0. The sample was stained with ethidium bromide and photographed under UV.

Results and discussion

Effect of Phx on cell proliferation and cell cycle progression in KB cells

Phx is a phenoxazine derivative whose primary structure is shown in Figure 1. We tested whether or not the drug has antitumor activity on KB cells. To examine the effect of Phx on cell proliferation, cultures of KB cells were added in medium with varing doses of Phx and the number of cells attached on the culture dish was determined after 1, 2 and 3 days (Figure 2). Without Phx, the cells proliferated linearly for 3 days. The proliferation of

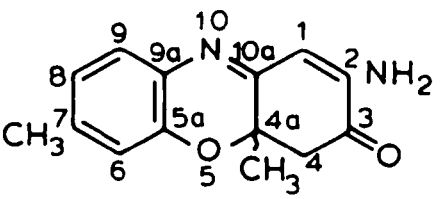

Figure 1. Structure of 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one.

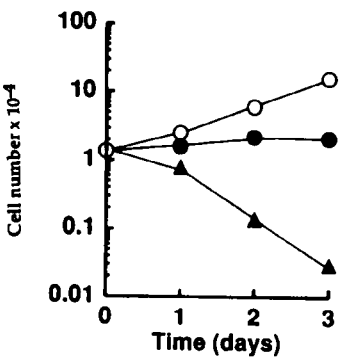

Figure 2. Effect of Phx on cell growth in KB cells. Cells were seeded at 10^4 cells/60 mm dish. After 1 day, cells were exposed to 25 μM (), 50 μM () Phx or no drug () and were cultured for the indicated times. Cell number was counted with a Coulter counter.

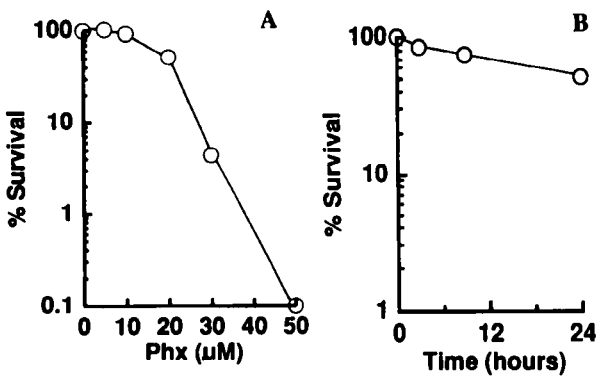

Figure 3. Effect of Phx on survival in KB cells. (A) Cells were continually exposed to various concentration of Phx. (B) Cells were exposed to 50 μM Phx for the indicated times and after removal of the drug they were cultured further. Percent survival was examined by a colony assay as described in Materials and methods.

KB cells was inhibited slightly at 25 μM Phx. KB cells degenerated and detached from the dishes of 1 day after addition of 50 μM Phx. Similarly, Phx inhibited the proliferation of mouse FM3A cells at nearly the same concentrations (data not shown).

The effect of Phx on the survival of KB cells was also examined by incubating cells in the medium with and without the drug, and determined by colony forming activity. As shown in Figure 3(A), Phx reduced the survival to 55% at a concentration of 20 μM, 4% at 30 μM and less than 0.1% at 50 μM. Although continuous exposure of cells to 50 μM Phx renders cells significantly inviable, short exposure is not so cytotoxic (Figure 3B). Cell viability was reduced only to 50% when cells were exposed to the drug for 24 h.

Since 50 μM Phx inhibited cell growth completely, we next examined by flow cytometry in which phase cell progression is affected (Figure 4A and B). To represent the population of KB cells in each cell cycle, the cells were divided into four fractions corresponding to the cells with reduced DNA content, in G_1 Phase, in S phase and in G_2/M phase, respectively (numbers 1, 2, 3 and 4 in Figure 4A). In the presence of Phx, the distribution of cell population in the cell cycle was not changed after 3 h. At 9 h, cells in S and G_2/M phase increased. At 24 h, cells with normal DNA content were reduced and cells with reduced DNA content increased significantly. These data are quantitatively expressed by counting the total cell number in each of the four fractions, and the changes of cell population were plotted at 3, 9 and 24 h after drug treatment (Figure 4B). Cells in G_1 were reduced with treatment time, while cells in S and G_2/M phase increased transiently at 9 h then decreased after 24 h. Cells with reduced DNA content increased after 9 h. The increase of cells in S and G_2 phase after 9 h would be due to a delay in cell cycle progression in S

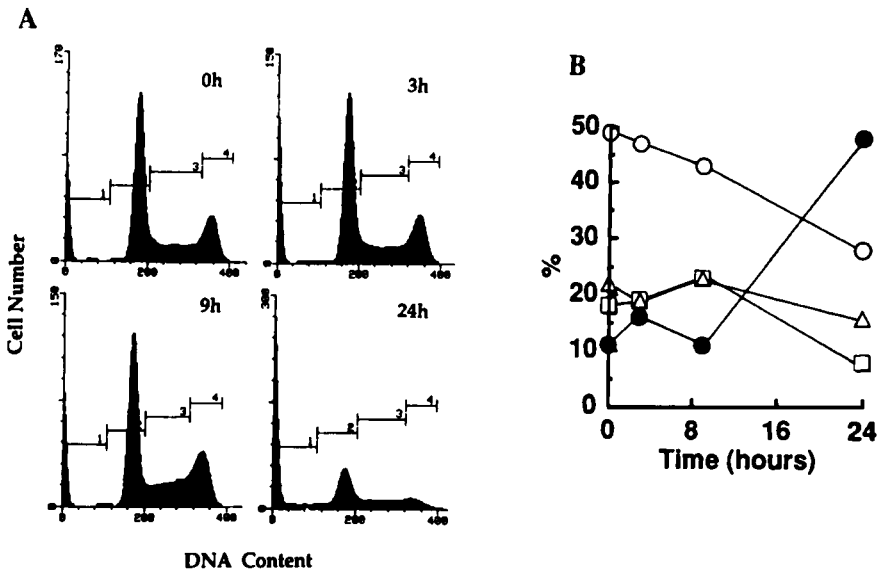

Figure 4. Effect of Phx on red cell cycle progression. Cells were seeded at 10^6 cells/140 mm dish. They were exposed to 50 μM Phx at 3, 9 and 12 h, and the distribution of the cells in the cell cycle was examined by flow cytometry (A). In (B), the percentages of cell numbers were plotted, which corresponds to cells in G_1 (), S (), G_2/M () and with reduced DNA content (), respectively.

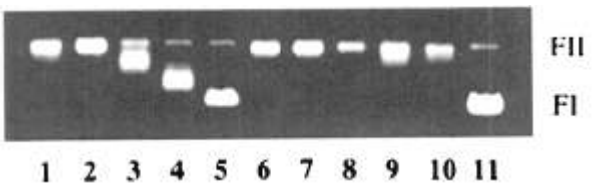

Figure 5. Assay of DNA intercalating activity of Phx. DNA intercalating activity was examined as Topo I-mediated DNA unwinding activity as described in Materials and methods. Relaxed coli E1 DNA (line 1) was treated with Topo I in the presence of *m*-AMSA at the concentrations of 0 (lane 2), 25 (lane 3), 125 (lane 4) and 500 μM (lane 5) or in the presence of Phx at concentrations of 25 (lane 6), 125 (lane 7) and 500 μM (lane 8). Relaxed coli E1 DNA was treated with 500 μM *m*-AMSA (lane 9) or 500 μM Phx (lane 10) in the absence of Topo I. Coli E1 DNA (lane 11). FI, supercoiled circular DNA; FII, relaxed circular DNA. After deproteinization, samples were examined by agar electrophoresis as described in Materials and methods.

phase because of reduced DNA synthesis, since Phx partially inhibits the corporation of [3H]-thymidine into cellular DNA (data not shown). Inhibitors of DNA synthesis have been shown to cause the accumulation of cells in S and G_2 phase.[10] Apparently, cells with a reduced DNA content increased at 24 h after Phx treatment. It is uncertain whether these cells represent a loss of cellular DNA or reduced DNA stainability. Gorczyca *et al.* showed that cells with reduced DNA content appeared in the presence of toxic drugs and these cells correspond to apoptotic cells.[11] We tested whether or not Phx induces apoptosis by examining the DNA ladders, which are one of the characteristics of apoptosis, and found that induction of cells with reduced DNA content does not lead to apoptotic cell death.

Some phenoxazine derivatives can overcome multidrug resistance; *N*-substituted phenoxazines increased accumulation of vinblastine.[12] Phenoxazine-related compounds increased accumulation of Vinca alkaloids in multidrug-resistant human tumor cells.[13] We examined whether or not Phx overcomes colchicine-resistant human epidermoid carcinoma (KB-Ch^r). The drug did not reverse the drug resistance (data not shown).

Test for DNA intercalator

Actinomycin D, a piperazine derivative, is known to be a DNA intercalator whose nature is presumably related to antitumor activity. Since Phx has a similar structure, we examined with the topoisomerase-mediated unwinding assay[14,15] whether Phx is a DNA intercalator. The principle of the unwinding assay is as follows. When a DNA intercalator is present, relaxed DNA is converted to positive DNA. Since Topo I can relax the negative and positive supercoiled DNA, the positive supercoils will be relaxed by the enzyme. After removal of the drug, the relaxed DNA will turn to negative supercoiled DNA, while a non-DNA intercalator does not change the DNA topology. Thus Topo I does not affect the DNA. As shown in Figure 5, with increasing concentrations of 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA), a DNA intercalator, the negative supercoiled DNA increased (lanes 3–5). In contrast, the supercoiled DNA did not increase in the presence of Phx (lanes 6–8), showing that Phx is not a DNA intercalator. Consistent with this observation is that the drug did not inhibit the activity of DNA Topo I and II (data not shown) although many DNA intercalators inhibit topoisomerases (16). From the data of flow cytometry, Phx is found to cause an accumulation of cells in S and G_2 phase, suggesting that the drug inhibits DNA synthesis. Since Phx does not intercalate in DNA, it remains to be resolved how Phx inhibits DNA synthesis. There are many possible mechanisms by which Phx inhibits DNA synthesis. One possibility is that the drug inhibits a DNA replication protein such as DNA polymerases, DNA ligase or DNA replication factor. It is also possible that Phx causes an imbalance of deoxyribonucleotide triphosphates by affecting ribonucleotide reductase or thymidylate synthase.

In summary, Phx, synthesized by the reaction of 2-amino-5-methylphenol with bovine hemolysates, has a relatively high solubility in water and has antitumor activity. Thus the drug has advantages for clinical application over chemically synthesized phenoxazines which are of poor solubility.

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