Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1057–1058

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

An Improved Method for the Rapid Preparation of 2-Amino-4,4a-dihydro-4a,7-dimethyl-3*H*-phenoxazine-3-one, a Novel Antitumor Agent

Akio Tomoda,[a,*] Sadao Arai,[b] Ryoji Ishida,[c] Takashi Shimamoto[d] and Kazuma Ohyashiki[d]

[a]*Department of Biochemistry, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan*
[b]*Department of Chemistry, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan*
[c]*Aichi Cancer Center Research Institute, 1-1 Kanokoden Chikusa-ku, Nagoya 464-0021, Japan*
[d]*Department of Internal Medicine, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan*

Received 21 November 2000; accepted 22 February 2001

Abstract—A simple and rapid preparation method for a novel antitumor agent, 2-amino-4,4a-dihydro-4a,7-dimethyl-3*H*-phenoxazine-3-one (Phx) was described. The procedure included (1) the reaction of bovine hemolysates with 2-amino-5-methylphenol, (2) one-shot denaturation of hemoglobin and proteins by methanol, and removal of the denatured hemoglobin and proteins, (3) concentration of the reaction products, and (4) purification by a Sephadex column. These procedures yielded Phx in 34% yield.

o-Aminophenol and its derivatives are metabolized to phenoxazinones during the reaction with human hemoglobin or erythrocytes.[1–4] Tomoda et al.[5] demonstrated that 2-amino-5-methylphenol was converted to 2-amino-4,4a-dihydro-4a,7-dimethyl-3*H*-phenoxazine-3-one (Phx, Fig. 1) by human hemoglobin and bovine hemoglobin, and noted that Phx was relatively soluble in water, though the chemically synthesized phenoxazine compounds are hardly soluble in water.

Phenoxazinone is a part of the chemical structure of actinomycin D, which is known to exert intensive anticancer activity on malignant tumors in children such as Wilms' tumor (kidney sarcoma), but also has adverse effects such as hematopoietic and immunosuppressive toxicity. Ishida et al.[6] found that it inhibited the in vitro growth of KB cells, a human epidermoid carcinoma cell line, in a dose-dependent manner. Furthermore, they demonstrated that the growth of methA tumor cells was suppressed by only 5 mg/kg body weight of Phx.[7]

To further investigate details of the antitumor activity and action mechanism of Phx, large amounts of it are required. Although we reported the preparation method for Phx,[5] the preparation was time-consuming and costly, in which a large quantity of CM Sephadex C-50 resin was required to remove hemoglobin. To overcome these disadvantages, we set out to improve the method for preparing Phx. In the present method, we improved the step of removal of hemoglobin and proteins in the hemolysates containing the reaction products. Namely, we introduced the one-shot denaturation of hemoglobin and proteins by methanol, instead of chromatographic trapping of hemoglobin and proteins and removal of residual hemoglobin and proteins by a Diaflow membrane, which consumed much time, in the previous method.[5] Secondly, we omitted the step using a SEP-PAC C-18 resin and thin layer chromatography for final purification of Phx, which is time-consuming.[5] Herein,

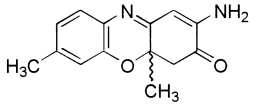

Figure 1. Structure of 2-amino-4,4a-dihydro-4a,7-dimethyl-3*H*-phenoxazine-3-one.

*Corresponding author. Fax: +81-3-3351-6466; e-mail: tomoda@tokyo-med.ac.jp

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00153-6

Table 1. Incorporation of [^{3}H]-thymidine into DNA of HAL-01 cell and HL-60 cell

	HAL-01 cell			HL-60 cell		
	Phx (μM)			Phx (μM)		
	0	100	200	0	100	200
1 h	0	0	0	0	0	0
2 h	25,000	17,000	9000	21,000	14,000	6000
4 h	34,000	18,000	10,000	25,000	15,000	9000
8 h	40,000	19,000	14,000	35,000	19,000	12,000

we adopted two steps of chromatographic purification of Phx, using Sephadex LH 20. These procedures yielded Phx in 34% yield.

Using Phx obtained following the present procedure, we studied the effect of Phx on cell growth and the incorporation of [^{3}H]-thymidine into acid insoluble fraction in a lymphoid leukemia cell, HAL-01 and a myeloid leukemia cell, HL-60 (Table 1). Phx inhibited the incorporation of [^{3}H]-thymidine into DNA of HAL-01 cell and HL-60 cell in a dose dependent manner,[8] being consistent with the results that the growth of these cells was suppressed by Phx, in vivo and in vitro.[9] Phx obtained by the new method had the same level of antitumor activity as that from the previous preparation method, indicating that the present method is superior to the previous method in terms of speed and cost of preparation.

Experimental

Bovine hemolysates were prepared by the method described in ref 10. 2-Amino-5-methylphenol (1.2 g) was dissolved in distilled water (100 mL), and then 0.2 N HCl solution (200 mL) was added. After complete dissolution of the compound, 0.2 N NaOH solution (200 mL) was added, little by little with stirring. The solution was neutralized (pH 7.0) by the addition of NaOH solution, and was immediately added to bovine hemolysates (3 L). The hemolysates including 2-amino-5-methylphenol were mixed well, and were incubated at 37 °C for 120 h, after which about 3 volumes of methanol were added to the hemolysates. Since hemoglobin and proteins in the hemolysates were denatured with the addition of methanol, these can be removed by centrifugation at 8000×*g* for 10 min. The supernatant was collected, and was concentrated in a large-scale rotary evaporator.

The concentrated solution including Phx (about 300 mL) was put on a column (7×100 cm) of Sephadex LH20 (equilibrated with 50% ethanol) and was eluted with 50% ethanol. The 4th and major band (yellowish brown) included Phx, which was collected, and concentrated in a small-scale rotary evaporator. The concentrated eluate was re-chromatographed through a Sephadex LH20 column (3 cm×30 cm, equilibrated with 50% ethanol), and was eluted with 50% ethanol. Ethanol and water were evaporated from the eluate, leaving purified Phx as a powder. Purified Phx also demonstrated a single band (yellowish brown) on a thin layer.[11]

The NMR data of Phx[12] showed that the compound was 2-amino-4,4a-dihydro-4a,7-dimethyl-3*H*-phenoxazine-3-one, the signals of which were identical with that reported previously.[5]

References and Notes

1. Tomoda, A.; Yamaguchi, J.; Kojima, H.; Yoneyama, Y. *FEBS Lett*. **1986**, *196*, 44.
2. Eckert, K. G.; Eyer, P. *Biochem. Pharmacol*. **1983**, *32*, 1019.
3. Tomoda, A.; Shirasawa, E.; Nagao, S.; Minami, M.; Yoneyama, Y. *Biochem. J*. **1984**, *222*, 755.
4. Tomoda, A.; Arisawa, M.; Koshimura, S. *J. Biochem*. **1991**, *110*, 1004.
5. Tomoda, A.; Hamashima, H.; Arisawa, M.; Kikuchi, T.; Tezuka, Y.; Koshimura, S. *Biochim. Biophys. Acta* **1992**, *1117*, 306.
6. Ishida, R.; Yamanaka, S.; Kawai, H.; Ito, H.; Iwai, M.; Nishizawa, M.; Hamatake, M.; Tomoda, A. *Anti-Cancer Drugs* **1996**, *7*, 591.
7. Mori, H.; Honda, K.; Ishida, R.; Nohira, T.; Tomoda, A. *Anti-Cancer Drugs* **2000**, *11*, 653.
8. The ability of DNA synthesis of HAL-01 cell (human lymphoid leukemia cell) and HL-60 cell (human myeloid leukemia cell) was determined by incorporation of [^{3}H]-thymidine into the acid-insoluble fraction. 5×10^4 cells were seeded in 1 mL into each tube and labeled with 1 μM Ci/mL of [^{3}H]-thymidine. The labeled cells were centrifuged at 600×*g* for 5 min, and the pelleted cells were suspended in phosphate-buffered saline (PBS). The process of resuspension and centrifugation was repeated twice. Then, 10% trichloroacetic acid (TCA) was added to equal volumes of resuspended cells with PBS. After 10 min standing at room temperature, acid-insoluble material was collected on glass fiber filter, washed with 5% TCA and ethanol, and then dried. The radioactivity on the filter was counted in the toluene base scintillation fluid.
9. Shimamoto, T.; Tomoda A.; Ishida, R.; Ohyashiki, K. *Clin. Cancer Res*. **2001**, in press.
10. Fresh bovine blood (1.6 L) obtained from a local abattoir, Tokyo, was treated with citrate as an anticoagulant, then centrifuged at 8000×*g* for 2 min. After removal of plasma and buffy coats, erythrocytes were suspended in 4 volumes of 0.9% NaCl solution, and centrifuged at 8000×*g* for 2 min. The pelleted erythrocytes were again resuspended with the same solution and centrifuged. Then, the erythrocytes were lysed with 5 volumes of distilled water. After standing for 10 min at room temperature, the lysates were centrifuged at 10,000×*g* for 20 min so as to remove erythrocyte membranes. About 3 L of hemolysates, including bovine hemoglobin, were obtained, and were used for the reaction with 2-amino-5-methylphenol.
11. Thin layer chromatography of purified Phx on a Kieselgel 60 F254 plate (Merck KGaA, Darmstadt, Germany) was performed in a solvent (chloroform/acetone = 3:1).
12. ^{1}H NMR and ^{13}C NMR spectra of purified Phx were taken on a Hitachi R-90H spectrometer, with tetramethylsilane as an internal standard.