2-Aminophenoxazine-3-one Suppresses the Growth of Mouse Malignant Melanoma B16 Cells Transplanted into C57BL/6Cr Slc Mice

Naoko MIYANO-KUROSUKI,^a Kunihiro KUROSUKI,^b Michiko HAYASHI,^b Hiroshi TAKAKU,^a Masaaki HAYAFUNE,^a Ken SHIRATO,^c Teruhiko KASUGA,^d Takahiko ENDO,^e and Akio TOMODA^{*,f}

^aDepartment of Life and Environmental Sciences, Faculty of Engineering, Chiba Institute of Technology; 2-17-1 Tsudanuma, Chiba 275-0016, Japan; ^bDepartment of Legal Medicine, School of Medicine, Toho University; 5-21-16 Ohmori-Nishi, Tokyo 143-8540, Japan; ^cLaboratory of Physiological Sciences, Graduate School of Human Sciences, Waseda University; 2-579-15 Mikajima, Tokorozawa 359-1192, Japan; ^d4th Department of Surgery, Tokyo Medical University; ^eDepartment of Forensic Medicine, Tokyo Medical University; and ^fDepartment of Biochemistry and Research Institute of Immunological Treatment, Tokyo Medical University; 6-1-1 Shinjuku, Tokyo 160-8402, Japan.

Received November 1, 2005; accepted August 11, 2006; published online August 18, 2006

Since phenoxazine is an essential structure of actinomycin D, which exerts a strong anticancer effect, we examined the anticancer effect of 2-aminophenoxazine-3-one (Phx-3) on mouse malignant melanoma B16 cells *in vitro* and *in vivo*. Phx-3 inhibited proliferation of the B16 cells in a dose-dependent manner *in vitro*. We further studied the *in vivo* effects of Phx-3 on mouse malignant melanoma B16 cells transplanted in female C57BL/6Cr Slc mice. Treatment with Phx-3 (0.5 mg/kg) completely suppressed the growth of mouse malignant melanoma B16 cells transplanted in mice as compared with the control group. Phx-3 was found to exert few adverse effects, in terms of bodyweight loss, changes in serum levels of blood biochemical parameters such as aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine, dysfunction of the liver and the kidney examined by pathological methods, piloerection and wasting, when mice were treated with a dose of 0.5 mg/kg. These results suggest that Phx-3 may be used to treat patients affected by malignant melanoma in future.

Key words phenoxazine; mouse malignant melanoma B16 cell; *in vivo* anti-tumor activity

In spite of the enormous medical struggle against malignant melanoma, the incidence of melanoma worldwide is increasing annually.^{1,2)} Since patients with malignant melanoma are resistant to chemotherapeutic agents,^{3,4)} the development of effective chemotherapeutic agents to treat malignant melanoma is urgent. For example, dacarbazine, the only compound that was approved for the treatment of malignant melanoma, exhibits at least 15–25% improvement rates.⁵⁾

We demonstrated that the relatively water soluble phenoxazines, such as 2-amino-4,4 α -dihydro-4 α ,7-dimethyl-2H-phenoxazine-3-one (Phx-1) and 2-amino-phenoxazine-3-one (Phx-3) produced by the reaction of bovine hemoglobin with *o*-aminophenol and its derivatives, exert anti-tumor effects on various cancer cells *in vitro* and *in vivo*.^{6–11)} The inhibition of the proliferation of cancer cells by Phx-1 and Phx-3 was accompanied by apoptosis.^{7,11)} Though the phenoxazine is the essential component of actinomycin D, which has strong anticancer effects, but also exerts adverse effects,¹²⁾ it was shown that Phx-1 caused strong anticancer effects against human leukemia cell line HAL-01 and Meth A cancer cells transplanted into mice with limited adverse effects.^{8,9)} Previous report showed that the proliferation of human malignant melanoma G-361 cells was extensively inhibited by Phx-3 *in vitro*, causing the G₁ arrest of the cells.¹¹⁾ These reports on the phenoxazines prompted us to investigate the *in vivo* anti-tumor effect of Phx-3 on mouse malignant melanoma cells transplanted in mice.

The present report describes that Phx-3 inhibits the proliferation of mouse malignant melanoma B16 cells *in vitro*, prevents the growth of the cells transplanted into C57BL/6Cr Slc mice, and causes few adverse effects on mice.

MATERIALS AND METHODS

Phx-3 Phx-3 were prepared by the reaction of *o*-aminophenol with bovine hemoglobin, as described previously.¹³⁾ Phx-3 was dissolved in ethanol before use, and then diluted with phosphate buffered saline (PBS) to appropriate concentrations. The chemical structure of Phx-3 was illustrated in Fig. 1, in comparison with that of Phx-1.

Cell Culture Methods and Cell Proliferation Assay Mouse malignant melanoma B16 cells were obtained from the JCRB cell bank (JCRB0202). These cells were maintained in Dulbecco's modified eagle's medium (DMEM) (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal calf serum (FCS) (Cansera International Inc., Ontario, Canada) and penicillin/streptomycin (10000 unit/ml; 10000 μ g/ml) (Invitrogen, Carlsbad, CA, U.S.A.). The tumor cells (10⁵ cells/ml/well) were pre-cultured for 24 h in a 24-well flat-bottomed plate at 37 °C in a 5% CO₂ humidified chamber. Then, various concentrations of Phx-3 (10, 30, 50, 100 μ M) were added and incubated for 1–3 d. After incubation, the medium of each well was discarded and 1 ml of fresh medium including 100 μ l of trypan blue was added to each well, and incubation was continued for 1 h. After incu-

Fig. 1. Chemical Structure of Phx-3 (A) Phx-1; (B) Phx-3

* To whom correspondence should be addressed. e-mail: tomoda@tokyo-med.ac.jp

bation, the number of viable cells was counted by microscopy.

A WST-1 Cell Counting Kit (Dojin East, Tokyo, Japan) was used to assess cell proliferation, and absorption was measured at 450 nm. The viability of cells was determined by the trypan blue dye exclusion test.

Animal Female C57BL/6Cr Slc mice were obtained at 6 weeks of age from Japan SLC (Hamamatsu, Japan). The mice were maintained in the Laboratory for Animal Experiments, Chiba Institute of Technology, with free access to Charles River solid rodent chow (Oriental Yeast, Tokyo) and water under filtered laminar air-flow conditions at $21 \pm 1^\circ\text{C}$, $60 \pm 5\%$ humidity and 12 h light a day. They were acclimatized for 7 d before the experiments.

Cancer cell line and transplantation 10^6 cells of mouse malignant melanoma B16 cells were inoculated s.c. into the flank of C57BL/6Cr Slc mice in a volume of 0.1 ml, on day 0 and then Phx-3 (0.5 mg/kg) was daily given i.p. for 12 consecutive days starting with the day of transplantation. The size of the subcutaneous tumor was measured with Vernier calipers in terms of two dimensions at right angles and expressed as volume (mm^3) calculated as follows: $4/3 \times \pi \times (\text{long diameter}/2) \times (\text{short diameter}/2)^2$.

Laboratory Examinations The animals were sacrificed by decapitation, 12 d after the transplantation of malignant melanoma B16 cells with or without Phx-3, and were subjected to pathological examination and assessment of serum levels of the blood biochemical parameters including aspartate aminotransferase (AST, or GOT), alanine aminotransferase (ALT, or GPT), blood urea nitrogen (BUN) and creatinine.

Pathological Examination Morphology of the liver, kidney and skin in the control mice and the mice transplanted with mouse malignant melanoma B16 cells and treated with 0.5 mg/kg Phx-3 was examined microscopically, after preserving the tissues in 10% formalin, treating them with paraffin, sectioning at nominal thickness of $5 \mu\text{m}$, and staining them with hematoxylin eodin (HE) for the liver and kidney and with silver ammonium for the skin (Fontana–Masson method).¹⁴⁾

Analysis of Serum Levels of Blood Biochemical Parameters Blood chemistry including the measurement of AST, ALT, blood urea nitrogen (BUN) and creatinine was examined by a Fuji Drychem 3500 automated analyzer (Fuji Medical System Co. Ltd., Tokyo).

Statistics The results were expressed as mean values $\pm$ S.E.M. The data for tumor size were ranked and subjected to the Kruskal–Wallis test, and then non-parametric Duncan's multiple range test (ranked multiple range test) to analyze the significance of differences between the control and the compound-treated groups. For the other data including body weight, we used the parametric Duncan's multiple range test after one-way analysis of variance (ANOVA) was used to assess the statistical significance of differences between the control and the compound-treated groups. A value of $p \geq 0.05$ was considered to indicate a significant difference in all statistical analyses.

RESULTS

Effects of Phx-3 on Tumor Proliferation *in Vitro* We studied the inhibitory effects of Phx-3 on the proliferation of

mouse malignant melanoma B16 cells *in vitro*. As shown in Fig. 2, Phx-3 inhibited the proliferation of mouse malignant melanoma B16 cells dependent on time and dose. The proliferation of the cells was inhibited by about 80% at 48 h, and almost completely (more than 90%) at 72 h, in the presence of $41.3 \mu\text{M}$ Phx-3. The present results demonstrate that Phx-3 may exert anticancer effects on mouse malignant melanoma B16 cells *in vitro*.

Effects of Phx-3 on Tumor Proliferation *in Vivo* Since Phx-3 showed anti-proliferative effects of mouse malignant melanoma B16 cells *in vitro*, we further examined the anticancer effects of Phx-3 on the cells transplanted into C57BL/6Cr Slc mice. The photos in Fig. 3 show the cutaneous (the upper column) and subcutaneous (lower column) appearances of the tumor after the transplantation of melanoma cells into the flank of mice with or without Phx-3. No tumor formation was observed in the mice receiving 0.5 mg/kg Phx-3, while tumor formation was extensive in the mice without Phx-3.

The changes in tumor size of mouse malignant melanoma B16 cells in the mice are shown in Fig. 4. It was found that Phx-3 at 0.5 mg/kg caused no increase of tumor in the flanks of mice.

Fig. 2. Anti-tumor Effects of Phx-3 on Mouse Malignant Melanoma B16 Cells *in Vitro*

The proliferation of mouse malignant melanoma B16 cells was studied *in vitro* at different concentrations of Phx-3 [0 (white bar), $0.413 \mu\text{M}$ (grayish bar), $41.3 \mu\text{M}$ (gray bar) and $41.3 \mu\text{M}$ (dark bar)] for 72 h. The number of the cells ($\times 10^4$) at 0, 24, 48 and 72 h was depicted as a function of different concentrations of Phx-3.

Fig. 3. The Cutaneous (the Upper Column) and Subcutaneous (the Lower Column) Outlook of the Tumor after the Transplantation of Mouse Malignant Melanoma B16 Cells into the Flanks of Mice, with or without 0.5 mg/kg Phx-3

NC: normal control with PBS and without transplantation of mouse malignant melanoma B16 cells into mice. PC: positive control with transplantation of mouse malignant melanoma B16 cells. Phx-3: Phx-3 (0.5 mg/kg) was administered i.p. for 12 consecutive days starting with the day of transplantation.

Fig. 4. Changes in Tumor Size of the Mouse Malignant Melanoma B16 Cells Transplanted into Mice, with or without Phx-3 for 12 d

○: Phx-3; △: PC. Data represent the mean ± S.E.M. *n* = 5.

Fig. 5. Effect of Phx-3 on Body Weight of Mice Transplanted with B16 Cells

○: Phx-3; △: PC; ■: NC. Data represent the mean ± S.E.M. *n* = 5.

Fig. 6. Microscopic Section (HE, ×200) of the Liver (A) and the Kidney (B) from a Mouse Treated with Phx-3 (0.5 mg/kg) after Subcutaneous Injection of Malignant Melanoma B16 Cells into the Flank

Table 1. Serum Levels of Blood Biochemical Parameters in the Control Mice and the Melanoma Transplanted-Mice Treated with Phx-3

	AST (GOT) (IU/l)	ALT (GPT) (IU/l)	BUN (mg/dl)	Creatinine (mg/dl)
Control	80 ± 29	29 ± 11	23.4 ± 9.8	0.2 ± 0.1
Phx-3 (+)	88 ± 25	33 ± 8	17.0 ± 5.8	0.2 ± 0.1

Control: control mice, Phx-3 (+): the melanoma transplanted-mice treated with Phx-3. *n* = 5 in each group. Data were expressed as mean ± S.D.

Fig. 7. Microscopic Section of Fontana–Masson–Stained Skin Tissue from a Positive Control Mouse with Melanoma (A) ×40; and (B) ×400, and from a Mouse Treated with Phx-3 after Transplantation of Malignant Melanoma B16 Cells; (C) ×100

The skin specimens were obtained from several sites where mouse malignant melanoma B16 cells were inoculated. The figures show the representative tissues of these specimens.

Figure 5 shows the body weight change monitored during the experiment. No significant difference was observed in body weight between the control and Phx-3 administered groups throughout the experiment. No mice died in any

group during the experiment. As shown in Fig. 6, no significant pathological changes were observed in the liver (Fig. 6A) and the kidney (Fig. 6B) of mice treated with Phx-3 (0.5 mg/kg) after subcutaneous injection of malignant melanoma B16 cells into the flank. Blood chemistry revealed that there were no significant differences in AST, ALT, BUN and creatinine in the serum of the control mice and the mice treated with Phx-3 (0.5 mg/kg) after subcutaneous injection of malignant melanoma B16 cells into the flank (Table 1).

Figure 7 shows the microscopic analysis of the Fontana-Mason-stained skin tissue from a positive control mouse with melanoma (Figs. 7A, B), and from a mouse treated with Phx-3 after transplantation of melanoma cells (Fig. 7C). As is seen in Figs. 7A and B, the tumor cell contained a fine dusting of melanin pigment within the cytoplasm in a positive control. On the contrary, a tumor was not formed in this tissue at all and the inoculated melanoma cells disappeared completely in the mouse treated with Phx-3 after transplantation of melanoma cells (Fig. 7C). These results are consistent with our finding shown in Figs. 3 and 4.

DISCUSSION

We examined the inhibitory effects of Phx-3, which was produced by the reaction of *o*-aminophenol with bovine hemoglobin,¹³⁾ on the growth of mouse malignant melanoma B16 cells *in vitro* and *in vivo*. *In vitro*, the proliferation of the cells was inhibited by Phx-3, dose and time dependently, and was almost completely inhibited by 41.5 μ M Phx-3 (Fig. 2). This result is comparable to our previous report on the anticancer activity of Phx-3 against human malignant melanoma G361 cells¹¹⁾ and on that of Phx-1 against various cancer cell lines.^{6–10)} We furthermore demonstrated that the growth of mouse malignant melanoma B16 cells transplanted into C57BL/6Cr Slc mice was completely inhibited by the administration of 0.5 mg/kg Phx-3 (Figs. 3, 4). This result was confirmed by the pathological examination shown in Figs. 7A–C. These results suggest that Phx-3 exerts the strong anticancer activity against mouse malignant melanoma B16 cells. In the light of the empirical findings that chemotherapeutic agents including 5-fluorouracil (5-FU) exert the anticancer activity at less than 1 μ M *in vitro*, and several mg/kg *in vivo*,⁸⁾ our present results in Figs. 2 and 5 revealed that Phx-3 exerts its anticancer activity against mouse melanoma B16 cells at extremely low concentrations (0.5 mg/kg Phx-3), in the mouse transplanted with B16 cells, compared with the case of B16 cells *in vitro* (41.5 μ M Phx-3). Similar results have been also obtained for Phx-1 administered to mice transplanted with Meth A carcinoma cells⁸⁾ or human leukemia cells.⁹⁾ A plausible explanation for the difference in anticancer activity *in vitro* and *in vivo* may be that Phx-3 was metabolized to more effective metabolites in mice and that Phx-3 might act synergistically with various cytokines in the body. For example, Hara *et al.*¹⁵⁾ found that the anticancer effects of Phx-1 were enhanced as much as hundred times, when Jurkat cells, a human T leukemic cell line, were treated with Phx-1 in combination with TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). These views are currently speculative, and should await further investigation.

It has been shown that many anticancer drugs exert adverse effects including the decrease of body weight, piloerec-

tion, and dysfunction of the inner organs. As shown in Fig. 5, no significant weight loss was indicated (Fig. 5). In addition, no pathological changes were observed in the liver and the kidney of mice treated with Phx-3 (0.5 mg/kg) after subcutaneous injection of malignant melanoma B16 cells into the flank (Figs. 6A, B), and there were no significant differences in AST, ALT, BUN and creatinine in the serum of the melanoma-transplanted mice with Phx-3 (Table 1). These results suggest that Phx-3 causes few adverse effects on mice. When 5 mg/kg 5-FU was administered to mice, these adverse effects were significantly observed in mice, causing the death.⁸⁾ Actinomycin D is shown to have anticancer effects, however exerts strong adverse effects.¹²⁾ Though the phenoxazine is an essential component of Actinomycin D, Eckert and Eyer¹⁶⁾ demonstrated that Phx-3 and several phenoxazines are not toxic to dogs. Our present results are in good agreement with those of Eckert and Eyer.¹⁶⁾ With regard to Phx-1, it caused strong anticancer effects against human leukemia cell line HAL-01 and Meth A cancer cells transplanted into BALB/c mice with limited adverse effects.^{8,9)} The few adverse effects of Phx-3 and Phx-1 may be attributed to the fact that the chemical structure of these phenoxazines (Fig. 1) is analogous to that of vitamin B₂, composed of the phenazine ring (isoalloxazine). The mechanism for few adverse effects of Phx-1 and Phx-3 should await further investigation, though the various biological effects of Phx-1 and Phx-3 including antitumor activities, immunosuppressive effects and antiviral effects *etc.*^{17–20)} have been recently reported.

Malignant melanoma is now one of the most common malignancy in the world, and a highly lethal disease.^{1,2)} Only dacarbazine is currently approved for the treatment among many chemotherapeutic agents, however, its objective response rate is 10–15%.^{3,21)} This agent is included for most combination regimens. Since we showed the anticancer effect of Phx-3 on mouse malignant melanoma B16 cells *in vitro* and *in vivo*, in the present study, and Phx-3 seems to have few adverse effects on mice, it may be possible to apply Phx-3 to the treatment of patients with malignant melanoma, in combination with dacarbazine or as a single dose, in future. Phx-3 may be expected to be useful as an anticancer agent against human malignant melanoma, which is extremely resistant to chemotherapy.^{1,2,21)}

Acknowledgments We would like to thank Professor J. Patrick Barron, International Medical Communication Center, Tokyo Medical University, for reviewing the present manuscript. Research was supported by funds from High-Tech Research Project for Private Universities, and a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology, Japan (2003–2007) and from the Ito Foundation.

REFERENCES

- 1) Jemal A., Devesa S. S., Hartge P., Tucker M. A., *J. Natl. Cancer Inst.*, **93**, 678–683 (2001).
- 2) Pawlik T. M., Sondak V. K., *Oncol. Hematol.*, **45**, 245–264 (2003).
- 3) Chapman P. B., Einhorn L. H., Meyers M. L., Saxman S., Destro A. N., Panageas K. S., Begg C. B., Agarwala S. S., Schuchter L. L. M., Ernstoff M. S., Houghton A., Kirkwood J. M., *J. Clin. Oncol.*, **9**, 2745–2751 (1999).

- 4) Eton O., Legha S. S., Bedikian A. Y., Lee J. J., Buzaid A. C., Hodges C., Ring S. E., Papadopoulos N. E., Plager C., East M. J., Zhan F., Benjamin R. S., *J. Clin. Oncol.*, **15**, 2045—2052 (2002).
- 5) Runkle G. P., Znik A. J., *Am. Fam. Physician*, **49**, 91—98 (1994).
- 6) Ishida R., Yamanaka S., Kawai H., Ito H., Iwai M., Nishizawa M., Hamatake M., Tomoda A., *Anti-Cancer Drugs*, **7**, 591—595 (1996).
- 7) Abe A., Yamane M., Tomoda A., *Anticancer Drugs*, **12**, 377—382 (2001).
- 8) Mori H., Honda K., Ishida R., Nohira T., Tomoda A., *Anticancer Drugs*, **11**, 653—657 (2000).
- 9) Shimamoto T., Tomoda A., Ishida R., Ohyashiki K., *Clin. Cancer Res.*, **7**, 704—708 (2001).
- 10) Koshiibu-Koizumi J., Akazawa M., Iwamoto T., Takasaki M., Mizuno F., Kobayashi R., Abe A., Tomoda A., Hamatake M., Ishida R., *J. Cancer Res. Clin. Oncol.*, **128**, 363—368 (2002).
- 11) Shimizu S., Suzuki M., Miyazawa K., Yokoyama T., Ohyashiki K., Miyazaki K., Abe A., Tomoda A., *Oncol. Rep.*, **14**, 41—46 (2005).
- 12) Hollstein U., *Chem. Rev.*, **74**, 625—652 (1974).
- 13) Shimizu S., Suzuki M., Tomoda A., Arai S., Taguchi H., Hanawa T., Kamiya S., *Tohoku J. Exp. Med.*, **203**, 47—52 (2004).
- 14) Masson F., *Am. J. Pathol.*, **4**, 181—211 (1928).
- 15) Hara K., Okamoto M., Aki T., Yagita H., Tanaka H., Mizukami Y., Nakamura H., Tomoda A., Hamasaki N., Kang D., *Mol. Cancer Ther.*, **4**, 1121—1127 (2005).
- 16) Eckert K. G., Eyer P., *Biochem. Pharmacol.*, **32**, 1019—1027 (1983).
- 17) Enoki E., Sada K., Qu X., Kyo S., Shahjahan Miah S. M., Hatani T., Tomoda A., Yamamura Y., *J. Pharmacol. Sci.*, **94**, 329—333 (2004).
- 18) Iwata A., Yamaguchi T., Sato K., Yoshitake N., Tomoda A., *Biol. Pharm. Bull.*, **28**, 905—907 (2005).
- 19) Fukuda G., Yoshitake N., Khan Z. A., Kanazawa M., Notoya Y., Che X. F., Akiyama S., Tomoda A., *Biol. Pharm. Bull.*, **28**, 797—801 (2005).
- 20) Musha S., Watanabe M., Ishida Y., Kato S., Konishi M., Tomoda A., *Biol. Pharm. Bull.*, **28**, 1521—1523 (2005).
- 21) Margolin K. A., Sondak V. K., “Cancer Management: A Multidisciplinary Approach,” PRR, Inc., New York, 2002, pp. 471—510.