Antiviral Activity of 2-Amino-4, 4 α -Dihydro-4 α -7-Dimethyl-3H-Phenoxazine-3-One on Poliovirus

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IWATA, A., YAMAGUCHI, T., SATO, K., IZUMI, R. and TOMODA, A. *Antiviral Activity of 2-Amino-4, 4 α -Dihydro-4 α -7-Dimethyl-3H-Phenoxazine-3-one on Poliovirus.* Tohoku J. Exp. Med., 2003, **200** (3), 161–165 — 2-Amino-4, 4 α -dihydro-4 α -7-dimethyl-3H-phenoxazine-3-one (Phx), which was produced by the reaction of bovine hemoglobin with 2-amino-5-methylphenol, inhibited the proliferation of poliovirus in Vero cells between 0.25 μ g/ml and 2 μ g/ml with maximal antiviral activity at 1 μ g/ml. These results suggest that Phx may be useful to prevent the proliferation of poliovirus infection. ——— 2-amino-4, 4 α -dihydro-4 α -7-dimethyl-3H-phenoxazine-3-one; poliovirus
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In order to overcome viral infections, many trials to develop antiviral drugs have been undertaken, while vaccination has been adopted to overcome several viral infections due to poliovirus, poxvirus, influenza virus etc. The usefulness of the antiviral drugs discovered so far was, however, restricted by the adverse effects of the drugs and appearance of drug-resistant viruses. Acyclovir and gancyclovir are clinically useful against herpes virus (Schaffer et al. 1978), but emergence of drug resistant variants after long-term treatment disturbs the

treatment of immunocompromised patients (Schmit and Boivin 1999). Azide thymidine is effective against HIV, but with marked adverse effects during long time administration (Yarchoan et al. 1989). Therefore, there is a persistent need for the antiviral drugs exerting strong antiviral and little adverse effects.

On the other hand, anthraquinones and anthraquinone derivatives including hypericin and emodin have been indicated to exert antiviral and virucidal effects to several viruses (Meruelo et al. 1988; Schinazi et al. 1990; Tang et al.

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1990). Andersen et al. (1991) reported that hypericin and emodin had antiviral activity against some enveloped viruses, but not against non-enveloped viruses such as poliovirus. Since the anthraquinones are a tricyclic chromophore, it would be expected that other tricyclic chromophores, for example, phenoxazines may show antiviral effects. However, antiviral effects of the phenoxazines have not been investigated hitherto, probably because of low biological activity such as anti-tumor effects of the chemically synthesized phenoxazines (Motohashi et al. 1991). Among phenoxazine compounds, actinomycin D has been exceptionally and extensively studied (Goldberg and Friedman 1971), but its clinical use is limited because of the adverse effects. Tomoda et al. (1992, 2001) found that 2-amino-4,4 α -dihydro-4 α -7-dimethyl-3H-phenoxazine-3-one (Phx) was produced by the reaction of human or bovine hemoglobin with 2-amino-5-methylphenol. Phx inhibited the proliferation of cancer cells such as Burkitt's lymphoblastoid cell lines, Raji and P3HR-1 (Koshibu-Koizumi 2002), human leukemia cell lines, HL-60 and HAL-10 (Shimamoto et al. 2001), and human lung carcinoma cell lines, A549 and H226 (Abe et al. 2001) in vitro, and showed little adverse effects in mouse (Mori et al. 2000; Shimamoto et al. 2001). Recently, Phx was demonstrated to exert immunosuppressive effects, because Phx down-regulates the expression of IgM in DT40 B cell line (Gao et al. 2002) and inhibits the incorporation of ^3H -thymidine into DNA of phytohemagglutinin-activated human T cells (Akazawa et al. 2002). Since it has been reported that emodin, a tricyclic chromophore, whose chemical structure is analogous to Phx, shows anti-tumor effect on hepatocellular carcinoma cells (Jing et al. 2002), and immunosuppressive effects (Huang et al. 1991), and antiviral effects (Andersen et al. 1991), we decided to investigate the antiviral effects of Phx. We found by chance that the proliferation of poliovirus was effectively inhibited by Phx. In this manuscript, we describe the

antiviral effects of Phx on poliovirus.

MATERIALS AND METHODS

Reagents

Phx was prepared by reaction of bovine hemoglobin with 2-amino-5-methylphenol, as previously described (Tomoda et al. 2001). Phx was dissolved in dimethyl sulfoxide (DMSO) to reach a concentration of 20 mM, before use.

African green monkey kidney cells (Vero cells), were kindly supplied by the Japanese Cancer Research Resources Bank (Tokyo). Cells were maintained in α -minimum essential medium (α -MEM) supplemented with 10% fetal calf serum (FCS, Sigma Co., Ltd., St. Louis, MO, USA), and 30 mg/liter kanamycin sulfate at 37°C under moisturized air containing 5% CO₂.

Determination of viral infectivity

The infectious titer of poliovirus suspension was determined using indicator cells, Vero cells (Johnson and Sarnow 1991). The cells were seeded in a 96-well microplate (Asahi Technoglass Co., Ltd., Tokyo) at a density of 2×10^5 to 3×10^5 cells per well in culture medium, and cultured at 37°C, for 2 days.

Then, Phx solution (20 mM) in DMSO diluted with α -MEM, was added to the cells in each well, to be final concentration of 0 $\mu\text{g/ml}$ (DMSO alone), 0.25 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$. After 1-hour incubation at 37°C, the supernatant was removed from the well by an aspirator. At this time, poliovirus suspensions, which had been serially diluted with α -MEM to obtain a 50% tissue culture infectious dose (TCID₅₀, defined as dilution ratio of the virus to generate 50% disruption of the cells) (LaBarre and Lowy 2001) were inoculated into Vero cells in each well. Cell were then incubated at 37°C, for 1 hour. After the supernatant was removed by an aspirator, various concentration of Phx solution was added to the cells in each well, and the cells were incubated at 37°C, for 2 days.

After 2 days, the disruption of Vero cells

was determined (Schmidtke 2001). Briefly, after removal of the supernatant with an aspirator, the remaining Vero cells were fixed to each plate, by treating with a fixing solution containing 0.9% NaCl and 4% paraformaldehyde, at 25°C for 2 hours. The fixing solution was removed with an aspirator, and the cells were stained with 0.5% crystal violet solution at 25°C for 1 hour. After staining, the cells were washed with 0.9% NaCl solution, 3 times, and were dried by air. Then, the dye incorporated into the cells was extracted with 100 μ l of 100% ethylene glycol, with stirring. Contents of the extracted dye were estimated by the optical density at 577 nm, using a plate reader (EL340 type, Biotech Instruments Co., Ltd., Tokyo). By these procedures, the disruption of Vero cells and the infectivity of poliovirus to the cells were estimated.

Effect of Phx on cell viability

Effect of Phx on the viability of Vero cells was examined in the presence of various concentrations of Phx, and without addition of poliovirus, according to the method as described above. There was no significant disruption of Vero cells at various concentrations of Phx up to 50 μ g/ml.

RESULTS AND DISCUSSION

As shown in Fig. 1, Phx inhibited the proliferation of poliovirus infected in Vero cells at all concentrations tested between 0.25 μ g/ml and 2 μ g/ml, and reached maximal antiviral activity at 1 μ g/ml. We observed that the proliferation of poliovirus was strongly inhibited after 1 hour treatment of Vero cells with Phx as described in Materials and Methods (Fig. 1), but was not inhibited, when poliovirus was previously treated with various concentrations of Phx and then inoculated into the Vero cells (data not shown). This result strongly suggests that Phx has not virucidal activity against poliovirus but has exerted antiviral activity through affecting unknown mechanism of the Vero cells. The present results also suggest that Phx may suppress the proliferation of poliovirus infected in humans, as the suppression of proliferation of the virus infected in the Vero cells may be interpreted as the prevention of virus infection in vivo. Taking account of the present results and that Phx exerted little adverse effects such as bodyweight loss, piloerection and myelosuppression in mice (Mori et al. 2000; Shimamoto et al. 2001), Phx may be applicable to the treatment or prevention of polio infection

Fig. 1. Antiviral effects of Phx on poliovirus.

in humans, in future.

Tang et al. (1990) reported that hypericin, a derivative of emodin exert antiviral activity against enveloped viruses such as HSV-1, influenza virus A and Mo-Mul V, but not against non-enveloped viruses such as poliovirus and adenovirus, at concentrations of 1.56 to 25 $\mu\text{g}/\text{ml}$. Interestingly, the chemical structures of Phx and emodin (1, 3, 8-trihydroxy-6-methylanthra-quinone) are similar, because these compounds are tricyclic chromophores with the methyl group at the same position; however, the former is not a semiquinone type, but the latter is a semiquinone, producing active oxygens (Huang et al. 1991). Such similarity and differences in the chemical structure seem to affect the differences in biological actions between these compounds. Namely, Phx and emodin have anti-cancer activity (Mori et al. 2001; Jing 2002; Koshibu-Koizumi et al. 2002), immunosuppressive activity (Huang et al. 1991; Akazawa et al. 2002; Gao et al. 2002), and vasorelaxing activity (our unpublished data; Huang et al. 1991). However, as we have shown, Phx does exert antiviral activity against poliovirus, and emodin does not (Andersen et al. 1991).

Whether Phx may exert antiviral activity against viruses other than poliovirus should be clarified in further studies. The mechanism for the inhibition of poliovirus is not clear at present.

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