Increase of Sensitivity and Uptake of Vinblastine by Reserpine in Rat Ascites Hepatoma

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Abstract—We investigated the effect of reserpine on the antitumor effect of vinblastine (VBL) with regard to the drug resistance of rat ascites hepatomas. The sensitivity to VBL was in the order of AH13 > AH44 > AH109A > AH66 cells in the in vitro growth-inhibitory test, and AH66 cells were inherently most resistant to VBL. The intracellular accumulation of VBL was lower in resistant cells than in sensitive cells. Reserpine increased the sensitivity to VBL in the order of AH66 > AH109A > AH44 > AH13 cells. The antitumor synergism was also observed in the in vivo experiments using AH44 and AH66. Reserpine enhanced the VBL accumulation more than 2 times in AH66 and AH109A cells, but slightly increased it in AH13 and AH44 cells. These results indicated that the synergistic effect of reserpine was more potent in relatively resistant cell lines to VBL, and the effect was caused by the enhancement of VBL accumulation. On the other hand, the enhanced growth-inhibitory effect and the accumulation of VBL in the presence of reserpine were not influenced by further preincubation with reserpine. Reserpine also did not influence the intracellular level of VBL increased by 2,4-dinitrophenol in a glucose deprived medium. Reserpine decreased the VBL extrusion from AH66 cells more strongly than that from AH44 cells. These results indicated the possibility that reserpine interfered with the VBL efflux process, while it might not influence the VBL influx process. In conclusion, reserpine potentiated the effect of VBL on resistant ascites hepatoma cells more than on sensitive cells, and the synergistic effect of reserpine could be explained by the inhibition of VBL efflux and VBL accumulation in tumor cells.

It is widely known that a tumor tissue consists of various subtype cells which have different sensitivities towards antitumor agents. The heterogeneity of tumor cells is one of the most important problems in cancer chemotherapeutics. We have been attempting to overcome this problem by the combined chemotherapy of antitumor agents with non-antitumor agents such as central nervous system depressants and sympathomimetics (1-6). Reserpine potentiated the antileukemic effect of several antitumor agents including vinblastine (VBL) on mice bearing leukemia L1210 (2, 3), and it seemed that the combination effect of reserpine was chiefly caused by the marked hypothermia in mice, while the cell growth was synergistically inhibited by the combined treatment in vitro (3). Our previous report showed that the antitumor effect of 1-γ-chloropropyl-2-chloromethylpiperidine hydrobromide (CAP-2), a nitrogen mustard type alkylating agent, on rat ascites hepatoma AH44 which was inherently resistant to alkylating agents was potentiated by reserpine, both in the in vitro and in vivo experiments (1). In the case of combined therapy in rats, it was thought that the synergism was provided by mechanisms other than hypothermia and depression because the dosage of reserpine used did not induce severe adverse reactions such as hypothermia and depression in rats.

In this report, we investigated the sensitivity to VBL in rat ascites hepatoma AH13, AH44,
AH66 and AH109A cells, and the influence of reserpine on the antitumor effect of VBL in these tumors. The influence of reserpine on the cellular accumulation of VBL was also investigated.

Materials and Methods

Cell culture: Rat ascites hepatoma AH13, AH44, AH109A and AH66 cells were provided by the Sasaki Institute, Tokyo. The cells were passaged weekly in the peritoneal cavity of female Donryu rats (Shizuoka Agricultural Cooperative Association for Laboratory Animals). The cells were washed with phosphate-buffered saline (PBS) to remove red blood cells and suspended in Eagle's minimum essential medium (MEM) containing kanamycin (100 µg/ml). The cells were incubated with reserpine (Sigma) and VBL (Shionogi) for 30 min after the preincubation in the absence or presence of reserpine for 3 hr at 37°C, washed with PBS, and suspended in Eagle's MEM supplemented with 10% fetal calf serum. The cells were plated in 35 mm plastic dishes (Falcon 3001) at 3×10^5 cells/ml and cultured for 2 days at 37°C in a CO₂ incubator. After the cultivation, the cell number was counted microscopically. In the case of AH66 and AH109A cells, the cells were counted after detaching them with a rubber policeman and trypsinization with 0.25% trypsin (Difco) for 5 min at 37°C. Reserpine was dissolved in dimethylsulfoxide (DMSO) to give a final concentration of 0.5% DMSO. DMSO was also added to the control group at the same concentration. The cytotoxic activity of VBL in the absence or presence of reserpine was evaluated by the growth rate (percent of control) and the 50% inhibitory concentration (IC50), which was obtained by plotting the logarithm of the concentration of VBL versus the growth rate of the treated cells. The initial cell number was subtracted in the calculation.

Uptake of VBL: Rat ascites hepatoma cells suspended at 5×10^5 cells/ml in Eagle's MEM supplemented with 10% fetal calf serum were incubated with 5 µg/ml of reserpine, 0.1 µCi of ^3H-VBL (9.9 Ci/mmol, Amersham) and 10^{-7} M of VBL for designated periods at 37°C following the preincubation for 10 min. After the incubation, the treated cells were collected by centrifugation (3,000 rpm for 5 min at 4°C) and washed twice with cold PBS. The cells were solubilized with Protosol (New England Nuclear), and the intracellular radioactivity was counted in a liquid scintillation counter.

Efflux of VBL: The cells incubated with ^3H-VBL for 30 min at 37°C were collected by centrifugation, washed, and suspended in fresh medium. The cells were incubated for designated periods, and the retained intracellular radioactivity was counted.

Influence of 2,4-dinitrophenol on uptake and efflux of VBL: AH66 cells were suspended in 5.8 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes)-buffered physiological saline (pH 7.2) containing 135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂ and 2.5 mM CaCl₂. In experiments for the uptake of VBL, the cells were preincubated in the presence or absence of 0.1 mM of 2,4-dinitrophenol (DNP, Wako) or 11.5 mM of glucose for 10 min at 37°C and further incubated with ^3H-VBL and cold VBL. The effect of reserpine on the uptake of VBL was examined in cells treated with DNP, which interferes with cellular energy generation. In experiments for the efflux of VBL, the cells were loaded with ^3H-VBL for 30 min and washed. These cells were suspended in fresh medium containing DNP (0.1 mM) and/or glucose (11.5 mM) and were incubated for the designated periods.

In vivo combined experiment: Female Donryu rats (6-week old) were intraperitoneally inoculated with 1×10^6 AH44 or AH66 cells. The rats were intraperitoneally administered VBL and reserpine (Daichi) once a day for 7 days from 3 days after the cell inoculation, and the experiment was terminated on day 60.

Results

Effect of reserpine on the growth-inhibitory action of VBL in AH13, AH44, AH109A and AH66 cells: The growth-inhibitory action of VBL and the combination effect of reserpine in each cell line were investigated in the in vitro system. Table 1 summarizes the IC50 values of VBL in the absence or presence of 2.5 to 10 µg/ml of...
reserpine. These results indicate that AH66 cells are most inherently resistant to VBL among these cell lines and the sensitivity of AH66 cells is one-sixth of that of AH44 cells and one-twentieth that of AH13 cells. The growth-inhibitory action of VBL was enhanced 66-fold in AH66 cells and 1.7-fold in AH13 cells by 10 μg/ml of reserpine. In AH66 cells, the effect of VBL was enhanced 3.4-fold even by 2.5 μg/ml of reserpine.

**Effect of reserpine on the uptake of VBL:** The time course of VBL uptake in AH13, AH44, AH109A and AH66 cells is indicated in Fig. 1. The amount of intracellular VBL at

| Reserpine (μg/ml) | IC50 (M) of VBL |
|-------------------|----------------|
|                   | AH13        | AH44        | AH109A       | AH66        |
| 0                 | 1.1×10⁻⁷    | 3.7×10⁻⁷    | 7.5×10⁻⁷     | 2.3×10⁻⁶    |
| 2.5               | N.D.        | N.D.        | N.D.         | 6.7×10⁻⁷ (3.4) |
| 5                 | N.D.        | 1.3×10⁻⁷ (2.8) | 2.1×10⁻⁷ (3.6) | 1.1×10⁻⁷ (21) |
| 10                | 6.3×10⁻⁸ (1.7) | 5.0×10⁻⁸ (7.4) | 5.5×10⁻⁸ (13) | 3.5×10⁻⁸ (66) |

Each IC50 value represents the mean of triplicate determinations, and numbers in parentheses represent increase (-fold) in cytotoxicity as compared to the values without reserpine. N.D.: not determined.

![Fig. 1](image)

**Fig. 1.** Effect of reserpine on the uptake of VBL in AH13, AH44, AH66 and AH109A cells. The cells were incubated with ³H-VBL in the presence (●—●) or absence (○—○) of 5 μg/ml of reserpine. Each point represents the mean±S.E. of triplicate determinations. * **Significantly different from the control at *P<0.05 and **P<0.01.
30 min in the absence of reserpine was 8.2, 5.6, 2.1 and 1.4 pmol/10^6 cells in AH13, AH44, AH109A and AH66 cells, respectively. When 5 µg/ml of reserpine was added to the cells just before the addition of ^3H-VBL, the uptake of VBL was slightly increased in AH13 and AH44 cells. In the case of the cells in which the uptake of VBL was much smaller, the uptake was increased about 2-fold in AH109A cells and about 4-fold in AH66 cells by reserpine.

Combination therapy with reserpine and VBL: The combination effect of reserpine and VBL was investigated in rats bearing AH44 or AH66 (Fig. 2). Reserpine did not induce hypothermia and did not prolong the lifespan of rats bearing each cell line at the dosage of 0.25 mg/kg by itself. When rats bearing AH44 were administered 0.0125 and 0.025 mg/kg of VBL, the mean of surviving days was 8.5 days in each case. The lifespan of the rats on combination therapy with VBL and reserpine was significantly prolonged, and the mean of surviving days was 16 days in the rats which were given 0.025 mg/kg of VBL and 0.25 mg/kg of reserpine. In the case of rats bearing AH66, rats were medicated with 0.05 and 0.1 mg/kg of VBL, and the values for the mean surviving days were 13.5 and 14.7 days, respectively. In the combination therapy with reserpine, the lifespan was prolonged to 24.2 and 32.8 days in the rats which were given 0.05 and 0.1 mg/kg of VBL, respectively. Then, to determine the mechanism for the combination effect of reserpine, the following experiments were carried out using these two cell lines (AH44 and AH66).

Effect of reserpine on the transport of VBL: At first, the influence of pretreatment of reserpine on the growth-inhibitory effect of VBL in AH44 and AH66 cells was investigated. Figure 3 shows the influence of pretreatment of reserpine on the combined growth-inhibitory effect. The growth of AH44

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**Fig. 2.** Effect of combination therapy with reserpine (Rp) and VBL against rats bearing AH44 and AH66. Female Donryu rats were inoculated with 1×10^6 AH44 or AH66 cells in the peritoneal cavity and intraperitoneally administered 0.25 mg/kg of Rp and VBL once a day for 7 days, from 3 days after the cell inoculation. Symbols: AH44) ---X untreated control, ■■■ Rp, O O 0.025 mg/kg VBL, ••• Rp and 0.025 mg/kg VBL, △△△ O 0.0125 mg/kg VBL, ▲▲▲ Rp and 0.0125 mg/kg VBL, AH66) ---X untreated control, ■■■ Rp, O O 0.1 mg/kg VBL, ••• Rp and 0.1 mg/kg VBL, △△△ O 0.05 mg/kg VBL, ▲▲▲ Rp and 0.05 mg/kg VBL.
cells was decreased to 70% of the control by \(10^{-7}\) M VBL alone, while the growth of AH66 cells was not affected by this concentration of VBL alone (column A). The cell growth was inhibited to 55% in AH44 cells and 60% in AH66 cells by the combined treatment with 5 \(\mu g/ml\) of reserpine (column B). The 3 hr pretreatment of reserpine, however, did not further affect the cell growth (column C). The influence of pretreatment of reserpine on the uptake of VBL is shown in Fig. 4. The amount of intracellular VBL increased immediately after the start of the incubation with VBL, but the accumulation rate decreased slightly in AH44 cells and decreased apparently in AH66 cells after 30 sec. When reserpine and VBL were simultaneously added to the cell suspension, reserpine (5 \(\mu g/ml\)) did not influence the early rapid phase of the accumulation rate of VBL observed in the initial 30 sec and accelerated the late accumulation rate. Further pretreatment with reserpine did not affect these accumulation rates of VBL.

Secondly, using AH66 cells which were the most resistant cell line, the effect of DNP and reserpine on the transport of VBL into the cells was investigated (Figs. 5 and 6). The cells, which were treated with DNP in

**Fig. 3.** Influence of pretreatment with reserpine on the growth-inhibitory action of VBL against AH44 and AH66 cells. The cells were preincubated without (A: \(\square\)) or with (B: \(\bigtriangleup\)) 5 \(\mu g/ml\) of reserpine for 3 hr. These cells were treated with \(10^{-7}\) M of VBL in the absence (A) or presence (B, C) of 5 \(\mu g/ml\) of reserpine for 30 min. Each column represents the mean±S.E. of triplicate determinations. *Significantly different from column A at \(P<0.01\).

**Fig. 4.** Influence of pretreatment with reserpine on the uptake of VBL in AH44 and AH66 cells. The cells were preincubated without (○—○) or with (△—△) 5 \(\mu g/ml\) of reserpine for 3 hr, and then they were incubated with \(3H\)-VBL in the absence (○—○) or presence (△—△) of 5 \(\mu g/ml\) of reserpine. Each point represents the mean±S.E. of triplicate determinations. ***Significantly different from the control at \(*P<0.05\) and **\(P<0.01\).

**Fig. 5.** Effect of DNP on the uptake and efflux of VBL in AH66 cells. (A): The cells were suspended in Hepes-buffered saline and incubated with \(3H\)-VBL in the presence of 11.5 mM of glucose (○—○) or 0.1 mM of DNP (△—△). The arrow indicates the addition of glucose (11.5 mM) into the incubation medium (△—△). (B): The cells were loaded with \(3H\)-VBL in Hepes-buffered saline for 30 min and washed at the arrow. These cells were suspended in fresh medium (△—△), DNP (0.1 mM)-containing medium (△—△), glucose (11.5 mM)-containing medium (○—○), or glucose- and DNP-containing medium (○—○). Each point represents the mean±S.E. of triplicate determinations. \(**:\) Significantly different from the glucose group at \(*P<0.05\) and \(**P<0.01\).
In glucose-free medium, accumulated VBL more than the cells incubated with glucose, but the accumulation was decreased by the addition of glucose into the incubation medium (Fig. 5A). Moreover, the efflux of VBL was suppressed by DNP in glucose-free medium, while in glucose containing medium, the suppression of the efflux by DNP was not observed (Fig. 5B). This result indicated that VBL was extruded from cells by an energy-dependent process. On the other hand, the accumulation of VBL in the cells treated with DNP and reserpine was to the same extent as that in the cells treated with DNP alone or with reserpine alone, and reserpine did not further enhance the VBL accumulation in the cells treated with DNP (Fig. 6).

**Effect of reserpine on the efflux of VBL:** The cells which were preloaded with VBL by incubation with ³H-VBL in the presence of reserpine for 30 min were incubated without or with reserpine, and the retained VBL was estimated. As shown in Fig. 7, the

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**Fig. 6.** Effect of reserpine on the uptake of VBL in the presence of DNP in AH66 cells. The cells were suspended in Hepes-buffered saline (pH 7.2) and preincubated with or without glucose (11.5 mM) or DNP (0.1 mM) for 10 min. These cells were further incubated with ³H-VBL for 20 min at 37°C. Reserpine (5 μg/ml) was added just before the addition of ³H-VBL. Each column represents the mean ± S.E. of triplicate determinations. *Significantly different from the control at P<0.01.

**Fig. 7.** Effect of reserpine on the efflux of VBL in AH66 cells. The cells were suspended in Eagle's MEM supplemented with 10% fetal calf serum and loaded with ³H-VBL for 30 min. These cells were washed, suspended in fresh medium, and incubated with (●) or without (○) 5 μg/ml of reserpine. Each point represents the mean ± S.E. of triplicate determinations. *Significantly different from the control at *P<0.05 and **P<0.01.
efflux of VBL from AH66 cells was suppressed in the presence of 5 μg/ml of reserpine, but in the case of the AH44 cells, the suppression was very slight.

Discussion

Recently, many trials to search for agents which can overcome the resistance of tumors to antitumor agents have been performed, and several non-antitumor agents have been mentioned as possible candidates (7–10). We have previously reported that reserpine, which is a major tranquilizer and an antihypertensive drug, potentiated the antitumor effect of several antitumor agents, including alkylating agents and VBL, on rat ascites hepatoma (1) and mouse leukemia L1210 (2, 3).

In this study, using rat ascites hepatoma, we investigated the correlation between the influence of reserpine on the antitumor effect of VBL and naturally acquired resistance of a tumor cell line to VBL. The sensitivity to VBL was in the order of AH13 > AH44 > AH109A > AH66 cells in the in vitro growth-inhibitory test, and AH66 cells was inherently most resistant to VBL. This sensitivity showed a close relationship to the intracellular accumulation of VBL as shown in Fig. 1. Namely, the amount of VBL in resistant AH66 cells at 30 min was 1.4 pmol/10^6 cells and then achieved the plateau level, but in the case of the most sensitive cell line AH13, the amount of intracellular VBL reached about 6-fold that of AH66 cells at 30 min and continued to increase. These results were in agreement with the reports by Ichimura (11) and Inaba et al. (12).

Reserpine potentiated both the growth-inhibitory effect and the chemotherapeutic effect of VBL, and the synergistic effect of reserpine was more potent in relatively resistant cells (Table 1 and Fig. 2). This effect of reserpine may be supported by the enhancement of intracellular VBL accumulation, the extent being larger in the cells relatively resistant to VBL than in the sensitive cells (Fig. 1). Skovsgaard (13) suggested that the mechanism of resistance of Ehrlich ascites tumor to Vinca alkaloids involved the energy-dependent extrusion of the agents and unspecific changes in the membrane that reduce the influx of the agents. Other investigators suggested that the resistance of mouse leukemia P388 and rat ascites hepatoma to daunorubicin, adriamycin and vincristine was attributable to the enhanced activity of the efflux of these antitumor agents from the cells, because the intracellular accumulation of vincristine was increased in glucose-free and DNP-containing medium, and the efflux of the antitumor agent was decreased in the same medium (12, 14). This study showed that the apparent uptake of VBL by AH66 cells was more increased in glucose-free and DNP-containing medium, and the efflux of VBL from the cells was decreased in that medium (Fig. 5). Furthermore, for the intracellular accumulation of VBL by reserpine, reserpine did not influence the early accumulation rate of VBL which was thought to indicate only the influx of VBL (Fig. 3). It was also observed that if reserpine was added to the culture 3 hr prior to VBL, the growth-inhibitory activity and the accumulation of VBL in AH44 cells and AH66 cells did not change in comparison with the simultaneous exposure to VBL and reserpine (Figs. 3 and 4). Moreover, reserpine did not further increase the accumulation of VBL in the glucose-free and DNP-containing medium (Fig. 6). Therefore, it was thought that the effect of reserpine on the enhancement of the growth-inhibitory activity of VBL and on the increase of the uptake of VBL were not due to the acceleration of the influx of VBL into the cells. Moreover, reserpine significantly accelerated the late accumulation rate of VBL which was observed about 30 sec after the start of uptake, and the extent was larger in VBL resistant AH66 cells than in sensitive AH44 cells. It may be possible to explain these phenomena from the facts that reserpine inhibited the efflux of VBL and the inhibitory effect was stronger in resistant cells than in sensitive cells (Fig. 7). From these findings, it seems that the overcoming of resistance produced by reserpine can attribute to the inhibitory effect of the drug on the efflux of VBL. There are few papers concerning the activity of reserpine against the active transport of drugs. It was shown
that reserpine was an uncoupler of mitochondrial oxidative phosphorylation like DNP (15) and inhibited the energized uptake of Ca\(^{++}\) by mitochondria (16), and it was also indicated that reserpine had a direct action as a Ca\(^{++}\)-antagonist on mammalian smooth muscle cells (17). In this report, it was observed that DNP did not affect the apparent uptake and efflux of VBL in the presence of glucose (Fig. 5), while reserpine enhanced the uptake of VBL and inhibited the efflux of VBL in Eagle's MEM which is glucose-rich (Figs. 1, 3 and 7). Recently, Tsuruo et al. (7, 8) have reported that the resistance of mouse leukemia P388 to vincristine was overcome by the Ca\(^{++}\)-channel antagonist verapamil and the calmodulin inhibitor trifluoperazine which inhibited the active extrusion of vincristine. It may be thought that the inhibitory effect of reserpine on the efflux of VBL was provided by its action as a Ca\(^{++}\)-antagonist, like verapamil. On the other hand, it is also worth deliberation that reserpine enhanced the growth inhibitory activity of the mitotic poison VBL (18), because reserpine is likely to possess a colchicine-like activity against microtubules (19). For a more complete elucidation of the mechanism, we must examine the effect of reserpine on the cellular components and the characteristics of the cells naturally acquired resistance to antitumor agents.

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