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# Saturable Function of P-Glycoprotein as a Drug-efflux Pump in Multidrug-resistant Tumour Cells

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## Abstract

P-glycoprotein acts as an active drug-efflux pump in multidrug-resistant tumour cells. We studied the capacity of P-glycoprotein to extrude drugs from the cells.

For nanomolar concentrations of vinblastine P388/ADR cells, which overexpress P-glycoprotein in the plasma membrane, accumulated vinblastine, at 37°C for 30 min, to a much lower extent than the sensitive cells (P388/S), while in the micromolar range the cellular concentration was similar for both types of cells. When cells were incubated with a low (10 nM) or high concentration (1 μM) of vinblastine while energy deprived, the vinblastine concentration increased only in the resistant cells incubated with the low concentration of vinblastine, and this increased level was lowered to the level under the normal conditions by addition of glucose. In contrast, the cellular concentrations in other cases were increased to the normal level by glucose. After cells were loaded with the low concentration of vinblastine, the cellular vinblastine was extruded more rapidly from the resistant cells than from the sensitive cells. The courses of vinblastine efflux from the cells loaded with the high concentration of vinblastine were similar in both types of cells. NA-382, a reported P-glycoprotein inhibitor, effectively increased the intracellular vinblastine and inhibited the drug efflux only from multidrug-resistant cells, P388/ADR and AH66 cells, which were incubated with the low concentration of vinblastine. Cellular uptake of NA-382 was also less in P388/ADR cells than in P388/S cells in culture with 10 nM but not 1 μM of the agent, and this low level was reversed to the level in the sensitive cells by 10 μM vinblastine.

These results indicate that P-glycoprotein as a drug-efflux pump works effectively under low extracellular concentrations of substrates, but does not under the high concentrations.

P-glycoprotein overexpressed in the plasma membrane of multidrug-resistant tumour cells acts as a drug-efflux pump in an energy-dependent manner (Riordan & Ling 1985; Hamada & Tsuruo 1988). P-glycoprotein has also been reported in several normal tissues (reviewed by Leveille-Webster & Arias 1995). Modification of the function of P-glycoprotein may be applicable to the drug therapy for multidrug-resistant tumours and drug delivery for other diseases. Therefore, it is important to measure the capacity of the drug-efflux pump for substrate drugs.

Most investigators have used highly sensitive methods for measurement of drug concentration, such as radioisotope-labelled compounds, and shown much evidence for the P-glycoprotein-dependent drug transport system. There are, however, unclear results for the P-glycoprotein function, in many cases using high drug concentrations with less sensitive methods for drug measurement, such as spectrophotometry or fluorometry. We thought this discrepancy may be based on the capacity of P-glycoprotein for drug-efflux pump action.

In this study, we indicate, using multidrug-resistant tumour cell lines, P388/ADR (Johnson et al 1976; Inaba et al 1979) and AH66 (Yoshida 1956; Miyamoto et al 1992), that the P-glycoprotein effectively functions with low drug concentrations outside the cells but is hardly effective in high drug concentrations.

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## Materials and Methods

### *Materials*

Vinblastine was purchased from Shionogi & Co., Osaka, Japan. 7-Oxo-*N*-ethoxycarbonylstaurosporine (NA-382) was kindly provided by the Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., Yokohama, Japan. [$^3$H]Vinblastine (374 GBq mmol$^{-1}$) was obtained from Amersham International, UK.

Vinblastine and NA-382 were dissolved in physiological saline and dimethylsulphoxide, respectively, and used after 200-fold dilution with the incubation medium.

### *Tumour cells*

The mouse leukaemia P388 (P388/S) and the adriamycin-induced multidrug-resistant cell line P388/ADR were passaged weekly through female BALB/c × DBA/2 (CDF1) mice (Nippon SLC, Hamamatsu, Japan). Rat ascites hepatoma cell lines, AH66 and AH66F, were also maintained in the peritoneal cavity of female Donryu rats (Nippon SLC). The tumour cells were harvested from tumour-bearing animals 6 to 7 days after transplantation.

### *Experiments on vinblastine accumulation and efflux*

Cells ($2 \times 10^6$ mL$^{-1}$) were incubated with varying concentrations of [$^3$H]vinblastine in normal Hanks' solution containing 5·6 mM glucose or in glucose-free Hanks' solution containing 10 mM sodium azide at 37°C. After appropriate times, the cells were washed, dissolved in 0·5 M sodium hydroxide, and neutralized. The radioactivity was counted in a Beckman LS-5800

liquid scintillation counter after the addition of a toluene:Triton X-100 (2:1, v/v) scintillation fluid mixture containing 0·2% 2,5-diphenyloxazole and 0·05% 1,4-bis[2-(5-phenyloxazoyl)]-benzene.

In the efflux experiments, cells were incubated with 10 nM or 1 μM vinblastine in glucose-free Hanks' solution containing 10 mM sodium azide at 37°C for 30 min, then washed, and further incubated in normal Hanks' solution at 25°C. After an appropriate time, the radioactivity remaining in the cells was measured, and the results were expressed as the percentage of retained vinblastine in the cells compared with the amount after the forced accumulation.

*Measurement of intracellular NA-382*

Cells were incubated with NA-382 in the absence or presence of 10 μM vinblastine at 37°C for 30 min. The intracellular concentration was measured using a high pressure liquid chromatography system with a spectrofluorometer (excitation wavelength 320 nm, emission wavelength 550 nm), by the method previously reported (Miyamoto et al 1995).

*Data analysis*

Experiments were performed at least three times. Statistical analysis was by Student's *t*-test and Welch's *t*-test.

## Results and Discussion

In multidrug-resistant tumour cells, anticancer drugs transported into the cells are extruded by a membrane-associated drug-efflux pump, P-glycoprotein, and the antitumour effect becomes diminished (Dano 1973; Inaba et al 1979). The vinblastine concentration in P388/S cells increased according to the extracellular drug concentration (Fig. 1). On the other hand, in the case of the resistant P388/ADR cells, which overexpress a 140 kDa P-glycoprotein in the plasma membrane (Hagiwara et al 1991), when cells were incubated with nanomolar concentrations of vinblastine, the cellular uptake of the anticancer drug was much less than in the sensitive cells. Under high vinblastine concentrations (micromolar range), the intracellular concentration was similar in both types of cells (Fig. 1). This result suggests a limited capacity of P-glycoprotein to extrude the drug, although vinblastine shows a high affinity for P-glycoprotein among the reported substrates (Akiyama et al 1988; Wakusawa et al 1992).

P-glycoprotein is an ATPase and pumps out drugs in an energy-dependent manner (Inaba et al 1979; Hamada & Tsuruo 1988). Cells incubated with 10 nM or 1 μM vinblastine when deprived of energy had the vinblastine concentration increased to a similar level in both P388/S and P388/ADR cells, according to the extracellular drug concentrations (Figs 2, 3). The cellular concentration in P388/ADR cells incubated with 10 nM vinblastine was decreased to the level under normal conditions by addition of 20 mM glucose (Fig. 3). In other cases, the cellular vinblastine level was increased to the normal

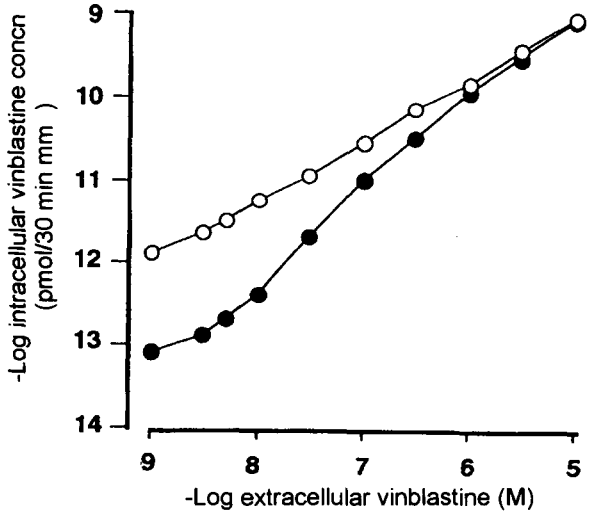


FIG. 1. Vinblastine uptake in P388 cells. P388/S cells (○) or P388/ADR cells (●) were incubated with varying concentrations of vinblastine in normal Hanks' solution at 37°C for 30 min. Data are the mean (s.e. is within the symbols) of three experiments performed in triplicate.

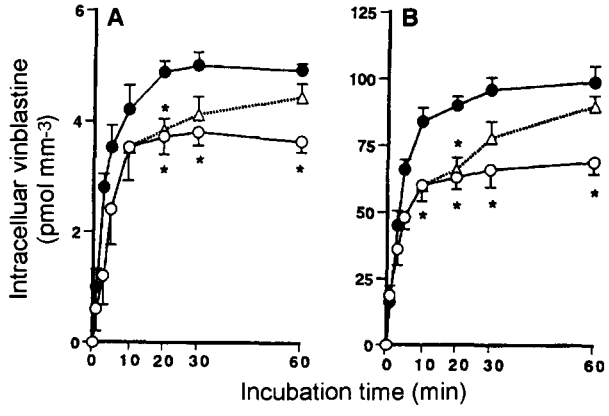


FIG. 2. Courses of the vinblastine concentration in P388/S cells. Cells were incubated with A. 10 nM or B. 1 μM vinblastine under energy-deprived conditions (○), and after 10 min 20 mM glucose was added to a part of culture (△) and incubated at 37°C during 60 min. Closed circles indicate the course in normal Hanks' solution. Data are the mean ± s.e. of three experiments performed in triplicate. $*P < 0.05$ compared with the value in normal Hanks' solution.

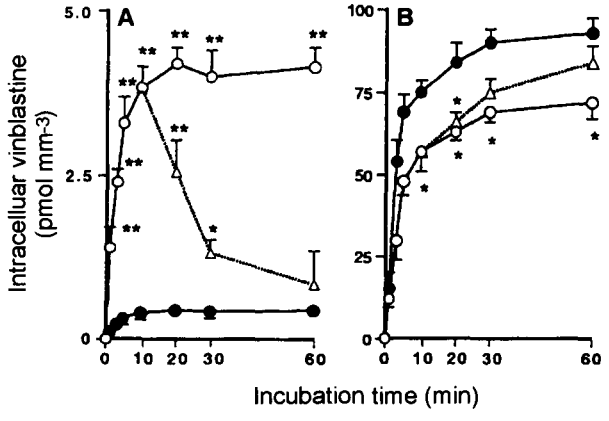


FIG. 3. Courses of the vinblastine concentration in P388/ADR cells. Cells were incubated with A. 10 nM or B. 1 μM vinblastine under energy-deprived conditions (○), and after 10 min 20 mM glucose was added to a part of culture (△) and incubated at 37°C during 60 min. Closed circles indicate the course in normal Hanks' solution. Data are the mean ± s.e. of three experiments performed in triplicate. $*P < 0.05$, $**P < 0.01$ compared with the value in normal Hanks' solution.

level by glucose. These results indicate that P388/ADR cells extrude the anticancer drug through an active efflux pump when there are low drug concentrations outside the cells, but not so with high drug concentrations, and also suggest that P388 cells have some inward transport system requiring energy.

Fig. 4 shows the vinblastine efflux rate in the cells. The clearance of vinblastine from the resistant cells was faster after they were loaded with 10 nM vinblastine than with 1 μM vinblastine. Next, we examined, using an inhibitor of P-glycoprotein, NA-382 (Miyamoto et al 1993), the capacity of P-glycoprotein as a drug-efflux pump in multidrug-resistant cells. The AH66 cell line expresses a 160 kDa P-glycoprotein and is resistant to vinblastine, and the AH66F line is a P-glycoprotein negative variant (Miyamoto et al 1992). NA-382 completely reversed the vinblastine accumulation under the low vinblastine concentration in both resistant cell lines, without influence on sensitive cell lines, and this agent did not affect the cellular concentration of vinblastine at the high vinblastine concentration (Table 1). Similarly, this agent significantly inhibited the efflux of vinblastine only from the resistant cells incubated with the low vinblastine concentration (Table 2).

Finally, the accumulation of NA-382, as a model of a non-anticancer drug, was examined in P388 cells. As shown in Table 3, the concentration of NA-382 in P388/ADR cells was significantly lower than in P388/S cells and was reversed to that in the sensitive cells by 10 μM vinblastine only when cells were incubated with 10 nM of the agent.

These results indicate that P-glycoprotein acts as a drug-efflux pump when there are low drug concentrations outside the cells, but does not effectively function in high drug concentrations. We suggest the function of P-glycoprotein is saturable according to the increase in the extracellular drug concentrations. It is necessary to perform successive combination chemotherapy so that low concentrations of anticancer drugs and high concentrations of multidrug-resistance modifiers

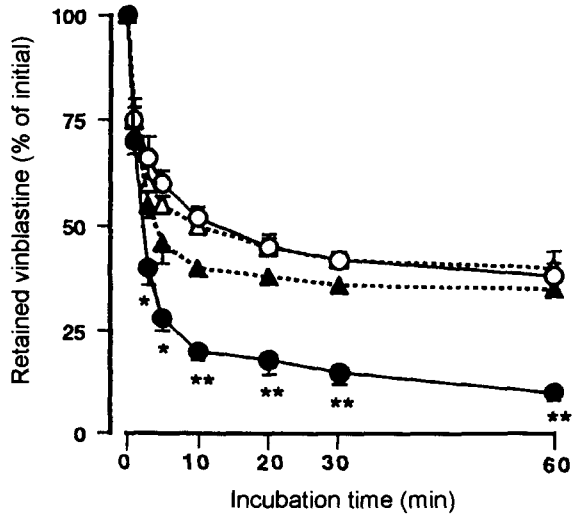


FIG. 4. Vinblastine efflux from P388 cells. P388/S cells (▲, △) or P388/ADR cells (●, ○) were incubated with 10 nM (▲, ●) or 1 μM vinblastine (△, ○) under energy-deprived conditions for 30 min, washed, and further incubated in normal Hanks' solution at 25°C for 60 min. Data are the mean ± s.e. of three experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$ compared with the course in P388/S cells.

Table 1. Effects of NA-382 on vinblastine accumulation by P388 cells and AH cells.

| Cell line | NA-382 (1 μM) | Extracellular vinblastine 10 nM: Intracellular vinblastine (pmol 30 $mm^{-1}$ $mm^3$) | Extracellular vinblastine 1 μM: Intracellular vinblastine (pmol 30 $mm^{-1}$ $mm^3$) |
|---|---|---|---|
| P388/S | − | 5·20 ± 0·52 | 97·5 ± 4·7 |
| | + | 5·24 ± 0·21 | 102·2 ± 6·2 |
| P388/ADR | − | 0·38 ± 0·12 | 93·9 ± 3·7 |
| | + | 4·84 ± 0·36* | 91·5 ± 4·5 |
| A-H66F | − | 2·16 ± 0·15 | 48·9 ± 2·5 |
| | + | 2·35 ± 0·24 | 49·5 ± 2·1 |
| A-H66 | − | 0·24 ± 0·05 | 46·9 ± 3·1 |
| | + | 2·44 ± 0·20* | 48·6 ± 2·5 |

Cells were incubated with 10 nM or 1 μM vinblastine in the absence (−) or presence (+) of 1 μM NA-382 in normal Hanks' solution at 37°C for 30 min. Data are the mean ± s.e. of three experiments performed in triplicate. *Significantly different from the vinblastine concentration in the absence of NA-382 at $P < 0.01$.

Table 2. Effects of NA-382 on vinblastine efflux from P388 cells and AH cells.

| Cell line | NA-382 (1 μM) | Loaded vinblastine 10 nM: Retained vinblastine (% of initial) | Loaded vinblastine 1 μM: Retained vinblastine (% of initial) |
|---|---|---|---|
| P388/S | − | 35·5 ± 3·8 | 39·0 ± 2·7 |
| | + | 39·1 ± 2·2 | 38·4 ± 3·5 |
| P388/ADR | − | 16·8 ± 2·7 | 41·6 ± 3·7 |
| | + | 38·3 ± 2·0* | 41·5 ± 2·9 |
| AH66F | − | 48·2 ± 5·3 | 52·6 ± 4·2 |
| | + | 49·5 ± 4·7 | 55·0 ± 3·1 |
| AH66 | − | 20·7 ± 2·2 | 55·6 ± 5·3 |
| | + | 54·7 ± 6·9* | 58·1 ± 5·0 |

Cells loaded with 10 nM or 1 μM vinblastine under energy-deprived conditions for 30 min were incubated in the absence (−) or presence (+) of 1 μM NA-382 in normal Hanks' solution at 25°C for 30 min. Data are the mean ± s.e. of three experiments performed in triplicate. *Significantly different from the retained vinblastine in the absence of NA-382 at $P < 0.01$.

Table 3. Effects of vinblastine on NA-382 accumulation by P388 cells.

| Cell line | Vinblastine (10 μM) | Extracellular NA-382 10 nM: Intracellular NA-382 (pmol 30 $mm^{-1}$ $mm^3$) | Extracellular NA-382 1 μM: Intracellular NA-382 (pmol 30 $mm^{-1}$ $mm^3$) |
|---|---|---|---|
| P388/S | − | 2·38 ± 0·46 | 140 ± 11 |
| | + | 2·51 ± 0·36 | 148 ± 12 |
| P388/ADR | − | 0·59 ± 0·03 | 157 ± 15 |
| | + | 2·20 ± 0·23* | 148 ± 10 |

Cells were incubated with 10 nM or 1 μM NA-382 in the absence (−) or presence (+) of 10 μM vinblastine in normal Hanks' solution at 37°C for 30 min. Data are the mean ± s.e. of three experiments performed in triplicate. *Significantly different from the concentration in the absence of vinblastine at $P < 0.01$.

should be maintained around the cancer tissues for an appropriate time (Miyamoto et al 1995). Recent clinical trials in combination therapy with chemosensitizers or P-glycoprotein inhibitors were very disappointing, because of the limited tolerance of the drugs used alone, which precluded attainment of potentially active levels in patients (Bellamy et al 1990; Erlichman et al 1993). On the other hand, several normal tissues, such as brain microvessel endothelium, canalicular cell membrane, digestive tract epithelium, adrenal, kidney, placenta, and so on express mdr1 gene or mdr3 gene-encoded P-glycoproteins (reviewed by Leveille-Webster & Arias 1995), although their physiological functions are now unclear. If nontoxic P-glycoprotein inhibitors are developed, drug therapy for not only multidrug-resistant cancers but also other diseases will be possible.

This study indicates that P-glycoprotein as a drug-efflux pump acts functionally only when there are low concentrations of the substrates outside the target cells. This evidence will provide information for the use of appropriate drug concentrations in investigations of P-glycoprotein and clinical drug therapy.

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