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Antitumour Effects and Pharmacokinetics of Combination of Vinblastine with a Staurosporine Derivative, NA-382, in P388/ADR-bearing Mice

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Abstract

The effects of a staurosporine derivative, *N*-ethoxycarbonyl-7-oxostaurosporine (NA-382), on the pharmacokinetics of vinblastine were evaluated, compared with those of verapamil, in multidrug-resistant P388/ADR-bearing mice.

At first, the in-vitro experiments indicated that NA-382 permeated into the cells better and were more effective in combined cytotoxicity with vinblastine and on accumulation of vinblastine than with verapamil in P388/ADR cells. In combined intraperitoneal injection with vinblastine (200 $\mu g\,kg^{-1}$) into P388/ADR-bearing mice, NA-382 in a suspension form (10 $mg\,kg^{-1}$) prolonged the life-span of the mice near to that of P388/S-bearing mice treated with vinblastine alone, but verapamil even at the maximum tolerated dosage (30 $mg\,kg^{-1}$) barely affected the in-vivo antitumour effect of vinblastine.

When simultaneously administered with vinblastine to P388/ADR-bearing mice, NA-382 maintained significantly higher vinblastine levels in the tumour cells for 24 h and gave a larger area under the time-intracellular vinblastine concentration curve (0 to 24 h) than those receiving vinblastine alone, with long retention of the agent in ascitic fluid. Verapamil increased the cellular vinblastine content for only 6 h, accompanying a rapid elimination of the agent from the ascitic fluid.

This study indicates that NA-382 is more effective against multidrug-resistance than verapamil, and its suspension is also advantageous for cancer chemotherapy of multidrug-resistant tumours.

A major obstacle to the successful treatment of cancer is drug resistance. Among many mechanisms of anticancer drug resistance, the multidrug resistance (MDR) associated with overexpression of P-glycoprotein in the plasma membrane has been extensively studied and numerous classes of MDR modifiers have been reported (Biedler & Meyers 1989). However, combination chemotherapy with these MDR modifiers has been very disappointing in clinical studies, because of the limited tolerance of the drugs by themselves, which precluded attainment of potentially active levels in patients (Bellamy et al 1990; Pennock et al 1991; Erlichman et al 1993). Furthermore, even in in-vivo experimental systems using excess dosages of the modifiers, the effect was generally inadequate.

The development of potent MDR modifiers devoid of other pharmacological activities and the improvement of treatment dosage forms of the drug based on the pharmacokinetics in the host animals confirm the MDR reversal hypothesis in the clinic. In the course of the former aim, we have developed a potent and less toxic MDR modifier, *N*-ethoxycarbonyl-7-oxostaurosporine (NA-382), from a series of staurosporine derivatives (Miyamoto et al 1993a,b). This study shows that 10 $mg\,kg^{-1}$ NA-382, which is one-twentieth of the maximum tolerated dose and has no significant pharmacological action in mice (Miyamoto et al 1993b), almost completely reversed the vinblastine resistance of P388/ADR in mice in combination with a simultaneous intraperitoneal injection of 200 $\mu g\,kg^{-1}$ vinblastine once a day for 10 days. On the other hand, verapamil barely affected the effect of vinblastine in mice at a single dose of 30 $mg\,kg^{-1}$, which is the maximum tolerated dose by intraperitoneal consecutive administrations. To solve the descrepancy between the in-vitro and in-vivo effects, we examined the pharmacokinetics of vinblastine and these MDR modifiers in P388/ADR-bearing mice.

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Materials and Methods

Materials

Vinblastine and verapamil were purchased from Shionogi & Co., Osaka, Japan, and Wako Pure Chemical Co., Tokyo, Japan, respectively. 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 verapamil were dissolved in physiological saline before use. Because NA-382 is a hydrophobic compound, it was suspended in 0·5% carmellose in physiological saline for in-vivo use, and was dissolved in dimethylsulphoxide and used after 200-fold dilution with the culture medium in the in-vitro experiments. The intraperitoneal injection volume of the drug solution or suspension was 10 mL kg^{-1}.

Tumour cells and culture

The mouse leukaemia P388 (P388/S) and adriamycin-induced

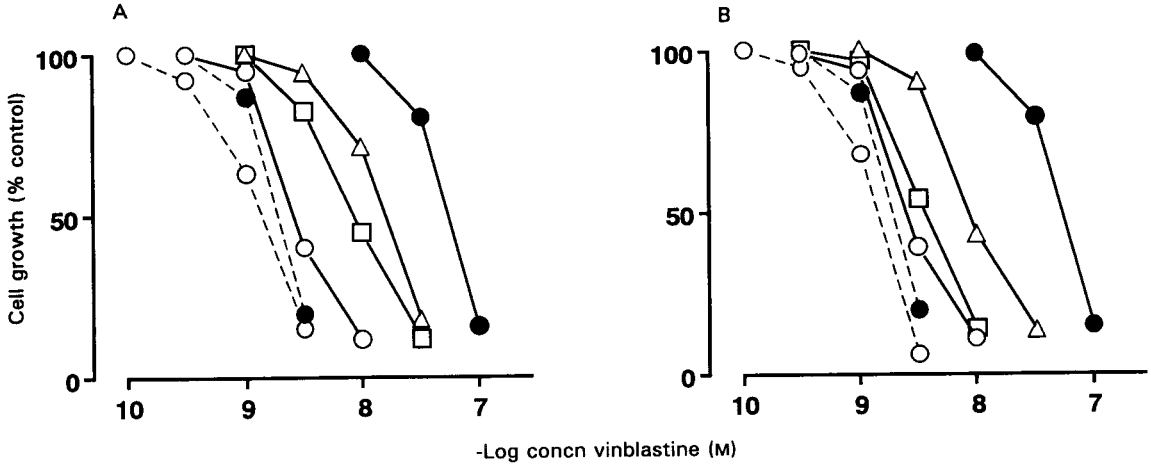

FIG. 1. In-vitro combined effects of verapamil and NA-382 on growth-inhibitory effect of vinblastine in P388/S cells (- - - - -) and P388/ADR cells (———). Cells were treated with different concentrations of vinblastine in the absence (●) or presence of A. 1 (△), 3 (□), or 10 (○) μM verapamil, or B. 125 (△), 250 (□), or 500 (○) nM NA-382.

MDR cell line P388/ADR (Inaba & Sakurai 1979) were kindly supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan. Cells were passaged weekly through female BALB/c × DBA/2 (CDF_1) mice (Nippon SLC, Hamamatsu, Japan), and harvested from tumour-bearing animals 6 to 7 days after transplantation. Cells were suspended in RPMI 1640 medium supplemented with 10% foetal bovine serum, 20 μM 2-mercaptoethanol, and 100 $\mu g\,mL^{-1}$ kanamycin at a density of $1{\cdot}5 \times 10^5$ cells mL^{-1} and cultured with or without agents at 37°C for 48 h in a CO_2 incubator. The survival of the cells was measured by the MTT assay (Carmichael et al 1987).

In-vivo combination therapy

Female CDF_1 mice (six in a group) were intraperitoneally inoculated with 1×10^6 P388/S or P388/ADR cells, and simultaneous intraperitoneal administrations of vinblastine (200 $\mu g\,kg^{-1}$) and NA-382 (10 $mg\,kg^{-1}$, one-twentieth of the maximum tolerated dose) or verapamil (30 $mg\,kg^{-1}$, the maximum tolerated dose by consecutive administration) were started 24 h after the cell inoculation once a day for 10 days.

In-vivo pharmacokinetic studies

Six or seven days after the intraperitoneal inoculation of 10^6 P388/S or P388/ADR cells into the mice, [^{3}H]vinblastine (200 $\mu g\ kg^{-1}$), NA-382 (10 $mg\,kg^{-1}$) or verapamil (30 $mg\,kg^{-1}$) was intraperitoneally injected, and 200 μL ascitic fluid was drawn from the peritoneal cavity at 10, 20, 30 min, 1, 2, 3, 6, 12, and 24 h after the drug injection. The ascitic fluid was rapidly centrifuged to separate the fluid and tumour cells. Cells were washed, dissolved in sodium hydroxide, and neutralized. Portions (40 μL) of the ascitic fluid and the solubilized cells were put into a toluene: Triton X-100 (2:1, v/v) scintillation mixture, containing 0·2% 2,5-diphenyloxazole and 0·05% 1,4-bis[2-(5-phenoloxazoly]benzene, and the radioactivity of [^{3}H]vinblastine was counted in a Beckman LS-5800 liquid scintillation counter.

For measurement of verapamil and NA-382, the intracellular drugs were extracted with acetone by ultrasonication. The ascitic fluid and the cell extract were dried under a nitrogen stream, dissolved in methanol, and injected in a volume of 50 μL into a high-performance liquid chromatography (HPLC) system, consisting of a Shimadzu LC-6A

Table 1. Combined antitumour effects of verapamil and NA-382 with vinblastine in P388/ADR-bearing mice.

Tumour	Modifier	Dose ($mg\,kg^{-1}$)	Modifier alone		Combined with 200 $\mu g\,kg^{-1}$ vinblastine	
			Survival days (mean ± s.d.)	%ILS[a]	Survival days (mean ± s.d.)	%ILS
P388/S	None	—	8·5 ± 0·4	—	16·1 ± 0·8	89·0
P388/ADR	None	—	10·5 ± 0·5	—	12·0 ± 0·5	14·3
	Verapamil	3	10·8 ± 0·4	2·9	12·2 ± 0·4	16·2
		10	11·0 ± 0·9	4·8	12·3 ± 0·8	17·1
		30	10·8 ± 0·8	2·9	13·8 ± 0·8	31·4
	NA-382	1	10·2 ± 0·4	−2·9	12·8 ± 1·0	21·9
		3	10·8 ± 1·0	2·9	15·1 ± 1·6	43·8
		10	10·3 ± 0·5	−1·9	19·0 ± 1·8*	80·9

Six mice in one group were inoculated intraperitoneally with P388/S or P388/ADR cells (10^6 cells/mouse) on day 0, and after 24 h vinblastine and the combined drug were administered intraperitoneally once a day for 10 days. [a] Percent increase in life-span (ILS) of the experimental group compared with the non-treated control group. *$P < 0{\cdot}05$ compared with the group treated with vinblastine alone.

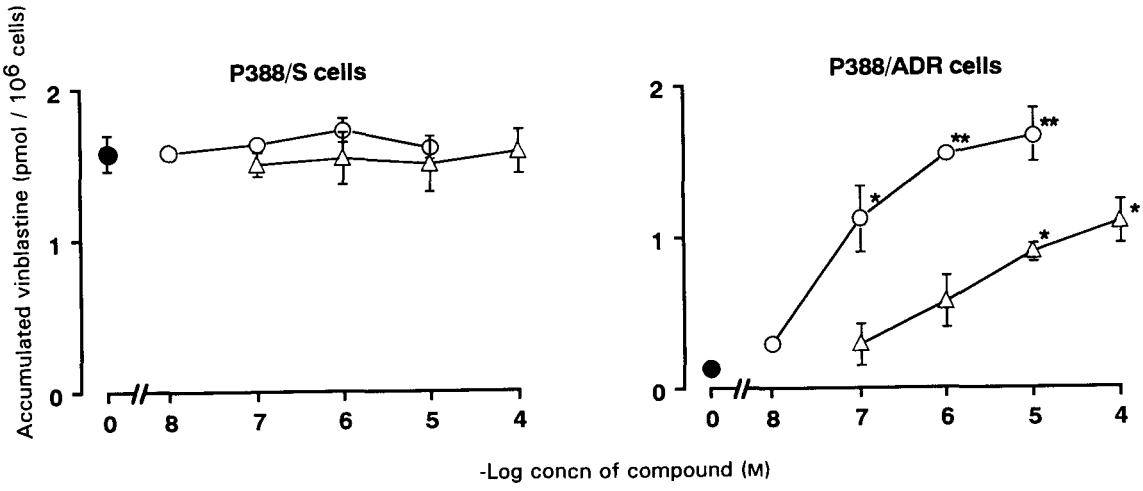

FIG. 2. In-vitro effects of verapamil and NA-382 on vinblastine accumulation in P388 cells. Cells were incubated with 10 nM vinblastine in the absence (●) or presence of different concentrations of verapamil (△) or NA-382 (○) for 30 min, and the intracellular vinblastine concentration was measured. $*P < 0.05$, $**P < 0.001$ compared with vinblastine alone.

liquid pump with a Waters μ-Bondasphere C_{18} (5μ-100A, 3·9 × 150 mm), a Yanaco M-315 UV spectrometer, a Shimadzu RF-550 spectrofluorometer, and a Shimadzu C-R6A data processor. Elution with mobile phase (80% methanol in 20 mM phosphate buffer, pH 7·4) and a flow rate of 1 mL min^{-1} were at 40°C in a Shimadzu CTO-6A column oven, and the effluent was detected at a wavelength of 278 nm for verapamil and at the excitation wavelength of 320 nm and the emission wavelength of 550 nm for NA-382. Standard curves were linear within the concentrations of verapamil and NA-382 used in this study. The limit of measurement was 400 pmol mL^{-1} verapamil and 50 pmol mL^{-1} NA-382.

In-vitro cellular accumulation

Cells (2×10^6 mL^{-1}) were incubated in the culture medium with 10 nM [^{3}H]vinblastine in the absence or presence of varying concentrations of verapamil or NA-382 at 37°C for 30 min. The cells were washed, dissolved in 0·5 M sodium hydroxide, and neutralized. The radioactivity was counted as described above. Intracellular verapamil and NA-382 accumulated for 30 min were also measured by an HPLC method as described above.

Data analysis

Experiments were carried out at least three times. Statistical analysis was using Student's *t*-test and the multiple comparative test of Dunnett (1955). Pharmacokinetic parameters were calculated using statistical moments analysis (Yamaoka et al 1978).

Results

Effects of verapamil and NA-382 on the antitumour effect of vinblastine

In the in-vitro experiments, P388/ADR cells were approximately 30-fold more resistant to vinblastine than the parent P388/S cells, and this resistance was decreased by verapamil and NA-382 in a concentration-dependent manner, being completely reversed with 10 μM verapamil and 500 nM NA-382 (Fig. 1). Table 1 shows the in-vivo combined effects of verapamil and NA-382 with vinblastine on P388/ADR-bearing mice. Although both verapamil and NA-382 did not show antitumour activity on P388/ADR-bearing mice by themselves, the combination of NA-382 and vinblastine markedly increased the life-span of the mice. NA-382 (10 mg kg^{-1}) together with vinblastine showed the same antitumour effect as vinblastine alone in P388/S-bearing mice. However, verapamil even at the maximum tolerated dose (30 mg kg^{-1}) barely influenced the effect of vinblastine on P388/ADR-bearing mice.

In-vitro effects of verapamil and NA-382 on vinblastine accumulation

When P388 cells were incubated with 10 mM vinblastine at 37°C, intracellular vinblastine increased and reached a

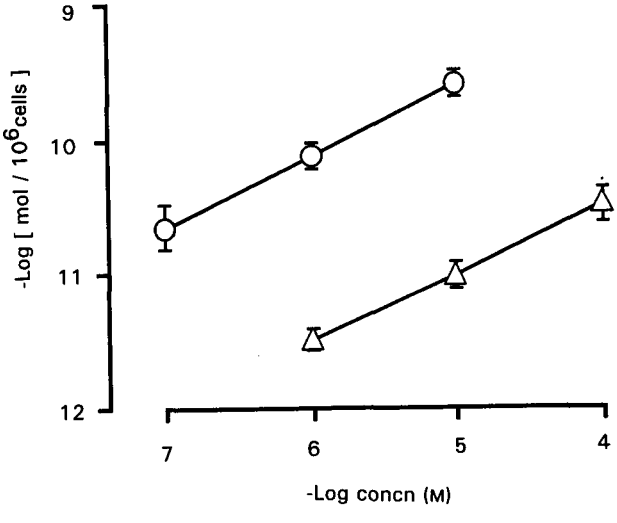

FIG. 3. Accumulation of verapamil and NA-382 by P388/ADR cells. Cells were incubated with different concentrations of verapamil (△) or NA-382 (○) for 30 min, and intracellular drugs were measured.

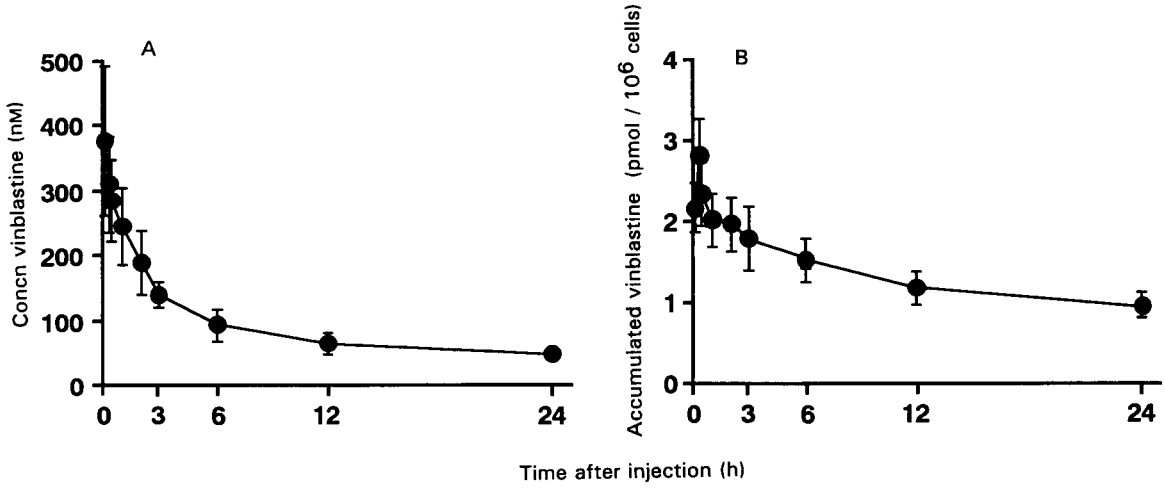

FIG. 4. Vinblastine concentrations in P388/S-bearing mice. Vinblastine (200 μg kg^{-1}) was injected intraperitoneally into the mice 6 to 7 days after the tumour cell inoculation, and vinblastine in the A. ascitic fluid, and B. P388/S cells was measured at the indicated times.

plateau within 30 min; the content in P388/S cells was about 10-fold that in P388/ADR cells. Verapamil and NA-382 increased the vinblastine accumulation for 30 min in a concentration-dependent manner in P388/ADR cells, but they did not influence the vinblastine accumulation in P388/S cells (Fig. 2). NA-382 was more effective on P388/ADR cells than verapamil on a molecular basis.

In-vitro accumulation of verapamil and NA-382 in P388/ADR cells

When cells were incubated with verapamil or NA-382 for 30 min, the cellular accumulation increased with the extracellular drug concentration (Fig. 3). The cellular accumulation of NA-382 was 20-fold that of verapamil. The results from Figs 2 and 3 indicate that the high potency of NA-382 for vinblastine accumulation in P388/ADR cells depends on its high permeation into the cells, in comparison with those of verapamil.

In-vivo kinetics of vinblastine combined with verapamil or NA-382 in P388-bearing mice

Figs 4 and 5 show the concentration–time curves of vinblastine in the ascitic fluid and in tumour cells after a single intraperitoneal injection at a dose of 200 μg kg^{-1} in combination with verapamil (30 mg kg^{-1}) or NA-382 (10 mg kg^{-1}). The concentration of vinblastine in the ascitic fluid from the tumour-bearing mice gave similar decay curves whatever the tumour cell lines and combinations with the MDR modifiers, the areas under the time–concentration curves from 0 to 24 h (AUC_{0-24h}) of vinblastine alone in the ascitic fluid of P388/S- and P388/ADR-bearing mice were 2258 and 2620 mM h, respectively, and the AUC_{0-24h} values for vinblastine in combination with verapamil and NA-382 in the ascitic fluid of P388/ADR-bearing mice were 2281 and 2205 mM h, respectively.

After an injection of vinblastine into the tumour-bearing mice, the vinblastine in P388/S cells was significantly higher

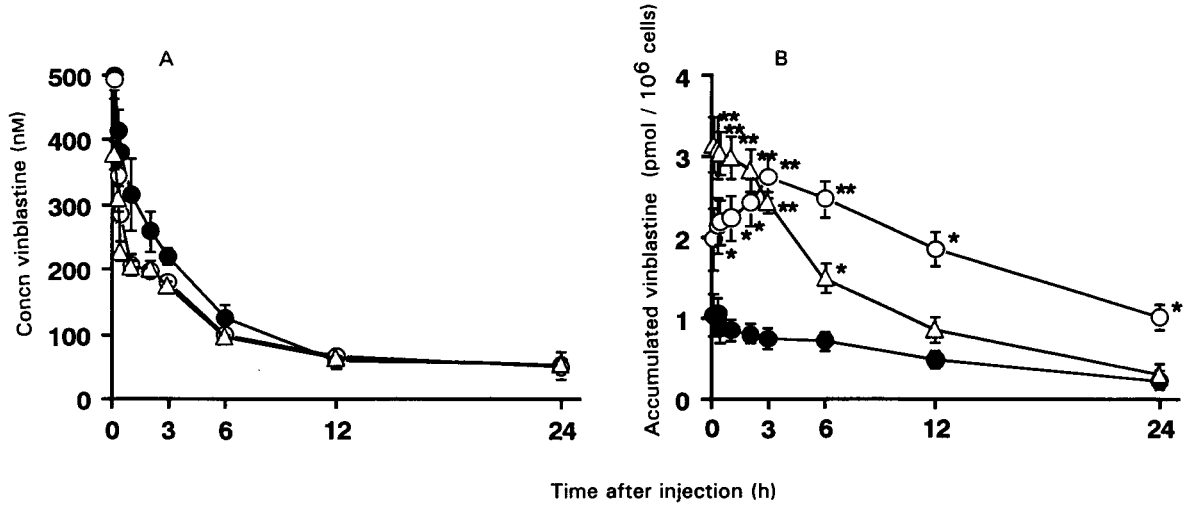

FIG. 5. Vinblastine concentrations after the combined treatment with verapamil or NA-382 in P388/ADR-bearing mice. Vinblastine (200 μg kg^{-1}) without (●) or with verapamil (30 mg kg^{-1} △) or NA-382 (10 mg kg^{-1} ○) was injected into the mice 6 to 7 days after the cell inoculation, and vinblastine in the A. ascitic fluid, and in the B. cells was measured at the indicated times. *$P < 0.05$, **$P < 0.01$ compared with vinblastine alone.

Table 2. Pharmacokinetic parameters of vinblastine in tumour cells after intraperitoneal administration of vinblastine and verapamil or NA-382.

Tumour cell	Treatment	AUC_{0-24h} (pmol h/1 × 10^6 cells)	MRT (h)	VRT (h^2)
P388/S cells	Vinblastine	29·5	8·67	41·0
P388/ADR cells	Vinblastine	17·2	9·22	42·1
	+ verapamil	31·2	7·29	38·1
	+ NA-382	46·5	9·79	47·9

AUC_{0-24} = area under the time–concentration curve from 0 to 24 h; MRT = mean residence time; VRT = variance of residence time.

than that in P388/ADR cells. When verapamil was simultaneously administered, the vinblastine in P388/ADR cells was significantly higher than in those receiving vinblastine alone up to 6 h, but thereafter decreased to the level of vinblastine alone. In combination with NA-382, although the peak time was delayed, vinblastine in P388/ADR cells was maintained at a significantly higher level than those receiving vinblastine alone for 24 h. Table 2 shows the pharmacokinetic parameters used to assess the effects of verapamil and NA-382 on the function of P-glycoprotein in P388/ADR cells. Both AUC_{0-24h} and mean residence time (MRT) in combinations with NA-382 were higher than those of the vinblastine alone and those of the combination of vinblastine with verapamil.

In-vivo kinetics of verapamil and NA-382 in P388/ADR-bearing mice

Fig. 6 shows the time–concentration curves of verapamil and NA-382 in the ascitic fluid of P388/ADR-bearing mice after the intraperitoneal injection of these agents. The concentration of verapamil in the ascitic fluid rapidly decreased and was not detectable 12 h after administration, at which time the intracellular level was also negligible. In the case of NA-382, the ascitic concentration increased during the first hour, reached a plateau, and gradually decreased to 600 nM after 24 h (Fig. 6), at which time the cellular content was 43 pmol 10^{-6} cells (not shown).

Discussion

This study indicates that NA-382 was more effective in increasing cytotoxicity of vinblastine and on accumulation of vinblastine in MDR P388/ADR cells than verapamil, with little influence on the sensitive P388/S cells in-vitro (Figs 1, 2). This difference between potencies of these MDR modifiers seems to be based on their permeation into the tumour cells (Fig. 3). When vinblastine (200 $\mu g\,kg^{-1}$) and verapamil (30 $mg\,kg^{-1}$) or NA-382 (10 $mg\,kg^{-1}$) were simultaneously injected into P388/ADR-bearing mice, NA-382 reversed the in-vivo vinblastine resistance of P388/ADR but verapamil was ineffective (Table 1).

Vinblastine is a time-dependent antitumour agent, acting at the late G_1 and S phases of the cell cycle followed by an arrest in the mitotic phase. Therefore, the effective vinblastine level in the cells (estimated to be > 1 pmol/10^6 cells) must be maintained over at least one doubling time of the cells, which was estimated as 12 h for these P388 cell lines from the in-vitro experiments. When verapamil was injected intraperitoneally, its concentration in ascitic fluid rapidly decreased, and it maintained the high vinblastine levels in P388/ADR cells for only 6 h (Figs 5, 6). This pharmacokinetic behaviour could be one reason why verapamil did not potentiate the in-vivo vinblastine effect. To maintain the effective cellular vinblastine level in the MDR cells, verapamil should be maintained at 10 μM outside the cells, by injections every 3 or 6 h or by continuous infusion.

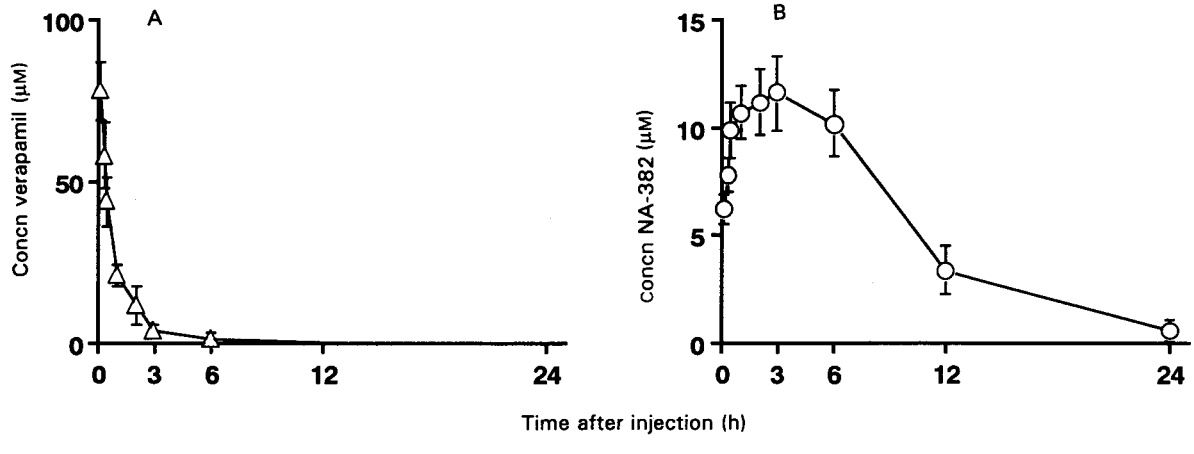

FIG. 6. Concentrations of A. verapamil and B. NA-382 in ascitic fluid of P388/ADR-bearing mice. Verapamil (30 $mg\,kg^{-1}$) or NA-382 (10 $mg\,kg^{-1}$) was injected intraperitoneally into the mice 6 to 7 days after the cell inoculation, and concentration of the agent in the ascitic fluid was measured at the indicated times.

However, this may be very difficult, because of adverse cardiac reactions. Indeed, a recent clinical study indicated that the blood level of patients given verapamil orally at 120 mg 6-hourly for 5 days in a course of combined chemotherapy reached only 850 nM and no statistically significant difference in response or survival was seen (Milroy 1993). On the other hand, when injected intraperitoneally into P388/ADR-bearing mice in a suspension, NA-382 was gradually dissolved, slowly eliminated from the ascitic fluid (Fig. 6), and retained in the ascitic fluid at a concentration of 600 nM and in the tumour cells at 43 pmol/10^6 cells even 24 h after the injection. Consequently, in combined injection with vinblastine this agent maintained the vinblastine level at greater than 1 pmol/10^6 P388/ADR cells for 24 h (Fig. 5). These long retention values of vinblastine and NA-382 should provide an antitumour effect against the MDR tumour. Thus, the antitumour combination effect of NA-382 on the vinblastine-resistant P388/ADR-bearing mice could be elucidated from in-vitro and in-vivo pharmacokinetic and pharmacodynamic studies of vinblastine and NA-382.

MDR-modifying drugs, having hydrophobic and cationic properties, bind to the intracellular or transmembrane domains of P-glycoprotein (Cornwell et al 1987; Akiyama et al 1988; Yusa & Tsuruo 1989; Wakusawa et al 1992) or cause conformational changes of the glycoprotein (Zordan-Nudo et al 1993; Pawagi et al 1994), decreasing the binding and extrusion of antitumour drugs from the cells. Some investigators have indicated that staurosporine increases drug accumulation in the MDR cells and suggested that this effect may be provided by both its direct action on the drug binding to P-glycoprotein and its inhibition of phosphorylation of P-glycoprotein through protein kinase C (Sato et al 1990; Ma et al 1991). However, we have indicated from structure–activity relationship studies of staurosporine derivatives that their effect on the MDR cells is based on their direct inhibitory action on the drug binding to P-glycoprotein, rather than on their ability to inhibit protein kinase C (Miyamoto et al 1993a, b; Wakusawa et al 1993). NA-382 is a hydrophobic alkaloid having low activity on protein kinases and low toxicity on the tumour cells and on whole animals. This compound seems to easily permeate the cell membrane and act on the P-glycoprotein.

This study indicates that NA-382 is a low toxic and potent MDR modifier and its intraperitoneal injection as a suspension is also advantageous in combined chemotherapy against ascitic tumours.

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