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Potentiation of the Vincristine Effect on P388 Mouse Leukemia Cells by a Newly Synthesized Dihydropyridine Analogue, PAK-200

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A newly synthesized dihydropyridine analogue, 2-[benzyl(phenyl)amino]ethyl 1,4-dihydro-2,6-dimethyl-5-(5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinan-2-yl)-1-(2-morpholinoethyl)-4-(3-nitrophenyl)-3-pyridinecarboxylate (PAK-200), at 1 μM completely reversed the resistance to vincristine in vincristine-resistant P388 mouse leukemia cells (P388/VCR), *in vitro*. PAK-200 at 2 μM inhibited the efflux of [^{3}H]vincristine from P388/VCR and increased the accumulation of [^{3}H]vincristine in P388/VCR to a level similar to that in P388 cells. P-Glycoprotein in membrane vesicles from P388/VCR cells was photolabeled with [^{3}H]azidopine. The labeling was completely inhibited by 10 μM PAK-200. The calcium antagonistic activity of PAK-200 was about 1000 times lower than that of another dihydropyridine analogue, nicardipine. Experiments with P388 and P388/VCR-bearing mice showed that PAK-200 enhanced the effect of vincristine on both leukemia cells *in vivo*. These results suggest that PAK-200 interacts with P-glycoprotein and reverses drug resistance in P388 mouse leukemia cells *in vitro*, and that PAK-200 has an ability to potentiate the effect of vincristine on P388 mouse leukemia cells *in vivo*.

Key words: Dihydropyridine analogue — PAK-200 — Multidrug resistance — P388 mouse leukemia cell

Multiple resistance to agents such as Vinca alkaloids and anthracyclines is called multidrug resistance (MDR[4]). MDR is one of the major obstacles to successful cancer chemotherapy. MDR cells were isolated from carcinoma cell lines and studied to elucidate the molecular basis for their resistance.[1,2] Expression of a 170 kDa transmembrane glycoprotein called P-glycoprotein (P-gp) has been observed in various multidrug-resistant cell lines.[2–5] P-gp is the product of MDR1 gene,[6,7] and is thought to function as a pump molecule that transport hydrophobic anticancer agents outside the cells.[8]

We have reported that the expression of P-gp is closely related to clinical drug resistance in some types of leukemia.[9,10] Agents that inhibit P-gp activity at concentrations with no cytotoxic effect may be useful in reversing MDR in clinical chemotherapy.

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[4] Abbreviations: MDR, multidrug resistance; PAK-200, 2-[benzyl(phenyl)amino]ethyl 1,4-dihydro-2,6-dimethyl-5-(5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinan-2-yl)-1-(2-morpholinoethyl)-4-(3-nitrophenyl)-3-pyridinecarboxylate; P-gp, P-glycoprotein; VCR, vincristine; ADM, adriamycin; T/C(%), percent increase in the mean survival, treated/control×100; T/V(%), mean survival time of group treated with PAK-200 and VCR divided by mean survival time of group treated with VCR alone; P388/VCR, P388 mouse leukemia cells resistant to VCR; P388/ADM, P388 mouse leukemia cells resistant to ADM; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Recently, some dihydropyridine analogues have been shown to reverse MDR *in vivo*.[11,12] The photoactive dihydropyridine calcium channel blocker, azidopine, photolabeled P-gp, and vinblastine and nimodipine inhibited this labeling.[13] P-gp seems to be an acceptor for some calcium channel blockers such as verapamil, diltiazem, and dihydropyridine analogues, that are reported to reverse MDR. The correlation between the reversing of drug resistance and the inhibition of [^{3}H]azidopine photolabeling of P-gp by dihydropyridine analogues suggests that P-gp plays a role both in MDR and in the reversing of MDR by dihydropyridine analogues.[14] Through a screening program, we have found some dihydropyridine analogues which inhibit the photolabeling of P-gp with [^{3}H]azidopine and also reverse drug-resistance in multidrug resistant KB-C2 cells *in vitro*. In this study, we investigated whether one such analogue, PAK-200, reverses drug-resistance in P388 mouse leukemia cells *in vivo* as well as *in vitro*.

MATERIALS AND METHODS

Chemicals PAK-200 was synthesized by Nissan Chemical Ind., Ltd. (Chiba). The structure and purity of the compound were determined by using the following procedures. The ^{1}H-NMR spectrum in $CDCl_3$ solution was recorded on a JEOL PMX60SI spectrometer; chemical shifts are given in ppm with tetramethylsilane as an

internal standard. The mass spectrum was obtained on a JEOL JMS D-300 instrument. Column chromatography was carried out on Merck silica gel 60 (70–230 mesh, American Society for Testing Materials).

The synthesis of PAK-200 was conducted as follows: 0.44 g (11.0 mmol) of NaH (60%) was added to a solution of 3.62 g (5.00 mmol) of 2-[benzyl(phenyl)-amino]ethyl 1,4-dihydro-2,6-dimethyl-5-(5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinan-2-yl)-4-(3-nitrophenyl)-3-pyridinecarboxylate in 18.1 g of DMF dried over 3A molecular sieves, and the mixture was stirred for 15 min at room temperature. Then 0.93 g (5.00 mmol) of 4-(2-chloroethyl)morpholine hydrochloride was added, and the mixture was maintained at 80°C for 6 h. The solvent was removed under reduced pressure and the residue was dissolved in 100 ml of $CHCl_3$ and washed with 100 ml of water. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate:ethanol=5:1) to give 0.91 g of PAK-200 as a yellow oil. NMR d 0.87 (s, 3H), 0.98 (s, 3H), 19–2.4 (m, 6H), 2.42 (s, 3H), 2.50 (d, J=2.2 Hz, 3H), 3.3–4.7 (m, 16H), 4.99 (d, J=14 Hz, 1H), 6.3–8.3 (m, 14H); MS (electron impact ionization), m/z 744 (5, M^+), 727 (6%), 595 (5%), 404 (9%), 210 (26%), 100 (100%). [^{3}H]Vincristine (VCR) (6.75 Ci/mmol) and [^{3}H]azidopine (40 Ci/mmol) were purchased from Amersham Corp., Arlington, Heights, IL. VCR, adriamycin (ADM), verapamil and nicardipine were obtained from Sigma Chemical Co., St. Louis, MO.

Cell lines and culture P388, P388/VCR and P388/ADM cell lines were kindly supplied by Dr. M. Inaba, The Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo). The cells were cultured in RPMI1640 medium (Flow Laboratories, Inc., Rockville, MD) and supplemented with 10% fetal calf serum (Flow Laboratories), 10 μM 2-hydroxyethyl disulfide (Aldrich Chemical Co., Inc., Milwaukee, WI) and 100 mg kanamycin/ml.

Cell survival by MTT assay MTT colorimetric assay was performed in a 96-well plate as an *in vitro* chemosensitivity test.[15] The assay is dependent on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. Equal numbers of cells were inoculated in each well with 90 μl of culture medium. After overnight incubation (37°C, 5% CO_2), 10 μl of VCR solution and 0.5 μl of sample solution were added to each well and the plate was incubated for 4 days. Then 10 μl of MTT (5.0 mg/ml PBS) was added to each well and the plate was incubated for a further 4 h. The resulting formazan was dissolved in 100 μl of 0.04 N HCl·2-propanol. The plates were placed on a plate shaker for 5 min and read immediately at 570 nm.

Accumulation and efflux of [^{3}H]VCR Cells were harvested 7 days after transplantation, washed 3 times with PBS, and suspended in RPMI 1640 medium with 10% fetal calf serum (final cell density, 7×10^6 cells/ml).

For the study of drug accumulation, cells (7×10^6 cells in each well) were incubated with 0.1 μCi of [^{3}H]VCR in the absence and presence of indicated concentrations of PAK-200 for 2 h. The cells were washed with ice-cold PBS three times, solubilized in 1% Triton X-100 in 10 mM phosphate buffer (pH 7.4), and harvested, then the radioactivity was counted.

For the efflux study, cells were incubated with 0.1 μCi of [^{3}H]VCR/ml in the absence or presence of 2 μM PAK-200 for 2 h, and washed with ice-cold PBS three times. These preloaded cells were resuspended in prewarmed fresh medium with or without 2 μM PAK-200 and incubated at 37°C for 60 min to induce outward transport of VCR.

At indicated times, 0.25 ml aliquots were transferred onto a 0.25 ml oil layer consisting of silicone and liquid paraffin at a volume ratio of 21:4 in a microtube and centrifuged.[11] After removal of both the supernatant above the oil layer and the oil layer, the cell pellet was dissolved in 1% Triton X-100, and the radioactivity was counted.

Animals and antitumor activity Male BALB/c×DBA/2 (CDF_1) mice weighing 20–23 g were purchased from Charles River Japan, Inc. (Tokyo). The mice were 5–6 weeks old at the time of tumor inoculation.

The PAK-200 was dissolved in ethanol with a small amount of HCl, diluted with sterile saline containing 0.2% Tween 80 and neutralized with 1 N NaOH to pH 7.0. All control experiments were done with the above solution but without PAK-200.

One million leukemia cells were inoculated ip. Antitumor agent was administered ip daily for 5 days starting the day after tumor inoculation. Six mice were used for each assay. Antitumor activity was evaluated in terms of the mean survival time for each group and expressd by the T/C (%) and the T/V (%) values.

Determination of calcium antagonistic activities The calcium-antagonistic activity of PAK-200 and nicardipine was determined by measuring the ability of the compounds to inhibit the contraction of a spirally sheared segment of thoracic aorta from rabbit in the presence of calcium.

The arteries, cut helically into strips, were fixed vertically between hooks under a tension of 2 g in an organ bath containing Kreb's-Henseleit solution (composition in mM: NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 4.9, glucose 11.1), which was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2. Before the start of the experiments, all preparations were allowed to equilibrate for 60 min in the

bathing solution, which was changed every 20 min. The preparations were contracted by exposure to high-potassium solution (composition in m*M*: KCl 50, NaCl 73.1). After the KCl-induced contractions had stabilized, each agent was added cumulatively. To assess and compare the relaxant activity, papaverine at a concentration of 10^{-4} *M* was added at the end of the experiments, and the relaxation served as a 100% reference value. The result was expressed as the negative logarithm of the dose required to give 50% of the maximum relaxation produced by 10^{-4} *M* papaverine.

Membrane vesicle preparation Membrane vesicles from KB-C2 cells and P388/VCR cells were prepared as described.[16] Protein concentrations were determined by the method of Bradford.[17]

Photoaffinity labeling Membrane vesicles (100 μg of protein) were incubated with 0.75 μM [^{3}H]azidopine (53 Ci/mmol) for 15 min at room temperature in the presence of the indicated concentrations of PAK-200. After continuous irradiation at 366 nm for 20 min at 25°C, samples were solubilized in a sodium dodecyl sulfate (SDS) sample buffer as described by Debenham *et al.*[18]

SDS gel electrophoresis Samples labeled with [^{3}H]-azidopine were adjusted to 50 m*M* Tris-HCl at pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 5% glycerol, and 0.1% bromphenol blue. Polyacrylamide gel electrophoresis was carried out according to Laemmli[19] in 8% gel without heating the sample.[20, 21] Proteins were stained with 0.25% Coomassie blue in 50% (w/v) trichloroacetic acid.

RESULTS

Reversal of drug resistance in P388/VCR and P388/ADM by PAK-200 We synthesized about 400 dihydropyridine analogues and investigated whether they reversed MDR in KB-C2 cells. Several analogues had MDR-reversing activity similar to or higher than that of cepharanthine or verapamil. PAK-200 (Fig. 1) was selected as an analogue for this study because it had the lowest calcium channel-blocking activity and was one of the most potent MDR-reversing agents. We first examined the cytotoxic effect of PAK-200 on P388 and P388/VCR cells by the MTT method.

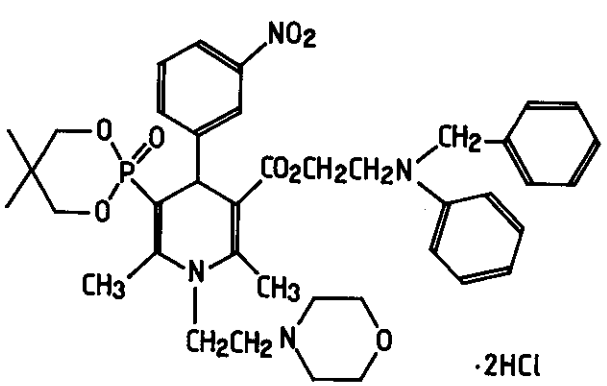

Fig. 1. Chemical structure of PAK-200.

PAK-200 at 5 μM had no cytotoxic effect on either P388 or P388/VCR cells. The D_{50} values (concentration reducing cell survival by 50%) of PAK-200 for P388 and P388/VCR cells were 12.5 μM and 13.5 μM respectively (data not shown). The dose-response curves of VCR and ADM with and without PAK-200 were assayed by the same method.

Table I summarizes the data on the *in vitro* effects of PAK-200 on the sensitivities to VCR and ADM of

Table I. Effect of PAK-200 on Drug Resistance in P388/VCR and P388/ADM *in vitro*

PAK-200 (μM)	D_{50} (ng/ml) to VCR[a]		D_{50} (ng/ml) to ADM	
	P388	P388/VCR	P388	P388/ADM
0	3.0	46	6.7	1400
1	1.7	2.7	3.5	—
2	0.8	1.0	2.4	50
5	0.3	0.4	—[b]	14

a) VCR, vincristine; ADM, adriamycin.
b) Not tested.

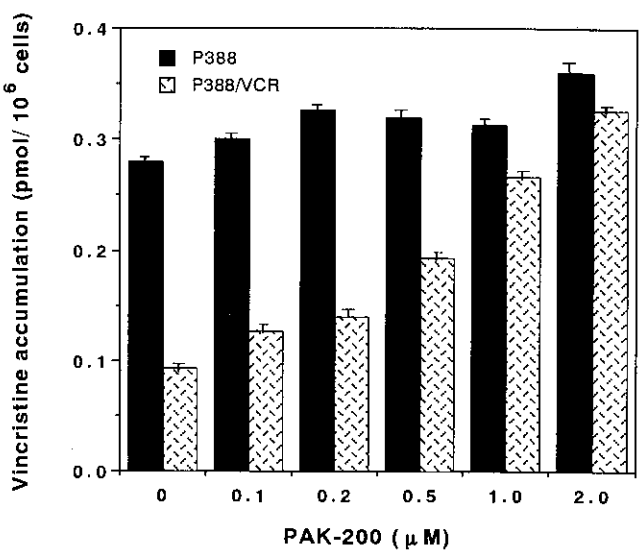

Fig. 2. Effect of PAK-200 on the accumulation of VCR in P388 and P388/VCR cells. Effects of PAK-200 on accumulation of VCR in P388 cells and P388/VCR cells at the indicated concentrations. Data represent the mean±SE of three experiments.

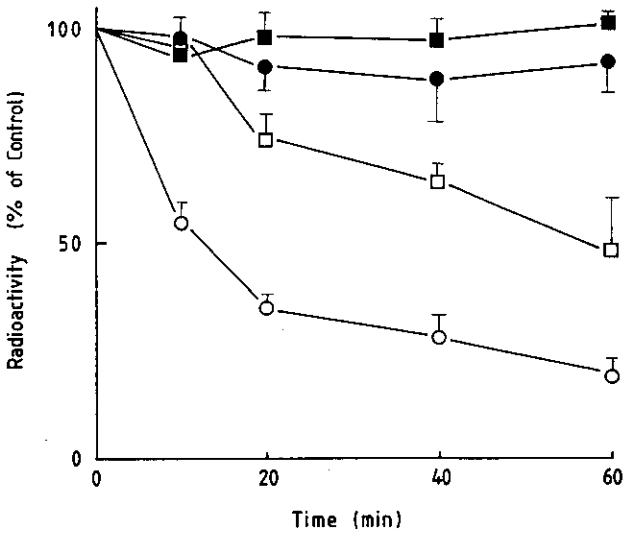

Fig. 3. Effect of PAK-200 on the extracellular transport of VCR. Release of VCR in the absence (●, ○) and presence (■, □) of 2 μM PAK-200 from P388 (●, ■) and P388/VCR (○, □) cells. Data represent the mean ± SE of three experiments.

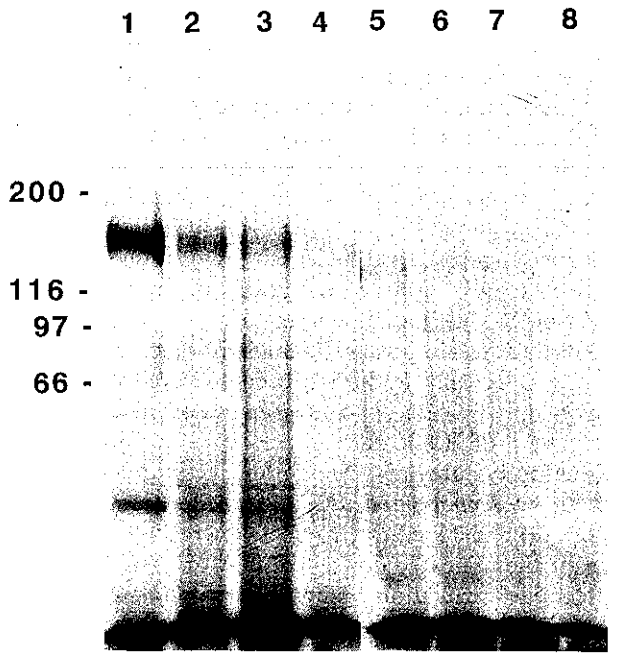

Fig. 4. Inhibition of [^{3}H]azidopine labeling of P-gp in membrane vesicles from KB-C2 and P388/VCR cells. KB-C2 vesicles (lanes 1–4) and P388/VCR vesicles (lanes 5–8) were incubated with [^{3}H]azidopine in the absence (lanes 1 and 5) and presence of PAK-200 at 1 μM (lanes 2 and 6), 10 μM (lanes 3 and 7), and 100 μM (lanes 4 and 8).

P388, P388/VCR and P388/ADM. PAK-200 at 1 μM completely reversed the resistance to VCR in P388/VCR. The resistance of P388/ADM to ADM was not completely reversed by PAK-200 at a nontoxic concentration for the cells. PAK-200 at 2 μM increased the sensitivity of drug-sensitive P388 to VCR and ADM by factors of 3.8 and 2.8, respectively.

Effect of PAK-200 on cellular accumulation and efflux of [^{3}H]VCR We then examined the effect of PAK-200 on the accumulation of VCR in P388 and P388/VCR cells (Fig. 2). The intracellular level of VCR in P388/VCR cells was 33% of that in P388 cells. The addition of PAK-200 at 2 μM enhanced the accumulation of VCR in P388/VCR cells about 3-fold to the level of that in P388 cells without PAK-200.

We examined whether the increased accumulation of VCR in P388/VCR cells with PAK-200 was due to the inhibition of drug efflux (Fig. 3). After incubation of the cells for 10 min in the absence of PAK-200, about 45% of VCR was lost from the P388/VCR cells, whereas almost all VCR was retained in the P388 cells that do not express P-gp. This efflux of VCR from P388/VCR cells was completely blocked for 10 min after the addition of PAK-200 to the culture medium. When P388/VCR cells were incubated for 60 min with or without PAK-200, about 55% or 80% of VCR was lost from the cells, respectively. PAK-200 did not significantly disturb the efflux of VCR from P388 cells.

Effect of PAK-200 on photoaffinity labeling of P-gp by [^{3}H]azidopine PAK-200 inhibited the efflux of VCR from P388/VCR cells. The analogue appears to interact with P-gp expressed in P388 cells to inhibit the pump activity of the protein. The photoactive dihydropyridine calcium channel blocker, azidopine, photolabeled P-gp in membrane vesicles from human KB-C2 cells. The labeling was almost completely inhibited by vinblastine and MDR-reversing dihydropyridine analogues.[14] We therefore studied the effect of PAK-200 on the photolabeling of P-gp in the vesicles from KB-C2 cells and P388/VCR cells (Fig. 4). The molecular weight of the labeled P-gp in membrane vesicles from KB-C2 was 160–175 kDa, and that from P388/VCR was 135–145 kDa. The P-gp expressed in the mouse leukemia cells was 25–30 kDa smaller than that in human MDR cells. PAK-200 at 10 μM completely inhibited the labeling of P-gp in membrane vesicles from P388/VCR cells, and considerably inhibited the labeling of KB-C2 P-gp.

Effect of PAK-200 on antitumor activity of VCR *in vivo* PAK-200 had relatively low toxicity. None of the five mice given 200 mg/kg PAK-200 died as a result. First, we tested a combination therapy of VCR and PAK-200

Table II. Effect of PAK-200 on Antitumor Activity of VCR in P388- and P388/VCR-bearing Mice[a]

Drugs and dosage	Survival time (days)	T/C (%)	T/V (%)
A. P388			
Control	9.3±0.5		
PAK-200, 80 mg/kg×2	9.5±0.5	102	
VCR, 100 μg/kg	18.8±1.3	202	
VCR, 100 μg/kg			
+PAK-200 20 mg/kg×2	18.5±1.2	198	98
40 mg/kg×2	23.7±3.9	254	126
80 mg/kg×2	26.7±6.1	286	142
B. P388/VCR			
Control	11.8±0.8		
PAK-200, 80 mg/kg×2	12.0±0.6	101	
VCR, 100 μg/kg	13.2±0.4	111	
VCR, 100 μg/kg			
+PAK-200 20 mg/kg×2	14.2±0.8	120	108
40 mg/kg×2	14.5±0.8	123	110
80 mg/kg×2	17.2±0.8	145	130

a) CDF_1 male mice were given ip implants of P388 (A) or P388/VCR (B) leukemia cells on day 0, and agents were given ip daily from day 1 to 5. Each group consisted of 6 mice.

for 5 successive days on P388/VCR mice (Table II). The effects of the combination therapy were dependent on the PAK-200 dose. PAK-200 alone at 80 mg/kg twice a day (160 mg/kg/day) did not significantly increase the life-span of P388- and P388/VCR-bearing mice, whereas VCR alone at a dose of 100 μg/kg/day showed 202% and 111% prolongation of life (T/C) of P388- and P388/VCR-bearing mice, respectively. When both agents were combined, the life span of P388- and P388/VCR-bearing mice compared with VCR alone increased 142% and 130%, respectively.

Calcium antagonism and hypotensive effect The calcium-antagonistic activities of PAK-200 and nicardipine were measured and compared. The antagonistic activity of PAK-200 ($pID_{50}=6.08$) was about 1000 times lower than that of nicardipine ($pID_{50}=9.15$).

The hypotensive effect of PAK-200 was also examined in conscious spontaneously hypertensive rats and compared with that of nicardipine. Intravenous injection of PAK-200 at a dose of 1.0 mg/kg caused a fall of systolic blood pressure by 13.4±3.5% (n=5). In contrast, administration of nicardipine at doses of 0.1 and 0.3 mg/kg caused a fall of systolic blood pressure by 28.5±2.2% (n=9) and 39.1±2.2% (n=9), respectively. The calcium-antagonistic and hypotensive effects of PAK-200 were markedly lower than those of nicardipine.

DISCUSSION

PAK-200 completely reversed VCR-resistance in P388/VCR cells at 1 μM (1/14 of the 50% growth-inhibitory concentration of PAK-200). The compound could not completely reverse the ADM-resistance in P388/ADM cells at a nontoxic concentration.

[^{3}H]Azidopine photolabeled P-gp expressed in P388/VCR cells as well as in KB-C2 cells, and the labeling was completely inhibited by 10 μM PAK-200. Potent MDR-reversing agents have a high affinity for P-gp.[14] The inhibition of the [^{3}H]azidopine photolabeling of P-gp in P388/VCR cells by PAK-200 suggests that P-gp plays a role in multidrug resistance and also in the reversal of the resistance by PAK-200 in mouse leukemia cells. The molecular weight of P-gp in mouse leukemia cells was 120–125 kDa, i.e., 25–30 kDa smaller than that in KB-C2 cells. This difference may be mainly attributable to the difference in sugar chains attached to the P-gp, since the deduced amino acid sequence of P-gp expressed in mouse and human is highly homologous.[6, 22]

The sensitivity of P388 cells to VCR and ADM was also increased by PAK-200. PAK-200 at 5 μM enhanced the sensitivity of P388 cells to VCR by a factor of 10, and increased the accumulation of [^{3}H]VCR in P388 cells. Since we could not detect any expression of P-gp in P388 cells with immunoblot analysis (data not shown), we assume that molecules other than P-gp are involved in these phenomena. Similar effects on drug-sensitive human KB cells of other dihydropyridine analogues, pyridines and synthetic isoprenoids have been observed.[16, 23, 24] They increased the accumulation of anticancer agents in drug-sensitive KB cells. Some MDR-reversing agents may affect not only P-gp but also other membrane molecule(s).[25, 26]

PAK-200 inhibited the efflux of VCR from P388/VCR cells. Increased accumulation of VCR in P388/VCR cells may be partially attributable to the inhibition of the drug efflux from the cells by PAK-200. The efflux rate of VCR from P388/VCR cells in the presence of 2 μM PAK-200 was considerably decreased but was still faster than that from P388 cells without PAK-200. On the other hand, PAK-200 at 2 μM increased the accumulation of VCR in P388/VCR cells to a level similar to that in P388 cells without PAK-200. In addition to inhibiting the efflux of VCR from P388/VCR cells, PAK-200 may increase the influx of VCR.

PAK-200 enhanced the VCR effect on P388 and P388/VCR cells *in vivo*. PAK-200 in combination with VCR prolonged the survival time of mice bearing P388 leukemia. Since P388 cells do not express detectable P-gp, PAK-200 might interact with other molecule(s) than P-gp in P388 cells and change the sensitivity of the cells to VCR. The T/C value and mean survival time in P388/

VCR-bearing mice given 100 μg of VCR/kg were 111% and 13.2 days, respectively. However, we obtained a maximum T/C value of 145% and a mean survival time of 17.2 days with a combination of 100 μg of VCR/kg and PAK-200. These results may indicate that VCR resistance in the mice inoculated with P388/VCR cells was partially overcome by PAK-200. Alternatively, PAK-200 may interact with other molecule(s) than P-gp and enhance the effect of VCR on P388/VCR cells *in vivo*. Further study is needed to elucidate which component is mainly involved in the life span increase of P388/VCR-bearing mice by PAK-200.

In conclusion, PAK-200 has two properties. It increases the sensitivity of P388 cells to VCR and reverses the MDR in P388/VCR *in vitro*. PAK-200 also potentiated the effect of VCR on both P388 and 388/VCR cells *in vivo*. These properties of PAK-200, together with its low calcium channel-blocking activity, should make PAK-200 suitable for clinical use.

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