Jpn. J. Cancer Res. 81, 1057–1064, October 1990

Reversal by Two Dihydropyridine Compounds of Resistance to Multiple Anticancer Agents in Mouse P388 Leukemia *in vivo* and *in vitro*

Akira Kiue,[1,3] Tetsuro Sano,[1] Aya Naito,[1] Haruaki Inada,[1] Ken-ichi Suzuki,[1] Masaya Okumura,[1] Junko Kikuchi,[2] Shin-ichi Sato,[2] Hiroshi Takano,[2] Kimitoshi Kohno[2] and Michihiko Kuwano[2]

[1]*Omiya Research Laboratory, Nikken Chemicals Co., Ltd., 1-346 Kitabukuro, Omiya, Saitama 330, and* [2]*Department of Biochemistry, Oita Medical School, Hasama-machi, Oita 879-56*

We investigated whether two representative 1,4-dihydropyridine derivatives, NK-250 and NK-252, could potentiate the antitumor activity of multiple anticancer agents including vincristine (VCR), vinblastine, vindesine and actinomycin D in drug-resistant tumor cells and their parental drug-sensitive tumor cells. NK-250 and NK-252 at 5–10 μM almost completely reversed VCR resistance in cultured VCR-resistant P388/VCR cells derived from the mouse drug-sensitive P388/S leukemia cell line and also potentiated the cytocidal activity of VCR in drug-sensitive P388/S cells. NK-250 and NK-252 at 1–10 μM inhibited the photoaffinity labeling by [^{3}H]azidopine of the cell-surface 170,000-molecular-weight P-glycoprotein. In chemotherapeutic experiments with leukemia-bearing mice, NK-250 or NK-252 was orally administered in combination with different drugs of the MDR phenotype administered intraperitoneally. The antitumor activity of the various combinations was found to be augmented in mice bearing P388/S- and P388/VCR-leukemia. Among the combinations examined, the combination of NK-250 and VCR was the most effective. These two 1,4-dihydropyridines, NK-250 and NK-252, are unique compounds because they were effective not only in circumventing the drug resistance, but also in potentiating the action of antitumor drugs against drug-sensitive tumors.

Key words: Multidrug resistance — Dihydropyridine derivative — *In vivo* circumvention

Development of simultaneous resistance to multiple anticancer drugs (multidrug-resistance: MDR)[4] is a major impediment to the successful chemotherapy of human tumors.[1] Expression of an MDR gene (MDR-1) appears to be involved in the appearance of clinical drug resistance.[2,3] Experimental therapeutic studies to screen MDR-reversing agents have been performed by targeting a membrane 170,000-molecular-weight P-glycoprotein (GP170) encoded by the MDR-1 gene.[4,5] Verapamil and other calcium channel blockers can enhance the antitumor activity of Vinca alkaloids and anthracyclines; MDR cells are usually resistant to such antitumor agents.[6–10] Outward transport (efflux) of these MDR-related agents in the MDR cells is mediated by an energy-dependent drug-efflux pump, GP170.[3,11] *In vivo* administration of these calcium channel blockers, however, causes cardiovascular disorders when their blood concentration is elevated to a level high enough to show the MDR-reversing effect.

One can thus expect potent MDR-reversing agents with reduced calcium channel-blocking activity to be useful for practical cancer chemotherapy. From this standpoint, dihydropyridines with weaker side effects have been searched for to see if they could overcome MDR. Some dihydropyridines have low calcium channel-blocking activity, but strong MDR-reversing activity *in vivo*[12] as well as *in vitro*.[13,14] In our laboratory, among many dihydropyridines tested, lipophilic 1,4-dihydropyridines were found to efficiently overcome MDR *in vitro*.[15] We have further screened 57 newly synthesized 1,4-dihydropyridine derivatives to see whether they could overcome vincristine (VCR) resistance in mice bearing VCR-resistant leukemia.[16] On intraperitoneal (ip) administration trials of such dihydropyridines, some of them with little calcium antagonistic effect or vasodilating activity could strongly potentiate the activity of VCR against drug-resistant leukemia *in vivo*.[16]

MDR-reversing agents are often screened *in vivo* by the ip administration of both reversing agent and antitumor drug in animals which have been inoculated ip with tumor cells. The reversing agents and antitumor drugs are preferably administered into sites different

[3] To whom correspondence should be addressed. Present address: Department of Biochemistry, Oita Medical School, Hasama-machi, Oita 879-56.

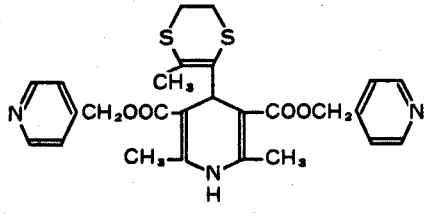
[4] The abbreviations used are: NK-compounds, 1,4-dihydropyridine derivatives; MDR, multidrug-resistance; VCR, vincristine sulfate; VLB, vinblastine sulfate; VDS, vindesine sulfate; ACD, actinomycin D; GP170, 170,000-molecular-weight glycoprotein; P388/S, P388 leukemia cells sensitive to anticancer drugs; P388/VCR, P388 leukemia cells resistant to VCR; T/C, mean survival time (in days) of the treated group divided by mean survival time of the control group; ip, intraperitoneal; po, oral.

from that used for inoculation of tumor cells. Other routes besides ip for the administration of the MDR-reversing agents should also be considered to develop practical therapeutic methods for clinical use. Our recent *in vivo* study has demonstrated that two 1,4-dihydropyridines (NK-compounds), NK-250 and NK-252, which have low calcium channel-blocking activity with very high affinity for GP170, can potentiate the activity of VCR in VCR-resistant leukemia-bearing mice when administered ip.[16]

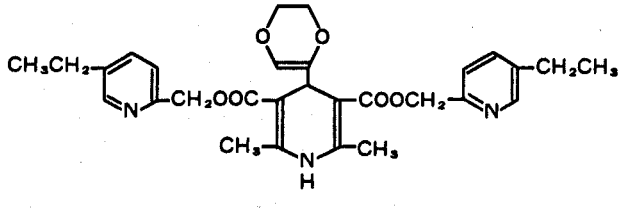
In this study, we investigated whether NK-250 and NK-252 could potentiate the effects of VCR, vinblastine (VLB), vindesine (VDS) and actinomycin D (ACD) in P388 leukemia-bearing mice when the dihydropyridine derivatives were orally (po) administered and the antitumor drug was administered ip.

MATERIALS AND METHODS

Cell lines and cell culture The P388 leukemia cell line sensitive to antitumor drugs, P388/S, and its subline, P388/VCR, resistant to VCR, were kindly supplied by Dr. M. Inaba, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo). Cells from P388/S or its resistant subline were grown in suspension in RPMI-1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, MD) in the presence of 10 μM 2-hydroxyethyl disulfide (Aldrich Chemical Co., Inc., Milwaukee, WI) and 100 μg/ml kanamycin.



NK-250



NK-252

Fig. 1. Chemical structures of 1,4-dihydropyridine derivatives (NK-compounds).

Drugs VCR used for *in vitro* assay was obtained from Sigma Chemical Co. (St. Louis, MO). For *in vivo* therapeutic experiments, we used VCR and VDS formulated for clinical use, which were purchased from Shionogi Co., Ltd. (Osaka), VLB from Kyorin Pharmaceutical Co., Ltd. (Tokyo) and ACD from Banyu Pharmaceutical Co., Ltd. (Tokyo). [^{3}H]Azidopine (40 Ci/mmol) was obtained from Amersham Japan Ltd. (Tokyo). Cepharanthine was obtained from Kaken Pharmaceutical Co., Ltd. (Osaka). NK-250 [bis(4-pyridylmethyl) 4-[2-(3-methyl-5,6-dihydro-1,4-dithiinyl)]-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate] and NK-252 [bis[2-(5-ethylpyridyl)methyl] 4-[2-(5,6-dihydro-*p*-dioxinyl)]-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate] were synthesized in Omiya Research Laboratory, Nikken Chemicals Co., Ltd. (Saitama). The chemical structures of NK-250 and NK-252 are shown in Fig. 1.

Animals Six- to 8-week-old male BALB/c×DBA/2 F_1 ($CD2F_1$) mice weighing 22–25 g were obtained from Charles River Japan, Inc. (Kanagawa). They were given food and tap water *ad libitum* and kept in a room conditioned at 22–24°C and 50–60% relative humidity, with 12 air changes per hour.

Cell growth inhibition assay *in vitro* Cells from P388/S or its resistant subline, P388/VCR, were harvested from tumor-bearing mice 6 to 7 days after transplantation and were suspended in RPMI-1640 medium with the antitumor agents and/or NK-compounds, seeded at a final density of 5×10^4 cells/ml, and incubated in a CO_2 incubator at 37°C for 2 days. Then 4 ml of 0.25% trypsin in calcium, magnesium-free Ringer's buffer solution was added to 1 ml of cell suspension and the mixture was incubated for 5 min at 37°C. The number of cells was counted with a model ZB1 Coulter counter.

Photoaffinity labeling of P-glycoprotein Membrane vesicles prepared from P388/VCR (120 μg of protein/assay) were incubated with [^{3}H]azidopine (1.5 μCi/assay) for 30 min at room temperature with or without NK-compounds and cepharanthine.[15-19] After irradiation for 15 min at 4°C, the samples were solubilized in SDS buffer as described previously.[16] Samples of half of the reaction mixture labeled with [^{3}H]azidopine were fractionated by electrophoresis on an SDS-polyacrylamide-urea gel.[16] The gel bed consisted of 5% polyacrylamide/4.5 *M* urea gel, pH 7.6, without a stacking gel.

Evaluation of antitumor activity *in vivo* $CD2F_1$ mice were inoculated ip with 0.2 ml of diluted ascites fluid containing 10^6 P388/S or 10^6 P388/VCR cells on day 0. Antitumor agents were given ip and NK-compounds were given po once daily for 5 or 10 days starting on day 1. Antitumor activity was evaluated in terms of the mean

survival time (in days) for each group and also in terms of the mean survival time of the treated group divided by the mean survival time of the control group (T/C values) (%). The data on mean survival time were analyzed by the two-tailed Student's t test and two-tailed Cochran's t test if the difference in distribution between the two groups to be compared was significant ($P<0.05$) by the F test. Antitutmor agents were dissolved in sterilized physiological saline. NK-compounds were suspended in sterilized 0.5% carboxymethyl cellulose sodium salt containing 0.1% Tween 80 as a vehicle.

RESULTS

Potentiation of VCR by NK-250 and NK-252 against drug-sensitive and drug-resistant cells in culture The drug sensitivity of P388/VCR cells was compared with that of its parental counterpart, P388/S cells, by assaying growth inhibition *in vitro* as reported by Inaba and Maruyama.[20] When the cells were exposed to VCR for 48 h, the 50% growth-inhibiting dose of VCR for P388/S was about 4 ng/ml, and that for P388/VCR was about 85 ng/ml; P388/VCR was thus 21-fold more resistant to VCR than P388/S (Fig. 2). The effects of NK-250 and NK-252 combined with various doses of VCR on the growth of P388/S and P388/VCR cells were compared. The combination of VCR with 5 μg/ml of NK-250 (Fig. 2A) and NK-252 (Fig. 2B) completely overcame the VCR resistance of P388/VCR.

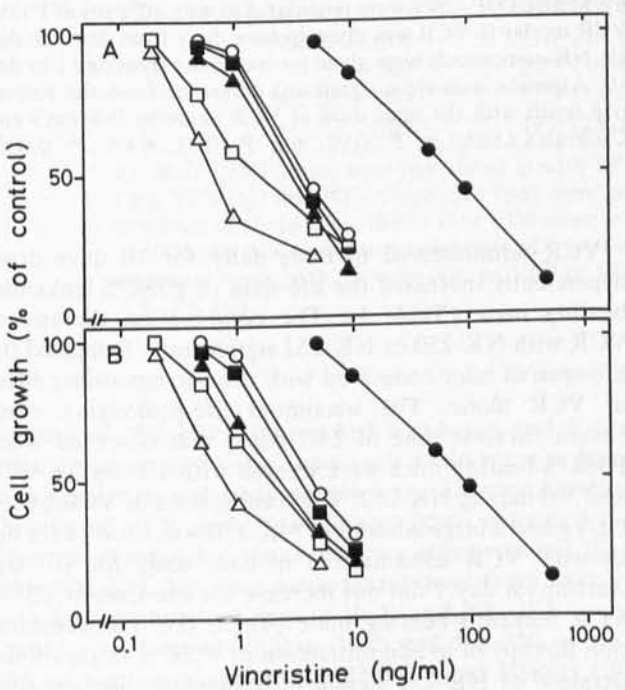


Fig. 2. Reversing effect of NK-250 (A) and NK-252 (B) on VCR-sensitive P388/S leukemia cells and on VCR-resistant P388/VCR leukemia cells *in vitro*. Sensitive P388/S cells were cultured with various concentrations of VCR in the absence (○) or presence of 5 (□) and 10 (△) μg/ml of NK-250 (A) or NK-252 (B) for 72 h. P388/VCR cells were cultured with VCR in the absence of (●) or presence of 5 (■) and 10 (▲) μg/ml of NK-250 (A) or NK-252 (B).

Photoaffinity labeling of P-glycoprotein by [^{3}H]-azidopine and its inhibition by NK-250 and NK-252 [^{3}H]Azidopine specifically labels GP170, which is overexpressed in membrane vesicles of multidrug-resistant cells. Agents which reverse MDR, such as verapamil and cepharanthine, interfere with this photoaffinity labeling. We investigated whether the NK-compounds inhibited the labeling by [^{3}H]azidopine of GP170 in membrane vesicles from P388/VCR cells. Consistent with previous reports,[4,16] cepharanthine at 100 μM almost completely inhibited the photoaffinity labeling (Fig. 3). NK-250 and NK-252 completely inhibited the photoaffinity labeling by [^{3}H]azidopine at 10 and 1 μM, respectively, suggesting specific interaction of the NK-compounds with GP170 (Fig. 3).

Combined effect of VCR and NK-250 or NK-252 on antitumor activity in P388/S- and P388/VCR-bearing mice Combined effects of VCR and NK-250 or NK-252 on antitumor activity were examined against both P388/S and P388/VCR leukemia-bearing mice. Figure 4A illustrates a therapeutic experiment with P388/S-bearing mice given NK-250 at 300 mg/kg po and VCR at 30 μg/kg ip. Figure 4B illustrates a therapeutic experiment with P388/VCR-bearing mice given NK-250 at 300 mg/kg po

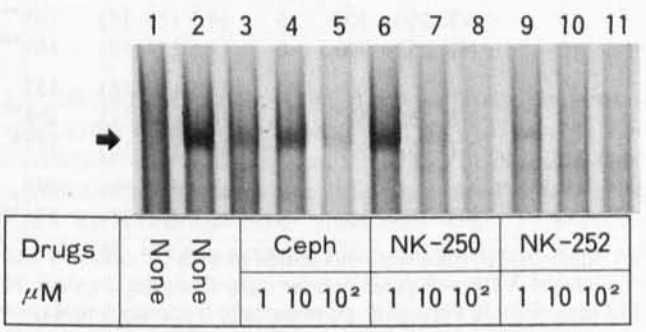


Fig. 3. Inhibition of [^{3}H]azidopine labeling of GP170 in membrane vesicles by NK-compounds. P388/VCR vesicles (120 μg of protein per lane) (lanes 2–11) were incubated with [^{3}H]azidopine in the absence or presence of the indicated drug concentrations. Autoradiograms were developed after one week of exposure. P388/S vesicles (lane 1) were also examined as a control. Cepharanthine (Ceph) was also tested. The arrow indicates the expected position of a 170-kDa band based on the use of molecular size markers.

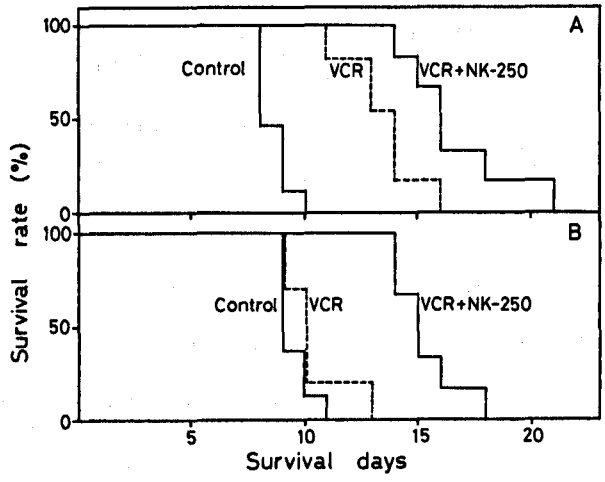


Fig. 4. Effect of NK-250 on antitumor activity of VCR in mice bearing VCR-sensitive P388/S (A) and VCR-resistant P388/VCR (B). Male $CD2F_1$ mice were inoculated ip with 10^6 P388/S or P388/VCR leukemia cells on day 0. VCR (ip) at 30 μg/kg/day and NK-250 (po) at 300 mg/kg/day were given once daily for 10 days starting on day 1. The control group consisted of 16 or 17 mice, the VCR alone group consisted of 10 or 11 mice and the group treated with VCR and NK-250 consisted of 6 mice.

Table I. Effects of NK-250 and NK-252 on Antitumor Activity of VCR in P388/S-bearing Mice[a)]

Dose of VCR (μg/kg/day)	Dose of NK-compounds (mg/kg/day)		Number of mice	Mean survival days (range)	T/C (%)
—		—	17	8.6 (8–10)	100
10		—	12	12.3 (11–13)	143
10	+NK-250	300	6	14.2 (13–15)	165***
10	+NK-252	300	6	14.5 (14–16)	169***
30		—	11	13.5 (11–16)	157
30	+NK-250	300	6	16.7 (14–21)	194**
30	+NK-252	300	6	15.2 (14–16)	177*
100		—	6	17.0 (15–19)	198
100	+NK-252	300	6	23.7 (21–27)	276***

a) Male $CD2F_1$ mice were inoculated ip with 10^6 cells of P388/S on day 0. VCR was given ip once daily from day 1 to day 10. NK-compounds were given po once daily from day 1 to day 10. Asterisks indicate a significant difference from the respective result with the same dose of VCR alone by Student's and Cochran's *t* tests: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

and VCR at 30 μg/kg ip. NK-250 and NK-252 *per se* had no antitumor effect on P388/S- and P388/VCR-bearing mice (data not shown).

Table II. Effects of NK-250 and NK-252 on Antitumor Activity of VCR in P388/VCR-bearing Mice[a)]

Dose of VCR (μg/kg/day)	Dose of NK-compounds (mg/kg/day)		Number of mice	Mean survival days (range)	T/C (%)
—		—	16	9.5 (9–11)	100
10		—	10	9.4 (9–11)	99
10	+NK-250	200	6	12.3 (12–13)	129***
10		300	6	13.5 (13–14)	142***
10	+NK-252	200	6	11.3 (11–12)	119***
10		300	6	11.5 (10–14)	121*
30		—	10	10.3 (9–13)	108
30	+NK-250	200	6	14.3 (13–16)	151***
30		300	6	15.3 (14–18)	161***
30	+NK-252	200	6	13.8 (12–18)	145**
30		300	6	14.3 (13–17)	151***
100		—	9	10.4 (9–12)	109
100	+NK-250	100	6	13.5 (12–15)	142***
100		200	5	16.2 (13–19)	171**
100		300	6	11.0 (5–17)	116
100	+NK-252	100	6	12.5 (10–15)	132*
100		200	6	14.3 (13–18)	151**
100		300	6	15.3 (14–17)	161***

a) Male $CD2F_1$ mice were inoculated ip with 10^6 cells of P388/VCR on day 0. VCR was given ip once daily from day 1 to day 10. NK-compounds were given po once daily from day 1 to day 10. Asterisks indicate a significant difference from the respective result with the same dose of VCR alone by Student's and Cochran's *t* tests: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

VCR administered ip once daily for 10 days dose-dependently increased the life-span of P388/S leukemia-bearing mice (Table I). The combination therapy of VCR with NK-250 or NK-252 significantly increased the life-span of mice compared with the corresponding effect of VCR alone. The maximum life-prolonging effect (mean survival time of 23.7 days) was observed when P388/S-bearing mice were treated with 100 μg/kg VCR and 300 mg/kg NK-252. The combination of VCR at 100 μg/kg and a large amount of NK-250 was toxic (data not shown). VCR administered ip once daily for 10 days starting on day 1 did not increase the life-span of P388/VCR leukemia-bearing mice (Table II). The combination therapy of ip administration of VCR with po administration of NK-250 significantly increased the life-span of mice compared with the corresponding effect of VCR alone. This antitumor-enhancing activity of NK-250 was dose-dependent at each dose of VCR. The maximum life-prolonging effect (mean survival time of 16.2 days) was observed when P388/VCR-bearing mice were treated with 100 μg/kg VCR and 200 mg/kg NK-250. The combination of VCR at 100 μg/kg and a large

Table III. Effect of NK-250 and NK-252 on Antitumor Activity of VLB and VDS in P388/S- and P388/VCR-bearing Mice[a)]

Drugs	Dose of NK-compounds (mg/kg/day)	P388/S Mean survival days (range)	P388/S T/C (%)	P388/VCR Mean survival days (range)	P388/VCR T/C (%)
Control	—	8.7 (6–10)	100	9.1 (7–10)	100
VLB (30 μg/kg/day)	—	12.2 (10–14)	140	9.2 (8–11)	101
+NK-250	300	13.3 (12–15)	153	13.0 (10–15)	143**
+NK-252	300	12.5 (9–16)	144	11.8 (10–14)	130*
VLB (100 μg/kg/day)	—	14.0 (8–16)	161	12.0 (8–16)	132
+NK-250	300	19.8 (19–22)	228**	15.2 (14–17)	167*
+NK-252	300	16.2 (13–20)	186	13.0 (12–14)	143
VLB (200 μg/kg/day)	—	13.8 (9–16)	159	12.7 (9–17)	140
+NK-250	200	20.2 (17–23)	232**	16.7 (10–20)	184*
+NK-252	300	18.2 (15–23)	209*	13.2 (12–15)	145
Control	—	8.7 (6–10)	100	9.4 (6–11)	100
VDS (10 μg/kg/day)	—	11.0 (8–13)	126	10.3 (10–11)	110
+NK-250	300	12.5 (11–15)	144	13.2 (12–14)	140
+NK-252	300	13.2 (9–15)	152	10.7 (9–12)	114
VDS (30 μg/kg/day)	—	12.8 (11–14)	147	10.0 (9–11)	106
+NK-250	200	15.0 (14–16)	172**	12.8 (11–16)	136**
+NK-252	200	15.6 (15–17)	179**	12.0 (11–14)	128**
VDS (100 μg/kg/day)	—	14.3 (9–21)	159	9.8 (9–11)	104
+NK-250	200	17.7 (15–21)	203	16.0 (13–18)	170**
	300	20.7 (16–25)	237*	11.4 (5–19)	121
+NK-252	200	17.5 (13–21)	201	12.2 (11–13)	130**
	300	19.0 (13–21)	218*	11.2 (7–13)	119

a) Male CD2F$_1$ mice were inoculated ip with 10^6 cells of P388/S or P388/VCR cell line on day 0. VLB (ip), VDS (ip) and NK-compounds (po) were given once daily from day 1 to day 5. The control group consisted of 12–18 mice, the VLB or VDS alone group consisted of 6 mice and the group treated with VLB or VDS and NK-compounds consisted of 5 or 6 mice. Asterisks indicate a significant difference from the respective result with the same dose of VLB or VDS alone by Student's and Cochran's *t* tests: * $P<0.05$, ** $P<0.01$.

amount of NK-250 (300 mg/kg) was toxic, and 3 of 6 mice demonstrated toxic signs such as decrease in locomotor activity and body weight loss. The combination therapy of VCR with NK-252 significant increased the life-span of mice, but the prolonging effect was less than with NK-250. No mice were found dead from toxicity with any combination dose of VCR and NK-252.

Combined effects of VLB or VDS and NK-250 or NK-252 on antitumor activity in P388/S- and P388/VCR-bearing mice VLB administered ip once daily for 5 days dose-dependently increased the life-span of P388/S leukemia-bearing mice, with mean survival times of 12.2 and 14.0 days at doses of 30 and 100 μg/kg VLB, respectively, but 13.8 days at 200 μg/kg VLB (Table III). The mean survival times of P388/VCR-bearing mice treated with VLB were 9.2, 12.0 and 12.7 days at 30, 100 and 200 μg/kg of VLB, respectively, suggesting weak cross-resistance of P388/VCR to VLB *in vivo*. The combination of VLB (ip) and NK-250 or NK-252 (po) significantly increased the life-span of P388/S- and P388/VCR-bearing mice compared with VLB alone (Table III). The maximum mean survival was 20.2 and 16.7 days, respectively, when P388/S- and P388/VCR-bearing mice were treated with 200 μg/kg VLB and 200 mg/kg NK-250. The prolonging effect of NK-252 on the survival time was less than that of NK-250.

VDS administered ip once daily for 5 days dose-dependently increased the life-span of P388/S leukemia-bearing mice, with mean survival tines of 11.0, 12.8 and 14.3 days at doses of 10, 30 and 100 μg/kg VDS, respectively (Table III). The mean survival times of P388/VCR-bearing mice were 10.3, 10.0 and 9.8 days when

Table IV. Effect of NK-250 and NK-252 on Antitumor Activity of ACD in P388/S- and P388/VCR-bearing Mice[a)]

Dose of ACD (μg/kg/day)	Dose of NK-compounds (mg/kg/day)	P388/S		P388/VCR	
		Mean survival days (range)	T/C (%)	Mean survival days (range)	T/C (%)
—	—	8.2 (8–9)	100	9.7 (8–11)	100
10	—	12.8 (10–15)	155	11.6 (10–14)	120
10	+NK-250 300	11.5 (10–13)	140	13.0 (12–14)	134*
10	+NK-252 300	14.3 (13–16)	174	11.8 (7–14)	122
25	—	14.1 (12–17)	171	12.8 (11–14)	132
25	+NK-250 300	15.8 (15–16)	192	15.8 (13–17)	163**
25	+NK-252 300	16.7 (15–18)	203	13.3 (12–16)	137
50	—	16.5 (14–18)	201	13.4 (10–16)	138
50	+NK-250 200	18.2 (17–19)	221**	15.7 (13–19)	162*
50	300	17.5 (15–22)	213	18.2 (15–20)	188***
50	+NK-252 200	17.3 (13–19)	211	14.8 (14–16)	153*
50	300	19.0 (17–21)	231**	17.0 (15–18)	175***

a) Male CD2F$_1$ mice were inoculated ip with 10^6 cells of P388/S or P388/VCR cell line on day 0. ACD (ip) and NK-compounds (po) were given once daily from day 1 to day 5. The control group consisted of 12 to 18 mice, the ACD alone group consisted of 6 to 12 mice and the groups treated with ACD and NK-compounds consisted of 5 or 6 mice. Asterisks indicate a significant difference from the respective result with the same dose of ACD alone by Student's and Cochran's *t* tests: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

they were treated with 10, 30 and 100 μg/kg of VDS, respectively, suggesting cross-resistance of P388/VCR to VDS *in vivo*. The combination of VDS (ip) and NK-250 or NK-252 (po) significantly increased the life-span of P388/S- and P388/VCR-bearing mice compared with VDS alone. When P388/S-bearing mice were treated with 100 μg/kg VDS and 300 mg/kg NK-250, and P388/VCR-bearing mice were treated with 100 μg/kg VDS and 200 mg/kg NK-250, the maximum mean survival times were 20.7 and 16.0 days, respectively. The effect of NK-252 was, however, less than that of NK-250. In the combination of 100 μg/kg VDS and 300 mg/kg NK-250, 3 of 6, and in the combination of 100 μg/kg VDS with 300 mg/kg NK-252, 1 of 6 P388/VCR-bearing mice died from toxicity on days 5 to 7 and on day 7, respectively.

Combined effect of ACD and NK-250 or NK-252 on antitumor activity in P388/S- and P388/VCR-bearing mice ACD administered ip once daily for 5 days dose-dependently increased the life-span of P388/S leukemia-bearing mice (Table IV). The mean survival times for P388/VCR-bearing mice treated with 10, 25 and 50 μg/kg ACD were 11.6, 12.8 and 13.4 days, suggesting rather weak cross-resistance of P388/VCR-bearing mice to ACD. The combination of ACD (ip) and NK-250 or NK-252 (po) significantly increased the life-span of P388/S- and P388/VCR-bearing mice compared with ACD alone (Table IV). When P388/VCR-bearing mice were treated with 50 μg/kg ACD and 300 mg/kg NK-250, the maximum life-prolonging effect, a mean survival time of 18.2 days, was observed. No mice were found dead from toxicity at any combination of doses of ACD with NK-250 and NK-252.

DISCUSSION

Resistance to antitumor drugs can be overcome *in vivo* by various agents including verapamil,[16] isoprenoid[21] and vitamin A[22] when these reversing agents are administered ip. The potentiation effects of these agents when combined with antitumor drugs have not yet been verified in practical cancer chemotherapy. Some 1,4-dihydropyridine derivatives can reverse MDR in cultured human cancer cell lines through interaction with GP170, resulting in increased accumulation of the antitumor drugs in drug-resistant cells.[15] Several of our newly synthesized 1,4-dihydropyridine derivatives have been further screened by determination of three different activities: *in vivo* VCR resistance-reversing activity in mice, affinity to GP170 and *in vitro* calcium antagonistic activity or *in vivo* hypotensive activity.[16] Six of the 57 dihydropyridine derivatives can potently reverse VCR resistance in leukemia-bearing mice, and 4 among the 6 compounds have much lower calcium-antagonistic and hypotensive activities than verapamil or nicardipine.[16]

The four NK-compounds including NK-250 and NK-252 at 1–10 μM almost completely inhibit the photoaffinity labeling by [^{3}H]azidopine of GP170 of membrane vesicles from VJ-300 cells.[16]

In this study, we selected NK-250 and NK-252 as the most favorable dihydropyridine derivatives. Concerning other derivatives of dihydropyridine, Kamiwatari *et al.*[13] and Yoshinari *et al.*[14] have independently reported that some derivatives of dihydropyridine can reverse MDR in culture. Their compounds have chemical structures which have very different substituents at the 3,4,5-positions of 1,4-dihydropyridine from NK-250 or NK-252, and their *in vivo* effects are not yet known. Shinoda *et al.*[12] have reported that another 1,4-dihydropyridine derivative, AHC-52, analogous to nifedipine can completely reverse VCR-resistance in P388/VCR cells and that the combination of VCR with AHC-52 causes a 206% increase in the T/C in P388/VCR-bearing mice. AHC-52, with less calcium-antagonizing activity than nifedipine, shows some structural similarity to NK-250 or NK-252, but in AHC-52 some of the 3,4,5-substituents of 1,4-dihydropyridine are completely different from those of NK-250 or NK-252. These independent studies of 1,4-dihydropyridine derivatives might reveal potent MDR-reversing agents with fewer side effects which could be suitable for practical use. In our present study, we demonstrate that NK-250 and NK-252 can overcome VCR resistance in an MDR cell line, P388/VCR, in culture (Fig. 2). Our previous study showed a more than 130% (T/V) increase in the life-span of P388/VCR-bearing mice when NK-250 and NK-252 were administered ip.[16] In this study, NK-250 or NK-252 was administered by the oral route. Combinations of ip administration of the antitumor drugs, VCR, VLB, VDS and ACD, and oral administration of NK-250 or NK-252 significantly enhanced the antitumor activity in P388/VCR-bearing mice (Tables I–IV), but only a slight (if any) effect appeared when adriamycin (ADM) was combined with the NK-compounds (unpublished data). NK-250 and NK-252 significantly potentiated VCR, ACD, VLB and VDS in P388/S-bearing mice (Tables I–IV), but not ADM (unpublished data).

Most agents that reverse MDR, such as cepharanthine, verapamil, reserpine and isoprenoids, inhibit the efflux pump activity which is mediated by cell-surface GP170 and the photoaffinity labeling of GP170 by [^{125}I]vindesine or [^{3}H]azidopine.[5, 17, 19, 23, 24] NK-250, NK-252 and other potent dihydropyridines inhibit the photoaffinity labeling by [^{125}I]vindesine or [^{3}H]azidopine of GP170 of membrane vesicles from MDR cell lines derived from human cancer KB cells, KB-C1 or VJ-300 cells.[15, 16] NK-250 and NK-252 also almost completely inhibited the photoaffinity labeling of GP170 of membrane vesicles of P388/VCR cells (Fig. 3). MDR-related antitumor drugs are expected to show high affinity to GP-170,[5, 17, 19, 23–25] but the specific affinity to GP170 of P388/VCR appears to differ among the antitumor drugs. Different affinity constants among the antitumor drugs for GP170 may affect the potentiation by NK-250 or NK-252 of the MDR-related antitumor drugs in P388/VCR-bearing mice. Other possibilities such as differences in drug metabolism and sites of action of the antitumor drugs, may also be involved in the dissimilar potentiation by NK-compounds.

NK-250 and NK-252 potentiated VCR, VLB, VDS and ACD in drug-sensitive P388/S leukemia-bearing mice *in vivo* as well as VCR in P388/S cells *in vitro*. However, drug-sensitive P388/S (this study) and KB[16, 26] cells show no significant expression of GP170. Our recent study demonstrated significantly enhanced accumulation of radioactive VCR in both drug-sensitive cells and their MDR counterpart cells.[27] However, the precise mechanism of the increase in antitumor activity of VCR or other antitumor drugs by the dihydropyridines in P388/S-bearing mice is not yet known. From our present study and the previous one[16] we consider that dihydropyridines such as NK-250 and NK-252 are unique agents in their ability to potentiate antitumor agents in drug-sensitive and drug-resistant tumors *in vivo* when administered ip and po. Further studies on the toxicology, pharmacology and drug metabolism of NK-250 and NK-252 are required before they can be put to clinical use.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan, and also by a research grant from the Princess Takamatsu Cancer Research Fund.

(Received May 23, 1990/Accepted July 28, 1990)

REFERENCES

1) Lane, M. Clinical problems of resistance to cancer chemotherapeutic agents. *Fed. Proc.*, **38**, 103–107 (1989).
2) Pastan, I. and Gottesman, M. M. Multiple-drug resistance in human cancer. *N. Engl. J. Med.*, **316**, 1388–1393 (1987).
3) Bradley, G., Juranka, P. F. and Ling, V. Mechanism of multidrug resistance. *Biochim. Biophys. Acta*, **948**, 87–128 (1988).
4) Akiyama, S., Cornwell, M. M., Kuwano, M., Pastan, I. and Gottesman, M. M. Most drugs that reverse multidrug

resistance also inhibit photoaffinity labeling of P-glycoprotein by a vinblastine analog. *Mol. Pharmacol.*, **33**, 144–147 (1988).
5) Akiyama, S., Yoshimura, A., Kikuchi, H., Sumizawa, T., Kuwano, M. and Tahara, Y. Synthetic isoprenoid photoaffinity labeling of P-glycoprotein specific to multidrug-resistant cells. *Mol. Pharmacol.*, **36**, 730–735 (1989).
6) Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.*, **41**, 1967–1972 (1981).
7) Tsuruo, T., Iida, H., Nojiri, M., Tsukagoshi, S. and Sakurai, Y. Circumvention of vincristine and adriamycin resistance *in vitro* and *in vivo* by calcium influx blockers. *Cancer Res.*, **43**, 2905–2910 (1983).
8) Rogan, A. M., Hamilton, T. C. and Young, R. C. Reversal of adriamycin resistance by verapamil in human ovarian cancer. *Science*, **224**, 994–996 (1984).
9) Tsuruo, T., Kawabata, H., Nagumo, N., Iida, H., Kitatani, Y., Tsukagoshi, S. and Sakurai, Y. Potentiation of antitumor agents by calcium channel blockers with special reference to cross-resistance patterns. *Cancer Chemother. Pharmacol.*, **15**, 16–19 (1985).
10) Kessel, D. and Wilberding, C. Anthracycline resistance in P388 murine leukemia and its circumvention by calcium antagonists. *Cancer Res.*, **45**, 1687–1691 (1985).
11) Roninson, I. B., Chin, J. E., Choi, K., Gros, P., Housman, D. E., Fojo, A., Shen, D-W., Gottesman, M. M. and Pastan, I. Isolation of human *mdr* DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proc. Natl. Acad. Sci. USA*, **83**, 4538–4542 (1986).
12) Shinoda, H., Inaba, M. and Tsuruo, T. *In vivo* circumvention of vincristine resistance in mice with P388 leukemia using a novel compound, AHC-52. *Cancer Res.*, **49**, 1722–1726 (1989).
13) Kamiwatari, M., Nagata, Y., Kikuchi, H., Yoshimura, A., Sumizawa, T., Shudo, N., Sakoda, R., Seto, K. and Akiyama, S. Correlation between reversing of multidrug resistance and inhibiting of [^{3}H]azidopine photolabeling of P-glycoprotein by newly synthesized dihydropyridine analogues in a human cell line. *Cancer Res.*, **49**, 3190–3195 (1989).
14) Yoshinari, T., Iwasawa, Y., Miura, K., Takahashi, I. S., Fukuroda, T., Suzuki, K. and Okura, A. Reversal of multidrug resistance by new dihydropyridines with lower calcium antagonistic activity. *Cancer Chemother. Pharmacol.*, **24**, 367–370 (1989).
15) Nogae, I., Kohno, K., Kikuchi, J., Kuwano, M., Akiyama, S., Kiue, A., Suzuki, K., Yoshida, Y., Cornwell, M. M., Pastan, I. and Gottesman, M. M. Analysis of structural features of dihydropyridine analogs needed to reverse multidrug resistance and to inhibit photoaffinity labeling of P-glycoprotein. *Biochem. Pharmacol.*, **38**, 519–527 (1989).
16) Kiue, A., Sano, T., Suzuki, K., Inada, H., Okumura, M., Kikuchi, J., Sato, S., Kohno, K. and Kuwano, M. Activities of newly synthesized dihydropyridines in overcoming of vincristine resistance, calcium antagonism, and inhibition of photoaffinity labeling of P-glycoprotein in rodents. *Cancer Res.*, **50**, 310–317 (1990).
17) Debenham, P. G., Kartner, K., Siminovitch, L., Riordan, J. R. and Ling, V. DNA mediated transfer of multiple drug resistance and plasma membrane glycoprotein expression. *Mol. Cell. Biol.*, **2**, 881–884 (1982).
18) Safa, A. R., Glover, C. J., Meyers, M. B., Biedler, J. L. and Felsted, R. L. Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. *J. Biol. Chem.*, **261**, 6137–6140 (1986).
19) Safa, A. R., Glover, C. J., Sewell, J. L., Meyers, M. B., Biedler, J. L. and Felsted, R. L. Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. *J. Biol. Chem.*, **262**, 7884–7888 (1987).
20) Inaba, M. and Maruyama, E. Reversal of resistance to vincristine in P388 leukemia by various polycyclic clinical drugs, with a special emphasis on quinacrine. *Cancer Res.*, **48**, 2064–2067 (1988).
21) Yamaguchi, T., Nakagawa, M., Shiraishi, N., Yoshida, T., Kiyosue, T., Arita, M., Akiyama, S. and Kuwano, M. Overcoming drug resistance in cancer cells with synthetic isoprenoids. *J. Natl. Cancer Inst.*, **76**, 947–953 (1986).
22) Nogae, I., Kikuchi, J., Yamaguchi, T., Nakagawa, M., Shiraishi, N. and Kuwano, M. Potentiation of vincristine by vitamin A against drug-resistant mouse leukemia cells. *Br. J. Cancer*, **56**, 267–272 (1987).
23) Cornwell, M. M., Pastan, I. and Gottesman, M. M. Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J. Biol. Chem.*, **262**, 2166–2170 (1987).
24) Safa, A. R. Photoaffinity labeling of the multidrug-resistance-related P-glycoprotein with photoactive analogs of verapamil. *Proc. Natl. Acad. Sci. USA*, **85**, 7187–7191 (1988).
25) Cornwell, M. M., Safa, A. R., Felsted, R. L., Gottesman, M. M. and Pastan, I. Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150- to 170-kDa protein detected by photoaffinity labeling. *Proc. Natl. Acad. Sci. USA*, **83**, 3847–3850 (1986).
26) Kohno, K., Kikuchi, J., Sato, S., Takano, H., Saburi, Y., Asoh, K. and Kuwano, M. Vincristine-resistant human cancer KB cell line and increased expression of multidrug-resistance gene. *Jpn. J. Cancer Res.*, **79**, 1238–1246 (1988).
27) Watanabe, Y., Takano, H., Kiue, A., Kohno, K. and Kuwano, M. Potentiation of etoposide and vincristine by two synthetic 1,4-dihydropyridine derivatives in multidrug-resistant and atypical multidrug-resistant human cancer cells. *Anti-Cancer Drug Des.*, **5** (1990), in press.