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ANALYSIS OF STRUCTURAL FEATURES OF DIHYDROPYRIDINE ANALOGS NEEDED TO REVERSE MULTIDRUG RESISTANCE AND TO INHIBIT PHOTOAFFINITY LABELING OF P-GLYCOPROTEIN

ICHIRO NOGAE,* KIMITOSHI KOHNO,* JUNKO KIKUCHI,* MICHIHIKO KUWANO,*†
SHIN-ICHI AKIYAMA,‡ AKIRA KIUE,§ KEN-ICHI SUZUKI,§ YOHJI YOSHIDA,‖
MARILYN M. CORNWELL,¶ IRA PASTAN¶ and MICHAEL M. GOTTESMAN¶

*Department of Biochemistry, Oita Medical School, Oita 879-56; ‡Cancer Research Institute, Kagoshima University School of Medicine, Kagoshima 890; §Omiya Research Laboratory, Nikken Chemicals Co., Ltd., Omiya 330; ‖Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan; and ¶Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

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Abstract—Synthetic dihydropyridine analogs were screened to determine whether they would reverse multidrug resistance of a multidrug-resistant human KB carcinoma cell line, KB-C1. Among twenty-four dihydropyridine analogs examined, thirteen almost completely overcame drug resistance (group A), nine partially overcame resistance (group B) and two did not reverse resistance (group C). The twenty-two compounds that reversed drug-resistance (groups A and B) were hydrophobic dihydropyridine derivatives. Three compounds that reversed resistance, NK-113, NK-138 and NK-194, increased the accumulation of [^{3}H]vincristine in the resistant KB-C1 cells, but not in the parental KB cells, nor in a revertant cell line, KB-C1-R2. NK-101 (group C), which did not reverse resistance, had no effect on drug accumulation. Enhanced efflux of vincristine from the resistant cells was inhibited completely by NK-194, but NK-194 did not affect vincristine influx. Nine of the twenty-four compounds were screened to determine whether they inhibited photoaffinity labeling of the cell surface protein gp170 (P-glycoprotein) in KB-C1 cells by *N*-(*p*-azido-[3-^{125}I]-salicyl)-*N'*-β-aminoethylvindesine ([^{125}I]NASV). All five compounds of group A, NK-138, NK-194, NK-200, NK-203 and NK-220, inhibited the photoaffinity labeling of gp170 at less than 10–100 μM, whereas NK-113 and NK-196 of group B inhibited the labeling at 100–200 μM. By contrast, NK-101 and NK-102 of group C did not inhibit labeling even at 2000 μM. These studies confirm the relationship among reversal of multidrug resistance, decreased efflux of vincristine, and inhibition of [^{125}I]NASV labeling of P-glycoprotein.

Expression of the multidrug-resistance gene *mdr* is correlated with acquisition of multidrug resistance in tumor cells [1–3]. The *mdr* gene encodes a plasma membrane glycoprotein of 170,000 molecular weight (gp170 or P-glycoprotein) which appears to be an efflux pump for certain anticancer agents, since multidrug-resistant cell lines show increased drug efflux activity [4–7]. Recent evidence suggests that expression of the human *mdr* gene may be responsible for drug resistance in clinical situations [8].

Our goal is to find a way to overcome multidrug resistance. Combination therapy of drug-resistant cells with anticancer agents plus a second agent which may block the enhanced efflux in multidrug-resistant clones has been studied [4–7, 9–16]. We recently proposed that cationic and amphipathic features may be essential in compounds that overcome drug resistance by blocking the efflux pump [13]. Most drugs that reverse multidrug resistance including verapamil, reserpine and cepharanthine inhibit photoaffinity labeling of P-glycoprotein (gp170) by [^{125}I]NASV** [16, 17]. Since dihydropyridine analogs are known reversers of drug resistance, and since many dihydropyridine derivatives are available, we have examined a large series of these analogs in order to determine (1) if there are any common structural features in the active agents, and (2) if the affinity of these agents for gp170 is correlated with the extent of their ability to overcome drug resistance.

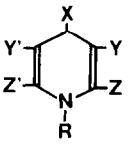
MATERIALS AND METHODS

Cell lines and cell culture. A multidrug-resistant subline, KB-C1 (clone KB-8-5-11-24 maintained in 1 μg/ml colchicine) was selected from human cancer KB cells with increasing concentrations of colchicine [18–20]. KB-C1-R2 is a spontaneous revertant of KB-C1, cloned from medium in the absence of colchicine for 3 months [13]. Cells were grown in monolayer in MEM [3] (Nissui Seiyaku Co., Tokyo, Japan)

† Correspondence: Dr. Michihiko Kuwano, Department of Biochemistry, Oita Medical School, Hazama-cho, Oita 879-56, Japan.

** Abbreviations: [^{125}I]NASV, *N*-(p-azido-[3-^{125}I]-salicyl-*N'*-β-aminoethylvindesine; MEM, minimal essential medium; D_{10}, concentration of drug that reduced the cloning efficiency to 10% of the control without drugs; and PBS, phosphate-buffered saline.

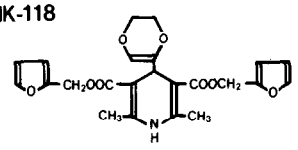
(a)



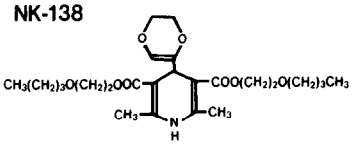
(b)

Group A

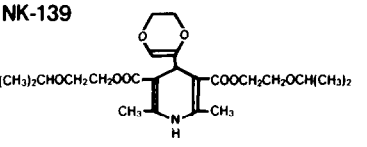
NK-118



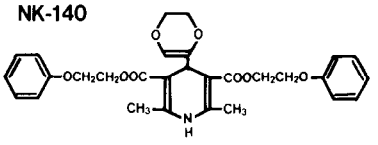
NK-138



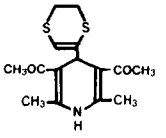
NK-139



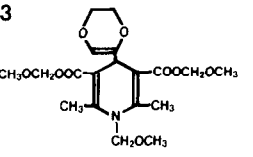
NK-140



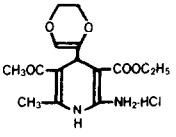
NK-149



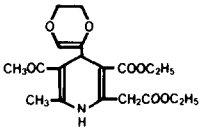
NK-163



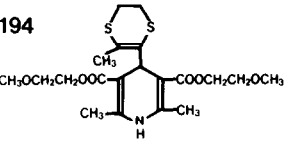
NK-180



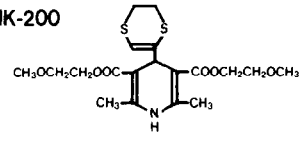
NK-187



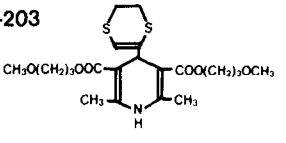
NK-194



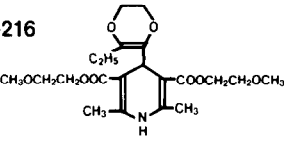
NK-200



NK-203



NK-216



NK-220

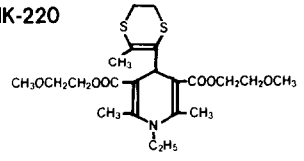


Fig. 1 (*continued on facing page*).

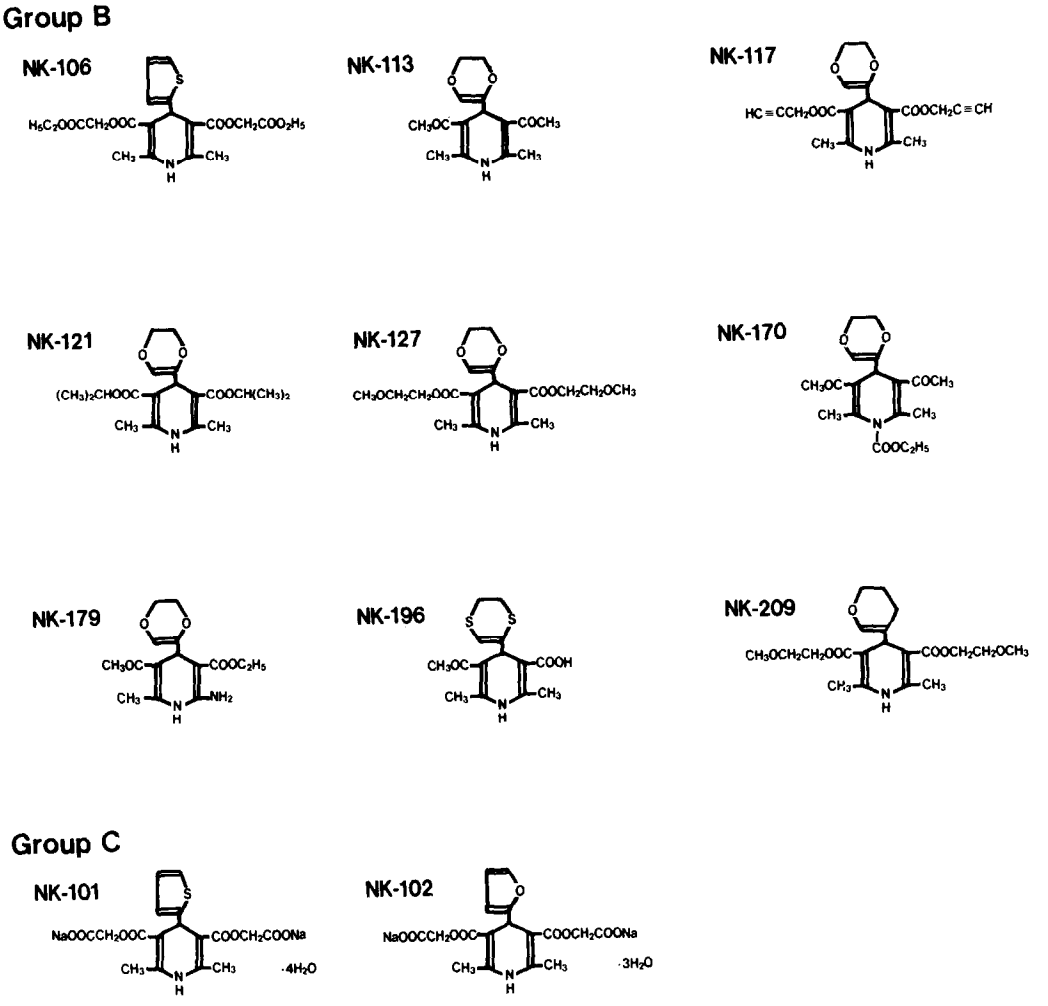


Fig. 1. Chemical structures of 1,4-dihydropyridine analogs. Shown are (a) the basic structure of 1,4-dihydropyridine and (b) the structures of its analogs.

containing 10% newborn calf serum (Microbiological Associates, Bethesda, MD), 1 mg/ml Bactopeptone (Difco Laboratories, Detroit, MI), 0.292 mg/ml glutamine, 100 μg/ml kanamycin, and 100 units/ml penicillin as described previously [12, 13].

Drugs and chemicals. Daunomycin, vincristine, vinblastine and actinomycin D were obtained from the Sigma Chemical Co., St. Louis, MO. [^{3}H]Vincristine (sp. act. 4.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA).

Cell survival by colony formation. Cell survival was determined by colony formation [12, 13]. KB or KB-C1 (300 cells) were plated in 60 mm dishes in the absence of any drug. Various drugs were added 16 hr later, and the colonies which appeared were scored after incubation for 10 days at 37°. Solutions of all drugs were freshly prepared before use in dimethyl sulfoxide. Relative resistance was determined by dividing the D_{10} of KB-C1 cells with or without dihydropyridine analogs by the D_{10} of KB cells without dihydropyridine analogs. The amount of each dihydropyridine analog alone inhibited less than 20% of the initial surviving activity of both KB or KB-C1 cells.

Drug accumulation. Cells (4×10^5/dish) were plated and incubated overnight at 37°. Then medium was replaced with serum-free MEM, and the cells were incubated with 34 nM [^{3}H]vincristine for 60 min with or without dihydropyridine analogs. Cells were washed once with cold PBS (g/liter: NaCl, 8.0; Na_2HPO_4 $12H_2O$, 2.9; KCl, 0.2; KH_2PO_4, 0.2) and then harvested. The cells were washed three times with cold PBS, and the cellular pellets were suspended in 0.7 ml of H_2O and mixed thoroughly with 7 ml of Scintisol EX-H (Wako Chemical Co., Osaka, Japan) before determination of radioactivity [12, 13].

Drug efflux and influx. Exponentially growing cells (4×10^5/dish) were plated and incubated overnight at 37°, and then placed in fresh medium. Cells were incubated with 34 nM [^{3}H]vincristine at 37° for 60 min [12, 13]. Each dish was washed three times with PBS and fresh serum-free medium added with or without dihydropyridine analogs, incubated for the indicated times at 37°, harvested, and counted.

Table 1. Effects of dihydropyridine analogs on drug resistance of KB-C1 cells*

Cell line	Dihydropyridine analogs	(μg/ml)	Relative resistance to DAU	VCR	VBL	Act-D
KB		0	1.0	1.0	1.0	1.0
	NK-138	10	0.9 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
	NK-194	50	0.8 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.2
	NK-113	10	1.0 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
	NK-101	150	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
KB-C1		0	13.3 ± 1.3	150.3 ± 2.1	52.4 ± 1.9	16.0 ± 1.4
	NK-138	10	1.3 ± 0.3	1.4 ± 0.2	1.5 ± 0.3	0.7 ± 0.1
	NK-194	50	1.2 ± 0.1	0.6 ± 0.1	0.8 ± 0.2	0.7 ± 0.1
	NK-113	10	3.4 ± 0.2	7.9 ± 1.4	6.7 ± 1.2	3.8 ± 0.4
	NK-101	150	13.1 ± 0.8	142.5 ± 3.8	48.6 ± 1.2	15.6 ± 0.6

* Abbreviations: DAU, daunomycin; VCR, vincristine; VBL, vinblastine; and Act-D, actinomycin-D. Values are means (± SE) of three experiments.

For the study of drug influx, cells were plated and incubated overnight, and the medium was changed to glucose-free, serum-free Hanks' balanced salt solution. Cells were incubated for 1 min at 37° with or without dihydropyridine analogs, and then 66 nM [^{3}H]vincristine was added for 1 min. Each dish was washed three times with ice-cold PBS, harvested, and then counted.

Membrane vesicle preparation. Membrane vesicles from KB and KB-C1 cells were prepared essentially as described [17, 21, 22] from cells grown under standard growth conditions [18, 19, 22]. Proteins were determined by the method of Bradford [23].

Photoaffinity labeling. Membrane vesicles were incubated with 3.8 μM [^{125}I]NASV (5×10^5 dpm) for 15 min at room temperature with or without dihydropyridine analogs [17, 24–26]. After irradiation of samples at 366 nm for 20 min at 25°, the samples were solubilized in sodium dodecyl sulfate ($NaDodSO_4$) sample buffer as described by Debenham *et al.* [27]. Samples labeled with [^{125}I]NASV were fractionated by electrophoresis on an $NaDodSO_4$/polyacrylamide/urea gel using a modification [16, 17] of the system described by Debenham, on a 5% polyacrylamide/4.5 M urea gel, pH 7.6, as previously described [17]. Proteins were stained with Coomassie blue (0.25% in 50% w/v) after the gels were fixed in trichloroacetic acid.

RESULTS

Reversal of multidrug resistance in human cancer cells by dihydropyridine analogs. The KB-C1 cells used in this study were 13- to 150-fold resistant to daunomycin, vincristine, vinblastine and actinomycin D (Table 1). We determined the abilities of twenty-four different dihydropyridine analogs to overcome multidrug resistance. Figure 1 shows the chemical structures of 1,4-dihydropyridine (Fig. 1a) and its twenty-four analogs (Fig. 1b). Dose–response curves of daunomycin, vincristine, vinblastine and actinomycin D with or without the dihydropyridine analogs were determined with the colony forming assay (see Figs. 2 and 3 and Table 1). Doses of

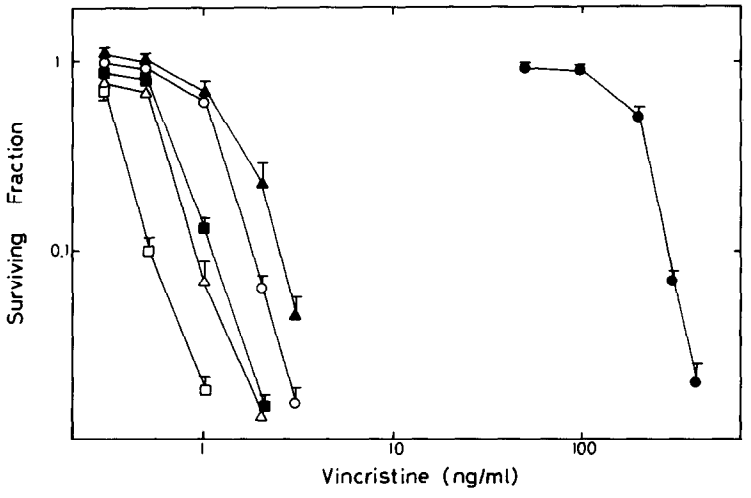


Fig. 2. Effects of NK-138 and NK-194 on resistance to vincristine. The effects of NK-138 and NK-194 on drug resistance in KB (○, △, □) and KB-C1 (●, ▲, ■) cells in the absence (○, ●) or presence of 10 μg/ml NK-138 (△, ▲) and 50 μg/ml NK-194 (□, ■) were examined by colony formation assay. Values are means of triplicate experiments.

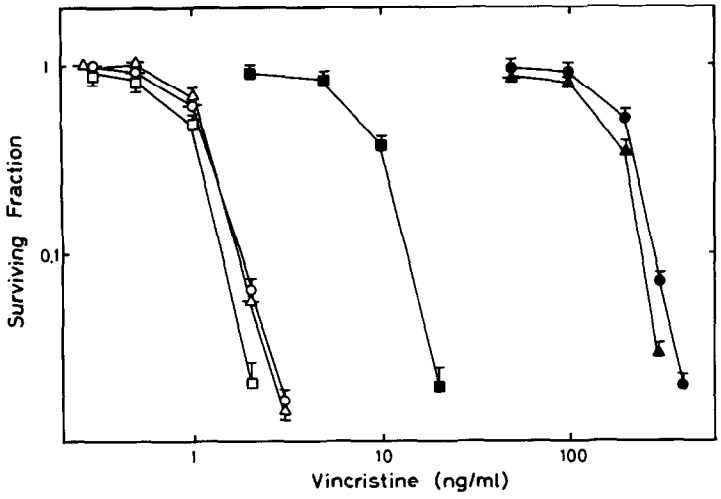


Fig. 3. Effects of NK-113 and NK-101 on drug resistance to vincristine. The effects of NK-113 and NK-101 on drug resistance in KB (□, △, □) and KB-C1 (●, ▲, ■) cells in the absence (○, ●) or presence of 100 μg/ml NK-113 (△, ▲) and 300 μg/ml NK-101 (□, ■) were examined by colony formation assay. Values are means ± SD of triplicate experiments.

dihydropyridine analogs used to test their abilities to overcome drug resistance were about one-fifth to one-tenth of the D_{10} values for these analogs. From the three repeated trials of dose–response curves with twenty-four dihydropyridine analogs, we classified them into three groups: group A—drugs that almost completely overcame drug resistance included thirteen dihydropyridine analogs, NK-118, NK-138, NK-139, NK-140, NK-149, NK-163, NK-180, NK-187, NK-194, NK-200, NK-203, NK-216, and NK-220; group B—drugs that partially reversed resistance included nine dihydropyridine analogs, NK-106, NK-113, NK-117, NK-121, NK-127, NK-170, NK-179, NK-196, and NK-209; and group C—drugs that did not overcome resistance included two dihydropyridine analogs, NK-101 and NK-102.

Figures 2 and 3 show examples of the colony formation assays used to test the reversing effect of the representative dihydropyridine analogs, NK-138, NK-194, NK-113 and NK-101. KB-C1 cells were about 150-fold more resistant to vincristine than KB cells. The combination of vincristine with 10 μg/ml NK-138 or 50 μg/ml NK-194 almost completely overcame vincristine resistance in KB-C1 cells, and the sensitivity of KB-C1 cells to vincristine became similar to that of KB cells in the presence of NK-138 or NK-194 (Fig. 2). NK-113 showed a partial ability to overcome vincristine resistance, while another

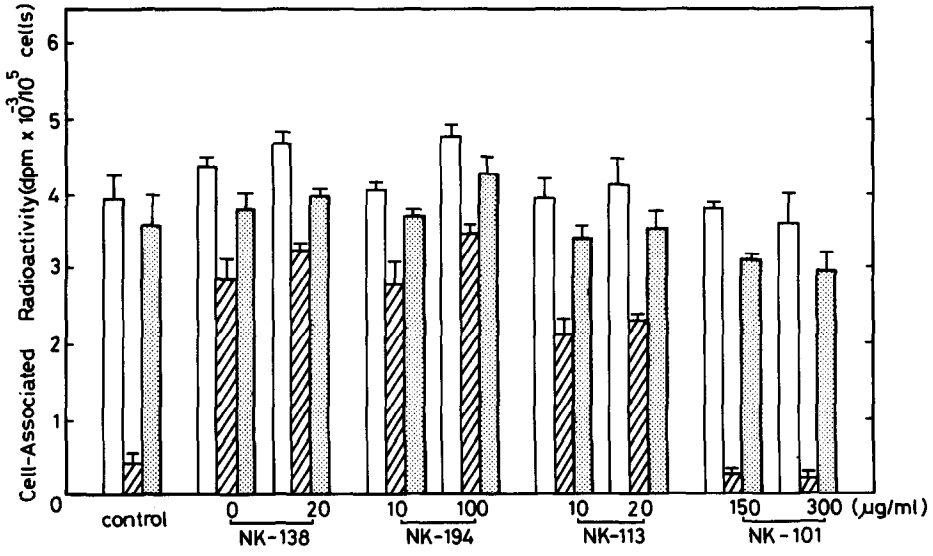


Fig. 4. Effects of dihydropyridine analogs on the accumulation of [^{3}H]vincristine in KB, KB-C1 and KB-C1-R2 cells. The intracellular levels of vincristine in the presence of two concentrations of dihydropyridine analogs in KB (□), KB-C1 (▨), and KB-C1-R2 (▩) cells were determined as described in Materials and Methods. Values are means ± SD of triplicate experiments.

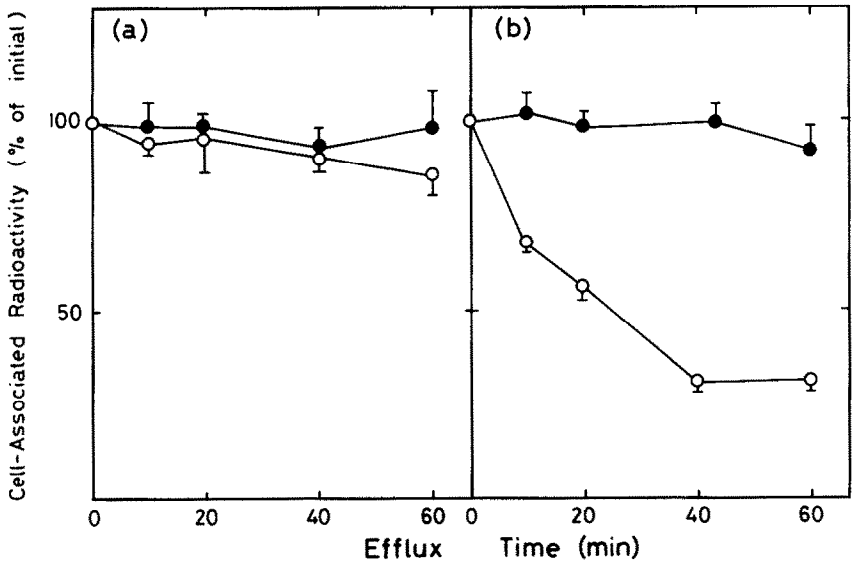


Fig. 5. Effect of NK-194 on efflux of vincristine. The release of vincristine in the absence (○) or presence (●) of 50 μg/ml NK-194 from KB (a) and KB-C1 (b) cells was followed. Values are means ± SD of triplicate determinations.

dihydropyridine analog, NK-101, failed to overcome resistance (Fig. 3). Table 1 summarizes data from dose–response curves of various combinations of NK-101, NK-113, NK-138 and NK-194 and daunomycin, vincristine, vinblastine and actinomycin D. NK-138 and NK-194 almost completely reversed the multidrug resistance of KB-C1 cells to daunomycin, vincristine, vinblastine and actinomycin D, whereas NK-113 only partially overcame this resistance. NK-101 did not reverse multidrug resistance (Table 1). We thus classified NK-138 and NK-194 as group A, NK-113 as group B, and NK-101 as group C.

Effect of dihydropyridine analogs on cellular accumulation of [^{3}H]*vincristine.* To examine how dihydropyridine analogs overcome multidrug resistance, we determined their effects on the accumulation of vincristine in KB and KB-C1 cells (Fig. 4). The intracellular level of vincristine in KB-C1 cells in the absence of dihydropyridine analogs was about one-tenth that of KB cells, whereas that in the revertant cell line, KB-C1-R2, was about the same as that in KB cells. The accumulation of vincristine in both KB and KB-C1-R2 cells treated with dihydropyridine analogs was not affected significantly. In contrast, NK-138 and NK-194 increased the accumulation of vincristine in KB-C1 cells about 8-fold. NK-101 did not increase accumulation of vincristine in KB-C1 cells and NK-113 had a partial effect (Fig. 4). The reversing effect on multidrug resistance by dihydropyridine analogs appears to be closely correlated with cellular levels of [^{3}H]vincristine in KB-C1 cells.

We next examined whether increased accumulation of anticancer agents in KB-C1 cells by NK-138 or NK-194 was due to inhibition of drug efflux. After incubation of the cells for 60 min in the absence of NK-194, about 70% of vincristine was lost from KB-C1 cells, whereas about 90% of vincristine was retained in KB cells. Addition of 50 μg/ml NK-194 to the culture medium almost completely inhibited the efflux of vincristine from KB-C1 cells (Fig. 5b). In contrast, NK-194 did not affect the efflux of vincristine from the parental drug-sensitive KB cells (Fig. 5a). Using [^{3}H]daunomycin, we also observed enhanced accumulation and decreased efflux in the presence of NK-138 or NK-194 (data not shown).

The effect of NK-194 on influx of vincristine was also examined. Using a method we have described previously [5], we assayed the cellular uptake of [^{3}H]vincristine for 1 min (Fig. 6). Figure 6 shows that there was no significant difference in the cellular uptake of vincristine between KB and KB-C1 cells in the absence or presence of NK-194.

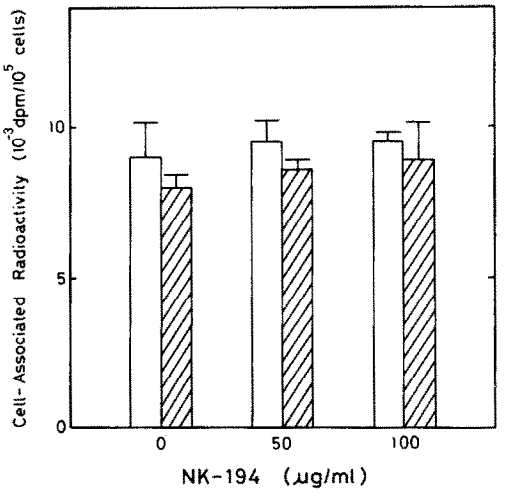


Fig. 6. Effect of NK-194 on influx of vincristine. The influx of vincristine (1 min uptake) was measured in the absence or presence of 50 or 100 μg/ml NK-194. Key: KB (□) and KB-C1 (▨). Values are means ± SD of triplicate experiments.

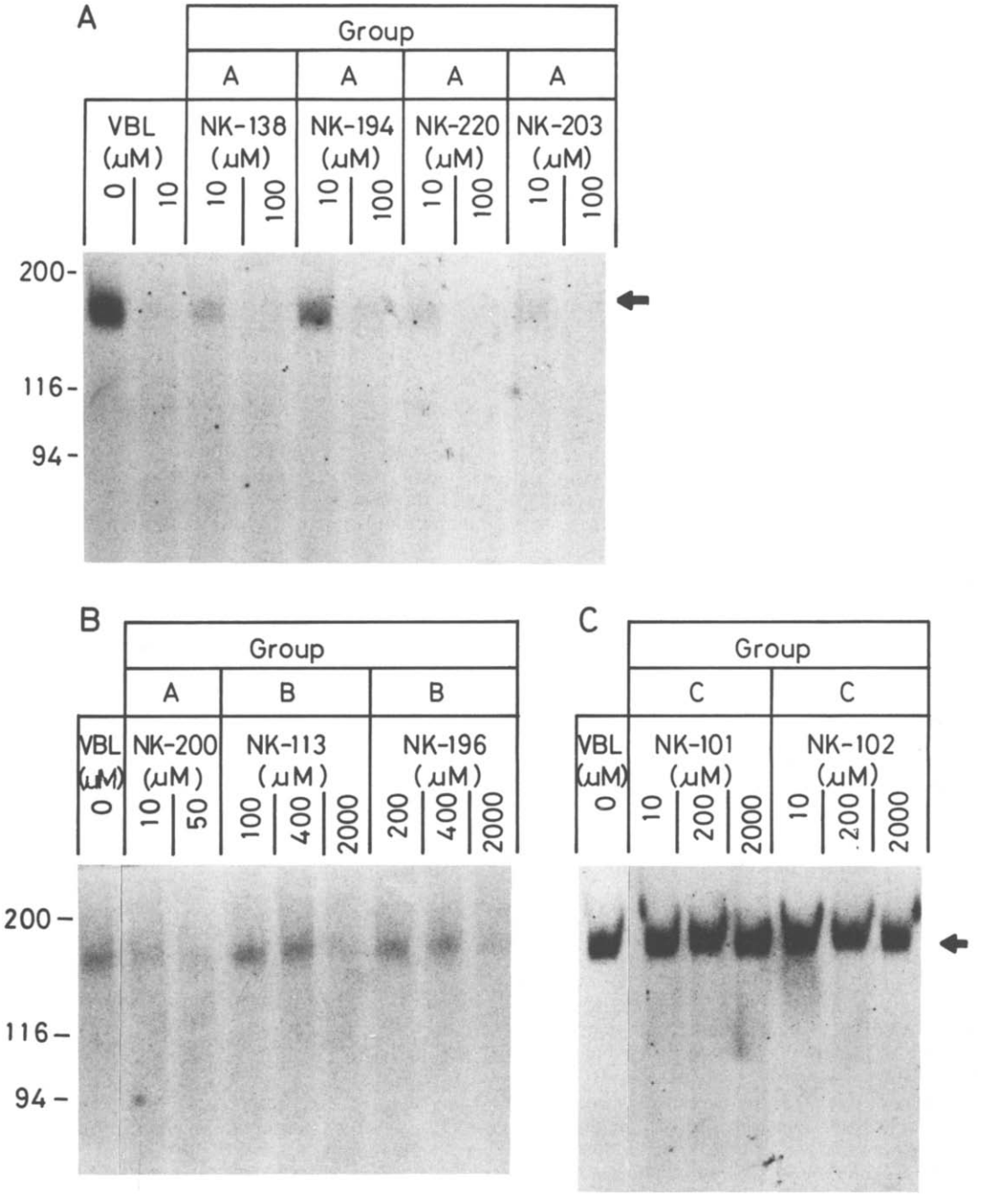


Fig. 7. Inhibition of [^{125}I]NASV labeling of the 150- to 170-kD P-glycoprotein in membrane vesicles by nine dihydropyridine analogs. KB-C1 vesicles (80 μg protein/lane) were incubated with [^{125}I]NASV in the absence or presence of the indicated drug concentrations. Autoradiograms were developed after a 96-hr exposure. Molecular size markers at the left are in kilodaltons. The arrow indicates the position of a 150- to 170-kD band seen by Coomassie blue staining. A, B and C, respectively, show classes of dihydropyridine analogs, group A, group B and group C respectively. Group A: drugs that almost completely overcame drug resistance; Group B: drugs that partially reversed resistance; Group C: drugs that did not overcome resistance. VBL: vinblastine.

Photoaffinity labeling of P-glycoprotein by [^{125}I]NASV. [^{125}I]NASV, a vinblastine analog, specifically labels the 150- to 170-kD P-glycoprotein which is overexpressed in membrane vesicles of drug-resistant KB-C1 cells, but no labeling with [^{125}I]NASV is found in vesicles from drug-sensitive parental KB cells or revertant cells [17, 21]. Agents which reverse multidrug resistance such as verapamil, quinidine, cepharanthine, and isoprenoids interfere with this photoaffinity labeling [16, 17, 28]. We thus examined whether dihydropyridine analogs inhibited the labeling of P-glycoprotein in membrane vesicles from KB-C1 cells by [^{125}I]NASV (Fig. 7). Figure 8 shows quantitation of the photolabeled

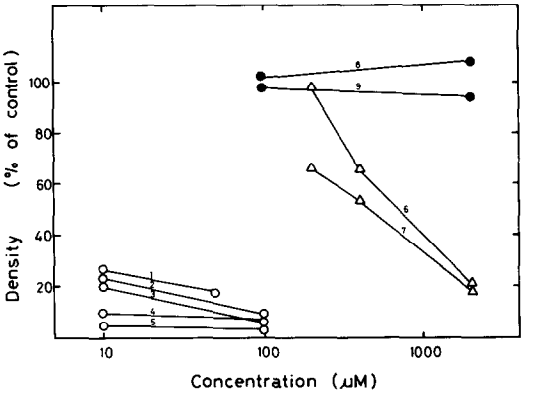


Fig. 8. Quantitative measurement of the inhibition of [^{125}I]NASV photolabeling. The density of the bands on the autoradiogram was determined. Data are expressed as a percent of the density of the photolabeling of P-glycoprotein in the absence of dihydropyridine analogs. Key: (○) group A, (△) group B, and (●) group C; (1) NK-200, (2) NK-194, (3) NK-138, (4) NK-220, (5) NK-203, (6) NK-113, (7) NK-196, (8) NK-101, and (9) NK-102.

gp170 band (Fig. 7) when measured by densitometric analysis. Compounds NK-138, NK-194, NK-200, NK-203 and NK-220 of group A inhibited the photoaffinity labeling of gp170 by [^{125}I]NASV at 10–100 μM (Figs. 7 and 8). NK-113 and NK-196 of group B did not inhibit the labeling at a concentration of 200 μM but inhibited it at 400–2000 μM. NK-101 and NK-102 of group C did not inhibit the labeling even at 2000 μM (Figs. 7 and 8).

DISCUSSION

Multidrug resistance is overcome by calcium channel blockers like verapamil [4, 5], phenothiazine calmodulin inhibitors like thioridazine, trifluoperazine, and chlorpromazine, [6], lysosomotropic amines like chloroquine and propranolol [14], some isoprenoids like *N*-solanesyldimethylbenzyl-ethylenediamine [12] and *N*-(*p*-methylbenzyl) decaprenylamine [29], and bisbenzylisoquinoline (biscoclaurine) alkaloids like cepharanthine [13]. In this study, we confirm the reversal effects of some dihydropyridine analogs on multidrug resistance. We have screened twenty-four dihydropyridine analogs whose chemical structures are given in Fig. 1. On the basis of their ability to overcome the multidrug resistance of the human KB carcinoma clone KB-C1, we classified them in three groups: A (good reversing ability), B (less active as reversing agents), and C (no reversing ability).

It is not possible to draw conclusions about the correlation between structure and activity from these studies. The most active reversing compounds in groups A and B were more hydrophobic than the group C molecules which contain hydrophilic moieties at positions y and y′. Determination of partition coefficients for some of these analogs suggest that activity is related to the overall hydrophobicity of the molecules (data not shown). More precise studies with more analogs are needed to formulate hypotheses about structure–activity relationship.

All the multidrug-resistance reversing agents in general inhibit drug efflux that is enhanced in multidrug-resistant cell lines. P-glycoprotein (gp170) is thought to be the efflux pump which regulates cellular levels of several anticancer agents [1–4]. Cornwell *et al.* [17] originally showed that a 150 to 170 kDa glycoprotein of the multidrug-resistant KB cells is specifically labeled with vinblastine analogs and that verapamil blocks the specific labeling. Calcium channel blockers which reverse multidrug resistance have been shown to bind to membranes from drug-resistant cells [28]. A dihydropyridine analog with calcium channel blocking action, azidopine, has been found to specifically interact with the gp170 by a photoaffinity labeling assay [26]. Akiyama *et al.* [16] have previously shown inhibition of photoaffinity labeling of gp170 by most agents that reverse multidrug resistance, irrespective of chemical structure. In this study, we have screened twenty-four dihydropyridine analogs which have similar structures. The five tested drugs of group A strongly inhibited the photolabeling and the two tested drugs of group C showed no inhibition, whereas two drugs of group B had intermediate inhibitory effects on photoaffinity labeling. The present study therefore confirms that inhibition of photoaffinity labeling by a vinblastine analog of P-glycoprotein in membrane vesicles will be a useful screen to identify agents that overcome drug resistance.

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