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A benzophenazine derivative, *N*-β-dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxy-benzo[a]phenazine-6-carboxamide, as a new antitumor agent against multidrug-resistant and sensitive tumors

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Summary. NC-190, a benzophenazine derivative (*N*-β-dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxy-benzo[a]phenazine-6-carboxamide), was effective against multidrug-resistant human and mouse tumor cells in vitro and in vivo. When vincristine (VCR)-resistant P388 leukemia-bearing mice were treated with an optimal dose of NC-190, four of six mice were cured, whereas treatment of mice with VCR resulted in only a marginal increase in life span. The compound also showed chemotherapeutic effect against Adriamycin-resistant P388 leukemia-bearing mice and was effective against various multidrug-resistant human and murine tumor cells in vitro. Its cytotoxicity to multidrug-resistant K562 cells was not enhanced by the addition of verapamil. The accumulation of NC-190 in multidrug-resistant K562 cells was slightly lower than that observed in sensitive K562 cells; the compound did not efficiently inhibit the binding of VCR to the plasma membrane of resistant cells, indicating that NC-190 has little affinity for P-glycoprotein. NC-190 inhibited the activity of DNA topoisomerase II. These observations suggest that NC-190 (1) is not transported out of resistant cells by P-glycoprotein and (2) inhibits DNA topoisomerase II activity in the cells, resulting in its likely effectiveness against various multidrug-resistant tumor cells.

Introduction

Cancer chemotherapy is often hampered by the emergence of drug-resistance during treatment. One of the major goals for chemotherapeutists is to discover new antitumor agents that are effective against drug-resistant tumors. When tumor cells acquire resistance to naturally occurring antitumor agents such as *Vinca* alkaloids and anthracycline antibiotics, they show cross(pleiotropic)-resistance to a variety of antitumor agents of natural origin. The mechanism of this pleiotropic drug resistance has been studied well during the last 3 years, and it was found that a glycoprotein termed P-glycoprotein, an efflux pump of antitumor agents, is an important component in rendering tumor cells resistant to various antitumor agents (for reviews see [4, 10, 11, 14]).

In an effort to discover antitumor agents that are effective against pleiotropic drug-resistant tumor cells, we found that a benzophenazine derivative, *N*-β-dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxybenzo[a] phenazine-6-carboxamide (NC-190) showed good therapeutic activity in vitro and in vivo against such tumor cells as well as drug-sensitive tumors. The compound has been reported to be more effective than Adriamycin (ADM) against i.p. inoculated L1210 leukemia, B16 melanoma, Lewis lung carcinoma, and M5076 reticulum cell sarcoma [7]. We found that NC-190 was effective against pleiotropic drug-resistant tumor cells, and we report the possible mechanism underlying the efficacy of this compound against resistant tumor cells.

Materials and methods

Drugs. NC-190 was provided by Taisho Pharmaceutical Co., Ltd. (Ohmiya, Saitama, Japan). All other antitumor agents were formulated for clinical use; ADM was obtained from Kyowa Hakko Co., Ltd. (Tokyo, Japan) and vincristine (VCR), from Shionogi Co., Ltd. (Osaka, Japan).

Tumor cells and culture. P388 leukemia cells were supplied by Simonsen Laboratories, Inc. (Gilroy, Calif), under the auspices of the National Cancer Institute (NIH, Bethesda, Md). P388 cells resistant to VCR (P388/VCR) and ADM (P388/ADM) were kindly supplied by the Mammalian Genetics and Animal Products Section, NCI, NIH. The human myelogenous leukemia K562 cell line was provided by Dr. Ezaki; sublines resistant to VCR (K562/VCR) and ADM (K562/ADM) were established in our laboratory [19, 20]. The human ovarian cancer line A2780 and its ADM-resistant subline (2780^{AD}) were provided by Dr. R. Ozols.

Abbreviations: NC-190, *N*-β-dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxy-benzo[a]phenazine-6-carboxamide; ADM, Adriamycin; VCR, vincristine; mAMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide

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Table 1. Cytotoxicity of NC-190 and ADM in human and mouse tumor cell lines sensitive and resistant to VCR, vinblastine, and ADM

Cell lines	IC_{50} (n*M*)[a]:			
	ADM		NC-90	
K562	11 ± 0.4		55 ± 10	
K562/VCR	350 ± 17	(32)[b]	180 ± 17	(3)
K562/ADM	3,100 ± 240	(282)	175 ± 9	(3)
CCRF-CEM	32 ± 4		209 ± 28	
CEM-VLB_{100}	224 ± 5	(7)	169 ± 6	(0.8)
A2780	1.5 ± 0.4		32 ± 6	
2780^{AD}	607 ± 57	(405)	779 ± 210	(24)
P388	4.0 ± 0.1		17 ± 4	
P388/VCR	50 ± 3	(13)	23 ± 2	(1.4)
P388/ADM	1,390 ± 430	(350)	445 ± 46	(26)

All data represent the mean (± SD) of three determinations

[a] Tumor cells ($2-4 \times 10^4$) were seeded in plastic tubes or cluster dishes. Graded concentrations of drugs were added; after 72 h of continuous drug exposure, the tumor cells were counted and the IC_{50} was determined as described in Materials and methods

[b] Numbers in parentheses represent the degree of resistance (*x*-fold) as compared with that of the corresponding parent cell lines

Table 2. Antitumor activity of NC-190, ADM, and VCR against P388 leukemia resistant to ADM and VCR

Drugs	Dose (mg/kg)	P388/ADM[a]:		P388/VCR[a]:	
		MS (days)	ILS (%)	MS (days)	ILS (%)
Control	–	9.7 ± 0.8	–	11.8 ± 1.2	–
NC-190	3.12	12.2 ± 0.4	25	22.5 ± 7.6	90
	12.5	15.8 ± 3.8	63	25.3 ± 4.8	114
	50	17.3 ± 1.6	79	>31[b]	>162(4/6[c])
ADM	0.8	10.8 ± 0.2	11		
	1.6	11.1 ± 0.1	11		
	3.2	11.4 ± 0.2	12		
VCR	0.1			10.5 ± 0.8	– 12
	0.2			11.3 ± 1.4	– 4
	0.4			13.0 ± 0.6	10

All data represent the mean ± SD

MS, mean survival

[a] P388 leukemia cells resistant to ADM (P388/ADM) and VCR (P388/VCR) (10^6 cells/mouse) were implanted i. p. into female CD_2F_1 mice (6 mice/group) on day 0, and drugs were given i. p. on days 1–5

[b] Observation period was 31 days; 2 of 6 mice had tumor in ascites

[c] Tumor-free survivors on day 31

Medicine Branch, NCI, NIH [12, 21]. The acute lymphoblastic leukemia cell line (CCRF-CEM) and its vinblastine-resistant subline (CEM-VLB_{100}) were provided by Dr. W. Beck, St. Jude Children's Hospital [1].

Drug treatment. For the drug treatment experiments, tumor cells (2×10^4 for P388, P388/VCR, and P388/ADM cells and 4×10^4 for K562, K562/ADM, K562/VCR, A2780, 2780^{AD}, CCRF-CEM, and CEM-VLB_{100} cells) were cultured at 37° C for 24 h in Corning 6-well tissue-culture clusters (for A2780 and 2780^{AD}, which grow on the surface of the dish) or for 5 h in Falcon 2054 culture tubes (for other cell lines, which grow in suspension) containing 2 ml growth medium (RPMI 1640 medium containing 5% fetal bovine serum and 100 µg/ml kanamycin) in a humidified atmosphere comprising 5% CO_2/95% air. The cells were then treated with graded drug concentrations, reincubated for 72 h in the presence of drugs, and counted with a model ZBI Coulter counter as previously described [15, 16]. Three samples were used for each drug concentration. In the control cultures, tumor cells grew exponentially during the incubation period. When the effect of verapamil was examined, the drug (final concentration, 3 and 10 µ*M*) was added before antitumor agents to the culture of K562/ADM and the cells were counted as above. The median concentration of drug necessary to inhibit the growth of tumor cells by 50% (IC_{50}) was determined by plotting the logarithm of the drug concentration vs the growth rate (percentage of control) of the treated cells.

Evaluation of antitumor activity. For evaluation of antitumor activity, 0.1 ml diluted ascites fluid containing 10^6 P388, P388/VCR, or P388/ADM cells were transplanted i. p. into CD_2F_1 mice [16, 18]. Drugs were dissolved in 0.9% NaCl solution and injected i. p. on days 1–5 after tumor inoculation. Six mice were used for each experimental group. Antitumor activity was evaluated by the mean survival (MS) of a group of mice and was also expressed by the increase in life span (ILS) in percent [22].

Cellular uptake of NC-190. Cellular uptake of the drug was measured in growth medium. K562/ADM cells (2×10^6) in Falcon 2054 culture tubes containing 1 ml growth medium with 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer were incubated with NC-190 tagged with carbon 14 ([^{14}C]-NC-190) (150 n*M*; sp. act., 5.2 mCi/mmol; provided by Taisho Pharmaceutical Co., Ltd.). After incubation at 37° C for 1 h, the intracellular NC-190 uptake was determined as previously described [17].

Binding inhibition assay of [^{3}H]-VCR to the plasma membrane. Inhibition of binding of [^{3}H]-VCR to a membrane preparation was measured by filtration methods as previously described [5, 6]. Plasma membrane prepared from K562/ADM cells (50 µg protein) was incubated at 25° C with 0.5 µM [^{3}H]-VCR and 3 m*M* adenosine triphosphate (ATP) in 10 m*M* TRIS-HCl (pH 7.4), 250 m*M* sucrose, and 5 m*M* $MgCl_2$ in a total volume of 50 µl. After 10 min, the reaction was stopped by the addition of 4 ml ice-cold buffer. Samples were collected by filtration on membrane filter (Millipore MF-membrane; pore size, 0.22 µm) pretreated with 3% bovine serum albumin solution and were then washed with another 4 ml ice-cold buffer. By this method, about 60% of membrane proteins were recovered on the filter. The filters were dried and radioactivity on each filter was measured.

Assay of DNA topoisomerase II. The standard topoisomerase II reaction mixture (20 µl) contained 50 m*M* TRIS-HCl (pH 7.9), 120 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 0.5 m*M* ethylene diamine tetraacetate (EDTA), 30 µg/ml bovine serum albumin, 0.5 m*M* ATP, 50 ng P4 knotted DNA and DNA topoisomerase II that had been partially purified from human myelogenous leukemia K562 [3]. After incubation at 30° C for 30 min, the reaction was terminated by the addition of 4 µl 10% (w/v) sodium dodecyl sulfate, 0.2% bromophenol blue. Reactions were loaded in 0.7% horizontal agarose gels and electrophoresed for 3 h at 6 V/cm, stained with 10 µg/ml ethidium bromide, destained in water, and photographed under UV illumination [13].

Results

Growth-inhibitory effect of NC-190 and ADM on sensitive and pleiotropic drug-resistant mouse and human tumor lines

The cytotoxicity of NC-190 to drug-resistant tumor lines is of interest for the further development of this agent for clinical use. K562/VCR, K562/ADM, CEM-VLB_{100}, 2780^{AD}, P388/VCR, and P388/ADM cells showed 32-, 282-, 7-, 405-, 13-, and 350-fold resistance to ADM, respectively, when the IC_{50} values of these drug-resistant

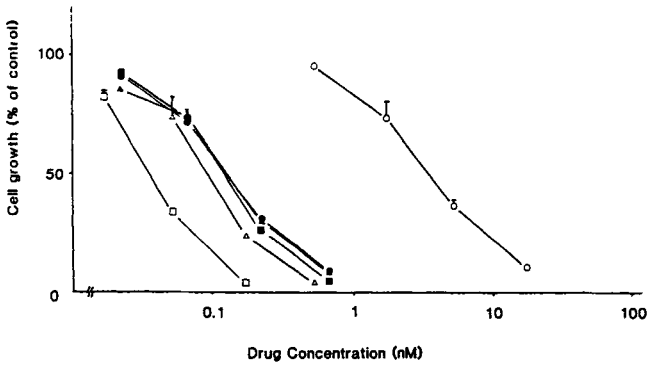

Fig. 1. Effect of verapamil on the cytotoxicity of ADM and NC-190 in multidrug-resistant K562 cells. The cells were incubated in the absence (○, ●) or presence of 3 μ*M* (△, ▲) or 10 μ*M* (□, ■) verapamil together with the indicated concentration of NC-190 (*closed symbols*) or ADM (*open symbols*). After 72 h, the cells were counted as described in Materials and methods

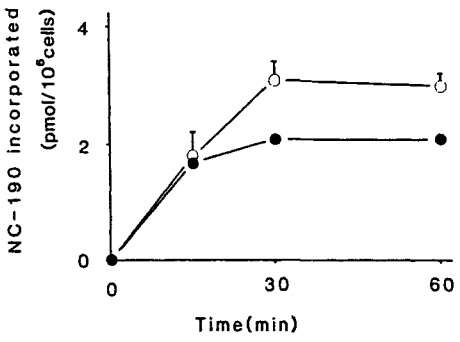

Fig. 2. Intracellular accumulation of NC-190. K562 (○) or K562/ADM (●) cells were incubated with 150 n*M* [^{14}C]-NC-190 for the time indicated and the radioactivities incorporated into the cells were counted as described in Materials and methods. Each point represents the mean of triplicate determinations; error bars represent the SD

sublines and their parent cell lines were compared (Table 1). These tumor lines showed only 3-, 3-, 0.8-, 24-, 1.4-, and 26-fold resistance to NC-190. NC-190 is more effective than ADM against various tumor cells, as described previously [7], and it is also more effective against pleiotropic drug-resistant mouse and human tumor lines. These observations suggest that NC-190 could be effective in vivo in animals and humans bearing pleiotropic drug-resistant tumors.

Chemotherapeutic effect of NC-190 in ADM- and VCR-resistant tumor-bearing mice

At doses ranging from 0.8 to 3.2 mg/kg, ADM given i.p. on days 1–5 after tumor inoculation showed marginal chemotherapeutic effect (ILS, <12%) in mice bearing P388 leukemia resistant to ADM (Table 2). NC-190 given on the same schedule resulted in 60%–80% ILS at doses of 12.5 and 50 mg/kg. An average of 2.0 g body weight loss was observed in mice given 50 mg/kg, which seems to be near the maximum tolerated dose of NC-190 on this schedule. Although these effects were not as strong as those previously observed in sensitive P388 leukemia-bearing mice [7], it is evident that NC-190 is effective against ADM-resistant tumor cells in vivo.

Similar results were also obtained with VCR in VCR-resistant P388 leukemia-bearing mice. At doses ranging from 0.1 to 0.4 mg/kg, VCR given i.p. on days 1–5 after tumor inoculation showed only marginal chemotherapeutic effect, if any, in mice bearing P388 leukemia resistant to VCR. NC-190 given at 50 mg/kg on the same schedule resulted in 4 of 6 mice being cured, as determined on day 31, when the experiment was terminated. The ILS of the two remaining tumor-bearing mice on day 31 was calculated to be >162%. Even at doses of 12.5 and 3.12 mg/kg, ILSs of 114% and 90%, respectively, were obtained. Thus, it is evident that NC-190 is effective against VCR-resistant tumor cells in vivo.

Effects of verapamil on the cytotoxicity of NC-190

Because NC-190 is effective against multidrug-resistant tumor cells, the possible interaction of this compound with P-glycoprotein is of interest. To investigate this, we examined the effect of verapamil on the cytotoxicity of NC-190 in multidrug-resistant tumor cells (Fig. 1). The ADM-resistant K562 (K562/ADM) subline used in this experiment showed an IC_{50} value of 3600 n*M*, which is approximately 300-fold higher than that obtained for K562 cells. NC-190 showed an IC_{50} value of 130 n*M*, which is only 3-fold higher than that obtained for K562 cells. Verapamil greatly enhanced the cytotoxicity of ADM in K562/ADM cells, and a 36- and 92-fold enhancement of ADM cytotoxicity occurred after incubation with 3 and 10 μ*M* verapamil, respectively, when the IC_{50} values of ADM were compared. However, at these concentrations verapamil did not enhance the cytotoxicity of NC-190 in K562/ADM cells. These results strongly suggest that NC-190 is not transported by P-glycoprotein.

Intracellular accumulation of NC-190 in K562 and K562/ADM cells

In the presence of 150 n*M* [^{14}C]-NC-190, the accumulation of NC-190 in K562 cells reached a plateau after incubation for 30 min at 37°C, and approximately 3.0 pmol NC-190 was incorporated into 10^6 cells (Fig. 2). The accumulation of NC-190 in K562/ADM cells was significantly lower than that in K562 cells; even so, 70% of that in K562 cells (2.1 pmol) was observed in K562/ADM cells. This efficient accumulation of NC-190 in K562/ADM cells was different from that previously observed with VCR or ADM in resistant cells [16, 17, 19, 20].

Table 3. Apparent inhibitory constants of antitumor agents on VCR binding to K562/ADM plasma membrane

Drugs	Ki^{app} (μM)[a]:
Vinblastine	0.08 ± 0.06
Actinomycin D	0.6 ± 0.4
ADM	14 ± 3
Colchicine	23 ± 9
NC-190	>400
Camptothecin	>400
5-Fluorouracil	>400

[a] Ki^{app} values were determined as described in Materials and methods

Inhibition of [^{3}H]-VCR binding to the plasma membrane of K562/ADM cells

We have previously described the ATP/Mg^{2+}-dependent, high-affinity binding of [^{3}H]-VCR to the plasma membrane of K562/ADM cells [5, 6]. This binding is closely associated with drug-transport mechanisms involving P-glycoprotein [5, 6]. Calcium channel blockers and other agents that are known to circumvent drug resistance could inhibit this [^{3}H]-VCR binding, as could unlabeled VCR, vinblastine, actinomycin D and ADM, to which K562/ADM cells exhibit cross-resistance. By using this system, the affinity of NC-190 for the plasma membrane was examined by determining the apparent inhibitory constant (Ki^{app}) of the compound (Table 3). The Ki^{app} values for vinblastine and ADM were 0.08 and 14 μ*M*, respectively, whereas the value for NC-190 was >400 μ*M*, indicating that the compound has very low affinity for the plasma membrane of K562/ADM cells and, presumably, for P-glycoprotein. This observation also supports our finding that NC-190 is not transported by P-glycoprotein.

Inhibition of DNA topoisomerase II activity

The inhibitory effect of NC-190 on the strand-passing activity of DNA topoisomerase II was measured using P4 knotted DNA (Fig. 3). At 25 and 125 μ*M*, NC-190 obviously inhibited this activity. 4′-(9-Acridinylamino)-methanesulfon-*m*-anisidide (mAMSA), which is known to be an inhibitor of DNA topoisomerase II, also inhibited the reaction at 25 μM. These results clearly indicate that NC-190 is an inhibitor of DNA topoisomerase II.

Discussion

A benzophenazine derivative, NC-190, is a newly synthesized antitumor agent. Phenazines are produced by *Streptomyces griseoluteus* [9, 23], and some phenazine derivatives have been found to possess antitumor activity against sarcoma 180 [2]. We have found that benzophenazine derivatives are even more active as antitumor agents and that NC-190 is the most active of these against various experimental tumors including P388 leukemia, L1210 leukemia, and B16 melanoma [7]. NC-190 possessed equal or greater activity against P388 leukemia than ADM at doses ranging from 0.1 to 100 mg/kg [7]; the compound is obviously superior to ADM in therapeutic dose range and antitumor activity.

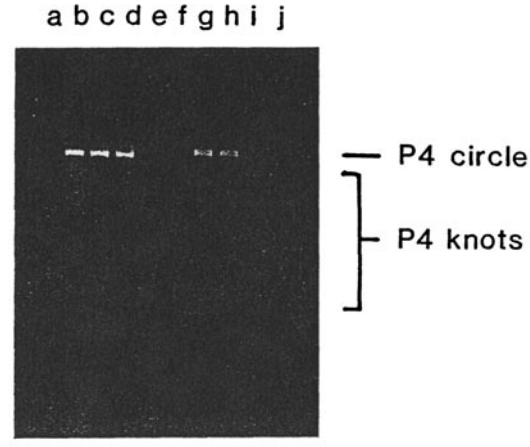

Fig. 3. Inhibition of strand-passing activity by NC-190. P4 knotted DNA and partially purified DNA topoisomerase II were incubated with or without NC-190 or mAMSA. *Lane a*, P4 knotted DNA control (no enzyme, no drug); *lane b*, no drug; *lanes c–f*, NC-190 (1, 5, 25, and 125 μ*M*, respectively); *lanes g–j*, mAMSA (1, 5, 25, and 125 μ*M*, respectively)

NC-190 was effective against multidrug-resistant human and mouse tumor cells; human K562 cells resistant to ADM showed only a 3-fold resistance to NC-190, whereas they showed a 280-fold resistance to ADM. The compound was also effective against P388 leukemia resistant to VCR and ADM in animals. This was especially evident in VCR-resistant P388 leukemia-bearing mice, all of which survived for 31 days; moreover, four of six rodents were free of ascites tumor by that time. P388/VCR was 13-fold more resistant to ADM. Because the degree of resistance in patients would be expected to be several-fold, these observations suggest possible efficacy for NC-190 in patients refractory to ADM and VCR therapy.

The reasons for the activity of NC-190 against multidrug-resistant tumor cells are interesting. The compound seems to have rather weak affinity to the plasma membrane of K562/ADM, presumably due to P-glycoprotein in the membrane. Verapamil did not potentiate the cytotoxicity of NC-190 to K562/ADM cells. These observations indicate that the compound could not be transported out of resistant cells by P-glycoprotein, which explains its effectiveness against multidrug-resistant tumor cells. However, the compound inhibited the activity of DNA topoisomerase II. As NC-190, a new type of antineoplastic agent, is an inhibitor of topoisomerase II, it could thus prove to be as effective as other inhibitors of topoisomerase II such as mAMSA [8] and etoposide [24] against multidrug-resistant tumor cells.

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