Jpn. J. Cancer Res. 83, 402–409, April 1992

Cell-killing Activity and Kinetic Analysis of a Novel Antitumor Compound NC-190, a Benzo[*a*]phenazine Derivative

Shiro Nakaike,[1,3] Takehiro Yamagishi,[1] Kayoko Nanaumi,[1] Susumu Otomo[1] and Shigeru Tsukagoshi[2]

[1]*Research Center, Taisho Pharmaceutical Co., Ltd., 1-403, Yoshino-cho, Ohmiya-shi, Saitama 330 and* [2]*Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 1-37-1, Kami-Ikebukuro, Toshima-ku, Tokyo 170*

A novel antitumor compound, *N*-β-dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxybenzo[*a*]-phenazine-6-carboxamide sodium salt (NC-190), was evaluated for antitumor activity *in vitro* against cultured tumor cell lines, and the kinetics of cell killing was elucidated. NC-190 strongly inhibited the growth of all of 3 murine tumor cell lines, 7 human tumor cell lines and 2 normal cell lines. With continuous exposure, the 50% inhibition concentrations were in the range of 0.005–0.06 μg/ml, except for KATO-III (2.15 μg/ml). By colony-forming assay, concentrations of NC-190 giving 90% cell kill (IC_{90}) at various exposure times were obtained with HeLa S3 cells. The plot of IC_{90}-exposure time on a log-log scale was linear for NC-190 with a slope of −1, which is typical for cell cycle phase-nonspecific agents. A 2 h treatment with NC-190 induced a rapid reduction in cell viability at doses of more than 3 μg/ml. At the dose where colony formation was completely inhibited, cell viability was persistently reduced to below 20% during the cell culture period. NC-190 cauced a dose- and time-dependent reduction in DNA synthesis. The inhibitions of RNA and protein synthesis were less than that of DNA synthesis. Spectroscopic studies of NC-190 mixed with calf thymus DNA demonstrated that NC-190 was capable of interacting with DNA. However, DNA thermal denaturation studies suggested that intercalation of NC-190 was weak in comparison with those of classical intercalating drugs.

Key words: NC-190 — Benzo[*a*]phenazine derivative — Antitumor activity — Cultured tumor cell — Kinetic analysis

N-β-Dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxybenzo[*a*]phenazine-6-carboxamide sodium salt (NC-190) is a unique compound with antitumor activity against animal tumors.[1] It has been shown that NC-190 is active in a number of *in vivo* tumor systems, including P388 and L1210 leukemias, B16 melanoma, Lewis lung carcinoma, sarcoma 180, M5076 reticulum cell sarcoma, Yoshida ascites hepatoma, and AH130 ascites hepatoma. Further studies have shown that NC-190 is active against adriamycin- and vincristine-resistant P388 leukemia (*in vitro* and *in vivo*), is hardly transported outside the resistant cells by P-glycoprotein, and inhibits DNA topoisomerase II activity of the tumor cells.[2] In the present paper, we report the growth-inhibitory and cell-killing effects of NC-190 in a variety of *in vitro* systems using cultured murine and human cells, which are associated with the inhibition of DNA synthesis. Furthermore, we conducted an analysis of the relationship between drug concentration and exposure time for 90% cell killing effect in order to determine the type of cell killing action of NC-190: that of cell cycle phase-nonspecific agents such as ACNU and mitomycin C,[3] or that of cell cycle phase-specific agents such as antimetabolites and Vinca alkaloids.[4] Based on the observation that NC-190 inhibited DNA synthesis, we have used spectroscopic studies to examine the potential interaction of the drug with DNA.

MATERIALS AND METHODS

Drugs NC-190 (molecular weight 456.4) was synthesized in our laboratory. Mitomycin C (MMC)[4] and adriamycin (ADM), formulated for clinical use, were obtained from Kyowa Hakko Kogyo Co., Ltd., Tokyo. 5-Fluorouracil (5-FU), ethidium bromide (EB) and calf thymus DNA were purchased from Sigma Chemical Co., St. Louis, MO. Drugs were dissolved in a culture medium or an appropriate buffer just before use. Calf thymus DNA was dissolved overnight at 4°C. [*Methyl*-^{3}H]-thymidine (5 Ci/mmol), [5-^{3}H]uridine (5 Ci/mmol), and L-[4,5-^{3}H]leucine (62 Ci/mmol) were purchased from Amersham International, plc (Amersham, UK).

[3] To whom all correspondence should be addressed.

[4] Abbreviations: MMC, mitomycin C; ADM, adriamycin; 5-FU, 5-fluorouracil; EB, ethidium bromide; IC_{50}, concentration necessary for 50% inhibition of cell growth; IC_{90}, concentration necessary for 90% cell kill; AUC, the area under the drug concentration-time curve; HPLC, high-performance liquid chromatography; SSC, standard saline citrate

Cell lines Three murine tumor cell lines, 7 human tumor cell lines, and 2 normal tissue cell lines were used. The cell lines were: L1210 leukemia (L1210), P388 leukemia (P388) and YAC-1 leukemia (YAC-1) established from murine tumors; KB oral epidermoid carcinoma (KB), HeLa S3 cervical carcinoma (HeLa), K562 chronic myelogenous leukemia (K562), KATO-III gastric carcinoma (KATO-III), LoVo colon adenocarcinoma (LoVo), MOLT-3 acute lymphoblastic leukemia (MOLT-3) and Raji Burkitt lymphoma (Raji) established from human tumors; and Chang liver and Chinese hamster ovary (CHO) established from normal tissue cells. L1210, P388, YAC-1, K562, KATO-III, MOLT-3, and Raji cells were cultured in RPMI-1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories, UK), 100 units/ml of penicillin and 100 μg/ml of streptomycin. KB, HeLa, and Chang liver cells were cultured in Eagle's minimum essential medium (GIBCO) supplemented with 10% fetal calf serum and 80 μg/ml of gentamycin. LoVo and CHO cells were cultured in Ham's F12 medium (GIBCO) supplemented with 10% fetal calf serum and 80 μg/ml of gentamycin. The cells were cultured in a growth medium at 37°C under a humidified atmosphere in a 5% CO_2 incubator. Experiments were performed with cells in the logarithmic phase of growth.

Determination of decay of NC-190 during the incubation in the culture medium To determine the decomposition of NC-190 in cell culture medium during the incubation period, the concentration of drug was measured by high-performance liquid chromatography (HPLC). HeLa cells (1×10^4 cells/ml) were cultured in 75 cm^2 tissue flasks at 37°C. After 24 h, a freshly prepared solution of NC-190 was added, giving a final concentration of 1 μg/ml. Immediately after addition, and at various times thereafter, aliquots (1 ml) were taken from the drug-containing medium. The concentrations of NC-190 were determined by HPLC. The HPLC conditions were: column, TSK-gel ODS 80TM (15 cm $\times$4.6 mm, Tosoh, Tokyo); mobile phase, acetonitrile-water-phosphoric acid (55:45:0.1) containing 10 m*M* sodium dodecylsulfate (SDS); flow rate, 1.0 ml/min; indicator wavelength, 290 nm.

Quantitative measurements of antiproliferative activity of NC-190 The cells were suspended in fresh growth medium at 2×10^4 cells/well in 6-well plates (Falcon Plastics, Oxford, CA) and incubated for 24 h at 37°C in a CO_2 incubator. The cells were then exposed to drugs for various periods of time, as described in "Results." In the case of time-response experiments using KB cells, 24-h-preincubated cells were exposed to NC-190 for various periods of time, then were transferred to fresh medium, and incubated further for a total of 96 h. The number of cells was counted with a ZM Coulter Counter (Coulter Electronics Ltd., UK). The IC_{50} (μg/ml) value was defined as that concentration of drug which achieved 50% reduction of growth in drug-treated cells, with respect to the controls. IC_{50} was calculated using the probit test. Viability of the cells was determined by trypan blue exclusion.

Colony-forming inhibition assay HeLa cells from an exponentially growing population were seeded into 35 mm plastic dishes and incubated in a CO_2 incubator. The number of cells seeded was adjusted so that the number of surviving cells was similar in each dish. After an attachment period of 4 h, the cells were treated with increasing concentrations of NC-190 for various periods of time. Following treatment, the drug was removed and fresh medium was added to the cultures. Further incubation was carried out for 11 days in the CO_2 incubator at 37°C. The cells were then fixed in formalin and stained with crystal violet before counting of the colonies. A survivor was defined as a cell giving rise to a colony containing more than 40 cells. The surviving fraction was calculated by dividing the colony number of the cells exposed to drug by that of the control. The IC_{90} value at each exposure time was determined using the probit test.

Effect on the incorporation of labeled precursors into nucleic acids and proteins For the dose-response studies, HeLa cells were cultured in 96-well microtiter plates (Falcon) at a cell concentration of 2×10^4 cells/well in 100 μl of medium. Following 24 h incubation to allow attachment of the cells, specified concentrations of NC-190 were added. To determine the macromolecular synthesis at 24 h after the drug addition, 20 μl of [^{3}H]-thymidine (50 μCi/ml), [^{3}H]uridine (100 μCi/ml), or [^{3}H]leucine (100 μCi/ml) was added to the appropriate wells 23.5 h after the drug addition, and incubations were continued for 1 h. The radioactivity incorporated into the trichloroacetic acid-insoluble fraction was counted in a liquid scintillation counter. For the time-response studies, 1 μg/ml of NC-190 was added after 24 h incubation of HeLa cells, and macromolecular sysnthesis was determined at 1, 2, 4, and 8 h after the drug addition. Labeled precursors were added 0.5 h before each specified time and cells were harvested 1 h after the addition of precursors.

Spectroscopy Wavelength scanning and DNA thermal denaturation studies were carried out with a Shimadzu UV-2100 spectrophotometer equipped with a thermoelectric control unit. Changes in spectra of drugs were recorded at room temperature, using 0.04 *M* Tris-HCl buffer (pH 7.9) containing 5% dimethylformamide in a 1.0 cm quartz cuvette. To determine the binding parameters of NC-190 and other control drugs to calf thymus DNA, the absorbance data at 490 nm (NC-190 and EB) or 480 nm (ADM) were used to calculate the Scatchard

plot, the apparent binding constant, K, and the apparent number of binding sites, ν, according to a method employed by Zunino *et al.*[5]

The thermal transition temperature (Tm) of calf thymus DNA was obtained from absorbance changes at 260 nm of drug-DNA mixture in 0.1×SSC (standard saline citrate) buffer contained 15 mM NaCl and 1.5 mM sodium citrate, pH 6.4, with 1% dimethylsulfoxide. The temperature of the cuvette was programmed from 50°C to 95°C at 0.5°C/min.

RESULTS

Stability of NC-190 in the culture medium The concentration of NC-190 in the culture medium in the presence of HeLa cells after incubation at 37°C for various periods was measured by HPLC. Table I shows the degradation kinetics of NC-190 in the culture medium in the presence of HeLa S3 cells. NC-190 was stable, and residual NC-190 amounted to 91% of the initial level even after 72 h incubation at 37°C.

Growth-inhibitory effect of NC-190 The growth-inhibitory effect of NC-190 on 10 tumor cell lines and 2 normal cell lines was tested and compared with those of 5-FU, MMC and ADM. Drugs were added after 24 h of cell implantation. The drug exposure time of each cell line was changed according to the cell growth rate of each. L1210, P388 and YAC-1 cells were exposed to drug for 48 h, while KB, HeLa, K562, LoVo, Raji, Chang liver and CHO cells were exposed for 72 h, and MOLT-3 and KATO-III were exposed for 96 and 144 h, respectively. Table II shows the mean IC_{50} values of several experiments. NC-190 inhibited cell proliferation in a variety of cell lines, with IC_{50} values usually in the range of 0.005–0.06 μg/ml. The IC_{50} value of NC-190 for KATO-III was about 100-fold greater than those for other cell lines. No difference of the sensitivity to NC-190 was found between tumor cell lines and normal cell lines. In addition, the results showed that NC-190 was almost as active as MMC in inhibiting the cell proliferation.

Mode of growth-inhibitory action of NC-190 KB cells were treated *in vitro* with graded concentrations of NC-190 for various times, then transferred to fresh medium, and incubated further for a total of 96 h. The growth-inhibitory activity of NC-190 was minimum with a short exposure time, but increased as the exposure time in-

Table I. Stability of NC-190 in the Culture Medium

Hours after addition[a]	NC-190 in the medium (μg/ml)[b]
0	0.98±0.05[c]
1	1.00±0.03
2	0.96±0.03
4	0.95±0.03
8	0.89±0.02
24	0.94±0.03
48	0.92±0.04
72	0.91±0.02

a) NC-190 was added at a concentration of 1 μg/ml to the culture of HeLa cells and incubated at 37°C.
b) Its concentraion was determined by HPLC.
c) Each value represents the mean±SE of triplicane cultures.

Table II. Comparison of Growth Inhibition of NC-190 and Othe Antitumor Agents against Various Cell Lines *in vitro*

Cell line[a]	IC_{50} (μg/ml)[b]							
	NC-190		5-FU[b]		MMC		ADM	
L1210	0.015	(1)	0.39	(2)	0.33	(2)	0.026	(1)
P388	0.028	(3)	0.21	(5)	0.044	(3)	0.0061	(3)
YAC-1	0.0045	(2)	0.057	(2)			0.0094	(1)
KB	0.028	(6)	0.62	(9)	0.010	(7)	0.0034	(8)
HeLa	0.039	(3)	2.62	(2)	0.039	(2)	0.0043	(2)
K562	0.016	(2)	6.99	(2)	0.027	(3)	0.013	(2)
KATO-III	2.15	(2)	1.15	(3)	0.080	(3)	0.25	(2)
LoVo	0.064	(3)	0.22	(2)			0.013	(1)
MOLT-3	0.011	(3)	0.89	(1)	0.033	(1)	0.0029	(1)
Raji	0.0099	(3)	1.34	(1)	0.029	(1)	0.0023	(1)
Chang liver	0.041	(2)	0.74	(2)			0.014	(2)
CHO	0.0089	(3)	0.55	(2)	0.049	(2)	0.034	(2)

a) Cells were incubated with the drugs for the periods described in the "Results" at 37°C.
b) Each value represents the mean of the number of tests indicated in parentheses.
c) 5-FU, 5-fluorouracil; MMC, mitomycin C; ADM, adriamycin.

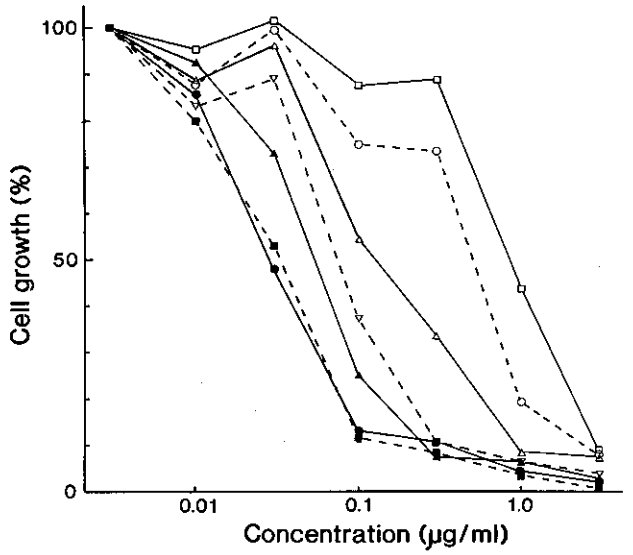

Fig. 1. Dose-response curves for NC-190 action on KB cell growth. Cells were incubated for 1 (□), 2 (○), 6 (△), 10 (▽), 24 (▲), 48 (■) and 72 h (●) with various concentrations of NC-190 at 37°C in 5% CO_2.

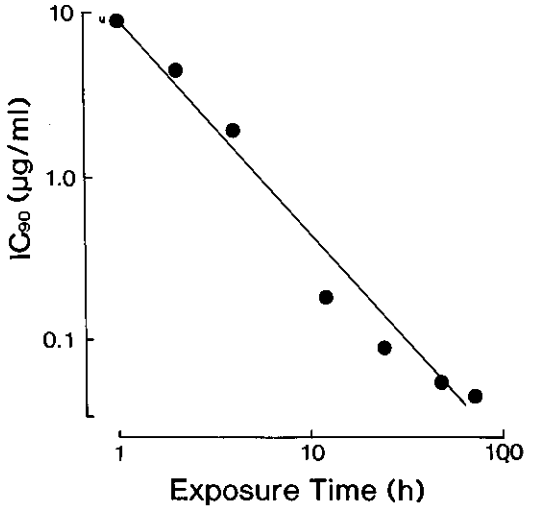

Fig. 3. Log-log relationship between IC_{90} value and exposure time for NC-190 against HeLa cells. IC_{90} values obtained from the concentration-survival curves for NC-190 in Fig. 2 were plotted against exposure times, each on a log scale.

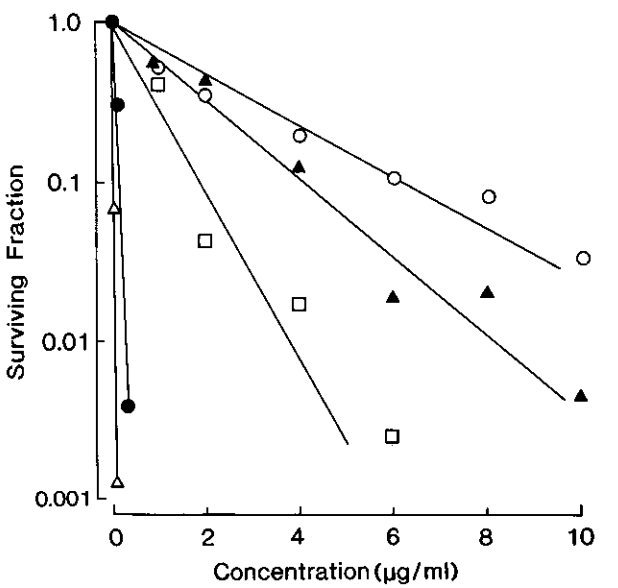

Fig. 2. Cytotoxic activity of NC-190 against HeLa cells as measured by monolayer clonogenic assay. Colony formation is shown as a function of NC-190 concentration following a 1 (○), 2 (▲), 4 (□), 12 (●) and 24 h (△) exposure to the drug.

creased. Further, NC-190 increased its activity as the concentration increased, indicating that NC-190 has both time- and concentration-dependent growth-inhibitory action on KB cells (Fig. 1). The IC_{50} values for 1, 2, 6, 10, 24, 48, and 72 h treatment were 0.749, 0.365, 0.155, 0.076, 0.061, 0.031, and 0.034 μg/ml, respectively.

Inhibition of colony formation An important issue that arises from the growth inhibition studies is whether NC-190 is cytostatic or cytotoxic. To obtain a quantitative estimate of the cytotoxic effects of NC-190, the effect of this compound on colony formation was determined. Fig. 2 shows the dose-response curves of the surviving fraction of HeLa cells treated with NC-190 for various periods. NC-190 had a profound effect on colony formation. After 24 h exposure, 0.3 μg/ml of NC-190 inhibited colony formation more than 99%, clearly demonstrating that exposure to NC-190 was cytotoxic.

The IC_{90} values for each exposure time were obtained and plotted on a log-log scale (IC_{90}-exposure time), as shown in Fig. 3. For NC-190, a long exposure time (i.e., 48 and 72 h) was approximately 100 times more effective than a short exposure time (i.e., 1 and 2 h) in inhibiting colony formation. IC_{90} values decreased with increasing exposure time, presenting a linear curve with a slope of -1, which is typical of cell cycle phase-nonspecific drugs[3,6] and implies an AUC (the area concentration-time curve)-dependent cell killing action of this drug against HeLa cells. The AUC was 5.17 μg·h/ml.

Changes in cell viability after 2 h treatment with NC-190 The ability of HeLa cells to exclude trypan blue as a function of NC-190 treatment was evaluated and compared with the clonogenicity. HeLa cells were exposed for 2 h to NC-190 at increasing concentrations, then

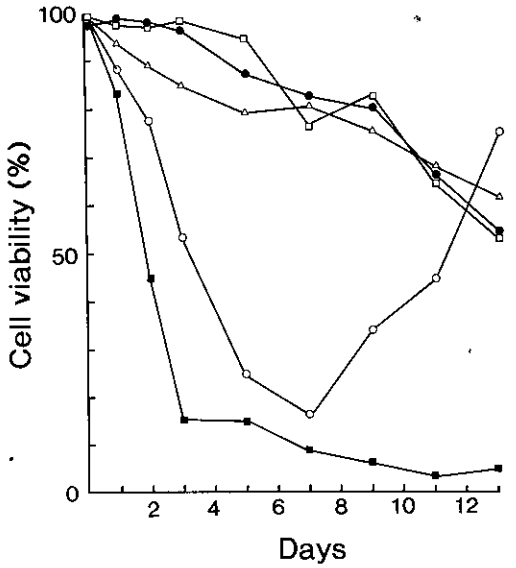

Fig. 4. Changes in cell viability, after 2 h treatment with NC-190. HeLa cells were exposed to 0 (□), 0.3 (●), 1 (△), 3 (○) and 10 μg/ml (■) of NC-190 for 2 h. Cell viability was determined by trypan blue exclusion.

transferred to fresh medium, and the percentage of cells which excluded trypan blue was measured for 13 days. The medium was not changed throughout the observation period. As shown in Fig. 4, there was a dose-dependent reduction in the proportion of viable cells as culture time increased. The viability of cells treated with 3 μg/ml of NC-190 was markedly reduced and reached the nadir of 16% viability by day 7, recovering to 75% by day 13. Cell kill increased more sharply in 10 μg/ml-treated cells, and had not recovered even by day 13, when the cell viability was 5%. The viability of control cells was gradually decreased from day 5 due to overgrowth and perhaps depletion of nutrients.

Inhibition of DNA, RNA, and protein syntheses The effect of NC-190 on the incorporation of ^{3}H-labeled precursors into DNA, RNA, and protein by HeLa cells is shown in Fig. 5. In a dose-response study, change was observed in the synthesis of macromolecules in cells exposed continuously for 24 h to various concentrations of NC-190. Pronounced DNA synthesis inhibition was observed from 0.1 μg/ml. Protein synthesis was also inhibited by NC-190 but to a lesser extent than DNA synthesis. RNA synthesis was not significantly inhibited.

The time-response relationship was established by exposing cells to 1 μg/ml of NC-190 for various times. Rapid inhibition of DNA synthesis was observed. NC-190 essentially shut down thymidine incorporation within 2 h, whereas uridine and leucine incorporations

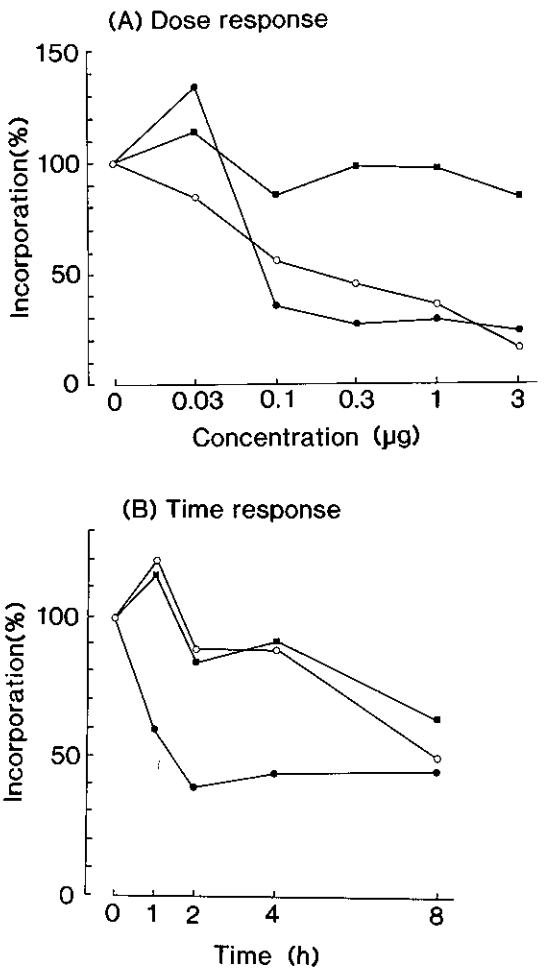

Fig. 5. Effect of NC-190 on the incorporation of labeled precursors into nucleic acids and proteins. A, Effect on the incorporation of [^{3}H]thymidine (●), [^{3}H]uridine (■) and [^{3}H]leucine (○) as a function of NC-190 concentration following 24 h exposure to drug. B, Effect on the incorporation of [^{3}H]thymidine (●), [^{3}H]uridine (■) and [^{3}H]leucine (○) as a function of the exposure time of cells to 1 μg/ml of NC-190.

were not inhibited until 8 h of incubation. These results indicate that NC-190 inhibits DNA synthesis after the drug addition, and that after long exposure, though protein synthesis inhibition occurs, RNA synthesis is less affected.

Interaction of NC-190 with DNA To check the DNA-interacting activity, 50 μ*M* (22.8 μg/ml) NC-190 was mixed with 0–2 m*M* calf thymus DNA and the spectrum of NC-190 was measured. The shifts in the spectra induced by the addition of DNA are shown in Fig. 6. The results demonstrate that calf thymus DNA can induce bathochromic shifts of the spectral peak and

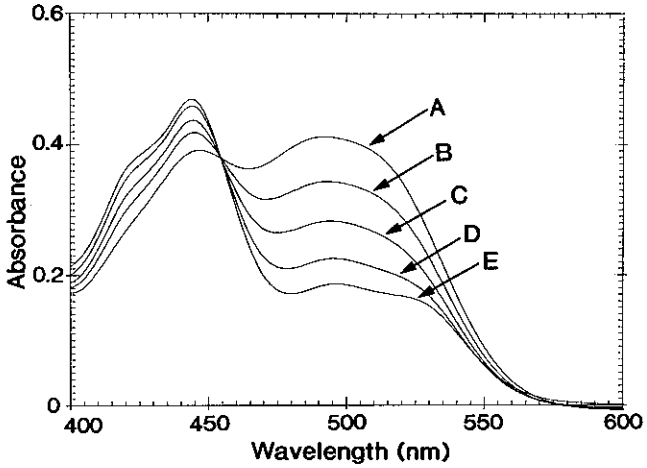

Fig. 6. Spectra of NC-190 at 50 μM in 0.04 M Tris-HCl buffer (pH 7.9) in the absence (A) or presence of 0.25 (B), 0.5 (C), 1 (D) and 2 (E) mM calf thymus DNA.

Table III. Binding Parameters of NC-190 with Calf Thymus DNA

Drug[a)]	K (M^{-1})	ν
NC-190	6.27×10^4	0.074
ADM	1.28×10^6	0.18
EB	1.03×10^6	0.21

a) ADM, adriamycin; EB, ethidium bromide.

Table IV. Effect of NC-190 on the Thermal Transition Temperature (*Tm*) of Calf Thymus DNA

Drug[a)]	Conc. (μM)	*Tm* (260 nm)	Δ *Tm* (260 nm)
None		67.5	—
NC-190	12.5	69.0	1.5
	25	68.7	1.2
	50	69.8	2.3
ADM	6.25	77.9	10.4
	12.5	81.3	13.8
	25	84.6	17.1
	50	88.4	20.9

a) ADM, adriamycin.

hypochromicity of NC-190. NC-190 also displayed an isosbestic point — a position where spectra cross consistently in the presence of increasing amounts of DNA; it was at approximately 450 nm. Changes in the absorbance at the spectral peaks of NC-190, ADM and EB were used to evaluate the binding parameters. Extrapolation of the Scatchard plot yields the apparent association constant, K, and the apparent number of binding sites per nucleotide, ν. The binding parameters are presented in Table III. This table summarizes the results of three different measurements. As a check of the method, the bindings of ADM and EB to DNA were measured. The results for ADM and EB-DNA interaction are in reasonable agreement with those reported elsewhere.[5,7] NC-190 showed apparent binding activity to DNA, but compared to ADM and EB, the affinity was weak and the number of binding sites per nucleotide was about one-third of those for ADM or EB.

The results mentioned above suggest that NC-190 may intercalate between base pairs in DNA. Therefore, the effect of NC-190 on the thermal transition of DNA was examined to check its ability to stabilize the double-helical structure of the macromolecule against thermal denaturation. The *Tm* of 0.1 mM calf thymus DNA in 0.1×SSC buffer containing 1% dimethylsulfoxide was 67.5°C. As shown in Table IV, ADM produced a marked elevation in the *Tm* of DNA dose-dependently. However, data in Table IV reveal that only small increases in *Tm* were found with NC-190.

DISCUSSION

As reported previously,[1] tumors sensitive to NC-190 *in vivo* include P388 leukemia and other ascites tumors, as well as subcutaneously implanted B16 melanoma and Lewis lung carcinoma. Activities in the latter system demonstrate that the compound is active when administered systemically against solid tumors.

The effect of NC-190 on cell proliferation and survival was determined by 2 different techniques. One approach based on cell counts measured the immediate effect of the drug on cell growth. The second technique, inhibition of colony formation, measured not only the immediate effect of the drug on cells but also the effect on the survival potential of the cells, since more than 4 consecutive doublings are required to form a macroscopically visible colony. In the cell growth assay, we selected 12 standard established cell lines of murine and human origin to check the inhibitory activity of NC-190. NC-190 showed growth inhibition against all tumor cells and normal cells tested *in vitro*. The IC_{50} values ranged from 0.005–0.06 μg/ml, except for KATO-III. The inhibition was both concentration- and time-dependent. At 2 h after the intravenous injection of 3 mg/kg to mice, the blood concentration of NC-190 was 0.14 μg/ml.[8] These results mean it is possible to get a sufficient concentration of NC-190 to inhibit tumor cell growth *in vivo*. The IC_{50} value of NC-190 for KATO-III cells was greater than those for other cell lines. KATO-III cells were also resistant to

adriamycin. KATO-III cells grow in aggregated clusters *in vitro*. Gupta[9] suggested that differences in the cytotoxicity of drugs between various cell lines were caused by differences in the cellular transport of drugs. Cellular transport in aggregated clusters might be one of the factors causing the drug sensitivity difference observed in this study. As NC-190 inhibited the growth of human stomach cancer xenografts, SC-2 and SC-9, transplanted into nude mice (unpublished results), the resistance of KATO-III to NC-190 seems to be cell line-specific but not stomach cancer-specific.

From the results of colony formation assay, NC-190 proved to be cytotoxic but not cytostatic. In order to correlate *in vitro* cytotoxicity quantitatively with *in vivo* antitumor activity and predict the therapeutic effectiveness of a drug from data on its *in vitro* cytotoxicity, it is important to analyze the relationship between the drug concentration and the exposure time necessary for a definite cell killing effect. Recent studies[3, 6, 10] on the kinetic analysis of drug effects *in vitro* have revealed that the cell-killing action of cell cycle phase-nonspecific agents such as MMC and cisplatin depends on the AUC. Accordingly, the plots for exposure time and corresponding IC_{90}'s of these drugs on a log scale were shown to be linear with a slope of -1 when they were stable in the culture medium. In contrast, the cell-killing action of cell cycle phase-specific agents such as antimetabolites and *Vinca* alkaloids is AUC-independent and shows a strong tendency toward time-dependence, presenting curves with a much steeper slope in the log-log graph for the exposure times and IC_{90}'s. As shown in Table I, NC-190 does not readily undergo degradation during exposure to cells in culture, and more than 90% of the initial concentration remained after 72 h incubation. Further, IC_{90} values of NC-190 for colony formation of HeLa S3 cells plotted on a log-log scale (IC_{90}-exposure time) showed a linear decrease with a slope of -1. From these results, we identified a cell cycle phase-nonspecific mode of action for NC-190. Therefore, it is concluded that NC-190 showed AUC-dependent cell-killing action.

In the colony formation test, values of the percent inhibition after 2 h treatment with 2, 4, 6, 8 and 10 μg/ml of NC-190 were 56, 88, 98, 98 and 99%, respectively. NC-190 at 3 μg/ml, which is about two-thirds of the IC_{90} value in the colony formation assay, reduced the cell viability by more than 80%, but the inhibition was not persistent and the cells had recovered from the damage by day 13 (Fig. 4). NC-190 at 10 μg/ml, which induced 99% inhibition in the colony forming assay, decreased the viability of cells by more than 90%, and the inhibition persisted throughout the observation period. These results indicate that more than 90% suppression of the cell viability throughout the incubation period is required to induce the complete inhibition of colony formation.

NC-190 also caused a dose- and time-dependent reduction in DNA synthesis, as measured by [^{3}H]thymidine incorporation. NC-190 at 0.1 μg/ml inhibited the DNA synthesis of HeLa cells by 67% after 24 h incubation. The IC_{50} value of NC-190 for KB cell growth with 24 h exposure was 0.061 μg/ml. HeLa cells showed almost the same sensitivity to NC-190 in cell growth assay (Table I). It seems reasonable that NC-190 inhibits DNA synthesis at a concentration comparable to that inhibiting *in vitro* cell growth.

NC-190 first inhibited DNA synthesis, and after a long exposure, protein synthesis was inhibited. The inhibition of RNA synthesis was less than that of protein synthesis even after 24 h exposure to NC-190. These results suggest that the primary inhibition of DNA synthesis by NC-190 is the cause of its antitumor activity. Tsuruo *et al.*[2] and Andoh *et al.*[11] reported that NC-190 inhibited the activity of DNA topoisomerase II. We are currently studying the mode of action of NC-190 in connection with enzymes related to the DNA synthesis pathway and cell cycle progression using flow cytometric analysis.

Because NC-190 inhibited DNA synthesis rather than RNA and protein syntheses, it appeared that NC-190 could function in a manner similar to that of antitumor drugs which interact directly with DNA, e.g., actinomycin D and adriamycin. This notion was also supported by the structure of NC-190, which is relatively flat and contains an aromatic moiety analogous to those of drugs which intercalate between base pairs in DNA. Therefore the potential interaction of NC-190 with DNA was examined by spectroscopic studies. Planar aromatic intercalating ligands are reported to display bathochromic shifts of spectral peaks in the presence of DNA.[12] The wavelength shifts are usually accompanied by a hypochromic effect on absorbance. That NC-190 can interact directly with DNA *in vitro* is clearly indicated by the results of the wavelength-scanning studies in which bathochromic shifts, a hypochromic effect and an isosbestic point were observed. However, the calculated binding parameters of NC-190 implied weaker binding as compared with classical intercalating agents such as ADM and EB. The finding that NC-190 elevated *Tm* of DNA very weakly means that the intercalating activity of this compound is weak and NC-190 does not stabilize the DNA double helix. As regards the binding of drugs to DNA, at least two modes of interaction can be distinguished; the "strongly" bound drugs are understood to be intercalated between base pairs of DNA, while the "weakly" bound drugs are thought to be attached to DNA by means of ionic interaction, probably with the phosphate backbone of nucleic acids.[5, 13, 14] ADM and other DNA-binding drugs are reported to compete for binding by either of the mechanisms. The results obtained in this report suggest that NC-190 may have a

"weak" binding mode of action predominantly, rather than "strong" binding. Taking into consideration the mode of binding of some intercalating drugs which structurally resemble NC-190,[13-15] it is suggested that the alkyl-amino residues of NC-190 may bind electrostatically to the outside of the double helix, while only a small portion of the aromatic system of the molecule intercalates between some specific base pairs. Detailed examinations such as DNA unwinding assays are in progress.

(Received October 30, 1991/Accepted January 28, 1992)

REFERENCES

1) Nakaike, S., Yamagishi, T., Samata, K., Nishida, K., Inazuki, K., Ichihara, T., Migita, Y., Otomo, S., Aihara, H. and Tsukagoshi, S. *In vivo* activity on murine tumors of a novel antitumor compound, *N*-β-dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxybenzo[a]phenazine-6-carboxamide sodium salt (NC-190). *Cancer Chemother. Pharmacol.*, **23**, 135–139 (1989).
2) Tsuruo, T., Naito, M., Takamori, R., Tsukahara, S., Yamabe-Mitsuhashi, J., Yamazaki, A., Oh-hara, T., Sudo, Y., Nakaike, S. and Yamagishi, T. A benzophenazine derivative, *N*-β-dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxybenzo[a]phenazine-6-carboxamide, as a new antitumor agent against multidrug-resistant and sensitive tumors. *Cancer Chemother. Pharmacol.*, **26**, 83–87 (1990).
3) Ozawa, S., Sugiyama, Y., Mitsuhashi, Y., Kobayashi, T. and Inaba, M. Cell killing action of cell cycle phase-nonspecific antitumor agents is dependent on concentration-time product. *Cancer Chemother. Pharmacol.*, **21**, 185–190 (1988).
4) Shimoyama, M. Cytocidal action of anticancer agents: evaluation of the sensitivity of cultured animal and human cancer cells. *In* "Comparative Leukemia Research 1973, Leukemogenesis," ed. Y. Ito and R. M. Dutcher, pp. 711–722 (1975). University of Tokyo Press, Tokyo.
5) Zunino, F., Gambetta, R., Di Marco, A. and Zaccara, A. Interaction of daunomycin and its derivatives with DNA. *Biochim. Biophys. Acta*, **277**, 489–498 (1972).
6) Ozawa, S., Sugiyama, Y., Mitsuhashi, J. and Inaba, M. Kinetic analysis of cell killing effect induced by cytosine arabinoside and cisplatin in relation to cell cycle phase specificity in human colon cancer and Chinese hamster cells. *Cancer Res.*, **49**, 3823–3828 (1989).
7) Gray, H. B., Jr., Upholt, W. B. and Vinograd, J. Buoyant method for the determination of the superhelix density of closed circular DNA. *J. Mol. Biol.*, **62**, 1–19 (1971).
8) Nakaike, S., Samata, K., Ichihara, T., Kohno, Y., Masuda, N., Otomo, S. and Tsukagoshi, S. Antitumor effect of NC-190. (4) Antitumor activity and tissue distribution in Lewis lung carcinoma bearing mice. *Proc. Jpn. Cancer Assoc., 47th Annu. Meet.*, 581 (1988).
9) Gupta, R. S. Species-specific differences in toxicity of antimitotic agents toward cultured mammalian cells. *J. Natl. Cancer Inst.*, **74**, 159–164 (1985).
10) Inaba, M., Mitsuhashi, J. and Ozawa, S. Kinetic analysis of 5-fluorouracil action against various cancer cells. *Jpn. J. Cancer Res.*, **81**, 1039–1044 (1990).
11) Andoh, T., Ikegami, Y., Ono, K., Ikeda, T., Yamagishi, T. and Nakaike, S. Inhibitory effects of the novel antitumor compound NC-190 on DNA topoisomerases. *Proc. Jpn. Cancer Assoc., 49th Annu. Meet.*, 410 (1990).
12) Porumb, H. The solution spectroscopy of drugs and the drug-nucleic acid interactions. *Prog. Biophys. Mol. Biol.*, **34**, 175–195 (1978).
13) Kapuscinski, J., Darzynkiewicz, Z., Traganos, F. and Melamed, M. R. Interactions of a new antitumor agent, 1,4-dihydroxy-5,8-bis[[(2-[(2-hydroxyethyl)amino]-ethyl]-amino]-9,10-anthracenedione, with nucleic acids. *Biochem. Pharmacol.*, **30**, 231–240 (1981).
14) Hartley, J. A., Reszka, K., Zuo, E. T., Wilson, D., Morgan, A. R. and Lown, J. W. Characteristics of the interaction of anthrapyrazole anticancer agents with deoxyribonucleic acids: structural requirements for DNA binding, interaction, and photosensitization. *Mol. Pharmacol.*, **33**, 265–271 (1988).
15) Double, J. C. and Brown, J. R. The interaction of amino-alkylaminoanthraquinones with deoxyribonucleic acid. *J. Pharm. Pharmacol.*, **27**, 502–507 (1975).