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A novel antitumor compound, NC-190, induces topoisomerase II-dependent DNA cleavage and DNA fragmentation

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Abstract A novel benzophenazine derivative, NC-190, is a potent antitumor compound. NC-190 has been shown to inhibit the DNA strand-passing activity of DNA topoisomerase II. We investigated further the mode of action of NC-190 against DNA topoisomerase II and DNA fragmentation. NC-190 inhibited the decatenation activity of purified topoisomerase II, but had only a weak inhibitory effect against topoisomerase I. A topoisomerase II-dependent DNA cleavage assay showed that NC-190 inhibited the enzyme activity by stabilizing a topoisomerase II–DNA cleavable complex. NC-190 induced growth inhibition, protein-linked DNA breaks, and DNA fragmentation in cultured HL-60 cells in a dose-dependent manner. These activities of NC-190 in HL-60 cells were comparable to those of etoposide (VP-16). These results demonstrate a good correlation among growth inhibition, topoisomerase II-dependent DNA cleavage, and DNA fragmentation induced by NC-190. A DNA unwinding assay showed that NC-190 had intercalating activity, but its activity appeared to be weaker than those of ethidium bromide and adriamycin. These results indicate that the mechanism by which NC-190 exhibits antitumor activity may be the inhibition of topoisomerase II.

Key words NC-190 · Topoisomerase II · Cleavable complex · DNA intercalation · Apoptosis

Introduction

NC-190, N-β-dimethylaminoethyl-9-carboxy-5-hydroxy-10-methoxy-benzo[*a*]phenazine-6-carboxamide sodium salt (molecular weight 456.4, Fig. 1) has strong antitumor activities in in vivo and in vitro tumor models [10, 11] and inhibits the strand-passing activity of topoisomerase II [17].

DNA topoisomerase II is a nuclear enzyme that alters DNA conformation by breaking and rejoining DNA strands, thereby playing an important role in many processes of DNA metabolism, including replication, transcription, recombination, and chromosome segregation at mitosis [19, 20]. Recent studies have demonstrated that topoisomerase II is the primary cellular target for a number of clinically important antitumor agents, such as etoposide (VP-16), adriamycin (ADM), mitoxantrone, amsacrine, and ellipticine [7, 14]. These agents trap topoisomerase II in an intermediary covalent complex with DNA, termed the 'cleavable complex', which can be detected as DNA double-strand breaks upon protein denaturant treatment. Recent results have demonstrated that apoptosis may be involved in cell death induced by various antitumor agents, including topoisomerase II inhibitors [5, 18].

In the study reported here, we investigated further the mode of action of NC-190 against DNA topoisomerase II and DNA fragmentation.

Materials and methods

Drugs

NC-190 was synthesized in our laboratory. Etoposide (VP-16,4′-demethylepipodphyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside) was purchased from Sigma and Nippon Kayaku Co. ADM, ethidium bromide (EB) and camptothecin were purchased from Sigma. [Methyl-^{3}H] thymidine and [α-^{32}P] ATP were obtained from Amersham International. The drugs were dissolved in dimethyl sulfoxide (DMSO).

Topoisomerases purification

Topoisomerase I was purified form mouse Ehrlich ascites tumor cells according to the method described by Ishii et al. [4].

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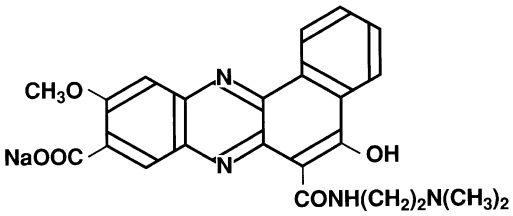

Fig. 1 Chemical structure of NC-190

Topoisomerase I-mediated DNA cleavage was measured, as described by Yamashita et al. [21]. One unit of activity was the amount of topoisomerase I that relaxed 0.2 μg of supercoiled pBR322 DNA.

Topoisomerase II was isolated from mouse Ehrlich ascites tumor cells and partially purified by hydroxylapatite and P-11 phosphocellulose column chromatography [9]. DNA topoisomerase II activity was determined by decatenation of catenated kinetoplast DNA (k-DNA) of trypanosomes [13]. One unit of activity was the amount of topoisomerase II that decatenated 0.12 μg of k-DNA.

Topoisomerase II-dependent DNA cleavage assay

An assay of the DNA–topoisomerase II cleavable complex formation was performed as described by Liu et al. [8]. Linear 3′-^{32}P-end-labeled pBR322 DNA (10 ng) was incubated at 37°C for 30 min with topoisomerase II in the presence or absence of drugs in 50 μl of reaction buffer. The reaction was stopped by the addition of SDS and EDTA. The cleavable complex was precipitated by KC1, washed and solubilized in water, and analyzed for radioactivity in a liquid scintillation spectrometer.

Cell culture and MTT assay

HL-60 human leukemia cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2/95% air.

The antiproliferative activity of NC-190 was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [1]. Exponentially growing HL-60 cells (2×10^3 cells/100 μl) were transferred to a 96-well flat-bottomed microtiter plate (Nunc) and incubated for 24 h. The cells were treated with graded concentrations of drugs for 72 h. After the treatment, 50 μl of 1 mg/ml solution of MTT in RPMI was added to each of the culture wells. After 4 h the plates were centrifuged at low speed for 5 min, the fluid content of each well was removed, and 100 μl DMSO added. The purple formazan product was solubilized by gently mixing the plate, and absorbances were measured at 540 nm using an automatic microspectrophotometer (Inter Med Immunoreader, NJ-2000). Background absorbance was subtracted from each well, and the percentage of control absorbance was considered to be the surviving fraction of cells. The 50% growth-inhibitory concentration (IC_{50}) was calculated using the probit test.

Cleavable complex formation in HL-60 cells

The method of Rowe et al. [12] was used. The DNA in logarithmically growing HL-60 cells (2×10^5 cells/ml) was labeled by adding [methyl-^{3}H] thymidine (specific activity, 44 Ci/mmol) to the medium, to reach a final concentration of 0.7 μCi/ml. After overnight incubation, cells were pelleted at 1000 rpm for 6 min in a Beckman centrifuge. The supernatant was removed and the cells were washed three times in phosphate-buffered saline and resuspended in fresh medium to reach a final concentration of 10^5 cells/ml. The cells were transferred in 1-ml aliquots into a 24-well microtiter plate (Inter Med), and incubated for another 2 h at 37°C. The cells were then treated with various concentrations of drugs for 2 h. The microtiter plate was centrifuged in a Beckman J-6M centrifuge at 2500 rpm for 3 min at room temperature. The medium was removed from each well and the cells were lysed by adding 1 ml of a prewarmed (65°C) lysis solution (1.25% SDS, 5 m*M* EDTA, pH 8.0, and 0.4 mg/ml salmon sperm DNA). The lysate was transferred to a 1.5-ml Eppendorf tube containing 250 μl 325 m*M* KCl. After vigorous mixing at the highest setting for 10 s, the sample was cooled on ice for 10 min and centrifuged at 15 000 rpm for 10 min at 4°C in a TOMY MRX-151 centrifuge. The pellet was resuspended in 1 ml of a wash solution (10 m*M* Tris-HCl, pH 8, 100 m*M* KCl, 1 m*M* EDTA, 0.1 mg/ml salmon sperm DNA) and maintained at 65°C for 10 min with occasional mixing. The suspension was cooled on ice for 10 min and recentrifuged. The pellet was washed again before resuspending in 200 μl water at 65°C. The suspension was then combined with 5 ml scintillation liquid and the radioactivity was determined.

Analysis of DNA fragmentation in HL-60 cells

DNA fragmentation of drug-treated HL-60 cells was analyzed by gel electrophoresis, as described by Sorenson et al. [15]. Briefly, each pellet of 10^6 HL-60 cells was suspended in 15 μl sample buffer (10 mg ribonuclease/ml, 15% Ficoll 70, 0.01% bromophenol blue in TBE). Next, the suspended pellet was transferred to a well of 2% agarose gel, lyzed and digested with proteinase K. Then the DNA was separated by electrophoresis. RNA was removed subsequently from the gel by incubation with ribonuclease A, and the DNA was visualized with EB.

DNA fragmentation was quantified using the thymidine release assay as described by Kolber and Hill [6]. In brief, HL-60 cells were labeled with [^{3}H] thymidine. After drug treatment, the amounts of thymidine released from the experimental samples were determined by adding Triton X-100 to each sample (final concentration, 0.04%), mixing, centrifuging for 5 min at 700 *g*, and recovering 100 μl supernatant for counting in a β-scintillation counter. Corrected DNA fragmentation was expressed as the amount of thymidine released as a percentage of total radioactivity.

DNA unwinding measurements

DNA unwinding activities of the drugs were determined using the method reported by Imamura et al. [3]. Briefly, treatment of closed-circular pBR322 DNA with topoisomerase I was carried out in 100 μl reaction mixture containing 140 m*M* sodium phosphate (pH 7.5), 6 m*M* Tris-HCl, 6 m*M* NaCl, 0.6 m*M* Na_2EDTA, 44 m*M* sucrose, an appropriate amount of DNA topoisomerase I, 2.9 μg of pBR322 DNA, NC-190 (5–50 μM), ADM (0.5–6 μM) or EB (0.25–3 μM). After incubation at 25°C for 30 min, the reaction mixture was extracted twice with phenol and once with chloroform. The aqueous phase was recovered, mixed with 20 μl Tris-buffer (40 m*M* Tris-HCl, 0.4 m*M* Na_2EDTA, 0.4 *M* NaCl, 20% surcose, 2% SDS, pH 8.0) and incubated at 45°C for 15 min. Samples were electrophoresed through a 1.4% agarose gel in 36 m*M* Tris-HCl (pH 7.8), 30 m*M* NaH_2PO_4, 1 m*M* Na_2 EDTA, stained with EB and photographed.

Results

Effects of NC-190 on the activities of topoisomerases

To determine the topoisomerase II inhibitory activity of NC-190, we used decatenation of k-DNA. Figure 2

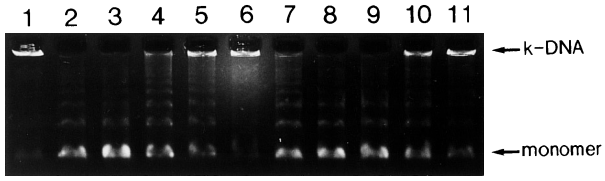

Fig. 2 Inhibition of topoisomerase II decatenation by NC-190. The decatenation assay was done in the presence of 2 units of topoisomerase II, as described in Materials and methods (*lane* 1 control kinetoplast DNA (k-DNA) 120 ng; *lane 2* control topoisomerase II; *lanes 3–6* same as lane 2, with 0.5, 2, 8, and 32 μM NC-190, respectively; *lanes 7–11* same as lane 2 with 0.5, 2, 8, 32 and 64 μM etoposide respectively)

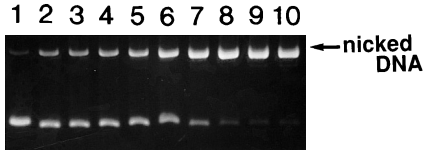

Fig. 3 DNA topoisomerase I-mediated DNA cleavage activities of NC-190. Supercoiled pBR322 DNA (0.2 μg) was incubated with 25 units topoisomerase I in the presence of drugs (*lanes* 3–10) followed by SDS/proteinase K treatment and then analyzed on an agarose gel containing 0.5 μg/ml EB (*lane 1* control pBR322 DNA; *lane 2* control topoisomerase I; *lanes 3–6* same as lane 2 with 0.5, 2, 8, and 32 μM NC-190, respectively; *lanes 7–10* same as lane 2 with 0.5, 2, 8, and 32 μM camptothecin, respectively)

shows that NC-190 inhibited topoisomerase II catalytic activity. Inhibition of decatenation was complete at 32 μM (Fig. 2, lane 6) and only partial at 2 μM (Fig. 2, lane 4). Etoposide also inhibited the decatenation activity of topoisomerase II, but the potency of etoposide in topoisomerase II inhibition was less than that of NC-190 (Fig. 2, lanes 7–11).

The effect of NC-190 on topoisomerase I activity was determined by a topoisomerase I-mediated DNA cleavage reaction, because a high concentration of NC-190 purturbs the electrophoretic patterns of pBR322 DNA due to its DNA binding activity. Topoisomerase I-mediated DNA cleavage was only slightly increased by the addition of NC-190 in the concentration range 0.5–32 μM (Fig. 3, lanes 3–6). Camptothecin, used as a positive control, induced complete DNA cleavage at concentrations above 2 μM (Fig. 3, lanes 7–10).

Topoisomerase II-dependent DNA cleavage activity

Most topoisomerase II inhibitory antitumor agents stimulate the formation of a cleavable complex between DNA and topoisomerase II [7, 14]. The effects of NC-190 and etoposide on topoisomerase II-mediated DNA cleavage were studied in vitro by reacting topoisomerase II with a ^{32}P-labeled pBR323 DNA fragment. The addition of NC-190 in the concentration range 0.3–30 μM induced topoisomerase II-mediated DNA

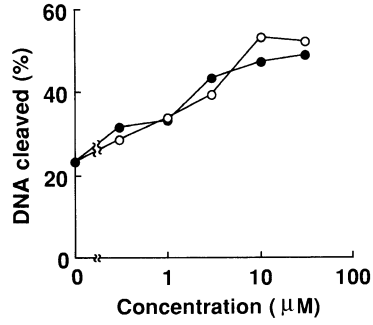

Fig. 4 Effects of NC-190 (●) and etoposide (○) on the formation of the topoisomerase II–DNA cleavable complex. Linear 3′-[^{32}P]-end-labeled pBR322 DNA (10 ng) was incubated at 37°C for 30 min with topoisomerase II with increasing concentrations of drugs, and the covalently linked topoisomerase II–DNA complex was precipitated with SDS-potassium as described in Materials and methods

cleavage in a concentration-dependent manner (Fig. 4). Etoposide also stimulated topoisomerase II-mediated DNA cleavage. The potency of NC-190 in inducing DNA cleavage was comparable to that of etoposide.

To determine the stability of the NC-190-induced cleavable complex, we tested the heat reversibility of topoisomerase II-mediated DNA cleavage activity induced by NC-190 and etoposide. Exposure of incubated reaction mixture with NC-190 and etoposide to 65°C for 15 min prior to the addition of SDS and proteinase K suppressed the DNA cleavage (data not shown). These results suggest that the mechanism of the topoisomerase II-dependent DNA cleavage induced by NC-190 is through the formation of the cleavable complex, as seen with etoposide, and the stability of the complex appears to be similar to that of etoposide.

Growth inhibitory activity of NC-190 in HL-60 cells

Exponentially growing HL-60 cells were treated with NC-190 or etoposide for 72 h. Both drugs inhibited the growth of HL-60 cells. The IC_{50} values of NC-190 and etoposide were 0.025 μg/ml (0.054 μM, $n = 3$) and 0.024 μg/ml (0.040 μM, $n = 3$), respectively (Fig. 5). The inhibitory activity of NC-190 against the growth of HL-60 cells is therefore comparable to that of etoposide.

Effect of NC-190 against cellular topoisomerase II–DNA, cleavable complex formation in HL-60 cells

The effect of NC-190 and etoposide on the formation of a cleavable complex in HL-60 cells was also determined using the K-SDS assay [12]. HL-60 cells were labeled with [^{3}H] thymidine, and treated with the drugs for 2 h. After drug treatment, the cells were lyzed, and the amount of protein–DNA complex was determined. Fig. 6 shows that NC-190 increased the amount of

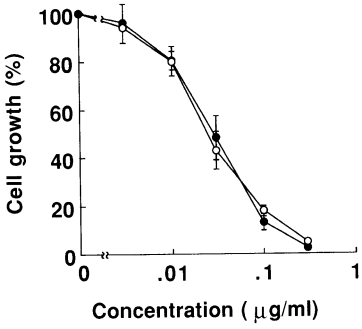

Fig. 5 Growth inhibitory activity of NC-190 against HL-60 cells. The antiproliferative activities of NC-190 (●) and etoposide (○) were determined using the MTT assay for 72 h of incubation. Each point represents the mean value ± SE from three experiments

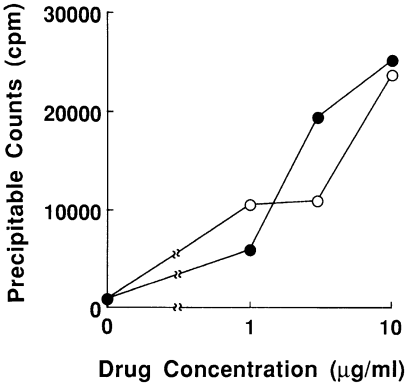

Fig. 6 Effects of NC-190 (●) and etoposide (○) on the protein–DNA complex formation in HL-60 culture cells. The formation of protein–DNA complexes was measured by the SDS precipitation assay. The total acid-precipitable counts per minute per assay was determined as 5.1×10^4

protein–DNA complex in HL-60 cells in a concentration-dependent manner. The potency of NC-190 in inducing the protein–DNA complex was similar to that of etoposide. Precipitable counts of NC-190- or etoposide-treated samples had decreased to control levels 2 h after drug washout (data not shown). Thus, NC-190 induced a topoisomerase II-DNA cleavable complex in HL-60 cells, and the complex formation was reversible after drug removal.

DNA fragmentation induced by NC-190 treatment in HL-60 cells

Recent results suggest that apoptosis may be involved in cell death induced by chemotherapeutic agents, including topoisomerase II-reactive drugs such as etoposide, amsacrine, and teniposide [5, 18].

We examined whether NC-190 induced apoptosis in HL-60 cells. Cells treated for 0–18 h with NC-190 at 10 μg/ml (22 μM) or etoposide at 10 μg/ml (17 μM) were lyzed and the patterns of DNA fragmentation

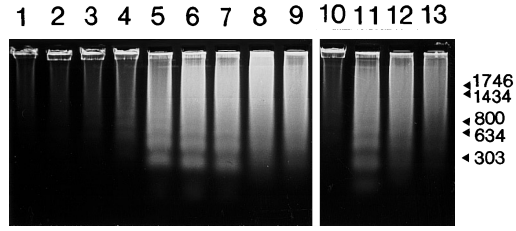

Fig. 7 Electrophoretic analysis of cellular DNA after treatment of HL-60 cells with NC-190 and etoposide. HL-60 cells were treated with 10 μg/ml (22 μM) NC-190 for 0, 0.5, 1, 2, 3, 4, 6, 12, and 18 h (*lanes* 1–9, respectively), or with 10 μg/ml (17 μM) etoposide for 1, 4, 12, and 18 h (*lanes 10–13*), respectively. DNA samples were prepared and electrophoresed as described in Materials and methods

were analyzed by gel electrophoresis (Fig. 7). By 1 h after the addition of NC-190 to the cells, the DNA from drug-treated cells (Fig. 7, lane 3) was indistinguishable from the DNA of control cells (Fig. 7, lane 1). In both samples, the DNA remained near the top of the gel. Between 2 and 6 h after the addition of NC-190, the amount of high molecular weight DNA decreased (Fig. 7, lanes 4–7) and the DNA cleavage band showed a pattern characteristic of an internucleosomal ladder, suggesting apoptosis. This DNA fragmentation remained evident at 12–18 h after the addition of NC-190 (Fig. 7, lanes 8, 9).

Trypan blue dye exclusion assays demonstrated that 99% of HL-60 cells excluded the trypan blue 12 h after the addition of NC-190. Thus, plasma membrane integrity was intact at a time when the genome had already sustained extensive damage.

Similar DNA cleavage was evident within 4 h after the start of treatment with 10 μg/ml (17 μM) etoposide (Fig. 7, lanes 10–13).

To determine DNA fragmentation quantitatively, we used the thymidine release assay, as described by Kolber and Hill [6]. This method confirmed the results described above, and DNA cleavage in HL-60 cells was found to be dependent on the concentration of the drugs (Fig. 8). The time course and degree of DNA cleavage in HL-60 cells induced by NC-190 was similar to those induced by etoposide.

DNA unwinding measurement

We have previously reported that NC-190 displays DNA binding activity [11]. In order to determine whether NC-190 can intercalate plasmid DNA, we compared the DNA unwinding activity of NC-190 and typical intercalators, such as EB and ADM. Agarose gel electrophoresis of closed-circular pBR322 DNA treated with an excess of topoisomerase I in the presence of increasing amounts of NC-190 showed that NC-190 unwound supercoiled DNA in a concentration-dependent manner (Fig. 9). The concentration range of

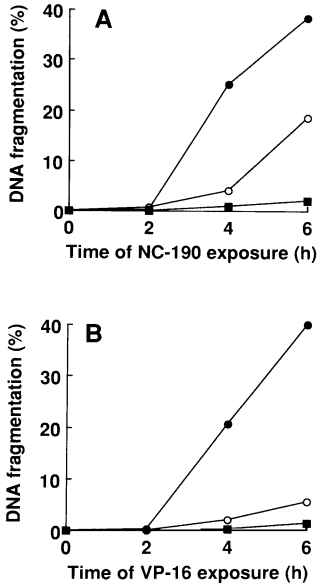

Fig. 8A, B The kinetics of drug-induced DNA fragmentation in HL-60 cells, expressed as the corrected percentage of DNA fragmentation plotted as a function of time. [^{3}H]-Thymidine-labeled HL-60 cells were incubated with NC-190 (**A**) or etoposide (VP-16) (**B**) at concentrations of 1 (■), 3 (○), and 10 (●) μg/ml for the times shown. The corrected percentage of DNA fragmentation was determined as described in Materials and methods

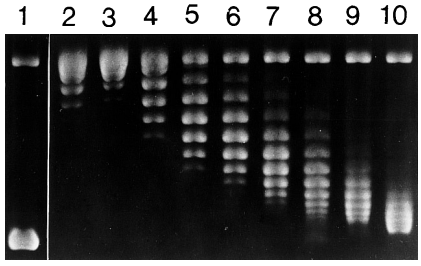

Fig. 9 Agarose gel electrophoresis of pBR322 DNA containing increasing numbers of superhelical turns. Effects of NC-190 on DNA unwinding assay. Unwinding measurements were done as described in Materials and methods (*lane 1* control pBR322 DNA; *lane 2* 10 units topoisomerase I; *lanes 3–10* same as lane 2 with 5, 10, 15, 20, 25, 30, 40, and 50 μM NC-190, respectively)

NC-190 which could induce an alteration of topoisomerase I-mediated DNA relaxation was 5–50 μM. For NC-190, a concentration about ten times higher than for EB was required to obtain the same number of superhelical turns of DNA (Fig. 10). This result suggests that NC-190 has intercalating activity, but that its activity is apparently weaker than those of EB and ADM.

Discussion

Most intercalative antitumor drugs, such as ADM, daunomycin, actinomycin D and mitoxantrone, also stabilize the topoisomerase II–DNA cleavable complex

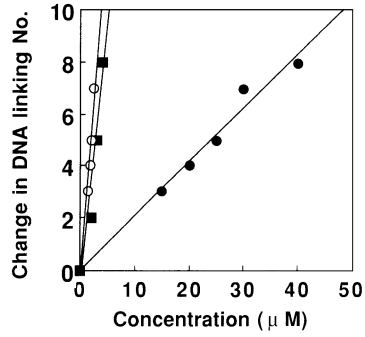

Fig. 10 Unwinding of pBR322 DNA by NC-190 (●), ADM (■), and EB (○) . The change in the DNA linking number produced by topoisomerase I in the presence of different concentrations of the drugs was determined as described in Materials and methods

[7, 14]. Some intercalators show two different types of effect on the formation of the topoisomerase II–DNA cleavable complex: induction at a low concentration, and suppression at a high concentration of the formation of the cleavable complex [16]. Considering this, Capranico et al. [2] have proposed that DNA intercalative topoisomerase II inhibitors be classified into three types: type 1, drugs which exhibit both induction of the cleavable complex at low concentrations and suppression at higher concentrations (e.g. ADM, ellipticines); type 2, drugs with low DNA binding affinities which exhibit only induction of the cleavable complex (e.g. amsacrine, 5-iminodaunorubicin); and type 3, drugs which exhibit only suppression of the cleavable complex (e.g. EB, 9-aminoacridine). In the present study, we found that NC-190 inhibited the activity of topoisomerase II through stabilizing the DNA–topoisomerase II cleavable complex, and did not suppress the formation of the cleavable complex in the concentration range 0.3–30 μM (Fig. 4). NC-190 had DNA intercalating activity, but its activity appeared to be weaker than those of EB and ADM (Figs. 9 and 10). Thus, NC-190 is clearly a type 2 intercalative topoisomerase II inhibitor.

We showed that NC-190 inhibited the growth of HL-60 cells, and induced topoisomerase II–DNA cleavable complexes and DNA fragmentation in HL-60 cells (Figs. 5–8). These activities of NC-190 were closely similar to those of etoposide. The results demonstrate a good correlation among growth inhibition, topoisomerase II-dependent DNA cleavage, and DNA fragmentation induced by NC-190 and by etoposide. These results indicate that the mechanism by which NC-190 exhibits antitumor activity may be through inhibition of topoisomerase II.

Recent results have suggested that apoptosis may be involved in cell death induced by chemotherapeutic agents including topoisomerase II-reactive drugs such as etoposide, amsacrine, and teniposide [5, 18]. The production of stabilized topoisomerase II–DNA cleavable complexes by topoisomerase II-targeted drugs is

thought to result in a cascade of events that leads to cell death [7, 14]. We have shown that NC-190 induced a characteristic pattern of internucleosomal DNA fragmentation in HL-60 cells, suggestive of apoptosis (Fig. 7). These results suggest that NC-190-induced stabilization of topoisomerase II–DNA cleavable complexes stimulates the induction of DNA fragmentation, which leads to cell death.

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