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ORIGINAL ARTICLE

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Establishment and characterization of a mouse FM3A cell mutant resistant to topoisomerase II-inhibitor NC-190

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Abstract We established an NC-190-resistant cell line, FM/NC-R, from the murine mammary carcinoma cell line FM3A and examined some of its characteristics. FM/NC-R cells were prepared by mutagen treatment followed by exposure to NC-190 in the culture medium. FM/NC-R cells were 76.5 times more resistant against NC-190 than FM3A cells as measured by their growth in vitro. FM/NC-R cells also showed cross-resistance to etoposide with NC-190. Neither NC-190 nor etoposide increased the lifespan of FM/NC-R-bearing mice at doses that prolonged the lifespan of FM3A-bearing mice more than four times. This resistance was not due to the change in the concentration of NC-190 in the cells, and there was no change in the expression of P-glycoprotein, a drug efflux pump in the cells. NC-190 and etoposide are inhibitors of DNA topoisomerase II, but there was no difference in cellular content of DNA topoisomerase II between the two cell lines as determined by Western blot analysis. The stabilization of DNA-DNA topoisomerase II cleavable complexes induced by NC-190 was lost in FM/NC-R cells. It was found that Gly881, which is located in the ATP binding site, was replaced by Arg in topoisomerase IIα of FM/NC-R cells. These results indicate that the NC-190-resistant cell line FM/NC-R contains a mutated DNA topoisomerase IIα.

Keywords NC-190-resistant · FM/NC-R cells · DNA topoisomerase II inhibitor · Mutated DNA topoisomerase IIα

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Introduction

We have demonstrated that the novel antitumor compound NC-190 (*N*-β-dimethylaminoethyl-9-carboxy-5-hydroxy-10-methoxy-benzo[*a*]phenazine-6-carboxamide sodium salt, molecular weight 456.4 Da) has potent antitumor activity against many tumor cell lines in vitro and animal tumor models in vivo [14, 15, 25]. We have also shown that NC-190 inhibits topoisomerase II (TopoII) by intercalating with DNA, and that this activity inhibits both DNA synthesis and cell cycle progression [11, 25, 27]. Furthermore, it has been shown that NC-190 as well as other TopoII inhibitors, cause cells to remain in the G_2/M phase of the cell cycle [26]. Because NC-190 has sufficient efficacy against Adriamycin- and vincristine-resistant P388 leukemia both in vitro and in vivo and it is likely to be effective against multidrug-resistant tumors, the drug is considered to be a new type of antitumor agent.

To study the antitumor mechanisms of NC-190 in more detail, we tried to prepare in vivo-established NC-190-resistant cell lines by repeated NC-190 administration to parental P388-bearing mice. However, such trials have been unsuccessful to date. We thus tried to make an NC-190-resistant cell line by the treatment of FM3A cells with the mutagen ethyl methanesulfonate (EMS) and further exposure of EMS-treated cells to NC-190 in a cell culture system. We report here the establishment of an NC-190-resistant cell line and its characterization.

Materials and methods

Isolation of NC-190-resistant cells from FM3A cells

To isolate NC-190-resistant cells, we used the mouse mammary carcinoma FM3A cell line. This cell line was cultured at 37°C in ES medium (Nissui Pharmaceutical Company, Tokyo, Japan) with 2% fetal bovine serum (GIBCO). ES medium was prepared from Eagle's minimum essential medium enriched with nine supplements: 0.2 m*M* each of seven nonessential amino acids (L-alanine,

L-asparagine, L-aspartic acid, L-glutamic acid, L-glycine, L-proline and L-serine), 1 m*M* sodium pyruvate and vitamin B_{12} (0.1 mg/l) [28]. FM3A cells propagated in suspension with a doubling time of about 12 h and reached saturation at a density of 2×10^6 cells/ml [16]. FM3A cells in the logarithmic growth phase (5×10^5 cells/ml) were diluted to 2.5×10^5 cells/ml in a 50-mm dish. They were mutagenized by treatment with 0.1% of EMS for 3 h, washed three times with ES medium, and cultured for 4 days in ES medium. The cells were then resuspended at 2×10^4 cells/ml in selective medium containing NC-190. The concentrations of NC-190 in the selective medium were gradually increased in a stepwise manner from 50 to 350 ng/ml over 9 months. The NC-190-resistant FM/NC-R cell line thus obtained was maintained through culture in ES medium containing 350 ng/ml of NC-190.

Assessment of drug resistance

Drug resistance of FM/NC-R cells was assessed using in vitro and in vivo assay systems. To measure in vitro growth inhibitory activities, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used as described previously [27]. After cells (2×10^4 cells/0.1 ml per well) were cultured in 96-well microplates with or without various concentrations of drugs for 48 h, 0.05 ml 0.1% MTT solution in phosphate-buffered saline (PBS) was added to each well followed by incubation for another 4 h. The medium was removed by centrifugation and the purple formazan product in the cells was measured at A_{540} by solubilization with DMSO. The IC_{50} (concentration necessary to cause 50% inhibition of cell growth) was calculated using the probit test. Drug resistance was expressed as the ratio of the IC_{50} of the drug for FM3A cells to its IC_{50} for FM/NC-R cells.

To test in vivo sensitivity, each cell line (1×10^5 cells) was transplanted intraperitoneally into C3H/He mice, and the drug was injected intraperitoneally once per day from day 1 to 5 after tumor cell inoculation. Antitumor activity was evaluated by the median survival time of each group, and also by the percentage increase in lifespan. Resistance to each drug was evaluated by the relationships between dosage and effect against each tumor.

Measurement of P-glycoprotein

To evaluate the level of P-glycoprotein recognized by C219 anti-P-glycoprotein antibody (Centocor Diagnostics, Malvern, Pa.), an anchored cell analysis was utilized [22]. FM3A and FM/NC-R cells (5×10^6 cells) in logarithmic phase were fixed in ice-cold 70% ethanol for 20 min, washed with PBS, and reacted with C219 at a final concentration of 17 μg/ml for 60 min. The cells were then stained with FITC-labeled goat anti-mouse antibody (Becton Dickinson) for 60 min in the dark in ice-cold water. After the cells had been washed with PBS containing 1% BSA, they were analyzed by flow cytometry (EPICS-ELITE; Coulter Electronics, Hialeah, Fl.).

Measurement of NC-190 uptake

The assay for [^{14}C]NC-190 uptake was performed by the method of Kakinuma et al. [6] with some modifications. Cells (2×10^6) were incubated in 0.5 ml ES medium containing 0.3 μ*M* [^{14}C]NC-190 (specific activity 977 MBq/mmol) for the indicated periods. Then 0.6 ml of each cell suspension was layered over an equal volume of a mixture of corn oil and dibutyl phthalate (3:10) and centrifuged in an Eppendorf microfuge for 1 min to separate the cells from the substrate in the aqueous phase. The cell pellet was solubilized in 1 ml 1% sodium dodecylsulfate by sonication and the radioactivity was counted in 10 ml scintillator (AQUASOL-2) by a liquid scintillation counter. The protein was determined by the method of Lowry et al. [12] using the cells precipitated by 5% trichloroacetic acid treatment. [^{14}C]NC-190 was synthesized in the Medicinal Research Laboratories, Taisho Pharmaceutical Company, and the method will be published elsewhere.

Western blot analysis of TopoII

The nuclear protein was prepared from 1×10^8 cells of each cell line according to the method of Sinha and Eliot [21], separated by 5% SDS-polyacrylamide gel electrophoresis [10], and transferred to nitrocellulose membrane at 70 mA for 3 h using a Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, Calif.) [9]. TopoII on the membrane was reacted with anti-TopoII polyclonal antibody (Code 2011-1, TopoGEN) and then stained with biotinylated secondary antibody using a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, Calif.) according to the accompanying manual.

TopoII-DNA complex assay

The DNA of each cell type in early log phase culture (2×10^5 cells /ml) was labeled by adding 25.9 kBq/ml [methyl-^{3}H]thymidine (1628 GBq/mmol, Amersham) to the medium. After incubation at 37°C for 12 h, cells were collected by centrifugation, washed three times with PBS, and resuspended in fresh medium at a final concentration of 1×10^5 cells/ml. Cells were incubated for 2 h at 37°C and then treated with various concentrations of NC-190 for 2 h at 37°C. The formation of covalent TopoII-DNA complexes was quantified by the method of Rowe et al. [19]. 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), and transferred to a 1.5-ml Eppendorf tube containing 0.25 ml 325 m*M* KCl. After vigorous vortexing at the highest setting for 10 s, the sample was cooled on ice for 10 min and centrifuged in an Eppendorf microfuge for 10 min at 4°C. The pellet was suspended in 0.2 ml H_2O (65°C), and the radioactivity in the suspension was determined by a liquid scintillation spectrometer.

Determination of nucleotide sequence of TopoII mRNA in FM3A and FM/NC-R cells

Total RNA was isolated from 2×10^7 cells by the method of Cathala et al. [2]. The cDNA fragment for TopoII was obtained by the reverse transcriptase PCR method [5]. A list of the primers used is available from the authors upon request. The nucleotide sequence was determined by the dideoxy chain-termination method using the ALFexpress system (Pharmacia Biotech, Piscataway, N.J.).

Results

Drug sensitivity of FM3A and FM/NC-R cells

The NC-190-resistant cell line, FM/NC-R, was obtained by EMS mutagenesis and by culturing the cells in the presence of NC-190. The sensitivity of FM3A and FM /NC-R cells to various antitumor drugs was examined (Table 1). The IC_{50} values of NC-190 against FM/NC-R cells and FM3A cells were 2.6 and 0.034 μg/ml, respectively. This finding indicates that FM/NC-R cells are resistant to NC-190 with an IC_{50} ratio (relative resistance) of 76.5. FM/NC-R cells showed cross-resistance to some other drugs with NC-190. The relative resistance of etoposide was 29.3, and it was high compared with those of six other antitumor drugs. Vincristine and Adriamycin exhibited partial resistance, with relative resistances of 6.40 and 5.27, respectively. FM/NC-R cells were slightly resistant to 5-fluorouracil. However, mitomycin C and cisplatin exhibited collateral sensitivity in both FM3A and FM/NC-R cells.

Table 1. Effect of NC-190 and other drugs on cell growth. The values shown are the mean of triplicate determinations

Drug	IC_{50} (μg/ml)		Relative resistance[a]
	FM3A cells	FM/NC-R cells	
NC-190	0.034	2.6	76.5
Etoposide	0.41	12	29.3
Vincristine	0.025	0.16	6.40
Adriamycin	0.091	0.48	5.27
5-Fluorouracil	0.18	0.42	2.33
Mitomycin C	0.4	0.31	0.71
Cisplatin	1.9	1.3	0.68

[a]IC_{50} of FM/NC-R cells/IC_{50} of FM3A cells

We estimated the in vivo sensitivities of FM3A and FM/NC-R cells to these antitumor drugs with an intraperitoneally transplanted tumor system. The results are shown in Table 2. NC-190 strongly increased the lifespan of FM3A-bearing mice at doses from 0.4 to 12.5 mg/kg. In contrast, there was no effect on the lifespan of FM/NC-R-bearing mice in the same dose range. Similarly, etoposide yielded an effective dose-dependent response in FM3A-bearing mice, but had no effect on FM/NC-R-bearing mice. Vincristine was also slightly more effective in FM3A-bearing mice than in FM/NC-R-bearing mice. Adriamycin was equally effective in both FM3A- and FM/NC-R-bearing mice at high dose (0.4 mg/kg), but was more effective in FM3A-bearing mice than in FM/NC-R-bearing mice at low dose (0.1 mg/kg). 5-Fluorouracil produced a similar response in both FM3A- and FM/NC-R-bearing mice. Mitomycin C and cisplatin produced more potent responses in FM/NC-R-bearing mice than in FM3A-bearing mice. These results indicate that NC-190-resistant FM/NC-R cells are resistant to TopoII inhibitors because the cells exhibited cross-resistance to etoposide and Adriamycin [20, 21].

Table 2. Antitumor effects of NC-190 and other drugs on FM3A- or FM/NC-R-bearing mice. The values shown are the data from seven mice in each group

Drug	Dose (mg/kg)	Increase in lifespan (%)[a]	
		FM3A cells	FM/NC-R cells
NC-190	0.4	89	3
	0.8	> 338	0
	1.6	> 338	1
	3.2	> 338	7
	12.5	> 338	15
Etoposide	0.4	89	5
	1.6	338	0
	6.5	> 338	7
Vincristine	0.4	44	5
	0.6	157	13
Adriamycin	0.1	> 338	14
	0.4	> 338	> 307
5-Fluorouracil	6.25	41	67
	25.0	45	89
Mitomycin C	0.2	170	> 307
	0.8	> 338	> 307
Cisplatin	0.8	12	7
	3.2	42	104

[a](Median survival time of drug-treated group×100/median survival time of control group)–100

P-glycoprotein levels in FM3A and FM/NC-R cells

In many antitumor drug-resistant tumor cell lines, one of the mechanisms of resistance is an increase in efflux of drug from the cells. The most common efflux protein related to the drug resistance is a P-glycoprotein [23]. We therefore examined the level of P-glycoprotein in FM3A and FM/NC-R cells by flow cytometry. As shown in Fig. 1, the representative cell population and median fluorescence were equivalent in these two cell lines, indicating that expression of P-glycoprotein was nearly equal. When human myelogenous leukemia K562 cells and Adriamycin-resistant K562/ADM cells [24] were analyzed under the same experimental conditions, expression of P-glycoprotein was greater in K562/ADM cells than in K562 cells (data not shown). These results indicate that the difference in drug sensitivities of FM3A and FM/NC-R cells is not due to the difference in expression of P-glycoprotein.

We then measured the level of intracellular accumulation of NC-190. As shown in Fig. 2, NC-190 accumulated to similar levels in both cell lines, and reached a maximum concentration within 60 min of incubation. The intracellular contents of [^{14}C]NC-190 at 2 h were 37.5 pmol/mg protein in FM3A cells and 39.8 pmol/mg protein in FM/NC-R cells. These results indicate that the resistance of FM/NC-R cells to NC-190 is not due to a reduction in the intracellular level of NC-190.

Activity of TopoII in FM3A and FM/NC-R cells in the presence of NC-190

We have previously reported that NC-190 inhibits TopoII [25]. We therefore compared the function and

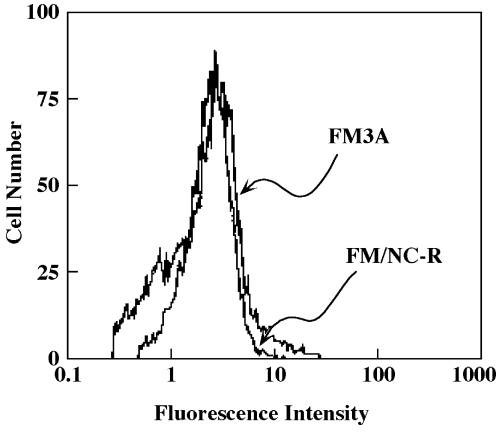

Fig. 1. Expression of P-glycoprotein in FM3A and FM/NC-R cells. Cells were fixed in ice-cold 70% ethanol and reacted with C219 anti-P-glycoprotein antibody. They were then stained with FITC-labeled goat anti-mouse antibody and analyzed by flow cytometry

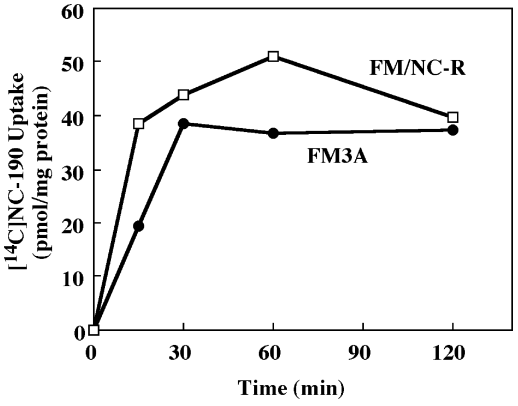

Fig. 2. [^{14}C]NC-190 uptake activities in FM3A and FM/NC-R cells. Cells were incubated with 0.3 μM [^{14}C]NC-190 for the indicated times. Intracellular uptake of NC-190 is expressed as amount per mg protein. Data are the means of duplicate determinations (*closed circles* FM3A cells, *open squares* FM/NC-R cells)

amount of TopoII in each cell line in the presence of NC-190.

The function of TopoII inhibitor was estimated by accumulation of cleavable complexes of DNA-TopoII induced by TopoII inhibitor. TopoII binds to DNA, and forms an intermediary complex to change DNA conformation by breaking and rejoining DNA strands. It has been reported that etoposide and Adriamycin make stable cleavable complexes and interfere with rejoining these complexes [7]. In a previous study, we have demonstrated that NC-190 induces formation of the TopoII-dependent DNA cleavable complexes [27]. We thus measured the level of cleavable complexes induced by NC-190. As shown in Fig. 3A, the level of cleavable complexes increased by 13-fold in FM3A cells when the cells were cultured with 10 μg/ml NC-190. In contrast, no increase in the level of cleavable complexes was observed in FM/NC-R cells cultured with NC-190. Similar results were obtained with etoposide.

The amount of TopoII was then determined by Western blot analysis. The results are shown in Fig. 3B. Lane 1 is the standard TopoII protein partially purified from Ehrlich cells, and lanes 2 and 3 are the nuclear protein from FM3A cells and FN/NC-R cells, respectively. In lanes 2 and 3, there was one main band at the same position as the standard, indicating that these bands represent TopoII. In these two lanes, no difference in the intensity of the TopoII band was observed suggesting that there was no difference in TopoII content between FM3A and FM/NC-R cells.

Next, the nucleotide sequences of TopoIIα mRNA in FM3A and FM/NC-R cells and their encoded amino acid sequences were determined. As shown in Fig. 4, Gly881, which is located in the ATP binding site of TopoIIα in FM3A cells, was replaced by Arg in TopoIIα of FM/NC-R cells. The remaining amino acid sequences were exactly the same in the DNA and ATP binding sites shown in Fig. 4 in these two cell lines. The results

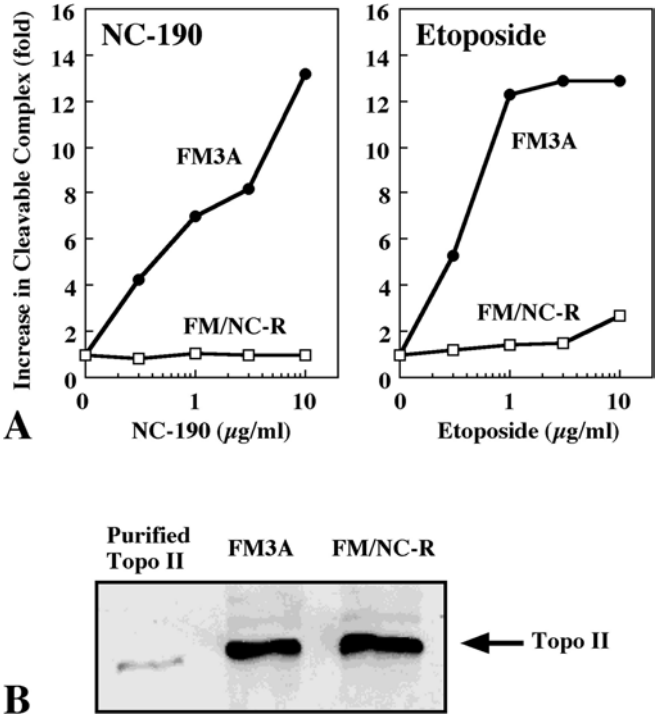

Fig. 3A, B. Increase in cleavable complexes of TopoII-DNA following culture with TopoII inhibitor (**A**) and the levels of TopoII (**B**) in FM3A and FM/NC-R cells. **A** Chromosomal DNA was labeled with [^{3}H]thymidine and the cells were then treated with NC-190 or etoposide. Amount of cleavable TopoII-DNA complex was measured as described in Materials and methods. Data are the means of duplicate determinations. **B** Western blotting of TopoII. *Lane 1* One unit of TopoIIα from Ehrlich ascites tumor cells. TopoII was partially purified as described previously [27]. *Lane 2* TopoIIα in 1×10^{8} FM3A cells. *Lane 3* TopoIIα in 1×10^{8} FM/NC-R cells

strongly suggest that TopoIIα in FM/NC-R cells became resistant to the inhibitor of TopoII through mutation at amino acid residue 881.

Discussion

To study the antitumor mechanism of NC-190 in more detail, we attempted to prepare NC-190-resistant cells. We were able to establish an NC-190-resistant cell line, termed FM/NC-R, from FM3A cells. The resistance index of FM/NC-R cells for NC-190 was 76.5 (Table 1). The FM/NC-R cell line also displayed resistance against NC-190 in the in vivo ascites tumor system (Table 2). NC-190 exhibited a strong effect against FM3A-bearing mice over a wide dose range from 0.4 to 12.5 mg/kg, but had no effect against FM/NC-R-bearing mice.

The NC-190-resistant cell line exhibited some degree of cross-resistance to various types of antitumor drugs in vitro (Table 1). In particular, its sensitivity to etoposide was markedly reduced, with a relative resistance of 29.3. Resistance to Adriamycin was less than that to etoposide. The reduced antitumor potency of these three agents against FM/NC-R-bearing mice compared with

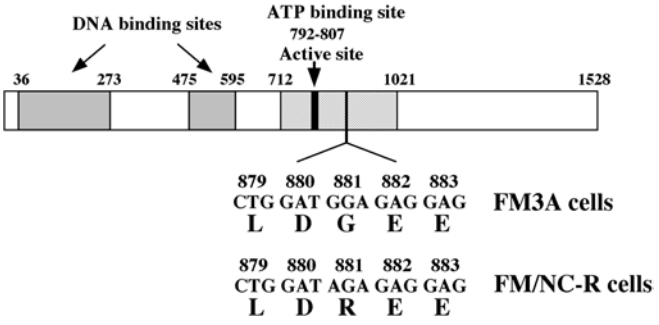

Fig. 4. Nucleotide sequence of TopoIIα cDNA and its encoded amino acid residues. The structure of TopoIIα its nucleotide sequence and its encoded amino acid residues 879 to 883 in FM3A and FM/NC-R cells are shown

parental FM3A-bearing mice (Table 2) strongly suggests that FM/NC-R cells are resistant to TopoII inhibitors.

Previously, we have reported that NC-190 inhibits the catalytic activity of TopoII [25]. TopoII is a nuclear enzyme which converts the topology of DNA through its binding to DNA double strands. Accumulation of DNA-TopoII complexes in HL-60 cells following treatment with NC-190 has been reported [27], indicating that NC-190 inhibits the function of TopoII. In this study, no increase in the level of DNA-TopoII complexes was observed in NC-190-treated FM/NC-R cells. These results suggest that the mechanism of resistance of FM/N-R to NC-190 is related to the function of TopoII.

Although Adriamycin also inhibits TopoII, FM/NC-R cells exhibited only a low level of resistance to Adriamycin. Capranico et al. have suggested that TopoII inhibitors can be classified into several categories according to their affinity for DNA and their effect on cleavable complex formation [1]. The difference in resistance to Adriamycin, etoposide and NC-190 of FM/NC-R cells may be because these TopoII inhibitors belong to different categories.

In many cases of drug resistance, the resistance is based on an increase in efflux of the drug from cells. The most common efflux protein related to drug resistance is P-glycoprotein [3, 4]. In this study, no difference in expression of P-glycoprotein was found between FM3A and FM/NC-R cells (Fig. 1). In addition, intracellular accumulation of NC-190 in FM/NC-R cells was slightly higher than that in FM3A cells (Fig. 2). These results indicate that the resistance against NC-190 of FN/NC-R cells is not due to an increase in efflux of NC-190 from the cells.

As mentioned above, FM/NC-R cells were resistant to TopoII inhibitor. The amount of TopoII in FM3A and FM/NC-R cells was nearly equal as determined by Western blot analysis (Fig. 3B), indicating that resistance is not based on a change in expression of TopoII. We found that there was no increase in DNA-TopoII complexes in response to NC-190 in FM/NC-R cells, while an increase was observed in FM3A cells. It has been reported that TopoIIα is associated in mammals with the centromere in a cell cycle- and species-specific manner and that TopoIIα is abundant compared with TopoIIβ [8, 18]. Thus, we sought to determine whether TopoIIα in FM/NC-R cells is mutated by the treatment of EMS. We actually found that Gly881, which is located in the ATP binding site, was replaced by Arg in TopoIIα of FM/NC-R cells. The locations of TopoIIα mutations thus far determined are amino acid residues 426 to 493 and 744 to 863 [8, 13, 17]. Gly881 is very close to the hotspot regions of TopoIIα. The results, taken together, support the idea that resistance to NC-190 of FM/NC-R cells is based on the mutation of TopoIIα.

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