A New Mechanism of Acquisition of Drug Resistance by Partial Duplication of Topoisomerase I

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ABSTRACT

Topoisomerase (topo)-I targeting antitumor agents are very effective in vivo against various human cancers. The indolocarbazole compound 6-N-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5H-indololo[2,3-a]pyrrolo-[3,4-c][carbazole-5,7-(6H)-dione (NB-506) is a potent inhibitor of the religation step of topo I reaction, like camptothecin (CPT). We established a NB-506-resistant cell line from murine leukemia cell line P388. This resistant cell line, P388/F11, exhibited 73-fold higher resistance to NB-506 and 3.5-fold higher cross-resistance to CPT than the parental cell line. No induction of cleavable complex formations induced by NB-506 and CPT were detected by K-SDS precipitation assays in P388/F11 cells. Analysis of nuclear extracts from P388/F11 cells revealed that the relaxation activity of topo I was one-quarter of that of the parental cells, and that the activity was resistant to induction of DNA cleavage by these drugs. Furthermore, Western blot and Northern blot analyses showed the expression of an abnormal-sized 170-kDa topo I protein and its 6.0-kb transcript and the absence of the normal topo I protein and transcript in P388/F11 cells. Analyses of the structure of the abnormal topo I transcript by reverse transcription-PCR and direct sequencing methods revealed that a large portion of the gene from codon 21 to codon 609 was duplicated in its coding region. This internal duplication resulted in in-frame fusion and, thus, production of a partially duplicated protein of 1357 amino acids. Finally, we expressed and purified the recombinant P388/F11 topo I in a baculovirus system. P388/F11 topo I showed similar catalytic activity to wild-type topo I, but reduced sensitivities to NB-506 and CPT. These results show that the altered sensitivity of duplicated topo I is involved in the NB-506 resistance of P388/F11 cells and indicate a novel resistant mechanism which involves duplication of the topo I gene.

INTRODUCTION

DNA topo1 is an enzyme that changes the topological state of DNA by catalyzing the transient breakage of a phosphodiester bond in a single DNA strand (1). It is a nuclear protein of about 100 kDa and has four domains: an unconserved amino-terminal domain, a conserved core domain, an unconserved positively charged linker domain, and a highly conserved COOH-terminal domain containing an active site tyrosine (2, 3). The core domain and the COOH-terminal domain are essential for the catalytic activity, and the creation of a single strand breakage involves the formation of a covalent bond between the 3′-end of the DNA strand break and the tyrosine residue in the COOH-terminal domain of topo I (1–3).

Topo I is involved in many biological processes, such as DNA replication and transcription, and probably also in recombination (1, 4, 5). Perhaps more importantly, topo I is a target for antitumor chemotherapy. CPT is a representative drug that targets topo I activity (6). The mechanisms of CPT cytotoxicity involve at least two successive steps.

First, the drug stabilizes the covalent enzyme-DNA intermediate by inhibition of the religation step of the topo I reaction, without affecting the cleavage reaction (7–10). Second, the cleavable complexes are converted to DNA double-strand breaks by interaction with moving replication forks (11, 12). Several studies suggested that these DNA breaks are responsible for the cytotoxicity of CPT (11, 13). Recent results on cancer chemotherapy with CPT derivatives have proved that topo I is a promising therapeutic target (14).

We developed a novel indolocarbazole compound, NB-506, that strongly inhibits topo I activity. NB-506 is an intercalating agent with a unique structure and, like CPT, stabilizes topo I-DNA cleavable complexes (15). Despite the inhibitory activities of NB-506 on DNA polymerase α and RNA polymerase II, the relatively strong effects of it on topo I and cleavable complex formation in cells at low concentration suggested that stabilization of the topo I cleavable complex is the main cell-killing mechanism. NB-506 showed strong antitumor effects on various human tumor xenografts (16, 17), and the derivative of it is to be subjected to clinical trials.

Many studies on CPT-resistant cell lines in vitro have been reported and provided a system for investigating the mechanism of action of the drug and of the appearance of resistant cells. The most common mechanism of CPT resistance is reduction of topo I expression, which results in decreased formation of cleavable complexes (18, 19). Another mechanism is topo I point mutations, which confer CPT resistance by affecting the drug-enzyme interaction (20–24). Other studies showed that rearrangement of the topo I gene is also involved in the resistance (19, 25, 26). However, the precise mechanism of the resistance is still unclear.

To elucidate the mechanisms of action of NB-506 and the appearance of drug resistance, we isolated and characterized a NB-506-resistant cell line from murine tumor cells.

MATERIALS AND METHODS

Materials. NB-506 was synthesized in our institute, as described previously (15). Topotecan, and rebeccamycin were synthesized in our institute. Adriamycin and CPT were purchased from Sigma Chemical Co. (St. Louis, MO). Cisplatin and etoposide were purchased from Nippon Kayaku Co. (Tokyo, Japan). [α-32P]dCTP and [3H]thymidine were obtained from Amersham Pharmacia (Uppsala, Sweden). Restriction enzymes were purchased from Takara Biomedicals (Osu, Japan).

Cell Culture and Isolation of the NB-506-Resistant Cell Line. The mouse leukemia cell line P388 was provided by Dr. T. Tsuruo (Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan) and grown in RPMI 1640 supplemented with 10% FCS, 420 mg/ml kanamycin, and 20 μM 2-mercaptoethanol.

The NB-506-resistant cell line P388/F11 was selected by stepwise and continuous exposure to NB-506. Exposure of cells was started at 0.11 μM NB-506 for 1 week. Then, the concentration of NB-506 was increased stepwise. After exposure for 20 weeks, the cells that could grow in medium containing 3 μM NB-506 were cloned by limiting dilution.

Drug Sensitivity Test. Exponentially growing cells were seeded at 1×10^5 cells into a 96-well tissue culture plate. After subculture for 24 h at 37°C, an equal volume of medium containing compound that was serially diluted at 1:3 was added to each well, and the cells were incubated for 72 h. Cell growth was measured by colorimetric tetrazolium-formazan assay (27).

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3 The abbreviations used are: topo, topoisomerase; NB-506, 6-N-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5H-indolo[2,3-a]pyrrolo-[3,4-c][carbazole-5,7-(6H)-dione; CPT, camptothecin; RT-PCR, reverse transcription-PCR; WT, wild-type.

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Accumulation of NB-506 in Cells. The accumulation of NB-506 in P388 and P388/F11 cells was determined by measuring the incorporation of [3,9-H]NB-506 (1.48 kBq/mmol) into the cells, as described previously (15).

Quantitative Analysis of DNA-Protein Complex Formation. The formation of cleavable complex in intact cells was quantified by K-SDS precipitation technique, as described previously by Yoshinari et al. (28).

Preparation of Nuclear Extracts. Crude nuclear extracts were prepared as described previously, except that salt extraction was performed with 0.6 M NaCl instead of 0.35 M NaCl (29). Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Topo I Enzyme Assays. The enzymatic activity of topo I was determined as relaxation of supercoiled pBR322 DNA, as described previously (30). One unit of topo I activity was defined as the amount of enzyme necessary for complete relaxation of 0.4 μg of pBR322 DNA. The effects of NB-506 and CPT on topo I activity were examined by DNA nicking assay (28). Briefly, nuclear extracts containing 80 units of topo I activity or 15 units of purified topo I were incubated with 0.4 μg supercoiled pBR322 DNA in reaction mixture [10 mM Tris-Cl, 50 mM KCl, 0.5 mM Tris-Cl, 0.5 mM DTT, and 30 μg/ml BSA (pH 7.6)] in the presence or absence of drugs for 10 minutes at 30°C. Reactions were stopped by the addition of SDS and further incubated with proteinase K. Nicked DNA was analyzed by 1% agarose gel electrophoresis containing 0.5 μg/ml of EtBr. Image was recorded with Gel Print2000i (Bio Image Corp., Ann Arbor, MI), and intensities of bands were quantified using a software MacBAS version 2.5 (Fuji Photo Film Co., Tokyo, Japan).

Western Blot Analysis of Topo I. Nuclear extracts were separated on 7.5% SDS polyacrylamide gel. After electrophoresis, the samples were transferred electrophoretically to a nitrocellulose filter. The membrane was probed with human serum from a scleroderma patient (Topogen Inc., Columbus, OH), followed by antihuman IgG conjugated with horseradish peroxidase as a secondary antibody (Amersham Pharmacia). The protein was detected with enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia).

Northern Blot Analysis of the Topo I Transcript. Total RNAs were extracted from P388 and P388/F11 cells with Isogen (Nippon Gene, Toyama, Japan), and mRNA was isolated by oligo-dT columns. Samples of 1 μg of polyadenylic acid RNA were separated on 1% agarose gel and transferred to nylon membranes. The membranes were probed with a 32P-labeled mouse topo I cDNA fragment spanning position 425 to 1721. The nucleotide positions shown are numbered according to Koikawa et al. (31).

Southern Blot Analysis of Topo I Gene. The genomic DNAs from P388 and P388/F11 cells were digested to completion with EcoI and XhoI. Digested DNAs (15 μg) were separated on 1% agarose gel and then transferred to nylon membranes. The membranes were probed with a 32P-labeled mouse topo I cDNA fragment (position 1456-1820).

PCR Analysis of the Topo I Transcript. The first strand cDNA was synthesized from 0.5 μg of mRNA with Superscript II reverse transcriptase (Life Technologies, Inc., Grand Island, NY) and oligo (dT)12-18 primers in 50 μl of reaction mixture at 37°C, after heat denaturation of RNA samples at 70°C, for 10 min according to the manufacturer’s protocol. Then, 2 μl of the first strand reaction mixture was subjected to PCR amplification using Takara Ex Taq (Takara Biomedicals) with 0.2 μM gene-specific primers. The PCR reaction was carried out for 30 cycles of 10 Sec for 15 cycles, 60°C for 1 min and 72°C for 1 min), followed by a 10 min extension at 72°C. The oligonucleotide primers used for amplification of the F11-topo I transcript were: 10F, 5’-GACCACTCCACAACGCATTCTC-3’ (position 10-29 of the mouse topo I cDNA sequence, sense); 627R, 5’-TTCCCCACATTCCACTTCT-3’ (position 627-647, antisense); 844F, 5’-AAAGATGGAGAAGAAATGA-3’ (position 844-865, sense); 256R, 5’-CCTTTCGCTCTTTGTTCCTC-3’ (position 256-277, antisense); 1162F, 5’-TGTACAGAAACGAGAAGGTT-3’ (position 1162-1182, sense); 1477F, 5’-AATGAGAAAGAAGAG-3’ (position 1477-1497, sense); 1782F, 5’-AAATGCTCCTCATACTACA-3’ (position 1782-1802, sense); 372R, 5’-GGGAGGAAAAATACCATCA-3’ (position 372-392, antisense); 510R, 5’-TCTCATACAAACAGCTTGATT-3’ (position 510-537, antisense); 1572R, 5’-AAGTCAAAATCCCAACACTAC-3’ (position 1572-1592, antisense); 2325R, 5’-AAACACACTGTTCTTTCAC-3’ (position 2325-2345, antisense).

Nucleotide Sequence Determination. The sequence around the fusion point of duplication of the F11 topo I transcript was determined from cloned fragments obtained by RT-PCR amplification. The 981-bp RT-PCR product generated with 1162F and 372R primers, which includes the fusion point, was cloned into a plasmid vector, pCRII (Invitrogen Corp., San Diego, CA). Sequencing was performed by the dyeoxy method using Sequense Version 2.0 (Amersham Pharmacia).

Cloning of Mouse Topo I cDNA from P388 Cells. The entire coding sequence of mouse topo I transcript was generated by RT-PCR from total RNA of parental P388 cell line. These fragments were cloned into pMw218 plasmid vectors (Nippon Gene), and the nucleotide sequence of the coding region was verified by sequencing analysis.

Constructions of WT and F11 Topo I in a Baculovirus Expression System. To obtain the transfer plasmid for recombinant WT topo I cDNA fragment containing the complete coding region of mouse topo I cDNA (position 1-2235) was attached to an oligonucleotide linker containing 6 × His tag at its amino-termini and inserted into EcoRI and BamHI site of a plasmid vector pBR/Bac9 to give the plasmid pBR/BacTOP-HIS. The resulting vector should produce a topo I fused to an amino-terminal 10 amino acid leader peptide (Met-Ala-His-His-His-His-His-Val-Asp) containing a 6 × His tag. The pBR/Bac9 is a low-copy transfer vector constructed by replacing the replication origin of pBacPAK9 (Clonetech Laboratories, Palo Alto, CA) by that of pBR322.

For obtaining the recombinant P388/F11 (F11) topo I plasmid, the SacI (position 1820) fragment, which encompasses duplication junction, was inserted into the unique SacI (position 1820) site of the WT topo I coding region of pBR/BacTOP-HIS to obtain plasmid pBR/BacTOP-F11-HIS. Recombinant baculovirus was produced by cotransfleting Spodoptera frugiperda (Sf9) cells with P388/F11 cells and transfected pBacPAK9 (Clonetech Laboratories, Palo Alto, CA) by that of pBR322.
with these recombinant plasmids and linearized BacPAK6 (Clonetech Laboratories) viral DNA by the calcium phosphate method (32). At 72–96 h after infection, the recombinant viruses were harvested from culture supernatants. The plaques were purified by the standard procedures recommended by Clonetech Laboratories. Sf9 cells were maintained in TC-100 medium (Life Technologies, Inc.) supplemented with 10% FCS and triptose phosphate broth at 27°C.

Purification of Recombinant WT Topo I. WT-topo I was prepared from Sf9 cells infected with the pBR/BacTOP-HIS, as described previously (33).

Purification of Recombinant F11 Topo I. Recombinant F11 topo I was purified from 1 x 10⁸ Sf9 cells 72 h after infection. The cells were harvested, washed with PBS (PBS without Ca²⁺ or Mg²⁺), and lysed in lysis buffer [10 mM sodium phosphate (pH 7.5), 5 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 0.25% NP4O, 1 M NaCl, 0.1 mM Pefablock SC, 10 mM leupeptin, 1 mM pepstatin A, and 3 mM 2-mercaptoethanol]. The mixture stood for 10 min on ice, then polyethylene glycol was added at a final concentration of 6%, and the polyethylene glycol precipitate was removed by centrifugation at 12,000 x g for 20 min. The supernatant of polyethylene glycol precipitate of the extract was directly loaded on Ni-NTA (Quiagen Co., Valencia, CA) column equilibrated with buffer A [20 mM sodium phosphate (pH 7.5), 10% glycerol, 0.5 M NaCl, 0.1 mM Pefablock SC, 10 mM leupeptin, 1 mM pepstatin A, and 3 mM 2-mercaptoethanol]. Elution was performed with linear gradient of imidazole. Topo I activity was eluted from the column at -75–110 mM imidazole. The pooled fractions were concentrated and loaded onto Superose12 (Amersham Pharmacia) equilibrated with buffer B [10 mM Tris-Cl (pH 7.6), 150 mM NaCl, 0.1 mM Pefablock SC, 10 mM leupeptin, 1 mM pepstatin A, and 3 mM 2-mercaptoethanol]. The 170-kDa topo I was eluted in 2 ml after the void volume. By this procedure, 170-kDa topo I was purified to near homogeneity. The fraction was pooled and stored at -80°C.

RESULTS

Establishment of NB-506-resistant Cells. NB-506-resistant cell lines were obtained from the mouse leukemia cell line P388 by continuous exposure to NB-506. After exposure for 20 weeks, we obtained the cells that grew in medium containing 3 μM NB-506. The resistant cell line P388/F11 was cloned by limiting dilution method from these cells. The growth of P388/F11 cells was inhibited by NB-506 with an IC₅₀ value of 4.3 μM, whereas the parental cells were inhibited with an IC₅₀ value of 59 nM (Table 1). Thus, the P388/F11 cells were 73-fold more resistant than the parental cells. This resistant phenotype was stable for at least 6 months during continuous culture without addition of NB-506. The doubling times of P388 and P388/F11 cells were similar, being 12.8 h and 13.0 h, respectively. There-

![Fig. 2](image1.png)

![Fig. 3](image2.png)
fore, the contribution of the growth rate to drug sensitivity was negligible.

The Cross-Resistances to Other Anticancer Agents. The P388/F11 cells showed cross-resistances to the other topo I inhibitors, CPT and topotecan (Table 1). The degrees of resistance of P388/F11 cells to these inhibitors were 3- to 5-fold those of parental cells and less than those of P388/F11 cells to NB-506. The P388/F11 cells showed 5.7-fold higher sensitivity to etoposide, an inhibitor to topo II (Table 1). Similar hypersensitivity has often been detected in resistant cell lines showing reduction in the amount or activity of topo I (34, 35).

Other antitumor agents with various mechanisms of action unrelated to topo I showed almost similar cytotoxicities against the two cell lines (Table 1). We also examined the accumulation of NB-506 in the P388/F11 cells, finding that it was 70% of that in the parental cells. This finding suggested little contribution of the accumulation to NB-506 resistance of P388/F11 cells (data not shown).

Topo I Activity and Drug Sensitivity in Nuclear Extracts. Next, we compared the topo I activities in 0.6 M NaCl nuclear extracts isolated from P388 and P388/F11 cells by DNA relaxation assay. The topo I relaxation activity of nuclear extracts from P388/F11 cells was approximately a quarter of that of extracts from parental cells (Fig. 2A). This reduction of total activity of topo I must contribute to the mechanism of resistance of P388/F11 cells. To determine whether the resistance could be explained by the reduction in topo I activity alone, we measured the sensitivity of topo I to NB-506 and CPT by DNA nicking assay using nuclear extracts containing the same units of topo I activity. In the presence of 80 units of topo I activity, formation of nicked DNA was induced dose-dependently by CPT and NB-506 in WT cells, but not in P388/F11 cells (Fig. 2B). This finding suggested that in addition to the 4-fold reduction of total cellular activity, the drug sensitivity of topo I was altered in P388/F11 cells. Thus, the absence of induction of DNA protein complex formation in P388/F11 cells was due to both qualitative and quantitative alterations of topo I.

Expression of Topo I Protein and Transcript. Topo I protein was detected by immunoblot analysis by using polyclonal antibody.
against human topo I (Fig. 3A). Normal 100-kDa topo I protein was detected in a nuclear extract of parental cells. On the other hand, normal-sized topo I protein was not detected in P388/F11 cells, but an unusual band migrating at a position of 170 kDa was detected. We also detected a size alteration of the topo I transcript in P388/F11 cells by Northern blot analysis (Fig. 3B). Parental cells contained a normal topo I transcript of 4.2 kb. On the other hand, only a 6.0-kb transcript was detected in P388/F11 cells. These correlated changes in both transcript and protein suggest the large structural alteration in the coding region of the topo I gene and that the 170-kDa protein detected in P388/F11 cells is an altered form of topo I.

**Tandem-duplicated Structure of Topo I cDNA in P388/F11 Cells.** To obtain more information on the structure of the large topo I transcript detected in P388/F11 cells, we performed RT-PCR amplifications of the transcript using several sets of primers in the whole coding region of topo I cDNA. Of these amplifications, an amplification with primers 1782F and 2325R, which correspond to the COOH terminus of the coding region, gave a weak 2.3-kbp band specific to P388/F11 (Fig. 4B, lane 9, arrowhead) in addition to the normal 564-bp band, and precise analysis of the fragment implied the tandem-duplicated structure of the fragment (data not shown).

Therefore, we investigated the possible duplication of the topo I transcript in P388/F11 cells. We designed primer sets that selectively amplify the duplicated sequence and performed RT-PCR analysis. As shown in Fig. 4, A and B, the RT-PCR with primer sets arranged in inverse orientation can amplify the fragments only when the corresponding region is duplicated. As expected, PCR amplification with the primer sets gave specific bands from P388/F11 (Fig. 4B, lane 9, arrowhead) in addition to the normal 564-bp band, and precise analysis of the fragment implied the tandem-duplicated structure of the fragment (data not shown).

To determine the fusion point of duplication on the transcript, we cloned and sequenced RT-PCR-generated fragments containing the fusion point. We found that the duplication resulted in in-frame fusion of the first nucleotide of codon 610 (position 1829) with the second nucleotide of codon 20 (position 59; Fig. 4, C and D). This fusion generated a novel histidine residue at this position. Finally, we determined the nucleotide sequence of the whole coding region of the transcript, except 9 bp from the ATG start codon, by direct sequencing. We confirmed almost all the structure of the P388/F11 topo I transcript, in which the 1771-bp region spanning position 59 to position 1829 is duplicated in tandem. The transcript containing in-frame duplication is capable of encoding a single polypeptide of 1357 amino acids, with a calculated molecular weight of \( M_r 161,000 \). This size is almost consistent with the molecular size detected in P388/F11 cells by Western blot analysis (Fig. 3A), suggesting the expression of the partially duplicated topo I protein in P388/F11 cells. We did not detect any point mutations in the sequenced region, including those previously reported to confer CPT resistance.

**Rearrangement of the Topo I Gene in P388/F11 Cells.** To investigate what mechanisms are involved in the partial duplication of the topo I transcript in P388/F11 cells, we examined the topo I gene structure by Southern blot analysis. Genomic DNAs from P388 and
P388/F11 cells were hybridized with a topo I cDNA probe corresponding to the region near the fusion point of duplication after digestion with EcoRI and XbaI. Rearranged bands of 5.5 kbp and a 0.4 kbp were detected for P388/F11 genomic DNA digested with EcoRI and XbaI, respectively (Fig. 5). These results show rearrangement of the topo I gene in P388/F11 cells and suggest that duplication of transcript was due to genomic rearrangement. Although certain bands detected in parental cells appeared to be absent in P388/F11 cells, it remained to be clarified whether WT allele is present in addition to the rearranged allele in P388/F11 cells.

Catalytic Activity and Drug Sensitivity of Recombinant F11 Topo I. To determine whether the duplicated F11 topo I enzyme has catalytic activity and the tandem duplication confers altered sensitivity on NB-506, we constructed full-length F11 topo I using cloned mouse cDNA fragments and expressed the recombinant F11 topo I in a baculovirus expression system. The purified WT or F11 recombinant protein was detected as almost a single band in SDS-PAGE analysis (Fig. 6A). The recombinant F11 topo I showed the same mobility as topo I protein detected in nuclear lysate of P388/F11 cells by the Western blot analysis, suggesting that the 170-kDa topo I detected in P388/F11 cells is identical with tandem-duplicated topo I (Fig. 6B).

DNA relaxation assay of purified topo I demonstrated that the F11 topo I had catalytic activity (Fig. 6C). The specific activities of WT and F11 topo I were $8.4 \times 10^5$ units/mg and $5.5 \times 10^5$ units/mg, respectively, indicating similar specific activities. Because these two preparations differed in purity and concentration, it was difficult to determine the exact amounts of the two molecules. Nevertheless, these data suggested that the F11 topo I, at least, have specific activity comparable with WT topo I.

The drug sensitivities of recombinant WT and F11 topo I were compared by DNA nicking assay in the presence of 15 units of topo I activity were incubated with 0.4 μg of supercoiled pBR322 DNA in the presence of the indicated concentrations of NB-506 and CPT. A, drug-induced nicking of DNA analyzed by 1% agarose gel electrophoresis. B, quantitative analysis of nicked DNA by image scanning. Percentages of nicked DNA in the total DNA are plotted against drug concentrations. Values are means for at least three independent experiments. ■, WT-topo I; ○, F11-topo I.

DISCUSSION

We isolated an NB-506-resistant cell line, P388/F11, by culturing the parental cell line in the presence of NB-506, but without mutagen treatment. The P388/F11 cells showed 73-fold higher resistance to NB-506 and 3- to 5-fold higher cross-resistance to CPT and topotecan than the parental cells. Decreased induction of topo I-mediated DNA cleavage by NB-506 was shown by analyses of whole cells, nuclear extracts, and recombinant enzymes. These results suggested that the alteration of topo I is the main reason for NB-506 resistance of P388/F11 cells. The results confirmed the previous suggestion that topo I is the primary target of NB-506 (15, 36). However, considering the relatively weak cross-resistance to CPT, some contribution of factors other than topo I alteration in the cellular resistance cannot be ruled out.

We found that the F11 topo I contains a duplication of residues 20 – 610, including a major portion of the core domain inserted at position 20 within the NH$_2$-terminal domain, a domain that is known to be dispensable for topo I activity (2). Hence, the F11 topo I can be envisioned as protein with a modified NH$_2$-terminal domain, but which otherwise contains all the domains that are essential for activity, located downstream of the duplicated regions. As expected, the
recombinant F11 topo I actually showed the relaxation activity comparable with WT topo I. Moreover, because topo I molecules other than 170-kDa topo I were hardly detected in P388/F11 cells, the duplicated topo I must be responsible for the essential functions in P388/F11 cells.

We also showed altered drug sensitivity of duplicated topo I by DNA nicking assay using purified recombinant enzyme. The recombinant F11 topo I showed reduced DNA cleavage in the presence of either CPT or NB-506. Several interpretations of these data are possible to explain the molecular mechanisms involved in the resistance of F11 topo I. One explanation is that the drug cannot bind to F11 topo I due to its duplicated structure. However, it is unclear whether the first core domain of the duplicated domains, which is distant from the active site, affects the structure of the putative drug binding site around active site tyrosine (3). Perhaps more likely, cleavage-religation equilibrium of F11 topo I enzyme may be shifted towards religation compared with WT topo I. The observation that F11 topo I showed reduced nicking activity in the absence of the drugs compared with WT topo I supports this notion (Fig. 7). Thus, the reduced drug stabilization of cleavage complex in F11 topo I may be explained by elevated rate of religation activity or decreased stability of the cleavable complex of F11 topo I (37). For further understanding of the resistance mechanism of F11 topo I, more precise enzymatic characterization of F11 topo I will be needed.

In conclusion, the partially duplicated F11 topo I showed the altered sensitivity to NB-506 and CPT. Thus, together with reduction of total topo I activity in P388/F11 cells, the decreased sensitivity of F11 topo I must be involved in the mechanism of resistance of P388/F11 cells to NB-506 and CPT. However, because the degree of drug resistance of purified F11 topo I is relatively weak as compared with the resistance detected in nuclear extract from P388/F11 cells, the involvement of other cellular factors affecting the F11 topo I sensitivity cannot be excluded.

Southern blot analysis of the topo I gene of P388/F11 cells suggested that the duplication of topo I in P388/F11 cells arose from genomic rearrangement of the topo I gene. Genomic rearrangement of the topo I gene has often been detected in CPT-resistant cell lines (19, 25, 26). The mechanisms of resistance for these cell lines, reported previously, were the reduced expression of topo I resulting from the rearrangement in one allele of the gene (19). However, our results demonstrated another mechanism for the resistance to topo I inhibitors involving the rearrangement of the topo I gene. Interestingly, the expressions of topo I proteins with different sizes from normal topo I have also been reported in some of CPT-resistant cell line (35, 38, 39). Although these abnormal topo I products were not well characterized in these studies, these facts support the possibility that rearranged topo I products are frequently involved in resistance to topo I inhibitors. P388/F11 cells and the CPT-resistant cell lines reported to contain the rearranged topo I were developed during continuous exposure to topo I inhibitors (19, 25, 26). Therefore, the relationship between the generation of topo I rearrangement and exposure to topo I inhibitors is of great concern. We did not determine the genomic breakpoint of topo I gene duplication in P388/F11 cells, so the molecular mechanism of duplication of F11 topo I is still unknown. However, topo I is suggested by several lines of evidence to be involved in illegitimate recombination (40–43). More importantly, cleavable complexes stabilized by topo inhibitors were shown to enhance events related to the illegitimate recombination. For example, CPT was shown to enhance sister chromatid exchange in treated cultured cells (44). From these findings, the illegitimate recombination mediated by drug-stabilization of the topo I-DNA complex is one of the possible mechanisms for the rearrangement of topo I detected in P388/F11 cells and other CPT-resistant cell lines.

In conclusion, our study is the first molecular analysis of rearrangement of topo I in resistant cells. We showed that the rearranged topo I product is catalytically active and involved in NB-506 and CPT resistance. Considering the possible effects of topo I inhibitors on recombination, rearrangement of the topo I gene, including tandem duplication, might be an important mechanism for resistance to topo I inhibitors in addition to known mechanisms. Further investigations on the mechanism of topo I rearrangement in P388/F11 cells will help in understanding the mechanism of development of resistance of drug-treated cancer cells.

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