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Advances in Brief

Use of Camptothecin-resistant Mammalian Cell Lines to Evaluate the Role of Topoisomerase I in the Antiproliferative Activity of the Indolocarbazole, NB-506, and Its Topoisomerase I Binding Site

Yoshimasa Urasaki, Gary Laco, Yuji Takebayashi,[1] Christian Bailly,[2] Glenda Kohlhagen, and Yves Pommier[3]

Laboratory of Molecular Pharmacology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892-4255

Abstract

NB-506 is a topoisomerase I (top1) inhibitor in clinical trials. In this study, we used a series of camptothecin (CPT)-resistant cell lines with known top1 alterations. We show that three mutations in different domains of the top1 enzyme that confer CPT resistance also confer cross-resistance to NB-506. The CPT-resistant cell lines and corresponding mutations were: human prostate carcinoma cells DU-145/RC1 (mutation R364H), Chinese hamster fibroblasts DC3F/C10 (mutation G503S), and human leukemia CEM/C2 cells (N722S). This result suggests that NB-506 and CPT share a common binding site in the top1-DNA complex. We next used these three cell lines and their parental cells to study the relationship between top1 poisoning by NB-506 and antiproliferative activity. We found that the CPT-resistant cells were only 2–10-fold resistant to NB-506, which suggests that NB-506 targets other cellular processes/pathways besides top1. This conclusion was further supported by the limited cross-resistance of top1-deficient murine leukemia P388/CPT45 cells (2-fold). Cross-resistance was also limited for J-109,382, an isomer of NB-506 that does not intercalate into DNA, indicating that the non-top1-mediated antiproliferative activity of NB-506 is not attributable to DNA intercalation. Together, these data indicate that NB-506 and indolocarbazoles are promising agents to overcome CPT resistance.

Introduction

The recently reported activity of CPT[4] derivatives in human cancers previously refractory to other treatments has prompted the development of novel top1 inhibitors. top1 inhibitors convert the cellular top1 enzyme into a cellular poison by inhibiting the religation step of the enzyme's DNA nicking-closing reaction, thereby trapping top1 in a covalent complex with DNA. The cytotoxic lesions probably result from stable top1 covalent complexes associated with double-strand breaks that are generated after collision of DNA and RNA polymerase machineries with the top1 cleavage complexes [see the recent paper by Strumberg *et al.* (1) and review in Ref. 2].

Non-CPT top1 inhibitors are been pursued actively (3). The indolocarbazoles are the most advanced group of compounds. Rebeccamycin was the first anticancer active indolocarbazole reported in 1987 by Bristol-Myers scientists (for recent and extensive reviews on indolocarbazoles as top1 inhibitors, see Refs. 4 and 5). Scientists at Banyu Tsukuba Research Institute (6) also reported that other indolocarbazole derivatives were effective top1 inhibitors. They discovered the indolocarbazoles from actinomycete extracts as top1 inhibitors using biochemical screening assays with purified enzyme (6). To increase the water solubility of these compounds, sugar derivatives were obtained, leading to NB-506 (7). Banyu presently has two water-soluble indolocarbazole derivatives (NB-506; also referred to as L-753,000) and more recently J-107088 (also referred to as ED-749) in clinical investigation (8, 9).

Although top1-mediated DNA cleavage *in vitro* and in cells has been demonstrated clearly and detailed structure-activity studies reported for indolocarbazoles [particularly by the collaborative group of Bailly, Riou, and Prudhomme–see references in Bailly (5)], to our knowledge, definitive evidence that top1 is the only (or primary) target of indolocarbazoles has not been provided. Some indolocarbazoles including NB-506 are DNA binders (intercalators), and it is possible that they interfere with other chromatin protein/processes besides top1 action.

In the present study, we used three CPT-resistant cell lines with known top1 mutations in different top1 domains (Fig. 1*A*; Ref. 2) and cells without detectable top1 (10–12) to compare NB-506 and CPT. We found that the top1 enzymes with point mutations are cross-resistant to NB-506, which suggests that NB-506 and CPT share a common binding site in the top1-DNA complex. However, growth inhibition experiments showed only partial resistance of the CPT-resistant cell lines to NB-506, which suggests that NB-506 has additional cellular targets besides top1.

Materials and Methods

Cell Culture, Chemicals, and Enzymes. Human prostate carcinoma DU-145, leukemia CEM cells, and their CPT-resistant subclones DU-145/RC1 (2) and CEM/C2 (13, 14) were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 10% FCS in a 5% CO_2 incubator at 37°C. The CPT-resistant DU-145 subline DU-145/RC1 was established by Dr. Pantazis Panayotis and colleagues (Brown University, Providence, RI). Murine leukemia P388 and its CPT-resistant subclone, P388/CPT45 mouse leukemia cells, were provided by Michael R. Mattern and Randall K. Johnson (SmithKline Beecham, King of Prussia, PA) and were cultured in RPMI 1640 containing 20% FCS and 10 μM β-mercaptoethanol (10, 11). Chinese hamster lung fibroblasts DC3F and its CPT-resistant subline DC3F/C10 (15) were grown in MEM with Earle's salt, supplemented with 10% FCS, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (ABI, Columbia, MD). No antibiotics was added to the medium. CPT was provided by Drug Synthesis and Chemistry Branch, Development Therapeutics Program, National Cancer Institute (Rockville, MD). NB-506 and J-109,382 were kindly provided by Dr. Tomoko Yoshinari (Banyu Pharmaceutical Co., Ltd., Tsukuba, Japan). Other drugs were purchased from Sigma (St. Louis, MO). [α-^{32}P]dGTP was purchased from New England Nuclear (Boston, MA). Human top1 was purified from Sf9 cells by using a baculovirus construct (16).

Cytotoxicity Assays. The MTT assay was used to determine drug sensitivity. Cells (3,000–15,000) were seeded as a suspension (100 μl/well) in 96-well microtiter plates. The cells were incubated at 37°C in the continuous presence of drug for 3 or 5 days. Cell viability was then assayed by adding of 50 μg of MTT dye (in PBS). After a 4-h incubation period, during which

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[1] Present address: Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, Japan.

[2] Present address: INSERM U 524, Institut de Recherche sur le Cancer, Place de Verdun, 59045 Lille Cedex, France.

[3] To whom requests for reprints should be addressed, at Laboratory of Molecular Pharmacology, Building 37, Room 4E28, National Cancer Institute, NIH, Bethesda, MD 20892-4255. Fax: (301) 402-0752; E-mail: pommier@nih.gov.

[4] The abbreviations used are: CPT, camptothecin; top1, topoisomerase I; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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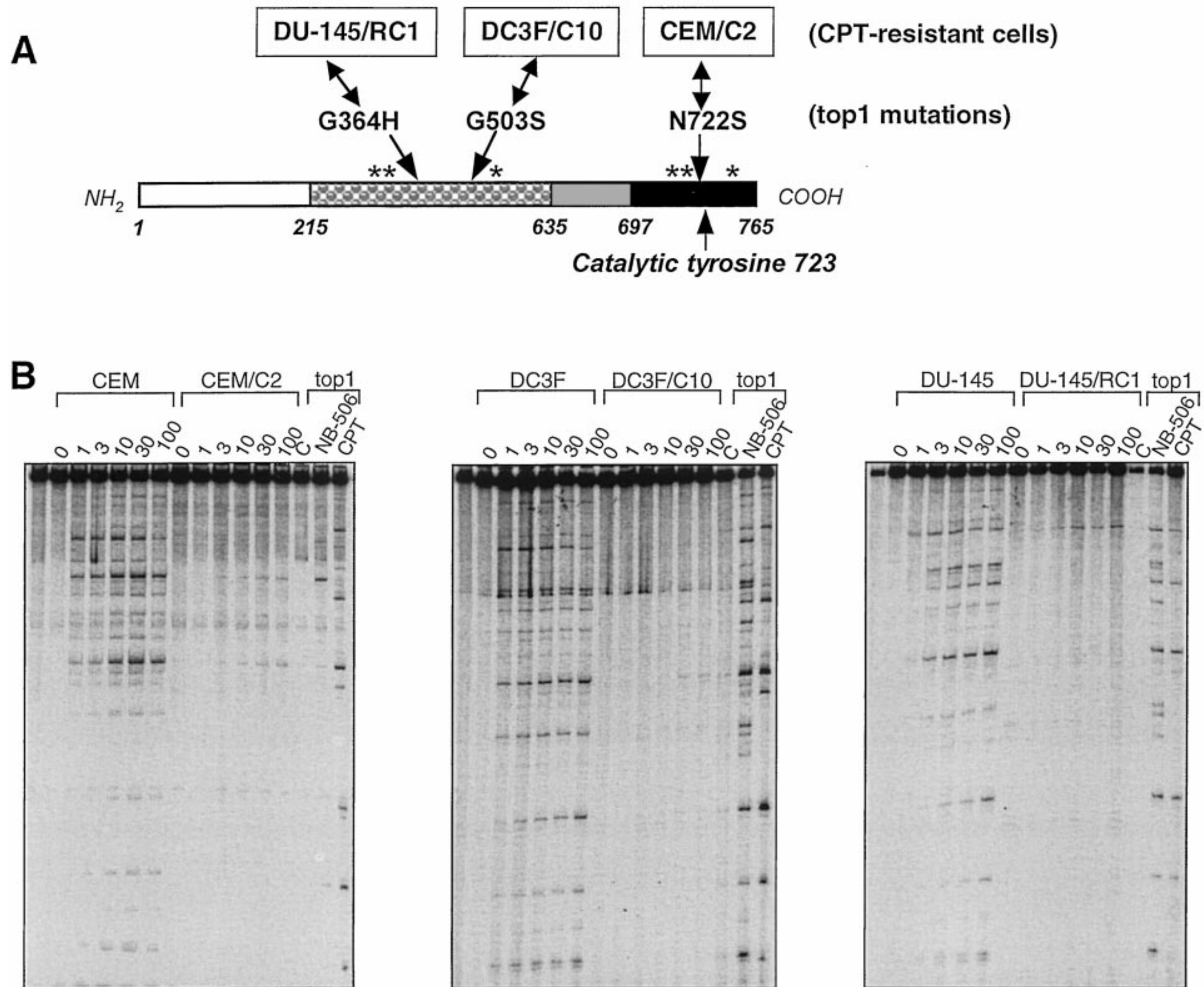

Fig. 1. Schematic representation of the top1 mutations in the CPT-resistant cell lines used and cross-resistance of CPT-resistant top1 mutants to NB-506. *A,* the COOH-terminal domain, the linker region, the core domain, and the NH_2 terminus domain are indicated from *right* to *left* as *black, gray, dotted,* and *white rectangles,* respectively. Mutations (and corresponding cell lines) used in the present study are indicated *above* the top1 schematic representation. *, position of other CPT resistance mutations (for details, see Ref. 2). *B,* nuclear extracts from parental and top1-mutant cells were used for DNA cleavage assay. ^{32}P-end-labeled *Pvu*II-*Hin*dIII 161-bp fragment from pBluescript was incubated with purified top1 or nuclear extracts (from *left* to *right,* CEM and CEM/C2, DC3F and DC3F/C10, DU-145 and DU-145/RC1, respectively) in the presence or absence of CPT or NB-506 at 25°C for 30 min. CPT was used at 1 μM, and the *numbers above* the *lanes* represent the concentrations of NB-506 in μM (from 1 to 100 μM). *C,* control without drug. Reactions were stopped with SDS (final concentration, 0.5%) and resolved in 7% sequencing gels. Imaging and quantification were performed with a PhosphorImager.

activated cells reduced the yellow MTT salt to its purple formazan, the stain was eluted into the medium by the addition of 100 μl of 2-propanol (containing 0.04 N HCl) or DMSO. Optical densities were quantified with an Emax microtiter plate reader at a test wavelength of 550 nm. Determinations for all experiments were made on triplicate, and the results were expressed as means and SDs.

Preparation of Nuclear Extracts. The method used is a modification of that described previously (17). Briefly, log-phase cultures containing 1×10^7 cells were washed twice at 4°C using nucleus buffer (150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, and 1 mM EGTA) and recovered by centrifugation at $200 \times g$ for 10 min. Cell pellets were resuspended in nucleus buffer containing 0.03% Triton X-100. After incubation at 4°C for 10 min, nucleus pellets were washed by ice-cold nucleus buffer twice. Salt extraction of the nuclear pellets was achieved by adjusting the final NaCl concentration to 0.35 M and by gentle mixing at 4°C for 30 min. After centrifugation at $12{,}000 \times g$ for 30 min, supernatants containing salt-soluble material were collected as nuclear extract.

Analysis of top1-mediated DNA Cleavage Sites. top1-mediated cleavage sites were sequenced in the 161-bp *Pvu*II-*Hin*dIII fragment of pBluescript (pSK) plasmid (Stratagene, La Jolla, CA). The fragment was singly end-labeled by a fill-in reaction. Briefly, linearized pSK (200 ng) was incubated with [α-^{32}P]dGTP in 1× labeling buffer [0.5 mM each dATP, dCTP, and dTTP in 50 mM Tris-HCl (pH 8.0),100 mM $MgCl_2$, and 50 mM NaCl] in the presence of 0.5 unit of the Klenow fragment of DNA polymerase I. Labeled DNA was purified by phenol chloroform extraction, followed by ethanol precipitation. For cleavage assays, labeled DNA (~50 fmol/reaction) was incubated with purified top1 or nuclear extract for 30 min at 25°C with or without drug in 1× reaction buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 15 μg/ml BSA]. Reactions were stopped by adding 0.5% SDS (final concentration), ethanol precipitated, and resuspended in loading buffer (80% formamide, 45 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue, pH 8.0). Reaction products were separated in a 7% denaturing polyacrylamide gels (7 M urea) in 1× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) for 2 h at 40 V × cm at 50°C. Imaging and quantitation were performed by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

top1-mediated DNA Cleavage Activity of NB-506 in the Presence of CPT-resistant Mutant top1 Enzymes. Fig. 1*B* shows the DNA cleavage sites induced by top1 in the presence of NB-506. Nuclear extracts from each of the cell lines with point mutations in different top1 domains (Fig. 1*A*), DU-145/RC1 (mutation R364H), Chinese hamster fibroblasts DC3F/C10 (mutation G503S), and human leukemia CEM/C2 cells (N722S) were used to test the activity of

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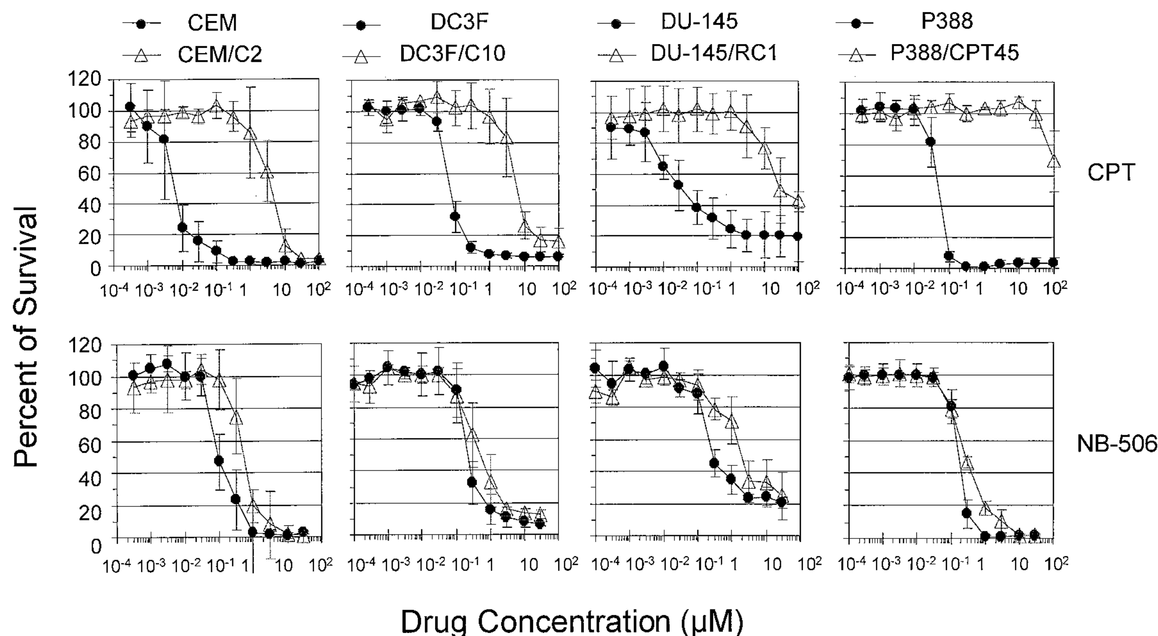

Fig. 2. Limited cross-resistance of CPT-resistant cell lines to NB-506. CEM, DC3F, DU-145, P388 (●), and their CPT-resistant derivative cell lines (CEM/C2, DC3F/C10, DU-145/RC1, and P388/CPT45; △) were treated with the indicated concentrations of CPT or NB-506. Cell survival was measured by MTT assay after 72 h of continuous drug exposure. Data were calculated from at least three independent experiments; *bars,* SD.

NB-506 in the presence of the CPT-resistant top1 enzymes (2, 13–15, 18). Comparison between the three mutant and parental cells showed that DNA cleavage activity was markedly reduced in the presence of the mutant top1 enzymes, even at 100 μM of NB-506. These results demonstrate that all three mutations that lead to CPT-resistance also render top1 resistant to NB-506.

For each panel, the three right lanes show a comparison between the DNA cleavage patterns observed in the presence of NB-506 and CPT. Consistent with previous results (19), the overall DNA cleavage patterns showed some similarities and differences between NB-506 and CPT. A number of cleavage sites were common to both drugs, whereas other sites appeared drug specific.

Drug Sensitivity to NB-506 in a CPT-resistant Cell Line. Because NB-506 showed high cross-resistance to mutant top1 enzymes in DNA cleavage assays, we evaluated whether the corresponding mammalian cells were cross-resistant to both CPT and NB-506 in cell proliferation assays. For this purpose, we used an additional cell line P388/CPT45. This cell line is cultured with 45 μM CPT and is highly CPT resistant. The resistance ratio to CPT was >2000 fold. Western blot analysis using monoclonal antibody against top1 does not detect top1 in P388/CPT45. This cell line was used recently to show that the top1 inhibition might contribute to the antiproliferative activity of 1-β-D-arabinofuranosylcytosine (12). The antiproliferative activity for NB-506 and CPT was evaluated by MTT assays (Fig. 2). Relative resistances were calculated for both drugs (Table 1). Cross-resistance to NB-506 was relatively small in all four cell lines, ranging from 2- to 9-fold. The top1-deficient cells (P388/CPT45 only) showed a 2-fold resistance to NB-506, whereas resistance to CPT was >2000-fold. These results demonstrate that NB-506 does not require the presence of top1 to exert its antiproliferative activity, and that top1 mutations that render the enzyme highly resistant to NB-506 confer only minimal resistance to the drug.

Table 1 *Antiproliferative activity of the indolocarbazoles (NB-506 and J-109,382) and CPT measured as IC_{50} ± SD by MTT assay*

	CPT (μM)	RR[a]	NB-506 (μM)	RR	J-109,382 (μM)	RR
CEM	0.01 ± 0.01		0.11 ± 0.04		0.027 ± 0.029	
CEM/C2	5.4 ± 2.9	540	0.61 ± 0.17	6	0.13 ± 0.10	5
DC3F	0.07 ± 0.01		0.20 ± 0.02		0.023 ± 0.004	
DC3F/C10	5.9 ± 1.8	84	0.71 ± 0.57	4	0.097 ± 0.047	4
DU-145	0.06 ± 0.06		0.23 ± 0.06		0.16 ± 0.11	
DU-145/RC1	21.7 ± 7.6	361	1.97 ± 0.85	9	1.75 ± 1.3	11
P388	0.05 ± 0.01		0.16 ± 0.04		0.017 ± 0.005	
P388/CPT45	>100	>2000	0.3 ± 0.1	2	0.11 ± 0.10	6

[a] RR, resistance ratio, calculated as the ratio of the IC_{50} in the CPT-resistant cells relative to the parental cell line.

Role of DNA Intercalation in the Antiproliferative Activity of NB-506. J-109,382 is an isomer of NB-506 in which the hydroxyl groups on the indolocarbazole ring are at positions 2 and 10 instead of 1 and 11 (Fig. 3). It was shown that DNA intercalation is not detectable with J-109,382 at concentrations where NB-506 has strong intercalating activity, whereas both compounds are effective top1 inhibitors (20). Thus, we used J-109,382 to examine the importance of intercalation for the antiproliferative activity of NB-506 (Fig. 3; Table 1). As in the case of NB-506, all of the CPT-resistant cells showed only limited resistance to J-109,382, which suggests that DNA intercalation is not likely to account for the top1-independent cytotoxicity of the indolocarbazoles, J-109,382 and NB-506.

Discussion

NB-506 is an important agent for cancer chemotherapy because it is in clinical trials and because it is the lead derivative for the indolocarbazoles, a new class of non-CPT top1 poisons. These agents were developed because of the remarkable activity of CPT derivatives in animal models and in clinical studies, and because of some of the known limitations of CPTs (21–23). These include rapid inactivation in human serum by E-ring opening that converts the α-hydroxylactone into a carboxylate, which is inactive against top1, and high binding to human serum albumin (24). Indolocarbazoles are among the most potent non-CPT top1 poisons and show excellent activity in preclinical models (reviewed in Refs. 4 and 5). The two main aspects of the present study are related to the drugs binding site(s) in the top1-DNA complex and to the role of top1 poisoning in the antiproliferative activity of NB-506.

Our observations are consistent with the possibility that indolocarbazoles bind to a site, which is at least in part common to the CPT

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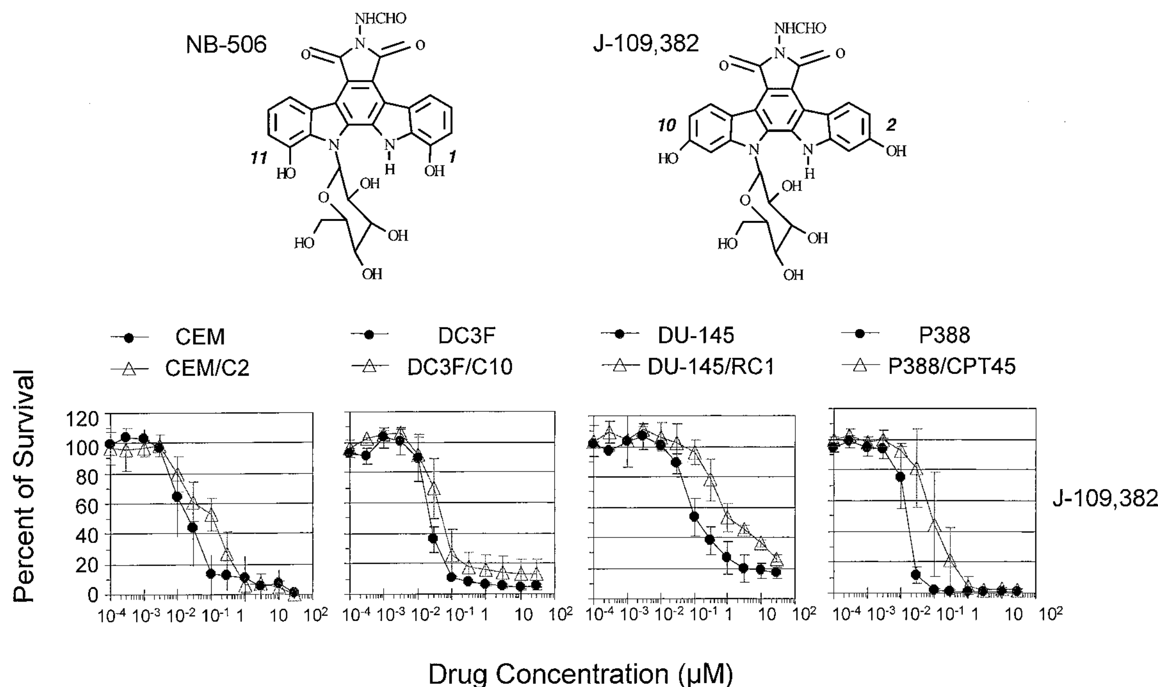

Fig. 3. Limited cross-resistance of CPT-resistant cell lines to J-109,382. CEM, DC3F, DU-145, P388 (●) and their top1-altered derivative cell lines (CEM/C2, DC3F/C10, DU-145/RC1, and P388/CPT45; △) were treated with the indicated concentrations of J-109,382. Cell survival was measured by MTT assay after 72 h of continuous drug exposure. Data were calculated from at least three independent experiments; *bars,* SD. Structure of NB-506 and J-109,382 are shown at the *top* of the figure.

binding site in the top1-DNA complex. This conclusion is based upon the fact that the three top1 point mutants examined (R364H, G503S, and N722S) were highly resistant to top1-mediated DNA cleavage. In the top1 protein structure (25, 26), these three residues are next to each other and to the DNA at the point of DNA cleavage. Another mutation, F361S, which is adjacent to the R364 residue, was also found recently to confer resistance to both NB-506 and CPT (27). It is also striking that a number of the top1-mediated DNA cleavage sites are common to both drugs, and that NB-506 and CPT share the same DNA base sequence preference (for guanine at the +1 position, immediately 3′ to the top1 cleavage site; Refs. 19 and 28). NB-506 differs from CPT by a more relaxed base preference at the −1 position (5′ from the DNA cleavage site; Ref. 19). Our interpretation of these observations is that both NB-506 and CPT share a common binding site at the enzyme-DNA interface. The corresponding drug binding pocket can be defined by the enzyme residues F361, R364, G503, and N722 and by the −1 and +1 bp on the DNA side. This conclusion is consistent with our previously proposed drug stacking model for top1 inhibition by CPTs (28). There is yet no structure for a drug bound in the top1-DNA complex. Two models have been proposed (25, 29). They are both consistent with the importance of the +1 bp (28, 30) and of the top1 amino acid residues outlined above. Hence, top1 poison represent a paradigm in which a small ligand (the top1 inhibitor) blocks a bimolecular biological process (the top1-DNA reaction) by enhancing the association of the two macromolecules (top1 and the DNA) in a ternary complex (drug-top1-DNA).

Although top1 is a target for NB-506, the antiproliferative activity of NB-506 must also result from interference(s) with other cellular processes. The fact that all three cell lines that expressed an NB-506-resistant top1 only showed <10-fold resistance to NB-506 in the cytotoxicity assays suggests that NB-506 has another target besides top1. This possibility is also stressed by the sensitivity of the top1-deficient cells (P388/CPT45; Refs. 10–12) to NB-506. DNA intercalation does not appear to be this other target, based on the lack of detectable intercalating activity of J-109,382 (20) and on the low cross-resistance profile of J-109,382 in the four cell pairs examined. Indolocarbazoles are among the most versatile molecules with regard to potential cellular targets. Staurosporine inhibits a range of protein kinases, and its 7-hydroxy derivative, UCN-01, was initially characterized as a protein kinase C inhibitor (31) and was later found to be a cell cycle checkpoint abrogator (32, 33). UCN-01 has been found recently to inhibit Chk1 (32), one of the key cell cycle checkpoint regulatory kinases. There is no evidence that NB-506 exhibits anti-protein kinase activity. In fact, early studies showed that NB-506 had no activity on protein kinase C at pharmacological concentrations (34) Determination of the cellular targets of NB-506 might be facilitated by the development of drug-resistant cell lines.

To our knowledge, only two cell lines have been reported. In the first case, the resistance of human lung cancer cells to NB-506 was attributed to a 10 times reduced expression of top1 (35). This cell line, SBC-3/NB, was found to be cross-resistant to the camptothecin derivative SN-38 but not to other anticancer agents, such as Adriamycin, etoposide, and vincristine. However, the resistance to SN-38 was weak (×22) compared with that measured with NB-506 (×454). In the second case, the cells have a unique type of top1 rearrangement that duplicated part of the NH_2 terminus domain. The corresponding top1 enzyme was resistant to NB-506 and CPT, as were the cells to the cytotoxicity of both drugs (36). These previously published studies suggested a role for top1 in the antiproliferative activities of NB-506. Our observations are consistent with this conclusion in the sense that top1 appears to be a target of NB-506 at low concentrations. However, it is clear that other cellular processes besides top1 and DNA intercalation are probably targeted by NB-506 at pharmacological concentrations. Therefore, NB-506 appears to be a promising agent to overcome CPT resistance.

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Use of Camptothecin-resistant Mammalian Cell Lines to Evaluate the Role of Topoisomerase I in the Antiproliferative Activity of the Indolocarbazole, NB-506, and Its Topoisomerase I Binding Site

Yoshimasa Urasaki, Gary Laco, Yuji Takebayashi, et al.

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