Antitumor Activities of a New Indolocarbazole Substance, NB-506, and Establishment of NB-506-resistant Cell Lines, SBC-3/NB

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ABSTRACT

The novel anticancer glucosyl derivative of indolo-carbazole (NB-506), an inhibitor of DNA topoisomerase I, exhibited strong in vitro cytotoxicity against various human cancer cell lines. In order to elucidate its cytotoxic mechanisms, we established nine NB-506-resistant sublines with different resistance ratios from human small cell lung cancer cells (SBC-3/P) by stepwise and brief exposure (24 h) to NB-506. Among them, SBC-3/NB#9 was 454 times more resistant to NB-506 than the parent cell line. The SBC-3/NB#9 cells showed cross-resistance only to topoisomerase I inhibitors, such as 11,7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin and 7-ethyl-10-hydroxy-camptothecin, and not to other anticancer drugs, such as vincristine, vinblastine, Adriamycin, etoposide, and teniposide. These results indicate that the difference on the effect of topoisomerase I was considered to be related to a resistance mechanism. The topoisomerase I activities of nuclear extracts eluted from SBC-3/NB#9 cells was only one-tenth of the parent cell activity. A Western blotting study indicated that this lower activity was due to a lower amount of DNA topoisomerase I. Furthermore, we found correlations between topoisomerase I activity and sensitivity to NB-506 in sublines with different degrees of resistance. Accumulation of [3H]-labeled NB-506 by SBC-3/NB#9 cells was only one-fifth of that by the parent cells, whereas intracellular accumulation of [3H]-labeled camptothecin by both cell lines did not differ. The reduction of accumulation was specific to NB-506, and this result may explain why the resistance ratio for NB-506 was higher than those for 11,7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin and 7-ethyl-10-hydroxy-camptothecin.

INTRODUCTION

The DNA topoisomerases are nuclear enzymes that catalyze the concerted breaking and rejoining of DNA strands, thereby controlling the topological states of DNA. Topoisomerase I catalyzes the passage of DNA strands through transient single-strand breaks, whereas topoisomerase II catalyzes the passage of DNA double strands through transient double-strand breaks. These topoisomerases are known to be involved in many DNA metabolic processes, including replication, recombination, transcription, and chromosome segregation at mitosis. In addition to their normal cellular functions, both topoisomerases I and II have generated extensive clinical interest as targets for cancer chemotherapy.

Eukaryotic topoisomerase II is the target for intercalating antitumor agents, such as 4'-[9-acridinylamino]-methanesulfon-m-anisidine, and Adriamycin, as well as nonintercalating agents like etoposide and teniposide. Euarkyotic topoisomerase I is the target for the antitumor plant alkaloid camptothecin (2-4) and its synthetic derivatives, such as CPT-11 (5) and topotecan (6). High levels of topoisomerase II in proliferating cells and very low levels in quiescent cells appear to explain the selective sensitivities of proliferative tumor cells to the cytotoxic effects of topoisomerase II-targeting drugs (3). In contrast, the intracellular levels of topoisomerase I have been reported to be largely unaffected by cultured cell growth conditions. However, the findings of Giovannella et al. (7) that topoisomerase I levels were elevated in advanced stage human colon cancer tissues compared with those in normal colon tissues lend support to the possibility that this enzyme also is an important target for antitumor drugs. In agreement with these results, recent clinical trials of camptothecin derivatives have shown that these drugs are potentially promising new antitumor agents (8-10).

Therefore, the identification of new drugs that induce the formation of cleavable topoisomerase I complexes is now viewed as a promising approach to finding clinically effective antitumor agents. In an attempt to discover new antitumor agents that target topoisomerase I, Kojiri et al. (11) screened Actinomycetes culture supernatants and isolated a novel indolocarbazole antibiotic, BE-13793C. Although BE13793C showed topoisomerase-inhibiting activity and good activity against Ehrlich ascites tumor cells, its low solubility in water hampered further evaluation. Therefore, they synthesized a glucosyl derivative of BE-13793C, NB-506 (the chemical structure of which is shown in Fig. 1), which was more water soluble. NB-506 was shown to have strong effects in inhibiting the growth of various experimental tumors, including solid tumors, not only in vitro but also in vivo. Its cytotoxicity was demonstrated to lead by the induction of topoisomerase I-mediated DNA cleavage (13).

In this study, we evaluated the activity of NB-506 against human tumor cells in vitro. Furthermore, we tried to establish NB-506-resistant cell lines and elucidate the likely mechanisms of their drug resistance, because development of drug resistance is likely to be a major limiting factor in determining the clinical success of NB-506.

MATERIALS AND METHODS

Materials. The indolocarbazole derivative, NB-506, and its tritium-labeled compound were obtained from Banyu Tsukuba Research Institute (Tsukuba, Japan). CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin and SN-38 (7-ethyl-1-hydroxy-CPT) were obtained from Yakult Co., Ltd. (Tokyo, Japan). CIpsatin, etoposide, and teniposide were gifts from Bristol Myers Japan (Tokyo, Japan), and 5-fluourouracil was supplied by Yakult Co., Ltd. (Tokyo, Japan). Adriamycin, vincristine, vinblastine, melphalan, and bleomycin were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), Shionogi Pharmaceutical Co. (Osaka, Japan), Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan), Sigma Chemical Co. (St. Louis, MO), and Nihon Kayaku Co., Ltd. (Tokyo, Japan), respectively. Plasmid pBR322 DNA was purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan), and tritium-labeled 12-[H]-camptothecin was purchased from Moravek Biochemicals, Inc. (Brea, CA).

Cell Culture and Isolation of NB-506-resistant Cell Lines. The cell line used as the parent for obtaining NB-506-resistant sublines was SBC-3/P, which is sensitive to NB-506 and CPT-11 and was derived from a human small cell lung carcinoma (kindly provided by Professor Kimura, Okayama University, School of Medicine, Okayama, Japan). The cells were propagated in RPMI 1640 supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 units penicillin/ml in an incubator under a humidified atmosphere of 5% CO2 and air, as described previously (14).
Each NB-506-resistant cell line was selected from a subculture that had acquired resistance to NB-506 as a result of stepwise and brief exposure to NB-506. Cultured SBC-3 cells were exposed to NB-506 at an initial concentration of 2.5 μg/ml for 24 h and then were washed and cultured in drug-free medium for 6 days. This treatment was repeated when the treated cells grew at the same proliferation rate as untreated cells, and the resulting cells were exposed to a concentration of the drug about three times higher than the medium for 6 days. This treatment was repeated when the treated cells grew at the same proliferation rate as untreated cells, and the resulting cells were cloned using the limiting dilution technique (Fig. 2).

Drug Sensitivity Test. The sensitivity and resistance of each cell line to NB-506 and other drugs were estimated using three different methods. (a) We evaluated the cytotoxic effect of continuous drug exposure on cell proliferation using the regrowth assay described previously (15). Duplicate 10-ml culture flasks, initially containing 2.5 × 10^6 cells/ml medium and the required drugs at various concentrations, were incubated for 7 days at 37°C under a humidified atmosphere of 5% carbon dioxide and 95% air, after which the cells were counted either with a TOA Medical Electronics Co., Kobe, Japan) or under a microscope, and the cell proliferation ratio of treated to control cultures was calculated. The antiproliferative activities were expressed as IC₅₀.

(b) For the soft agar colony inhibition assay (15), the cells were exposed to drugs in the agar throughout the growth period in vitro. The exponentially growing cells (9 × 10^6) were suspended in 2.7 ml RPMI 1640 containing the required drug, to which 0.3 ml hot agar solution (3%) was added. The resulting mixture was plated as the top layer on an underlayer of RPMI 1640 supplemented with 0.5% agar in a 35-mm plastic dish, and the plates were incubated under a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C for 9 days, after which the number of colonies on the triplicate control and drug-treated plates were counted by a computerized image analyzer ( Colony Analyzer CA-7A; Oriental Instrument Ltd., Tokyo, Japan).

(c) The MTT assay was performed as follows. Aliquots (100 μl) of an exponentially growing cell suspension (1 × 10^6 cells/ml) were seeded in 96-well microtiter plates, and 100-μl aliquots of the required drug solution (various concentrations) were added. After exposure to the drugs for 96 h at 37°C, 20 μl MTT solution (5 mg/ml in PBS) was added to each well. The plates were incubated at 37°C for an additional 4 h and centrifuged at 800 × g for 5 min.

The medium was aspirated from each well as completely as possible. DMSO (200 μl) was added to each well to dissolve the formazan, and the absorbances of the solution in each well were measured at 562 and 630 nm using Delta-soft ELISA analysis for a Macintosh computer interfaced to a Bio-Tek Microplate Reader (EL-340; Bio Metallics, Princeton, NJ). Wells containing only RPMI-FBS and MTT were used as controls. Each experiment was performed using six replicate wells for each drug concentration, and three independent experiments were carried out (16).

Accumulation Study. To evaluate drug accumulation, exponentially growing SBC-3/P and SBC-3/NB #9 cells were harvested. Cells (2 × 10^6) in 0.98 ml culture medium were preincubated at 37°C in a water bath for 45 min and then exposed to 75 μM [3H]NB-506 or camptothecin at 37°C in a humidified incubator with 5% CO₂. After various incubation times, the cells were collected by low-speed centrifugation, washed once with cold PBS, and recentrifuged. The resulting cell pellet was dissolved in 0.5 ml formic acid and 3 ml Celsol 1 solution was added (Nacalai Tesque, Kyoto, Japan) to each tube, and the radioactivity was measured with a liquid scintillation counter (LS6000TA; Beckman, Irvine, CA; Ref. 17).

Preparation of Nuclear Extracts and DNA Topoisomerase I Activity Assay. Crude nuclear extracts were prepared, as described by Deffie et al. (18). The cells were collected by centrifugation and washed twice with ice-cold nuclear buffer (NB; pH 6.5, 2 mM KH₂PO₄, 5 mM MgCl₂, 150 mM NaCl, 1 mM EGTA, and 0.1 mM DTT), recentrifuged, resuspended in 1 ml cold NB, and 9 ml cold NB containing 0.35% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride were added. The cell suspension was kept on ice for 10 min and washed with Triton X-100-free cold NB; then the nuclear protein was eluted for 1 h at 4°C with cold NB containing 0.35 M NaCl. A nuclear protein solution...
was obtained by centrifugation at 18,000 \( \times \) g for 10 min, and its protein concentration was determined using the method of Bradford (19) with bovine plasma \( \gamma \)-globulin as the standard.

The DNA topoisomerase I activity was determined by measuring the relaxation of supercoiled \( Escherichia \) coli DNA (pBR322), essentially as described by Liu and Miller (20). The reaction mixtures for measuring the total topoisomerase I activities in both cell lines comprised 100 mm KCl, 10 mm MgCl\(_2\), 1 mm DTT, 0.1 mm EDTA, 10\% glycerol, 50 mm Tris-HCl (pH 7.4), 1.7 \( \mu \)g pBR322 DNA, and crude nuclear extracts (0.001–2.0 \( \mu \)g protein).

The reaction mixtures for measuring the inhibition of DNA relaxation by NB-506 contained the specified concentrations of drug and amounts of nuclear extract (0.01 \( \mu \)g for SBC-3/P and 0.05 \( \mu \)g for SBC-3/NB#9) in addition to the above components. The reaction mixtures were incubated at 37°C for 20 min, and the reactions were terminated by adding 5 \( \mu \)l dye solution (2.5% SDS and 0.01\% bromophenol blue in water). These samples were applied to 0.7% agarose gel and electrophoresed for 3 h with a running buffer of Tris-acetate and 0.01\% bromophenol blue in water. These samples were stained with 2 \( \mu \)M ethidium bromide and photographed under transillumination with 300 nm UV light.

DNA Topoisomerase I Content. We used Western blotting analysis with topoisomerase I antiserum (20) to determine the cellular topoisomerase I contents. In brief, stored nuclear extracts (–80°C) were analyzed on 12.5% polyacrylamide slab gels containing SDS (21). Equivalent amounts of nuclear protein from each type of cell were applied to the gel and electrophoresed; then the proteins on the gels were transferred electrophoretically to nitrocellulose membranes (22), which were placed in 50 \( \mu \)M Tris/400 \( \mu \)M NaCl (pH 7.5) buffer containing 0.05\% Tween 20 and rinsed. All the rinses lasted 45 min with three changes of buffer, and all the incubations lasted 1 h. The membranes were rinsed and incubated with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, VA) in the above buffer containing 3\% chloro-4-naphthol and \( \mathrm{H}_2\mathrm{O}_2 \) in the above buffer containing 11\% methanol. The reaction was stopped by rinsing with water.

**RESULTS**

Cytotoxicity of NB-506 against Human Tumors and Their CPT-resistant Cell Lines in Vitro

The cytotoxicity of NB-506 was examined on various human tumors, namely human small cell (SBC-3 and H69) and non-small cell (PC-7, PC-9, and PC-14) lung cancer and human leukemia (K562 and RPMI8402) cell lines, and the results are shown in Table 1. The IC\(_{50}\) of NB-506 against RPMI8402, H69, and SBC-3 were 0.15, 0.14, and 0.046 \( \mu \)g/ml, respectively, which were significantly lower than those of CPT-11 (14). The IC\(_{50}\) for SBC-3 small cell lung cancer cell line, which, therefore, was used as the parent cell line for establishing NB-506-resistant cells.

Next, we examined whether RPMI8402/K5 and PC-7/CPT cells, which are resistant to CPT-11, were cross-resistant to NB-506 (Fig. 3). Both these cells showed cross-resistance to NB-506, which indicates that the cytotoxic mechanism of NB-506 is associated with topoisomerase I, because the CPT-11 resistance mechanism in these cells has been reported to be attributable to reduced topoisomerase I activity (14).

**Establishment of NB-506-resistant SBC-3 Cells.** Several cell lines resistant to NB-506 were established using the technique described previously for selecting of the PC-7/CPT sublines (14). Cultured SBC-3/P cells were exposed to NB-506 at an initial concentration of 2.5 \( \mu \)g/ml for 24 h, washed, and cultured in drug-free medium until cell proliferation resumed. Then, the resulting cells were exposed to about three times the previous drug concentration. This procedure, termed a drug treatment episode, was repeated when the cells showed the same proliferation ratio as untreated cultures. After the treatment episode with 10 \( \mu \)g/ml NB-506, the cultured cells were cloned using a dilution technique, which resulted in the establishment of four NB-506-resistant SBC-3 sublines (designated SBC-3/NB#1, #2, #3, and #4). Furthermore, SBC-3/NB#5 and #6 sublines were derived from a subcultured that acquired resistance to NB-506 by passing through three more successive episodes with 25, 50, and 100 \( \mu \)g/ml NB-506; and SBC-3/NB#7, #8, and #9 sublines were established from a subculture that was passed through an additional episode with 250 \( \mu \)g/ml NB-506 (shown in Fig. 2).

At concentrations of 0.01, 0.025, 0.05, and 0.25 \( \mu \)g/ml, NB-506 inhibited proliferation of the parent SBC-3 cells, determined using the regrowth assay, by 1, 25, 55, and 87\%, respectively. The concentration-response curves of the nine NB-506-resistant sublines against NB-506 are shown in Fig. 4, and the resistance ratios obtained from their concentration-response curves are summarized in Table 2. This table showed a tendency that the large number of treatment and the high concentration of NB-506 produced a high resistance. The SBC-3/NB#9 cells were the most resistant of these cell lines to NB-506; its IC\(_{50}\) was 21.3 \( \mu \)g/ml, which was 454-fold that against SBC-3/P cells. Therefore, SBC-3/NB#9 was selected for further study. The IC\(_{50}\) of NB-506 against subline SBC-3/NB#9f cells, which were cultured in drug-free medium for 10 weeks, was 21.0 \( \pm \) 0.52 \( \mu \)g/ml. Therefore, the acquired resistance to NB-506 was stable after prolonged growth in nonselective medium.

The colony formation assay (human tumor clonogenic cell assay) yielded an IC\(_{50}\) for NB-506 against SBC-3/P cells of 0.045 \( \mu \)g/ml (Fig. 5A), whereas that against SBC-3/NB#9 cells was 38 \( \mu \)g/ml, more than 800-fold that against the parent cells. The resistance ratio, determined using the MTT assay, was 4000-fold that of the parent cells (300 \( \mu \)g/ml for SBC-3/NB#9 versus 0.06 \( \mu \)g/ml for SBC-3/P; Fig. 5B).

**Characterization of NB-506-resistant Subline SBC-3/NB#9 Cells.** The doubling times of SBC-3/P and SBC-3/NB#9 cells were 15.2 and 16.4 h, respectively, their respective plating efficiencies were 1.3 and 1.2\%, and the parent and resistant cell diameters were 1.13 \( \pm \) 0.089 and 1.13 \( \pm \) 0.091 \( \mu \)m, respectively. None of these differences were significant. These findings were important for the study of mechanisms of resistance to NB-506, because the cytotoxic activity of NB-506 was cell cycle specific.

**Sensitivities of SBC-3/NB#9 Subline Cells to Other Anticancer Drugs.** Table 3 shows the cytotoxic effects (IC\(_{50}\) \( \mu \)g/ml) of various anticancer drugs against SBC-3/P and its resistant subline, SBC-3/NB#9. The IC\(_{50}\) of CPT-11 against SBC-3/P was 0.11 \( \mu \)g/ml, one-eighth that against SBC-3/NB#9. This subline also showed cross-resistance only to topoisomerase I inhibitors and none towards cisplatin, etoposide, teniposide, vinblastine, or vincristine. These results indicate that the resistance mechanism of these cells is unlikely to be that associated with typical multidrug resistance.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Cytotoxicity (IC(_{50}) ± SD; ( \mu )g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBC-3 (Small cell lung cancer)</td>
<td>0.046 ± 0.0083 0.11 ± 0.02</td>
</tr>
<tr>
<td>H69 (Small cell lung cancer)</td>
<td>0.14 ± 0.057 0.7 ± 0.11</td>
</tr>
<tr>
<td>PC-7 (Non-small cell lung cancer)</td>
<td>0.11 ± 0.0069 0.037 ± 0.0021</td>
</tr>
<tr>
<td>PC-9 (Non-small cell lung cancer)</td>
<td>6.9 ± 1.56 1.27 ± 0.061</td>
</tr>
<tr>
<td>PC-14 (Non-small cell lung cancer)</td>
<td>1.8 ± 0.14 2.6 ± 0.99</td>
</tr>
<tr>
<td>K562 (Leukemia)</td>
<td>0.83 ± 0.15 0.6 ± 0.11</td>
</tr>
<tr>
<td>RPMI8402 (Leukemia)</td>
<td>0.15 ± 0.04 0.76 ± 0.048</td>
</tr>
</tbody>
</table>

Table 1 In vitro cytotoxicity of NB-506 against various human tumor cell lines

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Figure 3. Cytotoxicity of NB-506, CPT-11, and SN-38 against RPM18402 and PC-7 and their corresponding CPT-11-resistant cells. The antitumor activities of the drugs were measured using the regrowth assay, as described in "Materials and Methods." Exponentially growing cells (3 x 10^6/10 ml) were incubated in medium containing the required drug at the indicated concentrations at 37°C for 7 days; after which, the cells were counted, and cell growth ratios were calculated. A, concentration-response curves of NB-506 and SN-38 against RPM18402 and K5. B, concentration-response curves of NB-506, CPT-11, and SN-38 against PC-7/P and PC-7/CPT.

**Intracellular Accumulation of NB-506.** The time course of NB-506 uptake by SBC-3/P cells is shown in Fig. 6A. When incubated with 10 μg/ml [3H]NB-506 (0.68 μCi), uptake by SBC-3/NB#9 cells was relatively slow, and steady-state was reached after 30 min, whereas SBC-3/P cells had not reached steady-state after 2 h. The rate constant determined from the initial uptake rate was 3.59 ± 0.10 min⁻¹ (mean ± SE) for the SBC-3/P cells and 0.89 ± 0.15 min⁻¹ for the SBC-3/NB#9 cells, which reflects the finding that the initial uptake by the NB-506-resistant cells (SBC-3/NB#9) was only 24.8% of that by the SBC-3/P cells. The accumulation rate constants of [3H]CPT for SBC-3/P and SBC-3/NB#9 cells were 53.3 ± 10.1 and 58.7 ± 5.8 min⁻¹, respectively (shown in Fig. 6B), and the cellular uptake of camptothecin by these two cell lines did not differ significantly. The decreased drug uptake by SBC-3/NB#9 compared with its parent cells was found to be specific for NB-506.

**Activity and Content of Topoisomerase I and the Effects of CPT-11 on Them.** The total cellular activities of DNA topoisomerase I of SBC-3/P and its NB-506-resistant sublines in the crude nuclear extracts eluted with 0.35 M NaCl were measured. The relaxation of pBR322 DNA incubated with different amounts of SBC-3/P and SBC-3/NB#9 cell nuclear protein extracts is shown in Fig. 7. In this experiment, the relaxation of supercoiled DNA catalyzed by topoisomerase I was monitored using gel electrophoresis. Relaxed forms were observed in the presence of only 0.005 μg SBC-3/P nuclear extract, whereas the SBC-3/NB#9 cell nuclear extract did not catalyze supercoiled DNA relaxation, even when 0.02 μg was used, which indicates that the topoisomerase I activity of SBC-3/NB#9 cells was about one-tenth that of SBC-3/P cells. The topoisomerase I activities of the other NB-506-resistant sublines also were lower; the amount of topoisomerase I protein required to relax all of the supercoiled DNA completely was 0.05 μg for the SBC-3/#6 and #9 sublines; 0.02 μg for SBC-3/NB#3, #4, #7, and #8; and 0.01 μg for SBC-3/NB#1 and #5. We concluded that the resistance of these sublines to NB-506 is due to topoisomerase I reduced activity.

To determine whether the reduced DNA topoisomerase I activities were due to reductions in the cellular contents, the DNA topoisomerase I contents of the nuclear extracts were measured using an immunoblotting assay. From the results of this study in Fig. 8, it appeared that the NB-506-resistant sublines contained a reduced amount of topoisomerase I protein in comparison to the parent cells. Furthermore, the degree of its reduction was correlated to the activity of topoisomerase I, indicating that the reduced activity was caused by reduction of amount of DNA topoisomerase I.

The effects of NB-506 on the catalytic activities of DNA topoisomerase I extracted from SBC-3/P and SBC-3/NB#9 cells were determined using 10 and 50 ng, respectively, of their nuclear extracts to adjust for their different DNA relaxation potencies in the absence
Table 2 Drug sensitivity of various clones of SBC-3 cells resistant to NB-506

<table>
<thead>
<tr>
<th>Clones</th>
<th>Cytotoxicity (IC50 ± SD; µg/ml)</th>
<th>Resistance ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBC-3/P</td>
<td>0.78 ± 0.16</td>
<td>17</td>
</tr>
<tr>
<td>SBC-3/#1</td>
<td>1.12 ± 0.28</td>
<td>24</td>
</tr>
<tr>
<td>SBC-3/#2</td>
<td>0.7 ± 0.38</td>
<td>15</td>
</tr>
<tr>
<td>SBC-3/#4</td>
<td>0.91 ± 0.25</td>
<td>19</td>
</tr>
<tr>
<td>SBC-3/#5</td>
<td>1.33 ± 0.06</td>
<td>28</td>
</tr>
<tr>
<td>SBC-3/#6</td>
<td>16.33 ± 2.89</td>
<td>347</td>
</tr>
<tr>
<td>SBC-3/#7</td>
<td>4.97 ± 0.15</td>
<td>106</td>
</tr>
<tr>
<td>SBC-3/#8</td>
<td>12.33 ± 2.08</td>
<td>262</td>
</tr>
<tr>
<td>SBC-3/#9</td>
<td>21.33 ± 2.08</td>
<td>454</td>
</tr>
<tr>
<td>SBC-3/#9f</td>
<td>19.09 ± 3.33</td>
<td>406</td>
</tr>
<tr>
<td>SBC-3/P</td>
<td>0.047 ± 0.0021</td>
<td></td>
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</tbody>
</table>

Fig. 5. Concentration-response curves of NB-506 on the growth of the SBC-3/P and SBC-3/NB#9, measured using the colony-forming and MTT assays. A, colony forming assay: exponentially growing cells (3 × 10^5/dish) were seeded in soft agar medium containing NB-506 at the indicated concentrations and cultured for 9 days, after which the colonies were counted with a computerized image analyzer. B, MTT assay: exponentially growing cells (1 × 10^5/well) were seeded in 96-well microtiter plates and exposed to NB-506. After a 4-day culture period, MTT was added to each well, the plates were incubated for a further 4 h, and then centrifuged; then the medium was removed, DMSO was added to each well to dissolve the pigment (formazan) crystals, and the absorbances of the solution were measured at 562 and 630 nm using a Bio-Tek Microplate Reader.

DISCUSSION

The new semisynthetic antitumor agent NB506 is derived from a novel indolocarbazole antibiotic produced by an Actinomycete (12). Recently, several indolocarbazole compounds were reported to show activity against experimental tumors in vivo. Two such compounds, 7-hydroxystaurosporine (UCN-01; Refs. 23 and 24) and 4'-benzoylstaurosporine (CGP 41 251; Ref. 25) appeared to exert antitumor activity via inhibition of protein kinases, including PKC, whereas rebeccamycin (26) and AT2433 (27) exerted their antitumor activity via interaction with DNA but demonstrated little inhibition of PKC (28). In a cell-free system, PKC activity was virtually unaffected by NB-506, which was postulated to act via inhibition of topoisomerase I-mediated DNA cleavage (29).

One of the aims of this study was to evaluate the efficacy of NB-506 by comparing it with another topoisomerase I inhibitor, CPT-11, currently undergoing Phases I and II clinical evaluation in adult and pediatric patients with malignant disease (8–10). Another aim was to establish an NB-506-resistant tumor cell line and elucidate its resistance mechanism, because the development of drug resistance...
Table 3  Cytotoxicity of various antitumor drugs against SBC-3/P and SBC-3/NB#9 cells

<table>
<thead>
<tr>
<th>Drugs</th>
<th>SBC-3/P IC50 ± SD (µg/ml)</th>
<th>SBC-3/NB#9 IC50 ± SD (µg/ml)</th>
<th>Resistance ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB-506</td>
<td>0.047 ± 0.0083</td>
<td>21.3 ± 1.53</td>
<td>454.00</td>
</tr>
<tr>
<td>CPT-11</td>
<td>0.11 ± 0.02</td>
<td>0.84 ± 0.14</td>
<td>7.60</td>
</tr>
<tr>
<td>SN-38</td>
<td>0.00016 ± 0.00012</td>
<td>0.0023 ± 0.00014</td>
<td>22.00</td>
</tr>
<tr>
<td>CDDP</td>
<td>0.094 ± 0.016</td>
<td>0.065 ± 0.012</td>
<td>0.69</td>
</tr>
<tr>
<td>VP-16</td>
<td>0.031 ± 0.011</td>
<td>0.042 ± 0.029</td>
<td>1.34</td>
</tr>
<tr>
<td>VM-26</td>
<td>0.0037 ± 0.0013</td>
<td>0.0035 ± 0.0035</td>
<td>0.94</td>
</tr>
<tr>
<td>ADM</td>
<td>0.0071 ± 0.00013</td>
<td>0.023 ± 0.005</td>
<td>3.24</td>
</tr>
<tr>
<td>VBL</td>
<td>0.00099 ± 0.00006</td>
<td>0.00047 ± 0.00007</td>
<td>0.80</td>
</tr>
<tr>
<td>L-PAM</td>
<td>0.17 ± 0.06</td>
<td>0.16 ± 0.057</td>
<td>0.91</td>
</tr>
<tr>
<td>5-FU</td>
<td>0.14 ± 0.006</td>
<td>0.14 ± 0.014</td>
<td>1.02</td>
</tr>
<tr>
<td>BLM</td>
<td>0.12 ± 0.021</td>
<td>0.14 ± 0.057</td>
<td>1.14</td>
</tr>
</tbody>
</table>

* CDDP, cis-diaminedichloroplatinum(II); VP-16, etoposide; ADM, Adriamycin; VBL, vinblastine; VCR, vincristine; L-PAM, L-phenylalanine mustard; 5-FU, 5-fluorouracil; BLM, bleomycin.

** p < 0.001

A) NB-506

B) NB-506

C) Camptothecin

Fig. 6. Uptake of NB-506 and camptothecin by SBC-3/P and SBC-3/NB#9 cells. Exponentially growing cells (2 × 10⁷/test) were preincubated at 37°C for 45 min and incubated with [³H]NB-506 or camptothecin at 37°C for the indicated time; after which, the cells were collected, washed with PBS, and dissolved in formic acid. Then the radioactivity was measured with a liquid scintillation counter. A, time course of NB-506 incorporation into SBC-3/P (○) and SBC-3/NB#9 (•) cells. Accumulation of NB-506 (B) and camptothecin (C) by SBC-3/P and SBC-3/NB#9 cells.

is likely be a major limiting factor in determining the clinical success of NB-506.

In transplantable rodent tumor models, such as P388, L1210, colon 26, and M5076, NB-506 was reported to show a broad spectrum and markedly high degree of antitumor activity (12). The life span of mice bearing P388 murine leukemia cells given 100 mg/kg/day NB-506 was prolonged by over 326%, and cures (60-day survivors) were observed in 4 of 5 (80%). Such high antitumor activity was not attained with any dose of CPT-11 (30, 31). Furthermore, the LD₅₀ of NB-506, administered i.p., in mice was 500 mg/kg, which was much higher than those of CPT-11 (177 mg/kg), etoposide (65 mg/kg), doxorubicin (14 mg/kg), mitomycin C (8 mg/kg), and cisplatin (15 mg/kg). This indicates that NB-506 is a drug with low toxicity and is a superior anticancer agent to CPT-11 against experimental animal tumors.

In this study using human cancer cell lines, NB-506 showed anti-tumor activity similar to or better than CPT-11. In particular, the cytotoxicity of NB-506 against human small cell lung cancer cell lines, such as SBC-3 and H69, was 5-fold that of CPT-11. Furthermore, we expected NB-506 to show cytotoxicity against CPT-11-resistant cells, because its structure and ability to intercalate double-stranded DNA differ from those of CPT-11. Regrettably, the CPT-11-resistant PC-7 and RPMI 8402 (K5) cell lines showed cross-resistance to NB-506 (Fig. 3). However, cells with the typical mdrl phenotype, such as the Adriamycin-resistant K562 and etoposide-resistant H69, were not cross-resistant to NB-506 (data not shown). These results indicate that NB-506 is superior to CPT-11 against human tumor cells as well as experimental animal tumor cells and pin our expectation of considerable cytotoxic effects on drug-resistant tumor cells.

A major limitation to cancer chemotherapy is the development of
resistance to chemotherapeutic drugs, which may be due to decreased drug uptake (17), efficient drug removal (mediated by P-glycoprotein), drug detoxification, e.g., by increased levels of cellular glutathione (16), enhanced DNA repair, gene amplification, and subsequent overexpression of the target molecule or drug target specificity changes. The resistance to topoisomerase inhibitors has been found to correlate with decreased topoisomerase activity (32). Topoisomerase inhibitors are cytotoxic by virtue of producing DNA lesions as a result of inhibiting topoisomerases during the course of DNA replication, and therefore, drug sensitivity is directly proportional to enzyme activity. We established a topoisomerase I inhibitor, CPT-11-resistant cell line (PC-7/CPT) from a human non-small cell lung cancer cell line, PC-7/P. The CPT-11-resistant cells exhibited about 10-fold resistance to CPT-11; their topoisomerase I activity, determined using the DNA relaxation assay, was lower, and the topoisomerase I from the resistant cells was 5 times more resistant to the inhibitory effect of CPT-11 compared with the parent cells (14). The topoisomerase I activity and level changes were demonstrated, using single-strand conformation polymorphism/PCR analysis, to result from topoisomerase I gene mutation (33). Thus, qualitative changes in topoisomerase I can lead to resistance to camptothecin and its derivatives (34, 35).

Fig. 8. Contents of topoisomerase I of SBC-3/P and its NB-506-resistant sublines. Topoisomerase I content was measured by Western blot assay described in "Materials and Methods." The resulting autoradiographs were quantified by scanning densitometry. The results were confirmed by normalization to β-actin signals. Contents of topoisomerase I were presented as the relative amount of parent cells.

Fig. 9. Inhibitory effects of NB-506 and CPT-11 on DNA relaxation catalyzed by topoisomerase I from SBC-3/P and SBC-3/NB#9 cells. The experiments are the same as those described in the legend to Fig. 7. Lanes 1 and 9, supercoiled and relaxed DNA, respectively. Lanes 2 and 10, no nuclear extract (negative controls). Lanes 2–8 and 10–16 were loaded in the presence of 0.05 and 0.01 μg SBC-3/NB#9 and SBC-3/P nuclear extracts, respectively. Lanes 2 and 10, no drug (positive controls); Lanes 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, and 16 contained 25, 10, 5, 2.5, 1, and 0.5 μg/ml NB-506 and CPT-11, respectively. A and B, the effects of NB-506 and CPT-11 on the topoisomerase I activities of SBC-3/P and SBC-3/NB#9 cells, respectively.
In this study, we developed, established and characterized nine NB-506-resistant sublines (SBC-3/NB#1—9) from a human small cell lung cancer cell line (SBC-3/P). Their resistance ratios ranged from 15- to 454-fold. Of these, SBC-3/NB#9 was selected for comparative single-strand conformation polymorphism (SSCP) analysis that showed no topoisomerase I gene changes had occurred (data not shown).

Changes in the cellular uptake/efflux of drugs are known to be common drug resistance mechanisms. In this experiment, we observed that less NB-506 was accumulated by the resistant than the parent cells, although the intracellular camptothecin accumulation by SBC-3/P and SBC-3/NB#9 did not differ. The SBC-3/NB#9 was distinguishable from multidrug-resistant cells, because the multidrug-resistant K562/ADM subline, which is known to overexpress P-glycoprotein, was distinguishable from multidrug-resistant cells, because the multidrug-resistant K562/ADM subline, which is known to overexpress P-glycoprotein (pp170), did not show cross-resistance to it. Therefore, the low intracellular drug accumulation by SBC-3/NB#9 compared with its parent cells appears to be attributable to reduced drug uptake, not a p-glycoprotein-mediated change in efflux. The SBC-3/NB#9 cells also were distinguishable from CPT-11-resistant cells (PC-7/CPT), the resistance mechanisms of which are considered to be a low affinity of topoisomerase I for CPT-11, as well as reduced enzyme activity and low levels. Therefore, SBC-3/NB#9 cells have at least two NB-506-resistance mechanisms, namely a low total topoisomerase I activity and low intracellular accumulation of NB-506. These multifactorial mechanisms contribute to the higher resistance of these cells to NB-506 and their relatively low degree of cross-resistance to CPT-11.

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Antitumor Activities of a New Indolocarbazole Substance, NB-506, and Establishment of NB-506-resistant Cell Lines, SBC-3/NB

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