Antitumor Activity of ER-37328, a Novel Carbazole Topoisomerase II Inhibitor

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Abstract

DNA topoisomerase II has been shown to be an important therapeutic target in cancer chemotherapy. Here, we describe studies on the antitumor activity of a novel topoisomerase II inhibitor, ER-37328 [12,13-dihydro-5-[2-(dimethylamino)ethyl]-4H-benzo[*c*]pyrimido[5,6,1-jk]carbazole-4,6,10(5H,11H)-trione hydrochloride]. ER-37328 inhibited topoisomerase II activity at 10 times lower concentration than etoposide in relaxation assay and induced double-strand DNA cleavage within 1 h in murine leukemia P388 cells, in a bell-shaped manner with respect to drug concentration. The maximum amount of DNA cleavage was obtained at 2 μM. Like etoposide, ER-37328 (2 μM) induced topoisomerase II-DNA cross-linking in P388 cells. A spectroscopic study of ER-37328 mixed with DNA demonstrated that ER-37328 has apparent binding activity to DNA. ER-37328 showed potent growth-inhibitory activity against a panel of 21 human cancer cell lines [mean (50% growth-inhibitory concentration) GI_{50} = 59 nM]. COMPARE analysis according to the National Cancer Institute screening protocol showed that the pattern of the growth-inhibitory effect of ER-37328 was similar to that of etoposide, but different from that of doxorubicin. Studies on etoposide-, amsacrine [4′-(9-acridinylamino)methanesulfon-m-anisidide (m-AMSA)]-, and camptothecin-resistant P388 cell lines showed that: (*a*) etoposide- and m-AMSA-resistant P388 cell lines were partially resistant to ER-37328 compared with the parental cell line; and (*b*) a camptothecin-resistant cell line showed no cross-resistance to ER-37328. In addition, ER-37328 overcame P-glycoprotein-mediated resistance. *In vivo*, ER-37328 produced potent tumor regression of Colon 38 carcinoma inoculated s.c., and its activity was superior to that of etoposide or doxorubicin. These results indicate that ER-37328 inhibits topoisomerase II activity through the formation of topoisomerase II-DNA cleavable complex and has potent antitumor activity both *in vitro* and *in vivo*.

Introduction

Topoisomerase II catalyzes DNA topological reactions via a DNA breakage/reunion mechanism. The DNA topological reactions allow the enzyme to segregate interlocked chromosomal DNA at mitosis (1–3) and to remove excess DNA supercoils generated during processes such as DNA replication, RNA transcription, and chromosome condensation (4–7). The breakage/reunion reaction of topoisomerase II can be interrupted by many anticancer drugs, such as etoposide, anthracyclines, and m-AMSA,[2] resulting in the accumulation of a topoisomerase II-DNA covalent intermediate, the cleavable complex (8). Accumulation of topoisomerase II-DNA cleavable complexes causes tumor cell death (8).

Six antineoplastic drugs (etoposide, teniposide, doxorubicin, daunorubicin, idarubicin, and mitoxantrone), which stabilize the topoisomerase II-DNA cleavable complex, are currently approved for clinical use in the United States (9). Etoposide has significant antitumor activity against a wide variety of neoplasms, including germ cell malignancies, lung cancer, non-Hodgkin's lymphomas, leukemias, Kaposi's sarcoma, neuroblastoma, and soft-tissue sarcomas (10, 11). Use of etoposide and cisplatin (or carboplatin) is standard therapy for patients with small-cell lung cancer (12). Etoposide is also a component of the standard treatment regimen (bleomycin, etoposide, cisplatin) for advanced testicular cancer (13). Doxorubicin is the most commonly used anthracycline (14). It is a drug of primary use in the treatment of patients with lymphomas, breast cancer and sarcomas. ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) and CHOP (cyclophosphamide, doxorubicin, and prednisone) are standard therapy for advanced Hodgkin's lymphoma and intermediate-grade non-Hodgkin's lymphoma, respectively (15, 16). CA (cyclophosphamide, doxorubicin) is a commonly used combination regimen for advanced breast cancer (17). Thus, topoisomerase II-inhibiting antineoplastic agents, which induce the topoisomerase II-DNA cleavable complex, are among the most effective antitumor drugs currently available for the treatment of human cancers and are key drugs in combination chemotherapy. However, these drugs are relatively ineffective against commonly occurring solid tumors such as non-small cell lung cancer, colon can-

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[2] The abbreviations used are: m-AMSA, amsacrine, 4′-(9-acridinylamino)-methanesulfon-m-anisidide; ER-37328, [12,13-dihydro-5-[2-(dimethylamino)ethyl]-4H-benzo[*c*]pyrimido[5,6,1-jk]carbazole-4,6,10(5H,11H)-trione hydrochloride]; GI_{50}, 50% growth-inhibitory concentration; NCI, National Cancer Institute; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MRP, multidrug resistance-associated protein.

cer, gastric cancer, and pancreatic cancer. In addition, most topoisomerase II-inhibiting agents are substrates for P-glycoprotein or MRP (18, 19). P-Glycoprotein or MRP-positive tumors are therefore less effectively treatable with topoisomerase II inhibitors. Heart damage, which is a characteristic toxicity of doxorubicin and other anthracyclines, limits their effective use (20). Accordingly, we thought that a structurally novel topoisomerase II inhibitor that has more potent antitumor activity than etoposide and doxorubicin against solid tumor cells *in vitro* and *in vivo*, would be a good candidate for a clinically useful antitumor drug.

We have screened a large number of structurally varied compounds for (*a)* the ability to inhibit topoisomerase II activity in relaxation assay; (*b*) the ability to induce DNA-protein cross-linking and double-strand DNA cleavage in cells as indicators of topoisomerase II-DNA cleavable complex formation (21); (*c*) antitumor activity against human solid tumor cell lines *in vitro*; and (*d*) antitumor activity *in vivo*. As a result of extensive screening, we found ER-37328.

In the present study, we describe the ability of ER-37328 to induce the topoisomerase II-DNA cleavable complex in cells, its growth-inhibitory effect on a panel of human solid tumor cell lines and its *in vivo* antitumor activity. In addition, the effect of ER-37328 on topoisomerase inhibitor-resistant cell lines and a multidrug-resistant cell line is also described.

Materials and Methods

ER-37328 and Other Compounds. ER-37328 and m-AMSA (2) were synthesized at Tsukuba Research Laboratories, Eisai Co., Ltd. SN-38 was obtained by the hydrolysis of irinotecan, which was purchased from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Etoposide was purchased from Bristol-Myers Squibb Co. Ltd. (Tokyo Japan). Doxorubicin was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). Camptothecin, cisplatin, and vincristine were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells. Murine colon cancer Colon 38, murine leukemia P388 and human lung cancer LX-1 were obtained from the Cancer Chemotherapy Center, Japan Foundation for Cancer Research (Tokyo, Japan). Human lung cancer PC-1 and human gastric cancer MKN-1, MKN-7, MKN-28, and MKN-74 were obtained from Immuno Biology Laboratory (Gunma, Japan). Human lung cancer A549 and human colon cancer WiDr were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Human gastric cancer HGC-27 and GT3TKB were obtained from Riken Cell Bank (Ibaraki, Japan). The other cell lines were purchased from American Type Culture Collection (Rockville, MD).

Isolation of Drug-resistant Cell Lines. P388/2A, P388/10A-1, P388/B-1, and P388/$V_{0.256}$ cell lines were isolated from P388 cells by stepwise selection with increasing concentrations of etoposide, m-AMSA, camptothecin, and vincristine, respectively. It was reported that P388/$V_{0.256}$ cell line overexpressed P-glycoprotein (22). P388/2A cell line was selected as an etoposide-resistant but not vincristine-resistant line.

Relaxation Assay. Topoisomerase II was purified from P388 cells as described by Saijo *et al.* (23). One unit of the enzyme was defined as the activity to relax completely 0.125 μg of supercoiled pBR-322 DNA at 30°C for 1 h. For the assay, 0.125 μg of supercoiled pBR-322 DNA (Takara Shuzo Co., Ltd., Tokyo, Japan) was relaxed with one unit of topoisomerase II in 20 μl of the assay buffer [50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 μM EDTA, 1 mM ATP, and 30 μg/ml BSA] in the presence or absence of an inhibitor at 30°C for 1 h. Samples were subjected to electrophoresis in 0.7% agarose gels with TBE buffer [89 mM Tris, 89 mM boric acid, and 2 mM EDTA (pH 8.0)]. The DNA was stained with ethidium bromide and photographed under UV light.

Detection of Double-Strand DNA Cleavage. Double-strand DNA cleavage was measured by using pulse-field gel electrophoresis as described by Okamoto *et al.* (24). P388 cells (1×10^6 cells/2 ml of culture medium) in the exponential growth phase were treated with each compound for 1 h at 37°C. The cells were washed with PBS, suspended in 100 μl of 50 mM EDTA (pH 8.0), mixed with an equal volume of 1.0% (w/v) low-temperature-melting agarose, poured into a sample plug caster, and left at 4°C for 60 min to solidify. The cells in the agarose block were treated with lysis buffer [10 mM Tris-HCl (pH 8.0), 500 mM EDTA, 1% Sarkosyl, 1 mg/ml proteinase K] at 50°C for 48 h, and washed three times with 50 mM EDTA (pH 8.0) for 3 days. Plugs about 3 mm long, cut from the agarose blocks, were loaded onto 1.0% (w/v) agarose gel in electrophoresis buffer [45 mM Tris-base, 45 mM boric acid, and 1.25 mM EDTA (pH 8.3)]. Electrophoresis was carried out by using a CHEF apparatus (horizontal gel chamber, a model 200/20 power supply and Pulsewave 760 switcher; Japan Bio-Rad Co., Tokyo, Japan). The gels were run at 200 V at 15–20°C in electrophoresis buffer with a switching time of 60 s for 15 h, followed by a switching time of 90 s for 8 h. The DNA was stained with ethidium bromide and photographed under UV light.

Detection of Topoisomerase II-DNA Cross-Linking. Topoisomerase II-DNA cross-linking was measured as described by Martinez *et al.* (25). P388 cells (2.5×10^6 cells in 2 ml of culture medium) in the exponential growth phase were treated with 2 μM ER-37328 or 50 μM etoposide and incubated for 1 h along with a negative control (no drug). Cells were centrifuged and lysed in 1 ml of TE [10 mM Tris-HCl (pH 8.0), and 1 mM EDTA] containing 1% Sarkosyl. The lysate was layered onto a CsCl step gradient (1 ml each of 4 different CsCl concentrations) and centrifuged in a Beckman SW50.1 rotor (31,000 rpm, 18 h, 25°C). Fractions (200 μl each) were carefully collected from the top by using a Pipetman. DNA concentration was determined by reading the absorbance at 260 nm. The DNA peak fractions, which contained the bulk of the DNA, were collected and the DNA concentration of each solution was adjusted. One hundred μl of the equal concentration of DNA solution was diluted with 200 μl of 25 mM sodium phosphate buffer (pH 6.5) and applied under vacuum to a nitrocellulose membrane, which had been presoaked in 28 mM sodium phosphate buffer (pH 6.5). The nonspecific binding sites were blocked by treatment with 5% Blotto [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5% nonfat dry milk] in TBS-T [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20]. The membrane was incubated with a rabbit polyclonal antibody to topoisomerase II

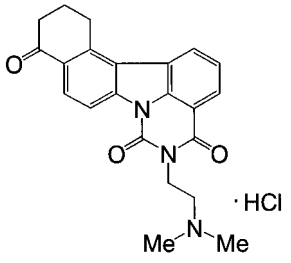

Fig. 1. Structure of ER-37328 {[12,13-dihydro-5-[2-(dimethylamino)-ethyl]-4H-benzo[*c*]pyrimido [5,6,1-jk]carbazole-4,6,10(5H,11H)-trione hydrochloride]}.

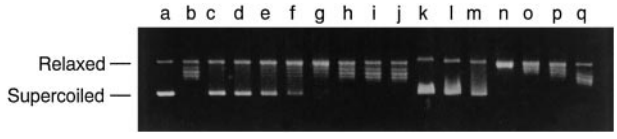

Fig. 2. Effects of ER-37328 and etoposide on the DNA-relaxing activity of P388 topoisomerase II. Supercoiled pBR-322 DNA was incubated with topoisomerase II purified from P388 cells in the presence or absence of an inhibitor. *Lane a*, no enzyme control; *Lane b*, enzyme control; *Lanes c–j*, 200, 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 μM etoposide; *Lanes k–q*, 10, 5, 2.5, 1.25, 0.63, 0.31, and 0.16 μM ER-37328.

(TopoGEN, Columbus, OH) as a primary antibody for 3 h. After three washings with TBS-T (10 min each), the membrane was incubated with goat antirabbit conjugated to horseradish peroxidase as a secondary antibody for 1 h. After washing with TBS-T, the final detection of the blot was carried out by using the ECL Western Blotting Detection system (Amersham, Arlington Heights, IL).

Absorption Spectra and Binding Measurement. Calf thymus DNA at various concentrations in 40 mM Tris-HCl buffer (pH 7.4) was placed in both sample and reference cuvettes (10 cm light path). ER-37328 or doxorubicin was then added to the sample cuvette to give a final concentration of 10 μM. Changes in spectra of drugs were recorded with a spectrophotometer. To determine the binding parameters of ER-37827 and doxorubicin to calf thymus DNA, the absorbance data at 340 nm (ER-37328) or 480 nm (doxorubicin) were used to obtain a Scatchard plot, affording the apparent binding constant, *K*, and the apparent number of binding sites, *v*, according to the method used by Zunino *et al.* (26).

Growth-inhibitory Effect. Exponentially growing solid tumor cells in 0.1 ml of medium were seeded in 96-well plates on day 0. On day 1, 0.1 ml aliquots of medium containing graded concentrations of test drugs were added to the cell plates. After incubation at 37°C for 72 h, the cell number was estimated by sulforhodamine B (SRB) assay, as described by Skehan *et al.* (27). Separately, the cell number on day 1 was also measured. GI_{50} values are the drug concentrations causing a 50% reduction in the net protein increase in control cells. The antitumor spectrum of ER-37328 was compared with those of other drugs by means of the NCI COMPARE analysis procedure (28). Exponentially growing P388 cells were seeded in 96-well plates on day 0. On day 1, 0.1 ml aliquots of medium containing graded concentrations of test drugs were added to the wells. After incubation at 37°C for 72 h, the cell number was determined by MTT assay (29). IC_{50} values were calculated as the drug concentrations that reduced the number of cells to 50% of the control number.

***In Vivo* Efficacy Study.** Female C57BL/6 mice (6 weeks of age; Charles River, Atsugi, Japan) were housed in barrier facilities on a 12 h light/dark cycle, with food and water ad libitum. About 30 mg of Colon 38 tumor tissue was inoculated s.c. into the flank of animals. Mice were randomized and separated into control (n = 8: Fig. 6A or *n* = 7: Fig. 6*B*) and treatment (*n* = 5 each) groups when the tumor volume reached approximately 300 mm^3 on day 1. ER-37328 was dissolved in 5% glucose. Etoposide was diluted in saline and doxorubicin was dissolved in saline. ER-37328 was administrated on day 1 (single dose) by i.v. injection into the tail vein, and etoposide and doxorubicin were given at the maximum tolerated dose on the reported administration schedule (30). Control animals received 5% glucose. Tumor volume and body weight were measured on the days indicated in the figures. Tumor volume was calculated according to the following equation: tumor volume (mm^3) = (length $\times$ $width^2$)/ (2).

Results

Inhibition of Topoisomerase II Activity. The effects of ER-37328 (Fig. 1) and etoposide on topoisomerase II activity were analyzed by means of relaxation assay (Fig. 2). ER-37328 and etoposide inhibited topoisomerase II activity at 2.5 μM and 25 μM, respectively, *i.e.,* ER-37328 exhibited a 10 times greater inhibitory effect than etoposide.

Induction of Topoisomerase II-DNA Cleavable Complex by ER-37328 in P388 Cells. In recent years, a diverse group of drugs has been reported which inhibit topoisomerase II activity but lack the ability to stabilize the cleavable complex (31). Therefore, we examined whether ER-37328 induced the topoisomerase II-DNA cleavable complex or not in cultured cells. First, the induction of double-strand DNA cleavage by ER-37328 was examined by using pulse field gel electrophoresis (Fig. 3), because the DNA of this complex becomes cleaved at the site of topoisomerase II attachment. The induction of double-strand DNA cleavage in P388 cells treated with ER-37328 for 1 h showed a in a bell-shaped relationship to drug concentration, and the maximum amount of double-strand DNA cleavage was detected at 2 μM. In contrast, etoposide induced double-strand DNA cleavage dose-dependently up to 50 μM. Second, to confirm the induction of topoisomerase II-DNA cleavable complex by ER-37328 directly, topoisomerase II-DNA cross-linking by ER-37328 was measured (Fig. 4). The DNA was collected from lysate of P388 cells exposed to 2 μM ER-37328 or 50 μ 77 etoposide for 1 h, by using a CsCl step gradient centrifugation method. The amount of topoisomerase II in the DNA fraction was analyzed by Western blot analysis using antibody to topoisomerase II. No topoisomerase II was detected in the DNA fraction in the absence of any drug. On the other hand, ER-37328 and etoposide increased the amount of topoisomerase II in the DNA fraction, indicating that these drugs have topoisomerase II-DNA cross-linking ability.

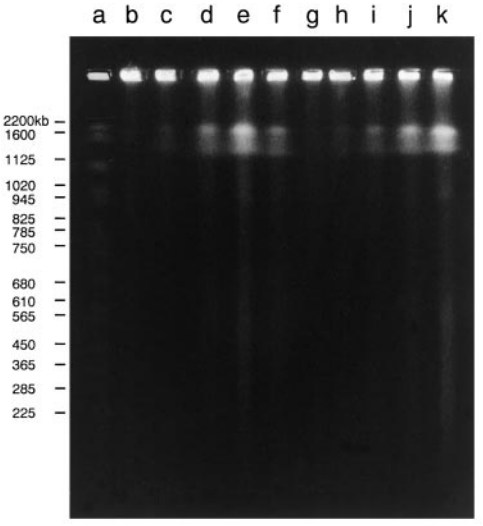

Fig. 3. Induction of double-strand DNA cleavage by ER-37328 and etoposide. P388 cells were incubated with various concentration of drugs for 1 h, and the cellular DNA was analyzed by using pulse field gel electrophoresis. *Lane a*, size markers; *Lane b*, no-drug control; *Lanes c–g*, 0.22, 0.67, 2, 6, and 18 μM ER-37328; *Lanes h–k*, 1.9, 5.6, 16.7, and 50 μM etoposide.

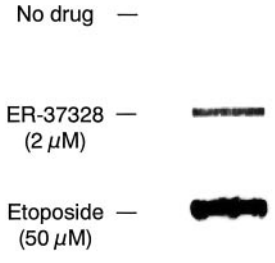

Fig. 4. Effects of ER-37328 and etoposide on the formation of topoisomerase II-DNA cross-linking in P388 cells. P388 cells were incubated with 2 μM ER-37328 or 50 μM etoposide for 1 h and lysed with Sarkosyl. The DNA was collected from the lysate by using a CsCl step gradient centrifugation method. DNA solution was applied to a nitrocellulose membrane under vacuum. Topoisomerase II was detected by Western blot analysis using antibody to topoisomerase II.

Interaction of ER-37328 with DNA. Some intercalating topoisomerase II inhibitors induce DNA-protein cross-links and double-strand DNA cleavage in a bell-shaped manner with respect to drug concentration (32–35). Therefore, the DNA-interacting activity of ER-37328 was examined. Ten μM ER-37328 was mixed with 0–74 μM calf thymus DNA and the spectrum of ER-37328 was measured. The addition of calf-thymus-DNA induced bathochromic shifts of the spectral peak and hypochromicity of ER-37328 with an isosbestic point at approximately 355 nm (data not shown). The apparent association constant, *K*, and the apparent number of binding sites per nucleotide, *v*, are presented in Table 1. ER-37328 showed apparent binding activity to DNA, but its binding affinity was two times lower than that of doxorubicin. The number of binding sites per nucleotide for ER-37328 was comparable with that for doxorubicin.

Table 1 Binding parameters of ER-37328 and doxorubicin with calf thymus DNA

Each drug (10 μM) was mixed with several concentrations of calf thymus DNA, and the spectrum of each drug was measured. Changes in the absorbance at 340 nm (ER-37328) or 480 nm (doxorubicin) were used to evaluate the binding parameters. Extrapolation of the Scatchard plot yields the apparent binding constant, *K*, and the apparent number of binding sites, *v* (mean ± SD, $n = 3$ or 4).

Compound	K ($\times 10^6$/mol)	v
ER-37328	4.2 ± 1.0	0.22 ± 0.01
Doxorubicin	9.7 ± 1.6	0.19 ± 0.02

Growth-inhibitory Effect. The growth-inhibitory effects of ER-37328 on 21 human solid tumor cell lines (8 lung, 7 colon, and 6 gastric cancer cell lines) were determined (Fig. 5). ER-37328 inhibited the growth of these cell lines with an average GI_{50} of 59 nM. This effect was about 20 times more potent than that of etoposide and comparable with that of doxorubicin and SN-38. The correlation coefficients with etoposide, doxorubicin, SN-38 (topoisomerase I inhibitor), and cisplatin, calculated according to the NCI COMPARE analysis procedure using ER-37328 as the benchmark (28), were 0.70, 0.56, 0.45, and 0.35, respectively. That is, the pattern of the growth-inhibitory effect of ER-37328 was similar to that of etoposide, but less similar to that of doxorubicin.

Cross-Resistance in Topoisomerase Inhibitor- and Vincristine-resistant P388 Cell Lines. The growth-inhibitory effects of ER-37328 on topoisomerase inhibitor-resistant cell lines were measured (Table 2). P388/2A cell line, which showed 44-fold resistance to etoposide, and P388/10A-1 cell line, which showed 530-fold resistance to m-AMSA, exhibited 7.1-fold and 8.4-fold resistance to ER-37328, respectively. P388/B-1 cell line, which showed 660-fold resistance to camptothecin, was not resistant to ER-37328. The growth-inhibitory effect of ER-37328 on P388/$V_{0.256}$ cell line, which showed a multidrug-resistance phenotype and overexpressed P-glycoprotein (22), was also measured. P388/$V_{0.256}$ cell line had 77-, 30-, and 19-fold lower sensitivity to vincristine, doxorubicin, and etoposide, respectively, than did the parental cell line. However, ER-37328 showed similar effects on P388/$V_{0.256}$ cells and the parental P388 cells.

***In Vivo* Efficacy Study.** The *in vivo* activity of ER-37328, etoposide, and doxorubicin against murine colon cancer Colon38 was tested (Fig. 6). Treatment of Colon 38 implanted into mice with ER-37328 induced a clear dose-dependent inhibition of tumor growth (Fig. 6*A*). Moreover, marked tumor regression was observed at 6 and 12 mg/kg. Body weight loss (14% loss) was observed at 12 mg/kg, but was recovered within 10 days after drug administration. No body weight loss was observed at 3 and 6 mg/kg. In contrast, treatment with etoposide (Fig. 6*A*) or doxorubicin (Fig. 6*B*) inhibited tumor growth, but did not induce tumor regression at the maximum tolerated dose on the schedule indicated (30).

Discussion

Topoisomerase II is the primary cellular target for a number of clinically important antitumor agents (8). These drugs stim-

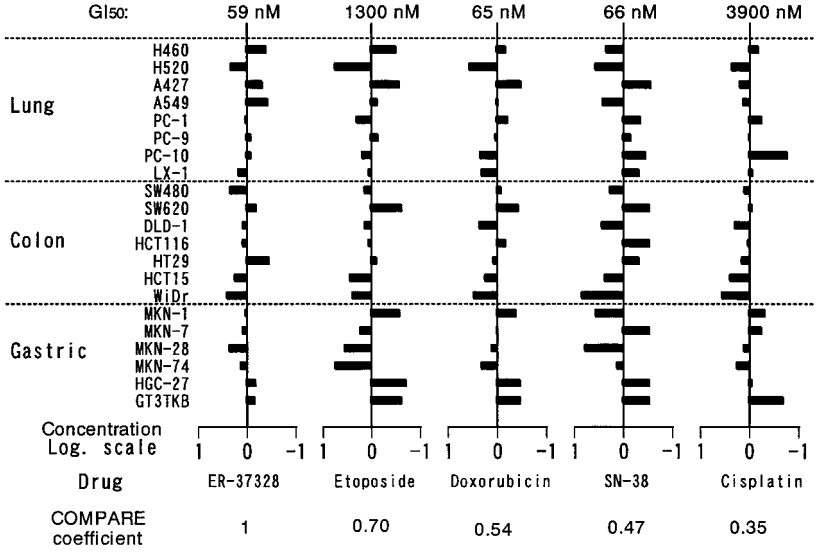

Fig. 5. Comparison of the GI_{50} mean graph profiles for ER-37328 with those of different anticancer drugs according to the NCI COMPARE analysis procedure. For each drug, the mean GI_{50} obtained for all of the cell lines is indicated at the top of the profile and is represented by a vertical bar through individual plots. *Horizontal bars*, (in a logarithm scale) the individual GI_{50} for each cell line relative to the mean value. *Negative values* (*on the right of the vertical reference*) indicate the most sensitive cell lines. *At the bottom of the graph* are indicated the overall similarities of the GI_{50} profiles between ER-37328 and other drugs, as calculated by the COMPARE analysis procedure.

Table 2 Cross-resistance in topoisomerase inhibitor- and vincristine-resistant P388 cell lines

Cells were incubated with drugs for 72 h. The cell number was determined by MTT assay. IC_{50} values were calculated as the drug concentrations that reduced the number of cells to 50% of the control number.

Compound	Relative resistance[a]			
	P388/2A	P388/10A-1	P388/B-1	P388/$V_{0.256}$
ER-37328	7.1	8.4	2.0 ± 0.5[b]	0.8 ± 0.1
Etoposide	44	110	2.7 ± 1.1	19 ± 5
m-AMSA	13	530	5.8 ± 1.6	NT[c]
Doxorubicin	6.6	4.0	1.6 ± 0.5	30 ± 4
Camptothecin	NT	NT	660 ± 110	NT
Vincristine	0.9	0.7	0.6 ± 0.1	77 ± 6

[a] Relative resistance, IC_{50} (resistant P388 cells)/IC_{50} (parental P388 cells).
[b] SDs are indicated when three separate experiments were performed.
[c] NT, not tested.

ulate the topoisomerase II-DNA cleavable complex that is presumed to be involved in the antitumor effect of the drugs. Therefore, the identification of new drugs that induce the formation of the cleavable complex has been viewed as a promising approach to find clinically effective anticancer agents. Indeed, several topoisomerase II inhibitors that are still under investigation and that have not yet been approved for general clinical use in the United States show antitumor activity in animals or clinical studies (36).

The studies reported here show that ER-37328 is a novel carbazole topoisomerase II inhibitor that stabilizes the topoisomerase II-DNA cleavable complex in cells and has potent antitumor activity *in vitro* and *in vivo*. The mean GI_{50} value obtained for 21 human solid tumor cell lines was 59 nM. *In vivo*, treatment with ER-37328 caused prominent regression of Colon 38 in mice. In addition, ER-37328 could overcome P-glycoprotein-mediated resistance.

The topoisomerase II-DNA cleavable complex can be measured in whole cells, because the stabilized configuration can be rendered irreversible by denaturation with detergents such as SDS (21). Denaturation produces a covalent linkage between DNA and topoisomerase II that can be detected as a topoisomerase II-DNA cross-link. Additionally, the DNA of this complex becomes cleaved at the site of topoisomerase II attachment so that double-strand DNA cleavage can also be used as an indicator of complex formation. Our data showed that ER-37328 induced double-strand DNA cleavage and topoisomerase II-DNA cross-linking in P388 cells. These results indicate that ER-37328 inhibits topoisomerase II by trapping the cleavable complex.

The double-strand DNA cleavage by ER-37328, unlike that by etoposide, showed a bell-shaped relationship to concentration. It has been reported that several intercalators that inhibit topoisomerase II activity, induced DNA-protein cross-link and/or DNA cleavage in a bell-shaped manner with respect to drug concentration (32–35). Therefore, the DNA-interacting activity of ER-37328 was examined. The result of the spectroscopic study indicates that ER-37328 has intercalative activity. High concentrations of intercalators may stabilize an intermediate in which topoisomerase II is dissociated from DNA or bound to DNA without inducing cleavage, or may induce DNA structure alterations that inhibit topoisomerase II binding to DNA, as described by Pierson *et al.* (34).

When the patterns of the growth-inhibitory effect of ER-37328, etoposide, doxorubicin, SN-38, and cisplatin in our tumor panel study were examined according to the NCI COMPARE analysis procedure (28), a high correlation was found between ER-37328 and etoposide. It is interesting that the COMPARE coefficient between ER-37328 and doxorubicin is low (coefficient, 0.54), although doxorubicin is also an intercalating topoisomerase II inhibitor. It was reported that doxorubicin has nuclear helicase inhibitory activity (37) and free-radical formation activity (38) in addition to topoisomerase II inhibition activity. These effects are thought to be partially involved in the cytotoxicity of anthracyclines, and might account for the low correlation between ER-37328 and doxorubicin. No significant correlation between ER-37328 and SN-38, a typical topoisomerase I inhibitor, was observed

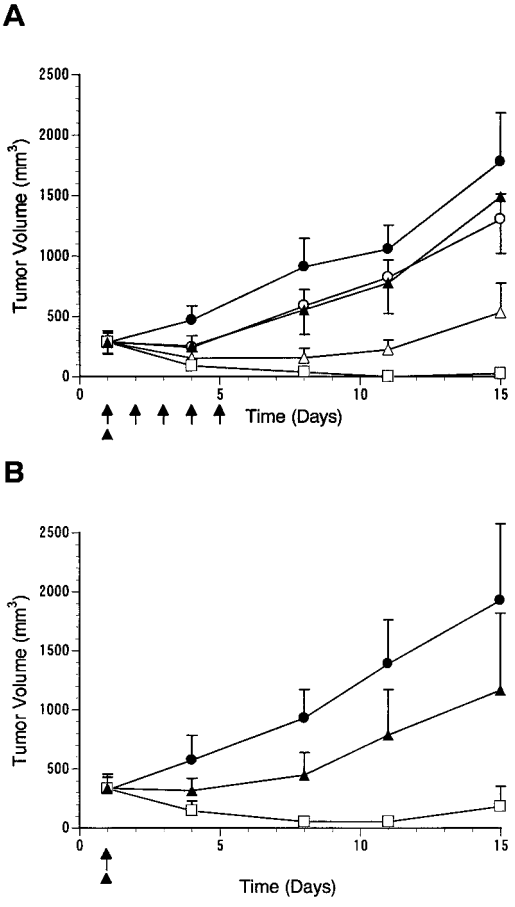

Fig. 6. *In vivo* effect on murine colon cancer Colon 38. Colon 38 was inoculated s.c. into the flank of mice as described in "Materials and Methods." Administration was started on day 1 when the tumor volume reached approximately 300 mm^3. Drugs were administered by tail vein injection at the time indicated by the *arrowhead* (ER-37328) or *arrow* (etoposide or doxorubicin). *A,* vehicle (●) or 3 (○), 6 (△), or 12 (□) mg/kg ER-37328 or 12 mg/kg/day etoposide (▲). *B*, vehicle (●), 12 mg/kg ER-37328 (□) or 12 mg/kg doxorubicin (▲). Data are expressed as tumor volume (mm^3); *bars*, SD.

and a camptothecin-resistant P388 cell line showed no cross-resistance to ER-37328, which suggested that ER-37328 does not have topoisomerase I-inhibitory activity.

Most topoisomerase II-inhibiting drugs are substrates for P-glycoprotein or for MRP (18, 19). This type of drug resistance limits the usefulness of topoisomerase II-inhibiting drugs in the clinic. In this study, we found that ER-37328 was effective against a multidrug-resistant cell line that overexpressed P-glycoprotein (the expression of MRP was not examined; Ref. 22), which indicated that ER-37328 is not transported out of cells by P-glycoprotein.

Finally, ER-37328 produced potent tumor regression of Colon 38 inoculated into mice. We would like to emphasize that its effect was superior to that of etoposide or doxorubicin, the most commonly used topoisomerase II inhibitors in the clinic.

In conclusion, ER-37328, a novel topoisomerase II inhibitor, shows strong antitumor activity *in vitro* and *in vivo* and overcomes P-glycoprotein-mediated resistance, and is, therefore, considered to be a promising candidate for the clinical treatment of tumors.

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