Effects of ER-37328 on Primary Tumor, Liver Metastasis, and Life Span in a Murine Colon 38 Orthotopic Transplantation Model

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Abstract
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) is a novel topoisomerase II poison with potent tumoricidal activity against solid tumor cells both in vitro and in vivo. Here, we describe studies on the effects of ER-37328 on the primary tumor, liver metastasis, and survival in a murine Colon 38 orthotopic transplantation model. When ER-37328 (10 mg/kg) was administered i.v. at 11 days or 20 days after transplantation, strong regression of the primary tumor was observed on both administration schedules. On the later schedule, ER-37328 completely blocked liver metastasis, whereas the mean number of metastases in the control group was 23.9. To examine the antitumor activity against Colon 38 at the liver in more detail, ER-37328 was administered to mice that had received an inoculation of Colon 38 tumor into the liver. ER-37328 showed strong tumor-regression activity against Colon 38 growing in the liver. In addition, administration of ER-37328 on a schedule of every 7 days four times caused a significant increase of 79% in life span in the orthotopic transplantation model, calculated by using mean survival times. Pharmacokinetic study revealed that ER-37328 was highly distributed to the tumor and organs. The ratios of the area under the concentration-time curves of ER-37328 in the tumor, lung, liver, and kidney versus plasma were 81, 77, 47, and 40, respectively. This high distribution to the tumor and liver may explain the potent antitumor activity of ER-37328 against Colon 38 tumor in the liver. In conclusion, the topoisomerase II poison ER-37328 is a promising candidate for clinical application against colon cancer.

Introduction
Six topoisomerase II poisons (etoposide, teniposide, doxorubicin, daunorubicin, idarubicin, and mitoxantrone) are currently approved for clinical use in the United States (1). These drugs are among the most effective antitumor drugs available currently for the treatment of human cancers, such as small-cell lung cancer, breast cancer, testicular cancer, lymphoma, and sarcoma (2–9), but are relatively ineffective against commonly occurring solid tumors, such as non-small-cell lung cancer, colon cancer, gastric cancer, and pancreatic cancer. Therefore, it is of interest to search for new topoisomerase II poisons with potent antitumor activity against non-small-cell lung cancer, colon cancer, gastric cancer, and pancreatic cancer.

ER-373282 is a novel topoisomerase II poison, which exhibits a strong tumoricidal activity both in vitro and in vivo against solid tumor cells (10).3 It showed more potent cell-killing activity than etoposide against a panel of human tumor cell lines exposed to both short-term (1 h) and long-term (72 h) drug treatment in vitro.3 In vivo, ER-37328 produced strong tumor regression of Colon 38 tumor inoculated s.c., whereas etoposide and doxorubicin, the most commonly used topoisomerase II poisons in the clinic, showed only growth-inhibitory activity (10). ER-37328 also demonstrated more potent antitumor activity than etoposide against human solid tumors inoculated s.c.3 In addition, most topoisomerase II poisons, such as anthracyclines and epipodophyllotoxins, are substrates for P-glycoprotein (11, 12), but ER-37328 is effective against a multidrug-resistant P388 subline overexpressing P-glycoprotein (10).

In recent clinical studies of anticancer agents, improvements in parameters such as clinical benefit, time to progression, overall survival, and quality of life have been considered as important outcomes (13, 14). To evaluate such parameters in an animal model, an orthotopic transplantation model is thought to be more appropriate than a s.c. model, which has generally been used for in vivo evaluation of anticancer agents, because it should better reflect the behavior of clinical tumors (15). An orthotopically transplanted colon cancer model has been found to be useful for evaluating drug effects on life span (16, 17). Moreover, inhibition of metastases would be more relevant to the clinical use of anticancer agents.

Received 6/24/02; revised 8/19/02; accepted 11/26/02.

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2 The abbreviations used are: 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; MTD, maximum tolerated dose; ILS, increase in life span; MST, mean survival time; AUC, area under the concentration-time curve; QD, single dose; Q7Dx4, every 7 days 4 times; RBW, relative body weight.

tasis, which is difficult to evaluate in a s.c. model, can also be evaluated in an orthotopic transplantation model (18).

We have reported previously that the effects of anticancer agents on primary tumor, liver metastasis, and life span can be evaluated rapidly in a murine Colon 38 orthotopic transplantation model (19, 20). In this study, we explored the usefulness of ER-37328 against colon cancer by using this orthotopic transplantation model.

Materials and Methods

Drugs. ER-37328 was synthesized at Tsukuba Research Laboratories, Eisai Co., Ltd. Etoposide was purchased from Bristol-Myers Squibb Co. Ltd. (Tokyo, Japan).

Animals. Female C57BL/6 mice and female BDF1 mice were obtained from Charles River (Atsugi, Japan), and were housed in barrier facilities on a 12 h light/dark cycle, with food and water ad libitum. Mice were used for experiments when they were 6–8 weeks old.

Tumor Cells. Murine Colon 38 carcinoma and M5076 ovarian sarcoma cells were supplied by the Cancer Chemotherapy Center, Japan Foundation for Cancer Research, (Tokyo, Japan).

Orthotopic Transplantation of Colon 38 Intact Tissue. Orthotopic transplantation of colon cancer intact tissue was conducted as described previously (19). Briefly, Colon 38 tumor growing s.c. in C57BL/6 mice was resected, and the tumor tissues were cut into pieces weighing 30 mg in HBSS after aseptic removal of necrotic portions. Mice were anesthetized with a 2.5% solution of a 1:1 mixture of 2,2,2-tribromoethanol and t-amyl alcohol (Tokyo, Japan). Etoposide was dissolved in 5% glucose and was administered by i.v. injection into the tail vein on day 18 at a dose of 10 mg/kg. Antitumor activity was determined by expressing the mean tumor volume of the test group (T) as a percentage of that of the control group (C; T/C × 100) on day 25. The tumor volume was calculated by the following formula: tumor volume (mm³) = 1/2 × (long diameter) × (short diameter)².

Pharmacokinetic Study. M5076 cells (1 × 10⁶), which were maintained in the abdominal cavity of C57BL/6 mice, were inoculated s.c. into BDF1 mice. When the tumor volume reached ~200 mm³, ER-37328 was administered i.v. via the tail vein at a dose of 12.5 mg/kg. After administration of ER-37328, blood, tumor, liver, kidney, and lung were obtained at 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h (n = 3). Blood samples were centrifuged immediately at 10,000 rpm for 5 min at 4°C to separate the plasma. Tissues were homogenized to 20% in distilled water. Plasma and the homogenates were deproteinized by using methanol containing 0.2% perchloric acid. The determination of ER-37328 was performed using a high-performance liquid chromatography-UV method. The AUCₜ, where T is the time of the last measurable concentration, was calculated by the trapezoidal method.

Statistical Analysis. The survival rates are presented as Kaplan-Meier curves. The survival curves of individual groups were compared by the log-rank test with Bonferroni adjustment. Values of P < 0.05 were considered statistically significant.

Results

Effect of ER-37328 on Orthotopically Transplanted Murine Colon 38 Tumor. The antitumor activities of ER-37328 and etoposide against orthotopically transplanted murine Colon 38 tumor were examined (Table 1). Both drugs were administered from day 11, when the tumor weight reached ~200 mg at the cecum, and antitumor activity was evaluated on day 20. ER-37328 showed strong antitumor activity against the tumor at the cecum with a T/C of 2% and also against the total tumor, which includes that at the cecum and peritoneum, with a T/C of 2%. Tumor weight at the cecum in mice treated with ER-37328 on day 20 was 14 mg, and this represents tumor regression. On the other hand, treatment with etoposide inhibited tumor growth (cecum, T/C = 66%; total, T/C = 60%), but did not induce tumor regression at the MTD on the schedule indicated (21). The effect on liver metastasis could not be judged, because the incidence of mice with liver metastasis and the number of liver metastases were low under this condition. Second, antitumor activity
of ER-37328 was examined against more advanced disease (Table 2). ER-37328 was administered on day 20, when the tumor weight had reached −1000 mg, and then on day 28, the effects of ER-37328 on tumor growth at the cecum, peritoneum, and on liver metastasis were evaluated. ER-37328 clearly regressed the tumor (−75% tumor regression) and completely blocked liver metastasis (control group, 23.9 ± 19.1; treatment group, 0 ± 0; mean ± SD).

**Effect of ER-37328 on Murine Colon 38 Tumor Growing at the Liver.** To estimate the usefulness of ER-37328 for colon cancer patients, it is important to examine whether ER-37328 can regress metastatic nodules in the liver. However, mice die of primary tumor-induced cachexia before the metastatic nodules grow sufficiently to allow evaluation of tumor regression activity in our orthotopic transplantation model. Therefore, the antitumor activity of ER-37328 against Colon 38 tumor implanted directly into the liver was examined (Fig. 1; Table 3). ER-37328 was administered on day 18, when the tumor volume had reached −500 mm³, and antitumor activity was evaluated on day 25. ER-37328 showed potent antitumor activity with a T/C value of 3%. In addition, ER-37328 potently regressed the tumor (−93% tumor regression).

**Effects of ER-37328 on Life Span in Mice Bearing Orthotopically Transplanted Murine Colon 38 Tumor.** Finally, the effects of ER-37328 on life span were examined in the orthotopic transplantation model. ER-37328 was administered from day 20 on two schedules, i.e., QDx1 and Q7Dx4. The results are shown in Fig. 2A and Table 4. In the ER-37328-treated groups, the ILS values on QDx1 and Q7Dx4 schedules were 26% and 79%, respectively. The effect on the Q7Dx4 schedule was statistically significant (P < 0.01 versus control). Severe body weight loss was not observed in the ER-37328-treated groups (Fig. 2B).

**Discussion**

Our results show that ER-37328 has promising antitumor activity in the Colon 38 orthotopic transplantation model. It inhibited primary tumor growth but showed only growth-inhibitory activity. ER-37328 characteristically shows potent cell-killing activity against tumor cell lines exposed to short-term drug treatment *in vitro*, as compared with etoposide (3). This may explain the more potent tumor-regression activity of ER-37328 than that of etoposide *in vivo*. The development of liver metastasis, which is the reason why long survival is generally not achieved after curative resection of the primary tumor in clinical colon cancer patients (15), was also potently inhibited by ER-37328. In addition, ER-37328 increased the life span of mice bearing orthotopically transplanted Colon 38 tumors.

### Table 1: Antitumor activity of ER-37328 and etoposide against orthotopically transplanted Colon 38 tumor

<table>
<thead>
<tr>
<th>Drug</th>
<th>Administration schedule</th>
<th>Tumor weight (mg)</th>
<th>No. of liver metastases per mouse (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 11 Mean ± SD</td>
<td>Day 20 Mean ± SD (T/C, %)</td>
</tr>
<tr>
<td>Control-1</td>
<td>Day 11</td>
<td>178 ± 68</td>
<td>0, 0, 0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Control-2</td>
<td>Day 11</td>
<td>178 ± 68</td>
<td>0, 0, 0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Day 11, 12, 13, 14, 15</td>
<td>925 ± 394 (100)</td>
<td>0, 0, 0, 1, 2, 2, 6</td>
</tr>
<tr>
<td>ER-37328</td>
<td>Day 11</td>
<td>1101 ± 448 (100)</td>
<td>(1.5 ± 2.0)</td>
</tr>
</tbody>
</table>

*"Total" includes tumor growing at the cecum and peritoneum.

### Table 2: Antitumor activity of ER-37328 against orthotopically transplanted Colon 38 tumor in advanced disease

<table>
<thead>
<tr>
<th>Drug</th>
<th>Administration schedule</th>
<th>Tumor weight (mg)</th>
<th>No. of liver metastases per mouse (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 20 Mean ± SD</td>
<td>Day 28 Mean ± SD (T/C, %)</td>
</tr>
<tr>
<td>Control-1</td>
<td></td>
<td>923 ± 304</td>
<td>0, 0, 0, 0, 0, 0, 0, 1</td>
</tr>
<tr>
<td>Control-2</td>
<td></td>
<td>958 ± 347</td>
<td>(0.1 ± 0.4)</td>
</tr>
<tr>
<td>ER-37328</td>
<td>Day 20</td>
<td>1504 ± 649 (100)</td>
<td>1, 4, 9, 14, 18, 25, 26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1866 ± 643 (100)</td>
<td>27, 30, 33, 35, 75 (23.9 ± 19.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>221 ± 99 (15)</td>
<td>0, 0, 0, 0, 0, 0, 0</td>
</tr>
</tbody>
</table>

*"Total" includes tumor growing at the cecum and peritoneum.
a One mouse was evaluated on day 26 because body weight loss exceeded 25%, and mean tumor weight was calculated by using tumor weight including that of this mouse.
b Liver metastasis was evaluated on day 26 because body weight loss exceeded 25%.

d Liver metastasis was evaluated on day 26 because body weight loss exceeded 25%.
38 tumor, and it drastically regressed Colon 38 tumor implanted into the liver.

Previously, Fidler et al. (22) reported that the chemosensitivity of tumor cells depends on the organ environment. For example, s.c. human colon tumor KM12L4 was sensitive to doxorubicin, whereas tumors growing in the cecum and liver were not. They also showed that P-glycoprotein expression was higher in KM12L4 cells harvested from cecum and liver tumors than in cells harvested from the tumor growing s.c., and they suggested that this may account, at least in part, for the observed differences in doxorubicin sensitivity. In addition, it was reported that colon tumors derived from clinical patients overexpress the mdr1 gene, which encodes P-glycoprotein (23–25). Therefore, it seems to be important to overcome P-glycoprotein-mediated resistance for the development of clinically useful anticancer agents against colon cancer. Most topoisomerase II poisons are substrates for P-glycoprotein (11, 12). This may partially explain the poor efficacy of topoisomerase II poisons against colon cancer in the clinic. In contrast, we reported previously that ER-37328 was effective against a multidrug-resistant cell line that overexpressed P-glycoprotein (10), and in the present study, ER-37328 was found to regress Colon 38 tumor transplanted into the cecum and the liver, as well as that transplanted s.c., as shown in our previous report (10).

To explore the reason why ER-37328 showed excellent antitumor activity against liver metastasis and tumor implanted into the liver, we examined the distribution of ER-37328 in the tumor, liver, and some other organs (Table 5). In this study, M5076 tumor was used instead of Colon 38 tumor, because Colon 38 tumor undergoes rapid necrosis and, therefore, it is difficult to measure the concentration of ER-37328 in the tumor. As a result, ER-37328 was highly distributed to the tumor and organs. The ratios of AUC of ER-37328 in the tumor, lung, liver, and kidney versus plasma were 81, 77, 47, and 40, respectively. This high distribution to the tumor and liver may explain the potent antitumor activity of ER-37328 against Colon 38 tumor in the liver. In addition, ER-37328 may be expected to show antitumor activity against metastasis to the lung, which is a major site of metastasis, in addition to the liver, in colon cancer patients, because of its high distribution to the lung. Funahashi et al.

<table>
<thead>
<tr>
<th>Drug Administration schedule</th>
<th>Tumor volume (mm³)</th>
<th>Day 18</th>
<th>Mean ± SD</th>
<th>Day 25</th>
<th>Mean ± SD (T/C, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-1 (n = 8)</td>
<td>—</td>
<td>506 ± 152</td>
<td>—</td>
<td>—</td>
<td>— (100)</td>
</tr>
<tr>
<td>Control-2 (n = 8)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1291 ± 369 (100)</td>
<td>— (100)</td>
</tr>
<tr>
<td>ER-37328 (n = 8)</td>
<td>Day 18</td>
<td>—</td>
<td>36 ± 36</td>
<td>—</td>
<td>36 ± 36 (3)</td>
</tr>
</tbody>
</table>

Fig. 1. Antitumor activity of ER-37328 against Colon 38 tumor transplanted into the liver. Colon 38 tumor was inoculated into the left lobe of the liver on day 0. ER-37328 was administered i.v. at a dose of 10 mg/kg on day 18, and the tumor volume was examined on day 25. Tumor volume in control groups was examined on day 18 (control-1) and on day 25 (control-2). The first, third, fifth, and seventh largest tumors in each group are shown in the figure.
(19) showed that lung metastasis developed at relatively low frequency as compared with liver metastasis in a Colon 38 orthotopic transplantation model. Therefore, it would be of interest to develop a lung metastasis model with high incidence by establishing highly lung-metastatic subpopulations of Colon 38 tumor and to examine the activity of ER-37328 against lung metastasis in the model.

The administration of ER-37328 on a Q7Dx4 schedule produced a significant ILS. This activity seems to be because of tumor regression activity of ER-37328 against the primary tumor on the cecum, because liver metastases formed only small nodes during the experimental period. In the clinic, standard treatment of colon cancer has been open surgical resection of the primary and regional lymph nodes for localized disease and chemotherapy for advanced disease. Therefore, a model in which mice die of liver metastasis may be preferable for evaluating the activity of drugs in terms of life span. We are currently developing such a model to examine additionally the activity of ER-37328 against liver metastasis.

In conclusion, the data presented here strongly suggest that ER-37328, a topoisomerase II poison, is a promising candidate for clinical application against colon cancer.

References
shima, M. Antitumor activity of 1 M tegafur-0.4 M 5-chloro-2,4-dihydroxy-pyridine-1 M potassium oxonate (S-1) against human colon carcinoma orthotopically implanted into nude rats. Cancer Res., 56: 2602–2606, 1996.


Molecular Cancer Therapeutics

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