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ORIGINAL ARTICLE

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Effect of E7010 on liver metastasis and life span of syngeneic C57BL/6 mice bearing orthotopically transplanted murine Colon 38 tumor

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Abstract *Purpose*: E7010 is an orally active sulfonamide antitumor agent showing good activity against various subcutaneously inoculated rodent tumors and human tumor xenografts. The purpose of this study was to evaluate the effect of E7010 on liver metastasis and life span of mice bearing orthotopically transplanted murine Colon 38 tumor. *Methods*: Orthotopic transplantation of murine Colon 38 tumor as intact tissue yielded hepatic metastasis with a high incidence in about 1 month in C57BL/6 mice, and the mice died in about 2 months with cachexia. In this model, the maximum tolerated dose of E7010 (100 mg/kg per day) was administered orally on various schedules, including for 14 days or daily until death, starting at 14 days after transplantation, or for 8 days from 21 days after transplantation. *Results*: E7010 showed tumor growth inhibition (T/C = 40%) at the orthotopic site similar to that at the subcutaneous site (T/C = 32%) when administered from 14 days after transplantation. When E7010 was started from 21 days after transplantation, it significantly decreased the number of hepatic metastases (control 17.1 ± 20.8, E7010 2.6 ± 5.3), although inhibition of tumor growth at the orthotopic site was only moderate (T/C = 60%). The administration of E7010 until death produced a significant increase in life span (control 49.8 ± 8.9 days, E7010 62.5 ± 6.1 days). Although the tumor weight of the E7010-treated group on the day of death was similar to that of the untreated group (control 1.166 ± 0.507 g, E7010 1.211 ± 0.632 g), there were significantly fewer liver metastases in the E7010-treated group (control 41.3 ± 31.1, E7010 2.0 ± 2.0). *Conclusion*: E7010 suppressed tumor growth at both primary and metastatic sites and increased life span in an orthotopic transplantation model of murine Colon 38 tumor in syngeneic C57BL/6 mice. Hepatic metastasis was inhibited more effectively than the growth of the primary tumor.

Key words E7010 · Antimitotic · Orthotopic transplantation · Survival · Liver metastasis

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Introduction

E7010 is an orally active, novel sulfonamide anticancer agent [19], which exhibits a broad spectrum of antitumor activity against human tumor xenografts [8]. It increases the percentage of mitotic cells and inhibits tubulin polymerization in a dose-dependent manner, and these activities correlate well with its cell growth-inhibitory activity [18]. Binding of E7010 to purified tubulin is inhibited by colchicine, but not by vincristine, although the binding properties are different from those of colchicine. Furthermore, E7010 is effective against tumor cells which are multidrug-resistant due to overexpression of P-glycoprotein. E7010-resistant P388 cell lines show no cross-resistance to vincristine or paclitaxel [18].

In a phase I study of single or 5-day repeated administration of E7010 [17], reduction of spinal cord metastasis in patients with uterine sarcoma, a minor response in patients with pulmonary adenocarcinoma and decreases in carcinoembryonic antigen and squamous cell carcinoma antigen in patients with stomach cancer and recurrent uterine cervical carcinoma, respectively, were observed. The dose-limiting toxicity in the single-dose study was peripheral neuropathy and that in the 5-day repeated-dose study was peripheral neuropathy together with intestinal paralysis. Pharmacokinetic analysis showed that E7010 has a favorable absorption and elimination profile and does not accumulate. The maximum allowable doses were 320 mg/mm^2 for the single-dose study and 200 mg/mm^2 for the 5-day repeated-dose study. A divided dose study is needed to see whether the blood level of E7010 can be better controlled.

Recent clinical studies of anticancer agents have tended to focus on parameters such as clinical benefit,

time to progression, overall survival and quality of life [14, 15]. Compared to the subcutaneous (s.c.) xenograft model which has generally been used for preclinical evaluation of anticancer agents, an orthotopic transplantation model may be more appropriate for such evaluations because it should better reflect the behavior of clinical tumors [3]. An orthotopically transplanted colon cancer model has been found to be useful for evaluating effect on life span [12, 16]. Moreover, inhibition of metastasis, which is difficult to evaluate in a clinical study, but is important to improve the outcome of cancer therapy, can also be evaluated [4] in an orthotopic transplantation model.

We have previously reported that orthotopically transplanted murine Colon 38 tumor rapidly metastasizes to the liver in syngeneic C57BL/6 mice [6], as occurs in colorectal cancer patients, and the mice die in about 2 months with small variation. Therefore, we considered that this was an appropriate model to evaluate the effects of drugs on metastasis of colon cancer and survival. In this study, we used the model to investigate the effects of E7010 on liver metastasis and survival, in addition to primary tumor growth.

Materials and methods

Drugs

E7010 was synthesized at Eisai Company (Tsukuba Research Laboratories, Ibaraki, Japan). E7010 was suspended in 0.5% methylcellulose and administered orally. 5-Fluorouracil (5-FU) was purchased from Kyowa Hakko Kogyo Company, Tokyo, Japan. It was diluted with 0.9% NaCl and administered orally. The control group was given 0.5% methylcellulose orally. The oral administration was accomplished by using a stainless steel gavage tube.

Animals

Female C57BL/6 mice were obtained from Charles River, Atsugi, Japan. They were given food (MF; Oriental Yeast Company) and UV-irradiated water ad libitum and maintained under specific pathogen-free conditions. They were used for experiments when they were 6 to 8 weeks old.

Tumor cells

Murine Colon 38 tumor was supplied by the Cancer Chemotherapy Center, Japan Foundation for Cancer Research, Tokyo, and maintained by serial s.c. inoculation in female C57BL/6 mice.

Orthotopic transplantation of Colon 38 intact tissue

Orthotopic transplantation of colon cancer intact tissue was conducted as described previously [6]. Briefly, Colon 38 tumor growing subcutaneously in C57BL/6 mice was resected and the tumor tissues were cut into pieces weighing 25 mg in Hank's balance salt solution after aseptic removal of necrotic portions. Mice were anesthetized with a 2.5% solution of a 1:1 mixture of 2,2,2-tribromoethanol (Aldrich, Milwaukee, Wis.) and *t*-amyl alcohol (Wako, Osaka, Japan). An incision was made in the left lower abdomen. Then the cecum was gently exposed and one of the tumor pieces was fixed onto the surface of the cecum with a 6-0 Dexon II suture (Davis-Geck, Manati, Puerto Rico). The cecum was returned to the abdominal cavity and the incision was closed with a Dexon II suture.

Experimental chemotherapy of subcutaneously inoculated Colon 38

About 25 mg of Colon 38 tumor was inoculated s.c. with a trocar into the right flank of each mouse on day 0. The animals were divided into vehicle- and drug-treated groups consisting of eight animals each on day 14 after transplantation. Each tumor was measured with a sliding caliper. The volume of the cuboid mass was calculated from the major dimension (L) and minor dimension (S) using the following equation: $tumorvolume = L \times (S)^2/2$. Antitumor activity in terms of tumor growth was determined by expressing the mean tumor weight of the test group (T) as a percentage of that of the control group (C) (T/C × 100).

Experimental chemotherapy of orthotopically transplanted Colon 38

Mice were transplanted orthotopically with about 25 mg of Colon 38 on day 0 and divided into vehicle- and drug-treated groups on either day 14 or day 21 after transplantation. E7010 was administered orally daily according to the indicated regimen for each experiment, at a dose of 100 mg/kg which was the maximum tolerated dose (MTD) with the 8-day daily schedule. 5-FU was administered orally daily at a dose of 30 mg/kg, which was the MTD in our experiment with the 8-day daily schedule. Mice were killed on the indicated days and the locally growing tumor and liver were resected. The excised tumors were weighed and the metastatic nodules in the liver were counted in a blind manner under a dissecting microscope after staining the liver with Bouin's solution.

For determination of life span, orthotopically transplanted mice were observed until 75 days after transplantation and were autopsied on the day of death or at the end of the observation period. If mice died with only local small tumor and death was accompanied by a loss of body weight, we judged the death as toxic. Antitumor activity in terms of tumor growth was determined by expressing the mean tumor weight of the test group (T) as a percentage of that of the control group (C) (T/C × 100). Efficacy in terms of life span was determined as the percentage increase in life span (ILS%) calculated in terms of mean survival times (MST): $ILS(\%) = [(MSTofthetestgroup/MSTofthecontrolgroup) - 1] \times 100$.

Statistical analysis

Differences in tumor weight and relative tumor volume were analyzed for significance using Student's *t*-test. The Mann–Whitney *U*-test and Kruskal–Wallis' *h*-test followed by the Steel test were used to compare the number of metastatic nodules in the livers. Differences in survival time were analyzed for significance using the log-rank test.

Results

Antitumor activity of E7010 against subcutaneously inoculated murine Colon 38 tumor

We first examined the antitumor activity of E7010 against subcutaneously inoculated murine Colon 38 tumor in syngeneic C57BL/6 mice. About 25 mg of tumor was inoculated s.c. on day 0. E7010 was administered orally at a dose of 100 mg/kg daily for 8 days from 14 days after inoculation, and then on day 23 the efficacy

of E7010 was determined. E7010 significantly inhibited s.c. tumor growth with a T/C of 41% for tumor volume and 32% for relative tumor volume compared to that on day 14 (Table 1). The body weight of mice in the treated group was not different from that on day 14 (data not shown).

Antitumor activity of E7010 against orthotopically transplanted murine Colon 38 tumor

We next examined the antitumor activity of E7010 against orthotopically transplanted murine Colon 38 tumor in syngeneic C57BL/6 mice. On day 0, about 25 mg of tumor tissue was transplanted onto the cecum. E7010 was administered orally at a dose of 100 mg/kg daily for 14 days from 14 days after transplantation, and then on day 28, the mice were autopsied and the effects of E7010 on tumor growth in both the cecum and peritoneum (tumor burden) and on liver metastasis were evaluated. E7010 significantly inhibited local growth with a T/C of 49% and tumor burden with a T/C of 40% (Table 2). The efficacy of E7010 in the orthotopic model was similar to that in the s.c. model. The number of liver metastases seemed to be reduced in the E7010-treated group, but it was difficult to evaluate the antimetastatic activity of E7010 because the incidence of mice with liver metastasis was low under this condition.

Therefore, we investigated whether E7010 inhibited liver metastasis of murine Colon 38 tumor in another regimen. E7010 was administered daily for 8 days from 21 days after transplantation, and then on day 36 mice were autopsied and the effect of E7010 was evaluated. E7010 decreased the tumor burden with a T/C of 60% but this was not statistically significant (Table 3). However, the number of liver metastases was significantly inhibited in the E7010-treated group (vehicle group 17.1 ± 20.8, E7010 group 2.6 ± 5.3, mean $\pm$ SD). Also, three of eight mice in the vehicle-treated group died with cachexia within 36 days, while all mice in the E7010-treated group were alive at this time.

Effect of E7010 on life span in mice bearing orthotopically transplanted murine Colon 38 tumor

In the experiment to evaluate the effect of E7010 on day 36 after transplantation, E7010 appeared to improve the survival time of mice bearing orthotopically transplanted tumor. Therefore, we examined the effect of E7010 on life span using this model with a consecutive administration schedule until 75 days after transplantation. We have previously reported that 5-FU administered orally at a dose of 30 mg/kg daily for 8 days from day 14 after transplantation decreases local tumor growth and inhibits liver metastasis on day 28 [6]. Therefore, we used 5-FU as a reference drug. The consecutive administration of 5-FU at the above dose from day 14 after transplantation completely blocked liver metastasis in mice bearing orthotopically transplanted murine Colon 38 (Table 4). Four of the eight mice treated with 5-FU were alive on day 75, but three mice died of toxicity (Table 5). Compared with the survival time of mice treated with the vehicle, that of mice treated

Table 1 Effect of 8-day daily administration of E7010 from 14 days after subcutaneous inoculation of murine Colon 38 tumor in C57BL/6 mice, evaluated on day 23 (values are means $\pm$ SD)

Drug	Dose (mg/kg)	Tumor volume (mm^3)			Relative tumor volume[a]
		Day 14	Day 23		
				T/C (%)	T/C (%)
Control	–	185.7 ± 146.4	667.0 ± 422.1	100	4.1 ± 1.8 100
E7010	100	181.6 ± 113.2	272.1 ± 189.9*	41	1.3 ± 0.6** 32

* $P < 0.05$, ** $P < 0.01$ vs control
[a] Tumor volume on day 23/tumor volume on 14

Table 2 Effect of 14-day daily administration of E7010 from 14 days after orthotopic transplantation of murine Colon 38 tumor in C57BL/6 mice, evaluated on day 28

Drug	Dose (mg/kg)		Incidence	Tumor weight (g) (mean $\pm$ SD)	T/C (%)	No. of hepatic metastases per mouse
Control	–	Cecum	8/8	0.830 ± 0.400	100	0, 0, 0, 0, 1, 2, 15, 29 (5.9 ± 10.7)
		Peritoneum	4/8	0.422 ± 0.280	100	
		Tumor burden[a]		1.041 ± 0.338	100	
E7010	100	Cecum	8/8	0.403 ± 0.220*	49	0, 0, 0, 0, 0, 1, 10, 13 (3.0 ± 5.3)
		Peritoneum	1/8	0.089	21	
		Tumor burden[a]		0.415 ± 0.248**	40	

* $P < 0.05$, ** $P < 0.01$ vs control
[a] Tumor burden includes tumor growing at the cecum and peritoneum

Table 3 Effect of 8-day daily administration of E7010 from 21 days after orthotopic transplantation of murine Colon 38 tumor in C57BL/6 mice, evaluated on day 36

Drug	Dose (mg/kg)	Tumor burden (g)		No. of hepatic metastases per mouse (mean ± SD)	Death with cachexia
		Mean ± SD	T/C (%)		
Control	–	1.790 ± 0.743	100	1[a], 2, 4, 7, 11, 18[a], 32[a], 62 (17.1 ± 20.8)	3/8
E7010	100	1.078 ± 0.398	60	0, 0, 0, 1, 12* (2.6 ± 5.3)	0/5

* $P < 0.05$
[a] Died on days 32, 35 and 36 after transplantation

Table 4 Effect of 60-day consecutive administration of E7010 and 5-FU from 14 days after orthotopic transplantation until death on hepatic metastasis of C57BL/6 mice bearing orthotopically transplanted murine Colon 38 tumor, evaluated on day 75

Drug	Dose (mg/kg)	Tumor burden (g)	Liver metastasis[a]			Toxic death
			No. of metastatic nodules per mouse	Mean ± SD	T/C (%)	
Control		1.166 ± 0.507	9, 15, 24, 34, 41, 49, 49, 109	41.3 ± 31.1	100	–
5-FU	30	0.816 ± 0.634	0, 0, 0, 0, 0, 0, 0, 0*[,b]	0	0	3/8
E7010	100	1.211 ± 0.632	0, 0, 1, 1, 2, 2, 5, 5*	2.0 ± 2.0	5	0/8

* $P < 0.01$
[a] Liver metastasis was evaluated on the day of death
[b] Four of eight mice were autopsied on day 75

Table 5 Effect of 60-day consecutive administration of E7010 and 5-FU from 14 days after orthotopic transplantation until death on life span of C57BL/6 mice bearing orthotopically transplanted murine Colon 38 tumor, evaluated on day 75 (*ND* not determined)

Drug	Dose (mg/kg)	Survival after transplantation (days)		Increase in life span (%)	Survivors at day 75
			Mean ± SD		
Control		37, 42, 42, 48, 53, 54, 55, 67	49.8 ± 8.9	–	0/8
5-FU	30	35[a], 41[a], 61[a], 65, ≥75, ≥75, ≥75, ≥75	ND	ND	4/8
E7010	100	57, 57, 58, 59, 61, 63, 71, 74*	62.5 ± 6.1	26	0/8

* $P < 0.01$ vs control
[a] Died of toxicity

with E7010 was significantly increased with an ILS of 26% (Table 5). Moreover, the number of liver metastases was significantly decreased in the E7010-treated group (vehicle group 41.3 ± 31.1, E7010 group 2.0 ± 2.0, mean ± SD), although the tumor burden (weight) on the day of death in the E7010-treated group was similar to that in the vehicle-treated group (Table 4). This result suggests that E7010 inhibited tumor growth more effectively at the metastatic site than at the primary site.

Discussion

We first examined the antitumor activity of E7010 against local tumor growth in the cecum in our model because it had been reported that the chemosensitivity of tumor cells is different depending on the site of inoculation in mice [5]. E7010 administered orally from 14 days after inoculation showed similar antitumor activity against the growth of Colon 38 tumor at both ectopic (s.c.) and orthotopic (cecum) sites. The drug was well distributed to various tissues (data not shown), and this might account in part for the similar effects at both sites.

In the evaluation of the effects on life span, the survival time of mice bearing orthotopically transplanted Colon 38 tumor was found to be significantly increased with an ILS of 26% following administration of E7010. The effects of other oral fluoropyrimidines, S-1 (15 mg/kg) and UFT (30 mg/kg), on the life span of nude rats bearing orthotopically transplanted human colorectal carcinoma KM12C cells have been examined [12]. The drugs were administered daily for 28 days from day 0 and the mean survival period in the control group was 73 days. S-1 and UFT both increased the life span of nude rats, with ILS values of 22% and 11%, respectively. Fluoropyrimidines are used for adjuvant chemotherapy in the treatment of colon cancer patients. The effect of E7010 on life span was similar to that of flu-

oropyrimidines in animal models, and thus E7010 may be of clinical value.

E7010 showed clear inhibitory activity against liver metastasis of orthotopically transplanted murine Colon 38 on day 36. Also, the numbers of metastases in the vehicle- and E7010-treated group were different, even though the tumor weight on the day of death was similar in the two groups in the life-span study. These results suggest that E7010 shows stronger antitumor activity against liver metastasis than against the primary tumor.

In clinical colon cancer patients, liver metastasis is the reason why long survival is generally not achieved after curative resection of the primary tumor [3]. A model in which the animals die of liver metastasis may be preferable for evaluating the effect of drugs on life span. Although the survival time of mice treated with E7010 was increased in our model, all mice treated died of cachexia, which seemed to be due to primary tumor growth, by day 75 after transplantation. Since E7010 had potently inhibited liver metastasis in mice at the time of death, the effect of E7010 on life span might be evaluated more appropriately by using a model in which mice die of liver metastasis. However, mice in which orthotopically transplanted Colon 38 tumor was resected 7 days after transplantation did not develop liver metastasis (data not shown). Establishing high-metastatic subpopulations of Colon 38 tumor so that metastasis would develop earlier may be necessary to produce an improved model for evaluating survival time, in which the primary tumor can be removed at an early stage and the mice subsequently die of liver metastasis.

We have previously reported that 5-FU at a dose of 30 mg/kg administered orally for 8 days from 14 days after transplantation, inhibits local tumor growth and liver metastasis of mice bearing orthotopically transplanted murine Colon 38 tumor on day 28 [6]. In the present study, when 30 mg/kg of 5-FU was administered consecutively, 5-FU completely inhibited liver metastasis until day 75 after transplantation. Further, four of eight mice survived for 75 days in the 5-FU group, while three of eight mice died of toxicity. With daily administration for 14 days from day 14 after transplantation, with evaluation on day 28, 5-FU inhibited tumor growth with a T/C of 9% and no mice died of toxicity (data not shown). Therefore, the dose of 30 mg/kg of 5-FU was toxic when administered consecutively, but was not toxic when administered for only 14 days. In order to determine the effect of 5-FU on life span more precisely, 5-FU should be administered at a lower dose. Although the E7010 dose of 100 mg/kg was the MTD in the 8-day administration schedule [8], that dose could also be administered for 60 days without toxicity, at least in our model.

Recently, three new anticancer drugs which affect tubulin polymerization and block cell cycle progression at the M phase, i.e. paclitaxel, docetaxel and vinorelbine, have entered clinical use for the treatment of ovarian, breast and non-small-cell lung cancer [1, 2, 7, 9, 11]. However, the efficacy of these drugs in gastrointestinal cancer remains to be determined, in part because of the high incidence of overexpression of MDR protein in gastrointestinal cancer [10], which blocks the effect of these drugs [13]. E7010 is a novel anticancer agent that inhibits tubulin polymerization and cell cycle progression at the M phase, and is active against MDR-overexpressing tumor and the parent tumor cells at similar doses [18]. In the study reported here we showed that E7010 significantly inhibited tumor growth in the cecum, increased life span and inhibited liver metastasis of murine colorectal carcinoma Colon 38. These results suggest that E7010 should be further examined for antitumor activity against gastrointestinal cancer.

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