Tumor Selective Effect of RS-1541 (Palmitoyl–Rhizoxin) in M5076 Sarcoma and Host Tissues *in Vivo*

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RS-1541 is a 13-O-palmitoyl derivative of rhizoxin, an inhibitor of tubulin polymerization. After intravenous administration of RS-1541 to mice bearing M5076 sarcoma, the maximal inhibitory effect of RS-1541 on DNA synthesis in the tumor was observed 24 h after administration, in agreement with the Cmax of rhizoxin produced from RS-1541, but not with the Cmax of RS-1541. The inhibitory effect after RS-1541 was much higher than that after rhizoxin itself. In the spleen, thymus and bone marrow, DNA synthesis was strongly inhibited by rhizoxin but not by RS-1541. After administration of RS-1541, no significant amounts of rhizoxin were detected in the tissues, except for the tumor. In acute toxicity tests, RS-1541 appeared to be less toxic than rhizoxin. These results indicate that RS-1541 possesses a high tumor-selective effect compared with rhizoxin, because of the selective production of rhizoxin in the tumor after administration of RS-1541.

KEY WORDS: RS-1541; rhizoxin; metabolic activation; tumor selectivity.

INTRODUCTION

Rhizoxin was discovered as an agent that induces rice seedling blight (1). The toxicity of rhizoxin has been shown to result from an inhibitory effect on tubulin polymerization (2), which thereby inhibits cell mitosis (3). This mode of action suggested the application of rhizoxin as an anti-tumor agent (4). In various *in vivo* tests, our laboratories found that RS-1541, a 13-O-palmitoyl derivative of rhizoxin (Fig. 1), exhibits much higher anti-tumor activity than rhizoxin (5). *In vitro,* however, substitution of a hydroxyl group at the 13-position of rhizoxin is known to markedly reduce the inhibitory effect of rhizoxin on tubulin polymerization (6).

In order to elucidate the increased activity of RS-1541 relative to rhizoxin *in vivo,* we previously examined the anti-tumor activities and pharmacokinetics of RS-1541 compared with those of rhizoxin, after single intravenous administration to mice bearing M5076 sarcoma, a spontaneous murine reticulum cell sarcoma (7). RS-1541 had a far more potent and prolonged anti-tumor activity than rhizoxin. RS-1541 bound preferentially to plasma lipoproteins and showed selective and sustained uptake by the tumor, while rhizoxin did not. Although the ester bond of RS-1541 was stable in plasma, a considerable amount of rhizoxin was detected in the tumor. The rhizoxin amount in the tumor after administration of RS-1541 was much higher than that after rhizoxin itself. The rhizoxin formation in the tumor *in vivo* was inhibited by chloroquine, an inhibitor of lysosomal enzymes, suggesting that RS-1541 was incorporated into the tumor via the endocytotic pathway after binding to lipoproteins, the uptake of which results in the formation of rhizoxin, the original anti-tumor agent, in the lysosomes.

In this study, we examined the cytocidal effect of RS-1541 compared with that of rhizoxin in mice bearing M5076 sarcoma, by measuring the compounds' inhibitory actions on ^{14}C-thymidine incorporation into DNA in several tissues. The acute toxicities of the compounds were also examined. We discuss the importance of rhizoxin formation in the tissue-selective action of RS-1541, based on the rhizoxin levels in tissues.

MATERIALS AND METHODS

RS-1541, Rhizoxin, and Other Reagents. Rhizoxin was isolated from culture broth of *Rhizopus chinensis* Rh-2 (8) in the Fermentation Research Laboratories of Sankyo Co. RS-1541 (13-O-palmitoyl rhizoxin) was synthesized by palmitoylation of rhizoxin, in the Bioscience Research Laboratories of Sankyo Co., Ltd. The structures of RS-1541 and rhizoxin are shown in Fig. 1. [2-^{14}C]-thymidine (1.85 GBq/mmol) was purchased from NEN Research Products, Japan. Polyoxyethylene (60) hydrogenated castor oil (HCO60) was obtained from NIKKOL, Tokyo. All other chemicals used were commercially available, reagent grade.

Animals. Female BDF$_1$ mice (F$_1$ from C57BL/6 female × DBA/2 male) were obtained from Charles River Japan, Tokyo, and allowed free access to water and food throughout the study. Each mouse was transplanted subcutaneously with 10^6 cells of M5076 sarcoma, a murine reticulum cell sarcoma, into their axillae at the age of 8–9 weeks. Experiments were conducted 13 days after inoculation.

Preparation of Dosing Solutions. RS-1541 (0.01–4.0 mg) was dissolved in 0.1 ml of dimethylacetamide, with each solution containing of HCO60 (0.02–8 mg) at twice the weight of the RS-1541. The solutions were further diluted in 9.9 ml of 0.15 M NaCl with sonication. Rhizoxin (1.0–4.0 mg) was dissolved in 0.2 ml of dimethylacetamide, and the solution was dispersed in 9.8 ml of 0.15 M NaCl containing 1%-Tween 80. Each dosing solution was administered through the tail veins of the animals at 0.01–4.0 mg/kg. ^{14}C-thymidine was intraperitoneally administered at 100 μCi/kg.

Incorporation of ^{14}C-Thymidine into DNA (9). Animal groups each consisting of 6 mice were administered RS-1541 or rhizoxin intravenously at 1 mg/kg, and killed by exsanguination through cardiac puncture under diethylether anesthesia after 2, 6, 24, or 48 h. One hour before exsanguination, ^{14}C-thymidine was intraperitoneally administered to each animal at 2 μCi/body. The spleen, thymus, small intestine, femurs and tumor were excised quickly and weighed. Bone marrow was obtained by washing the inner side of the femurs with 2 ml of 0.15 M NaCl. Each tissue was homogenized in

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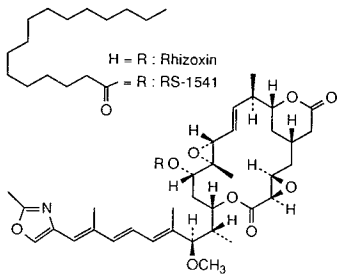

Fig. 1. Structures of RS-1541 and rhizoxin.

1 ml of 10% trichloroacetic acid (TCA), and centrifuged. The pellets were washed with another 1 ml of 10% TCA, and the pellets after centrifugation were dissolved in 1 ml of 1N NaOH to determine the radioactivity incorporated into DNA. The radioactivity was counted in an Aloka LCS-1000 liquid scintillation spectrophotometer. The DNA concentration of bone marrow samples was measured by the Burton method (10). The incorporated radioactivity was expressed as the amount of radioactivity per gram tissue (or per mg DNA in the case of bone marrow). The effects of RS-1541 and rhizoxin were expressed as the ratio (%) of incorporated radioactivity in the treated animals to that in the control animals.

Concentrations of RS-1541 and Rhizoxin in Plasma and Tissues. After single intravenous administration of RS-1541 or rhizoxin to tumor-bearing mice at 1 or 4 mg/kg, 3 animals were killed at each time point (5 and 30 min, and 2, 6, 24, and 48 h), and the plasma and tissues were collected. The samples were homogenized in a 4-fold volume of acetonitrile. The homogenate was centrifuged at 3,000 rpm for 10 min at 4°C, and the resultant supernatant fraction was subjected to high performance liquid chromatography (HPLC) under the following conditions; Column : Inertsil ODS-2 (4.6 × 150 mm) Gasukuro Kogyo, Japan, Detection : 310 nm, and Mobile phase : acetonitrile/tetrahydrofuran/H_2O (92/5/3) at 1.5 ml/min for RS-1541, acetonitrile/H_2O (60/40) at 1.0 ml/min for rhizoxin.

Pharmacokinetic Analysis. The plasma concentration data were fitted to the following equation by nonlinear iterative least squares regression (11): $Cp = A \cdot \exp(-\alpha t)$, where Cp is the plasma concentration, t the time, and A and α are constants. The area under the plasma concentration-time curve (AUC) was calculated by the equation : $AUC = A/\alpha$. The distribution volume (Vd) was calculated by the equation : Vd = Dose/A. The systemic clearance (CLp) based on the plasma concentration was calculated by the equation: CLp = Dose/AUC. The area under the tissue concentration-time curve (AUCt) and the mean residence time (MRT) were determined by the trapezoidal method.

Acute Toxicity. RS-1541, rhizoxin, placebo vehicle, or saline was administered intravenously at 1 or 4 mg/kg to female BDF_1 mice. Six mice were allocated to each animal group. On Days 1, 2, and 4, the animals were weighed, and blood samples were collected from the retro-orbital venous plexus to measure the numbers of red blood cells (RBC), white blood cells (WBC), and platelets (PLAT). The animals were killed by exsanguination from the carotid artery under diethylether anesthesia. Spleen, thymus, small intestine, and femur were excised quickly, weighed, and processed routinely for histological examination. Tissue preparations were fixed in 10% neutral buffered formalin, embedded in paraffin, stained by hematoxylin-eosin and examined by light microscopy.

RESULTS

Inhibition of DNA Synthesis. The inhibitory effects of RS-1541 and rhizoxin on ^{14}C-thymidine incorporation into DNA fractions of the tumor were measured at 1 mg/kg (Fig. 2a). After administration of rhizoxin, the incorporated radioactivity decreased to the lowest value (40% of control) after 2 h, and gradually recovered thereafter, reaching almost control level after 48 h. After administration of RS-1541, the radioactivity decreased to 18% of control at 24 h, and was still 25% at 48 h. RS-1541 (0.01–4 mg/kg) inhibited the ^{14}C-thymidine incorporation in a dose-dependent manner 24 h after administration (Fig. 2b). The half maximal inhibitory dose was about 0.25 mg/kg.

In the spleen, thymus, and bone marrow, rhizoxin significantly inhibited the incorporation of radioactivity after 6–24 h, but RS-1541 had no effect on these tissues (Fig. 3a,b,d). In the small intestine, rhizoxin had a maximal effect after 6 h, whereas RS-1541 showed a maximum after 24 h (Fig. 3c). However, the effects observed in the intestine were weak and recovered quickly compared with those in the tumor. Each tissue differs from the others in terms of the rate of recovery from inhibition caused by rhizoxin; the small intestine was the fastest and the thymus was the slowest.

Plasma and Tissue Concentrations of Rhizoxin and RS-1541. After intravenous administration of rhizoxin at 1 and 4 mg/kg, the plasma concentration decreased rapidly with respective half lives ($T_{1/2}$) of 0.21 h and 0.13 h (Fig. 4 and Table I). Both systemic clearance (CLp: 1.8×10^4 and 2.0×10^4 ml/h/kg) and distribution volume (Vd: 5.4×10^2 and 3.8×10^2

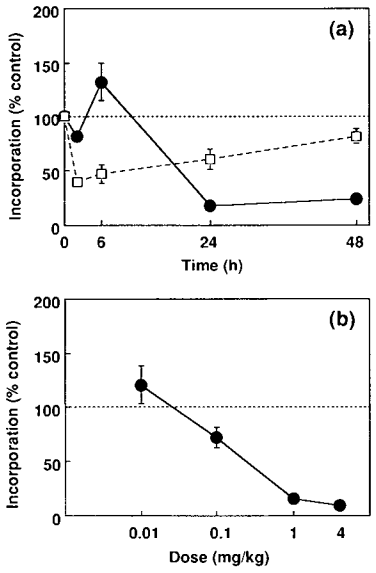

Fig. 2. Inhibitory effects of RS-1541 (●) and rhizoxin (□) at 1 mg/kg on incorporation of ^{14}C-thymidine into DNA fraction of the tumor (a) and the dose-dependent effect of RS-1541 (b). Incorporated radioactivity of the control animals was 204 ± 6 dpm/mg tissue. Each value represents the mean ± s.e. of 6 mice.

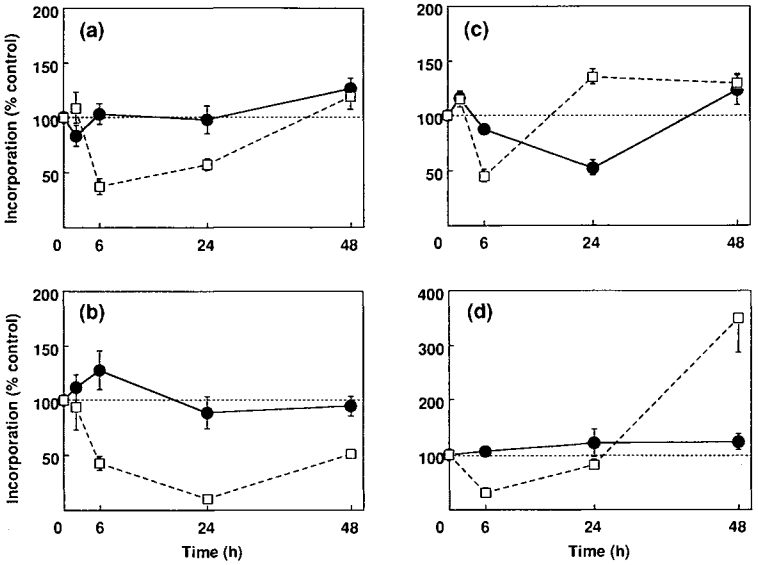

Fig. 3. Inhibitory effects of RS-1541 (●) and rhizoxin (□) at 1 mg/kg on incorporation of ^{14}C-thymidine into DNA fraction of the spleen (a), thymus (b), small intestine (c), and bone marrow (d). Incorporated radioactivities in the spleen, thymus, small intestine, and bone marrow of the control animals were 605 ± 82 dpm/mg tissue, 172 ± 10 dpm/mg tissue, 493 ± 12 dpm/mg tissue, and 28.5 ± 2.9 dpm/μg DNA, respectively. Each value represents the mean ± s.e. of 6 mice.

ml/kg) were very large. On the other hand, after administration of RS-1541 at 1 and 4 mg/kg, the plasma concentration decreased slowly with respective half lives of 6.7 and 6.3 h (Fig. 4 and Table I). The CLp values for RS-1541 (6.5 and 5.6 ml/h/kg) were 3,000 times less than those for rhizoxin. The Vd values (63 and 51 ml/kg) were small, being almost the same as the plasma volume of mice. After administration of RS-1541, rhizoxin was not detected in the plasma.

After administration of RS-1541 at 1 and 4 mg/kg, RS-1541 in the tumor reached the Cmax levels of 3.3 and 16.2 μg/g, respectively, at 6 h; and then decreased slowly (Fig. 5a). On the other hand, rhizoxin produced from RS-1541 in the tumor increased gradually and reached the Cmax levels of 0.77 and 1.36 μg/g for the doses, respectively, at 24 h (Fig. 5b). However, after administration of rhizoxin at 1 and 4 mg/kg, rhizoxin in the tumor immediately reached the respective Cmax levels of 0.37 and 1.25 μg/g, after 0.5 h; and then decreased rapidly (Fig. 5b). As shown in Table II, after administration of RS-1541, AUCt values of rhizoxin in the tumor (25.7 and 49.1 μg · h/g) were three to five times greater than those after administration of rhizoxin (4.8 and 18.6 μg · h/g). MRT values of rhizoxin in the tumor after RS-1541 (32.0 and 31.8 h) were also two to three times greater than those after rhizoxin (6.6 and 14.7 h).

Concentrations of RS-1541 and rhizoxin in various tissues 24 h after administration of RS-1541 at 4 mg/kg are shown in Fig. 6(b). RS-1541 was observed at high levels in the liver, spleen, and tumor. On the other hand, rhizoxin

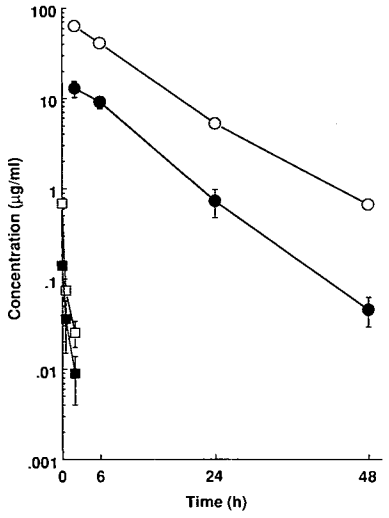

Fig. 4. Plasma concentrations of RS-1541 (●,○) and rhizoxin (■,□) after intravenous administration to mice bearing M5076 sarcoma at 1 mg/kg (closed) and 4 mg/kg (open). Each value represents the mean ± s.e. of 3 mice.

Table I. Pharmacokinetic Parameters for RS-1541 and Rhizoxin in Plasma After Intravenous Administration to Mice Bearing M5076 Sarcoma

	RS-1541		Rhizoxin	
Dose (mg/kg)	1	4	1	4
$T_{1/2}$ (h)	6.65	6.33	0.213	0.131
Ke (l/h)	0.104	0.109	3.25	5.30
Co (μg/ml)	16.0	78.9	0.185	1.07
AUC (μg · h/ml)	153	721	0.057	0.201
CLp (ml/h/kg)	6.53	5.55	1.76×10^4	1.99×10^4
Vd (ml/kg)	62.6	50.7	5.41×10^2	3.76×10^2

Results were obtained from the average plasma concentration of 3 mice in each time point and calculated by non-linear least squares methods.

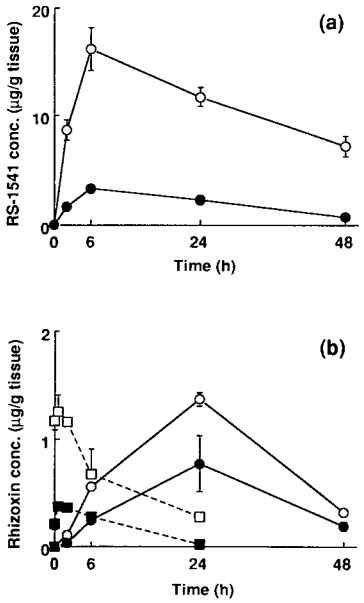

Fig. 5. Tumor concentrations of RS-1541 (a) and rhizoxin (b) after intravenous administration of RS-1541 (●,○) and rhizoxin (■,□) to mice bearing M5076 sarcoma at 1 mg/kg (closed) and 4 mg/kg (open). Each value represents the mean ± s.e. of 3 mice.

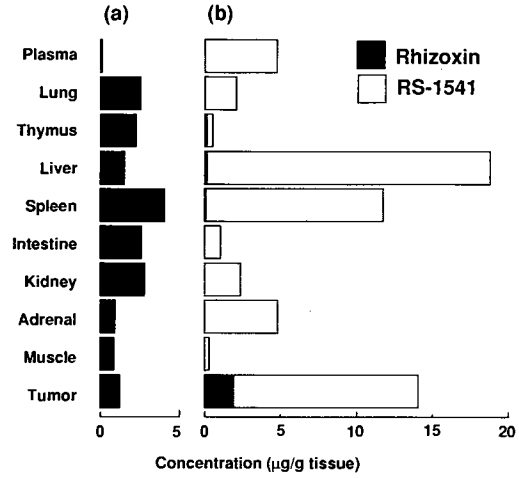

Fig. 6. Tumor selective formation of rhizoxin in mice bearing M5076 sarcoma. Concentrations of rhizoxin (solid bar) 0.5 h after administration of rhizoxin at 4 mg/kg (a). Concentrations of RS-1541 (open bar) and rhizoxin (solid bar) 24 h after administration of RS-1541 at 4 mg/kg (b). Each value represents the mean of 3 mice.

produced from RS-1541 was only significantly detected in the tumor. The rhizoxin concentrations after RS-1541 were not more than 0.1 μg/g in the liver, spleen, and thymus, and only small traces were found in the lung and intestine. However, 0.5 h after administration of rhizoxin at 4 mg/kg, concentrations of rhizoxin in most tissues were higher than in the tumor (Fig. 6(a)).

Acute Toxicity of RS-1541 and Rhizoxin in Mice. The results of acute toxicity tests on RS-1541 and rhizoxin in mice are summarized in Table III. No clinical signs of toxicity were observed in any groups after treatment with either compound. No reductions in body weight or decreases in RBC count were observed. Compared with the control group, mice injected with rhizoxin showed dose-dependent reductions in WBC and PLAT counts, and decreases in relative weights of the thymus. Mice injected with rhizoxin also had a small but significant decrease in spleen weight on Days 1 and 2. Mice given a high dose of rhizoxin showed an increase in small intestine weight. However, mice injected with RS-1541 showed no significant changes in blood cell numbers or organ weights.

Histologically, mice injected with rhizoxin showed dose-dependent atrophy of the lymphoid tissue of the thymus. In the spleen, a slight follicular atrophy on Day 1 and an extramedullary hematopoiesis on Day 4 were observed. A slight increase in apoptotic bodies in the small intestine, and a dose-dependent myelosuppression in the bone marrow were observed after rhizoxin. On the other hand, mice injected with RS-1541 showed only minimal changes in the small intestine and bone marrow at 4 mg/kg on Day 2.

Table II. Parameters for RS-1541 and Rhizoxin in Tumor After Intravenous Administration of RS-1541 or Rhizoxin to Mice Bearing M5076 Sarcoma

	RS-1541 administration		Rhizoxin administration	
Dose (mg/kg)	1	4	1	4
RS-1541				
Cmax (μg/g tissue)	3.33	16.2		
Tmax (h)	6	6		
AUCt (μg · h/g tissue)	129	1100		
MRT (h)	32.4	69.4		
Rhizoxin				
Cmax (μg/g tissue)	0.767	1.36	0.373	1.25
Tmax (h)	24	24	0.5	0.5
AUCt (μg · h/g tissue)	25.7	49.1	4.80	18.6
MRT (h)	32.0	31.8	6.36	14.7

AUCt and MRT were calculated by trapezoidal method.

DISCUSSION

RS-1541 showed a more potent and prolonged inhibition of ^{14}C-thymidine incorporation into DNA of tumors, compared with rhizoxin. This result is consistent with the results of our previous growth inhibition studies, in which we demonstrated the superiority of RS-1541 over rhizoxin in terms of anti-tumor activity against M5076 sarcoma in mice (7).

After administration of rhizoxin, the rhizoxin concentration in the tumor quickly reached Cmax at 0.5 h and then decreased rapidly, and the maximal inhibition of ^{14}C-thymidine incorporation was observed at 2 h, the earliest time point examined. Thus, almost no time-lag appeared to occur between the rhizoxin accumulation in the tumor and expression of its cytocidal effect.

After administration of RS-1541, on the other hand, RS-1541 reached Cmax after 6 h, and rhizoxin produced from RS-1541 reached Cmax after 24 h and then decreased slowly. The maximal inhibition of ^{14}C-thymidine incorporation was observed at 24 h, in agreement with the Cmax of rhizoxin but not with that of RS-1541. These results strongly suggest that rhizoxin produced from RS-1541 is responsible for the cytocidal effect of RS-1541.

Table III. Acute Toxicity of RS-1541 and Rhizoxin After a Single Intravenous Administration to Mice

		RS-1541						Rhizoxin					
Dose (mg/kg)		1			4			1			4		
Day		1	2	4	1	2	4	1	2	4	1	2	4
Body weights		—	—	—	—	—	—	—	—	—	—	—	—
Blood cell counts	RBC	—	—	—	—	—	—	—	—	—	—	—	—
	WBC	—	—	—	—	—	—	−2	−1	—	−3	−2	—
	PLAT	—	—	—	—	—	—	—	—	—	−1	−1	—
Organ weights	Thymus	—	—	—	—	—	—	−1	−3	—	−2	−3	−3
	Spleen	—	—	—	—	—	—	—	—	—	—	—	—
	Small intestine	—	—	—	—	—	—	—	—	—	—	—	+1
Histological findings	Thymus (At)	—	—	—	—	—	—	B	B	—	C	D	—
	Spleen (At)	—	—	—	—	—	—	B	—	—	B	—	—
	(He)	—	—	—	—	—	—	—	—	B	—	—	C
	Small intestine (Ap)	—	—	—	—	A	—	A	—	—	B	A	—
	Bone marrow (My)	—	—	—	—	A	—	B	B	—	C	C	A

Abbreviations: RBC = red blood cells; WBC = white blood cells; PLAT = platelets; At = atrophy; He = extramedullary hematopoiesis; Ap = increased apoptotic bodies; My = myelosuppression. Changes compared to control group: −3 = <50%; −2 = 50–70%; −1 = 70–85%; — = 85–115%; +1 = 115–130%; +2 = 130–150%; +3 = 150%<.
Grade of histological change: — = no effect; A = very slight; B = slight; C = moderate; D = severe.

Anti-tumor drugs have been classified into two types (12,13), i.e., type I drugs which are cell cycle phase-nonspecific and AUC-dependent, and type II drugs which are cell cycle phase-specific and time-dependent, from extensive *in vitro* colony-forming inhibition studies. Rhizoxin, an inhibitor of tubulin polymerization, is considered to belong to type II which includes *Vinka* alkaloids, and therefore, the duration of tumor concentration has a significant meaning. MRT values of rhizoxin in the tumor after administration of RS-1541 were several times greater than those after administration of rhizoxin itself. Thus, the strong effect of RS-1541 compared with rhizoxin was thought to be caused by the continuous exposure of the tumor cells to rhizoxin.

In the spleen, thymus and bone marrow, DNA synthesis was strongly inhibited after rhizoxin, but not after RS-1541. In the acute toxicity tests, we observed some undesirable effects with rhizoxin, such as decreases in WBC and PLAT numbers; loss in weight of spleen and thymus; and histological changes in some tissues, in a dose-dependent manner. However, RS-1541 had negligible effects. We showed that no significant amount of rhizoxin was detected in proliferating tissues except for the tumor after administration of RS-1541 to tumor bearing mice, in spite of high uptakes of rhizoxin by these tissues after administration of rhizoxin. The observed strong and selective cytotoxicity of RS-1541 in the tumor *in vivo* agrees well with the characteristic distribution profile of rhizoxin after RS-1541.

In the intestine, inhibition of ^{14}C-thymidine incorporation was observed after administration of RS-1541, irrespective of negligible detection of rhizoxin produced from RS-1541. No significant changes in organ weight were noted, nor abnormal histological findings, suggesting that ^{14}C-thymidine incorporation is quite sensitively affected prior to actual manifestation of toxicity.

In summary, we demonstrate in the present study, using M5076 sarcoma bearing mice, that RS-1541 possesses a high tumor-selective cytocidal effect compared with rhizoxin, and that the cytotoxicity is due to rhizoxin but not RS-1541 itself. RS-1541 appears to be converted to rhizoxin selectively in the tumor. Moreover, as we reported previously (7), RS-1541 binds to lipoproteins after administration, and shows selective and sustained uptake by the tumor, most likely through the LDL-receptor pathway. Therefore, the dual selectivity in distribution and activation probably contributes to the desirable nature of RS-1541 as an anti-tumor drug.

Although the involvement of lysosome in activation of RS-1541 has been suggested in M5076 sarcoma *in vivo* (7) and human gastric cancer cells, St-4, *in vitro* (14), the enzymatic mechanism for production of rhizoxin from RS-1541 needs further investigation. Also, selective distribution of RS-1541 should be demonstrated in various cancer cells to show a general effectiveness of RS-1541.

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