Enhanced Tumor Delivery and Antitumor Activity of Palmitoyl Rhizoxin Using Stable Lipid Emulsions in Mice

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Purpose. A highly lipophilic antitumor agent, 13-O-palmitoyl-rhizoxin (RS-1541), was incorporated into lipid emulsions of various sizes consisting of triglyceride ODO and surfactant HCO-60. Pharmacokinetics, toxicities, and antitumor activities were evaluated after intravenous administration to mice bearing subcutaneously inoculated M5076 sarcoma cells.

Methods. The levels of RS-1541 in the plasma and tissues including tumor, were determined by HPLC. The maximum tolerated dose (MTD) was estimated by toxic death and change in body weight. The decrease in tumor diameter was measured for antitumor activity.

Results. There existed large variations in pharmacokinetics of RS-1541, depending on the size of emulsion particles. Compared with a colloidal solution (reference solution), the small (110nm) and medium (230nm) size emulsions showed high concentrations of RS-1541 in the tumor, while the large emulsions (350nm–630nm) exhibited low concentrations. The MTD of RS-1541 was reduced, when incorporated in the emulsions larger than 220nm in size. At MTD, each size of emulsions (70nm–380nm) effectively retarded the tumor growth and increased survival time. The maximum effect was achieved for the 220 nm emulsions.

Conclusions. When particle size is properly selected, these emulsions could be promising and effective as an injectable carrier for lipophilic antitumor agents in order to enhance the tumor delivery and efficacies while reducing toxicities.

KEY WORDS: RS-1541(palmitoyl rhizoxin); emulsions; pharmacokinetics; toxicity; antitumor activity.

INTRODUCTION

Lipophilic antitumor agent rhizoxin (logP 2) exhibits its activity by the inhibition of tubulin polymerization(1). Its palmitoyl derivative RS-1541 (13-O-palmitoyl-rhizoxin) which is more lipophilic (logP 14), has been found to have greater *in vivo* antitumor activity than rhizoxin(2). To obtain more enhanced efficacy, we have incorporated RS-1541 into lipid emulsions consisting of triglyceride ODO and surfactant HCO-60. The surfactant HCO-60 has PEG-like unit (polyoxyethylene) which exhibits steric hindrance between liposomes and complements when attached on the liposome surface(3). Therefore, ODO/HCO-60 emulsions have pharmacokinetically different characteristics from conventional lipid emulsions prepared with soybean oil and lecithin. ODO/HCO-60 emulsions are stable to lipoprotein lipase and show low uptakes by mononuclear phagocytic system (MPS) and long circulation in the blood after i.v. injection(4). Dispositions of RS-1541 and emulsion lipid were simultaneously measured and distributions of RS-1541 in the plasma were analyzed with size exclusion chromatography. Thus, RS-1541 has been found to behave with the emulsion particles in the body, which is dependent on the size of the emulsions(5).

In the present study, we have evaluated the pharmacokinetics, toxicities, and antitumor activities of the emulsion formulations of RS-1541 in mice bearing the solid tumor M5076 sarcoma. Various emulsion sizes were investigated and compared with a colloidal solution. The colloidal solution, which forms micelle about 20nm in size, was used as a reference formulation, because water solubility of RS-1541 is extremely low.

MATERIALS AND METHOD

Materials

Rhizoxin (Fig.1) was isolated from the culture broth of *Rhizopus chinensis* Rh-2 at the research laboratories of Sankyo Co., Ltd. RS-1541 (13-O-palmitoyl rhizoxin, Fig. 1) was synthesized by palmitoylation of rhizoxin. The nonionic surfactant, a polyoxyethylene-(60)-hydrogenated castor oil (HCO-60) was obtained from Nikko Chemicals, Medium chain triglyceride, a dioctanoyl-decanoyl-glycerol (ODO) was purchased from Nisshin Seiyu, Tokyo, Japan. All other chemicals were commercially obtained and used as received.

Preparation of Emulsion Formulations

Oil in water (O/W) emulsions were prepared using a Microfluidizer with ODO as oil phase and HCO-60 as a surfactant(6). Various sizes of emulsions (70–630nm) were prepared by altering the concentrations of HCO-60 solution. Emulsion droplet size was measured by a dynamic laser light scattering, and expressed as mean diameter. Colloidal solution was prepared as follows; 10 mg of RS-1541 was dissolved in 1ml of 6% HCO-60/DMA, and diluted with 9ml of saline.

Animals

Female BDF_1 mice were obtained from Charles River Japan, Tokyo. A murine reticulum sarcoma M5076 cells (1×10^6 cells) were subcutaneously inoculated into the axillae of each mouse at the age of 7 weeks. Drug formulations were administered 13 days after the tumor implantation.

Pharmacokinetic Studies in Tumor Bearing Mice

After a intravenous administration of RS-1541 to tumor bearing mice at a dose of 5 mg/kg or maximum tolerated dose (MTD), the animals were lightly anesthetized and exsangui-

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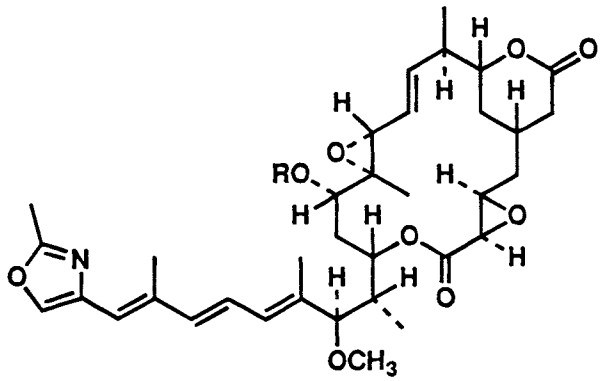
Abbreviations: RS-1541: 13-O-palmitoyl rhizoxin; ODO: dioctanoyl decanoyl glycerol; HCO-60: polyoxyethylene-(60)-hydrogenated castor oil; MTD: maximum tolerated dose.



R=H : Rhizoxin
$R=CH_3(CH_2)_{14}CO$: RS-1541

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

nated by cardiac puncture at different times. Three mice were used for each time point. Several organs including tumors were immediately excised, weighed, and subjected to the previously described HPLC assay(6) for RS-1541 and rhizoxin produced from RS-1541 by hydrolysis in the tissues.

Pharmacokinetic Analysis

The area under the concentration-time profiles (AUC), the mean residence time (MRT), and the steady-state volume of distribution (Vd) were determined by the trapezoidal method up to the final sampling time.

Toxicity Studies

Each formulation of RS-1541 was intravenously administered at a dose of 6 mg/kg to normal BDF_1 mice (7 weeks old and weighing 20–25 g). On each day for consecutive five days following drug administration, six mice in each group were weighed, anesthetized, and exsanguinated by cardiac puncture. The collected blood was added EDTA-2K and counted for white blood cells. The number of cells in a femur bone marrow in each mouse was also counted. For the evaluation of MTD, each formulation was intravenously injected to tumor bearing BDF_1 mice. Over eight days after the administration, deaths were noted and the body weights of mice were determined. Six mice were used for each group.

Evaluation of Antitumor Activity

The antitumor activities were examined under the same conditions as those for the pharmacokinetic experiments at MTD described above. Six mice were allocated to each group. The size of the tumor was measured every four to nine days for 60 days after the drug administration. The tumor diameter was measured as (a + b)/2, where a is the long axis and b the short axis of the solid tumor. For emulsions 240 nm in size and the colloidal solution, antitumor activities were evaluated also at the dose of 6.7mg/kg.

RESULTS

Pharmacokinetics of Emulsion Formulations of RS-1541 in Tumor Bearing Mice at a Dose of 5 mg/kg

Concentrations of RS-1541 and rhizoxin in the plasma, liver, and tumor following a intravenous injection of 5 mg/kg of RS-1541 in M5076 sarcoma bearing mice, are shown in Fig. 2. There existed large variations in the pharmacokinetics of RS-1541 and rhizoxin (the hydrolysis product of RS-1541) in the tissues, which was dependent on the mean size of emulsion particles used. Compared with the colloidal solution, the small size emulsions (110nm) showed higher concentrations of RS-1541 in the tumor as well as in the plasma, while it exhibited lower concentrations in the liver. The plasma and liver concentations of RS-1541 for medium size emulsions (230nm) were comparable with the colloidal solution, while those in the tumor were about twice that for the colloidal solution. In contrast, the large size emulsions (350nm–630nm) showed a much lower concentration of RS-1541 both in the plasma and tumor. The largest emulsions (630nm) showed relatively low RS-1541 concentrations both in the plasma and liver, probably because of rapid uptake of large particles by the lung(6). For all formulations of RS-1541 investigated, no significant amount of rhizoxin was detected in the plasma or the liver. Only in tumor tissues, however, was rhizoxin detected after RS-1541 administration, although those levels were lower than one-tenth of the RS-1541 levels. Similar to RS-1541, rhizoxin levels in the tumor were high for small size emulsions, and low for large size emulsions.

Toxicities of Emulsion Formulations of RS-1541 in Mice

Toxicities of RS-1541 in normal mice at a dose of 6 mg/kg are shown in Fig. 3. The body weight of mice gradually increased after the injection of emulsions at 220nm and 460nm in size, while they decreased both after the injection of a 90nm emulsion and a colloidal solution. For every formulation, the number of white blood cells gradually decreased. However, the extent of decrease was slight for the 220nm and 460nm emulsions, when compared with the colloidal solution. Moreover, the number of bone marrow cells decreased to a large extent, followed by the apparent recovery after the administration of each formulation. Also in this case, medium and large size emulsions exhibited less toxicity than the other formulations.

MTD in tumor bearing mice was determined as a maximum dose which did not cause toxic death and a change in body weight larger than 1.0g. The estimated MTD of 70nm, 100nm, 220nm, 380nm, and 600nm emulsions were 4.5mg/kg, 4.5mg/kg, 15mg/kg, 40mg/kg, and 60mg/kg, respectively, and that of the colloidal solution was 6.0mg/kg.

Pharmacokinetics of Emulsion Formulations of RS-1541 in Tumor Bearing Mice at MTD

The pharmacokinetics of RS-1541 and rhizoxin 6 hrs to 7days after a administration of RS-1541 were evaluated at MTD (Fig. 4, Table-I). In this long time scale, MRT(plasma) and Vd for RS-1541 increased with the size of emulsions. At MTD, in contrast to the case of a 5 mg/kg dosage, every emulsion formulation (110nm–470nm in size) showed higher RS-1541

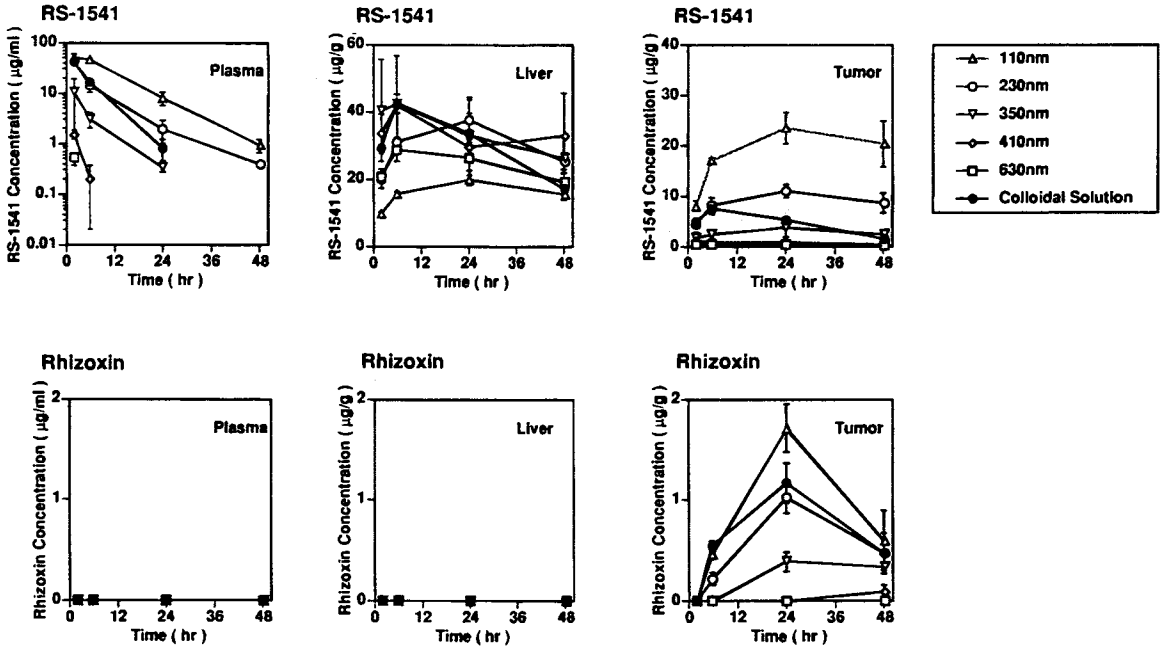


Fig. 2. Concentrations of RS-1541 (upper panels) and rhizoxin (lower panels) in the plasma, liver, and tumor after a single intravenous administration of various size of emulsion formulations and the colloidal solution of RS-1541 to mice bearing M5076 sarcoma at a dose of 5 mg/kg. Each value represents the mean ± S.E. of three mice.

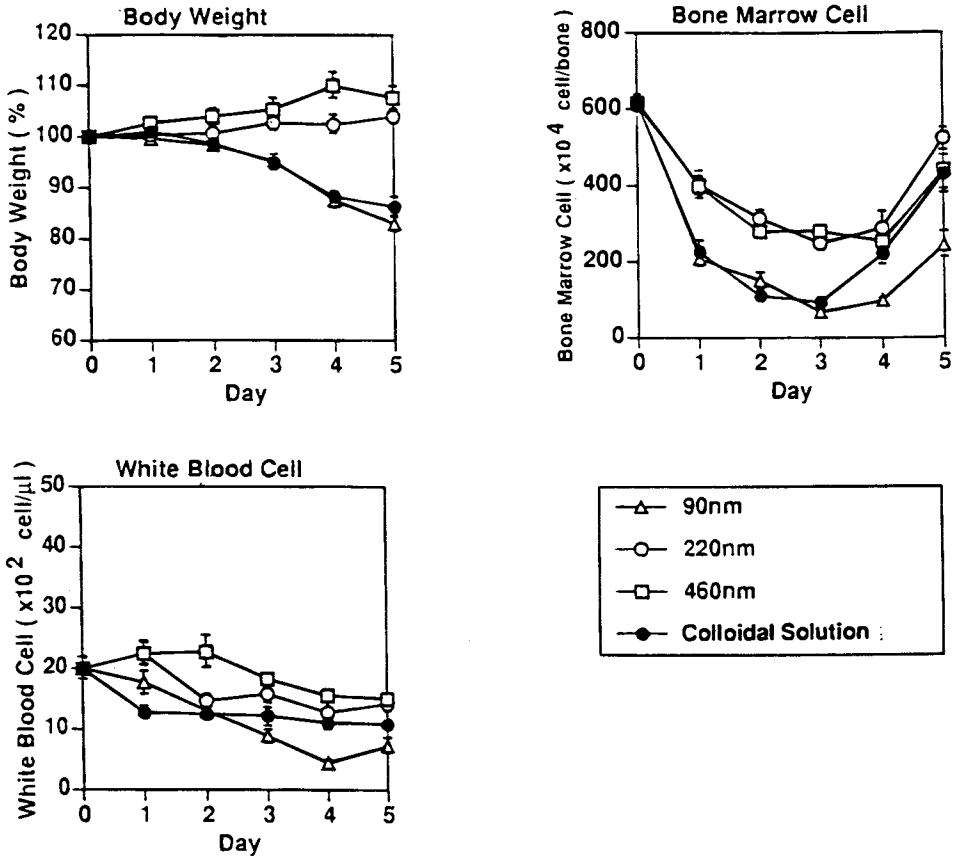


Fig. 3. Changes in the body weight and the number of white blood cells and bone marrow cells following a single intravenous administration of various size of emulsion formulations and the colloidal solution of RS-1541 to normal BDF_1 mice at a dose of 6 mg/kg. Each value represents the mean ± S.E. of six mice.

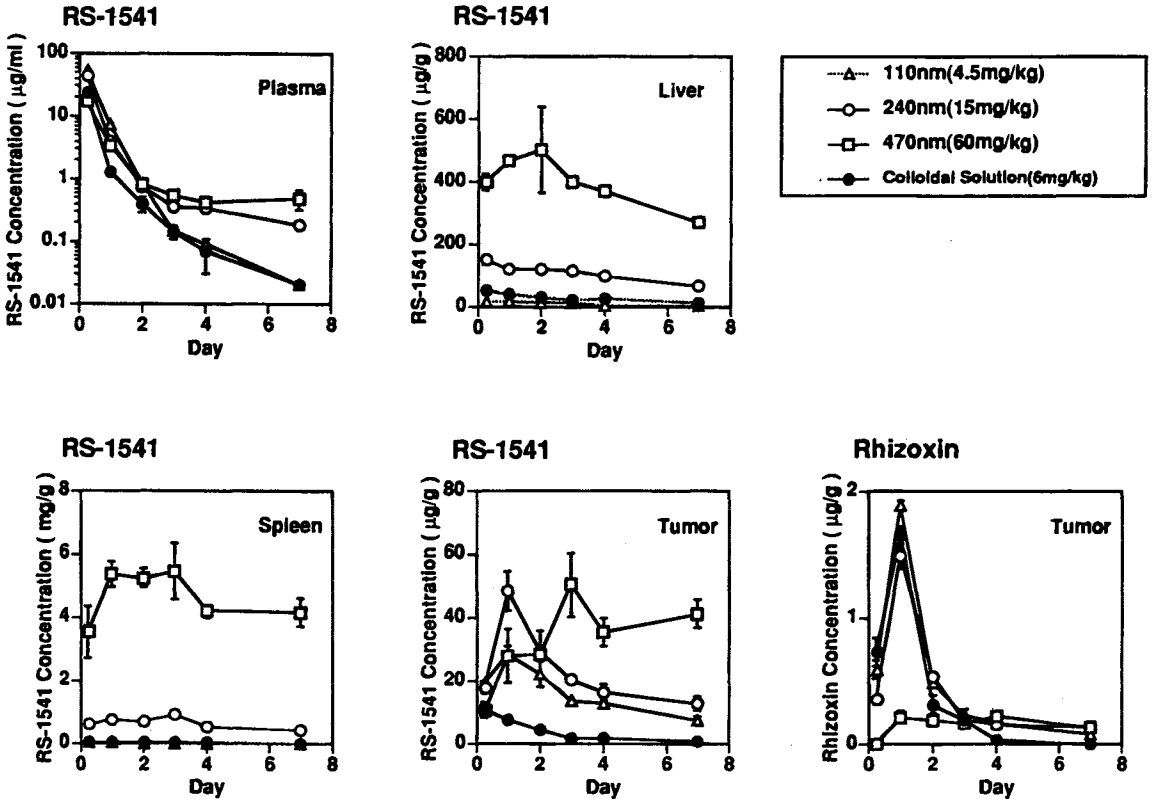


Fig. 4. Concentrations of RS-1541 in the plasma, liver, spleen, and tumor and rhizoxin in the tumor after a single intravenous administration of various size of emulsion formulations and the colloidal solution of RS-1541 to mice bearing M5076 sarcoma at MTD. Each value represents the mean ± S.E. of three mice.

Table I. Pharmacokinetic Parameters of RS-1541 and Rhizoxin after i.v. Injection of MTD of RS-1541 Emulsion Formulations to M5076 Bearing BDF1 Mice

AUC (μg·hr/ml or g)

Diameter	Dose (MTD) (mg/kg)	RS-1541 plasma	tumor	liver	spleen	Rhizoxin tumor
Colloidal Solution	6.0	519	518	3926	1766	62
110nm	4.5	1117	2547	1507	694	75
240nm	15.0	926	3760	17157	113495	66
470nm	60.0	436	6401	63319	827869	25

MRT (hr)

Diameter	Dose (MTD) (mg/kg)	RS-1541 plasma	tumor	liver	spleen	Rhizoxin tumor
Colloidal Solution	6.0	6.9	41.3	62.3	64.0	32.7
110nm	4.5	8.2	63.2	52.5	27.9	41.2
240nm	15.0	9.6	66.7	71.5	74.9	49.4
470nm	60.0	23.0	93.4	74.5	80.3	83.5

Vd (ml/kg)

Diameter	Dose (MTD) (mg/kg)	RS-1541
Colloidal Solution	6.0	79
110nm	4.5	33
240nm	15.0	155
470nm	60.0	3159

levels in the tumor than that of the colloidal solution. Both values of AUC and MRT in the tumor for the emulsions were higher than those for the colloidal solution. Moreover, for the emulsion 110nm and 240nm in size, the same values of rhizoxin in the tumor were also higher than those for the colloidal solution. For each formulation, rhizoxin was not detected in the plasma, liver, and spleen.

Antitumor Activities of Emulsion Formulations of RS-1541 in Tumor Bearing Mice

Every emulsion formulation (70nm–380nm in size) effectively retarded the tumor growth (Table-II). Compared with the colloidal solution, the tumor diameter was smaller for every size of emulsion, and was much smaller for 220nm and 380nm emulsions. The tumor growth delay was considerably increased after the treatment with 220nm and 380nm emulsions. The I.L.S. of the colloidal solution, 70nm emulsions, and 100nm emulsions was within the 60 to 70% range. The same value of 220nm and 380nm emulsions for I.L.S. was greatly increased, including more than half animals with complete remission on day 120. At the dose of 6.7 mg/kg, tumor growth delay and I.L.S. for 240nm emulsions were 43 days and 131%, respectively. The same values for the colloidal solution at the same dose were 30 days and 84%, respectively. Thus, activities of the medium size (240nm) emulsions were superior to those of the colloidal solution, when compared at the same dosage.

DISCUSSION

The use of a cytotoxic drug is generally restricted due to undesired dose-limiting toxicities in non-target organs(7). Concept of drug targeting has gained wide acceptance in the development of new antitumor agents as well as in improving the therapeutic efficacy of drugs on the market. The delivery of cytotoxic agents specifically to target cells would increase tumor cell death and decrease toxic effects on normal tissues. For this purpose, various drug carriers, such as liposomes(8), microcapsules(9), lipid emulsions(10), and macromolecules(11), have been extensively explored. Compared with other particle carriers, however, lipid emulsions are considered suitable for highly lipophilic antitumor agents because of high retention of such compounds within lipid particles. The entrapment of RS-1541 in ODO/HCO-60 emulsions is almost 100%, while that in liposomes with typical lipid composition is less than 1%. Thus, for RS-1541, we selected the lipid emulsions.

As a prodrug of rhizoxin, RS-1541 exhibits antitumor activity after hydrolized to rhizoxin only in the tumor tissues in mice. However, RS-1541 exhibits toxicities in the small intestines or bone marrow where rhizoxin can not be detected, suggesting activity of RS-1541 itself. In addition, the levels of RS-1541 in the tumor are much higher than those of rhizoxin after RS-1541 administration, and RS-1541 taken up by the tumor is the only source for rhizoxin in the tumor. Thus, the levels of RS-1541 as well as rhizoxin are of importance for the evaluation of emulsion formulations of RS-1541.

From the results obtained in this study, followings were clarified in comparison to the colloidal solution. I) Delivery of RS-1541 to the tumor is enhanced by the emulsions smaller than about 250nm in size. II) Toxicity is reduced by the emulsions larger than about 200nm. III) Antitumor activity at MTD is enhanced by every size of emulsions and the maximum activity is obtained by the medium size emulsions (220nm).

The emulsion particles could pass through the newly vascularized leaky capillary in the tumor tissues (12). The emulsions 470nm in size is too large to penetrate the capillary. However, RS-1541 was slowly transported to the tumor site with this size of emulsions (Fig. 4). The *in vitro* release of RS-1541 from the emulsion particles in the plasma, is very slow for every size of emulsions. After i.v. injection of emulsions smaller than 250nm, RS-1541 is stably retained in the emulsion particles in the blood and behaves with those particles. Whereas, in the case of large emulsions (> about 400nm), RS-1541 is rapidly taken up by MPS with the emulsion particles, and thereafter behaves as released form from the emulsions(5). Moreover, in the preliminary experiments using 14C-RS-1541 and 3H-ODO, *in vivo* tumor uptake rate was comparable between RS-1541 and ODO for emulsions smaller than 250nm, while that was different between RS-1541 and ODO for emulsions larger than 400nm. Thus, we consider that RS-1541 is delivered to the tumor in most part with emulsion particles after injection as the emulsions smaller than about 250nm, while RS-1541 is taken up by the tumor after released from the emulsion particles in case of large emulsions (> 400nm).

The emulsions larger than about 200nm in size reduced toxicity of RS-1541. Distributions of RS-1541 into toxicity-related tissues (bone marrow, intestine) are suppressed when

Table II. Antitumor Activity of RS-1541 Emulsion Formulations Against M5076 Sarcoma at MTD

Mean Diameter	Dose (MTD)[a] (mg/kg)	Tumor Diameter[b] (%)	Tumor Growth Delay[c] (day)	I.L.S.[d] (%)	Cure (on day 120)
Colloidal Solution	6.0	213	17	62	0/6
70nm	4.5	166	24	66	0/6
100nm	4.5	113	29	69	0/6
220nm	15.0	13	>61	>224	4/6
380nm	40.0	18	56	>195	3/6

[a] RS-1541 was given in each formulation to M5076 bearing BDF1 mice via a single i.v. injection at MTDs on day 13 after inoculation. (Six mice were used for each group.)

[b] Tumor diameter on day 44 divided by that on treatment day.

[c] Days required for the tumors to reach again the diameter on treatment day following a therapy.

[d] Increase in life span: ratio (%) of median survival days in a treatment group of mice to that in the control group (37 days).

injected as emulsions larger than 200nm (6). The medium size emulsins (220nm) exhibited more pronouced antitumor activity than the colloidal solution (Table-II), because of the enhanced tumor delivery (Fig. 2) and the reduced toxicity (Fig. 3) (increased MTD) of this size of emulsions. This result is consistent with the greater exposure (AUC and MRT) of the tumor tissues to both RS-1541 and rhizoxin at MTD (Fig. 4).

In summary, the present results have shown that ODO/HCO-60 emulsions would serve as a promising and effective carrier for lipophilic antitumor agent RS-1541, when the particle size is properly selected.

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