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ORIGINAL ARTICLE

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Intracellular activation and cytotoxic action of RS-1541 against cultured human tumor cells

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Abstract RS-1541, an acyl-derivative of rhizoxin (Fig. 1), is a potent antitumor compound. This agent showed cytotoxicity in vitro on some cultured human tumor cells, although it was less potent than rhizoxin. Rhizoxin exhibited antitumor effects by inhibiting the polymerization of tubulin, whereas RS-1541 did not inhibit tubulin polymerization in vitro. However, cell cycle analysis in vivo showed that the two agents had the same mode of action. The cytotoxicity of RS-1541 was enhanced when the initial cell density of the cells was increased. The cytotoxicity was also enhanced when the membrane fraction of St-4 cells, which were the most sensitive to RS-1541 among the cell lines tested, was added to the target cells. When St-4 cells were incubated with [^{14}C]-RS-1541, significant amounts of [^{14}C]-rhizoxin were produced within the cells. Further fractionation of the crude membrane showed that the activity that enhanced the cytotoxicity of RS-1541 (RS-1541-enhancing activity) belonged to the mitochondrial-lysosomal fraction, not to the microsomal fraction. Both the enhancing activity and the activity that converting [^{14}C]-RS-1541 to [^{14}C]-rhizoxin (RS-1541-converting activity) were inhibited by treatment with chloroquine, an inhibitor of lysosomal function. Cholesterol esterase derived from *Candida cylindracea* had RS-1541-enhancing *and* -converting activities. These data suggest that RS-1541 exerts its cytotoxic action after being converted to rhizoxin within the cells by a lysosomal enzyme such as cholesterol esterase.

Key words RS-1541 • Rhizoxin • Cytotoxicity

Abbreviations *DMSO* Dimethylsulfoxide • *PBS*(–) Ca^{2+}, Mg^{2+}-free phosphate-buffered saline; *HCO60* hydrogenated castor oil polyethylene glycol ether • *DMA* dimethylacetamide • *RSB* reticulocyte standard buffer, consisting of 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 10 m*M* TRIS-HCl, (pH 7.4) • *TLC* thin-layer chromatography • *ara-C* 1-β-D-arabinofuranosylcytosine • *LDL* low-density lipoprotein

Introduction

RS-1541 is the palmitoyl derivative of rhizoxin (Fig. 1), which is an antimitotic drug purified from the fungus *Rhizopus chinensis* [1, 2] and has been shown to have antitumor activities in vitro and in vivo, especially against vincristine-resistant tumors [12]. Many derivatives of rhizoxin have been synthesized for the purpose of improving the antitumor activity of rhizoxin, and their antitumor effects have been evaluated in murine experimental systems [5]. Among them, RS-1541 exhibited the most potent activity against solid tumors such as B16, colon 26, and M5076 [5, 9]. The therapeutic effects of RS-1541 on some tumors are superior to those of rhizoxin. Moreover, RS-1541 exhibits more potent activity against some human tumor xenografts than do all other drugs, including etoposide and cisplatin (unpublished data). RS-1541 is one of the promising compounds being evaluated clinically in Japan.

It has been shown that the cytotoxic action of rhizoxin like that of *Vinca* alkaloids [12], comes from its ability to inhibit microtuble assembly. Polymerization of tubulin isolated from porcine brains was completely inhibited by rhizoxin at a concentration of 10 μ*M* [8]. However, at concentrations of up to 1.2 m*M*, RS-1541

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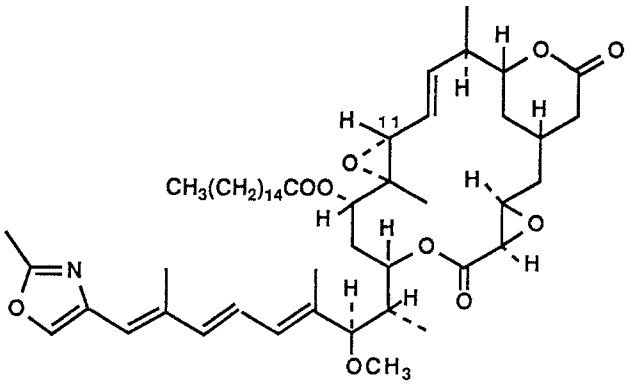

Fig. 1 Structure of RS-1541.

showed no effect on the polymerization of tubulin in vitro (unpublished data).

The purpose of this study was to explain the discrepancy between the in vivo and in vitro effects of RS-1541. In a previous paper [9], we had reported that (a) RS-1541 was stable in plasma and reached tumor tissues more effectively than rhizoxin and (b) when tumor-bearing mice were treated with RS-1541, rhizoxin was detected only in tumor tissue. Thus, we thought that a good approach for understanding the antitumor mechanisms of RS-1541 would be to examine the antitumor action of this agent at the cellular level. In the current study, using some human cell lines, we showed that RS-1541 exerted cytotoxicity when activated to rhizoxin within the tumor cells.

Materials and methods

Chemicals

Rhizoxin was isolated from *Rhizopus chinensis* Rh-2 [1, 2]. Rhizoxin was chemically acylated at the 13-hydroxyl group to obtain RS-1541. [^{14}C]-RS-1541 (1.22 MBq/mmol) was derived from [^{14}C]-rhizoxin, which was isolated from the broth of *R. chinensis* Rh-2 grown in the presence of [^{14}C]-methionine. Rhizoxin was dissolved in DMSO (10 mg/ml) and diluted with PBS(–) for use. RS-1541 was dissolved (10 mg/ml) with HCO60 (Nikko Chemicals) in DMA and diluted with PBS(–). Chloroquine was purchased from Sigma.

Cell Lines

Human ovarian-cancer cell line A2780, human lung-cancer cell line A549, human epidermoid-cancer cell line KB3-1, and human-colon cancer cell line HT-29 were supplied by the National Cancer Institute (NIH, Bethesda, Md.). A cultured line of human gastric tumor St-4 was established in our laboratory from a gastric tumor xenograft in nude mice originally provided by Dr. T. Kubota of Keio University. Tumor cells were maintained in plastic dishes (Corning Glass Works, Corning, N.Y.) in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 5% fetal bovine serum (Biocell) and kanamycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO_2.

Drug Treatment

Tumor cells were seeded in plastic dishes (35 mm, Corning) at a density of 0.5–10 × 10^4 cells/well. At 16–20 h after seeding, the cells were treated with graded concentrations of drugs and incubated for 72 h in the presence of drugs. After incubation, the medium was removed, then the cells were washed with PBS(–) and trypsinized with trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.05% trypsin, 0.02% EDTA). Next the cells were suspended in PBS(–) containing 1% fetal calf serum. The cells were suspended by pipetting and enumerated with a Model ZM Coulter Counter. The 50% growth-inhibitory concentrations (IC_{50} values) were determined by plotting the logarithm of the drug concentration versus the cellular growth rate [11].

Preparation of subcellular fractions of St-4 cells

The crude membrane and cytosol fractions were prepared by the method described by Hamada et al. [4] with some modifications. After trypsinization, St-4 cells (about 10^8 cells) were suspended in 1% FBS–PBS(–) and washed with PBS(–). Then the cells were resuspended in RSB to obtain a concentration of 10^6–10^7 cells/ml, and the suspension was set on an ice bath for 10 min. The swollen cells were disrupted with 20 strokes in a tightly fitting Dounce homogenizer. The homogenate was centrifuged at 1,000 *g* for 10 min at 4°C to remove the nuclei. The supernatant was centrifuged at 100,000 *g* for 30 min. The pellet was suspended in 5 ml of 10 m*M* TRIS-HCl (pH 7.4) and 250 m*M* sucrose to give the crude membrane fraction. The supernatant was recovered for use as the cytosol fraction. The mitochondrial-lysosomal and microsomal fractions were obtained as pellets by sequential centrifugation of the homogenate deprived of the nuclei at 12,000 *g* for 35 min and 100,000 *g* for 60 min at 4°C, respectively.

TLC for detecting RS-1541 and rhizoxin in tumor cells

Tumor cells were incubated with [^{14}C]-RS-1541 (1 μ*M*; 1.22 MBq/mmol) at 37°C for the indicated periods. After incubation, the cells were recovered as described above, then washed and resuspended with PBS(–). The cells were disrupted by sonication for 1 min at 4°C, and the metabolites were extracted by ethyl acetate. The extract was spotted on a TLC plate (20 × 20 cm, Merck 5715) and developed successively with benzene : isopropanol (16 : 1, v/v) and cyclohexane : ethyl acetate (1 : 1, v/v), containing 5% acetate. The plate was analyzed by a Fuji Imaging Analyzer (BA100).

Measurement of enzymatic activities

Acid phosphatase assay was performed using an Acid Phospha B-test Wako assay kit (Wako Pure Chemical Industries, Ltd). Cholesterol esterase activity was assayed by the method described by Yeaman et al. [13].

Cell cycle analysis

For flow cytometric analysis, CD2F1 mice were inoculated with 10^6 P388 leukemia cells, and RS-1541 or rhizoxin was given intraperitoneally at a dose of 1 mg/kg as a single injection at 5 days after tumor inoculation. The P388 cells were recovered from the peritoneal cavity at 12 or 24 h after drug administration. These cells were stained by propidium iodide after fixation with ethanol, treated with RNase A, and analyzed with a flow cytometer.

Results

Cytotoxic effect of RS-1541 on cultured human cell lines

The cytotoxicity of RS-1541 was examined using five human tumor cell lines. Tumor cells were seeded at two different inoculum sizes for each cell line and were treated with various concentrations of RS-1541 or rhizoxin. The IC_{50} values were determined as described in Materials and methods and are shown in Table 1.

RS-1541 was much less effective than rhizoxin against all the cell lines tested. The IC_{50} values obtained for RS-1541 differed among the cell lines, whereas those recorded for rhizoxin were almost constant. The RS-1541 IC_{50} varied depending on the initial cell density, whereas the rhizoxin IC_{50} did not. When cells were seeded at 1×10^5 cells/well, the cytotoxicities of RS-1541 were 2–3 times higher than those obtained when cells were seeded at 1×10^4 cells/well. The IC_{50} variation in relation to the inoculum size was further examined using St-4 cells, the most sensitive cell line tested. The IC_{50} value obtained for RS-1541 was decreased about 4-fold by increasing the initial cell density from 0.5 to 8×10^4 cells/well, whereas that of rhizoxin remained constant (Fig. 2). These observations could be explained by the activation of RS-1541 by the cells.

Activation of RS-1541 by the homogenate of tumor cells

The cellular activation of RS-1541 was examined using St-4 cells, which were most sensitive to RS-1541, and HT-29 cells, which were least sensitive to the drug. When a homogenate of St-4 cells equivalent to 1 or 3×10^5 cells was added to the assay system for the assessment of cytotoxicity against St-4 cells, the cytotoxicity was effectively increased (a 2.4-fold increase), whereas the same amount of homogenate of HT-29 was less effective (only a 1.5-fold increase; Fig. 3). Thus, the degree of activation by the cellular homogenate seems to correlate with the sensitivities of the cells.

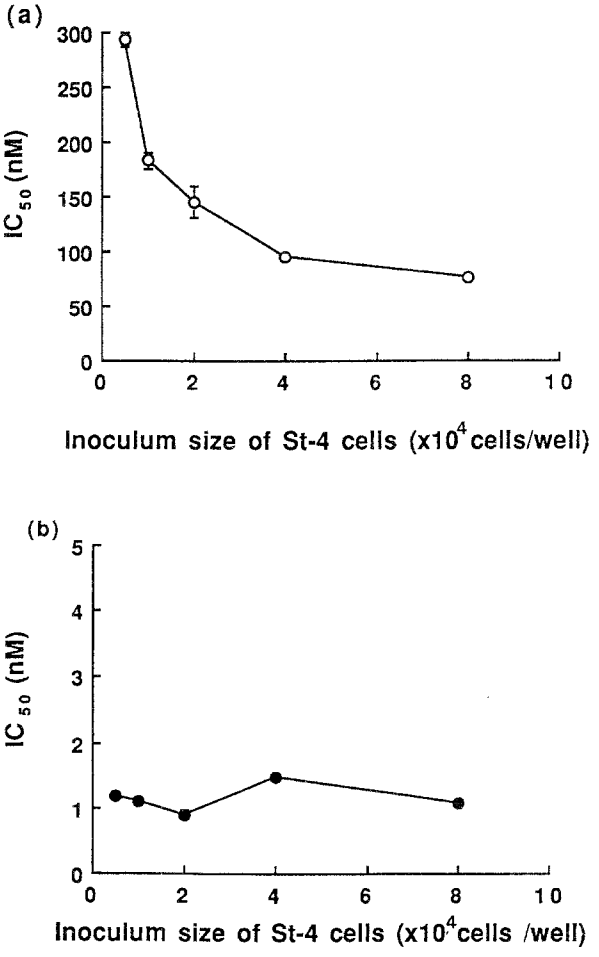

Fig. 2a, b Cytotoxic effects of RS-1541 and rhizoxin on St-4 cells with different cell densities. The cells were seeded at 0.5, 1, 2, 4, and 8×10^4 cells/well and were then incubated with **a** 50, 100, 200, and 400 n*M* RS-1541 or **b** 0.1, 0.3, 1, and 3 n*M* rhizoxin for 72 h. IC_{50} values were determined as described in Materials and Methods. Each point represents the mean value ± SD for three determinations

Table 1 Cytotoxicity of RS-1541 and rhizoxin against cultured human tumor cells. Tumor cells were inoculated at 10,000 or 100,000 cells/well and were then treated with 50, 100, 200, and 400 n*M* of RS-1541 or with 0.1, 0.3, 1, 3 n*M* rhizoxin. After 72 h of continuous drug exposure, the tumor cells were counted and the IC_{50} value was determined as described in Materials and methods[a]

Cell line	Initial cell density	RS-1541[a]	Rhizoxin[a]
A2780 (ovarian)	10,000 cells/well	251.7 ± 56	0.50 ± 0.18
	100,000 cells/well	86.3 ± 7.2	0.54 ± 0.01
A549 (lung)	10,000 cells/well	355.5 ± 121	0.67 ± 0.13
	100,000 cells/well	117.6 ± 39	0.61 ± 0.12
KB3-1 (epidermoid)	10,000 cells/well	164.0 ± 52	0.62 ± 0.14
	100,000 cells/well	67.4 ± 15	0.66 ± 0.20
St-4 (stomach)	10,000 cells/well	190.4 ± 3.2	0.56 ± 0.04
	100,000 cells/well	64.0 ± 5.5	0.52 ± 0.01
HT-29 (colon)	10,000 cells/well	478.7 ± 83	0.65 ± 0.01
	100,000 cells/well	208.0 ± 25	0.79 ± 0.04

[a]Mean value ± SD for three determinations (in n*M*).

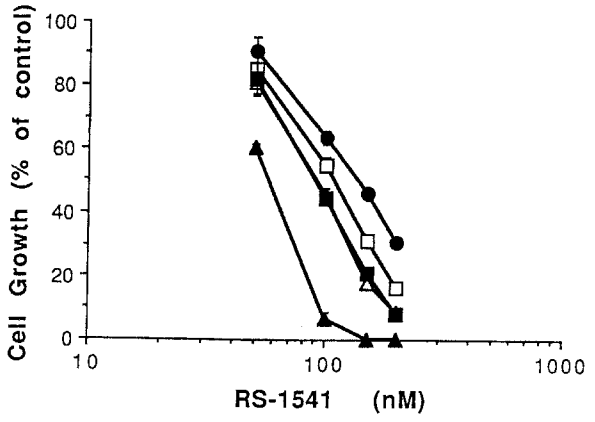

Fig. 3 Cytotoxic effect of RS-1541 in the presence of the cellular homogenate. St-4 cells were seeded and incubated with various concentrations of RS-1541 in the presence of the cellular homogenate of St-4 or HT-29 for 72 h. The homogenate of St-4, equivalent to $1x10^5$ (△) or 3×10^5 (▲) cells, and the homogenate of HT-29, equivalent to 1×10^5 (□) or 3×10^5 (■) cells, were used. The cytotoxicity of the control is also shown (●). Each point represents the mean value ± SD for three determinations

Activation of RS-1541 by the crude membrane fraction of St-4 cells

We fractionated the homogenate of St-4 cells by ultracentrifugation (100,000 *g* for 30 min) to obtain the crude membrane fraction and the cytosol fraction, and the ability of each fraction to activate RS-1541 was examined. When 30 or 100 μg (as protein) of each fraction was added to the assay, the membrane fraction enhanced the cytotoxicity of RS-1541 (Fig. 4), whereas the cytosol fraction did not. When 100 μg of the membrane fraction was used, the cytotoxicity was increased 4-fold. This ability was abolished by boiling for 5 min. On the other hand, neither the membrane fraction nor the cytosol fraction affected the cytotoxicity of rhizoxin (data not shown).

Identification of rhizoxin as a metabolite of RS-1541 in tumor cells

In an attempt to elucidate the mechanism of activation of RS-1541, we investigated the metabolism of RS-1541 in tumor cells using [^{14}C]-RS-1541. St-4 cells or HT-29 cells were treated with [^{14}C]-RS-1541 for 3, 7, or 22 h and the metabolites in each cell line were analyzed by two-dimensional TLC. A new radiolabeled spot appeared after incubation, the position of which was identical to that of rhizoxin, which shows strong cytotoxic activity. In a comparion of the two cell lines, significant amounts of rhizoxin were detected in the St-4 cells in a time-dependent manner, whereas smaller amounts of rhizoxin were detected in the HT-29 cells (Fig. 5). After 22 h of incubation, the production of rhizoxin from RS-1541 in St-4 cells (0.82 pmol/10^5 cells; conversion rate, 12.5%) was 10 times higher than

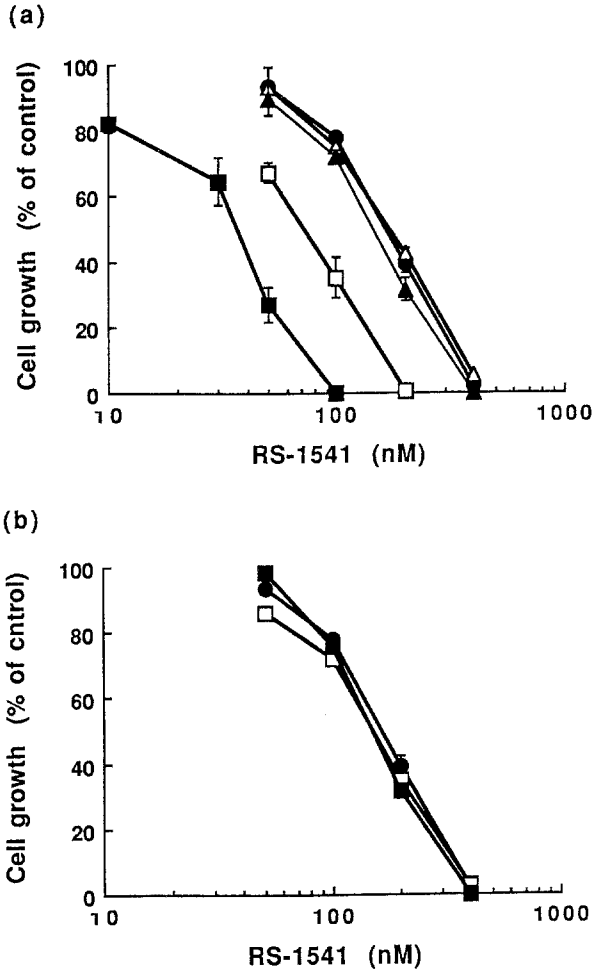

Fig. 4 a, b Cytotoxic effect of RS-1541 on St-4 cells in the presence of the **a** crude membrane or **b** the cytosol fraction of St-4 cells. St-4 cells were seeded and incubated with various concentrations of RS-1541 in the presence of 30 (□) or 100 (■) μg of each fraction. **a** Effects of 30 (△) or 100 (▲) μg of heated membrane on the cytotoxicity. The cytotoxicity of the control is also shown (●). Each point represents the mean value ± SD of three determinations

that in HT-29 cells (0.083 pmol/10^5 cells; conversion rate, 3.5%). Some other metabolites were detected, such as the lactone ring-opened form or the 2,3-epoxy group-opened form of rhizoxin, but none of these showed significant cytotoxicity (data not shown). The amounts of rhizoxin obtained in these experiments were not sufficient for analysis by high-performance liquid chromatography (HPLC). Therefore, we detected rhizoxin in the tumors of mice treated with RS-1541 by HPLC (data not shown). The above-mentioned results indicate that RS-1541 exerts its cytotoxic action when converted to rhizoxin within tumor cells.

Activation of RS-1541 by the mitochondrial-lysosomal fraction

To determine where RS-1541 is converted to rhizoxin, we separated the crude membrane into two fractions by

centrifugation. As shown in Table 2, the activity that enhanced the cytotoxicity of RS-1541 (RS-1541-enhancing activity), along with acid phosphatase activity and cholesterol esterase activity, was recovered in the pellet of 12,000 *g* centrifugation, which was the mitochondrial-lysosomal fraction, whereas the 100,000 *g* pellet, the microsomal fraction, had neither RS-1541-enhancing activity nor the other two enzymatic activities (acid phosphatase activity and cholesterol esterase activity).

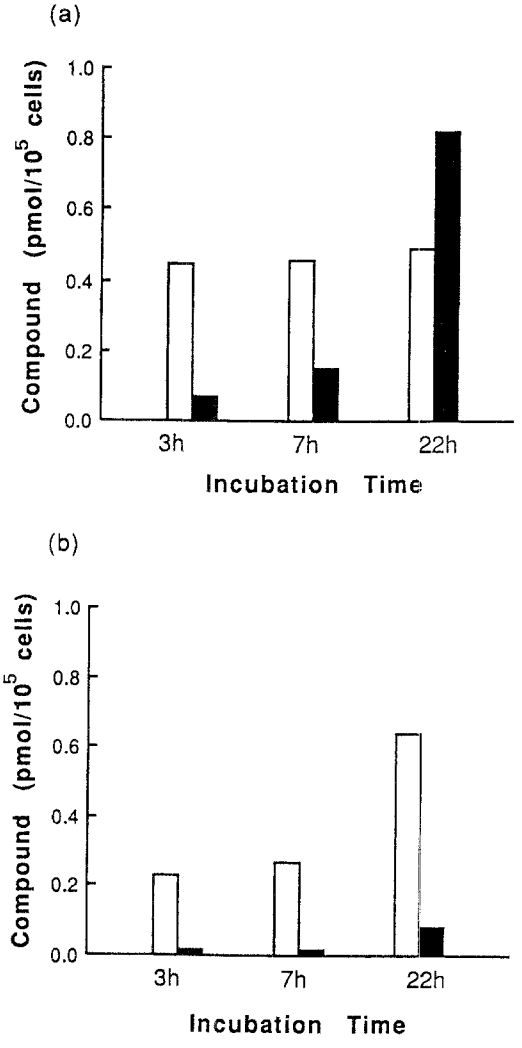

Fig. 5a, b Identification of [^{14}C]-rhizoxin within **a** St-4 or **b** HT-29 cultured with [^{14}C]-RS-1541. The cells were treated with 1 μ*M* [^{14}C]-RS-1541 for 3, 7, and 22 h at 37°C. Metabolites of each cell line were analyzed by two-dimensional TLC as described in Materials and methods (Open bars [^{14}C]-RS-1541, filled bars [^{14}C]-rhizoxin)

Activation of RS-1541 in lysosomes

To investigate whether RS-1541 was converted to rhizoxin in lysosomes, which are acidic organelles rich in hydrolytic enzymes, the effect of chloroquine, which is an inhibitor of lysosomal function, was examined. When St-4 cells were incubated with graded concentrations of RS-1541 or rhizoxin in the presence of chloroquine (1 or 3 μ*M*), the cytotoxicity of RS-1541, not of rhizoxin, was significantly reduced (Fig. 6). Moreover, the production of [^{14}C]-rhizoxin from [^{14}C]-RS-1541 was reduced from 13% to 6% in the presence of 30 μ*M* chloroquine (Fig. 7). These results suggest that RS-1541 is activated in lysosomes.

Activation of RS-1541 by cholesterol esterase

We examined whether cholesterol esterase, an enzyme rich in lysosome, could activate RS-1541. We found that RS-1541 showed strong cytotoxicity after preincubation with increasing amounts of cholesterol esterase of *Candida cylindracea* (Seikagaku Kogyo Co., Ltd.; Fig. 8). When we applied 6 units of the enzyme, the cytotoxicity of RS-1541 was enhanced over 10-fold. Moreover, when [^{14}C]-RS-1541 was treated with 6 units of this enzyme, a significant amount of [^{14}C]-rhizoxin was produced (data not shown). These results suggest that cholesterol esterase is a candidate for the enzyme that digests RS-1541 in lysosomes.

Cell-cycle analysis

Cell-cycle analysis by flow cytometry indicated that RS-1541 inhibited mitosis in tumor cells in a manner

Table 2 Subcellular distribution of RS-1541-enhancing activity in St-4 cells. The crude membrane fraction of St-4 cells was separated into two fractions by sedimentation following sequential centrifugation at 12,000 and 100,000 *g*, respectively. The RS-1541-enhancing activity and the activity of two enzymes are shown for each fraction

Fraction	As protein (μg)	RS-1541 enhancing activity IC_{50} (n*M*)[a]	ratio[b]	Cholesterol esterase activity[a,c]	Acid phosphatase activity[a,d]
12,000 *g* ppt	30	59.5 ± 3.1	0.36	22.4 ± 2.71	1.84 ± 0.02
	100	35.2 ± 2.4	0.21		
100,000 *g* ppt	30	166.1 ± 16	0.99	4.85 ± 0.12	0.42 ± 0.01
	100	143.6 ± 11	0.86		

[a]Data represented mean values ± SD for triplicate determinations
[b]Mean IC_{50}, with various fractions being divided by the mean IC_{50} of control (166.9 ± 28 n*M*)
[c]Cholesteryl oleate hydrolysis (pmol mg protein^{-1} min^{-1})
[d]Absorbance at 405 nm

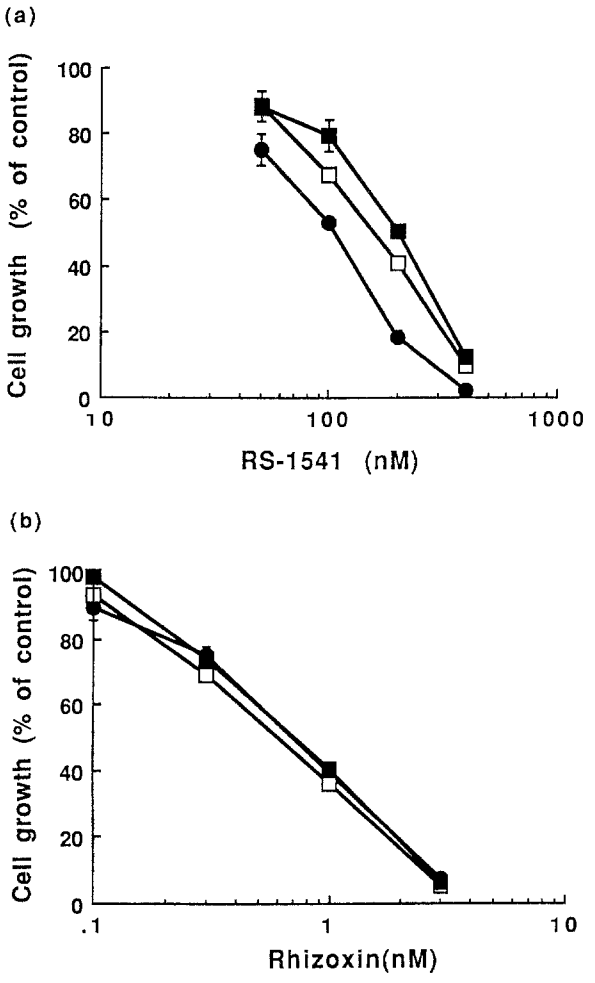

Fig. 6a, b Cytotoxic effect of RS-1541 and rhizoxin in the presence of chloroquine. St-4 cells were seeded and incubated with various concentrations of **a** RS-1541 or **b** rhizoxin with 1 (□) or 3 (■) μ*M* chloroquine for 72 h. The cytotoxicity of the control is also shown (●). Each point represents the mean value ± SD of three determinations

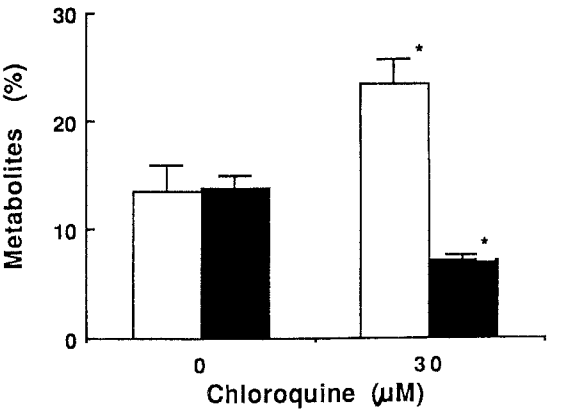

Fig. 7 Identification of [^{14}C]-rhizoxin within St-4 cells cultured with [^{14}C]-RS-1541 in the presence of chloroquine. St-4 cells were cultured with 1 μ*M* [^{14}C]-RS-1541 in the presence of 30 μ*M* chloroquine for 24 h at 37°C. The amount of [^{14}C]-rhizoxin was determined by two-dimensional TLC. The vertical axis shows the percentage of the total counts. Each bar represents the mean value ± SD for three determinations. (Open bars [^{14}C]-RS-1541, filled bars [^{14}C]-rhizoxin). *Significantly different from the value obtained without chloroquine ($P < 0.05$, student's *t*-test)

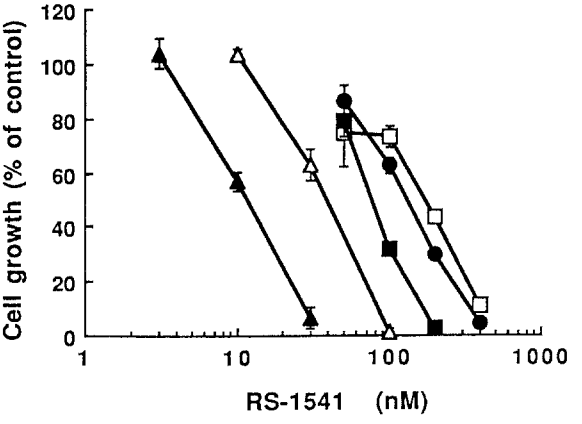

Fig. 8 Cytotoxic effect of cholesterol esterase-treated RS-1541 on St-4 cells. RS-1541 (1.16 μ*M*) was incubated with 0(□), 0.6(■), 2(△), and 6(▲) units of cholesterol esterase (*Candida cylindracea*) for 1 h at 25°C. Then the reaction mixtures were diluted with PBS(–) to the graded concentrations and the cytotoxicity was examined. The control cytotoxicity (●), i.e., no preincubation, is also shown. Each point represents the mean value ± SD for three determinations.

similar to that of rhizoxin. In this experiment, we used P388 cells as target cells because in vivo analysis can be performed using these. More than 50% of the P388 cells recovered from mice at 24 h after RS-1541 treatment were found to be blocked in the G_2-M phase as observed in rhizoxin-treated P388 cells (Fig. 9). This result suggests that RS-1541 and rhizoxin have the same mode of action, i.e., inhibition of mitosis.

Discussion

In the present study, we examined the cytotoxic effects of RS-1541, an acyl derivative of rhizoxin, on various human tumor cell lines in vitro. It was observed in all cell lines tested that the cytotoxicity of RS-1541 is weaker than that of rhizoxin, although the antitumor effects of RS-1541 in vivo are stronger than those of rhizoxin. Furthermore, RS-1541 showed no effect on the polymerization of tubulin in vitro. This result was expected from the observation that the 13-hydroxyl group, where the palmitoyl chain is introduced in RS-1541, is important for inhibiting tubulin polymerization [12]. However, cell-cycle analysis suggests that RS-1541 has the same mode of action as rhizoxin (Fig. 9).

Considering the above-mentioned results, there are two possible mechanisms for the cytotoxic action of RS-1541. One is the existence of target molecules for RS-1541 other than of tubulin; the other is the conversion of RS-1541 to another active compound. The

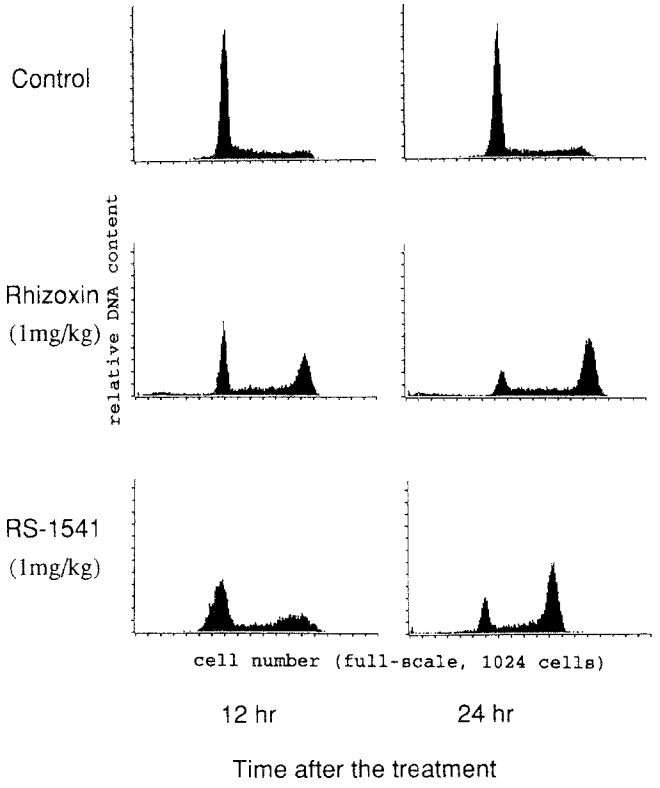

Fig. 9 Comparison of cell-cycle-phase distribution of P388 cells treated with rhizoxin or RS-1541. Cell-cycle analysis was performed as described in Materials and methods. (*x-axis*, Relative DNA content, *y-axis*, cell number—*full-scale* 1024 cells)

latter case is more likely, considering the data we obtained.

The IC_{50} value obtained for RS-1541 varied depending on the inoculum cell density. A similar observation has been reported from a study examining the cytotoxicities of N^4-acyl derivatives of ara-C [10]. In that investigation, the cells seeded at higher densities were less sensitive to the acyl-ara-Cs, indicating inactivation of the drugs by cellular factors. The observations made with acyl-ara-Cs suggested that RS-1541 could be activated by cellular factors.

Actually, [^{14}C]-rhizoxin, which is a strong inhibitor of microtubule assembly, was found in the cells exposed to [^{14}C]-RS-1541 on TLC analysis. Some uptake studies of the radiolabeled compounds showed that the amount of rhizoxin detected within the cells was enough to exert cytotoxic action (data not shown). Some other metabolites detected by TLC analysis, such as the lactone ring-opened form or the 2,3-epoxy group-opened form of rhizoxin, showed weak cytotoxicities (data not shown) as well as weak antitubulin activities [8].

Thus, we concluded that RS-1541 exerts its cytotoxic action through an activation process and that rhizoxin is the active form of RS-1541. The discrepancy between the in vivo and in vitro antitumor effects probably comes from the activation process of the prodrug.

The IC_{50} value recorded for RS-1541 varied among the tumor cell lines, whereas that obtained for rhizoxin did not. Between the two cell lines, St-4 and HT-29, St-4 cells, which were more sensitive than HT-29 cells to RS-1541, had more RS-1541-enhancing activity (Fig. 3). Furthermore, when the cells were incubated with [^{14}C]-RS-1541, larger amounts of [^{14}C]-rhizoxin were produced from [^{14}C]-RS-1541 within the St-4 cells than were produced within the HT-29 cells. The activity converting RS-1541 to rhizoxin (RS-1541-converting activity), which leads to the RS-1541-enhancing activity of a tumor cell, might in part contribute to the sensitivity of the cell to RS-1541.

The RS-1541-enhancing activity detected in the homogenate of St-4 cells was recovered in the crude membrane fraction, especially in the mitochondrial-lysosomal fraction, but not in the cytosol or microsomal fractions. Moreover, the conversion of RS-1541 to rhizoxin was inhibited by chloroquine, which is a known inhibitor of lysosomal function [3, 6, 7]. Lysosome is an organelle in which digestion of products derived from intra- and extracellular metabolism takes place. These results suggest that RS-1541 is activated in the lysosomes.

The RS-1541-enhancing activity was inactivated by heat treatment, which means that a heat-labile factor such as an enzyme is responsible for the activity. In addition, cholesterol esterase from *Candida cylindracea* could digest the palmitoyl chain of RS-1541 to produce rhizoxin effectively. We also found that RS-1541 bound to plasma lipoproteins and was effectively taken up by tumors in vivo, probably via the LDL pathway [9]. These observations suggest the possibility that an enzyme, such as acidic cholesterol esterase, activates RS-1541 in tumor cells.

It is presently hard to say whether only cholesterol esterase is really responsible for the activity exerted on RS-1541 to produce rhizoxin in tumors; nevertheless, this enzyme is a strong candidate, and its isolation from tumor tissue is under way. Should this indeed be the case, the enzyme activity would be a key factor governing the sensitivity of cells to RS-1541. RS-1541 is expected to show strong chemotherapeutic effects in vivo on tumors that have high levels of cholesterol esterase activity.

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