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Targeting of an antitumor agent, RS-1541 (palmitoyl-rhizoxin), via low-density lipoprotein receptor

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Abstract

RS-1541 is a 13-*O*-palmitoyl derivative of rhizoxin, an inhibitor of tubulin polymerization. RS-1541 has been shown to bind preferentially to plasma lipoproteins and to exhibit selective uptake by tumors in mice. We demonstrated that: (1) 99% of RS-1541 bound to human lipoproteins after incubation with human serum, among which the low-density lipoprotein (LDL) fraction attained 10%; (2) RS-1541/LDL complex was obtained by simply incubating human native LDL with a detergent-aided RS-1541 solution; (3) uptake of the [^{14}C]RS-1541/LDL complex in cultured human skin fibroblasts was dependent on the LDL receptor activities of the cells; (4) excess amounts of native LDL or 10 μM monensin, a proton ionophore, significantly inhibited the uptake; and (5) the detergent-aided solution of [^{14}C]RS-1541 showed very low cellular uptake. These results suggest the possibility of LDL as an endogenous targeting carrier of RS-1541 into tumor cells, which have higher LDL receptor activities.

Key words: Low-density lipoprotein; LDL receptor; RS-1541; RS-1541/LDL complex; Human skin fibroblast

1. Introduction

Rhizoxin was discovered as an agent that induces rice seedling blight (Noda et al., 1980). The toxicity of rhizoxin has been shown to result from the inhibition of tubulin polymerization (Takahashi et al., 1987a), which thereby inhibits cell mitosis (Tsuruo et al., 1986). This mode of action suggests the application of rhizoxin as an antitumor agent (Kerr et al., 1992). Our laboratories have found in various in vivo tests that RS-1541, a 13-*O*-palmitoyl derivative of rhizoxin, exhibits much higher antitumor activity than rhizoxin (Kobayashi et al., 1989). In vitro, however, substitution of the hydroxyl group at the 13-position of rhizoxin is known to markedly reduce the inhibitory effect of rhizoxin on tubulin polymerization (Takahashi et al., 1987b). We have previously compared the pharmacokinetics of RS-1541 with those of rhizoxin after intravenous administration to mice bearing M5076 sarcoma, a spontaneous murine reticulum cell sarcoma (Tokui et al., 1994).

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RS-1541 was found to bind preferentially to plasma lipoproteins and showed selective and sustained uptake by the tumor, while rhizoxin did not. A considerable amount of rhizoxin was detected in the tumor after administration of RS-1541, and the formation of rhizoxin in vivo was inhibited by chloroquine, an inhibitor of lysosomal enzymes.

One of the main problems in cancer chemotherapy is the lack of tumor selectivity. To circumvent this problem, several strategies have been explored by many investigators. Recently, low-density lipoprotein (LDL) has attracted attention as an endogenous carrier of antitumor agents to malignant cells, with a view toward reducing undesirable systemic effects (Vitols et al., 1990; Peterson et al., 1991; Filipowska et al., 1992; Lundberg, 1992; Samadi-Baboli et al., 1993). The basis for this idea is the knowledge that, compared to normal cells, growing tumor cells vigorously incorporate LDL via LDL receptors (Gal et al., 1981; Norata et al., 1984; Lombardi et al., 1989; Vitols et al., 1992), and release low molecular-weight substances by degrading LDL particles through the action of lysosomal hydrolyzing enzymes (Goldstein and Brown, 1977; Samadi-Baboli et al., 1990). Our previous results on RS-1541 in vivo (Tokui et al., 1993) were all consistent with the view that RS-1541 is taken up by the tumor via LDL receptors after binding to the plasma lipoproteins, which results in the formation of rhizoxin, the original anti-cancer agent, in the lysosome.

To obtain a rationale for the clinical use of RS-1541, we demonstrate in the present paper that RS-1541 binds to lipoproteins in human serum in vitro and that the cellular uptake of the RS-1541/LDL complex in human skin fibroblasts is dependent on the LDL receptor activity, which was increased by cultivating the cells in a lipoprotein-deficient medium.

2. Materials and methods

2.1. RS-1541, rhizoxin, and other reagents

Rhizoxin and [^{14}C]rhizoxin were isolated from culture broth of *Rhizopus chinensis* Rh-2 (Iwasaki et al., 1984) in the Fermentation Research Laboratories of Sankyo Co., Ltd. RS-1541 (13-*O*-palmitoyl rhizoxin) and [^{14}C]RS-1541 (specific activity, 650.8 kBq/mg) were synthesized by palmitoylation of rhizoxin and [^{14}C]rhizoxin, respectively, in the Bioscience Research Laboratories of Sankyo Co., Ltd. The structure and labeled positions of [^{14}C]RS-1541 are shown in Fig. 1. Na^{125}I was obtained from NEN Research Products, Japan. Polyoxyethylene(60) hydrogenated castor oil (HCO60) was from Nikkol, Tokyo. All other chemicals were commercially available, reagent grade.

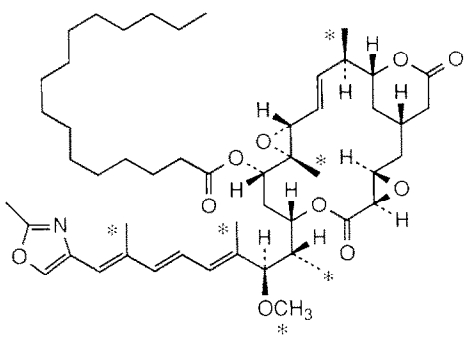

Fig. 1. Chemical structure and labeled position (*) of [^{14}C] RS-1541.

2.2. Plasma protein binding

Human serum, obtained from healthy volunteers, was incubated with [^{14}C]RS-1541 (2 μg/ml) for 0.5 h at 37°C. The density of the plasma was adjusted to 1.006, 1.063 or 1.215 g/ml by the addition of sodium bromide. Each mixture in duplicate was centrifuged at 50000 rpm for 40 h at 4°C. A 0.5 ml aliquot was collected from the uppermost layer of each tube, and was measured for radioactivity. The sample in the tube at 1.006 g/ml was regarded as the fraction of chylomicron (CM) and very low-density lipoprotein (VLDL); that at 1.063 g/ml was regarded as CM, VLDL and low-density lipoprotein (LDL); and that at 1.215 g/ml was regarded as CM, VLDL, LDL and high-density lipoprotein (HDL). The radioactivities associated with HDL, LDL and the combined fraction of CM and VLDL were calculated

by successive subtraction (Shireman and Wei, 1986).

2.3. Lipoproteins

Human LDL (density 1.019–1.063 g/ml) and HDL (density 1.063–1.215 g/ml) were prepared by differential ultracentrifugation (Havel et al., 1955) of serum samples collected from healthy volunteers. The lipoproteins were dialysed against buffer A (0.15 M NaCl, 0.24 mM Na_2EDTA, penicillin 100 IU/ml and streptomycin 100 μg/ml, pH 7.4). Iodination of LDL was accomplished according to the modified ICl method of Bilheimer (1972). The specific activity of ^{125}I-LDL was 342 cpm/μg protein.

2.4. Preparation of [^{14}C]RS-1541 / LDL complex

[^{14}C]RS-1541 (1.0 mg) was dissolved in 0.1 ml of dimethylacetamide containing 2.0 mg of HCO60, a detergent. This solution was further diluted in 9.9 ml of 0.15 M NaCl with constant application of sonication. The detergent-aided solution of [^{14}C]RS-1541 (20 μM) was incubated with human LDL (400 μg/ml) for 6 h at 37°C. The incubation mixture was analysed by gel filtration chromatography through a Superose 6HR 10/30 column (Pharmacia) and by 1% agarose gel electrophoresis. Precipitation of LDL was performed by the dextran sulfate/$MnCl_2$ method (Burstein and Scholnick, 1973). The incubation mixture was dialysed against buffer A, sterilized by filtration through a 0.22 μm Millex GV Millipore filter, and stored at 4°C as the [^{14}C]RS-1541/LDL complex, which was used within 1 week.

2.5. Cells

Normal human skin fibroblasts, CCD-27SK, were from American Type Culture Collection (Maryland), and maintained in plastic dishes (Corning Glass Works, NY) containing Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Biocell), penicillin 100 IU/ml and streptomycin 100 μg/ml, at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were seeded in 35-mm plastic dishes at a density of 3×10^4 cells/well. 48 h after seeding, the growth medium was replaced by medium containing 10% human lipoprotein-deficient serum (LPDS) or medium containing 10% LPDS and LDL (1.8 mg cholesterol/ml serum). These cells were cultured for a further 24 h and used for the uptake study. Henceforth, human skin fibroblasts with or without LDL receptor activities will be referred to as HSF[+] cells or HSF[−] cells.

2.6. Metabolism of ^{125}I-LDL by the cells

The cells were incubated with 5 μg of ^{125}I-LDL in 1 ml of medium containing 10% LPDS at 37°C. After 0, 1, 3, and 6 h, the medium was removed and each dish was washed four times with 1 ml of ice-cold buffer B (0.15 M NaCl, 50 mM Tris-HCl and 2 mg/ml bovine serum albumin, pH 7.4), followed by two washings with 1 ml of albumin-free buffer B. The cells were solubilized in 1 ml of 1 N NaOH and were measured for radioactivity and protein concentration (Lowry et al., 1951). The degradation of LDL was determined from the non-iodide trichloroacetic acid-soluble radioactivity in the incubation medium (Goldstein and Brown, 1974).

2.7. Cellular uptake of [^{14}C]RS-1541 / LDL complex

The cells were incubated with the [^{14}C]RS-1541/LDL complex or the detergent-aided solution of [^{14}C]RS-1541 at 1 μM as RS-1541 in 1 ml of medium containing 10% LPDS at 37°C. After incubation for 0, 1, 3, and 6 h, the medium was removed and the cells were washed as described above. The cells were solubilized and measured for radioactivity and protein concentration. Monensin, a proton ionophore (Blomhoff et al., 1984), was added to the cells 15 min before the uptake study.

3. Results

3.1. Serum protein binding of [^{14}C]RS-1541

Association of [^{14}C]RS-1541 with human serum proteins was demonstrated by ultracentrifugation.

Table 1
Distribution of [^{14}C]RS-1541 into human serum lipoproteins

Fractions	Distribution (%)
CM + VLDL	3.2 ± 0.6
CM + VLDL + LDL	13.2 ± 1.1
LDL (calculated)	10.0 ± 1.7
CM + VLDL + LDL + HDL	98.5 ± 0.3
HDL (calculated)	86.0 ± 0.9
Heavier proteins	0.6 ± 0.1
Total proteins	99.1 ± 0.4

Each value represents the mean ± S.E. ($n = 4$).
Combined flotation methods; aliquots of the serum were individually adjusted to $d = 1.006$, 1.063 or 1.215 g/ml and centrifuged for 40 h for isolation of CM + VLDL ($d = 1.006$), CM + VLDL + LDL ($d = 1.063$) or CM + VLDL + LDL + HDL ($d = 1.215$).

As shown in Table 1, 98.5% of the added radioactivity was recovered from the lipoprotein fractions. The amounts of radioactivity recovered in the fractions of HDL, LDL and combination of CM + VLDL were 86.0, 10.0 and 3.2%, respectively.

3.2. Formation of [^{14}C]RS-1541 / LDL complex

The detergent-aided solution of [^{14}C]RS-1541 (20 μM) was incubated with LDL (400 μg/ml) for 6 h at 37°C, and the mixture was analyzed by gel filtration chromatography. The radioactivity of the detergent-aided solution of [^{14}C]RS-1541 was mainly eluted at the void volume of the column. The radioactivity in the incubation mixture after 6 h was found to be associated mostly with LDL at 83.2%, as shown in Fig. 2. On performing agarose gel electrophoresis, a single protein band was observed with the same electrophoretic migration as native LDL. More than 80% of the radioactivity was precipitated with dextran sulfate/$MnCl_2$. The incorporation ratio of RS-1541 in LDL was 36 μg/mg protein, corresponding to 21 molecules of RS-1541 incorporated into each LDL particle (assuming the molecular mass of apolipoprotein B as 515 kDa). No aggregation or precipitation was noted after storage for 1 week at 4°C. The incubation mixture was used as the [^{14}C]RS-1541/LDL complex.

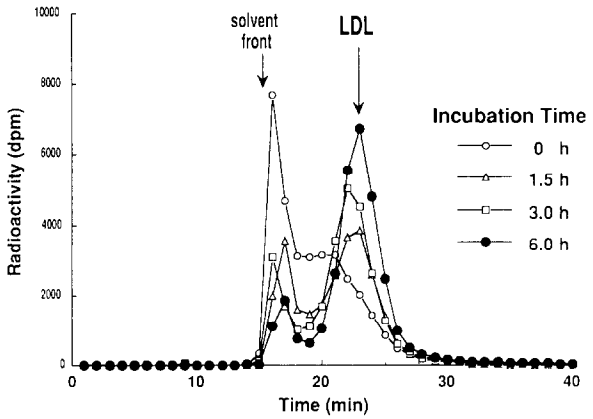

Fig. 2. Column elution profiles for the [^{14}C]RS-1541/LDL complex. The detergent-aided solution of [^{14}C]RS-1541 (20 μM) was incubated with human LDL (0.4 mg/ml) for 6 h at 37°C. At the indicated time, an aliquot of the mixture was applied to a Superose 6HR 10/30 column. The column was calibrated with human LDL.

3.3. Induction of LDL receptor activity of the cells

The cells were cultured for 24 h in medium containing LPDS (HSF[+] cells) or containing LPDS plus LDL (HSF[−] cells). The LDL receptor activities of these cells were evaluated by the uptake of ^{125}I-LDL at 5 μg/ml (Fig. 3). The

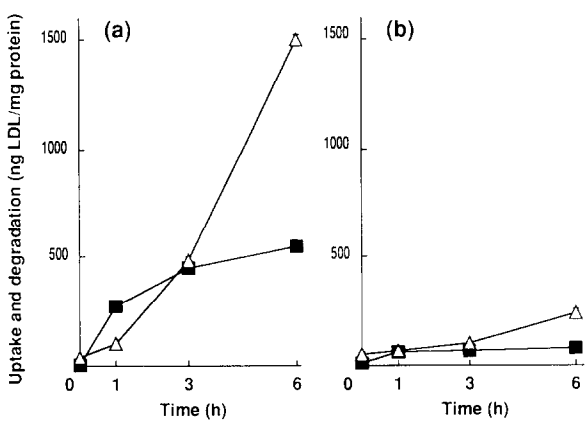

Fig. 3. Uptake and degradation of human ^{125}I-LDL by human skin fibroblasts. Cells were preincubated for 24 h in medium supplemented with (a) LPDS or (b) LPDS + LDL. The cells were incubated with 5 μg/ml of ^{125}I-LDL for 6 h at 37°C, and the uptake (■) and degradation (△) of ^{125}I-LDL were determined. Each value represents the mean ± S.E. ($n = 3$).

uptake of ^{125}I-LDL in HSF[+] cells at 6 h (548 ng/mg protein) was 7-fold higher than that in HSF[−] cells (82 ng/mg protein). The degradation of ^{125}I-LDL in HSF[+] cells was also much greater than that in HSF[−] cells. The doubling time of cell growth (approx. 24 h) was not changed by this treatment.

3.4. Uptake of [^{14}C]RS-1541/LDL complex by HSF[+] cells and HSF[−] cells

HSF[+] cells and HSF[−] cells were incubated with the [^{14}C]RS-1541 (1 μM)/LDL (20 μg/ml) complex or the detergent-aided solution of [^{14}C]RS-1541 (1 μM). In HSF[+] cells, the [^{14}C]RS-1541/LDL complex showed a 4.5-fold higher uptake than the detergent-aided solution of [^{14}C]RS-1541; the rate of uptake of the [^{14}C]RS-1541/LDL complex and that of the detergent-aided solution of [^{14}C]RS-1541 were 26.9 and 7.5 ng/mg protein/h, respectively (Fig. 4a). In HSF[−] cells, both the [^{14}C]RS-1541/LDL complex and the detergent-aided solution of [^{14}C]RS-1541 showed low uptake; the rate of uptake was 6.0 and 4.1 ng/mg protein/h, respectively (Fig. 4b).

Unlabeled LDL inhibited the uptake of the [^{14}C]RS-1541/LDL complex (20 μg/ml as LDL)

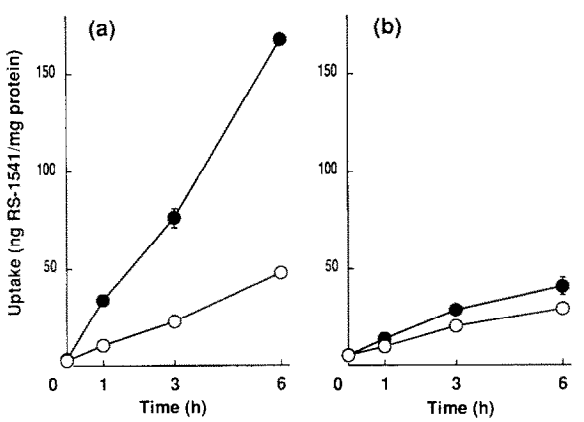

Fig. 4. Uptake of the [^{14}C]RS-1541/LDL complex by human skin fibroblasts. Cells were preincubated for 24 h in medium supplemented with (a) LPDS or (b) LPDS+LDL. The cells were incubated with the [^{14}C]RS-1541 (1 μM)/LDL (20μg/ml) complex (●) and the detergent-aided solution of [^{14}C]RS-1541 (1 μM) (○). Each value represents the mean± S.E. (n = 3).

Table 2
Effects of unlabeled LDL, monensin and low temperature on uptake of the [^{14}C]RS-1541/LDL complex by human skin fibroblasts

	Expt 1 (% of control)	Expt 2
[^{14}C]RS-1541/LDL complex [a]	100.0±2.6	100.0±1.2
+ unlabeled LDL (100 μg/ml)	42.4±1.0	
(500 μg/ml)	13.7±0.7	19.4±0.5
+ monensin (10 μM)		25.1±0.4
4°C	12.7±0.2	
[^{14}C]RS-1541 solution [b]		28.7±0.9

[a] [^{14}C]RS-1541 (1 μM)/LDL (20 μg/ml) complex.
[b] [^{14}C]RS-1541 (1 μM) solution.
Each value represents the mean±S.E. (n = 3).

by HSF[+] cells in a dose-dependent manner and reduced the uptake to less than 20% at 500 μg/ml (Table 2). 10 μM monensin, a proton ionophore, significantly decreased the uptake of the complex to 25.1% of initial values. Incubation at 4°C resulted in only 12.7% uptake compared to that at 37°C.

4. Discussion

The RS-1541/LDL complex was demonstrated to be incorporated into human skin fibroblasts via the LDL receptor. The cellular uptake of the RS-1541/LDL complex was dependent on the LDL receptor activities of the cells, and was inhibited by excessive amounts of LDL. Monensin, an agent that prevents internalization processes of endocytosis, significantly inhibited the uptake of the complex. The detergent-aided solution of RS-1541 showed very low cellular uptake.

Recently, it was reported that the neurotoxic side effects commonly seen during conventional vincristine therapy appeared to be reduced in cancer patients treated with a complex of vincristine with LDL (Filipowska et al., 1992), suggesting the possible low toxicity of an agent delivered by the LDL receptor pathway. However, the use of the LDL receptor pathway for the targeting of cytotoxic agents to cancer cells is not likely to produce absolute selectivity, since some normal cells, including liver and adrenal, also have a

high LDL receptor activity. Another objection against the use of LDL as a drug carrier is the competition due to the presence of endogenous LDL in blood. The elaborate preparation involved in an LDL complex of an antitumor agent will also be a barrier against clinical application.

It should be noted that the RS-1541/LDL complex is obtained by simply incubating human native LDL with the detergent-aided RS-1541 solution. We demonstrated that 99% of RS-1541 bound to human lipoproteins after incubation with human serum, among which the LDL fraction attained 10%. Therefore, the complex will be easily produced in human serum after administration of the detergent-aided solution of RS-1541 to patients, raising the possibility of LDL in plasma as an endogenous targeting carrier of RS-1541 into tumor cells, which have higher LDL receptor activities.

We have reported that RS-1541 binds preferentially to plasma lipoproteins and exhibits a selective and sustained uptake by tumors in mice (Tokui et al., 1994). A considerable amount of rhizoxin, an active metabolite of RS-1541, was detected in the tumor after administration of RS-1541. Interestingly, only a negligible amount of rhizoxin was observed in the liver due to further extensive metabolism (i.e., hydrolysis of lactone ring and epoxide).

Our results indicate that the chemical modification of a chemotherapeutic agent, conferring upon it high lipophilicity, will enable it to bind to lipoproteins after intravenous administration. These modifications have advantages for clinical use, as there is no need for elaborate preparation of an LDL complex of the agent, or for concern about competition between the LDL complex and endogenous LDL. We need further evidence for the participation of the LDL receptor pathway in manifestation of the cytotoxicity of the lipoprotein-bound RS-1541. The uptake and cytotoxicity of the RS-1541/LDL complex in human cancer cells are presently under investigation.

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