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ORIGINAL ARTICLE

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A possible mechanism for the long-lasting antitumor effect of the macromolecular conjugate DE-310: mediation by cellular uptake and drug release of its active camptothecin analog DX-8951

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Abstract DE-310, a new macromolecular prodrug, was designed to enhance the pharmacological profiles of a novel camptothecin analog (DX-8951f), and a single treatment with DE-310 exhibits a similar or greater therapeutic effect than do optimally scheduled multiple administrations of DX-8951f in several types of tumors. In this study, the drug-release mechanism by which DE-310 excites antitumor activity was investigated in Meth A cells, a malignant ascites model of murine fibrosarcoma. A single i.v. injection of DE-310 at the maximum tolerated dose (MTD) prolonged survival of Meth A-bearing mice by 300%. DX-8951 and glycyl-8951 (G-DX-8951), enzymatic cleavage products of DE-310, were detected in serum and ascites fluid, and also in the culture medium of Meth A ascites cells incubated in vitro with DE-310. The total amounts of DX-8951, G-DX-8951, and conjugated DX-8951 in Meth A tumor cells were three times higher than that in macrophages. Furthermore, DX-8951-related fluorescence was observed in Meth A ascites cells obtained from Meth A-bearing mice that had received DE-310 or CM-Dex-PA-DX-8951 that does not release free DX-8951. DX-8951-related fluorescence was also observed at the site of lysosomes in cells incubated in vitro with DE-310 at 37°C, but not in those incubated at 4°C. Drugs were released from DE-310 by cysteine proteinase prepared from Meth A tumor tissue. These results suggest that the mechanism by which DX-8951 is released from DE-310 in vivo is involved in the process of uptake of DE-310 into tumor or macrophages, digestion by intracellular lysosomal cysteine proteinase, and subsequent secretion of the drugs.

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Introduction

DX-8951f (DX-8951 monomethanesulfonate dehydrate) is a new camptothecin analog with a unique hexacyclic structure [1]. Compared with other camptothecin derivatives, DX-8951f is a highly effective topoisomerase I inhibitor and is the most potent cytotoxic agent against various tumor cell lines in vitro [2, 3]. Furthermore, DX-8951f exhibits remarkable antitumor activities against human solid tumors xenografted into nude mice, including CPT-11-resistant tumors [4, 5].

A new approach using macromolecular prodrugs is being pioneered to improve the efficacy of chemotherapeutic agents for cancer therapy. The macromolecular drug, designed as a drug delivery system to alter the pharmacokinetics of a low molecular weight drug, improves the antitumor efficacy and reduces toxicity of the agent. This improvement, which involves the tumor microenvironment, is referred to as the enhanced permeability and retention (EPR) effect [6–9]. The enhanced permeability is associated with the vascular architecture of tumor blood vessels and pathophysiological mediators. The retention effect is based on the lack of lymphatic clearance of macromolecules from tumor tissues. DE-310 is a novel construct formed by the linkage of DX-8951 to carboxymethyldextran polyalcohol (CM-Dex-PA), a class of polyhydroxylated carboxymethylpolysaccharides, via a Gly-Gly-Phe-Gly spacer. The CM-Dex-PA carrier with a molecular size higher than the renal filtration threshold is stable in plasma and is intended to reduce systemic drug clearance and degradation by the liver or the reticuloendothelial system. The CM-Dex-linked construct is acid-labile, and is known to be degraded by ''controlled Smith degradation'' of

polysaccharides, and is thereby degraded slowly in the lysosomal acidic environment [10].

A previous study by us has shown that a single dose of DE-310 produces high levels of DE-310 in plasma, which are maintained for a long time ($t_{1/2}$ 2.6 days) compared with the rapid decay of DX-8951f ($t_{1/2}$ 0.3 h) [11]. Furthermore, after reaching the target tissue, macromolecular prodrugs should release the active compound into tumor cells. To achieve such a tumor-selective delivery for the success of chemotherapy, over-expression of the tumor-associated enzymes can be exploited by tailoring the spacer of conjugated drugs. It has been shown that several classes of proteinases are overexpressed in many tumor cells, including cathepsins [12, 13]. PK-1, which comprises doxorubicin covalently bound to *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer by a peptidyl linker of Gly-Phe-Leu-Gly, is designed to generate the active antitumor drug under the action of cathepsin B [14]. The Gly-Gly-Phe-Gly spacer of DE-310 serves the important function of providing a low level sustained release of DX-8951 into the tumor tissue, without prematurely releasing the drug into the peripheral circulation. A single-dose of DE-310 exhibits similar or greater antitumor activity than multiple administrations of DX-8951f against various types of human tumor xenografts and murine solid tumors [11].

The objective of this study was to clarify the drug release mechanism of DE-310 using a malignant ascites model. It has been argued that the accumulation of malignant ascites in the peritoneal cavity may result from the impedance of peritoneal fluid drainage [15–17] and the enhancement of vascular permeability by various vascular mediators such as vascular endothelial growth factor (VEGF) [18–20]. From this point of view, a malignant ascites model is similar to a solid tumor model and may be a useful model for investigating the drug release mechanism in solid tumors. In a malignant ascites model, it was revealed that a single dose of DE-310 produced significant life-prolonging effects, and it was possible that free drugs released from DE-310 were responsible for the antitumor activity of DE-310. The observation of intracellular fluorescence derived from DE-310 was also consistent with the fact that DE-310 was incorporated into both Meth A tumor cells and macrophages by endocytosis and transferred to lysosomes. Furthermore, we demonstrated that drug release from DE-310 occurred by the action of lysosomal enzymes.

Materials and Methods

Compounds

DE-310 and CM-Dex-PA-DX-8951, synthesized by Daiichi Pharmaceutical Company (Tokyo, Japan), were carboxymethyldextran polyalcohol adducts of the antineoplastic agent DX-8951 [(1S,9S)-1-amino-9-ethyl-5-fluoro-1,2,3,9,12,15-hexahydro-9-hydroxy-4-methyl-10H, 13H-benzo[de]pyrano[3′,4′:6,7]indolizino[1,2-b]quinoline-10,13-dione]. The carboxymethylated polyalcohol was covalently bonded to the DX-8951 moiety via a tetrapeptide (GGFG) linker (DE-310) or without a linker (CM-Dex-PA-DX-8951). The average molecular weights (MW) of DE-310 and CM-Dex-PA-DX-8951 are each approximately 360,000 Da with a narrow polydispersity and a DX-8951 content of 5–7% w/w. For in vitro and in vivo use, DE-310 and CM-Dex-PA-DX-8951 were dissolved in pyrogen-free distilled water (Otsuka Pharmaceutical Company, Tokyo, Japan) (1 mg/ml as DX-8951 equivalents) and stored at 4°C overnight or at −20°C for a month. Concentrations of DE-310 and CM-Dex-PA-DX-8951 are expressed as DX-8951 equivalents.

Cells

Murine fibrosarcoma Meth A cells obtained from Hokkaido University (Sapporo, Japan) were maintained by serial intraperitoneal (i.p.) passage in syngeneic BALB/c mice (Nippon Charles River, Tokyo, Japan). Meth A ascites cells were collected from the peritoneal cavity of mice on day 7 after i.p. inoculation of Meth A cells. For the separation of Meth A ascites cells into Meth A tumor cells and macrophages, ascites cells were collected by washing with PRMI1640 medium (GIBCO-BRL, Grand Island, N.Y.) and cultured with RPMI1640 medium containing 10% fetal bovine serum (FBS; Hyclone, Logan, Utah) in 150-mm culture dishes (IWAKI, Tokyo, Japan). After 4 h of incubation, non-adherent cells were removed and suspended (1×10^7 cells/ml) in RPMI1640 medium containing 10% FBS (10% FBS-RPMI1640), followed by washing with RPMI1640 medium. The remaining adherent cells were washed with phosphate-buffered saline (PBS; GIBCO-BRL) twice and rinsed with a cold mixture of 2.5 m*M* EDTA (Kishida Kagaku, Osaka, Japan) and PBS. The adherent cells were removed with a cell scraper (Sumitomo Bakelite, Tokyo, Japan) and suspended in 10% FBS-RPMI1640.

Tissue homogenization

Meth A subcutaneous tumors was minced in 10 ml ice-cold buffer (250 m*M* sucrose, 25 m*M* MES, 1 m*M* EDTA, pH 6.5) for homogenization. The homogenate was centrifuged at 500 *g* for 11 min to precipitate a nuclear pellet which was washed in 20 ml of the above buffer and recentrifuged. The resulting supernatant and washing were pooled and centrifuged at 15,000 *g* for 20 min to yield a mitochondrial lysosomal pellet. The pellet was resuspended in 500 µl Britton Robinson buffer (40 m*M*, pH 4.5), frozen immediately, and stored at −70°C.

In vivo survival study

Meth A cells (1×10^6 cells/0.1 ml/mouse) were inoculated into the peritoneal cavity of syngeneic BALB/c mice

(day 0). Mice were given a single intravenous (i.v.) injection of DE-310 at doses of 11.4, 5.7, and 2.85 mg/kg as DX-8951 equivalents on day 1. Survival time was monitored daily until day 82. Increases in life-span (ILS) were calculated from the formula: ILS (%) = $[(MST_t/MST_c)-1]\times100$; where MST_t and MST_c are the median survival times of treated and control groups in days.

Pharmacokinetic study

Mice were treated with DE-310 at 11.4 mg/kg (DX-8951 equivalents) on day 5 after inoculation of Meth A cells into the peritoneal cavity. At 5 min and 30 min and 2, 4, 8, 24, and 48 h after injection of DE-310, mice were anesthetized with dimethyl ether and blood was collected from the heart. Ascites fluid was collected from the peritoneal cavity. The blood samples were left to clot for about 10 min, then centrifuged (11,000 *g*, 5 min, 4°C) and the serum was collected. The samples of ascites fluid and serum were subjected to HPLC analysis for determination of the concentrations of conjugated DX-8951, free DX-8951 and free G-DX-8951.

In vitro study for drug release of DE-310

Ascites cells were collected from Meth A-bearing mice and centrifuged at 1600 *g* for 5 min at 4°C. The cells suspended in RPMI1640 medium or supernatant were plated in 24-well plates and DE-310 dissolved in water was added to the wells. The plates were shaken with a Micromixer (Model no. MX-5, Sanko Junyaku Company, Tokyo, Japan) during the incubation time at 37°C under an atmosphere containing 5% CO_2. After 24 h of incubation, the concentrations of free DX-8951 and free G-DX-8951 in the culture medium were determined by HPLC.

In vitro study of cellular uptake of DE-310

Meth A cells and macrophages separated from Meth A ascites cells and suspended in 10% FBS-RPMI1640 were plated in non-coated 24-well plates (1×10^7 cells/well) for treatment with DE-310 (final concentration 20 μg/ml as DX-8951 equivalents). The plates were placed on the Micromixer and incubated at 37°C under an atmosphere containing 5% CO_2. After 24 h, the concentrations of free DX-8951, free G-DX-8951, and conjugated DX-8951 in the medium and cells were determined by HPLC.

In vitro study for drug release of DE-310 by tissue homogenate

A 10 μl quantity of tissue homogenate was added to 180 μl of the reaction buffer (Britton Robinson buffer, pH 4.5, 5.0, and 5.5, containing 1 m*M* EDTA, 10 m*M* cysteine, and 0.1% Brij35), followed by 10 μl DE-310 solution (33 μg/ml as DX-8951 equivalents). E-64 was also added to the prereaction mixture at 0.5 μ*M* (final concentration) for the inhibition study). These reactions were carried out for 20 h at 40°C, followed by the addition of 200 μl of an acetonitrile (CH_3CN)/0.1 *N* HCl 1:1 solution to the mixture to stop the reaction. Free DX-8951 and free G-DX-8951 were determined in the reaction mixtures by HPLC.

HPLC analysis

Samples (25 μl) of serum or ascites fluid were mixed with solvent comprising 90% (v/v) CH_3CN and 10% (v/v) 1 *N* HCl and centrifuged at 11,000 *g* for 10 min at 4°C. Each supernatant was injected onto the HPLC column for analysis, and the concentrations of free DX-8951 and free G-DX-8951 were determined. For determination of conjugated DX-8951, solvent comprising 90% (v/v) CH_3CN and 10% (v/v) 1 *N* HCl was added to cells, which had been washed three times with 150 μl saline (Otsuka) and centrifuged at 11,000 *g* for 10 min at 4°C, and the supernatant was mixed with 95 μl of thermolysin (lot no. SEQ7052; Wako Junyaku, Osaka, Japan) solution prepared at 2 mg/ml with 40 m*M* of Britton Robinson buffer (pH 6.0). After incubation at 40°C for 2 h, 10 μl reaction mixture was mixed with 990 μl of solvent comprising 50% (v/v) CH_3CN and 50% (v/v) 0.5 *N* HCl and centrifuged at 11,000 *g* for 10 min at 4°C, and the supernatant was analyzed. A standard curve was prepared with X (concentration of standard DE-310 solution as DX-8951 equivalents) vs Y [peak area of FG (Phenyl-Glycyl)-DX-8951 after thermolysin digestion]. The concentration of conjugated DX-8951 was calculated by referring to the peak area of free FG-DX-8951 determined by HPLC in samples and the standard curve.

Observation of DX-8951-related fluorescence

Mice inoculated 1 week before with Meth A cells, as described above, were inoculated (i.p.) with DE-310 or CM-Dex-PA-DX-8951. At 24 h after the injection of these conjugates, ascites cells were collected and cultured in glass bottomed dishes with 10% FBS-RPMI1640 at 37°C under an atmosphere containing 5% CO_2, followed by washing with PBS. Subsequently, ascites cells were immunostained by incubation with CD11b antibody labeled with fluorescence isothiocyanate (FITC) (Pharmingen, San Diego, Calif.) for 30 min at room temperature. After incubation, cells were washed twice with PBS containing 0.1% bovine serum albumin (BSA; Sigma, St Louis, Mo.) and 0.1% NaN_3 and suspended with mounting medium containing 0.1% NaN_3. Cells (1×10^6) were mounted on a slide glass and viewed by confocal laser scanning microscopy (CLSM; Leica, Germany).

Observation of endocytotic internalization

Ascites cells collected and prepared as detailed above were immunostained by incubation with FITC-labeled CD11b antibody for 30 min at room temperature. After incubation, cells were washed twice with PBS containing 0.1% BSA (Sigma) and 0.1% NaN_3 and suspended with mounting medium containing 0.1% NaN_3. Cells (1×10^6) were mounted on a slide glass and viewed by CLSM.

Intracellular distribution

Macrophages obtained from Meth A-bearing mice were cultured with 10% FBS-RPMI1640 in glass bottomed dishes and incubated with 10 µl LysoTracker Red DND-99 (Molecular Probes, Eugene, Ore.; final concentration: 500 n*M*), a lysosome marker, for 30 min and then incubated with DE-310 (final concentration: 200 µg/ml as DX-8951 equivalent) for 15 min and at 37°C under an atmosphere containing 5% CO_2. After removal of the medium, cells were washed with PBS and the glass bottomed dishes with RPMI1640 medium were viewed by CLSM.

Confocal laser scanning microscopy

Excitation wavelengths of 488 nm from an argon/krypton laser beam and 361 nm from a UV laser beam were used to illuminate the cells, and the emitted fluorescence was collected through 410–490 nm (DX-8951), 530 nm (FITC), and 590–640 nm (Lysotracker Red DND-99) filters.

Statistical analysis

The generalized Wilcoxon test was adopted to evaluate the life-prolonging effects of the drug. Statistical analysis was performed with SAS release 6.12 (SAS Institute Japan, Tokyo).

Results

In vivo antitumor activity in a malignant ascites model of murine fibrosarcoma Meth A

In a malignant ascites model with Meth A cells, all mice died on day 18 (Fig. 1). A single i.v. administration of DE-310 at the MTD of 11.4 mg/kg as DX-8951 equivalents induced a marked and significant life-prolongation effect with an ILS value of 307.7% ($P<0.001$ vs control, generalized Wilcoxon test). DE-310 doses of one-half and one-quarter the MTD also showed significant effects on survival with ILS values of 98.5% and 47.7% ($P<0.001$), respectively, without producing severe body weight loss.

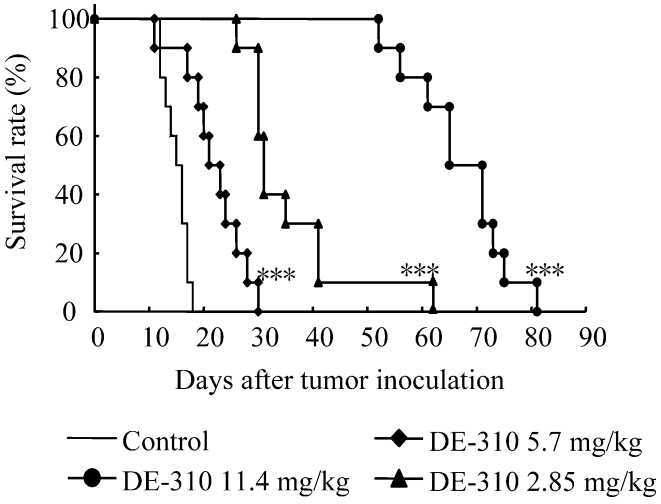

Fig. 1 In vivo life-span-increasing effects of DE-310 in Meth A-bearing mice ($n=10$). Mice inoculated i.p. with Meth A cells (1×10^6 cells) were treated i.v. with the doses of DE-310 shown in the figure beginning on day 1. Survival rate (%) = (number of mice that survived on day n/number of mice used) ×100. ***$P<0.001$ vs control, generalized Wilcoxon test

Pharmacokinetic studies in a Meth A malignant ascites model

Meth A-bearing mice received 11.4 mg/kg of DE-310 delivered i.v. and the concentrations of conjugated DX-8951 were determined in serum and ascites fluid at several time points after DE-310 injection (Fig. 2a). The concentration of conjugated DX-8951 in serum gradually decreased after dosing while that in ascites fluid increased, resulting in the same level as in the serum after 2 days. In both serum and ascites fluid, the concentration of free G-DX-8951 was two to four times higher than that of free DX-8951 at 8–48 h after administration of DE-310 (Fig. 2b). Furthermore, the concentrations of free DX-8951 and free G-DX-8951 in ascites fluid ranged from 4 to 15 times higher than those in serum at 8–48 h after the administration of DE-310.

In vitro study of drug release

Meth A ascites cells obtained from Meth A-bearing mice were incubated in vitro with DE-310 for 24 h. The concentrations of free DX-8951 and free G-DX-8951 detected in these Meth A ascites cells increased in a time-dependent manner (Fig. 3). The concentrations of free G-DX-8951 at 24 h and 48 h were 2.4 to 2.8 times higher than those of free DX-8951 (Fig. 3a). In contrast, neither free DX-8951 nor free G-DX-8951 was detected at 48 h in the supernatant of cultured ascites cells (Fig. 3b). Also, no free DX-8951 or free G-DX-8951 was detected in murine serum incubated with DE-310 (data not shown).

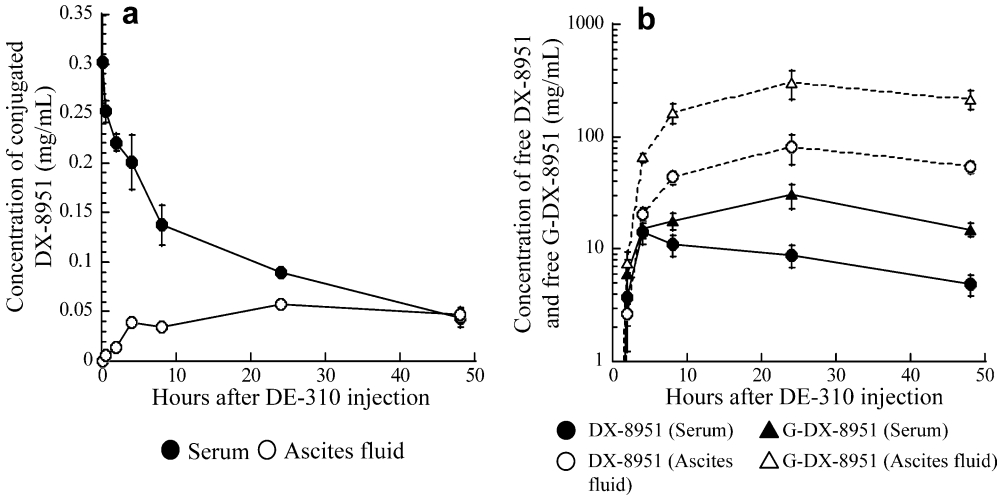

Fig. 2 The concentrations of conjugated DX-8951 (**a**), and free DX-8951 and free G-DX-8951 (**b**) after the i.v. injection of DE-310 into Meth A-bearing mice. Mice received DE-310 at 11.4 mg/kg (DX-8951 equivalents) on day 5 after Meth A cell injection. Each point represents the mean ± standard deviation ($n = 3$)

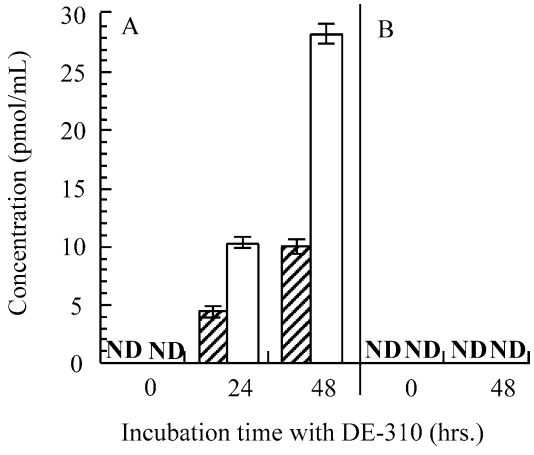

Fig. 3 The concentrations of free DX-8951 (*hatched bars*) and free G-DX-8951 (*open bars*) in samples from ascites cells of Meth A cells (**a**) and ascitic supernatant (**b**) incubated with 10 μg/ml of DE-310. The dose of DE-310 represents DX-8951 equivalents. Each column represents the mean ± standard deviation ($n = 3$) (*ND* not detected)

In vitro study of drug accumulation

Meth A ascites cells obtained from Meth A ascites-bearing mice were sorted into Meth A cells and macrophages. Each cell fraction was incubated in vitro with DE-310 for 24 h, and the concentrations of free DX-8951, free G-DX-8951, and conjugated DX-8951 were determined by HPLC. The sum of the three drug concentrations corresponds to the amount of DE-310 that was taken up into the cells. Given the total concentration based on the cell number (1×10^7 cells), the estimated amount of DE-310 incorporated in macrophages was three times higher than that in Meth A cells (Fig. 4a). Based on the macrophage population (11%) within the total amount of prepared ascites cells, the summed amount of incorporated DE-310 in whole Meth A ascites cells in mice was estimated to be three times higher than that in total macrophages (Fig. 4b).

Internalization of DE-310 in cells visualized by confocal laser scanning microscopy

As the intrinsic fluorescence of DX-8951 attached to a macromolecular carrier allowed us to follow the intracellular fate of DE-310, CLSM was used to assess whether or not the DE-310 was internalized within Meth A cells. Cells obtained from Meth A ascites-bearing mice after i.p. injection of DE-310 were immunostained with FITC-labeled CD11b antibody to differentiate the Meth A cells from macrophages. DX-8951-related fluorescence (blue color) was observed in Meth A cells and the macrophages were green in (Fig. 5a). To determine whether DX-8951-related fluorescence observed in Meth A cells and macrophages was derived from conjugated DX-8951, CM-Dex-PA-DX-8951, which does not release free DX-8951, was injected instead of DE-310 i.p. into Meth A ascites-bearing mice. DX-8951-related fluorescence was observed in both Meth A cells and macrophages as is also the case with DE-310 injection (Fig. 5b).

Endocytosis of DE-310 visualized by CLSM

Internalization of DE-310 and DX-8951f into Meth A ascites cells was examined at 37°C or 4°C (Fig. 6). After 10 min of incubation with DX-8951f, DX-8951-related fluorescence was observed in Meth A cells and macrophages at both 4°C and 37°C (Fig. 6a). In contrast, although DX-8951-related fluorescence was observed in cells incubated with DE-310 for 8 h at 37°C, it was not observed at 4°C (Fig. 6b).

Intracellular localization of DE-310

The CLSM images of macrophages incubated in vitro with DE-310 and a lysosome marker (Lysotracker Red DND-99) were analyzed (Fig. 7). The overlain image demonstrated yellow spots indicating colocalization of

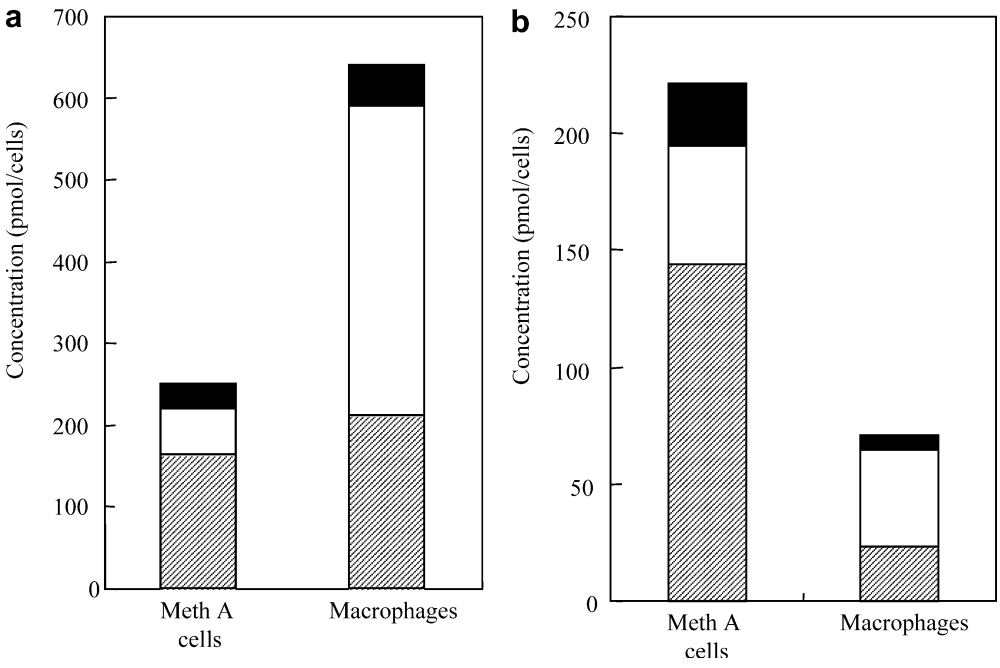

Fig. 4 The concentrations of free DX-8951, free G-DX-8951 and conjugated DX-8951 in Meth A cells and macrophages obtained from Meth A-bearing mice. The concentrations of free DX-8951 (*black*), free G-DX-8951 (*white*), and conjugated DX-8951 (*hatched*) were compared in the same number of cells (1×10^7 cells) (**a**) and on the basis of their constituent ratio in Meth A-bearing mice (**b**). The values represented by the columns are the means of three wells. Cells were incubated with DE-310 solution (final concentration 20 μg/ml) for 24 h

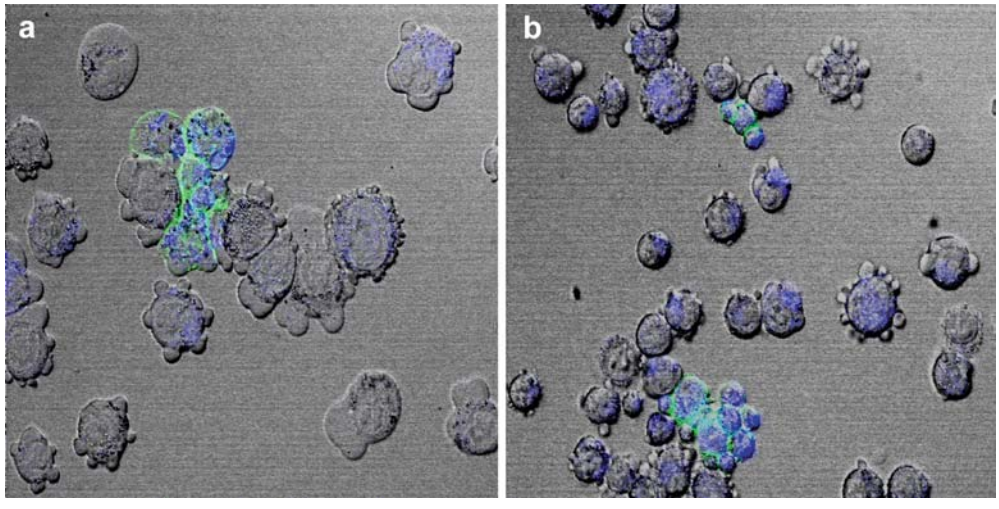

Fig. 5 CLSM images of ascites cells obtained from Meth A ascites-bearing mice treated i.p. with DE-310 (**a**) or CM-Dex-PA-DX-8951 (**b**). Composite images produced by projecting fluorescence derived from DX-8951 (*blue*) and FITC-labeled CD11b antibody for the membrane antigen of macrophages (*green*) onto the differential interference image of Meth A ascites cells. Mice received 0.2 ml of DE-310 or CM-Dex-PA-DX-8951 (10 mg/kg as DX-8951 equivalents) on day 5 after Meth A injection, and Meth A ascites cells were obtained 24 h after the injection

DX-8951 (green) and Lysotracker Red DND-99 (red). After 15 min of incubation, not only blue and red but also yellow spots were observed in macrophages. The intensity of these yellow spots was increased after 60 min of incubation.

Drug release of DE-310 induced by enzymes

Finally, the release rates of free DX-8951 and free G-DX-8951 from DE-310 incubated with the tissue homogenate prepared from Meth A tumor were determined (Fig. 8a). Enzymatic hydrolysis was tested at pH 4.5, 5.0, and 5.5. Under all conditions tested, free DX-8951 and free G-DX-8951 were detected. Maximum release was observed at pH 4.5. In addition, the release of free DX-8951 and free G-DX-8951 from DE-310 was completely inhibited in the presence of cysteine proteinase inhibitor E-64 at 1 m*M* (Fig. 8b).

Discussion

DE-310 is a novel macromolecular prodrug that links the camptothecin analog DX-8951 and the biodegradable carrier carboxymethyldextran polyalcohol via a peptidyl spacer (GGFG) for optimizing the delivery of the conjugate system. Preclinical studies of DE-310 have shown a long-lasting high level of its conjugate in blood, preferential tumor accumulation, and sustained release of both DX-8951 and G-DX-8951 in tumor tissue. This pharmacokinetic profile results in superior antitumor activity against various types of human xenografts and murine solid tumors after a single i.v. injection [11]. The

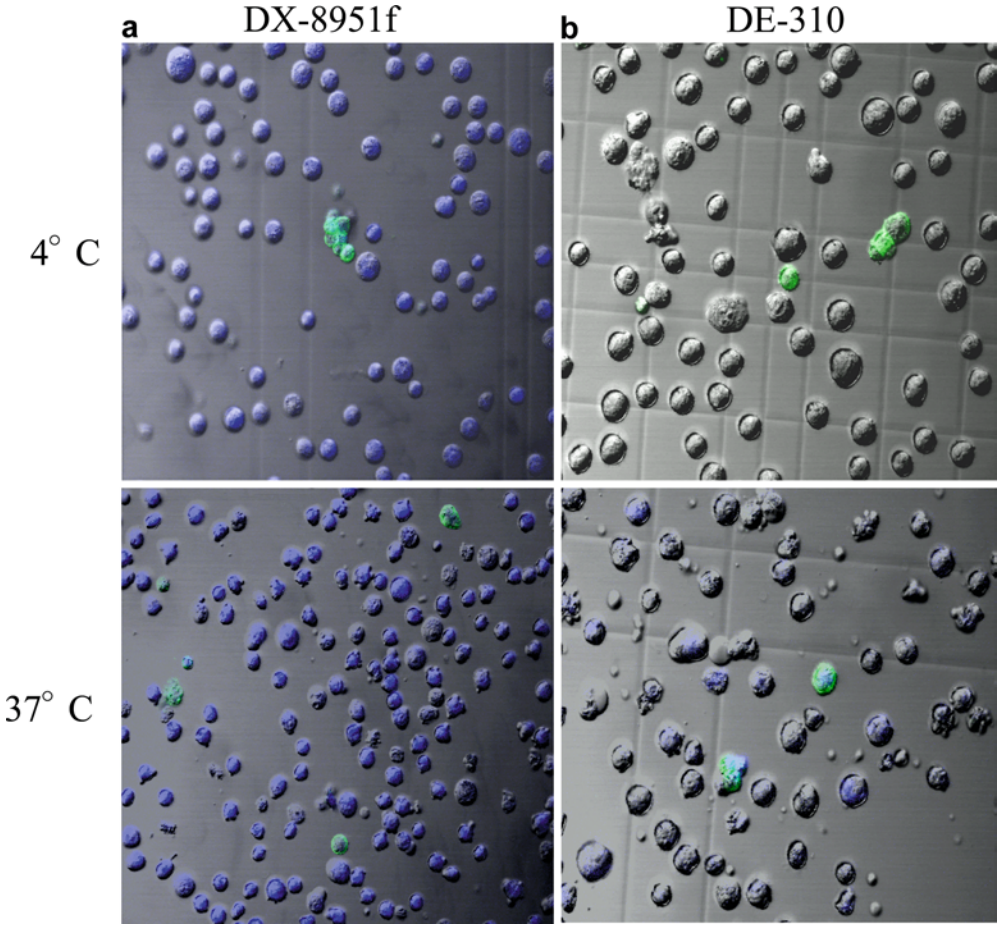

Fig. 6 CLSM images of Meth A ascites cells incubated in vitro with DX-8951f or DE-310. Composite images produced by projecting fluorescence derived from DX-8951 (*blue*) and FITC-labeled CD11b antibody (*green*) onto the differential interference image of ascites cells. Cells were incubated with DX-8951f (**a**) for 10 min or DE-310 (**b**) for 8 h at 4°C (*upper*) or 37°C (*lower*). The final concentrations of both DX-8951f and DE-310 were 500 μg/ml as DX-8951 equivalents (**a** ×20, **b** ×32)

purpose of this study was to delineate the mechanisms by which DE-310 exerts its pharmacological action at the cellular level. We believe that our studies will provide useful information on the therapeutic potential of DE-310 and a rationale for the design of drug-polymer conjugates.

In the present study, the effects of DE-310 on survival in the malignant ascites model of murine fibrosarcoma Meth A were first investigated. A single injection of DE-310 elicited a significant ILS of the animals in a dose-dependent manner (Fig. 1). After i.v. injection of DE-310 in this model, the concentration of conjugated DX-8951 increased in ascites fluid and decreased in serum (Fig. 2a). This kinetic change means that the DE-310 administered i.v. into Meth A-bearing mice is transferred from the tail vein vascular circulation to ascites fluid. Free DX-8951 and free G-DX-8951 (enzymatically cleaved products of DE-310) were detected in the ascites fluid and serum, and the concentrations of free drugs in ascites fluid was higher than those in serum (Fig. 2b). These free drugs were also detected when Meth A ascites cells isolated from Meth A-bearing mice were incubated in vitro with DE-310, but not in ascitic supernatant when administered after the same incubation (Fig. 3). These results suggest that DE-310 interacted with Meth A ascites cells and that the release of drugs from DE-310 mainly occurred in the peritoneal cavity of Meth A-bearing mice.

Basically, DE-310 does not exhibit cytotoxic activity in in vitro cell cultures [16], whereas DX-8951f shows potent cytotoxic activity against various murine and human tumor cell lines [2, 3]. These results may indicate that DE-310 is inactive against tumor cells and that it requires an activation mechanism for its antitumor activity to be elicited.

The improvements in potency of a drug by means of drug-polymer conjugation, especially in the case of camptothecin, have been reported. An example is T-0128, a 7-ethyl-10-aminopropyloxy-camptothecin (T-2513) bound to CM-Dex covalently through a peptidyl linker of Gly-Gly-Gly [21–24]. It was reported that T-0128 was incorporated into macrophages or macrophage-like tumor cell lines to exert antitumor activity, but not into carcinoma cell lines [22]. In contrast, PK-1, which consists of doxorubicin covalently bound to HPMA copolymer by the Gly-Phe-Leu-Gly linker, was taken up into tumor cells [14].

To reveal which cells were responsible for the uptake of DE-310, we next investigated the uptake of DE-310 in malignant ascites cells by CLSM, because ascites cells could be separated from tumor cells and macrophages easily. CLSM is a powerful tool for many branches of

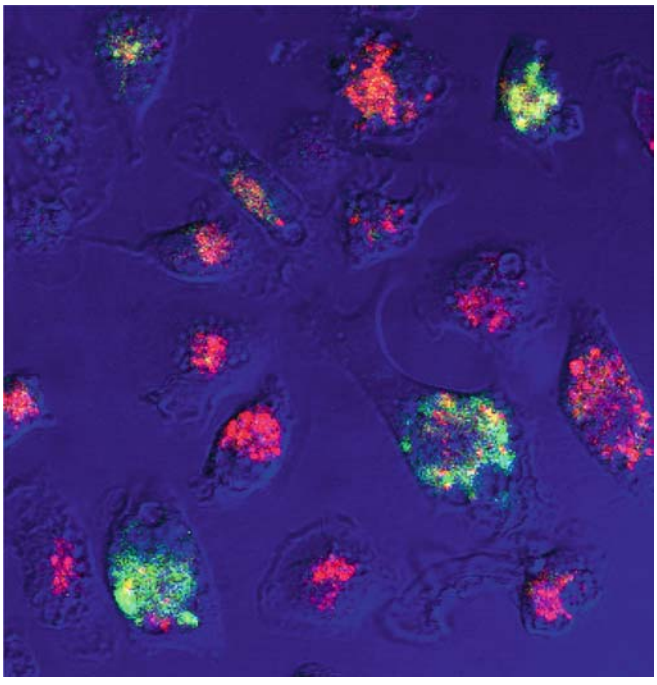

Fig. 7 CLSM images of macrophages incubated with DE-310 and a lysosome marker (Lysotracker Red DND-99). Composite images produced by projecting fluorescence derived from DX-8951 (*green*) and lysosome marker (*red*) onto the differential interference images of macrophages. Yellow color merged with DX-8951 and Lysotracker Red DND-99 fluorescence. Cells were preincubated with Lysotracker Red DND-99 (final concentration 500 n*M*) for 30 min and incubated with DE-310 (final concentration 200 μg/ml as DX-8951 equivalents) for 15 min

cell biology because of its high spatial resolution and its ability to demonstrate the three-dimensional features of biological materials [25]. Since CPT-11, which is semi-synthesized as a water-soluble derivate of camptothecin SN-38, emits fluorescence when activated by a UV laser beam, the intracellular distribution of CPT-11 has been analyzed by CLSM [26]. DX-8951 also emits fluorescence when activated by a UV laser beam in a similar manner to CPT-11 and SN-38. The intrinsic fluorescence of DX-8951 attached to a macromolecular carrier permitted us to observe the intracellular fate of DE-310 by CLSM. In Meth A ascites cells obtained from Meth A-bearing mice treated with DE-310, DX-8951-related fluorescence was observed in the cytoplasm (Fig. 5a). Meth A tumor cells could be discriminated from macrophages using FITC-labeled CD11b antibody [27]. DX-8951-related fluorescence was observed in both Meth A cells and macrophages immunostained with the antibody. In addition to DE-310, DX-8951-related fluorescence was also observed in cells when CM-Dex-PA-DX-8951, which does not release DX-8951, was administered to Meth A-bearing mice (Fig. 5b). These results indicate that DE-310 was incorporated into both Meth A tumor cells and macrophages in a conjugated form.

Compared with other small chemical antitumor agents, macromolecular drugs such as T-0128 and PK-1 may require some mechanism to enable them to traverse the cell membrane before exerting their pharmacological effects. It has been reported that PK-1 and T-0128 are taken up by endocytotic internalization [14, 22]. Endocytosis and pinocytosis have been shown to be affected at lower temperatures [28]. When cells were incubated at 4°C with DE-310, DX-8951-related florescence was not observed in either Meth A cells or macrophages, whereas DX-8951f was able to enter these cells (Fig. 6). These lines of evidence indicate that DE-310 was taken up by endocytosis not only into macrophages but also into Meth A tumor cells.

We further demonstrated by HPLC that DE-310 accumulated in Meth A cells and macrophages obtained and purified with Meth A ascites cells. It is conceivable that the combination of free DX-8951 and free G-DX-8951 in cells and culture medium, and conjugated DX-8951 in cells corresponds to the concentration of DE-310 incorporated into cells during incubation, since DE-310 was not degraded in the culture medium. When Meth A tumor cells and macrophages separated from Meth A ascites cells were incubated in vitro with DE-310, the concentration of DE-310 in macrophages was three times higher than that in Meth A cells for the same number of cells (1×10^7 cells) (Fig. 4a). In contrast, when the concentrations of DE-310 in Meth A cells and macrophages were calculated on the basis of their constituent ratios in Meth A ascites cells, the total amount of DE-310 taken up into Meth A cells was three times

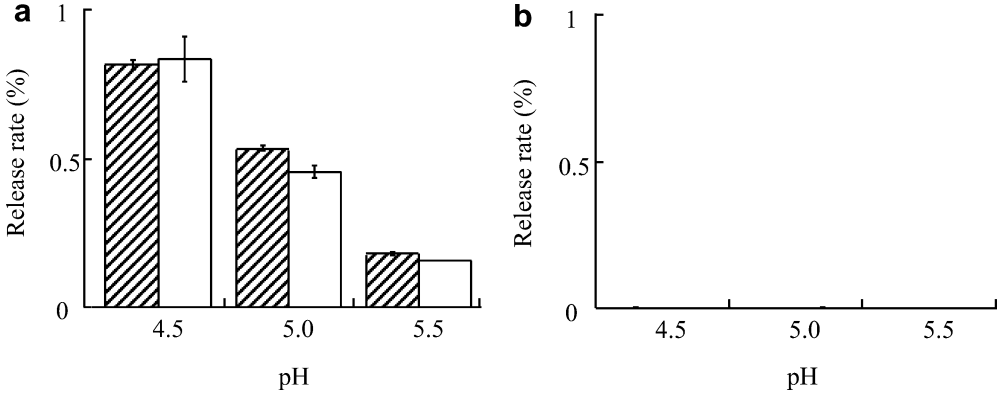

Fig. 8 Release rates of DX-8951 (*hatched bars*) and G-DX-8951 (*open bars*) from DE-310 in the presence of tissue homogenate at 40°C for 20 h without (**a**) or with E-64 (**b**) (final concentration 0.5 μ*M*)

higher than that into macrophages (Fig. 4b). These results suggest that Meth A tumor cells are key participants in the uptake and drug release of DE-310 in Meth A ascites cells. It has been reported that the cytotoxic effect of DX-8951 against Meth A cells is about 200 times higher than that of G-DX-8951. This result means that the antitumor activity of DE-310 against Meth A ascites was mainly due to the activity of DX-8951. As the cytotoxicity of DX-8951 and G-DX-8951 differed among human tumor cell lines, confirmation of their relationship in terms of their antitumor activities requires further study.

High structural flexibility and hydrophilicity of CM-Dex-PA, the carrier of DE-310, is thought to contribute to decreasing the endocytotic response of macrophages against conjugates. This property may differ from the non-PA-polysaccharide carrier of T-0128. Furthermore, it has been reported that tumor cells in ascites are activated by some types of cytokines and growth factors secreted from tumor-associated macrophages (TAMs) [29]. One possible interpretation for the difference of antitumor activity between the in vitro and in vivo situations might be the contribution of macrophages activated in vivo.

Endocytotic internalization involves membrane invagination with concomitant capture of macromolecules, followed by their transfer into the endosomal compartment of the cell. Most macromolecules are then directed, via a series of vesicle fusion events, to a secondary lysosomal compartment ensuring their continued exposure to an acidic environment [30]. One of the ways to monitor the subcellular trafficking of a macromolecular drug is to demonstrate its colocalization with known markers of cell organelles. We demonstrated that DX-8951-related fluorescence was colocalized with a lysosome marker and that their areas were enlarged in a time-dependent manner (Fig. 7).

Lysosomes contain a variety of lysosomal enzymes, which are capable of degrading macromolecules once they have entered cells. Lysosomal cysteine proteinases are proteolytic enzymes that in their mature form are localized in lysosomes and whose catalytic activity is based on a cysteine residue in the active site. These enzymes are active at pH values below 7 [31]. We demonstrated drug release from DE-310 using a homogenate prepared from Meth A tumor tissue. Free DX-8951 and free G-DX-8951 were detected when DE-310 was incubated with the homogenate under acidic conditions (pH 4.5, 5.0 and 5.5). Furthermore, drug release was completely inhibited by the cysteine proteinase inhibitor E-64 (Fig. 8). These results suggest that the release of drugs from DE-310 occurs by the action of lysosomal cysteine proteinase.

In summary, we have clarified that Meth A tumor cells and macrophages are key participants for the induction of DE-310 activity in Meth A-bearing mice. The likely model for the intracellular fate of DE-310 is illustrated (Fig. 9): DE-310 is internalized in cells by endocytosis as conjugate, and the endosomes containing DE-310 are next transferred to lysosomes, whereupon DX-8951 and G-DX-8951 are released from DE-310 by lysosomal enzymes.

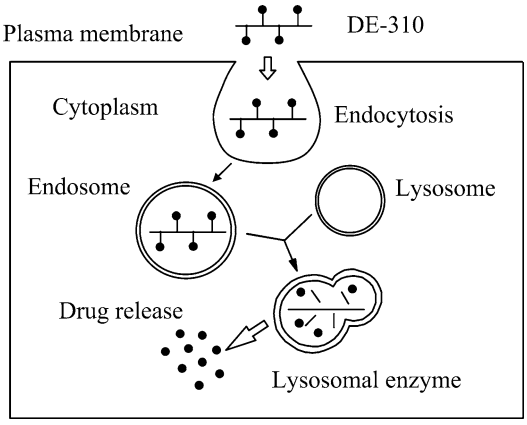

Fig. 9 Proposed drug-release mechanisms for DE-310 in cells

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