Relationship between Drug Release of DE-310, Macromolecular Prodrug of DX-8951f, and Cathepsins Activity in Several Tumors

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DE-310 is composed of the topoisomerase-I inhibitor DX-8951 (exatecan) and carboxymethyl dextran polyalcohol (CM-Dex-PA) carrier, which are covalently linked *via* peptidyl spacer (Gly-Gly-Phe-Gly). In this study, we investigated relationship between the cathepsin activity and the drug release of DE-310 by use of human liver origin cathepsin (B, L and H) and tumor cells (murine tumor cells (Meth A and M5076), and human tumor cells (HCT116, A549, PC-12, T98G, and HL-60)). Preliminary studies indicated that human liver cathepsin B produced Glycyl DX-8951 (G-DX-8951) from DE-310 more preferentially than DX-8951, whereas human liver cathepsin L produced DX-8951 preferentially. Release of drugs from DE-310 and cathepsin activities were measured in tumor cell types. The release of both DX-8951 and G-DX-8951 from DE-310 correlated well with cathepsin B activity of tumor cells. The release of DX-8951 was weakly, but not significantly, correlated with cathepsin L activity. In M5076 (high cathepsin activity) or Meth A (low cathepsin activity) xenograft models, the levels of DX-8951 and G-DX-8951 in M5076 were higher than in Meth A after single intravenous administration of DE-310. Our findings suggest that cathepsin B is primarily responsible for drug release from DE-310 in tumor.

Key words macromolecular prodrug; DE-310; camptothecin; cathepsin B; cathepsine L

Utilization of drug delivery systems is an attractive approach to improving efficacy and safety of anticancer drugs. Long-circulating macromolecular or particulate carriers are known to preferentially accumulate in solid tumors, due to the “enhanced permeability and retention (EPR)” effect,¹⁾ *i.e.*, leakiness of tumor angiogenic blood vessels and lack of effective lymphatic drainage. SMANCS (neocartinstatin polymer conjugate), Doxil (doxorubicin liposome), and Daunozome (daunorubicin liposome)^{2,3)} that exploit the EPR effect have already been launched in the market. Besides, PK1 (doxorubicin-HPMA polymer),⁴⁾ XYOTAX (paclitaxel-polyglutamate),⁵⁾ and NK911 (doxorubicin micelle)⁶⁾ are under clinical investigation.

DE-310 is a novel macromolecular prodrug of the topoisomerase-I inhibitor (1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-1,2,3,9,12,15-hexahydro-9-hydroxy-4-methyl-10*H*,13*H*-benzo[*de*]-pyrano[3',4':6,7]indolizino[1,2-*b*]quinoline-10,13-dione (DX-8951).⁷⁾ DX-8951 is covalently linked with carboxymethyl dextran polyalcohol (CM-Dex-PA) *via* the Gly-Leu-Phe-Gly (GGFG) tetrapeptide spacer (Fig. 1).⁷⁾ It has been demonstrated that a single dose of 11.4 mg/kg DE-310 exhibited stronger anti-tumor activity in mice than repeated doses (10 mg/kg daily for 5 d) of DX-8951f (exatecan; DX-8951 monomethansulfonate salt).⁸⁾ Taking into account that DX-8951f belongs to time-dependent anti-cancer drugs,⁹⁾ slow release of DX-8951 from DE-310 in addition to long-term retention in tumor tissues would have been beneficial in exhibiting such an effective anti-tumor activity.

The peptide spacer of DE-310 was designed to be cleaved by cysteine protease, cathepsins. Expression of cathepsins B, H, and L in tumor tissues and their involvement in tumor progression have been indicated.¹⁰⁾ Cathepsin B is expressed more highly in malignant tumors¹¹⁾ and is responsible for destruction of basal membranes, processing of matrix pro-

teases, and inactivation of protein inhibitors.¹²⁾ On the other hand, cathepsin H is highly expressed in colon cancer cells,¹³⁾ and cathepsin L is secreted in metastatic bone marrow tumor.¹⁴⁾ It is likely that the level of cathepsin species in tumor tissues would be one of the factors determining anti-tumor activity of DE-310.

In this study, we examined the drug release from DE-310 by cathepsins B, H, and L obtained from human liver to identify the products of hydrolysis and determine the rate of hydrolysis for each cathepsin species. Then, we examined *in vitro* drug release from DE-310 in different murine or human tumor cell types, in addition to the activity or expression of cathepsins B, H and L in the cells. Correlation between the drug release and cathepsin levels was analyzed to quantitatively delineate the mechanism of drug release from DE-310. In addition, tumor tissue distribution of DE-310 was investi-

Fig. 1. Structure of DE-310

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gated in two types of solid tumor bearing mice, *i.e.*, Meth A and M5076, which were different in cathepsin activity.

MATERIALS AND METHODS

Materials DE-310 is composed of carboxymethylated polyalcohol carrier, tetrapeptidyl spacer (Gly-Gly-Phe-Gly), and camptothecin analog DX-8951. DX-8951f is [(1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-1,2,3,9,12,15-hexahydro-9-hydroxy-4-methyl-10*H*,13*H*-benzo[de]pyrano[3',4':6,7]indolizino-[1,2-*b*]quinoline-10,13-dione] monomethanesulfonate (salt) dehydrate. These were synthesized by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). DE-310 contained 5–7%w/w DX-8951 and its molecular weight (MW) was *ca.* 340000 with a narrow polydispersity (weight average MW/number-average MW < 1.3). For use, DE-310 was dissolved in pyrogen-free distilled water (Otsuka Pharmaceutical Company, Tokyo, Japan) and stored 4°C overnight. Benzyloxycarbonyl-arginylarginine-4-methyl-7-coumarylamide (Z-Arg-Arg-MCA) and 1-arginine-4-methyl-7-coumarylamide (Arg-MCA), which are specific substrates for cathepsin B and cathepsin H, respectively,¹⁵⁾ were purchased from Peptide Institute, Inc. (Osaka, Japan). Cathepsin B (human liver, 3175 units/ml solution) was purchased from Sigma (St. Louis, MO, U.S.A.). Cathepsin L (human liver, 41.7 mU/ml solution) and Cathepsin H (human liver, 1330 mU/mg protein; 893.76 mU/ml solution) were purchased from Calbiochem (La Jolla, CA, U.S.A.). Cathepsin H was diluted to 446.88 mU/ml with 20 mM acetate buffer (pH 5.0) containing 1 mM of EDTA and 0.1% Brij 35. Cathepsin C (Bovine Spleen, 5.4 units/mg solid) was purchased from Sigma (St. Louis, MO, U.S.A.), diluted to 10.26 units/ml with 20 mM acetate buffer (pH 5.5) containing 1 mM of EDTA and 0.1% Brij 35.

Meth A (murine fibrosarcoma) was kindly obtained from Dr. Ichiro Azuma (Institute of Immunological Science, School of Medicine, Hokkaido University, Japan). HCT116 (human colon cancer) and T98G (human glioma) were obtained from American Type Culture Collection (VA, U.S.A.). A549 (human lung carcinoma) and PC-12 (human lung carcinoma expressed *p*-glycoprotein) were obtained from Toray Industries (Shizuoka, Japan). All of the cells were cultured in RPMI 1640 (Gibco-BRL; NY, U.S.A.) medium supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, U.S.A.). M5076 (murine ovarian sarcoma) was obtained from the Japanese Foundation for Cancer Research (Tokyo, Japan), and cultured in RPMI 1640 medium supplemented with 17% horse serum (HS; Hyclone Laboratories, Logan, UT, U.S.A.).

In Vitro Drug Release from DE-310 with Human Cathepsins A 10 μ l aliquot of 41.7 mU/ml human cathepsin solution was added to 180 μ l of Britton–Robinson buffer (pH 4.5, 5.5 or 6.5) containing 1 mM of EDTA and 10 mM of cysteine and 0.1% Brij 35. Then a 10 μ l aliquot of DE-310 solution was added to give a final concentration of 5 nmol/ml (DX-8951 equivalents). These reactions were done at 40°C for 20 h and stopped by the addition of 200 μ l of CH₃CN:0.1*N* HCl=1:1 solution to the mixture. The amount of DX-8951 and G-DX-8951 in the reaction mixture was measured by HPLC.

In Vitro Drug Release from DE-310 with Tumor Cells

Meth A, M5076, HCT116, T98G, A549, HL-60 and PC-12 were plated in non-coated 60 mm dishes (1×10⁷ cells/4 ml/dish) and then DE-310 solution was added to give a final concentration of 20 μ g/ml (DX-8951 equivalents). These dishes were incubated at 37°C for 24 h in air containing 5% CO₂. In the case of Meth A, M5076, and HL-60, the dishes were shaken with a micomixer (Model No. MX-5, Sanko Junyaku Company, Tokyo, Japan). At the end of incubation, the cells were centrifuged at 1000*g* for 10 min, and 500 μ l of the supernatant was taken for the HPLC assay. The cells were rinsed with saline 3 times and treated with 300 μ l of the 1:1 (v/v) mixture of 0.5 M HCl and acetonitrile. In the case of HCT116, A549, PC-12 and T98G, which are adherent cells, the culture medium was aspirated off and then the cells were rinsed with saline 3 times and lysed with 1 ml of the 1:1 (v/v) mixture of 0.5 M HCl and acetonitrile. In both cases, the cell lysate was centrifuged at 10000*g* for 10 min and the supernatant were analyzed by HPLC. Furthermore, release drugs in culture medium were determined. The culture media were collected, and added to same volume of 0.5 M HCl containing 50% (v/v) acetonitrile solution. The solutions were centrifuged at 10000*g* for 10 min, and the supernatants were analyzed by HPLC.

Cathepsins Activities in Tumor Cells The cell suspensions (1×10⁷ cells/ml) were homogenized by the handy micro homogenizer NS-310F (MICROTEC, Co., Ltd., Chiba, Japan) and then centrifuged (12000*g*, 4°C, 10 min) to yield the supernatant. The content of proteins leaked in the supernatant was determined by Bio-Rad protein assay dye reagent (Japan Bio-Rad Laboratories, Inc., Tokyo, Japan), using bovine serum albumin as a reference. The activities of cathepsins B and H in the supernatant were measured using the substrates Z-Arg-Arg-MCA and Arg-MCA, respectively, in a similar manner to the method of Barrett.¹⁵⁾ Briefly, to 2 μ l of the solution, 125 μ l of 400 mM phosphate buffer (352 mM KH₂PO₄, 48 mM Na₂HPO₄, 4 mM EDTA, pH 6.0), 365 μ l of 0.1% Brij 35 and 4 μ l of freshly prepared 1 M cysteine were added and preincubated for 5 min at 40°C. The reaction was started by the addition of 1 μ l of 10 mM substrate (Z-Arg-Arg-MCA or Arg-MCA) solution. Ten minutes later, 500 μ l of the solution containing 100 mM sodium chloroacetate, 30 mM sodium acetate and 70 mM acetic acid was added to stop the reaction. The amount of 7-amino-4-methyl-coumarin (AMC) liberated was measured by HPLC. Enzyme activities were calculated using a calibration curve established with AMC standard. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μ mol of AMC per minute from the substrates at 40°C. Expression of cathepsin L in the tumor cell was determined by a commercially available ELISA kit (RD laboratories, Diessen, Germany). The level of cathepsin L was expressed in mU of analyte per milligram of tissue protein.

Tumor Tissue Distribution of DE-310 in Meth A and M5076 Xenograft Models Meth A cells (1×10⁶ cells/0.1 ml/mouse) and M5076 cells (2×10⁶ cells/0.1 ml/mouse) were inoculated subcutaneously into the right flank of syngeneic BALB/c and C57BL/6 mice (Japan SLC, Inc., Shizuoka, Tokyo), respectively. When estimated tumor volume reached to approximately 500 mm³ after transplantation, the mice were injected intravenously with DE-310 at 11.4 mg/kg (DX-8951 equivalents, *n*=3). At 24, 48, and 72 h

Fig. 2. Release of DX-8951 and Glycyl DX-8951 from DE-310 Incubated with Cathepsin B (A), Cathepsin L (B), Cathepsin H (C), and without Any Enzymes (D) at 37 °C for 20 h

after administration of DE-310, tumor tissue was removed and weighed. Samples were stored at -30°C until analysis. The samples were mixed with $\text{CH}_3\text{CN}:0.5\text{ N HCl}=1:1$ solution at 4-fold volume (v/w) per tissue weight, and homogenized on ice using handy micro homogenizer to extract DX-8951, G-DX-8951 and conjugated-DX-8951. The homogenates were centrifuged (15000 *g*, 10 min), and the supernatant was analyzed to determine the amount of DX-8951 and G-DX-8951 by HPLC. Furthermore, for determining the amount of conjugated DX-8951, 5 μl of the supernatant was added to 95 μl of α -chymotrypsin (2 mg/ml) dissolved by Britton–Robinson buffer (pH 6.0), and incubated at 40°C for 2 h. After that, the reaction was stopped by adding 100 μl of $\text{CH}_3\text{CN}:0.5\text{ N HCl}=1:1$ solution and centrifuged (15000 *g*, 10 min). The supernatant was analyzed to determine the amount of total G-DX-8951 by HPLC, conjugated-DX-8951 calculated by deduction of G-DX-8951 from total amount of G-DX-8951.¹⁶⁾

HPLC Analysis The concentrations of DX-8951 and G-DX-8951 in the medium and cell extracts were determined by reversed-phase HPLC (CCPM-II pump, Tosoh, Tokyo, Japan) at 40°C implementing Symmetry Shield RP18 (4.6 $\times$ 100 mm; Waters, Milford, MA, U.S.A.). The mobile phase was a mixture of 36.5% of acetonitrile/methanol (2/1, v/v) and 64.5% of 0.1% trifluoroacetic acid at Symmetry Shield RP18 column, flowed at 1.0 ml/min. Detection was performed using the Waters 474 fluorescence detector (Waters, Milford, MA, U.S.A.) with excitation and emission wavelength of 375 nm and 445 nm, respectively. The peak area was calculated using a Millennium chromatography manager system (Waters, Milford, MA, U.S.A.). Measurement of AMC in cathepsins assay samples were also carried out with the same HPLC system, where excitation and emission wavelengths were set at 346 nm and 437 nm, respectively. The mobile phase of AMC was a mixture of 27% of acetonitrile/methanol (2/1, v/v) and 73% of distilled water.

RESULTS

***In Vitro* Drug Release from DE-310 with Human Cathepsins** Preliminary studies were carried out with cathepsins isolated from human liver. Figure 2 shows the release of DX-8951 and G-DX-8951 from DE-310 incubated with human cathepsins. For all cathepsins, the quantity of drug release decreased with increasing pH within the pH range tested. In cathepsin B, the release of G-DX-8951 was 10-fold higher than that of DX-8951 at pH 4.5 (Fig. 2A). On the contrary, cathepsin L catabolized the release of DX-8951 more preferentially (approximately 6-fold higher) than that of G-DX-8951 (Fig. 2B). As for cathepsin H, no DX-8951 was detected while only a small amount of G-DX-8951 was detected (<0.05 %) (Fig. 2C). When DE-310 was incubated in the absence of any enzymes, the drugs were not released at all (Fig. 2D).

***In Vitro* Drug Release from DE-310 with Murine and Human Cells** In order to investigate relationship between drug release from DE-310 and cathepsin activity, first, drug release from DE-310 was investigated in different tumor cells, *i.e.*, two types of murine (Meth A and M5076) and 5 types of human (T98G, A549, HL-60, PC-12, and HCT116) tumors cells. The release of both DX-8951 and G-DX-8951 were detected in all cell lines, where the total amount of DX-8951 and G-DX-8951 released varied 3.77–51.27 pmol/ 10^7 cells/24 h and 15.53–467.63 pmol/ 10^7 cells/24 h, respectively (Table 1). Here, most of the drugs released were detected in the incubation medium. The release of DX-8951 was the highest in M5076 cells, followed by T98G>A549 $\approx$ Meth A>HL-60>PC-12 $\approx$ HCT116. Although the release of G-DX-8951 was also highest in M5076 cells, it was remarkably low in A549 cells that exhibited a high activity towards the release of DX-8951. The release of the drugs did not occur when DE-310 was incubated with the cell-conditioned medium (data not shown).

Cathepsins Activity in Tumor Cells To delineate vari-

ability of the release of drugs from DE-310 in different tumor cells, the levels of cathepsins B, H and L were investigated (Table 2). Cathepsin B activity was much more variable than that of cathepsin H. The activity of cathepsin B was the highest in T98G cells followed by M5076>A549>PC-12>HCT116>Meth A>HL-60. Expression of cathepsin L

in human tumor cells was evaluated by ELISA involving human cathepsin L antibody, protein expression of cathepsin L per total amount of protein was determined in human tumor cells, except for murine tumors. Expression of cathepsin L per cellular proteins ranged from 0.90 to 2.89 mU/mg protein, which was the highest in T98G cells followed by A549>HCT116>PC-12>HL-60. On the other hand, cathepsin H activity ranged from 0.03 to 0.08, which was much lower than other cathepsins.

Table 1. The Drug Release from DE-310 with Several Tumor Cells *in Vitro*

Origin	Cell line	Source	DX-8951 (pmol/10 ⁷ cells)	G-DX-8951 (pmol/10 ⁷ cells)
Murine	Meth A	Cell lysate	0.32±0.09	1.28±0.24
		Medium	15.01±3.11	38.93±3.64
		Total	15.33±3.04	40.22±3.88
	M5076	Cell lysate	1.11±0.26	14.74±0.16
		Medium	50.17±7.86	452.89±16.11
		Total	51.27±7.66	467.63±16.26
Human	HCT116	Cell lysate	0.44±0.07	3.65±0.51
		Medium	3.33±1.04	31.6±1.91
		Total	3.77±1.01	35.25±1.89
	A549	Cell lysate	0.47±0.10	1.65±0.18
		Medium	17.17±4.33	13.87±5.49
		Total	17.64±4.23	15.53±5.37
	PC-12	Cell lysate	0.34±0.07	1.25±0.11
		Medium	5.14±1.17	50.53±0.93
		Total	5.49±1.24	51.76±0.84
	T98G	Cell lysate	1.99±0.40	16.21±0.90
		Medium	30.28±7.25	115.95±5.54
		Total	32.27±7.10	132.16±4.66
	HL-60	Cell lysate	BLQ	2.28±0.24
		Medium	5.60±0.73	42.38±6.15
		Total	5.60±0.73	44.66±0.32

DE-310 (20 µg/ml) were incubated with several tumors (1×10⁷ cells/dish) at 37 °C for 24 h (n=3). The release drugs were determined by HPLC (mean±S.D.). BLQ: Below limit of quantification.

Table 2. Cathepsin Activities in Various Tumor Cells^{a)}

Origin	Cell line	Cathepsin B (mU/mg protein)	Cathepsin H (mU/mg protein)	Cathepsin L (mU/mg protein)
Murine	Meth A	0.89±0.14	0.03±0.004	N.D. ^{b)}
	M5076	5.36±0.61	0.08±0.011	N.D. ^{b)}
Human	HCT116	1.30±0.16	0.04±0.005	2.08±0.03
	A549	4.25±0.50	0.04±0.007	2.26±0.15
	PC-12	3.67±0.64	0.03±0.003	1.11±0.08
	T98G	11.88±1.60	0.05±0.009	2.89±0.03
	HL-60	0.42±0.17	0.05±0.011	0.90±0.02

a) Cell suspensions (1×10⁷ cells/ml) were homogenized, and centrifuged to yield the supernatant. The content of proteins in the supernatant was determined by Bio-Rad protein assay dye reagent. The activities of cathepsins B and H in the supernatant were determined using the substrates Z-Arg-Arg-MCA and Arg-MCA, respectively, while that of cathepsin L was determined by ELISA kit. b) Not determined.

Correlation Analysis The relationship between activity of cathepsins and drug release using murine and human tumor cells was statistically investigated in Table 3. Here, cathepsin activity represents the total amount of the liberated drug detected in both incubation medium and cell lysate. The release of DX-8951 from DE-310 was highly correlated with cathepsin B activity in human tumor cells ($r=0.9421$, $p=0.0166$). The release of G-DX-8951 was also correlated with cathepsin B activity and released G-DX-8951 ($r=0.8494$, $p=0.0685$). As for cathepsin L, there was a positive association between expression and released DX-8951, though it was not statistically significant ($r=0.8047$, $p=0.1005$). Between the level of cathepsin L expression and released G-DX-8951, there was not a significant correlation. Furthermore, when we did similar analysis with added two murine tumor cells, the correlation coefficient between cathepsin B and released DX-8951 or G-DX-8951 decreased to 0.6110, 0.3430, respectively (data not shown). As a result, though there were a few examples, it suggested that species differences with murine and human existed in the correlation with cathepsin B and drug release.

Tumor Tissue Distribution of DE-310 Distribution of conjugated DX-8951, DX-8951, and G-DX-8951 in Meth A or M5076 tumor tissues was evaluated at 24, 48, and 72 h post intravenous injection of DE-310 (Table 4). At all time points, concentration of DX-8951 and G-DX-8951 was higher in M5076 xenograft models than in Meth A xenograft

Table 3. Correlations between Drug Release and Cathepsin Activity in Tumor Cells

Drug release vs. cathepsin activity	Correlation coefficient (r)	p value
DX-8951 vs. cathepsin B	0.9421	0.0166
G-DX-8951 vs. cathepsin B	0.8494	0.0685
DX-8951+G-DX-8951 vs. cathepsin B	0.9114	0.0312
DX-8951 vs. cathepsin L	0.8047	0.1005
G-DX-8951 vs. cathepsin L	0.4830	0.4098
DX-8951+G-DX-8951 vs. cathepsin L	0.5785	0.3069

Table 4. Tumor Distribution of Conjugated and Released DX-8951 in Meth A and M5076 Xenograft Models after Intravenous Injection of DE-310^{a)}

Tumor	Time (h)	DX-8951 (nmol/g)	G-DX-8951 (nmol/g)	Conjugated DX-8951 (nmol/g)	Percent released ^{b)}	
					DX-8951	G-DX-8951
Meth A	24	0.25±0.04	0.71±0.14	45.58±9.89	0.55	1.56
	48	0.11±0.02	0.27±0.04	28.06±2.45	0.39	0.96
	72	0.08±0.02	0.19±0.05	18.98±4.60	0.42	1.00
M5076	24	1.64±0.24	14.82±1.52	146.91±20.33	1.12	10.09
	48	0.57±0.06	4.52±0.64	76.64±12.18	0.74	5.90
	72	0.49±0.09	3.24±0.69	74.07±21.46	0.66	4.37

a) DE-310 (11.4 mg DX-8951 equivalent/kg) was intravenously administered in Meth A or M5076 bearing mice. Tumor tissue was excised at 24, 48, and 72 h post injection, and DX-8951 species in tumor tissues were determined. b) Percent released means the ratio of released drugs to conjugated DX-8951.

models. Even when the level of DX-8951 and G-DX-8951 was standardized with that of conjugated DX-8951, the level of both forms DX-8951, especially G-DX-8951, was greater in M5076 xenograft models.

DISCUSSION

DE-310 has a GGFG tetrapeptide spacer for linking DX-8951 with CM-Dex-PA. It was believed that the GGFG sequence was a substrate of lysosomal enzymes,^{17–19} as well as GFLG^{20,21} or some others.^{22–25} Nogusa *et al.*^{17–19} examined effect of peptide spacers on the release and anti-tumor activity of the drug, doxorubicin (DXR), conjugated with carboxymethyl-pullulan (CMPul). CMPul-GGFG-DXR showed the highest release of the drug, followed by CMPul-GFGG-DXR and CMPul-GGGG-DXR, when the conjugates were incubated with lysosomes for 24 h.¹⁷ Among the three conjugates, CMPul-GGFG-DXR indicated the strongest anti-tumor effect against rat bearing Walker-256, and Yoshida sarcoma.¹⁸ Their research group subsequently reported that anti-tumor activity of CMPul-GGFG-DXR was equivalent to that of CMPul-GFLG-DXR,¹⁹ which has a well-known GFLG spacer.^{20,21}

Our previous studies have shown that DE-310 elicits significant anti-tumor activity in various tumor models, whereas the activity occurred at lower doses and with a more profound effect than DX-8951 alone.⁸ High levels and prolonged retention of conjugated DX-8951, DX-8951, and G-DX-8951 have been observed in tumor tissues after intravenous injection of DE-310 in Meth A tumor-bearing mice.²⁶ Thus, liberated DX-8951s are believed to exhibit a cytotoxic effect in tumor tissues. Preclinical data indicated that the inhibitory activity of DX-8951 on topoisomerase-I activity is comparable to G-DX-8951 and that the *in vitro* cytotoxicity of DX-8951 is about 20–190 times more potent than G-DX-8951 (unpublished results). A clinical study demonstrated that G-DX-8951 concentration in tumor tissues are approximately ten fold greater than DX-8951, suggesting that G-DX-8951 might partially contribute to the cytotoxic activity of DE-310.²⁷

Structural requirements for drug release from macromolecular prodrugs have been discussed by Soyez *et al.*²⁸ who concluded that, whereas general trends could be found, the type of carrier and drug used greatly influences the degradation rate and pattern due to specificities of substrate-enzyme reactions. Taking into account the difference in cytotoxic effects between DX-8951 and G-DX-8951, the present study was initiated to investigate how these two forms appear from DE-310 hydrolysis.

Our previous investigation demonstrated that the release of the parent drugs from DE-310 were completely inhibited by a representative cysteine protease inhibitor E-64²⁹ in Meth A cell homogenate, indicating the involvement of cathepsins in this process. *In vitro* studies revealed (a) that cathepsin B produces both DX-8951 and G-DX-8951 while cathepsin L produces DX-8951 preferentially, (b) that the activity of cathepsins in tumor cells varies widely, and (c) that the release of G-DX-8951 from DE-310 showed a high correlation with cathepsin B activity in tumor cells, while that of DX-8951 did with cathepsins B and L. Thus, variability of cathepsins activity in cell types would affect the release rate of DX-

8951 or G-DX-8951 from DE-310 in the *in vivo* conditions. When Meth A and M5076 xenograft models were compared, tumor tissue concentration ratio of liberated drugs, especially G-DX-8951, was greater in M5076 xenograft model (Table 4). The *in vivo* result is reasonable, given that cathepsin B activity of M5076 cells was much greater than that of Meth A cells.

In the hydrolysis of HPMA-GFLG-nitroanilide,²¹ PK1,³⁰ and CMPul-GGFG-DXR^{17–19} by cathepsin B, glycyated forms were not produced from macromolecular prodrugs. When norfloxacin was coupled with chloroformate-activated dextran *via* GFLG spacer, on the other hand, G-norfloxacin was released in the presence of cathepsin B. Cathepsin B is a lysosomal thiol protease that has been found in the cells of many species. Besides endopeptidase activity, cathepsin B has also carboxydipeptidase activity.³¹ Soyez *et al.*²⁸ reported that NAP was released from HPMA-GFLG-NAP while F-NAP was released first from HPMA-GFLGF-NAP. Thus, the pattern of release appears to depend on the kind of drugs and spacers, relating to steric or electrostatic effect of their molecular structures.

The present study demonstrated that the release of drug from DE-310 depends on expression of cathepsins in tumor. Since the rate of drug release determines concentration–time course of liberated drug, it might be an important factor determining anti-tumor activity. The release of drug does not appear to be rate-limiting, since the tumor tissue concentration–time profiles of free DX-8951 and G-DX-8951 were parallel to that of conjugated DX-8951 (Table 4). When anti-tumor activity of DE-310 was concerned, however, appearance of G-DX-8951 would not be desirable. To avoid this problem, further design of spacer will be needed. Dubowchik *et al.*³² indicated that cathepsin B-mediated drug release was increased when self-immolative *p*-aminobenzylcarbonyl (PABC) spacer was inserted between DXR and lysosomal protease-sensitive peptides. In our on-going studies, PABC was inserted between DX-8951 and GGFG in DE-310, demonstrating that the release of free DX-8951 from the conjugate was more than 100 times greater than DE-310 in homogenates prepared from murine Meth A solid tumor. The experimental results will be reported elsewhere.

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REFERENCES

- 1) Maeda H., *Adv. Enzyme Regul.*, **41**, 189–207 (2001).
- 2) Matsumura Y., Maeda H., *Cancer Res.*, **46**, 6387–6392 (1986).
- 3) Janknecht R., *Support Care Cancer*, **4**, 298–304 (1996).
- 4) Vasey P., Kaye S. B., Morrison R., Twelves C., Wilson P., Duncan R., Thomson A. H., Murray L. S., Hilditch T. E., Murray T., Burtles S., Fraier D., Frigerio E., Cassidy J., *Clin. Cancer Res.*, **5**, 83–94 (1999).
- 5) Singer J. W., *J. Control. Release*, **109**, 120–126 (2005).
- 6) Nakanishi T., Fukushima S., Okamoto K., Suzuki M., Matsumura Y., Yokoyama M., Okano T., Sakurai Y., Kataoka K., *J. Control. Release*, **74**, 295–302 (2001).
- 7) Inoue K., Kumazawa E., Kuga H., Susaki H., Masubuchi N., Kajimura T., *Adv. Exp. Med. Biol.*, **519**, 145–153 (2003).
- 8) Kumazawa E., Ochi Y., *Cancer Sci.*, **95**, 168–175 (2004).
- 9) Kumazawa E., Tohgo A., *Expert Opin. Invest. Drugs*, **7**, 625–632 (1998).
- 10) Staak A., Tolic D., Kristiansen G., Schnorr D., Loening S. A., Jung K.,

- Urology*, **63**, 1089—1094 (2004).
- 11) Troy A. M., Sheahan K., Mulcahy H. E., Duffy M. J., Hyland J. M. P., O'Donoghue D. P., *Eur. J. Cancer*, **40**, 1610—1616 (2004).
 - 12) Sloane B. F., Moin K., Lah T. T., "Biochemical and Molecular Aspects of Selected Cancers," Vol. 2, ed. by Pretlow T. G., Pretlow T. P., Academic Press, San Diego, 1994, pp. 411—466.
 - 13) Schweiger A., Christensen I. J., Nielsen H. J., Sorensen S., Brunner N., Kos J., *Int. J. Biol. Markers*, **19**, 289—294 (2004).
 - 14) Katunuma N., Tsuge H., Nukatsuka M., Asao T., Fukushima M., *Arch. Biochem. Biophys.*, **397**, 305—311 (2002).
 - 15) Barrett A. J., *Biochem. J.*, **187**, 909—912 (1980).
 - 16) Shiose Y., Kuga H., Yamashita F., Hashida M., *J. Pharm. Biomed. Anal.*, **43**, 1290—1296 (2007).
 - 17) Nogusa H., Yano T., Okuno S., Hamada H., Inoue K., *Chem. Pharm. Bull.*, **43**, 1931—1936 (1995).
 - 18) Nogusa H., Yano T., Kajiki M., Gonsho A., Hamana H., Okuno S., *Biol. Pharm. Bull.*, **20**, 1061—1065 (1997).
 - 19) Nogusa H., Yano T., Kashima N., Yamamoto K., Okuno S., Hamana H., *Bioorg. Med. Chem. Lett.*, **10**, 227—230 (2000).
 - 20) Duncan R., Kopeckova R. P., Strohalm J., Hume I., Cable H. C., Pohl J., Llovd J. B., Kopecek J., *Br. J. Cancer*, **55**, 165—174 (1987).
 - 21) Rejmanova P., Kopecek J., Pohl J., Baudys M., Kostka Y., *Makromol. Chem.*, **184**, 2009—2020 (1983).
 - 22) Caiolfa V. R., Zamaï M., Fiorino A., Frigerio E., Pellizzoni C., d'Arqv R., Ghiqlieri A., Castelli M. G., Farao M., Pesenti E., Giqli M., Angeliucci F., Suarato A., *J. Control. Release*, **65**, 105—119 (2000).
 - 23) Conover C. D., Greenwald R. B., Pendri A., Gilbert C. W., Shum K. L., *Cancer Chemother. Pharmacol.*, **42**, 407—414 (1998).
 - 24) Zou Y., Wu Q. P., Tansey W., Chow D., Hung M. C., Charnsanqavej C., Wallace S., Li C., *Int. J. Oncol.*, **18**, 331—336 (2001).
 - 25) Okuno S., Harada M., Yano T., Yano S., Kiuchi S., Tsuda N., Sakamura Y., Imai J., Kawaguchi T., Tsujihara K., *Cancer Res.*, **60**, 2988—2995 (2000).
 - 26) Masubuchi N., *Pharmazie*, **59**, 374—377 (2004).
 - 27) Wente M. N., Kleeff J., Buchler M. W., Wanders J., Cheverton P., Langman S., Friess H., *Investigational New Drugs*, **23**, 339—347 (2005).
 - 28) Soye H., Schacht E., Vanderkerken S., *Adv. Drug Delv. Rev.*, **21**, 81—106 (1996).
 - 29) Ochi Y., Shiose Y., Kuga H., Kumazawa E., *Cancer Chemother. Pharmacol.*, **55**, 323—332 (2005).
 - 30) Duncan R., Rejmanova P., Kopecek J., Lloyd J. B., *Biochem. Biophys. Acta*, **678**, 143—150 (1981).
 - 31) Barrett A. J., Kirschke H., *Methods Enzymol.*, **80**, 535—561 (1981).
 - 32) Dubowchik G. M., Firestone R. A., *Bioorg. Med. Chem. Lett.*, **8**, 3341—3346 (1998).