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Pharmacokinetics of DE-310, a novel macromolecular carrier system for the camptothecin analog DX-8951f, in tumor-bearing mice

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To improve the distribution of the novel camptothecin analog DX-8951, DE-310, which is composed of DX-8951 covalently linked to a macromolecular carrier by a peptidyl (GGFG) spacer, was designed to exploit the Enhanced Permeability and Retention (EPR) effect. To compare the pharmacokinetics of a single dose of DE-310 with that of DX-8951f in Meth A tumor-bearing mice, the concentrations of conjugated DX-8951 (carrier-bound DX-8951), released DX-8951, and glycyl DX-8951 (G-DX-8951) were determined in plasma, liver, and tumor tissue. The concentrations of conjugated DX-8951 in tumor tissue were lower than those in plasma, with an AUC_{0-inf} of 1/6 that of plasma; however, the AUC_{0-inf} of released DX-8951 in tumor tissue was 30 times greater than that in plasma. The half-life ($t_{1/2}$) of conjugated DX-8951, released DX-8951, and G-DX-8951 in plasma, liver, and tumor tissue were 2–3 days. In contrast, after administration of ^{14}C-DX-8951f, the radioactivity in tumor tissue decreased in parallel with the decrease in plasma concentrations, with a $t_{1/2(0.083-3\,h)}$ of 0.6 h. These data show that higher levels and longer retention of conjugated DX-8951 and released DX-8951 occur in tumor tissue after the administration of DE-310 to Meth A tumor-bearing mice. These results indicate that DE-310 improves the pharmacokinetic profile of DX-8951f, particularly, its ability to target tumor tissue.

1. Introduction

DX-8951f, ([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 dihydrate: exatecan mesylate), is a water-soluble hexacyclic camptothecin derivative, synthesized by Daiichi Pharmaceutical Co., Ltd. DX-8951f demonstrates greater *in vitro* anti-tumor activity against a broader spectrum of tumors than other clinically relevant camptothecin analogues such as SN-38, topotecan, and camptothecin itself (Mitsui et al. 1995). Phase III clinical studies on DX-8951f are in progress in the US and Europe. A major dose-limiting toxic effect has been observed after administration of this compound, myelosuppression, in particular neutropenia and thrombocytopenia (De Jager et al. 2000). Although DX-8951f has shown clinically significant and beneficial anti-tumor effect in patients, because of its toxic effects, a great safety margin is required. The therapeutic index of DX-8951f is compromised by two factors common to most anticancer agents, toxicity and resistance. Thus, to improve its specific delivery to tumors and to decrease its dose-limiting myelotoxicity, a drug-polymer conjugate has been developed. Preparation of such a delivery system is an attractive means of reducing systemic toxicity while specifically delivery more drug to the tumor because this system can exploit the enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986). As shown in Fig. 1, DE-310 is composed of DX-8951 covalently linked to a macromolecular carrier (carboxymethyldextran polyalcohol, CM-Dex-PA) by a peptidyl (GGFG) spacer (Inoue et al. 2003). It has an approximate molecular weight of 300 kDa. Compounds with high molecular weight are thought to restrict uptake of the conjugate specifically into tumor during the process of endocytosis (Duncan et al. 1981), and consequently these conjugates display a lower volume of distribution and longer plasma half-life than their corresponding free drugs. DE-310 releases DX-8951 and glycyl-DX-8951 (G-DX-8951) in both tumor and normal tissue. In

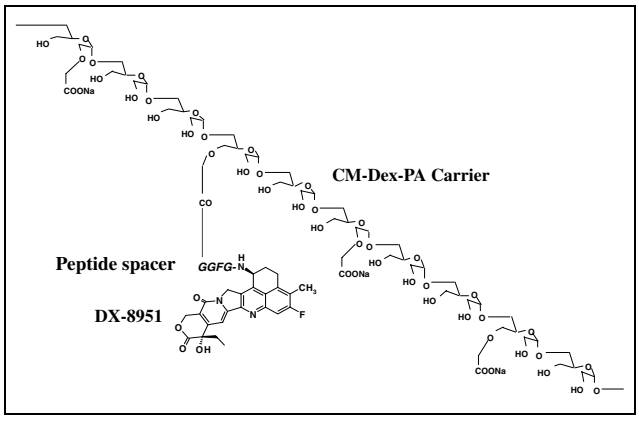

Fig. 1: Partial chemical structure of DE-310

this study, the pharmacokinetic properties of DE-310 were evaluated in Meth A tumor-bearing mice and were compared with those of DX-8951f and G-DX-8951.

2. Investigations and results

2.1. Pharmacokinetics of DE-310

DE-310 was intravenously administered at a dose of 5.7 mg equivalent to DX-8951/kg to Meth A tumor-bearing mice (n = 5). At 4 and 8 h, 1, 2, 3, 7, 10, 14, and 21 days after administration, blood, tumor, and liver were collected and concentrations of conjugated DX-8951, released DX-8951, and G-DX-8951 were determined. The conjugated DX-8951 concentration in tumor and liver tissue reached its maximum concentration (C_{max}) at 24 h and 4 h after administration, respectively. Thereafter, plasma and tissue concentrations declined with a $t_{1/2}$ of 2–3 days. Table 1 shows the ratio of the conjugated DX-8951 AUC_{0-inf} for the 3 tissues examined. The rank order of these ratios to tumor tissue was plasma (6.32) > tumor (1.00) > liver (0.34). Similarly, DX-8951 and G-DX-8951 concentrations in tumor tissue reached the C_{max} at 24 h after administration; however, those in liver reached the C_{max} at 8 h after administration. As shown in Table 1, the rank order of DX-8951 AUC_{0-inf} ratios to tumor tissue was tumor (1.00) > liver (0.34) > plasma (0.03); the rank order of the G-DX-8951 AUC_{0-inf} was liver (1.06) > tumor (1.0) > plasma (0.06). These results indicate that polymer-conjugated DX-8951 is preferentially taken up by tumor tissue rather than liver.

2.2. Pharmacokinetics of ^{14}C-DX-8951f

^{14}C-DX-8951 (Fig. 2) was intravenously administered at a dose of 6 mg/kg to Meth A tumor-bearing mice (n = 4). At 5, 30 min, 1, 3, 8, and 24 h after administration, blood samples, and the tumor, and liver were collected from each mouse, and the amount of radioactivity in each tissue was determined. As Fig. 3 shows, after administration of ^{14}C-DX-8951, the amount of radioactivity in the plasma, tumor, and liver decreased rapidly, with a $t_{1/2(0.083-3\,h)}$ of 0.6 h. The apparent terminal elimination half-life, $t_{1/2}$ (λz), of radioactivity was 7.8–17 h in plasma and tissues (data not shown). Radioactivity concentrations in tumor tissue reached its maximum at 30 min after administration, and decreased rapidly thereafter. The C_{max} and AUC_{0-inf} of radioactivity in the plasma was 1.8 and 0.84-fold of those in tumor, respectively (Table 2). The C_{max} and AUC_{0-inf} in liver were 12 and 7.3-fold of those in tumor, respectively. The rank order of radioactivity AUC_{0-inf} ratios to tumor tissue was liver (7.3) > tumor (1.0) > plasma (0.84).

Table 1: AUC_{0-inf} ratios (tissue/tumor) of conjugated DX-8951, released DX-8951, and G-DX-8951 in plasma, liver, and tumor after a single intravenous administration of DE-310 to Meth A tumor-bearing mice (equivalent to 5.7 mg DX-8951/kg)

Tissue	Conjugated DX-8951	DX-8951	G-DX-8951
Plasma	6.32	0.03	0.06
Tumor	1.00	1.00	1.00
Liver	0.34	0.34	1.06

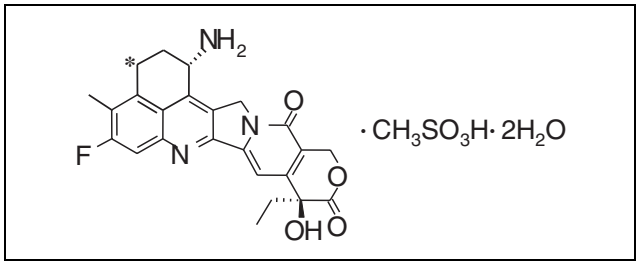

Fig. 2: Chemical structure and labeled position of ^{14}C-DX-8951f
*: ^{14}C-labeled position

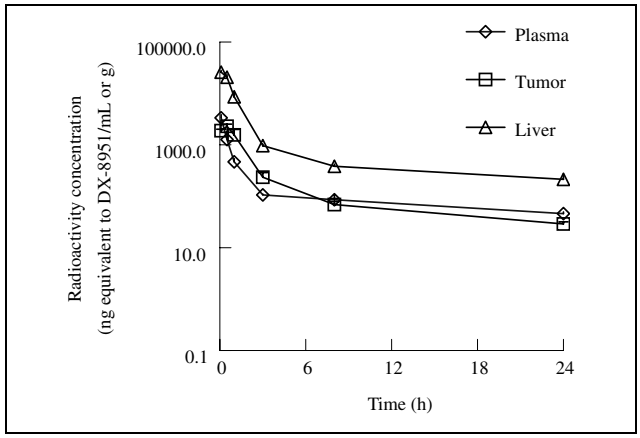

Fig. 3: Radioactivity concentration in plasma, tumor, and liver after a single intravenous administration of ^{14}C-DX-8951f at a dose of equivalent to 6 mg DX-8951/kg to Meth A tumor-bearing mice. Each point with a bar represents the mean + S.D. for four mice

Table 2: Pharmacokinetic parameters of radioactivity after a single intravenous administration of ^{14}C-DX-8951 to Meth A tumor-bearing mice (equivalent to 6 mg DX-8951/kg)

Tissue	C_{max}		AUC_{0-inf}	
	(ng/mL or g)	Ratio to tumor value	(ng · h/mL or g)	Ratio to tumor value
Plasma	4070	1.8	4960	0.84
Tumor	2300	1.0	5870	1.0
Liver	27000	12	42900	7.3

2.3. Pharmacokinetics of G-DX-8951

G-DX-8951 was intravenously administered at 6 mg/kg to Meth A tumor-bearing mice (n = 4). At 5, 30 min, 1, 3, 6, 10, and 24 h after administration, blood samples, tumor, and liver were collected from each mouse and concentrations of G-DX-8951 and released DX-8951 from G-DX-8951 were determined. Fig. 4 displays mean concentration versus time profiles of G-DX-8951 and DX-8951. C_{max} and AUC_{0-inf} calculated from mean concentration are shown in Table 3. At 5 min after administration, G-DX-8951 concentration reached its maximum in plasma, tumor, and liver. G-DX-8951 concentration declined in a bi-exponential manner showing a rapid distributive phase (–1 h) and a relatively slower elimination phase in all samples collected. The apparent terminal elimination half-life, $t_{1/2}$ (λz), of G-DX-8951 was 3.7–11 h in tissues (data not shown). In tumor tissue, G-DX-8951 concentration declined slowly in comparison to those in plasma and liver. The rank order of G-DX-8951 AUC_{0-inf} ratios to tumor tissue was liver (6.6) > tumor (1.0) > plasma (0.48) (Table 3). Similarly, DX-8951 concentrations declined more slowly in tumor tissue than in

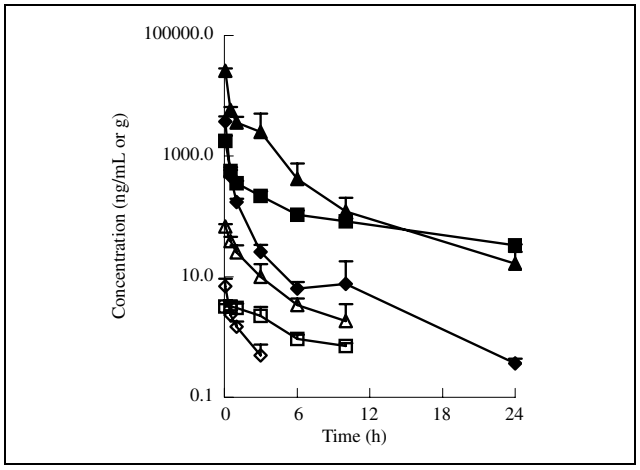

Fig. 4: Concentrations of G-DX-8951 and DX-8951 in plasma, liver, and tumor tissue after a single intravenous administration of G-DX-8951 at a dose of 6 mg/kg to Meth A tumor-bearing mice. Each point with a bar represents the mean + S.D. for four mice. —◆— G-DX-8951 (Plasma), —◇— DX-8951 (Plasma), —■— G-DX-8951 (Tumor), —□— DX-8951 (Tumor), —▲— G-DX-8951 (Liver), —△— DX-8951 (Liver)

Table 3: Pharmacokinetic parameters of G-DX-8951 and DX-8951 after a single intravenous administration of G-DX-8951 to Meth A tumor-bearing mice (6 mg/kg)

Analyte	Tissue	C_{max}		AUC_{0-inf}	
		(ng/mL or g)	Ratio to tumor value	(ng · h/mL or g)	Ratio to tumor value
G-DX-8951	Plasma	3740	2.1	1760	0.48
	Tumor	1780	1.0	3640	1.0
	Liver	26000	15	24200	6.6
DX-8951	Plasma	7.00	2.2	6.32	0.31
	Tumor	3.19	1.0	20.7	1.0
	Liver	68.3	21	116	5.6

plasma or liver. The rank order of DX-8951 AUC_{0-inf} ratios to tumor tissue was liver (5.6) > tumor (1.0) > plasma (0.31) (Table 3).

3. Discussion

Polymeric therapeutics afford the opportunity to inactivate the bound cytotoxic drug during transport to the tumor and thus reduce non-specific toxicity, restrict cellular uptake to the endocytic route and therefore improve tumor targeting by the EPR effect. DE-310 has verified this concept in animals (Fig. 5). CM-Dex-PA carrier was designed as high molecular weight, which is critical in determining the clearance of the polymer itself from the systemic circulation. The data in this study showed that high levels and prolonged retention of conjugated DX-8951 and released DX-8951 were observed in tumor tissue after the administration of DE-310 to Meth A tumor-bearing mice. These results indicate that DE-310 improved the pharmacokinetic profile of DX-8951f particularly regarding its ability of targeting to tumor tissue and resulted in a superior anti-tumor efficacy with a high therapeutic index after administration of DE-310 (Kumazawa et al. 2001). The concept of polymeric anticancer agents has been established clinically in the form of the *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates containing doxorubicin (PK1) (Vasey et al. 1999). This

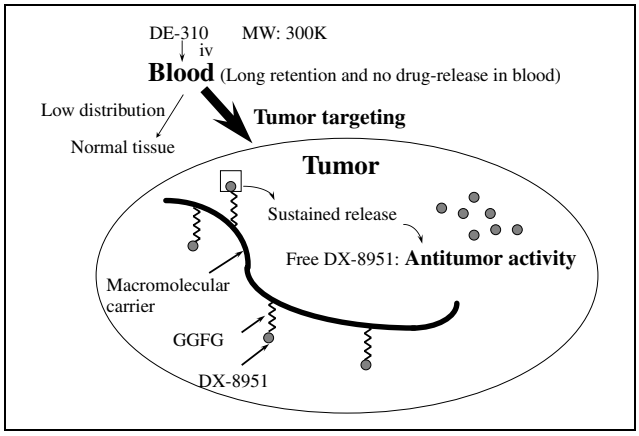

Fig. 5: Tumor targeting of DE-310 by EPR effect

conjugate displays tumor selective disposition in animal tumors (Seymour et al. 1994), and at the cellular level it is internalized via the endocytic route. The conjugate is inactive, hence non-toxic, and attachment of doxorubicin to the HPMA copolymer backbone via a peptidyl spacer mediates release intratumorally by the lysosomal thiol-dependent protease. DE-310 anti-tumor activity is significantly higher than that seen for DX-8951 in many animal tumor models, particularly in solid tumors where the EPR effect is operative (Kumazawa et al. 2001). The anti-tumor activity of G-DX-8951 is moderate in comparison to that of DX-8951f, however, G-DX-8951 is one of the major metabolite released from DE-310. Therefore the pharmacokinetic profile of G-DX-8951 should be investigated as well as DX-8951f. G-DX-8951 concentrations in tumor and liver showed the prolonged half-life rather than that observed in case of DX-8951f. Thus the difference of elimination manner from tissue was observed between these two released compounds. The results of toxicologic studies on mice and dogs suggested that DE-310 has no critical toxicity other than that of DX-8951f alone (Inoue et al. 2003). Phase I clinical study of DE-310 is now on going in US and Europe. The decreased myelotoxicity due to DX-8951 as seen in animals is also expected clinically in Phase I trials.

4. Experimental

4.1. Chemicals

DE-310, DX-8951 mesylate (DX-8951f), and G-DX-8951 were synthesized by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). ^{14}C-DX-8951f was synthesized by Daiichi Pure Chemicals Co., Ltd (Ibaraki, Japan). The specific radioactivity was 3.14 MBq/mg, and the radiochemical purity was 98.5% throughout the study. The chemical structure and labeled position are shown in Fig. 2. DW-8579 ($C_{23}H_{20}N_3FO_4$) and D91-7117 ($C_{29}H_{33}N_{34}$) were also synthesized by Daiichi Pharmaceutical Co., Ltd.; these compounds were used as internal standards (IS) for the determination of DX-8951 and G-DX-8951 concentrations during DE-310 and G-DX-8951 pharmacokinetic studies, respectively. All other chemicals were of analytical-reagent grade.

4.2. Animals

Murine Meth A fibrosarcoma cells (1×10^6 cells/0.1 mL/mouse) were subcutaneously transplanted into 5 week-old BALB/c mice. Tumor size was measured on the day of administration (actual measured tumor size range: 20.9–23.9 mm).

4.3. Pharmacokinetic study on DE-310 in Meth A tumor-bearing mice

DE-310 was intravenously administered at a dose of 5.7 mg equivalent to DX-8951/kg to Meth A tumor-bearing mice (n = 5). At 4 and 8 h, and at 1, 2, 3, 7, 10, 14, and 21 days after administration, blood was collected from the abdominal inferior vena cava under ether anesthesia.

4.4. Pharmacokinetic study on ^{14}C-DX-8951 in Meth A tumor-bearing mice

^{14}C-DX-8951 and non-labeled DX-8951f were dissolved in 5% glucose solution. The pH of the solution was adjusted to 3–4 with 0.1 N hydrochloric acid. This solution was further diluted with the 5% glucose solution to prepare a test solution with a concentration equivalent to 1.2 mg DX-8951/mL. The specific radioactivity of the test solution was 2.06 MBq/mL. A 6 mg equivalent to DX-8951/kg dose of ^{14}C-DX-8951 was intravenously administered to Meth A tumor-bearing mice (n = 4). At 5, 30 min, and at 1, 3, 8, and 24 h after administration, blood was collected from the abdominal inferior vena cava under ether anesthesia.

4.5. Pharmacokinetic study on G-DX-8951 in Meth A tumor-bearing mice

G-DX-8951 was intravenously administered at 6 mg/kg to Meth A tumor-bearing mice (n = 4). At 5, 30 min, and at 1, 3, 6, 10, and 24 h after administration, blood was collected from the abdominal inferior vena cava under ether anesthesia._

4.6. Tissue sample isolation procedures

The blood samples were centrifuged (1,700 × g for 10 min at 4 °C) to collect the plasma. After blood collection, the liver and the tumor from each mouse were collected. To each mouse liver and tumor, purified water (1 mL water per 0.35 g tissue) was added and the tissues were homogenized. Each homogenate was stored at −20 °C until extraction.

4.7. Measurement of conjugated DX-8951, DX-8951, and G-DX-8951 concentrations

The measurement method below was used in the pharmacokinetic study on DE-310 in mice. The analytical method to measure conjugated DX-8951 has been validated over a range of 0.11–279 μg/mL or g, and the analytical method for DX-8951 and G-DX-8951 has also been validated over a concentration ranges of 0.286–46.2 ng/mL or g for DX-8951 and 0.297–48.0 ng/mL or g for G-DX-8951. All the methods have sufficient intra-day and inter-day accuracy and precision. To determine the concentration of conjugated DX-8951, 300 μL of CH_3OH/phosphate buffer (80/20, v/v, pH 8.7) was added to a 50 μL aliquot of each plasma or tissue homogenate. After the samples were mixed thoroughly, each sample was centrifuged (22,000 × g for 10 min), 100 μL of supernatant was transferred to a fresh separate tube and 2 mg of thermolysin in 200 μL of a 0.1 M Tris-HCl (pH 8.5)/0.1 M $CaCl_2$ buffer (1/1, v/v) was added to each tube, which were then incubated at 50 °C for 2 h. After incubation, the reaction was stopped by adding 300 μL of a 0.5 N HCl/CH_3CN (1/1, v/v) solution, and 10 μL of each processed sample was separately loaded onto an HPLC with fluorescence detector (Hitachi; Tokyo, Japan). The HPLC conditions were as follows: Column: Inertsil ODS-2 (6.0 mm I.D. × 150 mm, 5 μm; GL Science; Tokyo, Japan); Column temperature: 45 °C; Mobile phase: A, 0.1% (v/v) trifluoroacetic acid (TFA)/CH_3OH/CH_3CN (12/2/1, v/v/v); B, CH_3OH/CH_3CN (2/1, v/v); Flow rate: 1.0 mL/min; Detection: Ex.: 365 nm, Em.: 445 nm; Gradient program: [Time (min)/B (%)]: 0/30 → 10/30 → 12/50 → 15/50 → 15.1/70 → 20/70 → 25/30. To determine the concentrations of DX-8951 and G-DX-8951, 100 μL of H_2O and 50 μL of the internal standard (DW-8579) in 1 mL of 1 N HCl/CH_3OH (1/99, v/v) were added to a 200 μL of each plasma or tissue homogenate. After the samples were mixed thoroughly, they were centrifuged (22,000 × g for 5 min), and all of each supernatant was transferred to fresh separate tubes. The samples were evaporated to dryness under a stream of nitrogen and reconstituted in 300 μL of 18% CH_3CN/50 mM phosphate buffer (pH 3.0, v/v). Each reconstituted sample was centrifuged (22,000 × g for 5 min) again. One hundred microliters of each supernatant was separately loaded onto the HPLC. The HPLC conditions were as follows: Column: Inertsil ODS-2 (6.0 mm I.D. × 150 mm, 5 μm); Column temperature: 45 °C; Mobile phase: A, 0.1% (v/v) trifluoroacetic acid (TFA)/CH_3OH/CH_3CN (12/2/1, v/v/v), B, CH_3OH/CH_3CN (2/1, v/v); Flow rate: 1.0 mL/min; Detection program: Ex.: 365 nm, [Time (min)/Em. (nm)]: 0/650 → 10/445 → 31/600 → 40/600; Gradient program: [Time (min)/B (%)]: 0/10 → 22/10 → 34/70 → 35/10 → 40/10.

4.8. Measurement of radioactivity

A portion of each plasma sample was mixed with about 10 mL of the liquid scintillator Aquasol-2 (Packard BioScience Co.; Meriden, CT) to measure the amount of radioactivity. A small portion (10–90 mg) of each liver was collected and separately dissolved in 1.5 mL of the tissue solubilizer Soluene-350 (Packard BioScience Co.) by heating at 55 °C for 12 h. After dissolution, approximately 10 mL of the liquid scintillator Hionic-Fluor (Packard BioScience Co.) was added to each dissolved homogenate to measure the amount of radioactivity.

4.9. Measurement of G-DX-8951 and DX-8951 concentration

The measurement method below was used in the pharmacokinetic study of G-DX-8951 in mice. The analytical method for DX-8951 and G-DX-8951 has been validated over concentration ranges of 0.150–500 ng/mL in plasma, and 0.577–1920 ng/g in liver and tumor. This method has sufficient intra-day and inter-day accuracy and precision. Two hundred microliters of each plasma sample and 500 μL of each liver and tumor homogenate were used. An aliquot of 0.1% (v/v) TFA/CH_3OH (60/40, v/v) and 10 ng/mL of the IS (D91-7117) was added to each sample. An 80 μL aliquot of 1 mol/L HCl was then added, mixed thoroughly, and each sample was allowed to stand for about 3 min. A 2 mL aliquot of 10 mmol/L phosphate buffer (pH 7.4) was then added, and the samples were mixed and centrifuged (1,700 × g for 5 min). Each supernatant was then loaded onto solid-phase extraction cartridge (OASIS® HLB cartridges, 60 mg/3 mL; Waters; Milford, MA) in a 25% (v/v) CH_3OH solution, and then eluted with 1 mL of 1 mol/L HCl/CH_3OH (1/99, v/v). The elutes were evaporated to dryness under a stream of nitrogen and reconstituted in 0.3 mL of 0.1% (v/v) TFA/CH_3OH (60/40, v/v). Each sample was transferred to a separate Ultrafree®-MC (30,000 NMWL Filter Unit; Millipore; Bedford, MA) and centrifuged (15,000 × g for 20 min). Each filtrate was then loaded onto an LC/MS/MS system API 3000™ (PE SCIEX and Agilent 1100 series (Agilent; Waldronn, Germany). The LC/MS/MS conditions were as follows: Ionization: ESI (Electrospray Ionization); Monitor mode: MS/MS selected reaction monitoring (SRM); Interface: Turbo IonSpray®; Monitor ion: G-DX-8951 Precursor ion = 493, Product ion = 419; DX-8951 Precursor ion = 436, Product ion = 375; IS Precursor ion = 488, Product ion = 401; Ion spray voltage (IS): 5000 V; Turbo gas temperature: 425 °C. HPLC conditions were as follows: Column: SYMMETRY® C18 (4.6 mm I.D. × 150 mm, 5 μm, Waters); Column temperature: 40 °C; Mobile phase: A, 0.1% (v/v) trifluoroacetic acid (TFA); B, CH_3OH; Flow rate: 0.8 mL/min. Gradient program: [Time (min)/B (%)]: 0/40 → 4.5/55 → 5.5/70 → 5.6 /40 → 10/40.

4.10. Data analysis

A non-compartmental analysis method was used to analyze the pharmacokinetic results with the WinNonlin ver 3.1. (Pharsight Co.; Cary, NC). The apparent terminal elimination half-life, $t_{1/2}$ (λz), was calculated from linear regression of the logarithm of the conjugated DX-8951, DX-8951, or G-DX-8951 concentration-time curve over the terminal linear disposition phase. The area under the concentration *versus* time curve ($AUC_{0\rightarrow inf}$) for plasma, liver, and tumor concentrations were calculated using the linear trapezoidal rule.

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