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Figure 2: Antitumour effects of DX-8951f and CPT-11 administered intravenously every 4th day for a total of four injections against human tumour xenografts in nude mice.

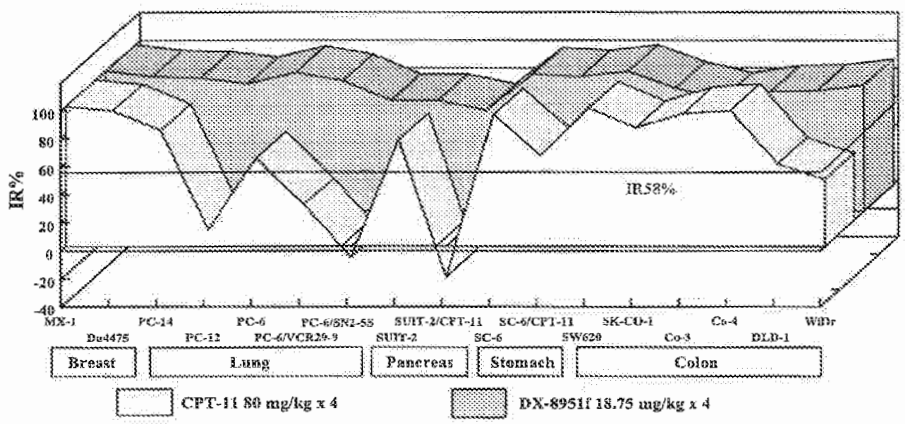


Table 1: Antitumour effects (IR%) of DX-8951f (18.75 mg/kg x 4) and CPT-11 (80 mg/kg x 4).

Compound	MX-1	Du4475	PC-14	PC-12	PC-6	PC-6/ VCR29-9	PC-6/ SN2-5S	SUIT-2	SUIT-2/ CPT-11
DX-8951f	100	96	95	91	99	93	79	79	72
CPT-11	100	97	83	12	63	30	-8	76	22

	SC-6	SC-6/ CPT-11	SW620	SK-CO-1	Co-3	Co-4	DLD-1	WiDr
DX-8951f	98	96	100	88	80	85	86	90
CPT-11	94	65	99	85	95	97	59	48

IR%: Percentage inhibition rate.
Dose levels of compounds were expressed as those of the anhydrous free base.

7. Antitumour effects on human solid tumours xenografted into nude mice

The antitumour activity of DX-8951f against xenografts of several different types of human tumours has been determined in nude mice. A total of four injections, scheduled every fourth day were compared with CPT-11 (administered with the same treatment regimen) [22,48]. CPT-11, like DX-8951f, was reported to show greater antitumour activity and improved safety with intermittent administration rather than with single dose administration [7]. Mice with an estimated tumour weight of 100 - 200 mg were treated with each compound. **Figure 2**, **Table 1** summarises the results of a series of experiments using a total of 17 human cancer lines (two breast cancers, five lung cancers, two pancreatic cancers, two gastric cancers and six colonic cancers). The chart shows the antitumour effects of DX-8951f and CPT-11 at the maximum tolerated doses of 75 mg/kg and 320

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mg/kg, respectively. The Y-axis shows the tumour-growth inhibition rates (IR). IR values equal to or greater than 58% are required for drug efficacy [49]. DX-8951f was effective (IR ≥ 58%) on all tumours examined, with excellent antitumour activity (IR ≥ 80%) demonstrated in 14 lines. CPT-11 exhibited antitumour activity with IR values over 58% against 12 lines, with IR values over 80% against only 8 of the same 17 human tumour lines. Furthermore, DX-8951f exhibited potent antitumour effects against an SN-38 resistant tumour (PC-6/SN2-5S), two CPT-11-resistant tumours (SUIT-2/CPT-11 and SC-6/CPT-11) and some P-glycoprotein-expressing tumours (PC-12, PC-6/VCR29-9 and DLD-1). In contrast, CPT-11 showed no activity [22,48]. These findings clearly indicate that DX-8951f has a greater spectrum of activity than CPT-11 against a variety of human tumour types. DX-8951f has also been found to be more active than SK&F104864 against SC-6 and SC-6/CPT-11, further suggesting the potential of DX-8951f in cancer treatment [48].

8. Antitumour effects in some metastasis models

The antitumour activity of DX-8951f has also been studied in a lung metastasis model of a murine lung carcinoma, 3LL, two liver metastasis models of a murine histiocytoma, M5076, and a human pancreas tumour, SUIT-2. 3LL and M5076 cells produce metastatic foci in the lung and liver, when transplanted intravenously into syngeneic mice. The mice eventually die from dyspnoea and liver dysfunction, respectively. In these models, DX-8951f has been shown to induce a significant prolongation in the survival period, even with single dose administration: increases in lifespan values against 3LL and M5076 ranged from 20 - 40% and 50 - 260%, respectively, at doses of 20 - 60 mg/kg [unpublished results]. DX-8951f was also tested in another liver metastasis model. SUIT-2 cells, following intrasplenic implantation, extensively grew in the spleen and liver of nude mice and the mice died due to ascite accumulation and liver dysfunction. DX-8951f was found to dose-dependently exhibit potent antitumour effects when injected intravenously 4 times at 5 day intervals for total doses of 10 - 40 mg/kg [22]. In particular, the growth of tumour cells in the liver and spleen and the degree of ascites were markedly inhibited in mice treated with the highest dose, with no visible tumour nodules or ascites observed in 3 /7 mice [22]. CPT-11 (administered at total doses of 60 - 240 mg/kg) dose-dependently inhibited tumour growth and ascites accumulation; antitumour activity was similar to, or reduced compared to DX-8951f [22].

9. Toxicological profiles in preclinical studies

The results of animal toxicological studies revealed that DX-8951f administration causes a decrease in leukocytes and platelets in mice, rats and dogs. Dose-limiting in clinical trials is predicted to be due to haematological toxicity. With regards to gastrointestinal toxicity, vomiting was observed in dogs, but was controllable with anti-emetics. Severe watery diarrhoea, which is frequently induced in rats and dogs following administration of CPT-11, has never been observed with DX-8951f [unpublished results].

10. Conclusions

DX-8951f, a water-soluble camptothecin analogue with a unique hexacyclic structure, is the most active inhibitor of topo I and tumour growth *in vitro* and *in vivo* of all the camptothecin derivatives. DX-8951f is extremely effective against several types of drug resistant tumours, where CPT-11 or SN-38 are ineffective. In addition, in some metastasis models, its antitumour effects and resulting life prolongation have been confirmed. DX-8951f is more efficacious and possesses broader effective dose ranges with multiple injections than with a bolus injection and intermittent administration. Based on these encouraging preclinical results, DX-8951f is expected to be clinically effective. The results of Phase I clinical trials of this compound, currently in progress in Europe, the USA and Japan, are awaited.

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DE-310, a novel macromolecular carrier system for the camptothecin analog DX-8951f: Potent antitumor activities in various murine tumor models

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DE-310 is a novel macromolecular conjugate composed of DX-8951f, a camptothecin analog, and a carboxymethyldextran polyalcohol carrier, which are covalently linked via a peptidyl spacer. In a murine Meth A (fibrosarcoma) solid tumor model, once daily×5 treatments (qd×5) with DX-8951f at the maximum tolerated dose (MTD) were required to shrink the tumor, and DX-8951f (qd×5) at 1/4 MTD was required to inhibit tumor growth. A single treatment (qd×1) with DE-310 at the MTD or 1/4 MTD shrank the tumor, with no body weight loss occurring at 1/4 MTD. Even at 1/16 MTD, DE-310 inhibited tumor growth. In a long-term assay, Meth A solid tumors disappeared in mice treated with DE-310 (qd×1) at the MTD and 1/2 MTD, and all 6 mice remained tumor-free on the 60th day after administration. Repeated injection (4 times) on schedules of every 3 days, 7 days or 14 days demonstrated that multiple treatment with DE-310 produced greater tumor growth delay than a single treatment with DE-310. Against 5 human tumor (colon and lung cancer) xenografts in mice, DE-310 (qd×1) was as effective as DX-8951f administered once every 4 days, 4 times. The life-prolonging activity of DE-310 was assessed in lung (3LL, Lewis lung carcinoma) and liver (M5076, histiocytoma) metastasis models. Against 3LL, DE-310 (qd×1) at the MTD to 1/3 MTD significantly prolonged survival, with an increase in life span (ILS) of 4.8- to 1.6-fold, respectively, over that in untreated control mice. Also, DE-310 (qd×1) significantly prolonged survival in the liver metastasis model of M5076. These results demonstrate that DE-310 is a promising agent for the treatment of cancer. (Cancer Sci 2004; 95: 168–175)

The effect of current therapeutic agents appears when the agent acts on the target organ in the body. Since drug molecules that are distributed to organs that are not targeted may cause side effects, treatment is generally performed using a dose at which efficacy is obtained without unacceptable side effects. However, most antitumor chemotherapeutic agents show clinical effects only at doses that cause side effects such as digestive toxicity and myelosuppression. Therefore, in the development of new antitumor chemotherapeutic agents, it is important to discover compounds having a stronger antitumor effect and weaker toxicity compared with current antitumor chemotherapeutic agents. For example, CPT-11 was found to exhibit higher antitumor activity with less toxicity than camptothecin (CPT) and showed promising activity in clinical evaluation.[1–8] However, it causes side effects such as severe watery diarrhea and there are marked inter-patient variations in both its effects and toxicity.[7,9,10] To obtain a more effective analogue with a lower toxicity, DX-8951f was designed.[11] DX-8951f is the most active inhibitor of topo I and tumor growth both *in vitro* and *in vivo* among the currently available CPT derivatives.[12–14] However, even with DX-8951f, there is no apparent increase in the selectivity to tumor tissue versus normal tissue and the half-life of the compound is relatively short ($t_{1/2}$ of 0.3 h mice).[15]

The ideal antitumor drug would have high antitumor activity but no toxicity, i.e., it would be a compound for which efficacy and side effects are completely separate, but it is difficult to achieve this with low-molecular compounds, as the history of antitumor chemotherapeutics shows. Various approaches for superior targeting of antitumor compounds have been investigated by many researchers, and there have been trials of macromolecular pro-drugs.[16,17] Based on the results obtained from examinations of macromolecular conjugates using doxorubicin in DDS Institute, Ltd., we started to study drug delivery using a macromolecular carrier based on a DX-8951-conjugate, in order to enhance the antitumor effect and to reduce the hematological toxicity of DX-8951f.

A novel macromolecular carrier system, based on the well-known enhanced permeability and retention (EPR) effect,[18] was designed to target DX-8951 to tumors and to provide sustained release of DX-8951 in tumors. We conducted extensive optimization studies of the peptidyl spacer, molecular weight of the carrier, DX-8951 content and degree of carboxymenthylation.[19] DE-310 is composed of DX-8951 and a biodegradable carrier, carboxymethyldextran polyalcohol (CM-Dex-PA), which are covalently linked by a peptidyl spacer (Gly-Gly-Phe-Gly, GGFG).[19] It has an average molecular weight of 340,000 (DX-8951 content: less than 8%, degree of carboxymethylation: ca. 0.3–0.4 per sugar residue).[19]

In the present study, we examined the therapeutic efficacy of DE-310 against a variety of human and murine tumors in mice.

Materials and Methods

Compounds. DE-310 (CM-Dex-PA-GGFG-DX-8951, Fig. 1A), a CM-Dex-PA adduct of the antineoplastic agent, DX-8951 [(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]. The carboxymethylated polyalcohol is covalently bonded to the DX-8951 moiety via a tetrapeptidyl spacer (Gly-Gly-Phe-Gly, GGFG). DX-8951f (Fig. 1B) 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) dihydrate. DE-310 and DX-8951f was synthesized in our laboratory. DE-310 and DX-8951f were dissolved in pyrogen-free distilled water and diluted using the same water. Dose levels of DE-310 and DX-8951f are expressed as those of DX-8951. Cisplatin (CDDP, Nippon Kayaku Co., Ltd., Tokyo) was diluted with pyrogen-free physiological saline. Cyclophosphamide (CPA, Shionogi Co., Ltd., Osaka) was dissolved in pyrogen-free distilled water and diluted using the same water.

Animals. Male, 6-week-old BALB/c mice, BALB/c-*nu/nu* nude mice, C57BL/6 mice were purchased from Japan SLC,

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Inc. (Shizuoka). They were housed in an exclusive experimental room under specific pathogen-free conditions, and given food sterilized by irradiation with γ-rays, in pelleted form (Oriental Yeast Co., Tokyo) or non-sterilized food, in pelleted form (Funabashi Farms, Chiba). Water treated with hypochloride or filtered water was available *ad libitum*.

Tumors. HCT116 was obtained from the American Type Culture Collection, Rockville, MD. PC-6 was obtained from Immuno-Biological Laboratories, Gunma. PC-12 was supplied by Toray Co., Ltd., Tokyo. HCT116/SN2-3 and PC-6/SN2-5S are drug-resistant cell lines established in our laboratory and isolated from HCT116 and PC-6, respectively, by stepwise selection with increasing concentrations of SN-38 (Yakult Honsha Co., Ltd., Tokyo). SN-38 is an active metabolite of CPT-11, a clinically available CPT analog. Differences in the cytotoxic effects of SN-38 against parental and resistant cell lines are indicated as "degree of resistance" and were calculated as follows: Degree of resistance=(GI_{50} for resistant cell line)/(GI_{50} for parental cell line). The degrees of resistance of HCT116/SN2-3 and PC-6/SN2-5S are 129 and 243, respectively. PC-12 is a cell line overexpressing P-glycoprotein.[13] HCT116, PC-6, HCT116/SN2-3 and PC-6/SN2-5S were cultured *in vitro* in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT or Bocknek, Canada), and then inoculated s.c. into nude mice and maintained as solid tumors by monthly or bi-monthly subcutaneous transplantation. Meth A was obtained from Hokkaido University, Sapporo. M5076[20] and 3LL[21,22] were obtained from the Japanese Foundation for Cancer Research, Tokyo. Meth A and M5076 were maintained by serial intraperitoneal passage in syngeneic mice. Ascitic fluid was collected 7 or 14 days after implantation, and tumor cells were washed twice with Hanks' balanced salt solution (HBSS). The cell suspension was inoculated into the peritoneal cavity of syngeneic mice. 3LL was serially maintained as a solid tumor by subcutaneous passage every 2 weeks in syngeneic mice.

Experimental designs. 1) Antitumor activity against murine Meth A. Meth A cells (1×10^6 cells/0.1 ml) were inoculated s.c. into the right flank of syngeneic BALB/c mice and compounds were injected using various schedules from 7 or 11 days after tumor inoculation. For the study of tumor growth-inhibitory effects, the antitumor activity was evaluated in terms of the tumor weight. Tumor masses were excised and weighed on the 21st day after tumor inoculation. Differences in tumor weight were statistically analyzed by using Dunnett's test. For tumor growth delay effects, growth delay, expressed as $T-C$, was defined as the difference in days between the median times (MT) required for the tumors of treated (T) and control (C) animals to reach an established tumor weight (ETW) approximately 3 or 5 times greater than that measured at the time of the original treatment.[23] The tumor growth delay was statistically analyzed by use of the Williams-Wilcoxon test. 2) Tumor growth-inhibitory effects against human cancer xenografts. Various human tumors maintained in nude mice were excised and cut into pieces approximately 2 to 3 mm in diameter in HBSS. A piece of tumor was transplanted s.c. into the right flank of nude mice using a trocar. When the mean ETW reached 100 to 170 mg between days 13 and 35 after tumor transplantation, the mice were randomly divided into experimental groups (5 or 6 mice per group) and were treated i.v. with DE-310 as a single dose (qd×1) or DX-8951f every 4th day for a total of 4 doses (q4d×4). After the first administration on day 0, the ETW and body weight of the mice were measured 2 to 5 times per week for 21 or 28 days. The tumor masses were then excised and weighed. The tumor weight was statistically analyzed by using Dunnett's test. 3) Life-prolonging effects in a liver metastasis model of M5076. M5076 cells (1×10^5 cells/0.2 ml) were inoculated i.v. via the tail vein of syngeneic C57BL/6 mice (day 0). Following an intravenous inoculation, the M5076 cells disseminated widely in the whole body and formed tumor colonies in various host organs. These tumor cells have been shown to be retained initially for 3 to 4 days in the lungs, then they detach, re-circulate, and grow mainly in the liver. Occasionally tumors form in other organs such as the spleen, ovary, bone marrow and brain.[20] Tumor colonies established in the liver grow rapidly and host mice usually die of hepatic dysfunction within 2 to 3 weeks after tumor cell inoculation. In the present experiment, mice were i.v. administered either a single dose of DE-310 or an intermittent dose (every 14th day for a total of 4 doses, q14d×4) of DE-310 beginning on day 8. A single dose of CDDP was i.v. administered on day 8. It was confirmed by preliminary histopathological examinations that tumor colonies were already established in the liver and other organs on day 8 after inoculation of M5076 cells (1×10^5 cells/0.2 ml). Survival time was monitored daily until termination of the study on day 130. Dead mice were immediately necropsied, and surviving mice were sacrificed and autopsied on day 130. Survival time was statistically analyzed by use of the Williams-Wilcoxon test. 4) Life-prolonging effects in a lung metastasis model of 3LL. For the preparation of single cell suspensions from 3LL solid tumors, the tumors were excised, and rinsed several times in HBSS. The necrotic portion was removed, and the tumors were cut into pieces and dispersed using 0.14% collagenase type I (Type I, Clostridiopeptidase A; EC3.4.24.3 from *Clostridium histolyticum*, Lot 80H0518, Sigma, St. Louis, MO) and 0.03% DNase (deoxyribonuclease I; EC3.1.21.1, Lot 33H9598, Sigma), as previously described in detail.[24,25] 3LL cells (5×10^5 cells/0.2 ml) were inoculated i.v. via the tail vein of syngeneic C57BL/6 mice (day 0). Following intravenous inoculation, the 3LL cells disseminated to the lungs and consistently formed tu-

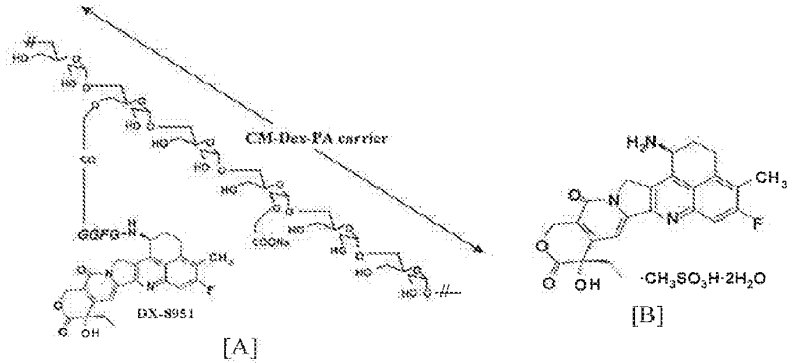


Fig. 1. Chemical structures of DE-310 (partial structure) [A] and DX-8951f [B].

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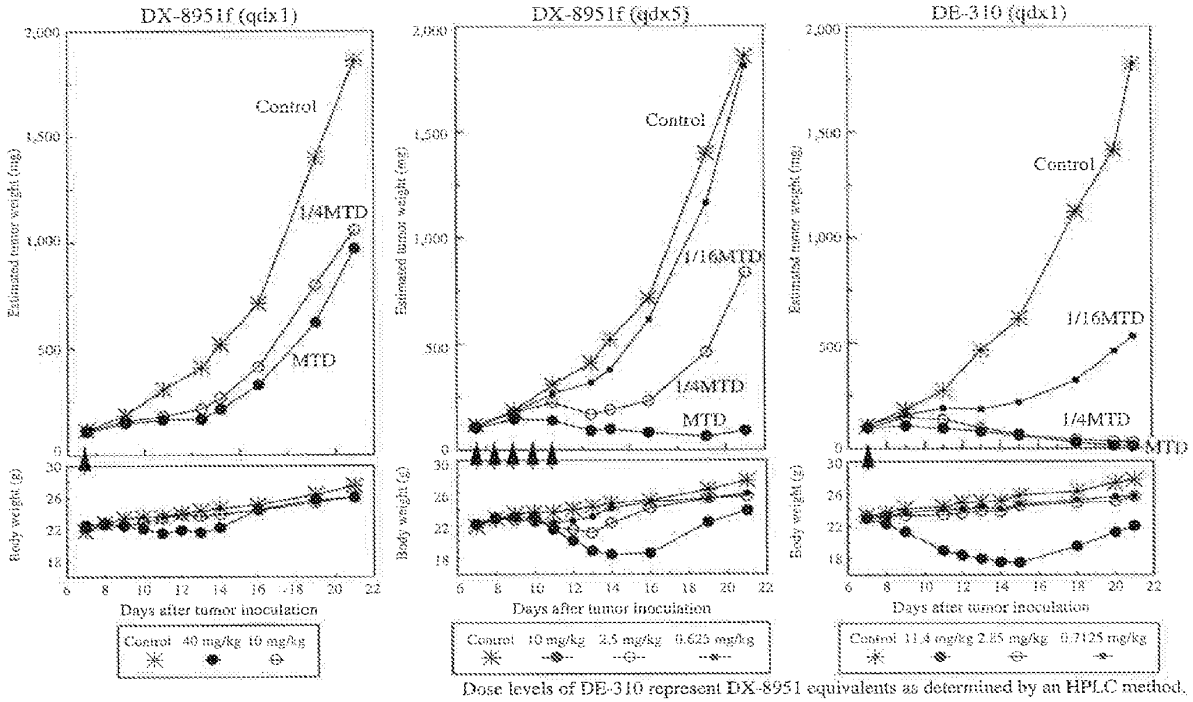


Fig. 2. Tumor growth curves and body weight changes in animals given DE-310 and DX-8951f as a single dose (qd×1) or once daily×5 doses (qd×5). The mice were divided into several groups (6 mice per group) on day 7. Estimated tumor weight and body weight for the MTD, 1/4 MTD and 1/16 MTD of each compound are plotted. Arrows indicate the days of treatment on each schedule. Points represent the mean value of surviving mice.

mor colonies. As a result of rapid tumor growth in the lungs, the host animals died of bleeding in the pleural cavity within 3 weeks after tumor cell inoculation.[21, 22, 24, 25] In the present experiment, mice were i.v. administered either a single dose or intermittent doses (every 7th day for a total of 4 doses, q7d×4) of DE-310, once daily times 5 doses (qd×5) of DX-8951f, or a single dose of CPA beginning on day 8. Survival time was monitored daily until termination of the study on day 123. Dead mice were immediately necropsied, and surviving mice were sacrificed and autopsied on day 123. Survival time was statistically analyzed by use of the Williams-Wilcoxon test.

Evaluations of activity and toxicity. The ETW was calculated using the formula[13]: $ETW=L\times W^2/2$, where L and W represent the length and the width of the tumor mass, respectively. The growth inhibition rate (IR) on the basis of tumor weight was calculated using the formula: $IR=(1-TW_t/TW_c)\times 100$ (%), where TW_t and TW_c represent the mean tumor weight of a treated group and that of the control group, respectively. When the IR was 58% or higher, the drug was considered effective.[26] Survival rates (SR) on day n were calculated from the following formula: SR (%)=(number of mice that survived on day n/number of mice used)×100. Increase in life span (ILS) was calculated from the following formula: ILS (%)=$[(MST_t/MST_c)-1]\times 100$, where MST_t and MST_c represent the median survival time of a treated group in days and that of the control group in days, respectively. When the ILS was above 50%, the drug was considered effective.[27] The rate of body weight loss (BWL) was calculated based on the following formula: BWL (%)=$(1-BW_n/BW_s)\times 100$, where BW_n and BW_s represent the mean body weight of mice on day n and that of mice on the day when administration started, respectively. BWL_{max} indicates the maximum value of BWL. BWL_{max} of less than zero (0) indicates no body weight loss. In the tables, the notations D and U indicate the number of mice that died of toxicity and the number of mice used, respectively.

Animal welfare. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co., Ltd.

Results

Comparison of antitumor effects of DE-310 and DX-8951f. As shown in Fig. 2, DE-310 with qd×1 at the maximum tolerated dose (MTD) and 1/4 MTD shrank the tumor, with no body weight loss occurring at 1/4 MTD. Even at 1/16 MTD, DE-310 inhibited tumor growth. In contrast, DX-8951f with qd×1 at the MTD and 1/4 MTD inhibited tumor growth moderately. In qd×5, DX-8951f at the MTD (10 mg/kg as total dose) and 1/4 MTD shrank the tumor and inhibited tumor growth, respectively.

Tumor growth delay effects of DE-310 with qd×1. Antitumor activity of DE-310 was examined in a longer-term study (about 3 months) because the short-term evaluation (14 days) showed substantial antitumor activity. As shown in Table 1, tumors in mice treated at the MTD and 1/2 MTD disappeared by the 15th day after administration, and all mice treated with those doses remained tumor-free up to day 67. Additionally, the rates of complete disappearance of tumor masses by DE-310 at 1/4 MTD and 1/8 MTD was 67%. DE-310 at 1/16 MTD and lower

doses exhibited dose-dependent tumor growth delay effects.

Tumor growth delay effects of DE-310 with various schedules. DE-310 with qd×1 caused the complete disappearance of tumor masses at several doses, as shown in Table 1. Therefore, in this examination with various schedules, treatment was started from 11 days. The size of tumor masses on day 11 was 1.6 times larger than in the case of Table 1 and Fig. 2. As shown in Fig. 3, DE-310 at 5.7 mg/kg with intermittent schedules exhibited a higher ratio of complete response or produced a greater tumor growth delay than when given with qd×1. BWL in the schedules of q3d×4 and q7d×4, which gave complete response, was very severe (25.1%) or moderate (11.1%), respectively. DE-310 at 2.85 mg/kg also resulted in a significant tumor growth delay in inverse proportion to the length of the administration interval.

Antitumor activity against colon cancer xenografts. As shown in Table 2, against parental HCT116, DE-310 and DX-8951f exhibited strong antitumor activity at the MTD to 1/3 MTD and at the MTD to 1/6 MTD, respectively. Antitumor activity of DE-310 at 1/6 MTD was similar to that of DX-8951f at 1/12 MTD. Against the resistant variant HCT116/SN2-3, DE-310 at 1/3 MTD and DX-8951f at 1/6 MTD produced moderate antitumor activity.

Antitumor activity against lung cancer xenografts. As shown in Table 3, DE-310 at the MTD and 1/6 MTD against parental PC-6 exhibited exceptional antitumor activity without any death from toxicity. Antitumor activity of DE-310 at the MTD against PC-6/SN2-5S was moderate. DX-8951f at the MTD against PC-6 showed strong antitumor activity, but was not effective at 1/6 MTD. Antitumor activity of DX-8951f at the MTD against PC-6/SN2-5S was moderate. Results against PC-

Table 1. Tumor growth delay effect of DE-310 with a single dose (qd×1) against Meth A solid tumors

Dose[1] (mg/kg)	Time to reach estimated tumor weight of 500 mg (days)			BWL_{max}[4] (%) [days]	F/U[5]
	Range	MT[2]	T−C[3]		
0	13–18	15.13	0	<0	0/6
11.4 (MTD)	>67	>67.08**	NA[6]	22.6 [13]	6/6
5.7	>67	>67.08**	NA	9.2 [11]	6/6
2.85	32–>67	>66.88**	NA	1.8 [9]	4/6
1.425	32–>67	>66.88**	NA	0.3 [8]	4/6
0.7125	18–>67	31.75*	16.63	<0	1/6
0.3563	15–20	19.67	4.54	<0	0/6
0.1781	13–18	15.25	0.13	0.2 [8]	0/6

The mice were randomized on day 7 and received an i.v. injection of DE-310. ** $P \leq 0.01$, * $P \leq 0.05$ vs. control (Williams-Wilcoxon test). Mean of estimated tumor weights of each group at the start of treatment were 107–118 mg.

1) Dose levels of DE-310 were expressed as those of DX-8951. The content of DX-8951 present in DE-310 was determined by HPLC.

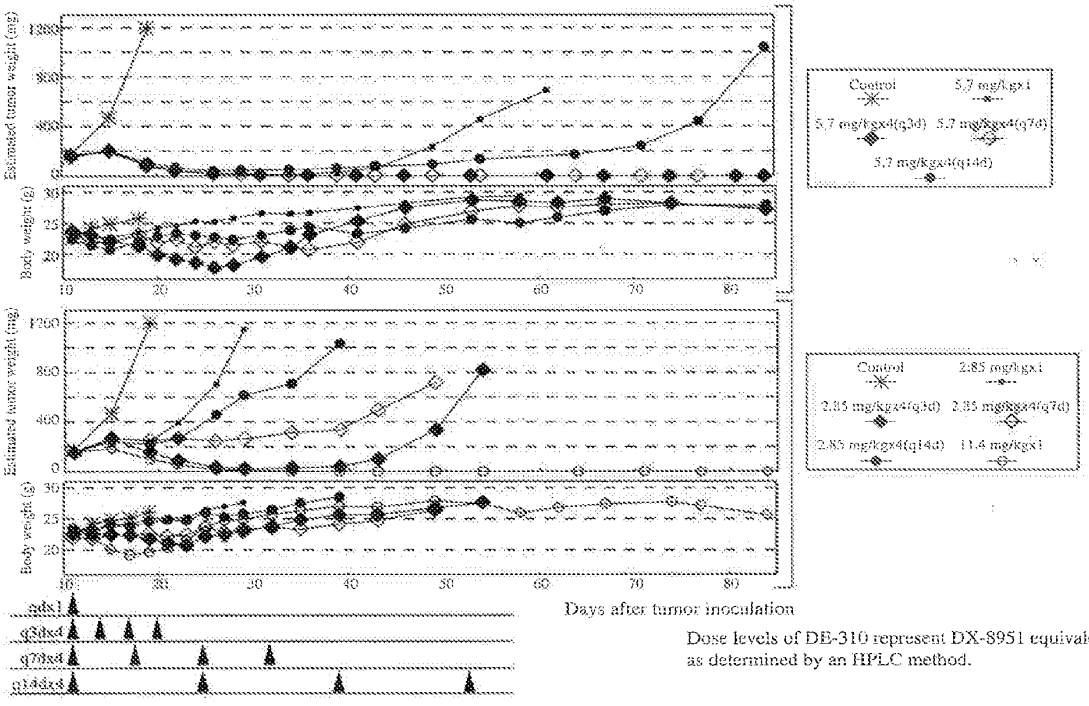
2) MT indicates median time taken for tumors to reach estimated tumor weight of 500 mg.

3) *T−C*, tumor growth delay, is the difference between the median time required for tumors in treated (*T*) and control (*C*) mice to reach an estimated tumor weight of 500 mg as approximately 5 times their untreated estimated tumor weight on day 7.

4) BWL_{max} is the maximum rate of body weight loss, with numbers in parentheses denoting the day. <0 indicates no body weight loss.

5) *F/U* indicates number of tumor-free mice on day 67/number of mice used.

6) NA means "not applicable."



Dose levels of DE-310 represent DX-8951 equivalents as determined by an HPLC method.

Fig. 3. Tumor growth curves and body weight changes in animals given DE-310 as a single dose and several repeated doses. The mice were divided into several groups (6 mice per group) on day 11 and received an i.v. injection of DE-310 with the schedule of a single dose (qd×1), every 3 days 4 times (q3d×4), every 7 days 4 times (q7d×4) or every 14 days 4 times (q14d×4). Arrows indicate the day of treatment. Points represent the mean value for 6 mice.

12 are shown in Table 4. DE-310 produced antitumor activity with IR values of 80% or higher at the MTD and 2/3 MTD. However, DX-8951f showed similar activity only at the MTD.

Antitumor activity in a liver metastasis model of murine histiocytoma. The results are shown in Table 5. Because two mice died following a single dose administration of DE-310 at 22.8 mg/kg, this dose was considered to be the MTD in this tumor model. DE-310 at the MTD to 1/2 MTD exhibited significant life-prolonging effects. The death of one mouse at 1/2 MTD was secondary to a poor nutritional state due to abnormal incisors. DE-310 at 1/4 MTD to 1/32 MTD also exhibited significant life-prolonging effects with ILS values of over 700%. Neither toxic death nor severe BWL was observed in these groups. Furthermore, 5 to 7 mice survived until day 130 and the ratio of surviving mice showing no macroscopic evidence of tumor tissues or nodules was 80 to 100%. Even at 1/64 MTD, the life-prolonging effect of DE-310 was statistically significant compared with the controls. DE-310 given intermittently for a total dose of 11.4 mg/kg exhibited a superior life-prolonging effect and more tumor-free survivors than did a single dose of 11.4 mg/kg. CDDP as positive control at the MTD to 1/3 MTD exhibited significant life-prolonging effects with ILS values of over 700%.

Antitumor activity in a lung metastasis model of murine lung carcinoma. The results are shown in Table 6. Because 2 mice died at 17.1 mg/kg, this dose was considered to be the MTD of DE-310 in this tumor model. DE-310 at the MTD to 1/12 MTD exhibited life-prolonging effects in a dose-dependent manner. DE-

Table 2. Anti-tumor effects of DE-310 and DX-8951f against human colon cancer HCT116 and HCT116/SN2-3 xenografts in nude mice

Compound	Schedule	Total dose[1] (mg/kg)	HCT116				HCT116/SN2-3			
			Tumor weight		BWL_{max}[2] (%) [day]	D/U[3]	Tumor weight		BWL_{max} (%) [day]	D/U
			Mean±SE (g)	IR (%)			Mean±SE (g)	IR (%)		
Control	—	0	2.810±0.408	0	0.3 [19]	0/6	1.944±0.457	0	7.6 [41]	0/6
DE-310	qd×1	11.4	0.012±0.004***	100	24.3 [20]	3/6[5b]	0.445±0.073***	77	30.4 [26]	3/6[5c]
	qd×1	8.55 (MTD)	0.024±0.007***	99	27.0 [23]	1/6[5b]	ND[4]			
	qd×1	5.70	0.051±0.014***	98	17.4 [19]	0/6	0.747±0.077**	62	14.0 [21]	0/6
	qd×1	2.85	0.277±0.040***	90	8.7 [20]	0/6	0.912±0.120*	53	7.1 [41]	0/6
	qd×1	1.425	0.589±0.083***	79	5.1 [17]	0/6	1.438±0.157	26	6.1 [43]	0/6
	qd×1	0.7125	1.028±0.189***	63	9.2 [41]	0/6	1.395±0.182	28	9.1 [41]	0/6
	qd×1	0.3563	1.742±0.184***	38	11.0 [41]	0/6	ND			
DX-8951f	q4d×4	75 (MTD)	0.007±0.003***	100	13.8 [26]	0/6	0.552±0.092***	72	16.6 [33]	0/6
	q4d×4	50	0.010±0.001***	100	10.4 [22]	0/6	0.603±0.086***	69	13.7 [33]	0/6
	q4d×4	25	0.016±0.005***	99	9.5 [25]	0/6	0.700±0.073***	64	10.1 [33]	0/6
	q4d×4	12.5	0.069±0.021***	98	4.7 [22]	0/6	0.872±0.180**	55	10.2 [43]	0/6
	q4d×4	6.25	0.477±0.064***	83	10.7 [22]	0/6	ND			

The mice were divided into several groups when the mean estimated tumor weight reached approximately 140 mg on day 13 (HCT116) or approximately 100 mg on day 15 (HCT116/SN2-3). Tumor weight was assessed on 28 days after the first administration (HCT116: day 41, HCT116/SN2-3: day 43). *** $P<0.001$, ** $P<0.01$, * $P<0.05$ vs. control (Dunnett's test).
1) Dose levels of DE-310 and DX-8951f were expressed as those of DX-8951. The content of DX-8951 present in DE-310 was determined by HPLC.
2) Maximum rate of body weight loss, with numbers in parentheses denoting the day. <0 indicates no body weight loss.
3) Number of mice that died of toxicity/number of mice used.
4) Not done.
5a, 5b, 5c) Death days were as follows: days 19, 19 and 21 (*5a*), days 25 (*5b*), days 20, 21 and 30 (*5c*).

Table 3. Anti-tumor effects of DE-310 and DX-8951f against human lung cancer PC-6 and PC-6/SN2-5S xenografts in nude mice

Compound	Schedule	Total dose[1] (mg/kg)	PC-6				PC-6/SN2-5S			
			Tumor weight		BWL_{max}[2] (%) [day]	D/U[3]	Tumor weight		BWL_{max} (%) [day]	D/U
			Mean±SE (g)	IR (%)			Mean±SE (g)	IR (%)		
Control	—	0	2.016±0.473	0	<0	0/5	0.799±0.146	0	4.8 [41]	0/6
DE-310	qd×1	8.55 (MTD)	0.002±0.001***	100	27.7 [33]	0/5	0.306±0.181	62	25.9 [41]	0/6
	qd×1	5.70	ND[4]				0.425±0.183	47	12.6 [42]	0/6
	qd×1	2.85	ND				0.705±0.176	12	0.9 [39]	0/6
	qd×1	1.425	0.045±0.013***	98	2.5 [30]	0/5	0.796±0.287	0	<0	0/6
	qd×1	0.7125	ND				0.818±0.176	−3	<0	0/6
DX-8951f	q4d×4	75 (MTD)	0.263±0.098***	87	18.5 [45]	0/5	0.381±0.161	52	16.0 [48]	0/6
	q4d×4	50	ND				0.299±0.101	63	11.3 [50]	0/6
	q4d×4	25	ND				0.410±0.141	49	5.6 [48]	0/6
	q4d×4	12.5	1.282±0.145	36	0.8 [29, 34]	0/5	0.559±0.184	30	0.6 [48]	0/6

The mice were divided into several groups when the mean estimated tumor weight reached approximately 150 mg on day 28 (PC-6) or 140–160 mg on day 35 (PC-6/SN2-5S). Tumor weight was assessed on 28 days after the first administration (PC-6: day 56, PC-6/SN2-5S: day 63). *** $P<0.001$, ** $P<0.01$ vs. control (Dunnett's test).
1) Dose levels of DE-310 and DX-8951f were expressed as those of DX-8951. The content of DX-8951 present in DE-310 was determined by HPLC.
2) Maximum rate of body weight loss, with numbers in parentheses denoting the day. <0 indicates no body weight loss.
3) Number of mice that died of toxicity/number of mice used.
4) Not done.

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Table 4. Anti-tumor effects of DE-310 and DX-8951f against human lung cancer PC-12 xenografts in nude mice

Compound	Schedule	Total dose[1] (mg/kg)	Tumor weight		BWL_{max}[2] (%) [day]	*D/U*[3]
			Mean±SE (g)	IR (%)		
Control	----	0	1.928±0.424	0	0.1 [18]	0/6
DE-310	qd×1	8.55 (MTD)	0.299±0.056***	85	24.5 [22]	0/6
	qd×1	5.70	0.378±0.057***	80	10.1 [22]	0/6
	qd×1	3.80	0.725±0.181**	62	4.6 [21]	0/6
	qd×1	2.53	1.179±0.151	39	0.9 [19]	0/6
	qd×1	1.70	1.014±0.156*	47	<0	0/6
DX-8951f	q4d×4	75 (MTD)	0.353±0.050***	82	9.7 [32]	0/6
	q4d×4	50	0.682±0.181**	65	8.4 [31]	0/6

The mice were divided into several groups when the mean estimated tumor weight reached approximately 170 mg on day 17. Tumor weight was assessed on 21 days after the first administration (day 38). *** $P<0.001$, ** $P<0.01$, * $P<0.05$ vs. control (Dunnett's test).
1) Dose levels of DE-310 and DX-8951f were expressed as those of DX-8951. The content of DX-8951 present in DE-310 was determined by HPLC.
2) Maximum rate of body weight loss, with numbers in parentheses denoting the day. <0 indicates no body weight loss.
3) Number of mice that died of toxicity/number of mice used.

Table 5. Anti-tumor effects of DE-310 and CDDP in a liver metastasis model of murine histiocytoma M5076

Compound	Schedule	Total dose[1] (mg/kg)	Survival (day)			BWL_{max}[2] (%) [day]	*D/U*[3]	*N/S*[4]
			Range	MST	ILS (%)			
Control	----	0	16–18	16.19	0.0	<0	0/10	----
DE-310	qd×1	34.2	16–18	17.60	8.7	31.2 [16]	10/10	----
	qd×1	22.8 (MTD)	26–>130	76.00**	369.4	11.6 [20]	2/10	2/2
	qd×1	17.1	50–>130	75.75**	367.9	5.4 [11, 20]	0/10	1/1
	qd×1	11.4	55–>130	81.00**	400.3	3.4 [9]	1/10	2/2
	qd×1	5.70	52–>130	>129.60**	>700.5	2.3 [9]	0/10	4/5
	qd×1	2.85	49–>130	>129.75**	>701.4	<0	0/10	6/6
	qd×1	1.425	48–>130	>129.86**	>702.1	<0	0/10	7/7
	qd×1	0.7125	70–>130	>129.86**	>702.1	<0	0/10	7/7
	qd×1	0.3563	47– >130	116.00**	616.5	<0	0/10	3/4
	qd×1	0.1781	21–127	33.00	103.8	<0	0/10	----
	q14d×4	11.4	81–>130	>129.94**#	>702.6	<0	0/10	8/8
	q14d×4	2.85	57–>130	>130.00**	>703.0	<0	0/5	4/4
CDDP	qd×1	15 (MTD)	16–>130	>129.94**	>702.6	20.9 [13]	1/10	7/8
	qd×1	10	52–>130	>129.94*	>702.6	6.5 [12]	0/10	5/8
	qd×1	5.0	74–>130	>130.00**	>703.0	2.3 [9]	0/10	7/9
	qd×1	2.5	20–>130	26.00**	60.6	<0	0/10	1/1
	qd×1	1.25	18–32	19.00	17.4	1.2 [9]	0/10	----

** $P\leq0.01$, * $P\leq0.05$ vs. control, # $P\leq0.05$ vs. single dose injection of the same total dose (Williams-Wilcoxon test).
1) Dose levels of DE-310 were expressed as those of DX-8951. The content of DX-8951 present in DE-310 was determined by HPLC.
2) Maximum value for rate of body weight loss (%), with numbers in parentheses denoting the day, <0 indicates no body weight loss.
3) Number of mice that died of toxicity/number of mice used.
4) Number of tumor-free mice/number of mice that survived by day 130 after tumor inoculation.

310 given intermittently for a total dose of 22.8 mg/kg exhibited a significant life-prolonging effect without toxic death. On the other hand, the life-prolonging effect of DX-8951f at the MTD was only 60% as the ILS value. CPA as a positive control exhibited a significant life-prolonging effect.

Discussion

Passive tumor-targeting with a macromolecular carrier system is based on the EPR effect. The EPR effect is a well-known feature of tumor tissue, including tumor blood vessels.[18] Moreover, the lymphatic clearance of macromolecules from tumor tissues does not occur. Accordingly, macromolecules remain in tumor tissues for a long period of time. DE-310 has a structure in which DX-8951 is combined with the biodegradable CM-Dex-PA carrier by a peptidyl spacer. The average molecular weight of DE-310 is 340,000 and the compound has a "stealthy" property.[19] These characteristics of DE-310 result in decreased excretion through the kidneys and decreased endocytosis in the liver/RES.[19] Accordingly, DE-310 exhibits prolonged high plasma-levels, and this phenomenon increases the EPR effect. After the distribution of DE-310 to the tumor tissue, DX-8951 is continuously generated from DE-310.[19]

In order to clarify the change in characteristics obtained by applying DX-8951 to drug delivery system (DDS), a comparison of the antitumor effects of DE-310 and DX-8951f was carried out using the Meth A model. Although DX-8951f at the MTD by qd×1 showed IR 50% at most, DX-8951f at the MTD

Table 6. Anti-tumor effects of DE-310, DX-8951f and CPA in a lung metastasis model of murine lung carcinoma 3LL

Compound	Schedule	Total dose[1] (mg/kg)	Survival (day)			BWL_{max}[2] (%) [day]	D/U[3]	N/S[4]
			Range	MST	ILS (%)			
Control	—	0	17–19	17.7	0.0	<0	0/10	—
DE-310	qd×1	22.8	15–16	15.2	−14.0	34.3 [15]	10/10	—
	qd×1	17.1 (MTD)	26–>123	84.0**	375.5	23.4 [12]	2/10	1/1
	qd×1	11.4	35–49	42.8**	142.0	17.6 [12]	0/10	—
	qd×1	5.70	26–32	28.4**	60.6	8.6 [12]	0/10	—
	qd×1	2.85	21–25	23.3*	32.1	3.7 [11]	0/10	—
	qd×1	1.425	18–23	21.0	18.9	0.8 [9]	0/10	—
	q7d×4	22.8	45–53	47.3**,##	167.5	14.8 [35]	0/10	—
	q7d×4	11.4	30–45	42.0**	137.7	28.0 [35]	0/10	—
DX-8951f	qd×5	10 (MTD)	26–37	28.4**	60.8	11.0 [15]	0/10	—
	qd×5	5.0	24–29	27.7**	56.6	5.6 [12]	0/10	—
	qd×5	2.5	23–28	25.0**	41.5	3.3 [15]	0/10	—
	qd×5	1.25	21–26	23.4*	32.3	2.1 [11]	0/10	—
CPA	qd×1	400 (MTD)	74–>123	>122.8**	>594.8	17.0 [11]	0/10	6/6
	qd×1	200	29–>123	43.0**	143.4	6.2 [9]	0/10	1/1
	qd×1	100	22–29	23.7*	34.0	2.4 [9]	0/10	—

** $P \leq 0.01$, * $P \leq 0.05$ vs. control, ## $P \leq 0.01$ vs. single dose injection of the same total dose (Williams-Wilcoxon test).
1) Dose levels of DE-310 and DX-8951f were expressed as those of DX-8951. The contents of DX-8951 present in DE-310 was determined by HPLC.
2) Maximum value for rate of body weight loss (%), with numbers in parentheses denoting the day, <0 indicates no body weight loss.
3) Number of mice that died of toxicity/number of mice used.
4) Number of tumor-free mice/number of mice that survived by day 123 after tumor inoculation.

Table 7. Comparison of the effective dose-ranges of DE-310 and DX-8951f against Meth A solid tumors

Compound	Schedule	MTD (mg/kg)	MED (mg/kg)	Therapeutic index
DE-310	qd×1	11.4	0.7125	16
DX-8951f	qd×1	40	>40	1>
	q4d×4	60	15	4
	qd×4	15	1.875	8
	qd×2/q4d×4	60	3.75	16
	q3h×3/q4d×4	60	3.75	16

Dose levels of DE-310 and DX-8951f were expressed as DX-8951. MTD: the maximum tolerated dose (total dose). MED: the minimum effective dose (total dose) which exhibited IR ≥58%. Therapeutic index: MTD/MED. qd×1: a single dose, q4d×4: every 4th day for a total of 4 doses, qd×4: once daily×4 treatments. qd×2/q4d×4: 4 cycles of daily doses for 2 days every 4th day, q3h×3/q4d×4: 4 cycles of 3 doses per day every 4th day.

and at 1/4 MTD by qd×5 showed a tumor regression effect and a tumor-inhibitory effect, respectively. Since DX-8951f has a time-dependent mode of cytotoxic action, it is possible to improve its antitumor action by changing the administration method. We were able to obtain an improvement in therapeutic index expressed as MTD/MED (dose which shows an antitumor effect of IR 58% and higher), as shown in Table 7.[26] That is, DX-8951f showed a therapeutic index of 16 when given by multiple administrations.

On the other hand, DE-310 at the MTD and at 1/4 MTD by qd×1 showed tumor regression, and even at 1/16 MTD, DE-310 showed a tumor growth-inhibitory effect. The therapeutic index of 16 obtained with multiple administrations of DX-8951f was achieved by a single dose of DE-310 corresponding to 1/5th of the dose of DX-8951f (compared as DX-8951 equivalents). Moreover, no body weight loss of Meth-A-bearing mice treated with DE-310 at 1/4 MTD was observed. These findings clearly suggest that the pharmacokinetics, antitumor effects and toxicity of DX-8951 are improved, enhanced and reduced, respectively, by applying to DDS.

The pharmacokinetics of DE-310 in a Meth A model have already been reported, as follows[9]: (I) The half-life times in the blood of ^{14}C-DX-8951f and ^{14}C-DE-310 (carrier-labeled and DX-labeled) given by qd×1 are about 20 min and 2.6 days, respectively. (II) The AUC of free DX-8951 in tumors following the administration of DE-310 is 3 times and 28 times higher than those in the liver and lung, respectively. (III) The half-life of free DX-8951 in tumors following the administration of DE-310 is 2 or 3 days. (IV) In mice treated with ^{14}C-DE-310 (carrier-labeled), 95% or more of the radioactivity is excreted within 42 days after administration. These results suggest that DE-310 has desirable characteristics, such as high retention in the blood, preferential accumulation in the tumor and sustained and low release of free DX-8951, and that the carrier of DE-310 is biodegradable. It is considered that the above pharmacokinetic results account well for the difference in antitumor effect between DX-8951f and DE-310.

Because DX-8951f administered every 4th day for a total of 4 injections showed an excellent antitumor effect against various human tumors xenografted in nude mice,[13] the antitumor effects of this schedule of DX-8951f and a single dose of DE-310 were compared. Considering that the half-life in the blood of the conjugated DX-8951 was 2 days (Meth A-bearing mice), the administration schedule of DE-310 may have been slightly disadvantageous compared with that of DX-8951f. DE-310, however, showed a remarkable effect against PC-6. In other human tumor xenografts too, the antitumor effect of DX-8951f given by intermittent administration was similar to that achieved by qdx1 of DE-310.

On the other hand, tumors in the clinical setting exist within organs and thus differ from human tumor xenograft models. Therefore, prolongation of the survival period with DE-310 was examined in a lung metastasis model and a liver metastasis model involving intravenous transplantation of tumor cells. In both metastasis models, DE-310 showed significant prolonga-

tion of survival. These findings showed that DE-310 was effective not only against s.c. transplanted tumors, but also against tumors in organs.

We have already obtained some results using Meth A ascites cells. Experiments suggest that DE-310 is internalized in tumor cells and macrophages by endocytosis as the conjugate, and endosomes containing DE-310 transfer to lysosomes, whereupon DX-8951 is released from DE-310 by lysosomal enzymes.[26] It was also observed that M5076 characteristically exhibits higher uptake of DE-310 into the cell and higher release of DX-8951 from DE-310 compared with Meth A or various human tumor cell lines (unpublished results). Therefore, tumor cell lines with high pinocytosis, such as histiocytoma, are likely to be good candidate tumors for DE-310 treatment.

Finally, the main dose-limiting toxicity of DE-310 was hematological toxicity, consistent with that previously reported for DX-8951f.[15, 28] However, DE-310 induced far less myelotoxicity than DX-8951f at doses that showed similar degrees of antitumor activity.[28] Moreover, we evaluated the antigenic potential of DE-310 in guinea pig models of active systemic anaphylaxis reaction (ASA) and passive cutaneous anaphylaxis reaction (PCA). The results suggest that DE-310 may have weak antigenic potential (positive ratio: 5%) (unpublished results).

The findings presented in this paper suggest that the pharmacokinetics of the CM-Dex-PA carrier are favorable, and this carrier may also be applicable to develop conjugates of other currently used antitumor drugs, in addition to DX-8951f.

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ORIGINAL ARTICLE

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Potent and broad antitumor effects of DX-8951f, a water-soluble camptothecin derivative, against various human tumors xenografted in nude mice

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Abstract *Purpose*: We have previously reported that DX-8951f, a water-soluble and nonprodrug camptothecin (CPT) derivative, exhibits both high in vitro potency against a series of 32 malignant cell lines and significant topoisomerase I inhibition. The purpose of this study was to evaluate the therapeutic efficacy of DX-8951f against human tumor xenografts in nude mice and to compare its activity with those of CPT-11 and other current CPT derivatives. *Methods*: The antitumor activity of DX-8951f against xenografts of several different types of human tumors was determined in nude mice using a schedule in which DX-8951f was administered intravenously every 4th day for a total of four injections. *Results*: Against both gastric adenocarcinoma SC-6 and its CPT-11-resistant variant, SC-6/CPT-11, DX-8951f demonstrated superior antitumor activity and antitumor activity over a broader range of doses than did CPT-11, SK&F104864 (hycamtin, topotecan) and GG-211 (GI147211). DX-8951f at 75 mg/kg was effective (growth inhibition rate IR ≧ 58%) against 15 of 16 lines of human cancers examined (6 colon cancers, 5 lung cancers, 2 breast cancers, 1 renal cancer and the above 2 gastric cancers), and exhibited excellent antitumor activity (IR ≧ 80%) against 14 of these lines. CPT-11 exhibited antitumor activity with IR values of 58% and higher against 11 lines and IR values of 80% and higher against only eight of the same 16 human tumors. DX-8951f was effective in inhibiting the growth of an SN-38-resistant tumor and some P-glycoprotein-expressing tumors, but CPT-11 was not. *Conclusions*: DX-8951f exhibited potent antitumor activity against various types of human tumor xenografts. Its in vivo antitumor effects were superior to those of current camptothecin analogs against certain tumors.

Key words DX-8951f · CPT-11 · Camptothecin derivatives · Human tumor xenografts

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Introduction

CPT-11, a DNA topoisomerase I inhibitor, has a mechanism of action different from other currently available therapeutic agents [13]. It exhibits antitumor activity against some human tumor xenografts in nude mice when administered intravenously (i.v.) as a single dose or when administered intermittently. CPT-11 is effective against human colon cancers Co-4 [14] and H-110 [5], lung cancers QG56 [14] and H-74 [5], and against breast cancer MX-1 [14]. Evaluation of phase II trials with CPT-11 has revealed a high response rate among small-cell lung cancers [17, 20], non-small-cell lung cancers [6,18], cervical cancers [23], ovarian cancers [22] and colorectal cancers [21]. The response rate to CPT-11 is 25% in colorectal cancer patients with prior radiotherapy or chemotherapy [21]. These findings demonstrate that the antitumor activity of CPT-11 observed in the preclinical studies also could be obtained clinically, and suggest that results of studies using human tumor xenografts in nude mice can be used to predict the clinical efficacy of future antitumor agents.

We previously reported that DX-8951f, a new camptothecin derivative, exhibits significant topoisomerase I inhibition and potent antitumor activity in vitro against human tumors derived from a variety of tissues. These effects of DX-8951f are greater than those of CPT-11 and SK&F104864 [19]. In the present study, we examined the therapeutic efficacy of DX-8951f against a variety of human tumor xenografts in nude mice and compared its activity with those of CPT-11 and other current camptothecin derivatives.

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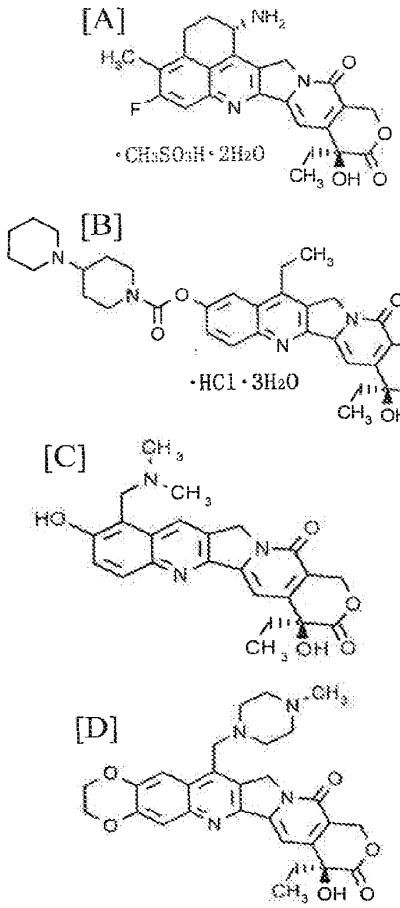


Fig. 1A–D Chemical structures of DX-8951f (A), CPT-11 (B), SK&F104864 (C) and GG-211 (D)

Materials and methods

Compounds

DX-8951f (Fig. 1A), (1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1*H*,12*H*-benzo [*de*] pyrano [3′,4′:6,7] indolizino [1,2-*b*] quinoline-10,13 (9*H*,15*H*)-dione monomethanesulfonate dihydrate, was synthesized entirely in our laboratory. CPT-11 (Fig. 1B) was provided by Yakult Honsha Co. (Tokyo, Japan). SK&F104864 (Fig. 1C) and GG-211 (Fig. 1D) were synthesized as described previously [11,15,16]. Prior to use, DX-8951f, CPT-11, SK&F104864 and GG-211 were dissolved in pyrogen-free distilled water (Otsuka Pharmaceutical Co., Tokyo, Japan) and diluted using the same water. Dose levels of the above compounds are expressed as those of the anhydrous free base. Vincristine (VCR, Shionogi Co., Osaka, Japan) was dissolved and diluted with pyrogen-free physiological saline (Otsuka Pharmaceutical Co.).

Animals

Male 6-week-old BALB/c-nu/nu nude mice were purchased from Japan SLC (Shizuoka, Japan). They were housed in an exclusively experimental room under specific pathogen-free conditions, and food sterilized by γ irradiation (Oriental Yeast Co., Tokyo, Japan) and water treated with hypochlorite were available ad libitum.

Tumors

The human tumor lines examined are listed in Table 1. SC-6 and MX-1 were supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan. Co-3 and Co-4 were kindly supplied by Dr. T. Kubota, School of Medicine, Keio University, Tokyo, Japan. SW620, SK-CO-1, DLD-1, WiDr, Du4475 and A-498 were obtained from the American Type Culture Collection, Rockville, Md. PC-12 was supplied by Toray Co., Tokyo, Japan. PC-6 and PC-14 were obtained from Immuno-Biological Laboratories, Gunma, Japan. SC-6/CPT-11, a CPT-11-resistant variant, was obtained by i.v. administration to SC-6-bearing nude mice of CPT-11 at 60 mg/kg once or twice per week for 2 months. PC-6/VCR29-9 and PC-6/SN2-5S were isolated from PC-6 by stepwise selection with increasing VCR and SN-38 concentrations, respectively. DLD-1, PC-12 and PC-6/VCR29-9 were confirmed to overexpress P-glycoprotein [19]. SC-6, SC-6/CPT-11, Co-3, Co-4 and MX-1 were maintained by sequential subcutaneous (s.c.) transplantation into nude mice. Other lines were cultured in vitro in RPMI-1640 medium (Gibco-BRL, N.Y.) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Utah, or Bocknek, Canada), and then inoculated s.c. into nude mice and maintained as solid tumors in the manner described above.

Evaluation of antitumor effects

Various human tumors maintained in nude mice were excised and cut into pieces approximately 2 to 3 mm in diameter in endotoxin-free (less than 30 pg/ml) Hanks' balanced salt solution (HBSS, Gibco-BRL). A piece of tumor was transplanted s.c. into the right flank of nude mice using a trocar. When the mean estimated tumor weight (ETW) reached 100 to 200 mg between days 10 and 40 after tumor transplantation, the mice were randomly divided into experimental groups (five or six mice per group) and were treated i.v. with a test compound every 4th day for a total of four injections (q4d × 4), every 4th day for a total of three injections (q4d × 3), every 7th day for a total of three injections (q7d × 3) or daily for 5 days (qd × 5). The ETW was calculated using the formula $ETW = L \times W^2/2$, where L and W represent the length and the width of the tumor mass, respectively. After the first administration on day 0, the ETW and body weight of the mice were measured two to five times per week for 22 or 28 days. The tumor masses were then excised and weighed. The growth inhibition rate (IR) on the basis of tumor weight was calculated using the formula $IR = (1 - TWt/TWc) \times 100$ (%), where TWt represents the mean of tumor weight of a treated group and TWc represents that of the control group.

The significance of differences in tumor weights between test and control groups was analyzed using Dunnett's test or the Tukey-Kramer test. When the IR was 58% and higher, the drug was evaluated as effective [4]. Conversely, when the IR was less than 58%, the drug was evaluated as ineffective even if statistical significance was shown. Furthermore, to evaluate the intensity of the side effects of compounds, the rate of body weight loss (BWL) and D/U were utilized as parameters of toxicity. BWL was calculated using the formula $BWL = (1 - BWn/BWs) \times 100$ (%), where BWn and BWs represent the mean body weights of mice on day *n* and on the day of initial administration, respectively. The maximum value of BWL was designated as BWLmax, and BWLmax less than zero indicates no body weight loss. D/U indicates the ratio of the number of mice that died of toxic effects to the number of mice used.

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Table 1 Human cell lines used in the experiments

Gastric cancer	SC-6[a]	Poorly differentiated adenocarcinoma
	SC-6/CPT-11[b]	CPT-11-selected variant of SC-6
Colon cancer	Co-3[c]	Well-differentiated adenocarcinoma
	Co-4[c]	Poorly differentiated adenocarcinoma
	SW620[d]	Adenocarcinoma
	SK-CO-1[d]	Adenocarcinoma
	DLD-1[d,g]	Adenocarcinoma
	WiDr[d]	Adenocarcinoma
Lung cancer	PC-12[e,g]	Differentiated adenocarcinoma
	PC-14[f]	Poorly differentiated adenocarcinoma
	PC-6[f]	Oat-cell carcinoma
	PC-6/VCR29-9[b,g]	VCR-selected variant of PC-6
	PC-6/SN2-5S[b]	SN-38-selected variant of PC-6
Breast cancer	Du4475[d]	Carcinoma
	MX-1[a]	Medullary tubular carcinoma
Renal cancer	A-498[d]	Carcinoma

[a] Supplied by Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo
[b] Drug-resistant cell lines established in our laboratory
[c] Supplied by Dr. T. Kubota, Keio University, School of Medicine, Tokyo
[d] Obtained from the American Type Culture Collection, Rockville, Md
[e] Supplied by Toray Co., Ltd., Tokyo
[f] Obtained from Immuno-Biological Laboratories, Gunma, Japan
[g] P-glycoprotein overexpression

Results

Antitumor effects of DX-8951f in various schedules against human SC-6 gastric cancer xenografts

To determine the schedule of administration used in further experiments, the therapeutic efficacies of DX-8951f when administered every 4th day for a total of four injections (q4d × 4), every 4th day for a total of three injections (q4d × 3) or every 7th day for a total of three injections (q7d × 3) against SC-6 xenografts were examined (Table 2). DX-8951f exhibited strong antitumor effects with IR values of 77% and higher at total doses of 25 and 50 mg/kg on all schedules. In a comparison of growth curves at 50 mg/kg (Fig. 2), it was tendentiously observed that the q4d × 4 schedule more readily caused the regressive effects of DX-8951f compared with the q7d × 3, and a schedule of q4d × 3 more frequently permitted regrowth of tumors than did the q4d × 4 schedule. Based on these findings, the q4d × 4 schedule was chosen as a schedule for intermittent application of DX-8951f, and the antitumor effects of the compound on the schedule against human tumor xenografts in nude mice were examined.

Antitumor effects against gastric cancer xenografts

The results are shown in Table 3. The antitumor effects of various camptothecin derivatives (DX-8951f at a total dose of 25 mg/kg, SK&F104864 at a total dose of

Table 2 Antitumor effects of DX-8951f in various schedules against human SC-6 gastric cancer xenografts in nude mice. A piece of SC-6 tumor block was inoculated into the right flank of BALB/c-nu/nu mice (day 0). Mice received multiple i.v. administrations on q4d × 4, q4d × 3 or q7d × 3 schedules from day 15 (experiment 1) or 14 (experiment 2). Tumor weight was assessed on day 43 (experiment 1) or 42 (experiment 2). Dose levels of DX-8951f are expressed as those of the anhydrous free base

Total dose (mg/kg)	Schedule	IR	BWLmax[a]		D/U[b]
		(%)	%	Day	
Experiment 1[c]					
50	q4d × 4	97	9.9	21	0/6
	q4d × 3	92	12.5	27	0/6
	q7d × 3	93	6.7	32	0/6
Experiment 2[d]					
25	q4d × 4	94	4.3	23	0/6
	q4d × 3	88	4.9	19	0/6
	q7d × 3	77	1.6	15	0/6
6.25	q4d × 4	58	2.8	15	0/6
	q4d × 3	45	< 0		0/6
	q7d × 3	50	< 0		0/6

$^{**}P < 0.01$, $^{*}P < 0.05$, Tukey-Kramer test
[a] Maximum rate of body weight loss (< 0 indicates no body weight loss)
[b] Number of mice that died of toxicity/number of mice used
[c] Tumor weight of control group: 4.797 ± 0.456 g
[d] Tumor weight of control group: 3.815 ± 0.398 g

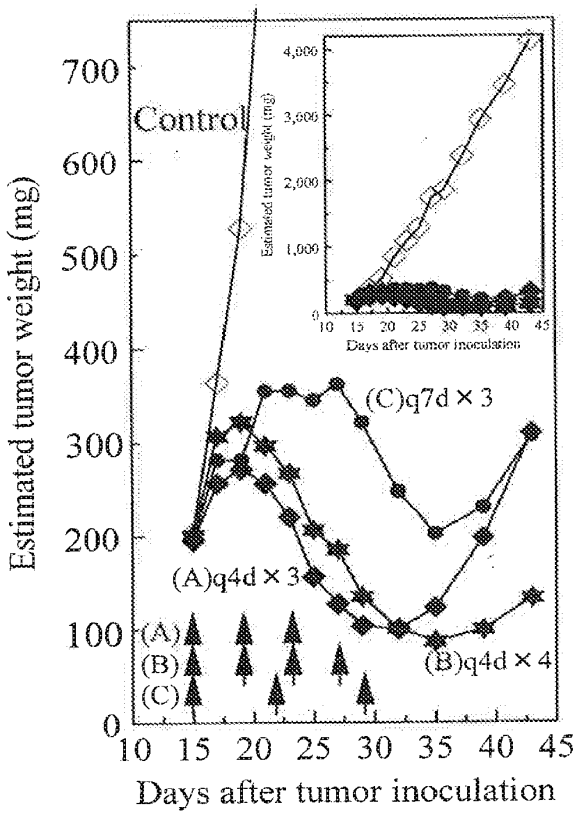


Fig. 2 Inhibitory effects of DX-8951f at 50 mg/kg as total dose in various schedules against human SC-6 gastric cancer xenografts in nude mice. Dose levels of DX-8951f are expressed as those of the anhydrous free base. The changes in estimated tumor weight observed in experiment 1 of Table 2 were plotted. *Arrows* indicate days of treatment on each schedule

30 mg/kg and GG-211 at a total dose of 25 mg/kg) against SC-6 xenografts were examined twice (experiments 1 and 2), and nearly equal IR values were obtained in the two experiments. The dose-effect curves, which represent the relationship between IR values and total doses of each compound, are shown in Fig. 3. DX-8951f exhibited a strong antitumor effect on SC-6, with IR values of 94% and higher at total doses of 75, 50 and 25 mg/kg without deaths due to toxicity, and with an IR value of 58% even at a total dose of 6.25 mg/kg, one-twelfth the highest dose. CPT-11 also exhibited an antitumor effect with IR values over 58% at all doses examined: an IR value of 94% was obtained at the highest total dose of 320 mg/kg, but the IR was reduced to 68% at a total dose of 160 mg/kg, half the highest dose. SK&F104864 at a total dose of 45 mg/kg showed an IR value of 81%, but resulted in deaths from toxicity (two of six mice). SK&F104864 failed to show an antitumor effect even at the highest nonlethal dose of 30 mg/kg. GG-211 was highly toxic at total doses of 75 and 50 mg/kg. GG-211 exhibited an antitumor effect with an IR value over 90% at a total dose of 25 mg/kg without deaths from toxicity, but was not effective at a total dose of 6.25 mg/kg (IR 39%), one-fourth the maximum tolerated dose (MTD).

Against SC-6/CPT-11, CPT-11 at a total dose of 320 mg/kg showed an IR value of 65%, but this value was low compared with its IR value of 94% against SC-6. This finding confirmed that SC-6/CPT-11 is resistant to CPT-11. DX-8951f showed a strong antitumor effect with IR values of 94% and higher at total doses of 75 and 50 mg/kg against both SC-6/CPT-11 and the parent tumor. SK&F104864 was not effective against SC-6/CPT-11 at total doses of 20 and 30 mg/kg, at which no mice died of toxicity. GG-211 at a total dose of 25 mg/kg resulted in an IR value of 69% against SC-6/CPT-11, but this effect was weaker than that against SC-6.

Antitumor effects against colon cancer xenografts

The results are shown in Table 4. DX-8951f at total doses of 75 and 50 mg/kg showed antitumor effects with IR values of about 80% against Co-3, Co-4 and DLD-1. Against Co-3 and Co-4 in particular, the compound exhibited antitumor effects with IR values of about 60% even at a total dose of 25 mg/kg. Furthermore, DX-8951f showed strong antitumor effects with IR values of about 90% against SK-CO-1 and WiDr at all doses examined and IR values of 96 to 100% at doses of 25 to 75 mg/kg against SW620. Thus, against all the above colon cancer lines, DX-8951f resulted in IR values of 80% and higher at 75 mg/kg and IR values above 58% at 50 mg/kg or, in selected tumors, at 25 mg/kg.

CPT-11 showed a strong antitumor effect against Co-3, Co-4 and SW620 with IR values of 90% and higher, and against SK-CO-1 with IR values of 62% and higher at total doses of 160 mg/kg or more. However, CPT-11 showed no or only a slight antitumor effect with IR values of 48% and 59% against WiDr and DLD-1 even at the highest dose of 320 mg/kg. The effects against these two tumor lines were weaker than those of DX-8951f.

Antitumor effects against lung cancer xenografts

The results for PC-12 and PC-14 are shown in Table 5. DX-8951f showed antitumor effects against these lung cancers with IR values of 91% and higher at total doses of 75 mg/kg and IR values of 60% to 70% at 50 mg/kg, without marked body weight loss. CPT-11 was not effective against PC-12. CPT-11 showed antitumor effects against PC-14 with IR values of 83% and 59% at total doses of 320 and 240 mg/kg. This effect was somewhat inferior to that of DX-8951f.

The results for PC-6 and its resistant variants are shown in Table 6, and the dose-effect curves for

Table 3 Antitumor effects of various camptothecin derivatives against human gastric cancer xenografts in nude mice. A piece of tumor block was implanted into the right flank of BALB/c-nu/nu mice (day 0). The mice were randomized and divided into several groups (six mice per group) when the mean estimated tumor weight reached 82 to 178 mg (SC-6 experiment 1, day 10; SC-6 experiment 2, day 14; SC-6/CPT-11, day 22). Groups of mice received i.v. injections of DX-8951f, CPT-11, SK&F104864 or GG-211 every 4th day for a total of four injections (q4d × 4). Tumor weight was assessed on the 28th day after the first administration. The tumor weights of the control groups were as follows: SC-6 experiment 1, 3.872 ± 0.335 g; experiment 2, 3.815 ± 0.398 g; SC-6/CPT-11, 1.122 ± 0.389 g. Dose levels of compounds are expressed as those of the anhydrous free base (*ND* not done)

Compound	Total dose[a] (mg/kg)	SC-6								SC-6/CPT-11			
		Experiment 1				Experiment 2							
		IR	BWLmax[b]		D/U[c]	IR	BWLmax[b]		D/U[c]	IR	BWLmax[b]		D/U[c]
		(%)	(%)	Day		(%)	%	Day		(%)	%	Day	
Control	0	0	< 0		0/6	0	< 0		0/6	0	< 0		0/6
DX-8951f	75 (18.75 × 4)	98***	3.9	16	0/6	ND				96**	22.1	37	0/6
	50 (12.5 × 4)	98***	3.1	16	0/6	ND				94**	6.9	31	0/6
	25 (6.25 × 4)	94***	2.6	15	0/6	94***	4.3	23	0/6	67*	1.2	23	0/6
	12.5 (3.125 × 4)	ND				ND				57	1.4	23	0/6
	6.25 (1.5625 × 4)	ND				58***	2.8	15	0/6	39	4.8	23	0/6
CPT-11	320 (80 × 4)	94***	3.9	12	0/6	ND				65	1.5	31	0/6
	240 (60 × 4)	77***	0.8	11	0/6	ND				59	1.2	28	0/6
	160 (40 × 4)	68***	< 0		0/6	ND				56	0.3	28	0/6
SK&F104864	45 (11.25 × 4)	ND				81***	9.6	19	2/6	65	22.5	31	3/6
	30 (7.5 × 4)	53***	< 0		0/6	53***	1.0	18	0/6	41	7.2	31	0/6
	20 (5 × 4)	28*	< 0		0/6	ND				28	0.4	24	0/6
	10 (2.5 × 4)	32*	< 0		0/6	ND				ND			
GG-211	75 (18.75 × 4)	Toxic	32.2	18	6/6	ND				ND			
	50 (12.5 × 4)	99***	30.9	22	5/6	ND				ND			
	25 (6.25 × 4)	95***	2.1	16	0/6	91***	3.9	19	0/6	69	17.7	38	0/6
	12.5 (3.125 × 4)	ND				ND				18	2.7	28	0/6
	6.25 (1.5625 × 4)	ND				36**	1.4	15	0/6	7	< 0		0/6

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; versus control by Dunnett's test

[a] Compounds were administered four times at 4-day intervals

[b] Maximum rate of body weight loss (< 0 indicates no body weight loss)

[c] Number of mice that died of toxicity/number of mice used

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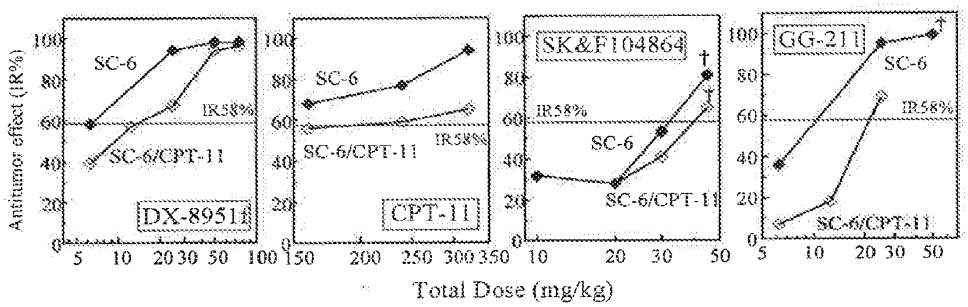


Fig. 3 Dose-effect curves for DX-8951f, CPT-11, SK&F104864 and GG-211 against human gastric cancers SC-6 (◆) and SC-6/CPT-11 (◇) in nude mice. Dose levels of the compounds are expressed as those of the anhydrous free base. Values of IR% are plotted against the total dose of each derivative. Data were obtained from Table 3. † indicates that some mice died of toxic effects

compounds against these tumor lines are shown in Fig. 4. DX-8951f exhibited antitumor effects against parental PC-6 at total doses of 25 and 50 mg/kg with IR values of 74% and 98%, respectively, without deaths from toxicity. It also showed strong antitumor effects against PC-6/SN2-5S and PC-6/VCR29-9 with IR values of 79% and 93% at a total dose of 75 mg/kg. CPT-11 showed a moderate antitumor effect (IR 63%) against PC-6 only at the highest dose of 320 mg/kg. CPT-11 was not effective against the resistant variants PC-6/SN2-5S and PC-6/VCR29-9. On the other hand, VCR at total doses of 3 and 2.25 mg/kg showed antitumor effects against PC-6 and PC-6/SN2-5S with IR values of about 70%. VCR was not effective against PC-6/VCR29-9.

Antitumor effects against breast cancer xenografts

The results are shown in Table 7. DX-8951f at total doses of 75 and 50 mg/kg, as well as CPT-11 at 320 and 160 mg/kg, showed strong antitumor effects against MX-1 and Du4475. Against MX-1, DX-8951f and CPT-11 caused complete regression of tumor masses.

Antitumor effects against renal cancer xenografts

The results are shown in Table 8. DX-8951f at total doses of 50 and 75 mg/kg exhibited no antitumor activity against A-498. CPT-11 also showed no antitumor effect against A-498.

Comparison of the efficacy of DX-8951f and CPT-11

The efficacies of DX-8951f and CPT-11 against human tumor xenografts of 16 lines examined are summarized in Table 9. The number of tumors against which DX-8951f or CPT-11 exhibited IR values of 80% and higher or IR values of 58% and higher at the MTD (75 mg/kg and 320 mg/kg, respectively) are shown as indexes of antitumor effects. Response rates are given in parentheses. DX-8951f exhibited antitumor effects with IR values of 58% and higher against 15 lines (94%) and with IR values of 80% and higher against 10 lines (88%). On the other hand, CPT-11 exhibited antitumor effects with IR values of 58% and higher against 11 lines (69%) and with IR values of 80% and higher against 8 lines (50%).

Discussion

We have previously reported that DX-8951f exhibits significant topoisomerase I inhibition and potent antitumor activity in vitro against human tumors derived from a variety of tissues, and that these effects of DX-8951f are greater than those of CPT-11 and SK&F104864 [19]. In the present study, we examined the therapeutic efficacy of DX-8951f against a variety of human tumor xenografts in nude mice. The schedule of administration was established as intermittent application (q4d × 4) based on experimental results (Table 2) and reports that CPT-11 [14], SK&F104864 [9] and GG-211 [3] by intermittent applications exhibit significant antitumor effects against human tumor xenografts.

First, the antitumor activity of DX-8951f against SC-6 gastric cancer xenografts was compared with those of CPT-11, SK&F104864 and GG-211 (Table 3). DX-8951f exhibited antitumor effects with IR values of 94% and higher at total doses of 75 (MTD), 50 and 25 mg/kg and with IR value of 58% even at one-twelfth the MTD, 6.25 mg/kg. When the therapeutic ratio [the ratio of MTD to minimum effective dose, MED (MTD/MED)] was compared, the ratio for DX-8951f (MTD/MED 12) was clearly higher than that of other camptothecin derivatives (MTD/MED less than 2). In addition, DX-8951f exhibited strong antitumor effects at total doses of 75 and 50 mg/kg against SC-6/CPT-11, as well as against the parent tumor. However, the efficacy of the other derivatives was less than this against SC-6/CPT-11. These findings indicate that the antitumor activity and the effective dose ranges of DX-8951f are superior to those of the current camptothecin derivatives against human tumor xenografts.

Table 4 Antitumor effects of DX-8951f and CPT-11 against human colon cancer xenografts in nude mice. A piece of tumor block was implanted into the right flank of BALB/c-nu/nu mice (day 0). The mice were randomized and divided into several groups (six mice per group) when the mean estimated tumor weight reached 100 to 200 mg (Co-3 day 15, Co-4 day 18, SW620 day 11, SK-CO-1 day 41, DLD-1 day 14, WiDr day 21). Groups of mice received i.v. injections of DX-8951f or CPT-11 every 4th day for a total of four injections (q4d × 4). Tumor weight was assessed on the 22nd day (Co-3) or the 28th day (Co-4, SW620, SK-CO-1, DLD-1, WiDr) after the first administration. The tumor weights of control groups were as follows: Co-3 1.504 ± 0.205 g, Co-4 2.386 ± 0.404 g, SW620 4.222 ± 0.762 g, SK-CO-1 0.508 ± 0.097 g, DLD-1 1.279 ± 0.127 g, WiDr 1.052 ± 0.138 g. The dose levels of the compounds are expressed as those of the anhydrous free base (*ND* not done)

Compound	Total dose[a] (mg/kg)	Co-3				Co-4				SW620			
		IR	BWLmax[b]		D/U[c]	IR	BWLmax[b]		D/U[c]	IR	BWLmax[b]		D/U[c]
		(%)	%	Day		(%)	%	Day		(%)	%	Day	
Control	0	0	9.0	35	0/4[d]	0	11.2	40	0/6	0	< 0		0/6
DX-8951f	75 (18.75 × 4)	80***	15.8	28	0/6	85***	15.4	34	0/6	100***	2.8	20	0/6
	50 (12.5 × 4)	78***	11.8	28	0/6	76***	11.9	32	0/6	99***	0.6	20	0/6
	25 (6.25 × 4)	59***	13.4	32	0/6	61***	13.3	32	0/6	96***	1.8	16	0/6
	12.5 (3.125 × 4)	47**	13.9	35	0/6	51**	15.5	40	0/6	ND			
	6.25 (1.5625 × 4)	43**	9.6	35	0/6	57***	18.4	40	0/6	ND			
CPT-11	320 (80 × 4)	95***	12.0	28	0/6	97***	9.2	27	0/6	99***	1.8	27	0/6
	240 (60 × 4)	89***	12.7	28	0/6	ND				99***	< 0		0/6
	160 (40 × 4)	91***	8.3	28	0/6	78***	10.9	34	0/6	94***	0.2	27	0/6
	80 (20 × 4)	86***	7.3	28	0/6	63***	17.3	40	0/6	ND			

Compound	Total dose[a] (mg/kg)	SK-CO-1				DLD-1				WiDr			
		IR	BWLmax[b]		D/U[c]	IR	BWLmax[b]		D/U[c]	IR	BWLmax[b]		D/U[c]
		(%)	%	Day		(%)	%	Day		(%)	%	Day	
Control	0	0	7.2	69	0/6	0	< 0		0/6	0	< 0		0/6
DX-8951f	75 (18.75 × 4)	88***	25.7	57	0/6	86***	5.6	28	0/6	90***	2.0	30	0/6
	50 (12.5 × 4)	89***	16.1	57	0/6	81***	2.2	28	0/6	93***	< 0		0/6
	25 (6.25 × 4)	88***	14.1	55	0/6	ND				ND			
CPT-11	320 (80 × 4)	83***	13.2	55	0/6	59**	1.2	26	0/6	48*	3.5	35	0/6
	240 (60 × 4)	76***	10.6	55	0/6	42*	1.9	26	0/6	36	< 0		0/6
	160 (40 × 4)	62**	9.2	55	0/6	ND				ND			

* $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$; versus control by Dunnett's test

[a] Compounds were administered 4 times at 4-day intervals

[b] Maximum rate of body weight loss (< 0 indicates no body weight loss)

[c] Number of mice that died of toxicity/number of mice used

[d] Two mice in the control group were eliminated because they died of tumor on days 32 and 35

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Table 5 Antitumor effects of DX-8951f and CPT-11 against human lung cancer xenografts in nude mice. A piece of tumor block was implanted into the right flank of BALB/c-nu/nu mice (day 0). The mice were randomized and divided into several groups (five or six mice per group) when the mean estimated tumor weight reached 108 to 202 mg (PC-12 day 12, PC-14 day 16). Groups of mice received i.v. injections of DX-8951f or CPT-11 every 4th day for a total of four injections (q4d × 4). Tumor weight was assessed on the 22nd day (PC-12) or the 28th day (PC-14) after the first administration. The tumor weights of the control groups were as follows: PC-12 5.027 ± 0.975 g, PC-14 6.586 ± 0.343 g. The dose levels of the compounds are expressed as those of the anhydrous free base

Compound	Total dose[a] (mg/kg)	PC-12				PC-14			
		IR	BWLmax[b]		D/U[c]	IR	BWLmax[b]		D/U[c]
		(%)	%	Day		(%)	%	Day	
Control	0	0	< 0		0/6	0	< 0		0/5
DX-8951f	75 (18.75 × 4)	91***	< 0		0/6	95***	2.6	25	0/5
	50 (12.5 × 4)	63**	< 0		0/6	71***	3.5	21	0/5
CPT-11	320 (80 × 4)	12	< 0		0/6	83***	< 0		0/5
	240 (60 × 4)	19	< 0		0/6	59***	< 0		0/5

** $P < 0.01$, *** $P < 0.001$; versus control by Dunnett's test
[a] Compounds were administered four times at 4-day intervals
[b] Maximum rate of body weight loss (< 0 indicates no body weight loss)
[c] Number of mice that died of toxicity/number of mice used

Table 6 Antitumor effects of DX-8951f, CPT-11 and VCR against human lung cancer xenografts in nude mice. A piece of tumor block was implanted into the right flank of BALB/c-nu/nu mice (day 0). The mice were randomized and divided into several groups (six mice per group) when the mean estimated tumor weight reached 124 to 163 mg (PC-6 day 21, PC-6/SN2-5S day 22, PC-6/VCR29-9 day 36). Groups of mice received i.v. injections of DX-8951f or CPT-11 every 4th day for a total of four injections (q4d × 4) or VCR daily for 5 days (qd × 5). Tumor weight was assessed on the 28th day after the first administration. The tumor weights of the control groups were as follows: PC-6 2.455 ± 0.267 g, PC-6/SN2-5S 1.822 ± 0.259 g, PC-6/VCR29-9 1.870 ± 0.377 g. The dose levels of the camptothecin derivatives are expressed as those of the anhydrous free base (*ND* not done)

Compound	Total dose[a] (mg/kg)	PC-6				PC-6/SN2-5S				PC-6/VCR29-9			
		IR	BWLmax[b]		D/U[c]	IR	BWLmax[b]		D/U[c]	IR	BWLmax[b]		D/U[c]
		(%)	%	Day		(%)	%	Day		(%)	%	Day	
Control	0	0	< 0		0/6	0	< 0		0/6	0	< 0		0/6
DX-8951f	75 (18.75 × 4)	99	28.8	30	4/6	79***	14.3	37	0/6	93**	15.2	52	0/6
	50 (12.5 × 4)	98***	17.9	35	0/6	65***	14.4	37	0/6	ND			
	25 (6.25 × 4)	89***	4.3	35	0/6	51**	< 0		0/6	ND			
	12.5 (3.125 × 4)	74***	< 0		0/6	45**	< 0		0/6	ND			
	6.25 (1.5625 × 4)	56***	3.4	22	0/6	55**	< 0		0/6	ND			
CPT-11	320 (80 × 4)	63**	3.9	22	0/6	−8	< 0		0/6	30	0.6	39	0/6
	240 (60 × 4)	39	< 0		0/6	3	< 0		0/6	ND			
	160 (40 × 4)	32	< 0		0/6	21	< 0		0/6	ND			
	80 (20 × 4)	−5	< 0		0/6	ND				ND			
VCR	3.0 (0.6 × 5)	73***	24.4	28	0/6	75***	22.2	28	0/6	5	19.6	42	0/6
	2.25 (0.45 × 5)	71***	22.9	28	0/6	73***	20.0	28	0/6	ND			
	1.5 (0.3 × 5)	62***	6.0	28	0/6	54**	2.8	28	0/6	ND			

** $P < 0.01$, *** $P < 0.001$; versus control by Dunnett's test
[a] Compounds were administered four times at 4-day intervals or daily for 5 consecutive days
[b] Maximum rate of body weight loss (< 0 indicates no body weight loss)
[c] Number of mice that died of toxicity/number of mice used

The antitumor activity of DX-8951f was further compared to that of CPT-11 using human colon, lung, breast and renal cancers. CPT-11 has been shown to exhibit high antitumor activity against human colon and lung cancers in previous preclinical [5, 14] and clinical [6, 17, 18, 20, 21] studies, and it was also effective against five of six colon cancer lines and two of five lung cancer lines in our study. DX-8951f exhibited marked activity against all lines of the above colon and lung cancers, including four lines (WiDr, PC-12, PC-6/VCR29-9 and PC-6/SN2-5S) against which CPT-11 was not effective. We have found that PC-12, PC-6/VCR29-9 and DLD-1 cells overexpress P-glycoprotein (P-gp) by Western-blotting analysis using an anti-P-gp monoclonal antibody [19]. P-gp, a plasma membrane protein, plays an important role in multidrug resistance as a drug efflux pump [2, 7, 25]. We have also shown that SK&F104864 and SN-38 (an active metabolite of CPT-11) [12, 14, 24] are significantly or moderately subject to P-gp-mediated resistance in vitro [19], as previously reported by others

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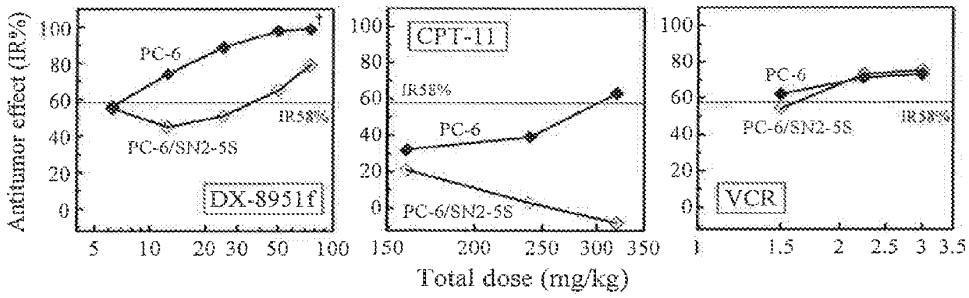


Fig. 4 Dose-effect curves for DX-8951f, CPT-11 and VCR against human lung cancers PC-6 (◆) and PC-6/SN2-5S (◇) in nude mice. Dose levels of camptothecin derivatives are expressed as those of the anhydrous free base. Values of IR% are plotted against the total dose of each compound. Data were obtained from Table 6. † indicates that some mice died of toxic effects

[1,8], but that DX-8951f is not affected by P-gp overexpression [19]. Against the above three cell lines, CPT-11 was not effective (PC-12, PC-6/VCR29-9) or exhibited antitumor activity with an IR value of 59% (DLD-1), but DX-8951f exhibited strong antitumor activity with IR values of 80% and higher. These results suggest that DX-8951f is less affected by P-gp than CPT-11 in vivo.

PC-6/SN2-5S, an SN-38-resistant variant derived from PC-6, was obtained by two courses of a stepwise selection procedure: PC-6/SN2-5 cells [10] were established from parental PC-6 cells in the first course by treatment with 0.04 to 2 ng/ml SN-38, and PC-6/SN2-5S cells were derived from PC-6/SN2-5 cells in the second course with 2 to 50 ng/ml SN-38. The mechanisms of resistance of PC-6/SN2-5 may be related to a decrease in intracellular accumulation of certain drugs, but may not be mediated by a classical efflux mechanism due to lack of expression of P-gp [10]. The mechanism of resistance of PC-6/SN2-5S has been speculated to be similar to that of PC-6/SN2-5, since the resistance patterns against various anticancer agents of these variants are similar and no expression of P-gp was detected in PC-6/SN2-5S cells. PC-6/SN2-5S cells exhibit significant resistance to

Table 8 Antitumor effects of DX-8951f and CPT-11 against human renal cancer xenografts in nude mice. A piece of tumor block was implanted into the right flank of BALB/c-nu/nu mice (day 0). The mice were randomized and divided into several groups (six mice per group) when the mean estimated tumor weight reached 134 mg on day 34. Groups of mice received i.v. injections of DX-8951f or CPT-11 every 4th day for a total of four injections (q4d × 4). Tumor weight was assessed on the 22nd day after the first administration. The tumor weight of the control group was 0.669 ± 0.055 g. The dose levels of the compounds are expressed as those of the anhydrous free base

Compound	Total dose[a] (mg/kg)	A-498			
		IR	BWLmax[b]		D/U[c]
		(%)	%	Day	
Control	0	0	< 0		0/6
DX-8951f	75 (18.75 × 4)	41	27.2	50	2/6
	50 (12.5 × 4)	28	16.6	49	0/6
	25 (6.25 × 4)	31	11.7	49	0/6
CPT-11	320 (80 × 4)	5	5.3	47	0/6
	240 (60 × 4)	32	5.0	43	0/6

[a] Compounds were administered four times at 4-day intervals
[b] Maximum rate of body weight loss (< 0 indicates no body weight loss)
[c] Number of mice that died of toxicity/number of mice used

Table 7 Antitumor effects of DX-8951f and CPT-11 against human breast cancer xenografts in nude mice. A piece of tumor block was implanted into the right flank of BALB/c-nu/nu mice (day 0). The mice were randomized and divided into several groups (5 or 6 mice/group) when the mean estimated tumor weight reached 100 to 200 mg (MX-1 day 20; Du4475 day 27). Groups of mice received i.v. injections of DX-8951f or CPT-11 every 4th day for a total of four injections (q4d × 4). Tumor weight was assessed on the 22nd day (Du4475) or the 28th day (MX-1) after the first administration. Tumor weights of control groups were as follows: MX-1 6.980 ± 1.686 g; Du4475 2.228 ± 0.395 g. Dose levels of compounds were expressed as those of the anhydrous free base

Compound	Total dose[a] (mg/kg)	MX-1				Du4475			
		IR	BWLmax[b]		D/U[c]	IR	BWLmax		D/U
		(%)	%	Day		%	%	Day	
Control	0	0	< 0		0/5	0	6.9	49	0/6
DX-8951f	75 (18.75 × 4)	100 ***	0.8	29	0/5	96 ***	6.0	37	0/6
	25 (6.25 × 4)	100 ***	1.4	21	0/5	78 ***	3.6	31	0/6
CPT-11	320 (80 × 4)	100 ***	< 0		0/5	97 ***	3.2	31	0/6
	160 (40 × 4)	98 ***	< 0		0/5	89 ***	1.4	31	0/6

*** $P < 0.001$; versus control by Dunnett's test
[a] Compounds were administered 4 times at 4-day intervals
[b] Maximum rate of body weight loss (%); with numbers in parentheses denoting the day; < 0 indicates no body weight loss
[c] Number of mice that died of toxicity / number of mice used

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Table 9 Comparison of antitumor effects of DX-8951f and CPT-11 against various human tumor xenografts in nude mice. Dose levels of compounds are expressed as those of the anhydrous free base. Values are no. (%) of tumors

Cancer	No. of tumors examined	DX-8951f (total dose 75 mg/kg)		CPT-11 (total dose 320 mg/kg)	
		≥IR80%	≥IR58%	≥IR80%	≥IR58%
Gastric	2	2 (100%)	2 (100%)	1 (50%)	2 (100%)
Colon	6	6 (100%)	6 (100%)	4 (67%)	5 (83%)
Lung	5	4 (80%)	5 (100%)	1 (20%)	2 (40%)
Breast	2	2 (100%)	2 (100%)	2 (100%)	2 (100%)
Renal	1	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	16	14 (88%)**	15 (94%)*	8 (50%)	11 (69%)

* $P < 0.10$, ** $P < 0.05$; versus CPT-11 by Fisher's exact probability test

SN-38 (240 times that of PC-6 cells), but markedly lower resistance to DX-8951f (15 times that of PC-6 cells) in vitro (unpublished data). The present results clearly indicated, in vivo as well, that CPT-11 was not effective against PC-6/SN2-5S tumors, but that DX-8951f inhibited the growth of the resistant tumors with IR values of 58% and higher (Table 6). These findings, taken together with the antitumor effects against SC-6/CPT-11 described previously, suggest that DX-8951f may be less affected by certain types of resistance to camptothecin derivatives.

We have also examined the survival activity, as evaluated by increased life span, of DX-8951f in metastasis models of murine lung cancer cell 3LL and histiocytoma cell M5076 by i.v. transplantation, confirming that the compound is effective (unpublished results). From the potency of DX-8951f in human tumor xenograft models and murine metastasis models, the compound is expected to be clinically effective. Phase I clinical trials of DX-8951f are in progress in Europe, the United States and Japan.

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Biochemistry

Sequence investigation of the major gastrointestinal tumor-associated antigen gene family, GA733

(oligonucleotide/intronless gene/exon shuffling/monoclonal antibody)

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ABSTRACT **The monoclonal antibody-defined, tumor-associated antigen GA733 was purified from the SW948 human colorectal carcinoma cell line and its partial amino acid sequence was determined. By using a synthetic oligonucleotide probe, two recombinants were isolated from a total human genomic library. We prove the existence of a family of GA733 genes. One of the genomic isolates is demonstrated to be an intronless gene, which is transcribed in pancreatic carcinoma cell lines and in placenta. The GA733 proteins were observed to contain sequences homologous to a repeat unit occurring 10 times in thyroglobulin and once in the HLA-DR-associated invariant chain. A more evolutionarily distant relationship was found with the α chain of the interleukin 2 growth factor receptor.**

Monoclonal antibodies (mAbs) CO17-1A and the related GA733 (1) have been extensively evaluated for the diagnosis and therapy of human gastrointestinal tumors. These mAbs were derived from the immunization of mice with a human colorectal adenocarcinoma cell line and a stomach adenocarcinoma cell line, respectively. CO17-A and GA733 mAbs bind to tumors of the gastrointestinal tract and also bind in varying degrees to normal epithelial tissues. GA733, unlike CO17-1A, has an extended specificity to carcinomas of other origins, such as the cervix, bladder, and lung (1). These independently derived mAbs immunoprecipitate a 40-kDa cell-surface glycoprotein and cross-inhibit each other's binding to antigen purified by GA733 mAb-affinity chromatography (2). The two mAbs manifest sequence identities in their heavy chain CDR3 regions (3). These findings indicate that the mAbs may bind to partially overlapping epitopes on the same antigen or, alternatively, the mAbs may recognize cross-reacting epitopes present on related antigens.

Both CO17-1A and GA733 mAbs have been shown to inhibit the growth of tumor xenografts in nude mice (4, 5), pointing out the potential suitability of these mAbs for the therapy of human tumors. When human subjects with gastrointestinal adenocarcinoma were inoculated with mAb CO17-1A (Ab1), many of the patients produced anti-idiotype antibody (Ab2) that reacted with the binding site of the Ab1 (6, 7). In theory, the administration of internal image Ab2 alone may effect an antitumor response.

By using experimental animals, the GA733 mAb (Ab1) has been used to obtain an Ab2 bearing the internal image of the GA733 tumor antigen; this Ab2 could elicit in two other species of animals an Ab3 that had Ab1-like binding specificity to purified tumor antigen (8). It has been shown in other systems that the immune response to antigen can be augmented by injection with antibodies to idiotype before exposure to antigen (9). Since purified GA733 antigen is not available in adequate quantities for immunization, having a molecular clone for the corresponding gene would be desirable, in that quantities of recombinant protein could then be generated. Recombinant immunogen would then be used for the development of protocols for the production of Ab3 in laboratory animals. Ultimately, the treatment of human tumors with a combined Ab2/recombinant tumor-associated antigen regimen could be realized.

In the present study, we determine the partial amino acid sequence of the GA733 antigen expressed in a colorectal carcinoma cell line and isolate a GA733-related gene that is expressed in pancreatic carcinoma cell lines.¶

MATERIALS AND METHODS

Purification and Sequencing of GA733 Antigen. The GA733 antigen was isolated by immunoaffinity chromatography from detergent extracts of SW948 tumors propagated in nude mice as described (2) except that the detergent was omitted from the basic buffer used to elute the antigen from the GA733 antibody column. The fractions judged by electrophoretic transfer (Western) blotting to contain GA733 antigen were pooled, dialyzed against 0.05 M NH_4HCO_3, and lyophilized. The protein was reduced, alkylated, and desalted using LH-20-Sephadex (1.4 × 5.5 cm) equilibrated with 88% formic acid/ethanol/water (20:50:30) (10). This material was judged to be pure by $NaDodSO_4$/PAGE and silver staining. Forty-kilodalton and 30-kDa species were observed, the latter probably representing a proteolytic breakdown product of the 40-kDa antigen.

Several amino-terminal sequence runs were performed (100–500 pmol of carboxymethylated 30-kDa GA733) on a gas-phase sequencer (Applied Biosystems) with on-line phenylthiohydantoin. Standard programs and reagents were used, except the reverse-phase column for phenylthiohydantoin amino acid analysis was a 5-μm, 2.1 × 250 mm, LC-18-DB column (Supelco, Bellefonte, PA).

Human Genomic Library Screening. The amino-terminal 18 residues of the 30-kDa fragment were used for the design of a 54-base oligonucleotide probe, based on preferred codon usage in humans (11). The DNA probe had a 70% G + C content and included a 10-base palindromic structure. The oligomer 5′-GTCGGGGTCGTACAGGCCGTCGTTGTTC-TGCAGGGCGCCCTCGGGCTTGGCCCT-3′ was synthe-

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Abbreviations: PAMs, point accepted mutations; mAb, monoclonal antibody; IL-2, interleukin 2.

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¶The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04152).

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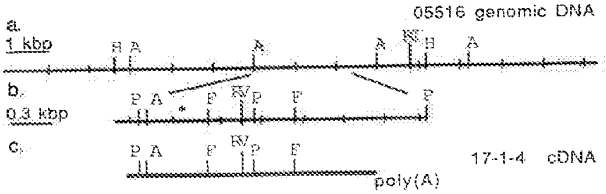


FIG. 1. Relationship between the GA733-1 chromosomal gene and full-length cDNA. (*a*) Restriction map for the 14.3-kbp genomic insert. Direction of transcription is from left to right. (*b*) The 2.2-kbp genomic region that was sequenced. The site of hybridization of the 54-base oligonucleotide probe is shown (*). (*c*) Restriction map of the placental cDNA clone. Enzyme abbreviations are indicated as follows: H, *Hin*dIII; A, *Apa* I; RI, *Eco*RI; P, *Pst* I; F, *Fsp* I; and RV, *Eco*RV.

sized by automated phosphoramidite chemistry on an Applied Biosystems model 380A DNA synthesizer. Full-length 54-mer was isolated by denaturing PAGE and C_{18} chromatography as described (12).

A total human genomic library constructed by Lawn *et al.* (13) and obtained through the American Type Culture Collection (ATCC no. 37333) at the third amplification was plated and transferred to nitrocellulose filters in duplicate. One-half microgram of oligomer was 5′ phosphorylated in a reaction mixture containing 280 μCi of [γ-^{32}P]ATP (5000 Ci/mmol; 1 Ci = 37 GBq) and T4 polynucleotide kinase (12). The reaction mixture was adjusted to contain 0.02 M EDTA/0.5% $NaDodSO_4$ and the labeled oligomer was separated on a Sephadex G-25 column. Prehybridization and hybridization conditions for the use of the 54-base oligonucleotide were identical to those described for a 90-base probe (12).

Structural Analysis of the Genomic Isolate. A restriction map for the 14.3-kilobase-pair (kbp) genomic insert was based on electrophoretic analysis of partial digestion products of the Charon 4A recombinant using ^{32}P-end-labeled oligonucleotides complementary to the phage left and right cohesive ends (Collaborative Research, Waltham, MA).

Dideoxynucleotide sequencing was carried out (14) using T7 DNA polymerase (United States Biochemical, Cleveland) by the standard method in parallel with a method that substitutes dITP for dGTP.

The predicted amino acid sequence was evaluated for homology to known protein sequences using the program

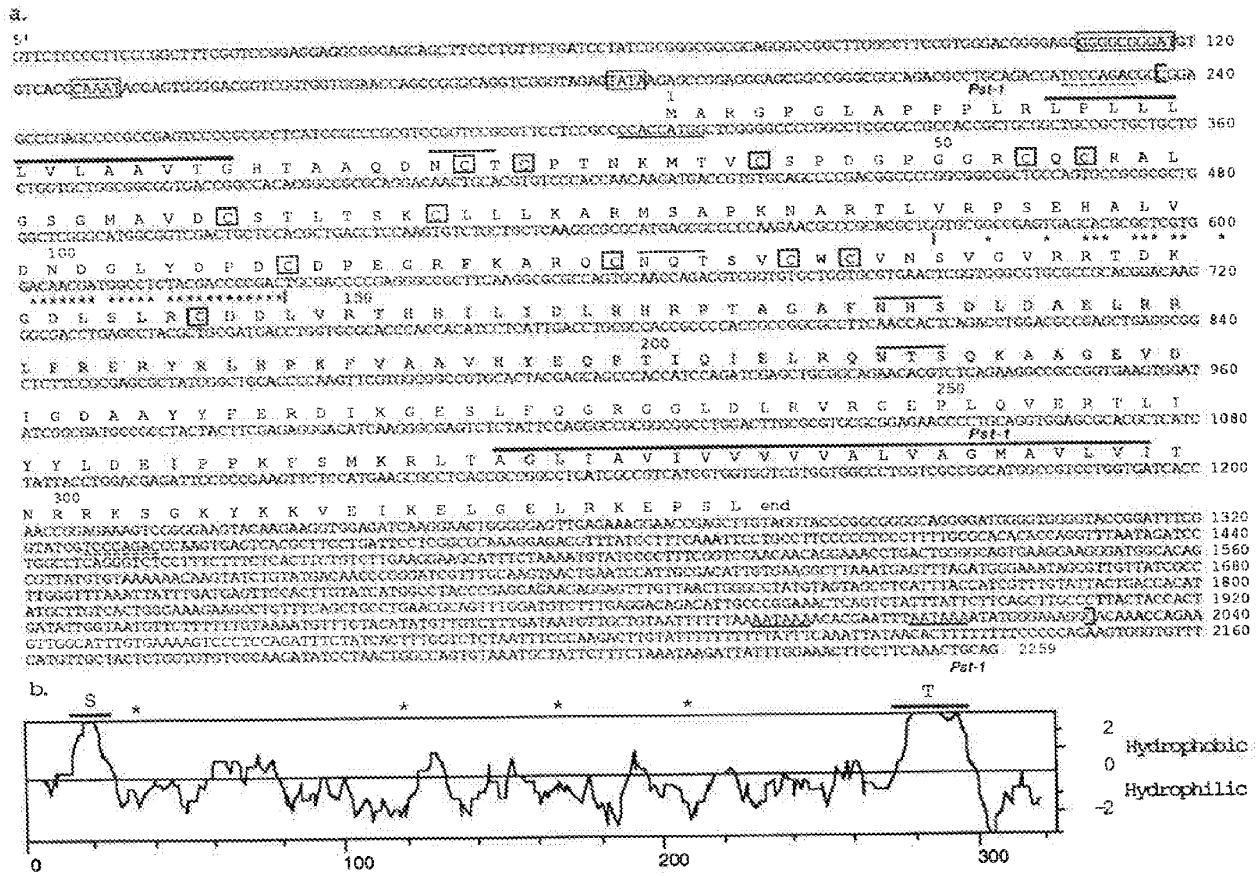


FIG. 2. (*a*) Complete DNA sequence of the GA733-1 chromosomal gene. The region of hybridization of the 54-base oligonucleotide probe (vertical lines) and the positions of base pairing (*) are indicated. Consensus DNA sequences include a putative Sp-1 binding site, a CAAT box, and a TATA box in the promoter region (boxes); a sequence for the initiation of translation (underline); and two possible poly(A) signals (underline). An 8-bp direct repeat flanks the coding region (dashed lines). The predicted protein is characterized by a signal sequence (overline), 12 cysteine residues (boxes), 4 potential N-linked glycosylation sites (overline), and a single 23-residue transmembrane domain (overline). Positions corresponding to the 5′ and 3′ ends of a full-length GA733-1 cDNA clone are marked (brackets). (*b*) Kyte–Doolittle plot of the 323-amino acid sequence of GA733-1. Structural features illustrated are the putative hydrophobic signal peptide (S), the potential N-linked glycosylation sites (*), and the hydrophobic transmembrane domain (T).

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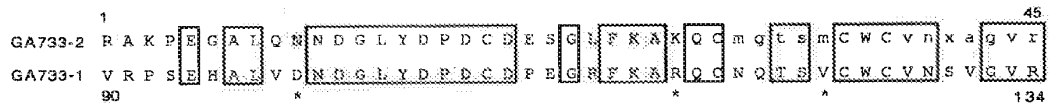


FIG. 3. Sequence comparison of the two GA733 proteins. Lowercase letters indicate provisional assignments; position 41 is undetermined (x). Sequence identities (boxes) and chemically conserved substitutions are illustrated (*).

FASTP (15) to search release 15.0 of the NBRF protein database. Sequences with the highest score were further evaluated with the program ALIGN, using the mutation data matrix [250 point accepted mutations (PAMs)] (16). Unless otherwise indicated, the pairwise alignments were done using a gap penalty of 20. Alignment scores are expressed as standard deviation (SD) units above the mean score of 100 random runs. A score between 3 and 8 is indicative of a possible relationship; scores >8 are considered highly significant.

RNA Preparation and Hybridization Analysis. Cytoplasmic poly(A)$^+$ mRNA was purified as described (17) from the human colorectal carcinoma cell lines SW948 and SW707 and from the pancreatic carcinoma cell lines BXPC-3 and Capan-2 (ATCC). mRNAs were denatured and electrophoresed by the method of Lehrach (18), transferred to nitrocellulose filters, and hybridized to a gel-purified (12), nick-translated 0.85-kbp *Pst* I genomic fragment derived from GA733-1.

RESULTS

The sequence of the amino-terminal 45 residues of the 30-kDa fragment of GA733 was established. The oligonucleotide probe identified two different recombinants from the human total genomic library, as determined by restriction enzyme analysis and by intensity of hybridization signals. Here we analyze in detail the sequence of the initial genomic isolate, 05516, and designate this gene GA733-1; the antigen isolated from the SW948 cell line will be referred to as GA733-2.

Identification of a GA733-Related Gene. To target the initial DNA sequence characterization, 05516 DNA (Fig. 1*a*) was analyzed by Southern blotting and found to contain a 0.85-kbp *Pst* I restriction fragment that hybridized to the oligonucleotide probe (Fig. 1*b*). Analysis with the program BESTFIT (19) indicated that the oligomer hybridized with the *Pst* I fragment at 35 (65%) of 54 positions, with a distribution of base pairing indicative of a related sequence (Fig. 2*a*).

An extended DNA sequence analysis of flanking restriction fragments (Fig. 1*b*) identified a (G + C)-rich promoter region, including three GGGCGG hexanucleotides (Fig. 2*a*). In the context of a decanucleotide, one GC box is identical to that of the simian virus 40 GC box IV, which has been characterized as a medium affinity site for the transcription factor Sp-1 (20), although direct experiments have not been carried out to determine if the GA733-1 promoter is in fact Sp-1 responsive. The promoter also has an atypical CAAT box (21) and a canonical TATA box.

A 323-amino acid protein is predicted with a molecular mass of 35,710 Da, which is consistent with it being a member of a family of 40-kDa glycoproteins. The probable relationship between the 40-kDa and 30-kDa forms of GA733-2 that were observed during the antigen purification may be explained by the finding that the amino-terminal 45 residues of the 30-kDa form of GA733-2 correspond to GA733-1 sequences located 90 residues from the proposed amino terminus. The 40-kDa form probably contains the amino terminus and during isolation, the amino-terminal 90 residues (≈10 kDa) are cleaved, giving rise to the 30-kDa breakdown product. This interpretation is consistent with the finding of 40 kDa being blocked, whereas 30 kDa was not.

A Kyte–Doolittle plot (22) of the predicted protein suggests the features of an integral membrane protein (Fig. 2*b*). A classic signal sequence (23) is predicted with charged residues in the precore sequence, a 13-residue hydrophobic core, and a postcore region containing amino acids with small, uncharged side chains as candidate signal peptidase cleavage sites (Fig. 2*a*). Assuming that the signal peptidase recognition site is T-A-A ↓ , where cleavage (↓) would be located after the fourth amino acid following the core sequence, a 244-amino acid extracellular domain is predicted. A clustering of 12 cysteine residues and 4 potential N-linked glycosylation sites are present in the extracellular domain. A single 23-residue transmembrane domain is followed by a 26-residue cytoplasmic domain, 9 of which are positively charged.

cDNA clones 1.8 kbp in length have been isolated; these clones are probably full-length, as their length correlated with the results of Northern blot experiments (see below). Based on restriction analysis (Fig. 1*c*) and preliminary DNA sequence (S.W., K. Huebner, J.J.P., H.K., and A.J.L., unpublished data), it has been determined that GA733-1 is an intronless gene. The 5′ end residue of the full-length cDNA corresponds to a position in the gene sequence that is 53 bases from the TATA box (Fig. 2*a*), although the actual RNA start site has not been ascertained by a primer-extension experiment. The 3′ end of the cDNA is 13 residues after one of two possible poly(A) signals.

Examination of the DNA sequence presented in Fig. 2*a* using the program REPEAT (19) detected several 8-base direct repeats. One in particular—TCCCAGAC—occurs directly before the probable RNA start site and again in the 3′ untranslated region before the poly(A) addition site.

Homology of the Two GA733 Proteins. The region of 05516 flanking the site of hybridization was evaluated by comparing the open reading frame defined by the oligonucleotide with extended amino acid sequence data derived from native protein. The GA733-1 amino acid sequence was shown to be identical to that of native GA733 antigen sequence at 19 of the first 30 positions, with conserved substitutions occurring at 3 other positions (Fig. 3). In addition, a rare C-W-C amino acid sequence at positions 36–38 of the native protein is also

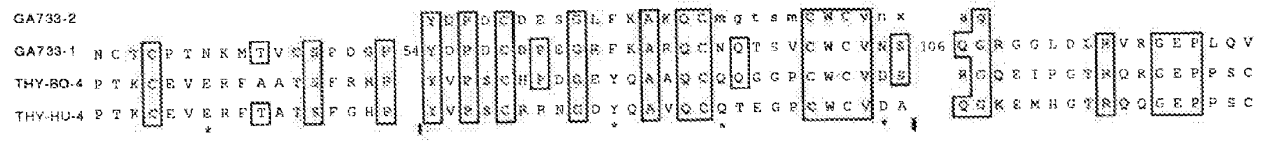


FIG. 4. Homology of thyroglobulin and the two GA733 proteins. GA733 sequences are aligned with the fourth type I repeat unit of bovine and human thyroglobulins (THY-BO-4; THY-HU-4), respectively. Only identities (boxes) and chemically conserved substitutions (*) between GA733 and thyroglobulin sequences are shown. Vertical bars indicate 27 of the 29-residue overlap detected by FASTP. The gapped version was generated by the comparison of GA733-1 sequences with those encoded by exon 8 of the human thyroglobulin gene using the program ALIGN; the other sequences were aligned manually.

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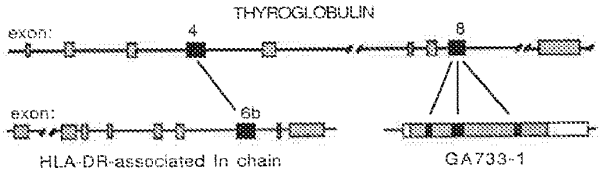


FIG. 5. Relationship between the present-day structures of the human genes for thyroglobulin, the HLA-DR-associated invariant (In) chain, and GA733-1. The exon/intron organization of part of the thyroglobulin gene and all of the HLA-DR-associated In chain gene are illustrated. Exon homologies among genes are indicated (dark boxes). The GA733-1 coding sequence region (filled boxes) and 5′ and 3′ untranslated sequences (open boxes) are shown.

present at corresponding positions in the GA733-1 sequence. Using ALIGN, the alignment score of the first 30 residues of GA733-2 and the GA733-1 sequence was 17 SD units. Thus, our initial genomic isolate together with protein sequence data have proven that GA733 is a member of a family of at least two closely related genes.

Homology of GA733-1 to Other Sequences. Both the GA733-1 and GA733-2 sequences were discovered to be homologous to the human and bovine thyroglobulin type I repeat unit (Fig. 4). The alignment scores of GA733-1 with a 29-residue segment of human and bovine type I repeats are 11 and 14 SD units, respectively, thus establishing the high statistical significance (16) of this homology.

Sequences encoded by each of the 16 known 5′ end exons of the human thyroglobulin gene (24) were aligned to the entire 323-amino acid sequence of GA733-1. Exon 8, which encodes the fourth repeat unit in its entirety, gave the highest alignment score. Two gaps were incorporated into the 61-residue, exon 8-encoded sequence. Even taking these two gaps into account with the high gap penalty of 20, the alignment score was 9.6 SD units.

The GA733 genes are related to the HLA-DR-associated invariant chain gene (25, 26), in that exon 6b of this gene (27) is homologous to the second type I repeat of thyroglobulin, and the second and fourth type I repeats derive from an ancestral 60-amino acid unit (24). The relationships among these three genes are depicted in Fig. 5.

The database search also showed evidence of homology between GA733 sequences and the α subunit of the interleukin 2 (IL-2) growth factor receptor (28). The boundary of the homology coincides with the beginning of exon 2 of the human gene and extends over 45 residues (Fig. 6). The corresponding murine sequences were taken from the cDNA sequence (29). When the homology with the human exon 2 was analyzed by ALIGN, the highest alignment score attainable was 7.5 SD units, using a gap penalty of 40 and matrix bias of +3. This indicates a possible relationship. The GA733 sequences were more distantly related to exon 4, which has common ancestry with exon 2 (28), as 9 of 18 positions identical or chemically similar among the GA733 genes and human exon 2 remain so in exon 4 (Fig. 6).

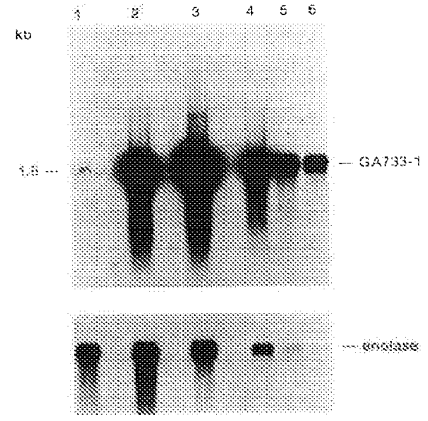


FIG. 7. Northern blot analysis of GA733-1 mRNA in gastrointestinal tumor cell lines. (*Upper*) The nitrocellulose filter contained 2.5 μg each of poly(A)$^+$ mRNAs from the colorectal carcinoma cell line SW948 (lane 1) and the pancreatic carcinoma cell lines BXPC-3 and Capan-2 (lanes 2 and 3, respectively). Capan-2 mRNA was diluted 1:10, 1:50, and 1:100 (lanes 4–6). (*Lower*) The filter was dehybridized and then hybridized with a control enolase probe.

Expression of GA733-1 in Gastrointestinal Tumor Cell Lines. Two pancreatic carcinoma cell lines were observed by Northern blot analysis to express larger amounts of a 1.8-kb mRNA species (Fig. 7 *Upper*, lanes 2 and 3) relative to the colorectal carcinoma cell line SW948 (Fig. 7 *Upper*, lane 1). When the Capan-2 pancreatic carcinoma mRNA was diluted 1:100 (lane 7), the hybridization signal was still more intense compared to that of SW948. This apparent difference in the level of GA733-1 mRNA observed in these two cell types was normalized to enolase mRNA levels, which were observed to be constant in both types (Fig. 7 *Lower*). However, taking into account the relatedness of the GA733 genes, this experiment may not distinguish between transcription of only cross-hybridizing GA733-2 mRNA in SW948 or transcription of GA733-2 in addition to a low level GA733-1 mRNA in these cells. In similarly controlled experiments, GA733-1 mRNA was present in placenta and was not detected in the SW707 rectal carcinoma cell line or in the SK-mel-37 melanoma cell line.

DISCUSSION

A chromosomal gene encoding an integral membrane glycoprotein, termed GA733-1, was isolated and proven to be closely related to the gastrointestinal tumor-associated antigen GA733-2. Paradoxically, although the oligonucleotide probe was designed from the amino acid sequence of purified GA733-2 protein, the probe hybridized most strongly to the related GA733-1 sequence. A possible explanation for this is that the 18 codons used to design the oligomer may be interrupted by an intron in the GA733-2 gene.

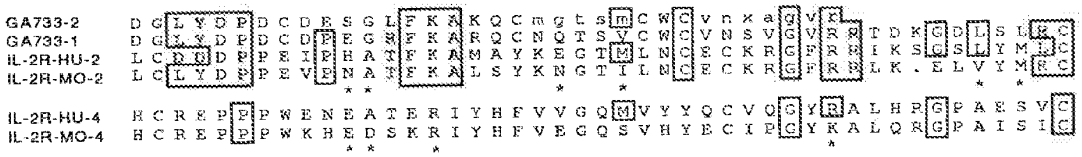


FIG. 6. Homology of the GA733 proteins to the α subunit of the IL-2 receptor. GA733 sequences are aligned with 45 of the 63 residues of the second exon of the human (HU-2) IL-2 receptor gene and with corresponding murine sequences (MO-2). A single-residue gap is inserted in the mouse sequence (.). Sequences encoded by the fourth exon of the human (HU-4) IL-2 receptor gene and the corresponding murine sequences (MO-4) are shown below.

That GA733-1 is a functional intronless gene is rather uncommon. In contrast, inactive retropseudogenes are a relatively frequent occurrence. Although no evidence for the remnants of a poly(A) tail were observed in the genomic sequence, a flanking direct repeat was observed. Thus, retrotransposition (30) is a possible mechanism for the gene duplication in this family, with truncation occurring in the 3' untranslated sequence of the precursor cDNA. This can be evaluated more definitively when the organization of the genes for the other members of this family are determined.

The phenomenon of exon shuffling (31) has probably played a role in the evolution of the GA733 genes, as portions of these genes were found to be homologous to exon 8 of thyroglobulin. The exon shuffling event must have preceded the gene duplication event(s) within the GA733 gene family. After the acquisition of sequences encoding the 60-amino acid domain, insertional events may have ensued, resulting in the segmentation of the domain into three parts. Although statistically significant, the functional significance of this relationship is unknown, as the function of the type 1 repeats in the thyroglobulin molecule is not understood. A comparative analysis of the present-day sequences of the thyroglobulin, HLA-DR-associated invariant chain, and the GA733 genes suggests that two exon shuffling events have occurred.

The GA733 sequences with homology to the IL-2 receptor overlap with those that are related to thyroglobulin. Although exon 2 of the IL-2 receptor and exon 8 of thyroglobulin both derive from ancestral 60-amino acid repeating units (24, 32), no statistically significant relationship could be demonstrated between them. A possible relationship between these two ancestral units is recognizable only in the context of their relationship to GA733 sequences and may be limited to a structural motif present within the two ancestral units.

The homology of GA733-1 to the IL-2 receptor is intriguing, in that it coincides with exon 2 of the IL-2 receptor, which encodes sequences involved in growth factor binding (33). Although conclusions cannot be drawn as to the biological significance of this sequence relationship, it raises the possibility that the GA733 tumor-associated antigen gene family may function as growth factor receptors, perhaps binding growth factors related in structure to IL-2. This seems plausible in light of the finding that the human genome contains sequences homologous to the gene for IL-2 (34).

The isolation of other members of the GA733 gene family will allow a determination of their tissue specificity and their binding reactivities to a group of related mAbs. The generation of additional mAbs of different specificities will also be possible. The availability of recombinant protein or of synthetic peptides for B- and T-cell epitopes will provide immunogen for possible therapeutic applications. The relatedness of GA733-1 to the gastrointestinal-associated antigen GA733-2 and the observed expression of the GA733-1 antigen in pancreatic carcinoma cell lines identifies this gene product as a potential target for immunotherapeutic approaches to this cancer.

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Medical Sciences

Human trophoblast cell-surface antigens defined by monoclonal antibodies

(choriocarcinoma/fetal cells/fluorescence-activated cell-sorter)

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ABSTRACT **A series of monoclonal antibodies has been raised against the human choriocarcinoma cell-line, BeWo. Four antigens, Trop-1, -2, -3, and -4, are defined on normal and malignant trophoblast cells. Trop-1 and Trop-2 appear to be specifically expressed on syncytio- and cytotrophoblasts, whereas Trop-3 and Trop-4 are also detected on various tumor cell lines, normal lymphocytes, and monocytes. Anti-Trop-1 and anti-Trop-2 antibodies might prove useful for detection and isolation of fetal trophoblast cells circulating in pregnant women's blood and for diagnosis and therapy in patients having choriocarcinomas and other germ-cell neoplasms.**

The trophoblast is a fetal-derived tissue located at the interface between the fetus and the maternal circulation. It develops from the inner layer of young proliferative cells, called cytotrophoblasts, from which originates the outer layer of syncytiotrophoblasts that are in direct contact with the maternal circulation. Trophoblast cells sometimes give rise to tumors called choriocarcinomas.

The passage of fetal nucleated cells into the mother has been documented, such cells having been detected in maternal blood samples either directly (1) or after enrichment with a fluorescence-activated cell sorter (FACS) (2, 3). The histological type of these cells, however, has not been determined, and it is not known whether they form a homogenous population. The proximity of trophoblast cells to mothers' blood makes them good candidates for at least some of the fetal cells found in maternal circulation and, indeed, syncytiotrophoblast cells have been observed in maternal lungs (4). Much smaller cytotrophoblast cells might not be trapped in the lung capillary network and would continue to circulate. Specific antibodies to trophoblasts could be used to identify FACS-sorted cells or as fluorescent reagents to label them so they can be separated with the FACS. Xenogeneic antisera have been raised to human trophoblast cells (5), but using these for isolating rare trophoblasts from maternal blood cells is greatly hampered by their evident complexity, which requires that they be extensively absorbed.

The emergence of hybridoma technology, which overcomes the technical limitations of conventional serology (6), has permitted us to raise monoclonal antibodies to human trophoblast cells. This communication deals with the generation and characterization of a series of such monoclonal antibodies to various antigens expressed on normal and malignant trophoblast cells. Four new antigens, named Trop-1, Trop-2, Trop-3, and Trop-4, are defined with these antibodies.

MATERIALS AND METHODS

Cell Culture. The BeWo, JEG, Reid, and JAR choriocarcinoma cell lines were obtained from Howard Sussman of Stanford University and from Janice Chou of the National Institutes of Health. They were grown in monolayers in 50% Waymouth's medium (Irvine Scientific, Santa Ana, CA)/40% balanced salt solution/10% newborn calf serum (Irvine Scientific) supplemented with $NaHCO_3$ to 7.5% final concentration and glutamine. The HT1080C and HeLaS3 cell lines were obtained from Douglas Wallace, Stanford University and grown as monolayers in glutamine-supplemented RPMI 1640 (Irvine Scientific)/15% newborn calf serum. The adherent cells were suspended by using 0.25% trypsin/EDTA (GIBCO). All the other cell lines (including the hybridomas) were grown in suspension in RPMI 1640/15% newborn calf serum.

Production of Monoclonal Antibodies. Two BALB/cN female mice were immunized and boosted 3 weeks later with 10^6 cells of the BeWo choriocarcinoma cell line injected intraperitoneally. Three days after the boost, spleen cells were harvested and fused to NS-1 myeloma cells as described (7) at a 4:1 ratio in the presence of 50% polyethylene glycol 1500 (BDH Chemicals, Poole, England). After the fusion, 4×10^5 cells per well were incubated in 0.2 ml of hypoxanthine/aminopterin/thymidine.

Initial Screening of Hybrid Production and Cloning. On the 11th day after the fusion, all 150 cultures showed growth. Culture supernates were harvested and screened for antibodies to the immunizing BeWo cell line by using a radioimmunoassay; 2×10^4 BeWo cells were plated in wells of microtiter plates (Costar, Division of Data Packaging, Cambridge, MA), and allowed to adhere to the plastic overnight, and then washed with radioimmunoassay buffer. The cells were incubated for 1 hr with 20 μl of supernate and then washed three times. Anti-BeWo antibodies were detected by adding, for 1 hr, 30,000 cpm of an ^{125}I-labeled goat anti-mouse immunoglobulin antiserum absorbed on a human immunoglobulin immunosorbent. Incubations were at room temperature. After three more washes, the cells were lysed in the presence of 1% Nonidet D. The lysates were harvested with a cotton tip swab and assayed in a gamma scintillation counter (Micromedic Systems, Division of Rohm and Haas, Philadelphia, PA). Individual viable cells from positive cultures of interest were directly deposited into microculture wells with the FACS. In a previous report (8), the FACS was used to sort individual hybrids on the basis of their binding of fluorescent microspheres coupled with antigen. In the present case, however, the cells were selected on the basis of viability only. Growing clones were tested 1 week later for production of the desired monoclonal antibody. Monoclonal antibody isotypes were determined by reaction with mouse allotype-specific I-labeled monoclonal antibodies (9). Clones 162-21.2, 162-25.3, and 162-28.2 are IgG2a (*Igh-1a* allotype), and clones 162-43.4 and 162-46.2 are IgG1 (*Igh-4a* allotype) (10).

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Abbreviation: FACS, fluorescence-activated cell sorter.

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Table 1. Distribution of Trop antigens on normal and tumor cells

Target cell	Trop-1 162-21.2	Trop-2 162-25.3 and 162-46.2	Trop-3 162-10.2	Trop-4 162-28.2 and 162-43.4
Choriocarcinoma				
BeWo	+	+	+	+
JEG	+	+	+	+
Reid	+	+	ND	ND
JAR	+	−	ND	ND
Fibrosarcoma				
(HT1080C)	+	−	+	+
Cervix carcinoma				
(HeLa S3)	−	−	+	+
Colon carcinoma				
HT18	−	−	ND	ND
HT29	−	−	ND	ND
Melanoma				
(Shroet)	−	−	ND	ND
Neuroblastoma				
(TG8)	−	−	ND	ND
Erythroleukemia				
(K562)	+	−	+	+
Lymphoid				
Ramos (B lymphoma)	−	−	+	+
Molt-4 (T)	−	−	+	+
Normal blood				
Lymphocytes	−	−	+*	+
Monocytes	−	−	+	+
Platelets	−	−	+	−
Erythrocytes	−	−	−	−

The reactivity of anti-Trop monoclonal antibodies was tested against various target cells by indirect immunofluorescence and FACS analysis (or by radioimmuno binding assay). Target cells were incubated with 50 μl of antibody containing hybridoma supernate, and the reaction was revealed by incubation with a fluoresceinated (or ^{125}I-labeled) anti-mouse immunoglobulin. Intensity of fluorescence (or radioactivity) bound per target cell was compared with background fluorescence (or radioactivity) obtained from target cells incubated with anti-mouse immunoglobulin only. Results were scored as positive (+) or negative (−). ND, not done.
* Two subsets with different antigen densities.

Indirect Immunofluorescent Staining. Target cells (2×10^5) obtained from cultured cell lines or mononuclear cells from Ficoll-Paque (Pharmacia) preparations of human peripheral blood samples were incubated for 45 min on ice in 100 μl of RPMI 1640 containing 50 μl of hybridoma supernate. After two washes, the cells were incubated in the presence of a saturating amount of a fluorescein-coupled goat anti-mouse immunoglobulin antiserum absorbed on human immunoglobulins. The cells were washed twice, resuspended, and immediately analyzed on a FACS.

Blocking Studies. For investigating the proximity or identity of two antigenic determinants recognized by two monoclonal antibodies of distinct isotypes, the target cells were incubated first with 50 μl of one monoclonal antibody for 30 min and then with 50 μl of the second monoclonal antibody for another 30 min. After washing, the reaction of the latter with the target antigen was detected with a fluorescent goat anti-mouse IgG2a or IgG1 specific for the second monoclonal antibody used.

Enzymic Treatments. For studies of the enzyme sensitivity of the antigenic determinants recognized by the monoclonal antibodies, 2×10^5 target cells were incubated in RPMI 1640 at 37°C in the presence of various enzymes (see *Results*) and then washed and processed for indirect immunofluorescent staining. Enzymes used were Pronase and neuraminidase (Calbiochem/Behring), trypsin and α-chymotrypsin (Worthington), and a mixture of 17 glycosidases (from *Turbo cornutus*, Miles).

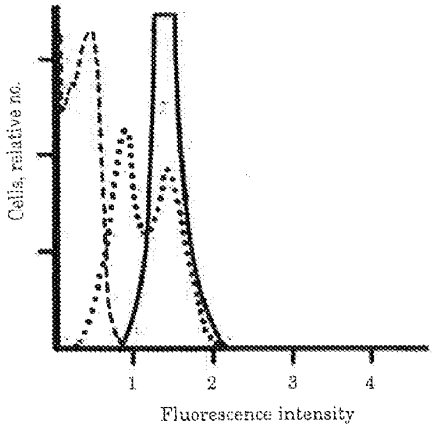


FIG. 1. Immunofluorescence staining of peripheral blood lymphocytes. Target cells were incubated for 30 min with 50 μl of medium (----), hybridoma supernate containing anti-Trop-3 162-10.2 (·····), or anti-Trop-4 162-28.2 (——) antibodies. The reaction was revealed by a fluoresceinated goat anti-mouse immunoglobulin antiserum and analyzed on a FACS. Fluorescence units are arbitrary and given on a logarithmic (base 10) scale.

FACS Analysis. Cells were analyzed as described (11) with a modified FACS-II (Becton Dickinson FACS Systems, Sunnyvale, CA) fitted with a logarithmic amplifier (12).

Tissue Section Immunoperoxidase Staining. Portions of placentas were obtained from early abortions carried out for rea-

Table 2. Blocking studies with anti-Trop monoclonal antibodies

Blocking antibody	Staining antibody	Mean fluorescence intensity	Blocking
	Experiment 1		
—	—	1.7*	
—	—	1.7†	
—	162-46.2	2.1*	
162-25.3	162-46.2	1.8*	Yes
162-21.2	162-46.2	2.1*	No
—	162-25.3	2.7†	
162-46.2	162-25.3	2.0†	Yes
	Experiment 2		
—	162-21.2	3.3†	
162-46.2	162-21.2	3.2†	No
—	—	1.5*	
—	—	1.3†	
—	162-28.2	3.4†	
162-43.4	162-28.2	2.2†	Yes
—	162-43.4	2.8*	
162-28.2	162-43.4	1.4*	Yes

Target cells were incubated for 30 min with 50 μl of hybridoma supernatant containing blocking antibody. Supernate (50 μl) containing the staining antibody was then added to the incubation medium. After 30 min more, the reaction was revealed with a fluoresceinated goat anti-mouse IgG1 or IgG2a. The cells were analyzed on the FACS. Mean fluorescence intensities are given on a logarithmic (base 10) scale. Antibodies are of the IgG1 (162-46.2 and 162-43.4) or IgG2a (162-25.3, 162-21.2, and 162-28.2) isotype.
* Stained with fluoresceinated anti-γ_1.
† Stained with fluoresceinated anti-γ_{2a}.

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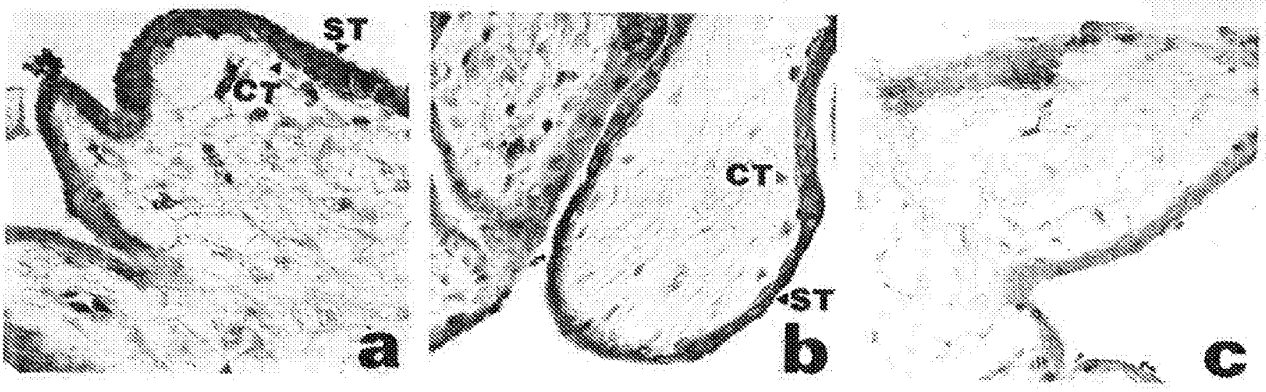


FIG. 2. Placenta section immunoperoxidase staining. Formalin-fixed paraffin-embedded sections of a 10-week placenta were stained with anti-Trop-1 (*a*), anti-Trop-2 (*b*) and 11-4.1 (control) (*c*) antibodies and then incubated with a peroxidase-coupled anti-mouse immunoglobulin. Both cytotrophoblast (CT) and syncytiotrophoblast (ST) stain positively with anti-Trop-1 and anti-Trop-2 antibodies.

sons independent of this study. Normal liver, kidney, spleen, thymus, and lymph nodes were obtained from portions of surgical specimens not required for pathologic examination. Tissue was frozen, cut, and stained as described (13) except that the last stage applied was peroxidase-coupled rabbit anti-mouse immunoglobulin (Dako, distributed by Accurate Chemical and Scientific, Hicksville, NY), which was followed by diaminobenzidine (Sigma) at 1 mg/ml in phosphate-buffered saline with 10 μl of 30% H_2O_2 per ml. After a 2- min incubation, the slides were washed, incubated with 0.5% $CuSO_4$ in isotonic saline for 5 min, washed, dehydrated, and mounted in Permabond. Formalin-fixed paraffin-embedded placenta was stained in the same way. Undiluted hybridoma culture supernates were used for this staining.

RESULTS

We examined the cellular reactivity of six distinct anti-BeWo monoclonal antibodies by indirect immunofluorescence staining and FACS analysis using a series of human tumor and lymphoid cell lines and normal peripheral blood mononuclear cells (Table 1). Three antibodies, 162-10.2, 162-28.2, and 162-43.4, stained all cell lines tested, as well as blood lymphocytes and monocytes. However, these antibodies detect two different antigens; distinct peripheral blood lymphocyte FACS staining profiles were obtained with 162-10.2 on the one hand and with 162-28.2 and 162-43.4 on the other hand (Fig. 1). The FACS staining profiles on all cells tested with 162-28.2 and 162-43.4 were similar. As each antibody also blocked the staining of the target with the other (Table 2), both antibodies probably react with the same or proximal determinants of the same antigenic molecule. This antigen, called Trop-4, does not distinguish lymphocyte subpopulations. In contrast, Trop-3, detected by 162-10.2, is present on about half of the lymphocytes with a considerably higher density than on the other half (Fig. 1).

The other three monoclonal antibodies appear to be trophoblast-specific (Table 1). 162-21.2 reacts with an antigen designated Trop-1. 162-25.3 and 162-46.2 both react with a second antigen referred to as Trop-2. They crossblock each other but do not block the staining of Trop-1 with 162-21.2 (Table 2). Trop-1 is present on all four choriocarcinoma cell lines and also, at a much lower density, on the fibrosarcoma and the K562 cell lines. It is absent from normal blood cells and various other tumor cell lines (Table 1). The expression of Trop-2 is restricted to three of the four choriocarcinoma cell lines (Table 1). It could not be detected on any of the other normal or tumor cells tested.

Sections of placenta were stained with all six monoclonal antibodies and with 11-4.1, a mouse immunoglobulin (of 2a subclass) that does not react with human tissues as a control. All four Trop antigens were detectable on both frozen and fixed paraffin-embedded sections. Fig. 2 illustrates staining with anti-Trop-1 and anti-Trop-2 antibodies. Study of frozen sections of normal adult liver, kidney, spleen, thymus, and lymph nodes with anti-Trop-1 and anti-Trop-2 antibodies did not produce any detectable staining.

We were unable to immunoprecipitate any I-labeled protein from lactoperoxidase-labeled choriocarcinoma cells or cell membranes. However, the Trop-3 antigen could be precipitated with the 162-10.2 antibody from I-labeled peripheral blood lymphocytes. $NaDodSO_4$/polyacrylamide gel electrophoresis analysis indicates that Trop-3 consists of a single chain protein of $M_r \approx 200{,}000$ (data not shown). An indication that the other three Trop antigens are also associated with proteins was obtained by protease and glycosidase treatment of cells bearing these antigens. Results shown in Table 3 indicate that the staining for Trop-1 and Trop-3 antigens was virtually abrogated, while the staining for Trop-2 and Trop-4 was greatly decreased, after incubating 2×10^6 cells for 2 hr with 500 μg of Pronase. Treatment of the cells with 500 μg of trypsin drastically decreased the staining for Trop-1, Trop-2, and Trop-3, whereas Trop-4 was hardly affected. The staining for all four antigens was partially decreased after treatment with 500 μg of α-chymotrypsin. No decrease in staining was obtained by pretreating the cells with neuraminidase or a mixture of 17 glycosidases (data not shown).

Table 3. Percentage decrease of staining after treatment of target cells with proteases

	Treatment		
Antigen	α-Chymotrypsin	Trypsin	Pronase
Trop-1	38	81	99
Trop-2	42	86	61
Trop-3	41	91	100
Trop-4	48	7	90

Target cells were incubated for 2 hr with 500 μg of enzyme and, the reaction was stopped by washing the cells with serum-containing medium. Cells were then divided into aliquots and stained with anti-Trop antibodies, and the reaction was revealed by incubation with a fluoresceinated goat anti-mouse immunoglobulin antiserum and subjected to FACS analysis.

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Ex. 1002

DISCUSSION

We have reported the production of monoclonal antibodies raised against the human neoplastic trophoblast cell line BeWo. Four newly recognized antigens, called Trop-1, Trop-2, Trop-3, and Trop-4, have been defined with six of these antibodies on the cell surfaces of normal and malignant trophoblast cells. Two main cell distribution patterns have been observed by testing the reactivity of the monoclonal antibodies against a panel of human cell lines and peripheral blood cells. The expression of the Trop-1 and Trop-2 antigens appeared to be virtually restricted to cells of trophoblast origin as they reacted only with normal trophoblast cells and choriocarcinomas, with the exceptions of the fibrosarcoma cell line HT1080C and the erythroleukemia cell line K562, which expressed a low density of Trop-1. Conversely, monoclonal antibodies to Trop-3 and Trop-4 antigens displayed a wide range of reactivity and were positive with various solid tumor and lymphoid cell lines and with normal nucleated peripheral blood cells. Interestingly, FACS analysis of immunofluorescent staining showed that, whereas Trop-4 was homogenously distributed, Trop-3 on the contrary was not evenly expressed on peripheral blood lymphocytes and monocytes; about one-half of the total lymphocyte and monocyte populations expressed a higher antigen density (as much as a 4-fold effect in some individuals) than the other half.

In previously reported studies with xenogeneic antitrophoblast antisera, trophoblast surface antigens were classified into two major groups (5). The first group (TA1) included antigens present on trophoblast and some tumor cell lines; the second group (TA2) included antigens widely expressed on normal and tumor tissues. It is difficult to compare monoclonal antibodies with conventional antisera because of the complexity of the latter. It appears, however, that the anti-Trop-1 and anti-Trop-2 monoclonal antibodies, on one hand, and the anti-Trop-3 and anti-Trop-4 monoclonal antibodies, on the other hand, define antigens related to the TA1 and TA2 groups, respectively.

Enzymatic studies indicate that all four antigens are associated with polypeptides. The staining for all four was greatly decreased after a 2-hr incubation of the target cells with large amounts (500 μg) of proteases. By titrating the Pronase sensitivity of the four antigens, we found that Trop-3 was the most Pronase-sensitive antigen; its staining was abolished by treating the target cells for 60 min at 37°C with as little as 30 μg of Pronase, whereas Trop-2 and Trop-4 were comparatively more Pronase resistant and Trop-1 showed an intermediate sensitivity. A protein consisting of a single chain of $M_r \approx 200{,}000$ was precipitated from peripheral blood lymphocytes with anti-Trop-3 antibodies.

The function these antigens fulfill at the surface of trophoblast and other cells is currently unknown. In this regard, it is noteworthy that among the six monoclonal antibodies, only the two anti-Trop-4 antibodies added to the culture medium of a mixed lymphocyte reaction inhibit the allogeneically stimulated proliferation.

We foresee several possible clinical applications for trophoblast-specific reagents such as the anti-Trop-1 and anti-Trop-2 monoclonal antibodies described here. They could be used to investigate the passage of fetal trophoblast cells into maternal circulation during pregnancy and, if present, to sort them with a FACS for possible prenatal diagnostic use. They could also be coupled to radionuclide probes to detect and then deliver local irradiation to primary tumors and metastases in patients with choriocarcinomas and other germ-cell neoplasms. They might also be able to immunologically target cytotoxic agents coupled to the antibodies or antibody fragments as has been described (14, 15). Finally, studies performed in the rat have indicated that xenogeneic antitrophoblast antisera, when injected into mated female rats after being absorbed with lymphoid cells, showed an abortifacient activity (16). Thus, the anti-Trop-1 and anti-Trop-2 monoclonal antibodies described here could have useful investigative, diagnostic, and therapeutic applications.

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Engineered antibody–drug conjugates with defined sites and stoichiometries of drug attachment

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The chimeric anti-CD30 IgG_1, cAC10, conjugated to eight equivalents of monomethyl auristatin E (MMAE) was previously shown to have potent antitumor activity against CD30-expressing tumors xenografts in mice. Moreover, the therapeutic index was increased by lowering the stoichiometry from 8 drugs/antibody down to 2 or 4. Limitations of such 'partially-loaded' conjugates are low yield (10–30%) as they are purified from mixtures with variable stoichiometry (0–8 drugs/antibody), and heterogeneity as the 2 or 4 drugs are distributed over eight possible cysteine conjugation sites. Here, the solvent-accessible cysteines that form the interchain disulfide bonds in cAC10 were replaced with serine, to reduce the eight potential conjugation sites down to 4 or 2. These Cys→Ser antibody variants were conjugated to MMAE in near quantitative yield (89–96%) with defined stoichiometries (2 or 4 drugs/antibody) and sites of drug attachment. The engineered antibody–drug conjugates have comparable antigen-binding affinities and *in vitro* cytotoxic activities with corresponding purified parental antibody–drug conjugates. Additionally, the engineered and parental antibody–drug conjugates have similar *in vivo* properties including antitumor activity, pharmacokinetics and maximum tolerated dose. Our strategy for generating antibody–drug conjugates with defined sites and stoichiometries of drug loading is potentially broadly applicable to other antibodies as it involves engineering of constant domains.

Keywords: auristatin/CD30/conjugate/engineered antibody

Introduction

Antibody conjugation to potent cytotoxic drugs is a promising way to enhance the antitumor activity of antibodies and reduce the systemic toxicity of drugs, as evidenced by numerous examples of preclinical efficacy and at least six antibody–drug conjugates currently in clinical development (Lambert, 2005; Wu and Senter, 2005). Clinical demonstration of the antibody–drug conjugate concept is provided by the approval of gemtuzumab ozogamicin (Mylotarg) for the treatment of acute myeloid leukemia (Bross *et al.*, 2001). Gemtuzumab ozogamicin is a humanized anti-CD33 IgG_4 conjugated to calicheamicin, a highly cytotoxic natural product that induces double-stranded DNA cleavages (Hamann *et al.*, 2002a,b). Other drugs that have been commonly conjugated to antibodies include auristatins and maytansinoids, which potently inhibit tubulin polymerization (Lambert, 2005; Wu and Senter, 2005).

Many different antibodies conjugated to auristatins have robust antitumor activity in mouse tumor xenograft studies including ones that target: CD30 (Doronina *et al.*, 2003; Francisco *et al.*, 2003), sialyl Lewis^y (Doronina *et al.*, 2003), E selectin (Bhaskar *et al.*, 2003), CD20 (Law *et al.*, 2004), EphB2 (Mao *et al.*, 2004), TMEFF2 (Afar *et al.*, 2004) and CD70 (Law *et al.*, 2006). For example, the chimeric anti-CD30 IgG_1, cAC10 (Wahl *et al.*, 2002), was conjugated via its eight solvent-accessible cysteine residues to monomethyl auristatin E (MMAE) in near quantitative yield (Doronina *et al.*, 2003). This homogeneous conjugate with 8 drugs/antibody was highly efficacious in SCID mouse xenograft models of anaplastic large cell lymphoma and Hodgkin's disease with a therapeutic index (curative dose/maximum tolerated dose) of 30–60 (Doronina *et al.*, 2003; Francisco *et al.*, 2003). A further 2- to 3-fold improvement in therapeutic index was achieved by reducing the drug stoichiometry from 8 drugs/antibody down to 2 or 4 drugs/antibody (Hamblett *et al.*, 2004). A potential drawback of such 'partially-loaded' conjugates is heterogeneity in the stoichiometries and sites of drug attachment. For example, a conjugate with a mean stoichiometry of 4 drugs/antibody is a mixture with 0, 2, 4, 6 or 8 drugs/antibody (Hamblett *et al.*, 2004; Sun *et al.*, 2005). Conjugates with uniform stoichiometry of 2 or 4 drugs/antibody were obtained by purification of corresponding mixtures, albeit with substantial reduction in yield and remaining heterogeneity as the 2 or 4 drugs were distributed over eight possible conjugation sites.

Here, we have generated antibody–drug conjugates with defined sites and stoichiometries of drug loading as a possible way to enhance the clinical potential of this class of antibody therapeutics. The solvent-accessible cysteine residues in the antibody cAC10 were replaced with a homologous residue, serine, to generate variants with either two or four remaining accessible cysteines. The Cys→Ser variants were then used to generate homogeneous and precisely defined antibody–drug conjugates that were then compared extensively *in vitro* and *in vivo* with more heterogeneous antibody–drug conjugates derived from prior methods (Hamblett *et al.*, 2004; Sun *et al.*, 2005).

Materials and methods

Cells and reagents

The anaplastic large cell lymphoma line, Karpas-299 (CD30 positive), and the non-Hodgkin's lymphoma cell line, WSU (CD30-negative), were obtained from the Deutsche

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Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweig, Germany). L540cy (CD30 positive), a derivative of the Hodgkin's disease cell line L540 adapted to xenograft growth, was developed by Dr Harald Stein (Institüt für Pathologie, University Veinikum Benjamin Franklin, Berlin, Germany). Cell lines were grown in RPMI-1640 media (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum.

Mutagenesis and subcloning

The construction of cAC10, a chimeric form of the murine monoclonal antibody, AC10, has previously been described (Wahl *et al.*, 2002). Mutants of cAC10 were generated in pBluescript vectors containing cDNAs for either cAC10 heavy (pBSSK-AC10H) or light (pBSSK-AC10L) chain using a Quikchange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and pairs of complementary oligonucleotide primers. The plus strand primers for the heavy chain mutants were (mutated codons underlined):

C220S, 5′-GTTGAGCCCAAATCTTCTGACAAAACTCACACATGCCC-3′;

C226S, 5′-GACAAAACTCACACATCCCCACCGTGCCCAGC-3′;

C226S:C229S, 5′-GACAAAACTCACACATCCCCACCGTCCCCAGCACCTGAACTC-3′.

Sequential rounds of mutagenesis were performed to gene rate the C220S:C226S and the C220S:C226S:C229S mutants. Similarly, the plus strand primer for the light chain mutant was C218S, 5′-CTTCAACAGGGGAGAGTCTTAGACGCGTATTGG-3′ (mutated codon underlined). The cAC10 heavy chain variant cDNAs were excised from pBluescript using *Xho*I and *Xba*I and ligated into the mammalian expression vector pDEF38 (Running Deer and Allison, 2004) downstream of the CHEF EF-1α promoter. The cAC10 light chain variant cDNAs were excised from pBluescript with *Mlu*I and cloned into similarly cleaved pDEF38 downstream of the CHEF EF-1α promoter.

Antibody expression

The cAC10 variants were stably expressed in a CHO-DG44 cell line as previously described for the cAC10 parent antibody (Wahl *et al.*, 2002). Briefly, 50 μg of each of the heavy and light chain expression constructs in vector pDEF38 were linearized with *Pvu*I and then coelectroporated into CHO-DG44 cells (Urlaub *et al.*, 1986). Stable cell lines expressing the cAC10 variants (Table I) were selected in hypoxanthine and thymidine deficient EX-CELL™ 325 media (JRH Biosciences Inc., Lenexa, KS, USA). High titer clones identified by antigen-binding ELISA were recovered by limiting dilution cloning and cultured in spinner flasks (2.5 l) or WAVE bioreactors (5–10 l, WAVE Biotech LLC, Bridgewater, NJ, USA).

Antibody purification

The cAC10 parent and variant antibodies were purified by protein A followed by anion exchange chromatography using a FPLC (ÄKTAexplorer, GE Healthcare, Piscataway, NJ, USA). Briefly, the antibody-containing conditioned media were concentrated ~10-fold and buffer-exchanged into phosphate-buffered saline (PBS), pH 7.4 by tangential flow filtration (Millipore, Bedford, MA, USA). The concentrated samples were treated with 0.5% (v/v) Triton X-100 (Sigma, St Louis, MO, USA) with gentle stirring overnight at 4°C for endotoxin removal. Samples were loaded on to a protein A column (GE Healthcare) pre-equilibrated with PBS, pH 7.4. The column was washed with PBS, pH 7.4, 2–3 column volumes (CV) 0.5% v/v Triton X-100, 1 M NaCl in PBS, pH 7.4 then with PBS, pH 7.4 until a stable baseline was reached. Bound antibody was eluted from protein A with 30 mM sodium acetate, pH 3.6 and then dialyzed against 20 mM Tris–HCl, 10 mM NaCl, 1 mM EDTA, pH 8.0 (buffer A). The pooled antibody was then loaded on to Q sepharose (GE Healthcare) pre-equilibrated with buffer A and washed with 2–3 CV buffer A, 5–10 CV buffer A containing 0.5% (v/v) Triton X-100 and 5 CV buffer A. Antibodies were eluted from Q sepharose by either step or linear NaCl gradient from 10–500 mM NaCl in buffer A and dialyzed against PBS, pH 7.4. Purified antibodies were analyzed by SDS–PAGE and by TSK-Gel G3000SW HPLC size exclusion chromatography (Tosoh Bioscience, Montgomeryville, PA, USA).

Table I. Cys→Ser antibody variants

Variant name[a]	Location of Cys→Ser mutations[b]	Competition binding to Karpas-299 (IC_{50}, nM)[c]
C8	None (parent)	2.8 ± 0.1
C2v1	L214, H220, H226	2.2 ± 0.4
C2v2	H220, H226, H229	2.6 ± 0.4
C4v1	L214, H220	3.2 ± 0.4
C4v2	H226, H229	2.4 ± 0.1
C4v3	H220, H226	nd[d]

[a]cAC10 variants are identified by the number of solvent-accessible cysteine residues and, where necessary, a variant number. E.g. C2v1 denotes a cAC10 variant containing two solvent-accessible cysteine residues (Fig. 1).
[b]L, light chain; H, heavy chain; numbering scheme of Kabat *et al.* (1991).
[c]Mean (± SEM) for three or more independent experiments.
[d]nd, not determined. Antibody variant C4v3 was excluded from the remainder of this study as size exclusion chromatography suggested significant aggregation, and SDS–PAGE analysis was not consistent with the behavior anticipated for one interchain disulfide bond between heavy chains).

Antibody conjugation

Conjugation of cAC10 Cys→Ser antibody variants with either 2 (variants C2v1–E2 and C2v2–E2) or 4 (variants C4v1–E4 and C4v2–E4) equivalents of MMAE involved antibody reduction with a few (2.5–4) equivalents of tris(2-carboxyethyl)phosphine (Acros Organics, Geel, Belgium) and conjugation to maleimidocaproyl-valine-citrulline-*p*-aminobenzoyl-MMAE (vcMMAE) (Doronina *et al.*, 2003) without removal of excess tris(2-carboxyethyl)phosphine. The extent of reduction was assessed prior to drug addition by purifying a small amount of the reduction reaction through a PD-10 column (GE Healthcare) and titrating the number of antibody-cysteine thiols with 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellman, 1958). The reduced antibodies were reacted with vcMMAE for 60 min at 0°C and excess *N*-acetylcysteine (Acros Organics) then added to quench any unreacted vcMMAE. The reaction mixture was then diluted 5-fold with water and then loaded on to a hydroxyapatite column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with 10 mM sodium phosphate pH 7.0, 10 mM NaCl. The column was washed with 5 CV of the same buffer and the conjugate eluted with 100 mM sodium phosphate, pH 7.0 and 10 mM NaCl. The

conjugates were concentrated and buffer-exchanged into PBS using Amicon Ultrafree centrifugal filter units (Millipore, Bedford, MA, USA).

The generation of cAC10 conjugates with a mean stoichiometry of 4 drugs/antibody, C8–E4 mixture (C8–E4M, range of 0–8 drugs/antibody), and 2 drugs/antibody, C8–E2 mixture (C8–E2M), have been described (Hamblett *et al.*, 2004; Sun *et al.*, 2005). C8–E2M was subjected to hydrophobic interaction chromatography to isolate conjugates loaded with a uniform stoichiometry of 4 drugs/antibody (C8–E4) and 2 drugs/antibody (C8–E2) as previously described (Hamblett *et al.*, 2004).

Conjugate analysis

Antibody–drug conjugates were analyzed to determine the stoichiometry of drug loading using the molar extinction coefficients at wavelengths of 248 and 280 nm for the antibody (9.41×10^4 and 2.34×10^5 M^{-1} cm^{-1}, respectively) and drug (1.50×10^3 and 1.59×10^4 M^{-1} cm^{-1}, respectively), as previously described (Hamblett *et al.*, 2004). The location of drug attachment to the antibody heavy and light chains was investigated by reverse phase HPLC using a PLRP-S column (Polymer Laboratories, Amherst, MA, USA; No. 1912-1802: 1000 Å, 8 μm, 2.1 × 150 mm) and solvents A [0.05% (v/v) trifluoroacetic acid in water] and B [0.04% (v/v) trifluroacetic acid in acetonitrile] at a flow rate of 1 ml/min at 80°C. The solvent conditions were as follows: isocratic 25% solvent B (3 min), linear gradient to 50% solvent B (25 min), linear gradient to 95% solvent B (2 min), linear gradient to 25% solvent B (1 min) and isocratic 25% solvent B (2 min). Prior to chromatography, antibody–drug conjugate samples (10–20 μl, 1 mg/ml) were reduced with 20 mM DTT at 37°C for 15 min to cleave the remaining interchain disulfide bonds.

Endotoxin measurement

Endotoxin levels in antibody and antibody–drug conjugate preparations were determined by quantitative kinetic or endpoint Limulus amebocyte lysate assay using a chromogenic substrate as described by the vendor (Cambrex, Walkersville, MD, USA). Briefly, the sample was mixed with the Limulus amebocyte lysate and substrate reagent, and the absorbance at 405 nm measured over time (WinKQCL, Cambrex) or after 15 min incubation for kinetic and endpoint assays, respectively. The sample-related inhibition or enhancement of the Limulus amebocyte lysate endotoxin reaction was also analyzed by spiking serial dilutions of the sample with constant endotoxin concentrations for the endotoxin measurement in the sample allowing simultaneous detection of quality and quantity of sample and sample-induced interference. The endotoxin level in samples was then determined from the endotoxin standard curve generated by each assay method, which is linear over a concentration range. All purified antibody and antibody–drug conjugates had ≤0.05 endotoxin units/mg protein, which is at least 5-fold below the endotoxin level judged acceptable for *in vivo* studies.

In vitro characterization of antibody variants and drug conjugates

Competition binding of the cAC10 antibody variants and their corresponding drug conjugates was undertaken to assess the impact of mutations and drug conjugation upon antigen binding. Briefly, cAC10 was first labeled with europium using a protocol from the vendor (Perkin-Elmer, Boston, MA, USA). CD30-positive Karpas-299 cells were then combined with serial dilutions of the cAC10 parent antibody, variants or corresponding antibody–drug conjugate in the presence of 1 μg/ml europium-labeled cAC10 in staining medium [50 mM Tris–HCl pH 8.0, 0.9% (w/v) NaCl, 0.5% (w/v) bovine serum albumin, 10 μM EDTA] for 30 min on ice then washed twice with ice-cold staining medium. Labeled cells were detected using a Fusion HT microplate reader (Perkin-Elmer). Data were baseline-corrected and reported as the percent of maximum fluorescence as calculated by the sample fluorescence divided by the fluorescence of cells stained with 1 μg/ml cAC10-europium alone.

Growth inhibition of CD30-positive Karpas-299 or L540cy cells or CD30-negative WSU non-Hodgkin's lymphoma cells treated with cAC10 Cys→Ser variant conjugates was determined by incubating conjugates with cells for 92 h followed by incubation with 50 μM resazurin for 4 h at 37°C. Dye reduction was measured using a Fusion HT microplate reader. Data were analyzed by a non-linear least squares fit to a four-parameter logistic equation using Prism v4.01 (GraphPad Software Inc, San Diego, CA, USA).

Pharmacokinetics

The pharmacokinetic properties of the antibody–drug conjugates (C2v1–E2, C2v2–E2, C8–E2, C4v1–E4, C4v2–E4, C8–E4 and C8–E4M) were evaluated in SCID mice. Groups of SCID mice (three mice/group) were administered with 10 mg/kg of test material based on the antibody component by tail vein injection. Blood samples were collected from each mouse via the saphenous vein at 1 h, 4 h, 1 day, 2 days, 4 days, 7 days, 15 days, 21 days, 28 days, 36 days, 42 days and 50 days post-injection and serum isolated. Serum concentrations of antibody–drug conjugates were measured by antigen-binding ELISA as described previously (Hamblett *et al.*, 2004). The time course of serum concentrations for each animal was plotted and non-compartmental pharmacokinetic parameters were calculated with WinNonlin version 4.0.1 (Pharsight, Mountain View, CA, USA).

Xenograft models

A total of 5×10^6 Karpas-299 or L540cy cells were implanted into the right flank of C.B-17 SCID mice (Harlan, Indianapolis, IN, USA) to establish a subcutaneous disease model of anaplastic large cell lymphoma or Hodgkin's disease, respectively (Doronina *et al.*, 2003; Francisco *et al.*, 2003; Hamblett *et al.*, 2004). Tumor volume was calculated using the formula, $(A \times B^2)/2$, where A and B are the largest and second largest perpendicular tumor dimensions, respectively. Tumor-bearing mice were randomly divided into groups of 8–10 animals when the mean tumor volume was 100 mm^3. Mice groups were treated with a single intravenous dose of an antibody–drug conjugate or alternatively left untreated. Conjugate doses of 6.0 and 12.0 mg/kg for the 2 drugs/antibody conjugates and 3.0 and 6.0 mg/kg for the 4 drugs/antibody conjugates were used for the L540cy xenograft studies. Conjugate doses of 0.5, 1.0 and 2.0 mg/kg for the 2 drugs/antibody conjugates and 0.5 and 1.0 mg/kg for the 4 drugs/antibody conjugates were used for the Karpas-299 xenograft models. Animals were euthanized when tumor volumes reached ~1000 mm^3.

Maximum tolerated dose

Groups of three rats (Sprague–Dawley) (Harlan) were injected with 40, 60 or 80 mg/kg of C2v1–E2, C2v2–E2 and C8–E2 and 20, 30 or 40 mg/kg of C4v1–E4, C4v2–E4, C8–E4 and C8–E4M via the tail vein to determine the single dose maximum tolerated dose. Rats were monitored daily for 14 days, and both weight and clinical observations were recorded. Rats that developed significant signs of distress were euthanized.

Results

Construction and expression of antibody Cys→Ser variants

The parent antibody for this study, cAC10, is a chimeric IgG_1 that binds to human CD30 (Wahl *et al.*, 2002). Antibody cAC10, has four solvent-accessible interchain disulfide bonds that are readily reducible and conjugated in near quantitative yield to vcMMAE, a thiol-reactive auristatin derivative (Doronina *et al.*, 2003). This homogeneous antibody–drug conjugate comprising the cAC10 parent antibody with 8 drugs/antibody is designated here as C8–E8 (Fig. 1A). The solvent-accessible cysteines in cAC10 were systematically replaced with a homologous residue, serine, to generate antibody variants with either four (C4v1, C4v2 and C4v3) or two (C2v1 and C2v2) remaining accessible cysteines (Table I and Fig. 1A). These engineered antibody variants were used to generate drug conjugates with defined stoichiometries and sites of drug attachment.

All antibody variants were stably expressed in CHO-DG44 cell lines at titers of 25–125 mg/l. The antibody variants were purified from 2.5 to 10 l cultures by protein A affinity and ion exchange chromatography (Table I) and then analyzed by size exclusion chromatography and SDS–PAGE. All variants electrophoresed under reducing conditions gave rise to two major bands consistent with the presence of heavy and light chains (data not shown). Under non-reducing conditions, all antibody variants except C4v3 gave electrophoretic patterns (Fig. 1B) consistent with their anticipated interchain disulfide bonding pattern (Fig. 1A). Antibody variant C4v3 was excluded from the remainder of this study on the basis of its unanticipated electrophoretic behavior and a size exclusion chromatography profile that suggested significant aggregation.

Preparation of antibody–drug conjugates

The cAC10 parent antibody (C8) was partially reduced to yield a mean of 2 or 4 free thiols/antibody and then reacted with vcMMAE. The corresponding conjugates, C8–E2M and C8–E4M, have a mean loading of 2 drugs/antibody and 4 drugs/antibody, respectively. C8–E2M and C8–E4M are mixtures of conjugates loaded with 0, 2, 4, 6 or 8 drugs/antibody (Hamblett *et al.*, 2004; Sun *et al.*, 2005). Conjugates with uniform stoichiometry of either 2 drugs/antibody (C8–E2) or 4 drugs/antibody (C8–E4) were purified from the C8–E2M mixture by hydrophobic interaction chromatography as previously described (Hamblett *et al.*, 2004; Sun *et al.*, 2005). MMAE conjugates of the Cys→Ser variants were generated by reduction of the corresponding antibodies followed by reaction with vcMMAE.

For each antibody–drug conjugate the observed drug loading stoichiometry by spectrophotometric (Hamblett *et al.*, 2004) and reverse phase HPLC analyses (Sun *et al.*, 2005) closely matched those expected (Table II). Peak area analysis following size exclusion chromatography suggests that all antibody–drug conjugates were >98% monomeric. The yield of the Cys→Ser variant conjugates (89–96%) was similar to the heterogeneous conjugate, C8–E4M (95%), but greatly improved over the more homogeneous conjugates C8–E4 (11%) and C8–E2 (27%) purified from C8–E2M (Table II). Somewhat higher

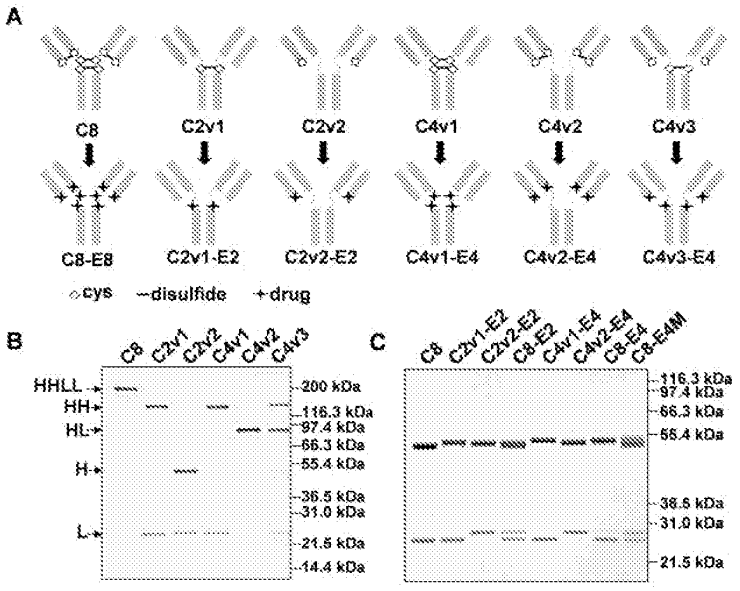


Fig. 1. Design and SDS–PAGE analysis of antibody Cys→Ser variants and corresponding drug conjugates. (**A**) Schematic representation of antibody variants and drug conjugates highlighting the location of solvent-accessible cysteines (diamonds), interchain disulfide bonds (–) and subsequently conjugated drugs (+). Antibodies and antibody–drug conjugates are identified by their variant name (see Table I), and loading stoichiometry with the drug, MMAE. For example, C8–E8 denotes the conjugate in which all eight solvent-accessible cysteine residues in the cAC10 parent antibody (C8) are conjugated to MMAE (E8). (**B**) SDS–PAGE analysis of antibody variants under non-reducing conditions. Arrows indicate the mobility of antibody heavy–light chain tetramer (HHLL), heavy chain dimer (HH), heavy–light chain dimer (HL), heavy chain (H) and light chain (L). (**C**) SDS–PAGE analysis of antibody–drug conjugates under reducing conditions.

Table II. *In vitro* characterization of antibody–drug conjugates

cAC10 drug conjugate[a]	Conjugate yield (%)[b]	Drugs/antibody: method 1, method 2[c]	Monomer (%)[d]	Competition binding to Karpas-299 (IC_{50}, nM)[e]	Karpas-299 cytotoxicity (IC_{50}, nM)[e]	L540cy cytotoxicity (IC_{50}, nM)[e]
C2v1–E2	92.7	2.0, 1.9	98.4	2.9 ± 0.3	0.26 ± 0.12	0.28 ± 0.03
C2v2–E2	88.9	2.1, 1.8	98.5	2.5 ± 0.1	0.46 ± 0.30	0.27 ± 0.02
C8–E2	27.4[f]	2.0, 2.0	99.7	2.8 ± 0.2	0.32 ± 0.21	0.28 ± 0.02
C4v1–E4	90.6	4.0, 3.8	99.2	3.2 ± 0.1	0.07 ± 0.02	0.13 ± 0.01
C4v2–E4	96.0	4.1, 3.8	99.0	2.8 ± 0.2	0.07 ± 0.02	0.12 ± 0.01
C8–E4	10.8[f]	4.0, 4.0	99.5	2.4 ± 0.3	0.07 ± 0.01	0.18 ± 0.04
C8–E4M	95	4.4, 4.4	98.8	3.0 ± 0.4	0.03 ± 0.01	0.07 ± 0.02

[a]Antibody–drug conjugates are identified by their cAC10 variant name (see Table I), loading level with the drug, MMAE, and whether the drug stoichiometry is uniform or variable (M). E.g. C8–E4M and C8–E2M denotes the parent antibody, cAC10, loaded with a mean stoichiometry of 4 drugs/antibody (range of 0–8 drugs/antibody) and 2 drugs/antibody (range of 0–8 drugs/antibody), respectively.
[b]Yield of conjugate obtained as a percentage of purified antibody. The free drug in all conjugate preparations was below the detection limit (<0.05%).
[c]Methods 1 and 2 refer to the ratio of absorbance at wavelengths of 248 and 280 nm (Hamblett *et al.*, 2004) and reverse phase HPLC analysis under reducing conditions, respectively (Fig. 2).
[d]Estimated from the peak areas in size exclusion chromatography.
[e]Mean (± SEM) for three or more independent experiments.
[f]Percentage yield after hydrophobic interaction chromatography of C8–E2M based on the starting cAC10 protein. Yields of up to 30% C8–E4 have been achieved by using C8–E4M as a starting point for purification (S.Alley, unpublished data).

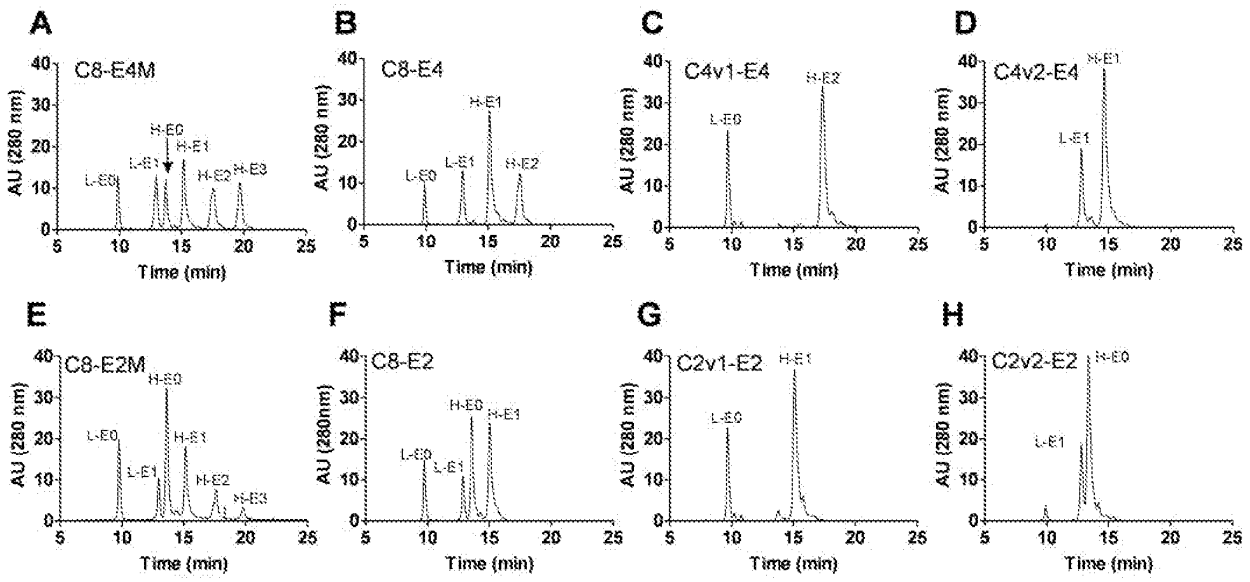


Fig. 2. Reverse phase HPLC analysis of antibody–drug conjugates under reducing conditions. (**A**) C8–E4M, (**B**) C8–E4, (**C**) C4v1–E4, (**D**) C4v2–E4, (**E**) C8–E2M, (**F**) C8–E2, (**G**) C2v1–E2 and (**H**) C2v2–E2 (see Table II). Peaks were identified by the ratio of their absorbance at wavelengths of 248 and 280 nm (see Materials and methods). L-E0 and L-E1 are used to denote light chains loaded with 0 or 1 equivalents of MMAE, respectively, whereas H-E0, H-E1, H-E2 and H-E3, indicate heavy chains loaded with 0, 1, 2 or 3 equivalents of MMAE, respectively.

yields (up to 30%) of C8–E4 have been achieved by using C8–E4M as a starting point for purification (S.Alley, unpublished data). SDS–PAGE analysis of the antibody–drug conjugates under reducing conditions fully resolves light chains loaded with 0 or 1 equivalents of MMAE (L-E0 and L-E1, respectively) and partially resolves heavy chains loaded with 0, 1, 2 or 3 equivalents of MMAE (H-E0, H-E1, H-E2 and H-E3, respectively), as shown previously (Doronina *et al.*, 2003). For each Cys→Ser variant conjugate, a single light and heavy chain band with the anticipated mobility was observed (Fig. 1C). In contrast, more complex electrophoretic patterns were observed for C8–E2 and C8–E4M and to a lesser extent for C8–E4 (Fig. 1C).

Reverse phase HPLC under reducing conditions was used to further evaluate antibody–drug conjugate heterogeneity as this method can fully resolve all light and heavy chain species (Sun *et al.*, 2005). C8–E4M (Fig. 2A) is the most heterogeneous conjugate containing all six possible species: L-E0, L-E1, H-E0, H-E1, H-E2 and H-E3. Purification of C8–E4M to generate C8–E4 reduces the heterogeneity down to four species: L-E0, L-E1, H-E1 and H-E2 (Fig. 2B). The homogeneity of cAC10 Cys→Ser variant conjugates is demonstrated by the presence of the anticipated single major light and heavy chain peaks: L-E0 plus H-E2, and L-E1 plus H-E1, for C4v1–E4 (Fig. 2C) and C4v2–E4 (Fig. 2D), respectively. Similarly for the 2 drugs/antibody conjugates C8–E2M (Fig. 2E) is the most heterogeneous containing all six possible species. Purification of C8–E2 (Fig. 2F) from C8-E2M reduces heterogeneity to L-E0, L-E1, H-E0 and H-E1. In contrast, C2v1–E2 (Fig. 2G) and C2v2–E2 (Fig. 2H) each gave rise to a pair of major peaks: L-E0 plus H-E1 and L-E1 plus H-E0, respectively.

In vitro characterization of antibody–drug conjugates

Competition binding experiments revealed that neither Cys→Ser mutations (Table I) nor vcMMAE conjugation (Table II) impaired antigen binding by the cAC10 antibody. Next the cytotoxicity of cAC10 Cys→Ser variant conjugates were assessed on CD30 positive (Karpas-299 and L540cy) and negative (WSU non-Hodgkin's lymphoma) cell lines. The C2v1–E2 and C2v2–E2 conjugates have very similar potency to C8–E2 on both CD30 positive cell lines (Table II). In addition, C4v1–E4, C4v2–E4, C8–E4M and C8–E4 conjugates displayed similar activity on both CD30 positive cell lines tested (Table II). Thus, defining the site and stoichiometry of drug attachment did not significantly impact the *in vitro* cytotoxic activity of the antibody–drug conjugates. Increasing the drug stoichiometry from 2 to 4 drugs/antibody increased the potency (reduced IC_{50} values), consistent with previous observations (Hamblett *et al.*, 2004; Sun *et al.*, 2005). CD30 negative WSU non-Hodgkin's lymphoma cells were insensitive to all cAC10 antibody–drug conjugates (data not shown).

Pharmacokinetics

SCID mice were dosed with various antibody–drug conjugates to determine how the sites and stoichiometries of drug loading would affect the pharmacokinetic properties. Immune deficient (SCID) rather than competent mice were chosen for consistency with the efficacy experiments (see below) and to avoid an antibody response to the antibody drug conjugates. SCID mice lack endogenous IgG and so administered IgG may potentially bind to endogenous Fc receptors on a variety of tissues and normal cells. Whilst this possibility was not explored in this study, it seems unlikely to have a major influence, since similar pharmacokinetic properties have been observed for chimeric antibodies in immune competent and Balb/c mice (Zuckier *et al.*, 1994).

For cAC10 conjugated with MMAE, decreasing the loading from 8 drugs/antibody to 4 or 2 drugs/antibody was previously shown to decrease the clearance and increase the pharmacokinetic area-under-the-curve (Hamblett *et al.*, 2004). We observed similar differences between the conjugates loaded with 2 drugs/antibody and 4 drugs/antibody. For example, the exposure of C2v1–E2, C2v2–E2 and C8–E2 was increased compared to C4v1–E4, C4v2–E4, C8–E4 and C8–E4M as determined by the area-under-the-curve (Table III). Also, clearance values for conjugates loaded with 2 drugs/antibody were lower than for those loaded with 4 drugs/antibody (Table III). Notably, there were only small differences in the pharmacokinetic area-under-the-curve or clearance values between C4v1–E4, C4v2–E4, C8–E4 and C8–E4M (Fig. 3A and Table III). Likewise, the pharmacokinetic parameters of C2v1–E2, C2v2–E2 and C8–E2 were similar (Fig. 3B and Table III).

Table III. Antibody–drug conjugate tolerability and pharmacokinetic parameters

cAC10 drug conjugate[a]	Maximum tolerated dose (mg/kg)[b]	Terminal half-life (days)	Area-under-the-curve (μg-day/ml)	Clearance (ml/day/kg)
C2v1–E2	40	13.1 ± 3.7	2210 ± 360	4.6 ± 0.8
C2v2–E2[c]	60	16.6	2460	4.4
C8–E2	40	15.9 ± 2.3	2790 ± 150	3.6 ± 0.2
C4v1–E4	20	17.4 ± 3.3	1800 ± 80	5.6 ± 0.2
C4v2–E4	<20	14.8 ± 1.3	1060 ± 220	9.7 ±2.2
C8–E4	20	11.8 ± 1.3	1700 ± 120	5.9 ± 0.4
C8–E4M	<20	13.1 ± 4.4	1420 ± 440	7.6 ± 2.7

[a]See Table II for antibody–drug conjugate nomenclature.
[b]The single dose maximum tolerated dose in Sprague–Dawley rats was defined as the highest dose that did not induce >20% weight loss or severe signs of distress.
[c]One mouse was found dead on Day 22 and eliminated from the pharmacokinetic analysis.

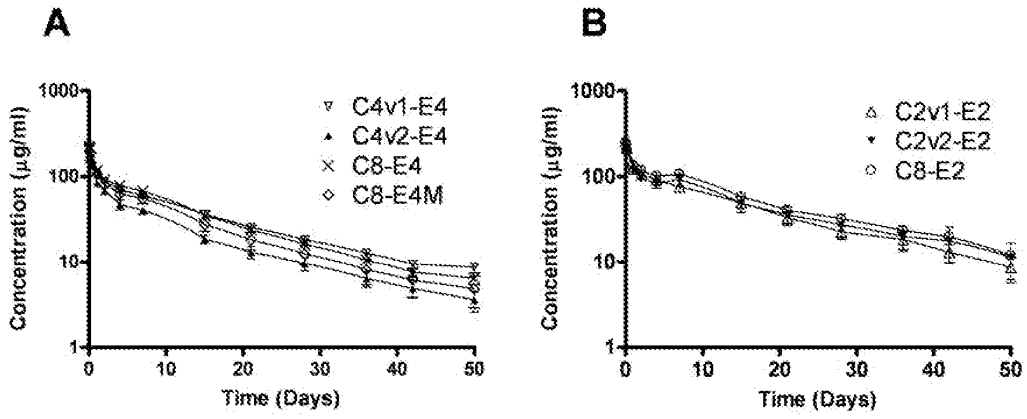


Fig. 3. Pharmacokinetics of antibody drug conjugates loaded with 4 drugs/antibody (**A**) and 2 drugs/antibody (**B**). SCID mice were treated with 10 mg/kg of conjugate and serum samples were collected and analyzed by ELISA to determine antibody–drug conjugate concentrations.

Antitumor activity of antibody Cys→Ser variant conjugates in vivo

The single dose efficacies of the cAC10 Cys→Ser variant drug conjugates were compared to conjugates of the parent antibody in established (~100 mm^3) subcutaneous xenograft models of Hodgkin's disease (L540cy) and anaplastic large cell lymphoma (Karpas-299) in SCID mice. A tumor that decreased in size such that it was impalpable was defined as a complete regression, whereas a complete regression that lasted beyond 100 days post-tumor implant was defined as a 'cure'. Treatment of L540cy xenograft models with C4v1–E4, C4v2–E4, C8–E4 and C8–E4M resulted in comparable responses with complete regressions being achieved at both 3.0 and 6.0 mg/kg for each antibody–drug conjugate (Fig. 4A and B). Treatment of Karpas-299 models with conjugates containing 4 drugs/antibody at doses of 0.5 and 1.0 mg/kg showed very similar and potent efficacy for all antibody–drug conjugates (data not shown). Responses of L540cy xenografts to treatment with C2v1–E2 and C2v2–E2 were comparable and complete regressions were induced at both 6.0 and 12.0 mg/kg doses (Fig. 4C and D). C8–E2 was slightly more potent than C2v1–E2 and C2v2–E2 with cures achieved at both dose levels (Fig. 4C and D). Karpas-299 xenograft models treated with single doses of the conjugates containing 2 drugs/antibody showed similar response trends with 3 of the 10 animals achieving complete regressions for C2v1–E2 and C2v2–E2 and 8 of 10 complete regressions for C8–E2 at a 1.0 mg/kg dose (data not shown).

Maximum tolerated dose

The maximum tolerated dose was defined as the highest dose that did not induce >20% weight loss or severe signs of distress. Previously, the single dose maximum tolerated dose of C8–E2 in BALB/c mice was shown to be >250 mg/kg (Hamblett *et al.*, 2004; Sun *et al.*, 2005)—the highest dose that could be readily tested. Sprague–Dawley rats were chosen here to assess the single dose tolerability of each antibody–drug conjugate. These rats are more sensitive than mice to the anti-CD30 MMAE conjugates and so permit more precise determination of the maximum tolerated dose for conjugates with 2 drugs/antibody. The cAC10 antibody (Wahl *et al.*, 2002) binds to human CD30 but does not cross-react with the corresponding antigen from rats or mice. Thus, antigen-independent but not antigen-dependent toxicities of cAC10 drug conjugates can be explored in these species.

All conjugates loaded with 2 drugs/antibody were tolerated by rats at higher doses than those with 4 drugs/antibody, consistent with previous studies of C8–E2 and C8–E4 tolerability in mice (Hamblett *et al.*, 2004; Sun *et al.*, 2005). Minor differences in tolerability were observed between C2v1–E2,

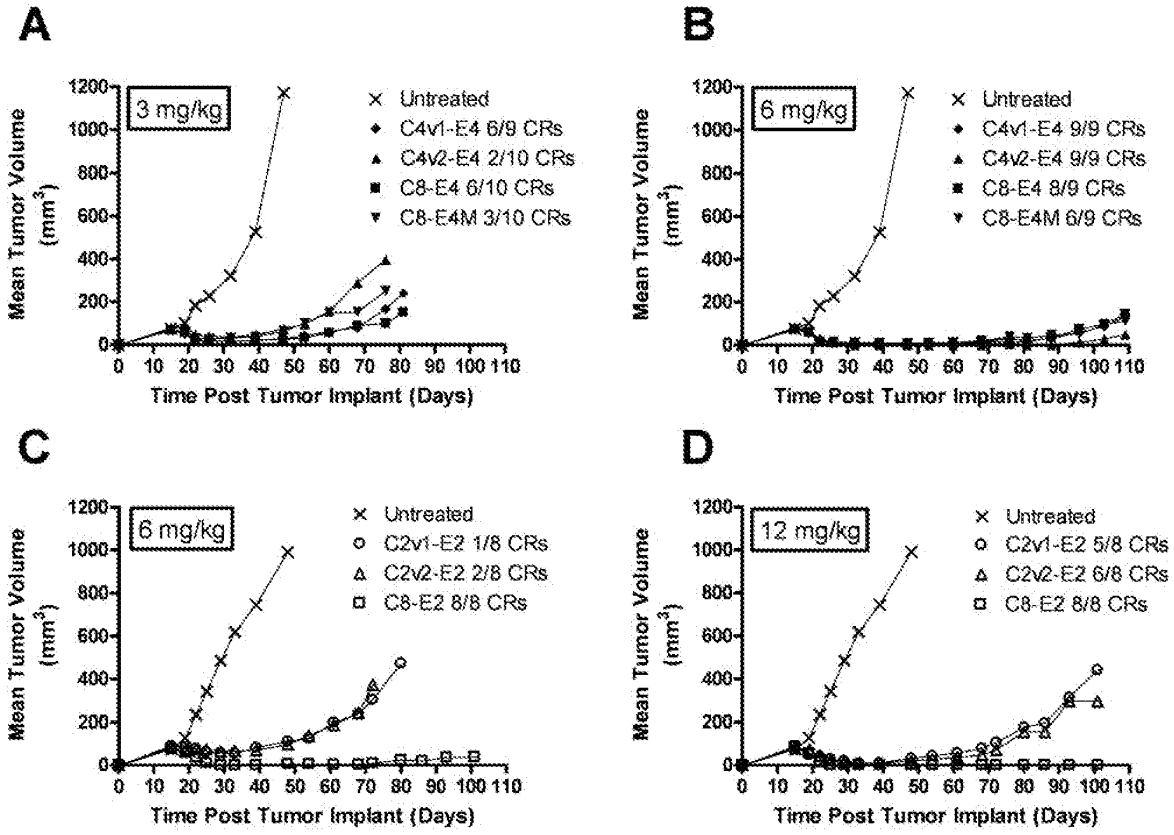


Fig. 4. Single dose efficacy studies on SCID mice bearing L540cy subcutaneous xenografts. Mice were treated 12 days post-tumor implant with a single dose of C4v1–E4, C4v2–E4, C8–E4 and C8–E4M at 3.0 mg/kg (**A**) and 6.0 mg/kg (**B**). Mice were treated 12 days post-tumor implant with a single dose of C2v1–E2, C2v2–E2 and C8–E2 at 6.0 mg/kg (**C**) or 12.0 mg/kg (**D**).

C2v2–E2 and C8–E2, which were dosed at 40, 60 and 80 mg/kg. The 40 mg/kg dose of each conjugate was well tolerated whilst the 60 mg/kg dose was only well tolerated by rats treated with C2v2–E2 (Table III). The conjugates containing 4 drugs/antibody were each dosed at 20, 30 and 40 mg/kg. Again there were only small differences in tolerability between conjugates. Animals injected with the 20 mg/kg dose of C4v1–E4 and C8–E4 experienced no adverse effects while several animals in the groups treated with the 20 mg/kg doses of C4v2–E4 and C8–E4M showed signs of distress and one from each group was sacrificed on Day 9. Based on these data the maximum tolerated doses for C4v1–E4 and C8–E4 were determined to be 20 mg/kg while the maximum tolerated doses for C4v2–E4 and C8–E4M were determined to be <20 mg/kg (Table III). The slightly greater toxicity of C8–E4M than C8–E4 likely reflects, at least in part, the drug loading stoichiometry of 4.4 drugs/antibody and 4.0 drugs/antibody, respectively (Table II), and the known correlation of drug load and toxicity (Hamblett *et al.*, 2004; Sun *et al.*, 2005).

Discussion

Desirable attributes of antibody–drug conjugates for targeted therapy include linkers between antibody and drug that are stable in circulation but readily cleavable within the target cells to release active drug, high therapeutic index (curative dose/maximum tolerated dose) and homogeneous composition to facilitate drug development. An antibody–drug conjugate that comes close to meeting these criteria is the anti-CD30 antibody, cAC10, conjugated to auristatin E (MMAE) via the eight solvent-accessible cysteine residues through linkers that are cleavable by lysosomal proteases such as cathepsin B (Doronina *et al.*, 2003; Francisco *et al.*, 2003). Reduction in the drug stoichiometry from 8 drugs/antibody to 4 drugs/antibody or 2 drugs/antibody increased the therapeutic index by ≥2-fold (Hamblett *et al.*, 2004), albeit at the expense of reduced conjugate yield and increased heterogeneity (drug attachment site and stoichiometry).

Here, we have developed a protein engineering approach to generate homogeneous antibody–drug conjugates in high yield with precisely definable site and stoichiometry of drug attachment. The chosen strategy was to systemically replace the solvent-accessible cysteine residues with serines. These cysteine residues commonly form interchain disulfide bonds in IgG. Thus, success of our strategy relies upon biosynthetic assembly of IgG, as well as IgG structure and function under physiologic conditions, to be independent of the presence of disulfide bonds. Several prior observations suggest that this would likely be the case. First, biosynthetic assembly of antibody light and heavy chains relies upon non-covalent interactions and does not require a disulfide between light and heavy chains as shown by studies with engineered $F(ab')_2$ fragments (Rodrigues *et al.*, 1993). Moreover, removing the interchain disulfide bond impacted neither the antigen binding affinity of a $F(ab')_2$ fragment nor its pharmacokinetic properties (Rodrigues *et al.*, 1993). Second, removal of the inter-heavy chain disulfide bonds of an IgG_1 did not impact expression nor binding to antigen but did impair effector functions (Gillies and Wesolowski, 1990). Third, the pharmacokinetic properties of cAC10 IgG in mice were not significantly altered by reduction and carboxymethylation of all four interchain disulfide bonds (K.J.H. and J.A.F., unpublished data).

Antibody variants were generated with either six (data not shown), four or two remaining accessible cysteines that were then conjugated to the drug, MMAE, in near quantitative yield. These naturally occurring cysteine residues are distant from the antigen-binding variable domains and, as anticipated, neither their replacement with serines (Table I) nor drug conjugation (Table II) impacted antigen binding. Removal of two, three or all four of the antibody interchain disulfide bonds has minimal effect on the antibody multimerization state as judged by size exclusion chromatography. In addition, similar *in vitro* potencies of the various 2 drugs/antibody conjugates (C2v1–E2 and C2v2–E2 versus C8–E2) and 4 drugs/antibody conjugates (C4v1–E4 and C4v2–E4 versus C8–E4 and C8–E4M) (Table II) suggests that the engineered conjugates are stable at least over the 4 day time course of the assay.

The engineered conjugates with 4 drugs/antibody, C4v1–E4 and C4v2–E4, have comparable antitumor activity to the parental conjugates, C8–E4 and C8–E4M, in both L540cy (Fig. 4) and Karpas-299 (not shown) xenograft models and exhibit only small differences in tolerability by rats (Table III). The antitumor efficacy of the engineered conjugates with 2 drugs/antibody, C2v1–E2 and C2v2–E2, is slightly lower than for the parental conjugate, C8–E2, with only minor differences in tolerability by rats. Additionally, broadly similar pharmacokinetic properties were found for the conjugates with 2 drugs/antibody (C2v1–E2, C2v2–E2 and C8–E2) and amongst those with 4 drugs/antibody (C4v1–E4, C4v2–E4, C8–E4 and C8–E4M). Thus, efficacy, tolerability and pharmacokinetics of the engineered antibody–drug conjugates are impacted to only a minor extent by the chosen alternative sites of drug attachment. In contrast, reducing the stoichiometry of drug loading from 4 to 2 drugs/antibody reduces efficacy and increases tolerability with little net effect on the therapeutic index (Fig. 4 and Table III), consistent with previous observations (Hamblett *et al.*, 2004). Surprisingly, the most heterogeneous conjugate C8–E4M (0–8 drugs/antibody with a mean of 4 drugs/antibody and variable sites of drug attachment) has similar *in vitro* and *in vivo* properties to the most homogenous conjugates, C4v1–E4 and C4v2–E4 (fixed stoichiometry of 4 drugs/antibody at defined sites). Thus, from this study in conjunction with prior work (Hamblett *et al.*, 2004; Sun *et al.*, 2005), the stoichiometry of drug attachment is a more critical determinant of antibody–drug conjugate potency and tolerability than is the site of drug attachment and conjugate homogeneity.

Comparable *in vitro* and *in vivo* properties notwithstanding, the engineered antibody–drug conjugates offer significant advantages for drug development over their purified parental counterparts (C8–E2 and C8–E4) in terms of improved yield (>90% versus ≤30%) and reduced heterogeneity of sites of drug attachment. The engineered conjugates (C4v1–E4 and C4v2–E4) were produced in similar yield to the unpurified parental counterpart, C8–E4M) but are much more homogenous—a potentially significant advantage from a drug manufacturing standpoint. The precise definition of drug location and stoichiometry in the engineered antibody–drug conjugates described here contrasts with the heterogeneity of many antibody–drug conjugates [reviewed by Wu and Senter (2005)] including gemtuzumab ozogamicin (Mylotarg), the only FDA-approved antibody–drug conjugate. Gemtuzumab ozogamicin has a mean stoichiometry of 2–3 drugs/antibody attached via lysine residues with 50% of the antibody

being unconjugated (Hamann *et al.*, 2002b) (gemtuzumab ozogamicin prescribing information).

Prior engineering of IgG for site-specific conjugation to cysteine residues involved the *addition* of cysteine residues rather than the *removal* of naturally occurring cysteines described here. For example, an extra cysteine residue was introduced at several different locations in the C_H1 domain of an IgG_4 (Lyons *et al.*, 1990). Of these added non-hinge cysteines, the fully accessible ones were reversibly blocked, whereas less accessible cysteines were only partially (~50%) blocked and available thiols were site-specifically radiolabeled. Selective reduction of the blocked thiols proved difficult with only 50% thiol recovery being achieved without reduction of the hinge disulfide bonds. More recently, a cysteine residue was introduced at position 442 of the C_H3 domain of an IgG_4 (Stimmel *et al.*, 2000). Again the cysteine residue was reversibly blocked but in this case mild and selective reduction conditions were devised and site-specific labeling achieved with a radionuclide chelator. The performance of such engineered conjugates *in vivo* has yet to be reported. Introducing cysteine residues into antibody domains can sometimes promote interchain and intermolecular disulfide bonds that are undesirable for antibody–drug conjugate applications. For example, installing an additional cysteine residue at position 444 in the C_H3 domain led to the formation of disulfide-linked IgG dimers with enhanced effector functions (Caron *et al.*, 1992; Shopes, 1992), whereas a cysteine installed at position 119 in a C_H1 domain created a compact and tethered IgG with an additional disulfide bond between the two heavy chains (Shopes, 1993).

Reduced IgG interaction with Fcγ receptors may limit antibody uptake by non-target organs and improve tumor localization (Hutchins *et al.*, 1995)—a potentially advantageous property for antibody–drug conjugate applications. Removal of the hinge region interchain disulfide bonds from an IgG_1 has been reported to greatly impair antibody-dependent cellular cytotoxicity, consistent with impaired Fc–Fcγ receptor interactions, and also reduce complement-dependent cytotoxicity (Gillies and Wesolowski, 1990). The parent antibody, cAC10, is inefficient in supporting antibody-dependent cellular cytotoxicity so we are currently exploring the impact of Cys→Ser mutations and drug conjugation on the effector functions of other antibodies.

Generation of C8–E2 or C8–E4 using partial reduction methods results in antibody–drug conjugates where the majority of MMAE is linked to cAC10 via the cysteines involved in forming the heavy–light chain disulfide bond and the hinge region interchain disulfides are left intact (Sun *et al.*, 2005). While methods have been developed to produce C8–E2 and C8–E4 with the majority of MMAE linked to the hinge region (Sun *et al.*, 2005), recombinant generation of C2v1–E2, C2v2–E2, C4v1–E4 and C4v2–E4 results in antibody–drug conjugates lacking all interchain disulfide bonds and thus reduces the potential for internalization by non-target tissues mediated by interactions with Fcγ receptors.

Much research has focused on optimizing drugs and linkers for antibody–drug conjugates (Lambert, 2005; Wu and Senter, 2005). Consideration of drug stoichiometry has also been shown to enhance the therapeutic window of cAC10vcMMAE antibody–drug conjugates (Hamblett *et al.*, 2004; Sun *et al.*, 2005). Here, we report that the antibody component of the antibody–drug conjugate can also be optimized to complement advances in drug and linker technologies and generate highly potent and homogeneous product candidates. The strategy developed here for generating antibody–drug conjugates with defined sites and stoichiometries of drug loading is potentially broadly applicable to other antibodies as it involves engineering of constant domains. Indeed, we have successfully used this approach to generate antibody–drug conjugates of a humanized anti-CD70 antibody (C.F.M., S.C.A. and P.C., unpublished data).

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A New Water-soluble Camptothecin Derivative, DX-8951f, Exhibits Potent Antitumor Activity against Human Tumors *in vitro* and *in vivo*

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CPT-11, a semisynthetic derivative of camptothecin, exhibited strong antitumor activity against lymphoma, lung cancer, colorectal cancer, gastric cancer, ovarian cancer, and cervical cancer. CPT-11 is a pro-drug that is converted to an active metabolite, SN-38, *in vivo* by enzymes such as carboxylesterase. We synthesized a water-soluble and non-pro-drug analog of camptothecin, DX-8951f. It showed both high *in vitro* potency against a series of 32 malignant cell lines and significant topoisomerase I inhibition. The anti-proliferative activity of DX-8951f, as indicated by the mean GI_{50} value, was about 6 and 28 times greater than that of SN-38 or SK&F 10486-A (Topotecan), respectively. These three derivatives of camptothecin showed similar patterns of differential response among 32 cell lines, that is, their spectra of *in vitro* cytotoxicity were almost the same. The antitumor activity of three doses of DX-8951f administered i.v. at 4-day intervals against human gastric adenocarcinoma SC-6 xenografts was greater than that of CPT-11 or SK&F 10486-A. Moreover, it overcame P-glycoprotein-mediated multi-drug resistance. These data suggest that DX-8951f has a high antitumor activity and is a potential therapeutic agent.

Key words: Camptothecin — Topoisomerase I — P-Glycoprotein — CPT-11 — Topotecan

Camptothecin (CPT) was isolated from the Chinese tree, *Camptotheca acuminata* and its structure was identified by Wall *et al.*[1] It inhibits type I DNA topoisomerase (topoisomerase I)[2–4] and has strong antitumor activity against several experimental tumors,[5] but in clinical trials in the early 1970s, failed to show meaningful activity and proved to cause severe and unpredictable cystitis.[6, 7]

The outcome of a great amount of effort to find new derivatives of CPT with higher antitumor activity and less toxicity has been the production of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin hydrochloride trihydrate (CPT-11), a semisynthetic analogue of CPT which has significant antitumor activity against various experimental tumor models in mice.[8] Clinical evaluation of CPT-11 has revealed that it is active against lymphoma,[9] small-cell lung cancer,[10] non-small-cell lung cancer,[11] colorectal cancer,[12] gastric cancer,[13] ovarian cancer, and cervical cancer.[14] CPT-11 is a pro-drug that is converted *in vivo* to 7-ethyl-10-hydroxycamptothecin (SN-38), which has an antitumor activity at least 100 times that of CPT-11 *in vitro*[15, 16] and is responsible for much of the efficacy and toxicity of CPT-11. CPT-11 has exhibited high antitumor activity in clinical studies, but side effects such as leukopenia and diarrhea have been observed with a large interpatient variation.[17] The polymorphism of enzymes such as carboxylesterase, which has been demonstrated to convert CPT-11 to SN-38 in rat serum,[18] may cause the interpatient variation.

We intended to synthesize water-soluble CPT derivatives retaining potent topoisomerase I-inhibitory activity and broad-spectrum antitumor activity, and having little or no interpatient variation in efficacy or toxicity. We have already reported that hexacyclic derivatives of CPT showed superior topoisomerase I inhibitory activity.[19] Also, we have synthesized about 150 hexacyclic CPT analogues and selected a new non-pro-drug compound of camptothecin, DX-8951f, as a candidate for further development (Fig. 1).

In this study, we examined the antitumor activities of DX-8951f *in vitro* and *in vivo*, and its inhibitory activity against topoisomerase I.

MATERIALS AND METHODS

Cell lines *In vitro*: Human cell lines (Table I) were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah) and 2 m*M* L-glutamine (Sanko Junyaku Co., Ltd., Tokyo). A mouse cell line, P388, was maintained in the former medium supplemented with 20 μM 2-mercaptoethanol (Nacalai Tesque, Inc., Kyoto). Cell cultures were passaged once or twice weekly.

[1] To whom requests for reprints should be addressed.

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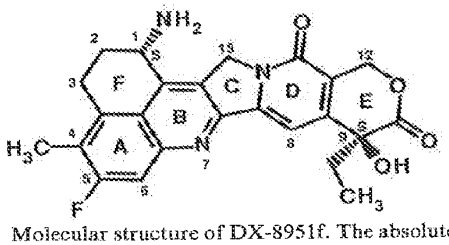


Fig. 1. Molecular structure of DX-8951f. The absolute configuration at position 1 of DX-8951f is *S*.

In vivo: Poorly differentiated gastric adenocarcinoma SC-6 was supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo). It was maintained by sequential s.c. transplantations into nude mice.

Animals Male athymic nude mice (BALB/c-nu/nu), aged 5 or 6 weeks, were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu). They were housed in an exclusive experimental room and were given sterilized food and water *ad libitum*.

Drugs SK&F 104864-A (SK&F) was synthesized as described previously.[20] Camptothecin (CPT), CPT-11 and SN-38 were provided by Yakult Honsha Co., Ltd. (Tokyo). DX-8951f, (1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1*H*,12*H*-benzo[*de*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione methanesulfonate was totally synthesized in our laboratory.[21] For *in vitro* usage, SK&F, CPT, SN-38 and DX-8951f were each dissolved in dimethyl sulfoxide (DMSO) and diluted with the medium described above, and for the *in vivo* experiment, SK&F, CPT-11 and DX-8951f were each dissolved in distilled water. Dose levels of SK&F, CPT, CPT-11, SN-38 and DX-8951f were expressed in terms of the free bases. Vincristine (VCR) was purchased from Shionogi Co., Ltd. (Osaka). Cisplatin (CDDP) was purchased from Nippon Kayaku Co., Ltd. (Tokyo). Verapamil was purchased from Eisai Co., Ltd. (Tokyo).

***In vitro* cytotoxicity** Growth inhibition experiments were carried out in 96-well flat-bottomed microplates (Falcon, Oxnard, California), and the amount of viable cells at the end of the incubation was determined by MTT assay, essentially as described by Mosmann.[22] Thus, 500–20,000 cells/well in 150 μl of medium were plated and grown for 24 h (P388, CCRF-CEM and K562 cells for 4 h), the drug (in 50 μl medium/well), or the medium alone as a control, was added, and the cells were cultured for an additional 3 days. After addition of MTT (20 μl/well, 5 mg/ml in phosphate-buffered saline), the plates were incubated for 4 h and centrifuged at 800*g* for 5 min, then the medium was removed and the blue dye formed was dissolved in 150 μl of DMSO. The absorbance was measured at 540 nm using a Microplate Reader model 3550 (Bio-rad, Richmond, California). Each data point on the growth curves represents the average of results from four wells. The cell population density at time 0 (the time at which drugs were added) was also measured. Growth inhibition of 50% (GI_{50}) was calculated from $100\times[(T-T_0)/(C-T_0)]=50$, which is the drug concentration causing a 50% reduction in optical density in control cells during the drug incubation (C: control optical density, T: test optical density, T_0: optical density at time zero). Differential growth inhibition was designated by Δ (Fig. 2), which was calculated from $\log(1/GI_{50})-\text{mean}[\log(1/GI_{50})]$ as described previously.[23]

***In vivo* antitumor activity** Tumor masses maintained in nude mice were excised, cut into fragments (ca. 3-mm

Table I. Cell Lines Used in *in vitro* Experiments

Breast cancer (human)		
DU-4475[a]	MCF-7[a]	MDA-MB-157[a]
MDA-MB-231[a]	MDA-MB-361[a]	MDA-MB-415[a]
MDA-MB-435S[a]	MDA-MB-468[a]	SK-BR-3[a]
Colon cancer (human)		
HCT116[a]	SK-CO-1[a]	SW480[a]
SW620[a]	T84[a]	WiDr[a]
Stomach cancer (human)		
AGS[a]	HS746T[a]	MKN28[b]
NUGC-3[c]		
Non-small cell lung cancer (human)		
A549[a]	PC-10[e]	PC-12[d]
PC-13[e]	PC-14[e]	PC-6[e]
PC-7[e]	PC-9[e]	
Small cell lung cancer (human)		
QG90[d]		
Ovarian cancer (human)		
HOC-21[f]		
Leukemia (human)		
CCRF-CEM[a]	K562[g]	
Leukemia (mouse)		
P388[h]		
MDR overexpressing cell (human)		
PC-6/VCR (VCR-selected MDR variant of PC-6)[i]		

a) Obtained from the American Type Culture Collection, Rockville, MD.
b) Supplied by Dr. A. Hoshi, National Cancer Center, Tokyo.
c) Supplied by Japanese Cancer Research Resources Bank, Tokyo.
d) Supplied by Toray Co., Ltd., Tokyo.
e) Obtained from Immuno-Biological Laboratories, Gunma.
f) Supplied by Dr. S. Sekiya, Chiba University Medical School, Chiba.
g) Supplied by Dr. J. Yata, Tokyo Medical and Dental University, Tokyo.
h) Supplied by Dr. T. Tashiro, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo.
i) Established in our laboratory.

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cubes), and transplanted s.c. into nude mice. When the estimated tumor volume (TV) in the mice had grown to between 100 and 300 mm^3, the animals were divided into experimental groups of 6/cage and were treated i.v. with a test drug every 4 days for a total of three doses. The TV was calculated from $TV = L \times W^2/2$ where L and W represent the length and the width of the tumor mass, respectively. After the first administration on day 0, the TV and body weight of the mice were measured two or three times a week for 26 days. Then the tumor masses were excised and weighed. The rate of inhibition of tumor growth on the basis of TV (IR) was calculated from $IR = (1 - RV_t/RV_c) \times 100$, where RV_t represents the mean ratio of TV on day n to that on day 0 of a treated group and RV_c indicates that of the control group. The largest value for IR was designated as IR_{max}, which indicates the greatest effect of each drug. The tumor growth inhibition rate on the basis of tumor weight (IR_{tw}) was calculated from $IR_{tw} = (1 - TW_t/TW_c) \times 100$, where TW_t indicates the mean tumor weight of a treated group and TW_c represents that of the control group. IR_{tw} was statistically analyzed using the Williams-Wilcoxon test (a nonparametric test). The rate of body weight reduction (ΔBW) was calculated from $\Delta BW = (1 - BW_n/BW_0) \times 100$, where BW_n and BW_0 represent the mean body weights of mice on day n and day 0, respectively. The maximum value of ΔBW was designated as ΔBW_{max}.

Topoisomerase I assay Topoisomerase I was prepared from P388 cells by the methods of Ishii *et al.*[24] with minor modifications as described elsewhere in detail.[25] Assay of topoisomerase I activity and calculation of the concentration of each test compound required for 50% inhibition of topoisomerase I activity (IC_{50}) were performed as previously described.[25]

RESULTS

***In vitro* potency against a series of 32 malignant cell lines** The cytotoxicity of DX-8951f, SN-38, SK&F and CPT against 31 human cancer cell lines, including breast cancer, gastro-intestinal cancer, lung cancer, ovarian cancer and leukemia, and against mouse leukemia P388 was assessed. DX-8951f showed strong antiproliferating activity, and the mean GI_{50} values against breast cancers, colon cancers, stomach cancers, and lung cancers were 2.02 ng/ml, 2.92 ng/ml, 1.53 ng/ml, and 0.877 ng/ml, respectively (Table II). Comparing the mean GI_{50} values of the compounds, DX-8951f was about 6 times as potent as SN-38, and about 28 times as potent as SK&F. DX-8951f also showed superior cytotoxicity against human ovarian cancer, leukemia, and mouse leukemia.

To confirm that the spectrum of anticellular activity of DX-8951f was similar to that of SN-38, we also evaluated the patterns of cytotoxicity of the drugs in terms of the value of Δ that was calculated from $\log(1/GI_{50}) - \text{mean}[\log(1/GI_{50})]$ (Fig. 2). The three camptothecin derivatives had similar patterns.

Table II. *In vitro* Cytotoxicity against a Series of 32 Cell Lines

Cell line	GI_{50} (ng/ml)			
	DX-8951f	SN-38	SK&F	CPT
Breast				
DU4475	0.808	1.55	13.8	4.41
MCF-7	0.322	0.64	3.01	1.98
MDA-MB-157	0.163	1.11	2.79	1.31
MDA-MB-231	8.74	35.3	211	45.6
MDA-MB-361	2.52	7.56	263	18.1
MDA-MB-415	3.64	4.32	12.4	14.5
MDA-MB-435S	1.16	5.33	24.0	10.8
MDA-MB-468	0.328	1.36	7.73	5.45
SK-BR-3	0.518	1.94	39.5	10.3
Colon				
HCT116	0.347	1.29	9.39	3.86
SK-CO-1	0.125	0.592	2.22	0.651
SW480	3.00	13.8	65.5	30.7
SW620	0.375	1.78	5.86	3.41
T84	12.1	66.4	435	86.1
WiDr	1.55	6.82	37.3	6.06
Stomach				
AGS	0.335	1.45	6.69	2.84
HS746T	4.47	10.2	57.6	26
MKN28	1.07	14.7	52.6	20.6
NUGC-3	0.240	0.841	5.39	2.66
Lung				
A549	0.914	14.9	45.5	5.69
PC-10	0.778	14.0	50.8	7.89
PC-12	1.15	12.5	40.8	8.34
PC-13	0.368	1.76	8.47	4.04
PC-14	1.82	13.7	110	29.8
PC-6	0.269	0.828	3.19	1.53
PC-7	1.43	2.98	28.2	16.5
PC-9	0.324	2.43	11.1	4.22
QG90	0.844	3.23	19.6	11.9
Others				
CCRF-CEM	0.382	0.901	4.08	3.02
K562	10.4	108	191	54.3
P388	0.728	2.91	12.7	5.73
HOC-21	5.80	32.8	124	41.2
Mean				
Breast	2.02	6.57	64.1	12.5
Colon	2.92	15.1	92.5	21.8
Stomach	1.53	6.80	30.6	13.0
Lung	0.877	7.37	35.3	9.99
Others	4.33	36.2	82.9	26.1
All	2.09	12.1	59.5	15.3

GI_{50} was determined by MTT assay, as described in "Materials and Methods."

Topoisomerase I-inhibitory activity In comparison with CPT and the other CPT analogues, SN-38 and SK&F,

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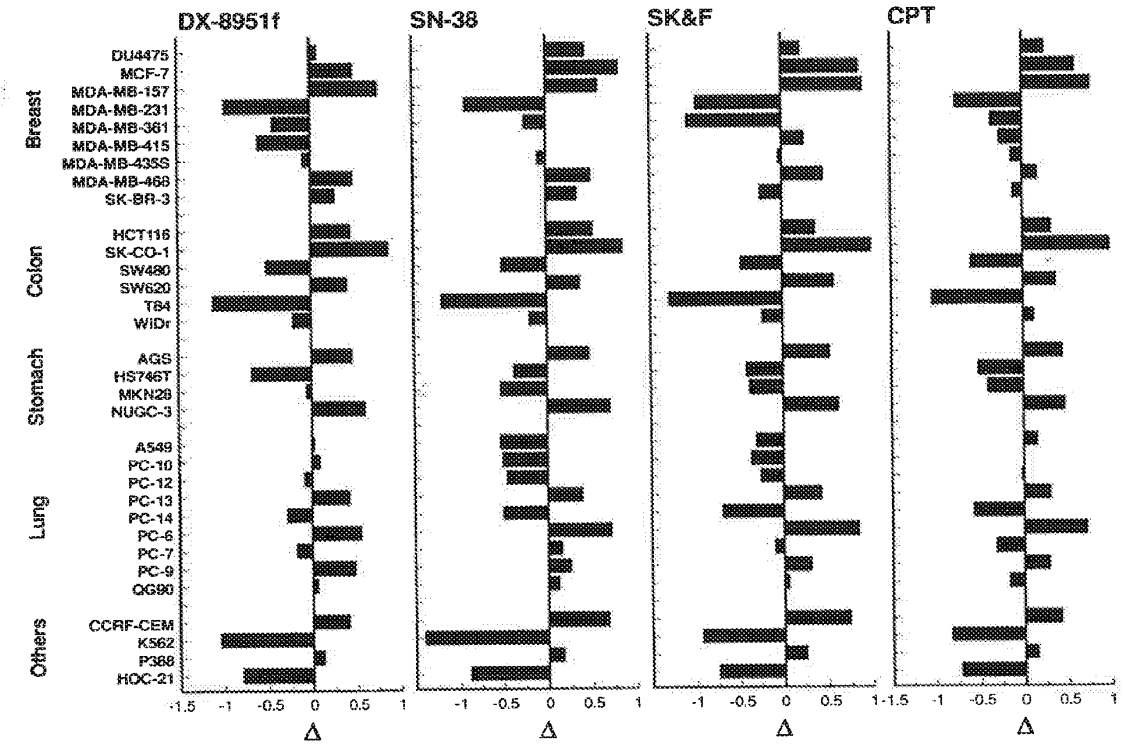


Fig. 2. Pattern of activity against a panel of 32 malignant cell lines. GI_{50} was determined by MTT assay, as described in "Materials and Methods." Δ was calculated from $\log(1/GI_{50}) - \text{mean}[\log(1/GI_{50})]$. Δ indicates the relative sensitivity of each cell line among the 32 cell lines. A cell line having a larger Δ value is more sensitive to the agent, and Δ 0 is the average sensitivity.

DX-8951f was the most potent topoisomerase I inhibitor. DX-8951f was about 3 times as potent as SN-38, and about 10 times as potent as SK&F (Table III).

DX-8951f can overcome P-glycoprotein-mediated resistance To investigate the effect of P-glycoprotein (Pgp) overexpression on the cytotoxicity of DX-8951f, we assessed the cytotoxicity of DX-8951f against a human non-small cell lung cancer cell line, PC-6, and its variant, PC-6/VCR, which was isolated from PC-6 by stepwise selection with increasing VCR concentrations. Overexpression of Pgp was detected by Western blotting analysis with anti-Pgp monoclonal antibody C219 (data not shown). Two independent experiments were done. PC-6/VCR was 380 to 530 times as resistant to VCR, and 3 times as resistant to SK&F as PC-6 (Table IV). On the other hand, Pgp overexpression had little or no effect on the cytotoxicity of SN-38, DX-8951f and CDDP. We also investigated the effect of verapamil on Pgp-overexpressing cells. Verapamil (5 μg/ml) was used with each agent. It greatly affected the cytotoxicity of VCR against PC-6/VCR cells, and enhanced the cytotoxicity of SK&F by about twice. However, verapamil did not affect the cytotoxicity of DX-8951f. DX-8951f had the same degree of activity as the non-MDR agent CDDP against PC-6/VCR.

Table III. Inhibitory Effects on Topoisomerase I Activity

Agent	IC_{50} (μg/ml)
DX-8951f	0.975
SN-38	2.71
SK&F	9.52
CPT	23.5

Topoisomerase I was prepared from P388 cells.

Antitumor activity of DX-8951f against human tumor xenograft As Table V shows, DX-8951f suppressed the growth of human gastric adenocarcinoma SC-6 xeno-

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Table IV. Cytotoxicity against Pgp-overexpressing Cells

Agent	Exp. 1			Exp. 2				
	VR (−)[a] (GI_{50} ng/ml)		Degree of resistance[c]	VR (−)[a] (GI_{50} ng/ml)		Degree of resistance[c]	VR (+)[b] (GI_{50} ng/ml)	VR (+)/ VR (−)[d]
	PC-6	PC-6/VCR		PC-6	PC-6/VCR		PC-6/VCR	
DX-8951f	0.0896	0.0690	0.8	0.179	0.182	1.0	0.156	0.86
SN-38	0.655	0.751	1.1	0.723	1.23	1.7	0.800	0.65
SK&F	1.73	4.36	2.5	2.20	6.37	2.9	2.40	0.38
VCR	0.284	107	380	0.215	114	530	0.225	0.002
CDDP	138	69.0	0.5	113	93.9	0.83	86.5	0.92

GI_{50} was determined by MTT assay, as described in "Materials and Methods."
a) Cells were exposed to each agent in the absence of verapamil.
b) Cells were exposed to each agent in the presence of 5 μg/ml verapamil.
c) (GI_{50} for PC-6/VCR)/(GI_{50} for PC-6) in the absence of verapamil.
d) (GI_{50} for PC-6/VCR in the presence of verapamil)/(GI_{50} for PC-6/VCR in the absence of verapamil).

Table V. Antitumor Activity against Human Tumor Xenograft, Gastric Adenocarcinoma SC-6

Agent	Total (mg/kg)	IR_{max}[a] (%)	IR_{tw}[b] (%)	ΔBW_{max}[c] (%)	No. of toxic deaths
DX-8951f	3.325	54 (12)	43	3.8 (6)	0/6
	6.25	72 (14)	58*	2.6 (6)	0/6
	12.5	82 (17)	73**	5.9 (6)	0/6
	25	95 (17)	90**	7.0 (6)	0/6
	50	96 (17)	92**	12.5 (12)	0/6
CPT-11	173	82 (17)	66**	2.5 (6)	0/6
	260	89 (17)	75**	4.8 (6)	1/6
SK&F	26.6	69 (14)	49*	10.4 (9)	1/6
	40	92 (14)	81**	22.3 (8)	3/6

a) Maximal tumor growth-inhibition rate calculated from the estimated tumor volume; numbers in parentheses indicate the number of days after initial drug administration on which IR_{max} was reached.
b) Tumor growth-inhibition rate calculated from the tumor weight: * $P<0.01$, ** $P<0.001$ as compared with untreated controls.
c) Maximal rate of body weight reduction, the numbers in parentheses denoting the day of ΔBW_{max}.

grafted into nude mice. When mice were treated i.v. with DX-8951f three times at 4-day intervals, significant inhibition of tumor growth was observed over a wide dose range (3.325 mg/kg to 50 mg/kg of total dose) without toxic death. Its antitumor activity was dependent on the total dose, and at the highest dose of 50 mg/kg, the tumor growth inhibition rate was 92%, while the inhibition rate of CPT-11 at around the maximum tolerated dose (MTD), a total dose of 260 mg/kg, was 75%. The tumor growth inhibition rate of SK&F was only 49% at a total dose of 26.6 mg/kg, which was thought to be close to the MTD.

DISCUSSION

In an investigation of water-soluble and non-pro-drug CPT derivatives with strong antitumor activity, we have succeeded in obtaining DX-8951f, a hexacyclic compound having an amino group and a fluorine atom at positions 1 and 5, respectively. It is more potent than SN-38 or SK&F in terms of cytotoxicity. The superior efficacy of DX-8951f has led to its becoming a candidate for further development.

To determine the anticancer spectrum of DX-8951f, its anticellular activity against a series of 32 malignant cell lines was tested *in vitro*. The *in vitro* activities of DX-8951f were stronger than those of the CPT derivative SN-38, the active metabolite of CPT-11, or of SK&F, and showed the smallest GI_{50} values against all of the 32 cell lines used. This series of 32 malignant cell lines was used to test various compounds, and similar patterns of sensitivity were seen for compounds that had similar chemical structures or mechanisms of action to those found in the National Cancer Institute's drug screening program[33] (data are not shown). The pattern of activity of DX-8951f was similar to that of SN-38, and DX-

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8951f had strong topoisomerase I-inhibitory activity. These data suggest that DX-8951f may have the same mechanism of action as CPT-11.

It was reported that SK&F is subject to Pgp-mediated resistance.[26, 27] In our case, although the degree of resistance was low, SK&F was considered to be affected by Pgp, but SN-38 and DX-8951f were not. Overexpression of Pgp has been commonly observed in colorectal carcinoma.[28] In clinical trials CPT-11 was active against colorectal cancer[12] but SK&F was not.[29, 30] Though there might be a considerable difference beween CPT-11 and SK&F, we consider that overexpression of Pgp is an important factor influencing the response in patients with colorectal cancer. DX-8951f was obviously less affected by Pgp compared to SK&F, and equally or less affected compared to SN-38. Therefore, DX-8951f may exhibit activity against Pgp-overexpressing tumors.

Three i.v. doses of DX-8951f administered at 4-day intervals showed strong antitumor activity against human gastric adenocarcinoma SC-6 xenografts transplanted into nude mice. Although both DX-8951f and CPT-11 were significantly effective against SC-6, the effective total dose range of DX-8951f was much wider than that of CPT-11. Moreover, the tumor growth inhibition ratio of DX-8951f was greater than those of CPT-11 and SK&F. The maximum body weight reduction and number of toxic deaths indicate that the toxicity of DX-8951f may be weaker than that of CPT-11 or SK&F at effective dose levels.

In conclusion, DX-8951f exhibits potent antitumor activity both *in vitro* and *in vivo*. Furthermore, it may overcome Pgp-mediated multi-drug resistance, and is expected to reduce the interpatient variations in side effects and efficacy since it is not a pro-drug. DX-8951f is therefore considered to be a promising candidate drug for the treatment of various cancers.

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Whether HER2-positive non-breast cancers are candidates for treatment with Ado-trastuzumab emtansine?

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Abstract

The National Comprehensive Cancer Network (NCCN) has recommended Ado-trastuzumab emtansine (T-DM1) as a preferred agent for patients with human epidermal growth factor receptor-2 (HER2)-positive metastatic breast cancer and prior trastuzumab therapy. Overexpression of HER2 was reported in other cancer types such as bladder, gastric and urogenital carcinosarcomas similar to what is discovered in breast cancer. Some preclinical studies demonstrated the potential anti-tumor effects of T-DM1 in HER2-positive non-breast cancers. There is a paucity of data over the clinical evaluation of T-DM1 in human studies of non-breast cancer patients. We review some preclinical and ongoing clinical studies that assessed the efficacy of T-DM1 administration in the treatment of non-breast HER2 positive malignancies. Performing large and well-designed trials in this area is matter of interest and highly recommended.

Keywords: Ado-trastuzumab emtansine, cancer, human epidermal growth factor receptor 2, recombinant humanized monoclonal antibody

INTRODUCTION

Overexpression of human epidermal growth factor receptor 2 (HER2) is reported approximately in 15–20% primary human breast cancers.[1] HER2 and other member of HER superfamily, consist of tyrosine kinase receptors which have roles in the proliferation's regulation and epithelial cells' survival.[2] HER2 is a proto-oncogene encoded by ErbB2 located on chromosome 17. It is one of the members of HER family and has four plasma membrane-bound receptors of tyrosine kinases that transmit extracellular signals to initiate cellular signaling pathways via mitogen-activated protein kinase, phosphoinositide 3-kinase, phospholipase C, protein kinase C, and signal transducer and activator of transcription. Recently, there are some reports that HER2 is expressed in many tissues, including the breast, gastrointestinal tract, kidney, and heart. It has been suggested that possession of HER2 lead to cell proliferation and also apoptosis suppression. This irregularity induces excessive/uncontrolled cell growth and tumorigenesis.[3,4,5]

In the past, patients who suffer HER2-positive breast cancer had poor outcome;[6] however, after introducing trastuzumab, a recombinant humanized monoclonal antibody that binds to the extracellular subdomain of HER2, the destinies have changed.[7] It is worth noting that not all the patients take advantages of trastuzumab administration, approximately 15% of patients relapse after treatment, indicating trastuzumab resistance eventually emerges in the patients. Several resistance mechanisms have been noted to be responsible including changes in receptor–antibody interaction, induction of the downstream pathways due to increasing in signaling from either other members of the HER family or other receptors, and finally constitutive activation of downstream elements.[8,9]

Resistance to trastuzumab as a HER2 receptor antagonist, causing concerns, and in recent years, great efforts were applied in developing therapeutic agents to either potentiate the effect of trastuzumab or target cells which have become resistant to trastuzumab.

Ado-trastuzumab emtansine (T-DM1), is an antibody-drug conjugate, in which trastuzumab linked to the fungal cytotoxic agent, mertansine. This agent was approved for the HER-positive metastatic breast cancer. Antibody-drug conjugate such as T-DM1 are composed of antigen-specific antibodies bounds to cytotoxic agents in order to selectively transfer drugs to tumor cells and minimize systemic toxicity.[10,11]

T-DM1 consists of the monoclonal antibody trastuzumab linked to a potent microtubule inhibitor (emtansine), leading to a targeted delivery of chemotherapy agent to cells that overexpress HER2.[12]

A derivative of maytansine, emtansine (also known as DM1), is a cytotoxic agent that its mechanism of action is, microtubule inhibition similar to vinca alkaloids. Off note, emtansine are more potent than vincristine and vinblastine *in vitro* and failed to show therapeutic effect at tolerable doses.[13]

T-DM1 binds to the HER2 receptors, and makes the HER2 receptor and drug-antibody conjugate internalized via endocytosis. Inner of cells, emtansine separates from the attached antibody by lysosomal degradation. Hence, emtansine binds to its target, tubulin, and causes cell cycle arrest and apoptosis. The role of trastuzumab is targeted delivery of emtansine and inhibition of HER2 signaling pathways.[14,15]

T-DM1 has primarily been evaluated as a single agent in patients with metastatic HER2-positive breast cancer. There are several Phase II studies on which T-DM1 was administered every 3 weeks as a single agent.[16,17,18]

Afterwards, the Phase III clinical trial, trastuzumab emtansine for HER2-positive advanced breast cancer (EMILIA), was released. This study was the only competed Phase III clinical study, in which T-DM1 administration was randomly compared to lapatinib plus capecitabine in patients with HER2-positive, unresectable, locally advanced, or metastatic breast cancer who were previously treated with trastuzumab and a taxane. The results were in favor of the positive effects of T-DM1 administration in overall survival and median duration of response.[19]

According to the package labeling, appropriate candidates for T-DM1 include patients who have received prior therapy for metastatic disease or those who have developed recurrent disease during or within 6 months of completing adjuvant therapy.[20]

The National Comprehensive Cancer Network has recommended T-DM1 as a preferred agent for patients with HER2-positive metastatic breast cancer and prior trastuzumab therapy.[21]

According to promising and hopeful results of T-DM1 administration in patients with HER2-positive metastatic breast cancer, this agent attracts attention in using in other HER2-positive non-breast cancers. New investigation unleashed the presence of HER2 receptors in other malignant organs such as bladder, gastric, and urogenital carcinosarcomas.

In this article, we reviewed the available published data from preclinical and clinical trials over the use of T-DM1 in HER2-positive non-breast cancers. This review focuses on encouraging researchers and clinicians to perform future investigation in this era.

ADO-TRASTUZUMAB EMTANSINE IN HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2-POSITIVE GASTRIC CANCER

One of the most frequent and fatal human malignancies all over the world is gastric cancer.[22] Since it is asymptomatic in preliminary phase, many patients suffered from gastric cancer are diagnosed when cancer develop beyond curative strategy. By recent development in molecular biology of HER2 and analyses in genomics and proteomics finding, it has now been discovered that HER2 is implicated in other severe forms of cancer, especially gastric cancer.[23] The frequency of HER2-positive overexpression in gastric cancer estimated around 20% and is associated with poor clinical outcome.[23] Hence, inhibiting the HER family signal transduction is likely contribute to improved survival of patients surfed from gastric cancer.

One of the milestone studies over the use of anti-HER2 antibody in the treatment of gastric cancer is trastuzumab for gastric cancer (ToGA) study.[24] ToGA was an open-label, international, Phase III, randomized controlled trial undertaken in 122 centers worldwide. In this study, patients with gastric or gastro-esophageal junction cancer who have overexpression of HER2 protein were included. Participants were randomly assigned to receive a standard chemotherapy regimen or chemotherapy in combination with intravenous trastuzumab. The ToGA trial is a landmark in the treatment of gastric cancer, since following the successful completion of the study, trastuzumab, in combination with chemotherapy, approved for this indication. Results showed that median overall survival was 13·8

months (95% confidence interval [95% CI] 12–16) in those received trastuzumab plus chemotherapy compared to 11·1 months[10,11,12,13] in those received chemotherapy alone (hazard ratio 0·74; 95% CI 0·60–0·91; $P = 0·0046$). Prolonged overall survival in trastuzumab administrated group, led to the guidelines recommendation that HER2 testing should be routinely performed in patients with metastatic or recurrent gastric cancer.[25] Due to promising results by trastuzumab combination therapy in HER2-positive gastric cancer, consequently T-DM1 became the corn stone of consideration.

In gastric cancer, T-DM1 has been evaluated in several preclinical, *in vitro* and *in vivo*, HER2 positive cell culture.[26,27] For the first time in 2011, Barok *et al*.[26] studied the effects of T-DM1 on HER2-positive human gastric cancer cells and xenograft tumors. *In vitro* study, four HER2-positive gastric cancer cell lines including (N-87, OE-19, SNU-216, and MKN-7) were applied. Results showed that except in SNU-216 cells, T-DM1 has superior inhibitory activity against cancer cells in comparison to trastuzumab. *In vivo* study, using same cell lines in xenograft tumors, similar positive antitumor effects was found by T-DM1. In this study, T-DM1 has a promising antitumor effect in HER2-positive gastric cancer cell lines by direct effects including apoptotic cell death and improper mitosis as well as antibody-mediated cellular cytotoxicity, even in tumors which had developed resistance to trastuzumab.

In another preclinical study, T-DM1 as a single agent showed statistically significant antitumor activity in three HER2-rich expression tumor models evaluated (NCI-N87, SCH and 4-1ST). In addition, the combination of T-DM1 and pertuzumab, a HER2 dimerization inhibitor, in xenografted gastric tumors induced significant tumor regression. The possible mechanisms involved in tumor regression of combination treatment are enhancement of antibody-dependent and increase in cellular cytotoxicity by AKT, v-akt murine thymoma viral oncogene homolog 1, and signal inhibition.[27]

Nowadays, in a clinical trial which is underway, the efficacy and safety of T-DM1 compared with standard taxane therapy in patients with HER2-positive advanced gastric cancer was examined.[28] In this multicenter, randomized, adaptive Phase II/III study, patients will be randomized to one of the three arms, 3.6 mg/kg T-DM1 every 3 weeks, 2.4 mg/kg T-DM1 every week, or taxane therapy (docetaxel or paclitaxel per investigator choice), for at least four cycles (12 weeks). Patients will receive study protocol treatment until disease progression, unacceptable toxicity, or withdrawal. Considered endpoints of the study are overall survival, progression free survival, duration of response, and time to gastric cancer symptom progression, as well as safety.

Using T-DM1 in HER2-positive gastric cancer is in infancy, lots of well-designed randomized control trials must be handled to clarify the real indication, proper doses, and duration of treatment by this agent.

ADO-TRASTUZUMAB EMTANSINE IN HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2-POSITIVE BLADDER CANCER

Urothelial bladder carcinoma is a major global health concern responsible for 165,000 deaths annually. Over the two past decades, there has been no statistically significant improvement in 5-year patients' survival rates for locally advanced and metastatic condition.[29] In recent study, several mutations in the extracellular and tyrosine kinases domains of HER2, was reported in urothelial bladder

cancers. HER2 amplifications were detected in 9% of bladder cancer specimens.[30] It has been suggested that having HER2 expression correlated with inferior recurrence-free survival.[31,32]

Furthermore, identifying of HER2 mutations and the role of this pathway in bladder carcinogenesis is desirable. More heavily reason back to the use of anti-HER2 agents, (e.g., trastuzumab, lapatinib, ado-trastuzumab emtansine, and pertuzumab) in the bladder carcinoma's treatment.

In a Phase II study done by Hussain *et al*.,[31] the safety and efficacy (response rates, time to disease progression, survival) of trastuzumab, carboplatin, gemcitabine, and paclitaxel in advanced urothelial carcinoma with HER2 over-expression were evaluated. These patients achieved a remarkable response rate of 70% (31 out of 44 patients; 5 complete responses, and 26 partial responses), and a median time to progression and overall survival of 9.3 and 14.1 months, respectively. The primary end point of the study was cardiac toxicity that was higher than projected.

Today, it is believed that novel strategies targeting HER2 lead to outcomes of HER2 positive bladder cancer's improvement. An encouraging preliminary study was designed by the aim of evaluating the effect of T-DM1 compared to trastuzumab in different cell lines and xenograft models of HER2 over expressing in bladder cancer. In this study, RT4V6 cell line was used as a highest HER2 expressing bladder cancer cell line. In comparison to trastuzumab, T-DM1 showed higher growth inhibition. T-DM1 made apoptosis of RT4V6 cells after G2/M arrest on cell cycle analysis. The most interesting finding was that HER2 expression was higher in cell lines with acquired cisplatin resistance's cell line. Moreover, in an orthotopic bladder cancer xenograft model, tumor growth of cisplatin resistant cells, RT112, was significantly inhibited by T-DM. These inhibitory effects are suggested to be via the induction of apoptosis compared to treatment with control IgG or trastuzumab.[33]

Taken together, although there is a paucity of data around the administration of HER2-possitive bladder cancer, but when we want to looking for emerging strategy, in bladder cancer treatment, targeting this pathway must be considered. However, T-DM1 showed promising antitumor effects in preclinical models of HER2 over expressing bladder cancer, but running several well-designed clinical trial is warranted to prove the potential positive effects of T-DM in affected patients.

ADO-TRASTUZUMAB EMTANSINE IN HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2-POSITIVE UTERINE AND OVARIAN CARCINOSARCOMAS

Carcinosarcoma, also characterized as malignant mixed Müllerian tumors, are one of the rarest and challenging tumors among all gynecological malignancies. These tumors compromise in any genital tract anatomical organs of the female body such as endometrium, ovary, cervix, vulva, vagina or fallopian tube. But among that, endometrium is the most common affected site.

On the other hands, uterine sarcoma accounts for 3–5% of all corpus uteri malignancies with an incidence in the United States of 1–4/100,000 women. On the other hands, ovarian carcinosarcoma has a worse prognosis than uterine carcinosarcoma, with a median survival rate of 8–32 months and recurrence rates of 50–100%.[34,35]

This high rate of tumor recurrence, resistance to therapy, and the poor median survival of patients suffered from carcinosarcoma propose a need to identify more effective treatment modalities. By identifying carcinomatous component-induced tumor growth, it is suggested that therapeutic interventions targeting cellular pathways that drive epithelial cell proliferation may potentially be beneficial against these kind of tumor. It has been reported that HER2/neu is overexpressed and amplification in uterine carcinosarcoma ranged from 17% to 43%.[34]

Having HER2, raising the possibility that anti-HER2 therapies may be an effective modalities in the treatment of carcinosarcoma.

For the first time, English *et al*.[36] evaluated the T-DM1 against HER2-positive uterine serous carcinoma *in vitro* followed by developing a supportive carcinosarcoma *in vivo* model.

In this study, fifteen primary uterine serous carcinoma cell lines were assessed for HER2 protein expression by the aim of immunohistochemistry and flow-cytometry. They found that near 33% of uterine serous carcinoma were positive for HER2 gene amplification and protein overexpression. *In vitro*, T-DM1, and trastuzumab cytostatic and apoptotic activities were evaluated by flow-cytometry-based proliferation assays and showed that T-DM1 was notably more effective than trastuzumab in inhibiting cell proliferation and inducing apoptosis in HER2 overexpression's cell line. Moreover, in mice model, T-DM1 treatment led to highly reduction in tumor size and longer survival comparing to trastuzumab and control group. Furthermore, T-DM1 showed effective antitumor activity in both xenograft and cell lines models. It may represent a novel treatment option for HER2-positive uterine serous carcinoma patients with disease refractory to trastuzumab and traditional chemotherapy.

The positive effects of T-DM1 against primary HER2 positive and HER2 negative carcinosarcoma cell lines were documented in another report. Nicoletti *et al*. in 2015,[37] in a similar experimental study noted that T-DM1 was dramatically more effective than trastuzumab in inhibiting cell proliferation and in inducing G2/M phase cell cycle arrest in the HER2 expressing cell lines *in vitro* and *in vivo*.

According to new and promising finding from these two study, we can conclude that T-DM1 at least may represent a novel treatment option for the subset of HER2 positive carcinosarcoma patients with disease refractory to chemotherapy. Although these results must be reconfirm in several well-designed clinical studies.

CONCLUSION

Antibody-mediated therapy for the treatment of various human malignancies has proved efficacy in the past 30 years, and it is now one of the most successful strategies for targeted treatment of patients suffering from hematological malignancies and solid tumors.[38]

Not so far days, when trastuzumab, as a recombinant humanized monoclonal antibody, has been introduced to HER2 positive breast cancer treatment, hope opens door for these group of poor prognosis cancers. But todays concerns were raised along to trastuzumab resistance that was induced by malignant cells. Furthermore, resistance to HER2-directed therapies remains a challenge.[8] Evidence

suggests that combinations of HER2-directed agents by other cytotoxic ones may show additive or synergistic effects and lead to improving outcome.[39]

By developing the antibody–drug conjugates technique, the opportunity came up to take advantage of selectively delivery of potent cytotoxic drugs by monoclonal antibodies to antigen-expressing tumor cells.

Base on the success results from experimental studies, recently, Food and Drug Administration was approved brentuximab vedotin (Adcetris®) and T-DM1 (Kadcyla®), for clinical applications of cancer treatment. Other new agents are rapidly expanding.[39]

The clinical studies demonstrated that T-DM1 is associated with improved efficacy and safety compared with traditional chemotherapy. Several clinical trials showed the beneficiary effects of T-DM1 in the HER2-positive metastatic setting and in patients with prior exposure to trastuzumab and lapatinib.[40]

The use of T-DM1 in other malignances with HER2 overexpression and gene-amplification except than breast cancer is of key interest. New technical development has demonstrated that HER2 overexpression is seen in some other malignancies such as gastric cancer, bladder cancer, uterine and ovarian carcinosarcomas. It has suggested that up to 20%, 9%, 30% of gastric, bladder, and uterine carcinosarcomas, respectively, overexpress HER2 and is associated with significantly worse outcomes.[23,31,34] In comparison to HER2 positive breast cancer by near 30% overexpression,[5] these quantities are large enough to be considering in treatment modalities.

By developing the new concern around trastuzumab resistance and on the other hand, documented efficacy of T-DM1 as better therapeutic option, T-DM1 is now in the spotlight.

This review has discussed just some of the limited data showing the preclinical benefits of T-DM1 for treating HER2-positive non-breast cancer. All *in vitro* and *in vivo* study confirmed that T-DM1 administration led to prolonging overall survival and progression-free survival. Hence, by considering these promising results, it is time to assessed clinical responses by designing Phase I/II clinical trials. If future clinical results can demonstrate the similar results as preclinical data, then new era in the treatment of any HER2-positive non-breast cancer and survival prolongation will be created.

In March 2011, based on the results of the ToGA trial,[24] trastuzumab was intensively approved in gastric cancer. The introduction of trastuzumab made new term in gastric cancer entitled “HER2-positive gastric cancer,” similar to HER2-positive breast cancer. This discovery gives us a clue that HER2-positive non-breast cancers are developing and identifying. Finding new agents for treatment of them are important future issues.

As an example, new advancing in the knowledge of urothelial carcinoma pathophysiology and underlying molecular mechanisms showed the most relevant molecular pathways that might demonstrate therapeutic potential.[34] The most important relevant overactive signaling networks are fibroblast growth factor receptor, PI3K/AKT/mTOR, and HER2. Any development in identifying

the signaling networks on each cancer types leads to use of another possible effective treatment modality in the future. Furthermore, if clinical trials reach to same prominent results as we reviewed. In the near future, we will see the routine use of trastuzumab conjugated agents such as T-DM1 in chemotherapy regime of other HER2-positive non-breast malignancies.

We strongly recommend managing well-designed control trials to clarified future additional data, over the more precise number and roles of HER2 receptors as a therapeutic target in HER2-positive non-breast malignancies. We need to know many details about the best administrated regime in HER2-positive non-breast malignancies. While we are in the beginning of way and it is soon for any important decision.

AUTHORS' CONTRIBUTION

Azadeh Moghaddas was responsible for literature review, data collection, and preparing and editing the manuscript draft. Ali Borhani helped in data gathering.

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Conflicts of interest

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Original article

TROP2 expression as prognostic marker for gastric carcinoma

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ABSTRACT

Background: In gastric cancer the recurrence rate is unacceptably high, even after R0 resection and (neo)adjuvant chemotherapy. Therefore, there is an urgent need for identification of predictive and/or prognostic biomarkers to select high-risk patients who might benefit from additional therapies. Expression of TROP2 has been shown to be associated with tumour aggressiveness and poor prognosis in patients with various epithelial cancers.

Aims: To investigate TROP2 expression in gastric cancer and its correlation with clinicopathological features and disease outcome.

Methods: Expression of TROP2 was investigated by immunohistochemistry of tumour specimens from 104 patients who underwent resection for gastric cancer. Parameters found to be of prognostic significance in univariate analysis were verified in a multivariate Cox regression model.

Results: TROP2 was found to be overexpressed in 58 (56%) tumour samples. Significantly higher expression of TROP2 could be detected in intestinal-type carcinomas ($p = 0.03$). In intestinal-type gastric cancer, TROP2 overexpression was significantly correlated with shorter disease-free survival (DFS) ($p = 0.03$). Among the total group, TROP2 overexpression was predictive for poor disease-free ($p<0.01$) and overall ($p = 0.03$) survival in lymph node positive patients. Multivariate Cox regression analysis revealed TROP2 overexpression to be an independent prognostic marker for poor DFS in the subgroup of patients with intestinal-type gastric cancer irrespective of lymph node involvement.

Conclusion: Results show that TROP2 is an independent prognostic marker for disease recurrence in intestinal type gastric cancer. Due to its wide distribution TROP2 may become an attractive therapeutic target in a subgroup of patients with gastric cancer.

Gastric cancer represents the second leading cause of cancer mortality worldwide. While surgical resection provides the only means of cure, the prevalence of advanced stage disease at presentation reflects the high mortality rate of this malignancy. But even after potentially curative resection, the five-year survival rates of 35–45% are still modest.[1 2] The positive impact of adjuvant chemoradiotherapy on survival in patients with resected carcinoma was reported in 2001 by Macdonald *et al*.[3] However, the majority of patients develop tumour recurrence. Therefore, there is a need for new potential markers with therapeutic options to better tailor treatment. Cancer immunotherapy and molecular target therapy aimed at cancer specific genes describe a new way of treatment of different cancer entities.[4 5]

The human trophoblast cell-surface antigen TROP2 (also termed GA733-1, M1S1, EGP-1) is encoded by the *TACSTD2* gene which has been mapped to the human chromosome 1p32.[6 7] The *TACSTD* gene family encodes for two different integral membrane glycoproteins, termed TACSTD2 (TROP2) and TACSTD1 (EpCAM). The *TACSTD2* gene encodes a protein with several structural features in common with the transmembrane glycoprotein TACSTD1.[8] TROP2, originally identified on human trophoblast and choriocarcinoma cell lines, was subsequently shown to be highly expressed by the majority of human malignant tumours.[11 12] The function of TROP2 is not well understood although it has previously been suggested to play a role in regulating the growth of carcinoma cells.[13 14] The cytoplasmic tail of TROP2 can be phosphorylated on serine 303 and contains a phosphoinositide binding sequence with a HIKE-like structure. Mutations in this sequence are found in different human genetic diseases and in cancer.[15] We recently showed that TROP2 overexpression was a prognostic marker for poor survival in oral squamous cell carcinoma.[16] As a consequence of its wide distribution on epithelial tumours, TROP2 seems to be an attractive target for immunotherapy. In the present study we examined the expression of TROP2 in the tissue of patients with resected gastric cancer and its correlation with clinicopathological features.

MATERIALS AND METHODS

Patients and tissue samples

The study was conducted according to the regulations of the local ethics committee. A total of 104 patients with gastric cancer resected between 1993 and 2004 at the Department of Visceral, Transplant and Thoracic Surgery, Medical University Innsbruck, were included in this retrospective study. Clinical data were obtained by reviewing the charts as well as from our computerised documentation system (ChiBASE). All data regarding date and cause of death were confirmed by the "Tumorregister Tirol", a cancer register maintained by the Tyrolean government. Tumours were histologically classified according to the Lauren classification into intestinal and diffuse gastric adenocarcinomas and were staged according to the International Union Against Cancer classification (UICC). Adjuvant or additive chemotherapy was recommended to patients with UICC stage III and IV. For subgroup analysis, patients were divided into early stage disease (UICC IA+IB) and advanced stage disease (UICC stage

Table 1 Patient characteristics

Clinicopathological variable	Gastric cancer (n = 104) n (%)
Sex	
Male	70 (67)
Female	34 (33)
Age	
Mean	67
Median	70
Range	30–94
Tumour site	
Proximal third	30 (29)
Middle third	14 (13)
Distal third	57 (55)
Total stomach	3 (3)
Depth of wall invasion (T stage)	
pT1	16 (15)
pT2	60 (58)
pT3	25 (24)
pT4	3 (3)
Lymph node metastasis	
N0	46 (44)
N1, N2	58 (56)
Distant metastasis	
M0	94 (90)
M1	10 (10)
Residual disease	
R0	97 (93)
R1/R2	7 (7)
Lauren classification	
Intestinal	88 (85)
Diffuse	16 (15)
Stage (UICC)	
IA	15 (14)
IB	22 (21)
II	30 (29)
IIIA+IIIB	19 (18)
IV	18 (17)
Histological grade	
Well	3 (3)
Moderate	33 (32)
Poor	68 (65)
TROP2 overexpression	
Yes	58 (56)
No	46 (44)

>II). Table 2 summarises demographic data and tumour characteristics.

Immunohistochemistry

The expression of TROP2 was determined by immunohistochemistry using the purified goat polyclonal antibody against the recombinant human TROP2 extracellular domain at a dilution of 1:50 (AF650, R&D Systems, Minneapolis, Minnesota, USA). Briefly, 5 μm sections were cut from paraffin blocks, mounted on adhesive-coated glass slides (Instrumedics, Missouri, USA), deparaffinised and rehydrated with xylene and graded alcohol series. Endogenous peroxidase was blocked with methanol containing 30% hydrogen peroxide for 20 min. Pretreatment consisted of a 20 min incubation period in a pronase solution (water bath at 37°C). After several washes, sections were incubated in a biotinylated anti-goat antibody for 30 min (1:100, Ab6740, Abcam, Cambridge, UK). Labelling for TROP2 was completed using the ABC reagent (Vectastain Elite ABC Kit Standard PK-6100, Vienna, Austria) for 30 min at room temperature. Visualisation of immunolabelled structures was achieved by incubating sections in diaminobenzidine/H_2O_2 solution containing 30% hydrogen peroxide until coloration was visible (usually 3–4 min). Finally, slides were counterstained with Mayer's hemalaun solution (Merck, Darmstadt, Germany), followed by clearing and mounting. Positive and negative controls were included in each run. Representative micrographs of tumours with predominant membranous staining of TROP2 are shown in fig 1.

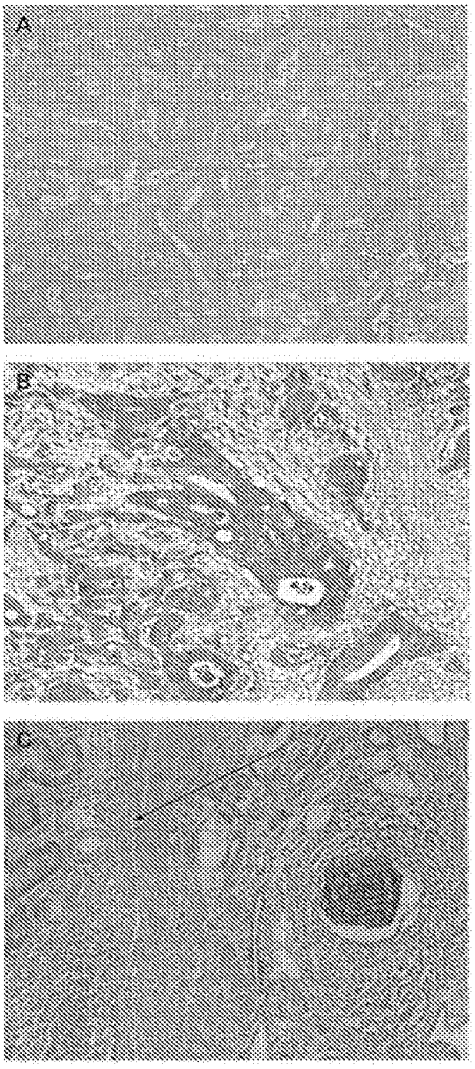


Figure 1 Immunohistochemical staining for TROP2 expression in human gastric cancer tissues, original magnification ×100. Note the homogenous membranous immunostaining of tumour cells. (A) Gastric cancer without immunostaining. (B) Gastric cancer with strong (score 12) expression. (C) Normal gastric mucosa with immunostaining (arrow).

Table 2 Correlation of TROP2 with conventional clinicopathological parameters in patients with intestinal-type and diffuse-type gastric cancer

	Total patients	p Value		TROP2 overexpression		
				No	Yes	
	n	DFS	OS	n (%)	n (%)	p Value
Intestinal-type carcinoma						
Sex						
Male	63	0.60	0.53	23 (26)	40 (45)	0.32
Female	25			12 (14)	13 (15)	
Histological grade						
Well	3	0.51	0.06	2 (2)	1 (1)	0.48
Moderate	32			14 (16)	18 (20)	
Poor	53			19 (22)	34 (39)	
Depth of wall invasion (T stage)						
pT1	16	0.001	0.01	6 (7)	10 (11)	0.95
pT2	52			20 (23)	32 (36)	
pT3	18			8 (9)	10 (11)	
pT4	2			1 (1)	1 (1)	
Lymph node metastasis						
No	36	0.001	0.01	12 (14)	24 (27)	0.38
Yes	52			23 (26)	29 (33)	
Distant metastasis						
No	79	0.003	0.001	33 (38)	46 (52)	0.25
Yes	9			2 (2)	7 (8)	
Resection margin						
R0 vs.	85	0.001	0.63	35 (41)	50 (59)	0.15
R1+R2	3				3 (100)	
Stage (UICC)						
I	32	0.01	0.40	11 (13)	21 (24)	0.43
II+III+IV	56			24 (27)	32 (36)	
Diffuse-type carcinoma						
Sex						
Male	7	0.48	0.81	4 (25)	3 (19)	0.37
Female	9			7 (44)	2 (12)	
Histological grade						
Well	3	0.13	0.15	1 (6)	5 (31)	0.48
Moderate	15			10 (63)		
Poor						
Depth of wall invasion (T stage)						
pT1		0.001	0.13			0.01
pT2	8			8 (50)		
pT3	7			2 (12)	5 (31)	
pT4	1			1 (6)		
Lymph node metastasis						
No	10	0.79	0.61	8 (50)	2 (12)	0.21
Yes	6			3 (19)	3 (19)	
Distant metastasis						
No	15	0.001	0.05	10 (63)	5 (31)	0.48
Yes	1			1 (6)		
Resection margin						
R0 vs.	12	0.68	0.85	9	3	0.35
R1+R2	4			2	2	
Stage (UICC)		NF	NF			
I	5			5 (31)	5 (31)	0.06
II+III+IV	11			6 (38)		

DFS, disease-free survival; OS, overall survival; NF, not feasible.

Evaluation of TROP-2 expression

Two independent pathologists (PM and HM) evaluated TROP2 expression in a blinded fashion using light microscopy. Discordant cases were re-evaluated to achieve a consensus. Antigen expression was defined as the presence of specific staining on the surface of tumour cells. TROP2 overexpression was evaluated by calculating a total immunostaining score as the product of a proportion and intensity score. The proportion score described the estimated fraction of positive stained tumour cells (0 = none; 1 = <10%; 2 = 10–50%; 3 = 51–80%; 4 = >80%). The

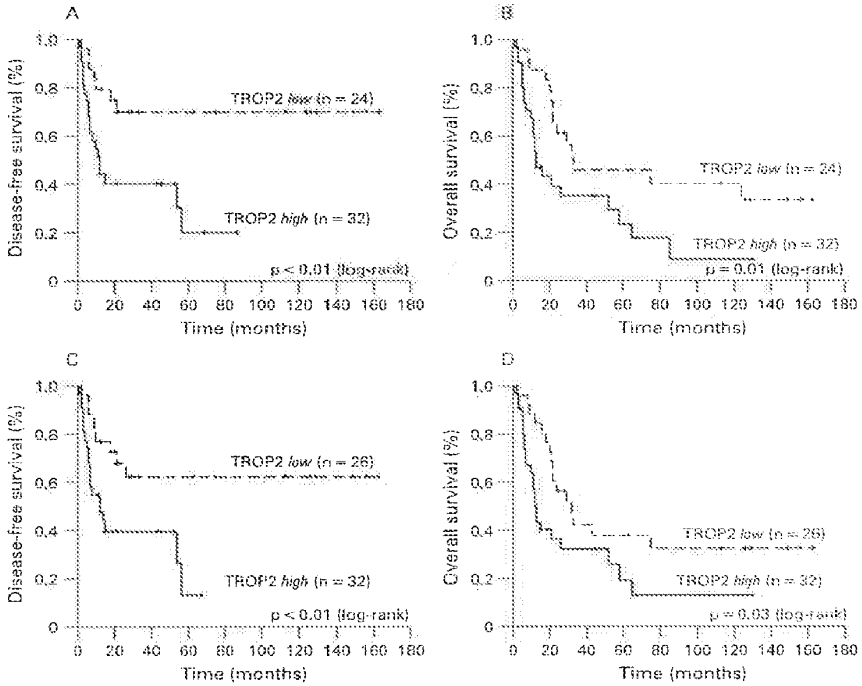


Figure 2 (A) Prognostic significance of human trophoblast cell-surface antigen (TROP2) overexpression in 56 patients with advanced intestinal type cancer regarding disease-free survival by Kaplan–Meier analysis. *high*, patients with TROP2 overexpressing tumour (n = 32); *low*, patients with tumour (n = 24) without TROP2 overexpression. Patients with *low* TROP2 expression had a significantly better disease-free survival rate than patients with *high* TROP2 expression as defined by log-rank test (p<0.01). (B) Prognostic significance of human trophoblast cell-surface antigen (TROP2) overexpression in 56 patients with advanced intestinal type gastric cancer regarding overall survival by Kaplan–Meier analysis. *high*, patients with TROP2 overexpressing tumour (n = 32); *low*, patients with tumour (n = 24) without TROP2 overexpression. Patients with *low* TROP2 expression had a significantly better overall survival rate than patients with *high* TROP2 expression as defined by log-rank test (p = 0.01). (C) Prognostic significance of human trophoblast cell-surface antigen (TROP2) overexpression among all gastric cancer patients with lymph node metastasis (n = 58) regarding disease-free survival by Kaplan–Meier analysis. *high*, patients with TROP2 overexpressing tumour (n = 32); *low*, patients with tumour (n = 26) without TROP2 overexpression. Patients with *low* TROP2 expression had a significantly better disease-free survival rate than patients with *high* TROP2 expression as defined by log-rank test (p<0.01). (D) Prognostic significance of human trophoblast cell-surface antigen (TROP2) overexpression among all gastric cancer patients with lymph node metastasis (n = 58) regarding overall survival by Kaplan–Meier analysis. *high*, patients with TROP2 overexpressing tumour (n = 32); *low*, patients with tumour (n = 26) without TROP2 overexpression. Patients with *low* TROP2 expression had a significantly better overall survival rate than patients with *high* TROP2 expression as defined by log-rank test (p = 0.03).

intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score ranged from 0 to 12. Regarding the total score the tissue samples were bimodally distributed with the nadir at a total score of 3–4. Therefore, TROP2 "overexpression" was arbitrarily defined as a total score >4 as described previously.[7]

Statistical methods

Statistical analysis was performed using SPSS V.10.0 (SPSS, Chicago, Illinois, USA). Survival curves were calculated using the Kaplan and Meier method and compared by the log-rank test. Follow-up time was censored if the patient was lost to follow-up. Patients who died without documented disease recurrence were considered censored for disease-free survival but were included as deaths for overall survival analysis. The association between TROP2 expression and clinicopathological variables were assessed by the χ^2 test. Factors shown to be of prognostic significance in the univariate models were evaluated in a multivariate Cox regression model. A p value <0.05 was considered statistically significant.

RESULTS

Surgical outcome

All patients underwent surgical intervention with curative intent. Total gastrectomy was performed in 47 patients whereas 57 patients had undergone subtotal gastrectomy. Gastric cancer with early stage disease (UICC IA+IB) was noted in 37 (36%) cases, while advanced cancer (UICC>II) was found in 67 (64%) cases. Chemotherapy was recommended to patients with UICC stage III and IV. In six patients neoadjuvant chemotherapy was used to facilitate radical resection. Among all patients, complete resection (R0) of the tumour was possible in 97 (93%) cases.

Histological subtypes

Intestinal-type gastric cancer was diagnosed in 88 (85%) cases whereas 16 (15%) patients had a diffuse carcinoma. Median overall survival time for patients with intestinal-type carcinoma was 52 months (range 1–163); it was 16 months (range 1–54) for diffuse-type carcinoma. For diffuse-type gastric cancer patients median disease-free survival was 14 (range 1–55) months (not reached in intestinal-type gastric cancer)

Table 3 Univariate survival analysis for the total cohort regarding disease-free and overall survival (log-rank)

	No n (%)	DFS p Value	OS p Value
Sex			
Male	70 (67)	0.27	0.88
Female	34 (33)		
Histological grade			
Well	3 (3)	0.19	0.62
Moderate	33 (32)		
Poor	68 (65)		
Lauren classification			
Intestinal	88 (85)	0.009	0.008
Diffuse	16 (15)		
Depth of wall invasion (T stage)			
pT1	16 (15)	0.001	0.006
pT2	60 (58)		
pT3	25 (24)		
pT4	3 (3)		
Lymph node metastasis			
No	46 (44)	0.001	0.03
Yes	58 (56)		
Distant metastasis			
No	94 (90)	0.001	0.001
Yes	10 (10)		
Resection margin			
R0 vs	97 (93)	0.01	0.03
R1+R2	7 (7)		
Stage (UICC)			
I	37 (36)	0.001	0.006
II+III+IV	67 (64)		
TROP2 overexpression			
Yes	58 (44)	0.16	0.97
No	46 (56)		

DFS, disease-free survival; OS, overall survival.

Histological subtype significantly correlated with gender, age, grade, resection margins and overexpression of TROP2, whereas no correlation was found for depth of invasion, nodal stage and the presence of distant metastases. TROP2 overexpression could be detected in 60% of intestinal-type cancer and 31% of diffuse type ($p = 0.03$), respectively.

Immunohistochemistry

Normal gastric epithelial cells showed no to weak TROP2 staining. In contrast, in cancer cells a moderate to strong homogeneous membranous expression of TROP2 was noted in 58 (56%) carcinoma specimens (fig 1). Strong TROP2 expression (score 9–12) was found in 23 of 104 (22%) cases, whereas moderate expression (score 6–8) occurred in 35 (34%) cases and weak or no expression (score 0–4) in 46 (44%) cases. Thus, according to the above mentioned criteria, TROP2 was found to be overexpressed in 58 (56%) of 104 cases.

TROP2 expression and correlation with clinicopathological features

By univariate analysis we compared TROP2 overexpression to sex, histological grade, T stage, lymph node metastases, presence of distant metastases, resection margin and UICC stage (table 2).

In diffuse-type gastric cancer patients TROP2 overexpression correlated significantly with the depth of wall invasion of the tumour. In contrast, no correlation between TROP2 overexpression and clinicopathological parameters was found in carcinoma of intestinal-type.

Using the method of Kaplan and Meier, patients with intestinal-type carcinoma had a significantly better disease-free ($p<0.01$) and overall survival ($p<0.01$) than patients with diffuse-type gastric cancer. It should be noted that TROP2 overexpression significantly correlated with shorter disease-free survival ($p = 0.03$) in the subgroup of patients with intestinal-type gastric cancer.

Moreover, in patients with advanced intestinal-type gastric cancer (UICC ≥II), TROP2 overexpression was significantly associated with poor disease-free ($p<0.01$) (fig 2A) and overall survival ($p = 0.01$) (fig 2B). In contrast, TROP2 overexpression was not predictive for disease-free and overall survival in patients with intestinal type gastric cancer in an early tumour stage (UICC IA+IB). Interestingly, among the total group, TROP2 overexpression was significantly associated with poor disease-free ($p<0.01$) (fig 2C) and overall survival ($p = 0.03$) (fig 2D) in patients with but not in patients without lymph node metastases.

Univariate survival calculations for the total cohort revealed depth (T stage) of wall invasion ($p<0.01$), nodal status ($p<0.01$), presence of distant metastasis ($p<0.01$), completeness of resection (R status) and UICC stage ($p = 0.001$) as significant predictors of disease-free survival, whereas gender ($p = 0.27$) and histological grade ($p = 0.19$) were not. There was a trend towards inferior disease-free survival in patients with TROP2 overexpression. Regarding overall survival, T stage ($p<0.01$), nodal status ($p = 0.03$), presence of distant metastasis ($p<0.01$), R status ($p = 0.03$), histological grade ($p = 0.02$) and UICC stage ($p = 0.006$)were of prognostic significance, whereas gender and TROP2 overexpression were not (table 3).

For multivariate analysis, all statistically significant variables evaluated in the univariate analyses were included in a Cox proportional hazard analysis. Remarkably, in intestinal type carcinoma TROP2 overexpression ($p<0.01$; relative risk 6.39; 95% CI 2.20 to 18.55) along with depth of wall invasion, lymph

Table 4 Multivariate analysis (Cox regression) of several prognostic markers in relation to survival of patients with intestinal-type gastric cancer

	DFS		OS	
	p Value	RR* (95% CI)	p Value	RR (95% CI)
Depth of wall invasion				
T1+T2 vs T3+T4	0.0001	7.9 (2.2 to 18.5)	NS	
Lymph node metastasis	0.0002	11.9 (3.2 to 44.1)	NS	
Distant metastasis	NS		NS	
Resection margin (R0 vs R1+R2)	0.02	5.5 (1.2 to 23.9)	NS	
TROP2 overexpression	0.0006	6.3 (2.2 to 18.5)	NS	

DFS, disease-free survival; OS, overall survival; RR, relative risk; NS, not significant.

node metastases and involvement of free resection margin were independent prognostic factors for disease-free survival (table 4).

For patients with diffuse-type carcinomas multivariate analysis was omitted due to small sample size.

DISCUSSION

The present study showed the expression of TROP2 and its prognostic value in gastric cancer. For the first time, our data provide evidence that TROP2 plays an important role in this aggressive malignancy. Significantly higher expression of TROP2 could be detected in intestinal-type gastric cancer than in diffuse-type carcinoma ($p = 0.03$), although a comparison of the two groups is disputable because of the different number of cases. In intestinal-type gastric cancer TROP2 overexpression was detectable in 53 of 88 (60%) specimens and was an independent prognostic marker for disease-free survival. In univariate analyses TROP2 overexpression significantly correlated with poor disease-free and overall survival in advanced intestinal-type carcinoma. Interestingly, among all patients TROP2 overexpression significantly correlated with poor disease-free and overall survival in lymph node positive patients but not in lymph node negative patients.

TROP2, encoded by the single-exon gene *TACSTD2* has been shown to play an important role in tumour growth. We recently reported that TROP2 overexpression predicts poor survival in oral squamous cell carcinoma.[16] In addition, Nakashima *et al* showed that TROP2 is overexpressed in oesophageal cancer and suggested that its expression could be related closely to malignant transformation.[18] The results of this study provide evidence that TROP2 overexpression predicts poor disease-free and overall survival, particularly in patients with intestinal type gastric cancer. Interestingly, a multistage model of carcinogenesis has been described for intestinal gastric carcinoma which is similar to the adenoma–carcinoma sequence reported for colon cancer.[19] Our data are in line with results from Ohmachi *et al* who indicated that TROP2 was strongly expressed in colorectal cancer, correlating with aggressiveness and poor prognosis.[20]

Moreover we showed that TROP2 overexpression was predictive for disease recurrence and poor overall survival in lymph node positive gastric cancer patients and advanced intestinal type carcinoma.

Although TROP2 has been previously suggested to function as a cell–cell adhesion receptor in cancer cells, recent data are indicative of a function in cell signal transduction and tumour cell growth.[21] Recently, Wang *et al* found evidence that TROP2 has a functional role in tumourigenesis. They showed that a series of colon carcinoma cell lines require TROP2 expression for tumourigenic growth. By knocking down TROP2 in certain colon cancer cell lines, the number of formed cell colonies was dramatically reduced compared to the control cell lines. Furthermore, suppression of TROP2 expression inhibited the invasive behaviour of a certain cell line. Conversely, increasing TROP2 expression led to increased anchorage-independent growth of colon cancer cell lines.[22] TROP2 is highly homologous to the cell–cell adhesion molecule Ep-CAM.[17] Due to immunogenic properties of Ep-CAM in cancer patients, active and passive immunotherapeutic agents have been developed.[23] In resected colon cancer patients, significant reduction in mortality and relapse rates can be induced following treatment with the Ep-CAM-specific monoclonal antibody edrecolomab.[24] The immunogenic properties have been recently described by Mangino *et al* who showed TROP2 to be a target molecule efficiently recognised by human cytotoxic T lymphocytes,[25]

Take-home messages

- TROP2 is an integral membrane glycoprotein that is highly expressed by the majority of human malignant tumours.
- TROP2 overexpression was predictive for poor disease-free and overall survival in lymph node positive gastric cancer patients.
- In intestinal-type gastric cancer, TROP2 overexpression was significantly correlated with shorter disease-free survival.
- In patients with advanced intestinal-type gastric cancer (UICC ≥II), TROP2 overexpression was significantly associated with poor disease-free and overall survival.
- In intestinal-type carcinoma, TROP2 overexpression along with depth of wall invasion, lymph node metastases and involvement of free resection margin were independent prognostic factors for disease-free survival.

Furthermore, a humanised monoclonal antibody (hRS7) was generated with promising results in an in vivo breast cancer model.[26]

In summary, we have found evidence that TROP2 is associated with gastric cancer growth. Due to its wide distribution TROP2 may become an attractive therapeutic target in a subgroup of patients with gastric cancer. Moreover, based on the predictive value for recurrence, TROP2 may also practically be used as an immunohistochemical marker for a more aggressive treatment strategy.

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ORIGINAL ARTICLES

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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

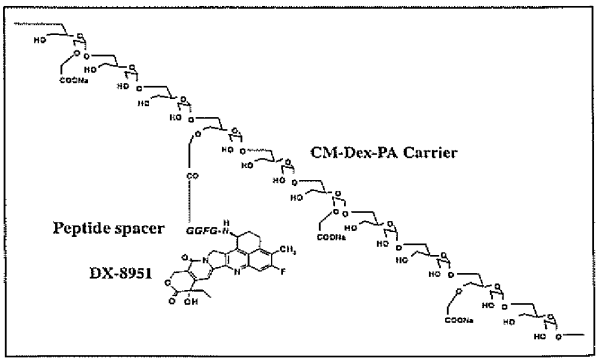


Fig. 1: Partial chemical structure of DE-310

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

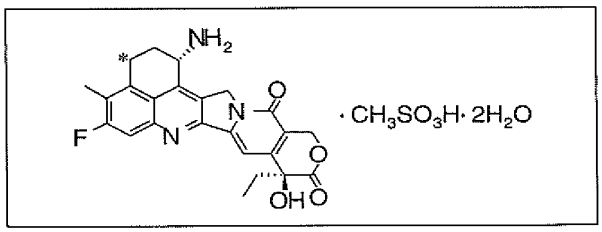


Fig. 2: Chemical structure and labeled position of ^{14}C-DX-8951f
*: ^{14}C-labeled position

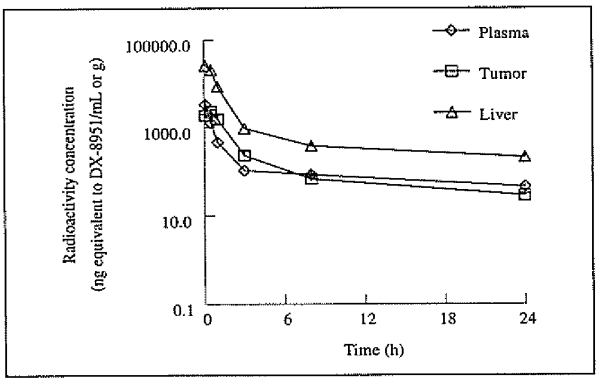


Fig. 3: Radioactivity concentration in plasma, tumor, and liver after a single intravenous administration of ^{14}C-DX-8951f at a dose of equivalent to 6 mg DX-8951/kg to Meth A tumor-bearing mice. Each point with a bar represents the mean + S.D. for four mice

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

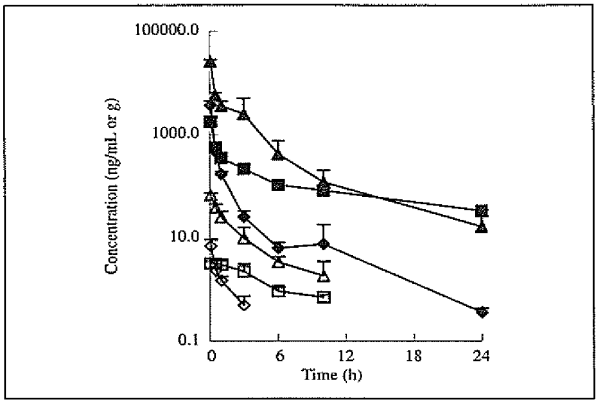


Fig. 4: Concentrations of G-DX-8951 and DX-8951 in plasma, liver, and tumor tissue after a single intravenous administration of G-DX-8951 at a dose of 6 mg/kg to Meth A tumor-bearing mice. Each point with a bar represents the mean + S.D. for four mice. —◆— G-DX-8951 (Plasma), —◇— DX-8951 (Plasma), —■—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

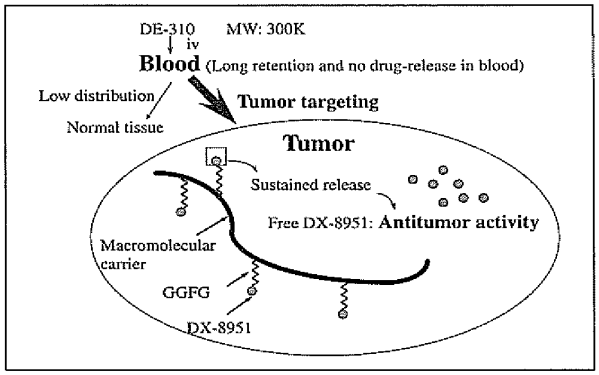


Fig. 5: Tumor targeting of DE-310 by EPR effect

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-inf}) for plasma, liver, and tumor concentrations were calculated using the linear trapezoidal rule.

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LETTERS

Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3

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Oncogenic tyrosine kinases have proved to be promising targets for the development of highly effective anticancer drugs. However, tyrosine kinase inhibitors (TKIs) against the human epidermal growth factor receptor (HER) family show only limited activity against HER2-driven breast cancers, despite effective inhibition of epidermal growth factor receptor (EGFR) and HER2 *in vivo*[1–8]. The reasons for this are unclear. Signalling in *trans* is a key feature of this multimember family and the critically important phosphatidylinositol-3-OH kinase (PI(3)K)/Akt pathway is driven predominantly through transphosphorylation of the kinase-inactive HER3 (refs 9, 10). Here we show that HER3 and consequently PI(3)K/Akt signalling evade inhibition by current HER-family TKIs *in vitro* and in tumours *in vivo*. This is due to a compensatory shift in the HER3 phosphorylation–dephosphorylation equilibrium, driven by increased membrane HER3 expression driving the phosphorylation reaction and by reduced HER3 phosphatase activity impeding the dephosphorylation reaction. These compensatory changes are driven by Akt-mediated negative-feedback signalling. Although HER3 is not a direct target of TKIs, HER3 substrate resistance undermines their efficacy and has thus far gone undetected. The experimental abrogation of HER3 resistance by small interfering RNA knockdown restores potent pro-apoptotic activity to otherwise cytostatic HER TKIs, re-affirming the oncogene-addicted nature of HER2-driven tumours and the therapeutic promise of this oncoprotein target. However, because HER3 signalling is buffered against an incomplete inhibition of HER2 kinase, much more potent TKIs or combination strategies are required to silence oncogenic HER2 signalling effectively. The biologic marker with which to assess the efficacy of HER TKIs should be the transphosphorylation of HER3 rather than autophosphorylation.

Selective inhibitors of the ABL tyrosine kinase are effective in putting nearly all patients with BCR-ABL-driven leukaemia in chronic phase into complete remission[11]. This proof of concept brings new hope to the treatment of other tyrosine-kinase-driven cancers. Another important tyrosine kinase family is the HER family, consisting of EGFR, HER2, HER3 and HER4. A subset of breast cancers are driven by overactive EGFR or HER2 tyrosine kinases and an abundance of data from *in vitro* and mouse models suggests that continued activity of these tyrosine kinases drives cancer progression[12–14]. While the complexities of this tyrosine kinase family are not yet fully understood, their oncogenic signalling functions should, in theory, be amenable to silencing by TKIs. Several orally bioavailable HER-family TKIs are in preclinical and clinical development. Although in *in vitro* biochemical assays these agents differ in their relative activities against individual HER kinase family members, in cell-based assays they are effective at inhibiting both EGFR and HER2 and equally effective at suppressing the growth of EGFR- and HER2-driven tumour cells[15–19]. They are also effective at inhibiting EGFR and HER2 phosphorylation in patients' tissues and tumours[5–8]. But these agents show very limited clinical anti-tumour activity[1–5]. Their clinical development to this point has been driven largely by the detection of modest delays in tumour progression. The failure to reverse cancer progression despite an apparent inhibition of HER kinase function has created an enigma in the concept of TKI cancer therapy that we have been exploring.

It is through heterodimerization and transphosphorylation that the HER family performs its signalling functions. Importantly, downstream PI(3)K/Akt pathway signalling is predominantly mediated through the transphosphorylation of the kinase-inactive member HER3 (refs 9, 10). We have previously reported that sensitivity to HER-family TKI therapy correlates with the inhibition of PI(3)K/Akt pathway signalling[15,20]. We and others have also reported that failure to inhibit PI(3)K/Akt signalling leads to TKI resistance[20–22]. But in contrast to reports from *in vitro* models, Akt activity is not inhibited in most patients on HER TKI therapy[5,6,8]. This led us to look more closely at the inhibition of PI(3)K/Akt signalling.

To investigate this discrepancy, we studied the durability of Akt inhibition by TKI, with surprising results. Although gefitinib inhibits Akt signalling in HER2-driven cancer cells (as previously reported[15]) this inhibition is not durable. Akt signalling resumes after a transient inhibition despite continued drug therapy (Fig. 1a, b). In light of this finding, we looked at the broader HER-family signalling activities over a period of 96 h after continuous exposure of BT474 breast cancer cells to gefitinib at concentrations that non-selectively inhibit EGFR and HER2. TKI treatment effects a sustained inhibition of EGFR and HER2 phosphorylation and a durable inhibition of downstream MAPK (mitogen-activated protein kinase) and JNK (Jun N-terminal kinase) pathway signalling (Fig. 1a). However, dephosphorylation of the kinase-inactive family member HER3 is transient. HER3 signalling resumes and persists despite continued drug exposure and effective suppression of EGFR and HER2 (Fig. 1a, b). The reactivation of HER3 signalling explains the reactivation of Akt signalling, because HER3 is the principal HER-family member that binds PI(3)K and drives Akt signalling in these tumours[9,10]. TKI-refractory Akt signalling remains sensitive to PI(3)K inhibitors as expected (not shown).

These time-dependent findings are not due to drug degradation because the drug is replenished daily in these studies and HER3/Akt signalling resumes despite repeatedly refreshing drug supply up to and beyond the point of resumption of Akt signalling (not shown). There is no significant expression of HER4 before or after drug treatment in these cells (data not shown). These findings are not unique to BT474 and SkBr3 cells and have been confirmed in other HER2-overexpressing breast cancer cells, including MDA-453, AU565, MDA-361 and HCC1954 cells (Supplementary Fig. 1). These findings

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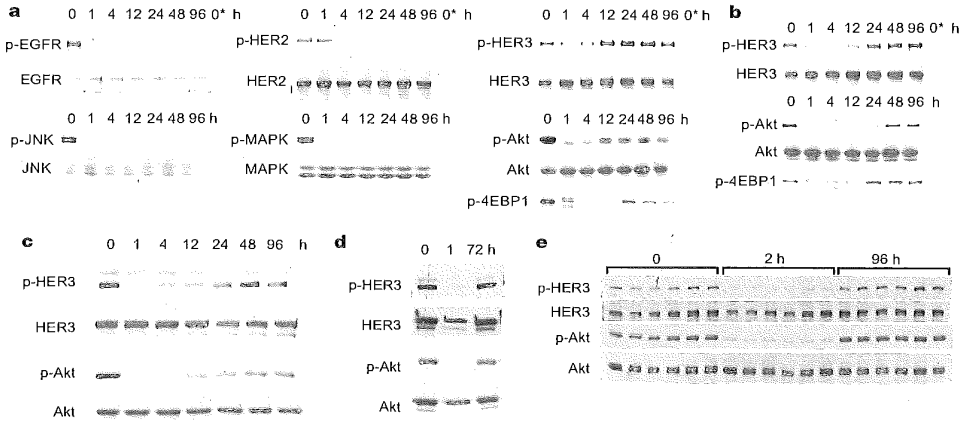


Figure 1 | HER TKIs fail to induce sustained inhibition of HER3 signalling in HER2-driven breast cancer cells. **a**, BT474 cells were treated with 5 μM gefitinib for the indicated times and assayed for expression and phosphorylation of the indicated proteins. Lane 0* is an IgG immunoprecipitation control. **b**, Data of interest shown from the identical experiment done in SkBr3 cells. **c**, SKBr3 cells were treated with 5 μM erlotinib for the indicated times and lysates immunoblotted with the indicated antibodies. **d**, SKBr3 cells were treated with 20 μM AG825 for the indicated times, with the drug being replenished 1 h before the 72 h harvest. **e**, Mice bearing HCC1569 human breast cancer xenografts were treated with gefitinib at 150 mg kg^{-1} once daily. Animals were killed 2 h after the first dose or 2 h after the fourth dose (96 h total treatment) and tumours were rapidly harvested for immunoblotting with the indicated antibodies. The six lanes corresponding to each time point represent six different animals. p, phosphorylated.

are not unique to gefitinib and are seen with other HER TKIs, including agents with *in vitro* selectivity profiles favouring EGFR or HER2, such as erlotinib or AG825 (Fig. 1c, d). Nor are these findings artefacts of the *in vitro* models. Treatment with gefitinib of mice that have various HER2-driven xenograft tumours similarly fails to durably suppress HER3 and Akt signalling, despite a transient suppression (Fig. 1e and Supplementary Fig. 2). This is not due to ineffective drug biodistribution, because in these models gefitinib was dosed three times higher than doses known to achieve sustained xenograft tumour concentrations of above 2–4 μM and averaging 6–10 μM (ref. 23). We had previously established that inactivation of PI(3)K/Akt signalling is mechanistically linked to HER TKI sensitivity in HER-family-driven cancers, so we felt that the failure of these drugs durably to inactivate PI(3)K/Akt signalling was entirely

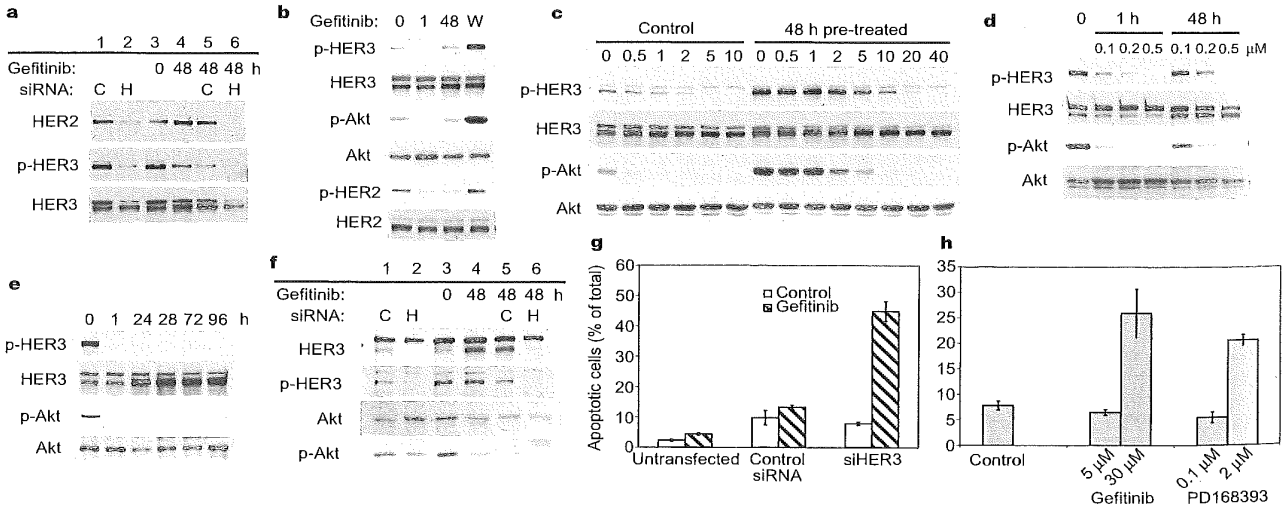


Figure 2 | Forward shift in HER3 phosphorylation–dephosphorylation equilibrium following extended HER TKI treatment. **a**, BT474 cells were transfected with anti-HER2 (H) or control (C) siRNA and harvested 64 h after transfection (lanes 1, 2). Additional arms were treated with 48 h of gefitinib untransfected (lanes 3, 4) or following siRNA transfection (lanes 5, 6). **b**, SKBr3 cells were treated with 5 μM gefitinib for 0, 1 or 48 h. Arm W was treated for 48 h, washed, and incubated in drug-free media for one more hour. **c**, SKBr3 cells were treated with the indicated concentrations of gefitinib for one hour (left side). Additional arms were treated with 5 μM gefitinib for 48 h and subsequently treated with the indicated concentrations of gefitinib for one additional hour (right side). **d**, SKBr3 cells were treated with the indicated concentrations and durations of PD168393. **e**, SKBr3 cells were treated with 2 μM PD168393 for the indicated times. **f**, SKBr3 cells were transfected with anti-HER3 (H) or control (C) siRNA and harvested four days after transfection (lanes 1, 2). Additional arms were treated with gefitinib or control in untransfected cells (lanes 3, 4) or following siRNA transfection (lanes 5, 6). **g**, In parallel with **f**, SkBr3 cells were either left untransfected, or transfected with anti-HER3 or control siRNA followed by 5 μM gefitinib or control for 48 h. Apoptotic cells were identified by their sub-G1 DNA content. **h**, SKBr3 cells were treated as indicated for 48 h. Apoptotic cells were identified by Annexin V expression. Error bars, s.d.

consistent with their limited clinical activities. Therefore we set out to study the molecular mechanism by which HER3 evades TKI therapy.

TKI-refractory HER3 phosphorylation is due to HER2, because it can be suppressed by anti-HER2 siRNA transfection (Fig. 2a). Although cross-talk between receptor families can occur, we have found no evidence for non-HER-family tyrosine kinases mediating TKI-refractory HER3 phosphorylation. The reactivation of HER3 signalling is not associated with the induction of any new tyrosine kinases and remains resistant to the broad-spectrum kinase inhibitor staurosporine (not shown). This apparent desensitization of HER3 signalling to TKIs is due to a forward shift in the equilibrium of the HER3 phosphorylation–dephosphorylation reactions, establishing a new steady-state HER3 phosphorylation level despite significant inhibition of HER2 kinase and autophosphorylation activity by TKIs. This forward shift becomes clearly evident in the form of HER3 and Akt superphosphorylation when drug inhibition is withdrawn during the new steady state (Fig. 2b). HER3 phosphorylation remains suppressible by TKI in the new steady state, but much higher concentrations are required to completely dephosphorylate HER3 because the un-inhibited HER3 phosphorylation state is significantly higher in the new steady state (Fig. 2c, compare left and right). Therefore drug-refractory HER3 phosphorylation is due to resistance at the level of the substrate HER3, and is driven by residual HER2 kinase activity. Similar characteristics apply to the more potent irreversible TKIs[24]. The irreversible TKI PD168393, which inhibits all HER-family kinases, when used at partially or near-maximal inhibitory concentrations, induces a similar desensitization of HER3 to continued drug therapy (Fig. 2d, 0.1–0.2 μM doses). However, at fully inactivating concentrations both reversible (Fig. 2c, 40 μM dose) and irreversible (Fig. 2e) TKI can durably suppress HER3 and Akt signalling.

The biological consequence of drug-refractory HER3 and Akt signalling is tumour cell survival. In fact, the anti-proliferative activity of TKIs is reversible and tumour cells resume proliferative growth after drug withdrawal. If drug-refractory HER3 signalling is averted by anti-HER3 siRNA, TKI treatment of HER2-driven cancer cells leads to apoptotic tumour cell death (Fig. 2f, g, and Supplementary Fig. 4). This is the expected outcome of effective oncoprotein inactivation, and recapitulates the apoptotic fate of oncogene withdrawal seen in reversible transgenic models of HER2 tumorigenesis[14]. Sustained inhibition of HER3 signalling using TKIs at their fully inactivating doses (from Fig. 2c–e) also leads to apoptotic tumour cell death not seen with doses that allow HER3 escape (Fig. 2h).

The TKI-induced forward shift in the HER3 phosphorylation–dephosphorylation steady state is due to increased HER3 substrate concentration driving the forward reaction, and decreased phosphatase activity impeding the reverse reaction. Increased HER3 substrate concentration occurs through a significant increase in HER3 expression at the plasma membrane where the phosphorylation reaction occurs (Fig. 3a, b). Unlike HER2, which is predominantly localized to the plasma membrane, the HER3 pool is largely within intracellular compartments, but there is also some membrane expression[25]. The TKI-induced forward shift in HER3 steady-state phosphorylation is driven by HER3 relocalization to the plasma membrane and can be suppressed by inhibitors of vesicular trafficking (Fig. 3c, d). The HER3 dephosphorylation rate is also slowed after 48 h of TKI exposure (Fig. 3e). The retarded HER3 dephosphorylation rate may be due to reduced access to cytosolic protein tyrosine phosphatases as a result of altered endocytic trafficking, or it may be due to inhibition of protein tyrosine phosphatases. In support of the latter, TKI therapy increases the concentration of cellular reactive oxygen species (Fig. 3f), which are known to inhibit protein tyrosine phosphatases and are thus emerging as an important regulator of their activity[26,27]. Consistent with this, drug-refractory HER3 signalling can be suppressed by concomitant treatment with certain anti-oxidants (Fig. 3g).

The changes in steady-state HER3 signalling that evolve with TKI treatment are driven by the loss of Akt signalling and probably involve Akt-mediated negative feedback signalling. Consistent with this, HER3 signalling does not escape TKI treatment when a constitutively active Akt is transfected (Fig. 4a). Conversely, inhibition of

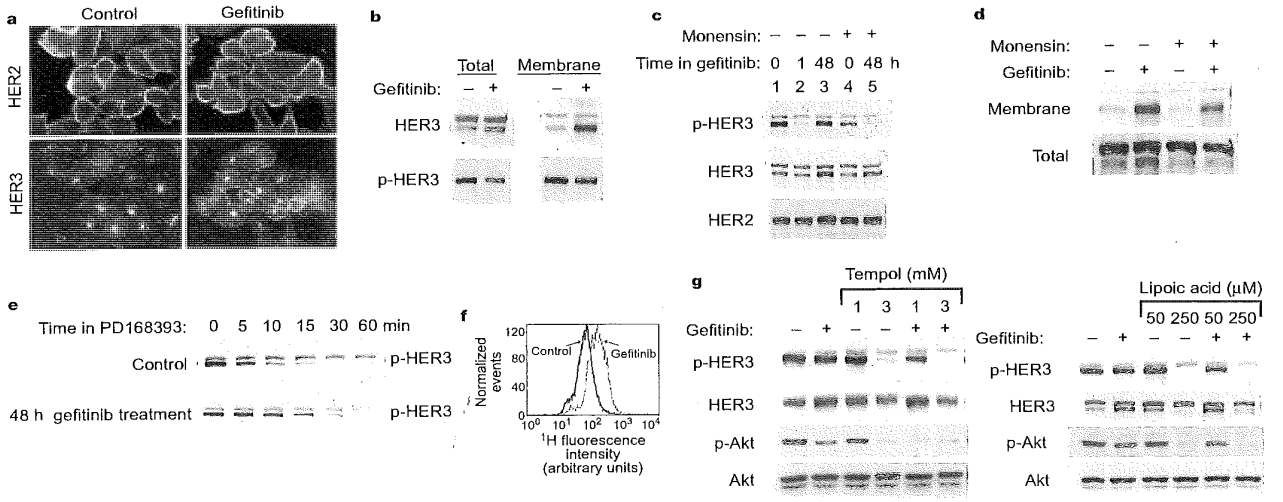


Figure 3 | Mechanism of HER3 reactivation after extended HER TKI treatment. a, SKBr3 cells were treated with 5 μM gefitinib or control for 48 h and stained with anti-HER2 or anti-HER3 antibodies for immunofluorescence microscopy. **b**, In addition, cell surface proteins in control or 48 h pre-treated cells were biotinylated, precipitated and immunoblotted as indicated. **c**, Control or gefitinib pre-treated (5 μM, 48 h) SKBr3 cells were treated with 20 μM monensin for the final 6 h and analysed by western blotting. **d**, SkBr3 cells were treated with gefitinib for 48 h and with 20 μM monensin for the final 6 h. Membrane and total HER3 was immunoblotted from cell surface proteome pulldowns (above) or total lysates (below). **e**, The dephosphorylation rate of p-HER3 following 48 h of gefitinib treatment was determined immediately after initiation of fully inactivating concentrations of the irreversible HER TKI PD168393 (2 μM). **f**, Reactive oxygen species were quantified in control or gefitinib pre-treated (5 μM, 48 h) SKBr3 cells, as described in the Methods. **g**, SKBr3 cells were treated with 5 μM gefitinib or in combination with the indicated concentrations of the anti-oxidants Tempol or α-lipoic acid for 48 h.

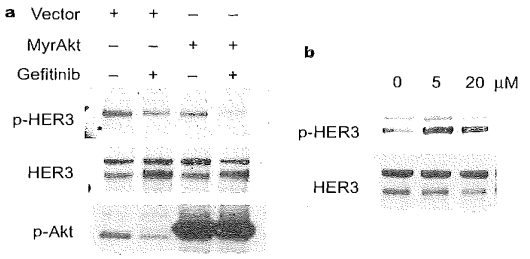


Figure 4 | Akt regulates HER3 signalling via negative feedback signalling. **a**, 2×10^6 SKBr3 cells were transiently transfected with 5 μg of pcDNA3-myristoyl-Akt plasmid or control pcDNA3 plasmid. The following day 5 μM gefitinib was added for an additional 48 h where indicated. Lysates were immunoblotted as indicated. **b**, SKBr3 cells were treated with 5 or 20 μM LY294002 for 8 h and lysates immunoblotted as indicated.

Akt signalling by a PI(3)K inhibitor leads to a compensatory increase in HER3 phosphorylation (Fig. 4b). Complete inactivation of HER2 kinase with high doses of TKIs induces the maximum feedback signalling and HER3 redistribution, but owing to the complete inactivation of HER2 kinase, HER3 signalling cannot be restored and so the feedback loop fails to rescue Akt activity.

Standard preclinical models have notoriously overestimated the clinical potential of HER TKIs, so we challenged the traditional approach to evaluating TKIs in these models. Traditionally, signalling inhibitors are thought to have a continuous suppressive effect through rapid and sustained inhibition of their direct molecular targets and downstream signalling events. This notion of drug therapy may be too simplistic. Clearly, continuous exposure to a growth factor stimulus does not produce continuous high-output downstream signalling. Rather, it leads to a sequence of signalling events, programmed by negative and positive feedback signalling, until establishment of a new steady state in the presence of continued stimulus. We find here that continuous exposure to TKIs similarly leads to a sequence of signalling events that manifest over time, until a new steady state is reached.

With this new perspective, we report that HER3 and PI(3)K/Akt signalling is not effectively inhibited by current TKIs. In particular, the allocation of kinase and signalling functions to different members within the HER family allows the signalling substrate HER3 to restore signalling activity despite significant inhibition of HER2 kinase, in effect buffering HER3-mediated PI(3)K/Akt signalling against an incomplete loss of HER2 kinase function. This inherent signal buffering capacity allows tumour cells to evade the pro-apoptotic effects of TKIs, making such TKI therapy considerably less effective. To treat HER-driven cancers much more effectively may thus require drugs with much higher potency or drugs that completely inactivate HER kinase function. Irreversible TKIs, although more potent, are subject to similar limitations. Owing to their reactive groups and reduced selectivity, many irreversible agents cannot be delivered at completely inactivating doses. Future highly selective irreversible inhibitors may turn out to be more effective. Until highly specific and fully inactivating drugs can be designed, combination treatment strategies designed to undermine the resiliency of HER-family signalling may offer the most promising approach in the near future. In addition, inhibition of autophosphorylation activity deceptively overstates the efficacy of TKIs and is a poor *in vivo* biologic marker. A better biological marker of efficacy with which to guide future therapies should be HER3 transphosphorylation.

The signal buffering capacity endowed by the separation of kinase and signalling functions to different family members in the HER kinase family attests to an evolutionary advantage conferred by the loss of catalytic activity in the HER3 protein kinase. This may be one of the reasons why approximately 10% of human kinases appear to be catalytically inactive[28].

METHODS

Cell culture and reagents. PD168393 was synthesized as previously described[29]. Commercially available gefitinib and erlotinib were purified for *in vitro* use. Reagent sources are detailed in Supplementary Information. For immunofluorescence studies, cells grown on fibronectin-coated cover slips were treated as indicated, fixed in 4% paraformaldehyde, permeabilized, and stained with the indicated primary antibodies and fluoroscein isothiocyanate (FITC)-conjugated secondary antibodies. Cells were visualized using a Zeiss Axioplan 2 fluorescence imaging microscope.

Apoptosis. Cells were seeded at 300,000–500,000 per well in 12-well or 6-well clusters. Apoptotic cells were identified and quantified by analysis of Annexin V binding using the Annexin V-FITC apoptosis detection kit (Calbiochem) according to the manufacturer's instructions, or by their sub-G1 DNA content and quantified by fluorescence-activated cell sorting (FACS) analysis as previously described[30]. All experimental arms were done in duplicate and displayed as averages with standard of deviation (s.d.) error bars.

Transfections. Cells were seeded at a density of 300,000 cells per well in 12-well plates and transfected the following day. For siRNA transfections 100–300 nmol of siRNA (Dharmacon) was premixed with Lipofectamine2000 in Opti-MEM media and then added to each well. For plasmid transfections, 2 μg of plasmid DNA was premixed with Lipofectamine2000 in Opti-MEM media and added to wells for 6 h.

Cell surface biotinylation. Cells were chilled on ice and rinsed twice with ice-cold PBS. Freshly prepared sulpho-NHS-SS-biotin (labelling reagent) was added to the final concentration of 0.5 mg ml^{-1} in PBS. After 45 min incubation at 4 °C, cells were lysed for immunoprecipitation.

Reactive oxidation species assay. Cells were rinsed twice with PBS and incubated with 10 μM of freshly prepared H2DCFDA in phenol-red-free media for 45 min at 37 °C. Cells were then trypsinized and reactive oxygen species levels were detected by flow cytometry.

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Expression of c-*erb*B3 protein in primary breast carcinomas

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Summary Expression of c-*erb*B3 protein was investigated in 104 primary breast carcinomas comprising nine comedo ductal carcinoma in situ (DCIS), 91 invasive ductal carcinomas and four invasive lobular carcinomas using two monoclonal antibodies, RTJ1 and RTJ2. Of the 91 invasive ductal carcinomas, seven contained the comedo DCIS component adjacent to the invasive component. An immunohistochemical technique was used to evaluate the association between expression of c-*erb*B3 and clinical parameters and tumour markers such as epidermal growth factor receptor (EGFR), c-*erb*B2, cathepsin-D and p53 in archival formalin-fixed paraffin-embedded tumour tissues. Our results indicated that RTJ1 and RTJ2 gave identical staining patterns and concordant results. It was found that the overexpression of c-*erb*B3 protein was observed in 67% (6/9) of comedo DCIS, 52% (44/84) of invasive ductal carcinomas, 71% (5/7) of carcinomas containing both the in situ and invasive lesions and 25% (1/4) of invasive lobular carcinomas. A significant relationship ($P < 0.05$) was observed between strong immunoreactivity of c-*erb*B3 protein and histological grade, EGFR and cathepsin-D, but not with expression of c-*erb*B2, p53, oestrogen receptor status, lymph node metastases or age of patient. However, we noted that a high percentage of oestrogen receptor-negative tumours (58%), lymph node-positive tumours (63%) and c-*erb*B2 (63%) were strongly positive for c-*erb*B3 protein. We have also documented that a high percentage of EGFR (67%), c-*erb*B2 (67%), p53 (75%) and cathepsin-D-positive DCIS (60%) were strongly positive for c-*erb*B3. These observations suggest that overexpression of c-*erb*B3 protein could play an important role in tumour progression from non-invasive to invasive and, also, that it may have the potential to be used as a marker for poor prognosis of breast cancer.

Keywords: c-*erb*B3; breast cancer; immunohistochemistry

C-*erb*B3, a new member of the *erb*B type I family of tyrosine kinase receptors, has been mapped to human chromosome 12q11–13 (Kraus et al, 1989). However, the related epidermal growth factor receptor (EGFR) is located on chromosome 7p12–13 (Spurr et al, 1984) and the c-*erb*B2 gene on 17p12–21.3 (Coussens et al, 1985; Popescu et al, 1989). The gene for the c-*erb*B3 receptor is transcribed into a 6.2-kb mRNA, which is translated into the protein and modified by glycosylation to give a mature protein of about 160 kDa (Plowman et al, 1990; Prigent and Gullick, 1992). Recently, it has been shown that heregulin/neu differentiation factor (NDF) binds to and stimulates the tyrosine kinase activity of c-*erb*B3 (Carraway et al, 1994).

Expression of c-*erb*B3 has been detected in normal human adult and fetal tissues using polyclonal antibodies (Prigent et al, 1992). Rajkumar et al (1993) have described the production of a monoclonal antibody (RTJ1) that is specific for c-*erb*B3 protein and reported positive immunostaining in a series of gastrointestinal tract tumours. Other studies have also demonstrated overexpression of c-*erb*B3 protein in breast carcinomas (Lemoine et al, 1992*a*; Gasparini et al, 1994; Quinn et al, 1994; Travis et al, 1996), pancreatic cancers (Lemoine et al, 1992*b*), gastric cancers (Sanidas et al, 1993), cervical cancer (Hunt et al, 1995; Rajkumar et al, 1995), prostate cancer (Poller et al, 1992), bladder cancer (Rajkumar et al, 1996) and ovarian carcinomas (Mandai et al, 1994; Simpson et al, 1995).

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Expression of EGFR and c-*erb*B2 receptors and the other members of the *erb*B family has been extensively studied in many different tumours, and it has been found that their overexpression is associated with poor prognosis (Lofts and Gullick, 1991; Gullick, 1991). Elevated levels of EGFR and c-*erb*B2 have been found in breast carcinomas and are often found to be associated with poor prognosis (Sainsbury et al, 1987; Costa et al, 1988). Currently, there is little information available regarding the expression of c-*erb*B3 protein in formalin-fixed and paraffin-embedded primary breast carcinomas. As breast cancer is an important disease in Malaysia, especially in younger women, we used this study to evaluate the c-*erb*B3 expression in Malaysian breast cancer patients. The indirect immunoperoxidase method was used to examine the expression of c-*erb*B3 protein in breast carcinoma tissues fixed in formalin. We used two different types of monoclonal antibodies, RTJ1 (IgM) and RTJ2 (IgG1), which were raised against the same 49.3-kDa synthetic peptide to detect the c-*erb*B3 protein. Here, we report the expression of c-*erb*B3 protein in different histological types of primary breast carcinomas and relate it to several clinical parameters, such as oestrogen receptor status, lymph node status, histological grade and patients' age, and tumour markers such as EGFR, c-*erb*B2, cathepsin-D (oestrogen-regulated protein) and p53 (tumour-suppressor gene).

MATERIALS AND METHODS

Patients and tissues

A consecutive series of 29 and 75 primary breast carcinomas were obtained, respectively, from Subang Jaya Medical Centre, Subang

Jaya, Selangor, Malaysia, and Hospital Kuala Lumpur, Kuala Lumpur, Malaysia. Tumours in most of the patients were detected symptomatically and only a small proportion were diagnosed mammographically, with further histological confirmation in both the hospitals. Patients were treated by wide local excision with axillary node dissection if the tumour size was less than 4 cm. Mastectomy was performed when the tumour was more than 4 cm in size. The median tumour size was 2 cm. No treatment had been given before the tumour was excised. The median number of nodes retrieved in axillary dissection was 12 nodes for Hospital Kuala Lumpur and ten nodes for Subang Jaya Medical Centre. In our study, lymphatic invasion was seen in almost all cases. Vascular invasion was observed in five cases. The tumours were fixed in buffered formalin for 12–18 h after surgery. Paraffin sections of tissues were cut at 5 µm thickness for histological and immunohistochemical evaluation. Full clinical information was available for each patient. Of the 104 cases of primary breast carcinomas, nine were ductal carcinoma in situ (DCIS) of comedo type, 84 were invasive ductal carcinomas, seven contained both the in situ and the invasive component and four were invasive lobular carcinomas. Forty-one of the patients showed lymph node metastases and 54 showed no lymph node involvement. Patients were grouped into two groups based on age at the time of diagnosis: age < 50 years and age ≥ 50 years. Sixty-six patients were diagnosed at age below 50 and 38 were diagnosed at the age of 50 and above. All the invasive ductal carcinomas were graded using the method described by Elston (1987). Eighteen of the invasive ductal carcinomas were well differentiated (grade I), 43 were moderately differentiated (grade II) and 30 were poorly differentiated (grade III). Invasive lobular carcinomas were graded according to the modified criteria (Elston, 1987) of Bloom–Richardson (Bloom and Richardson, 1957). All four invasive lobular carcinomas were poorly differentiated.

Immunohistochemistry

The staining method employed was an indirect immunoperoxidase system using a standard streptavidin–biotin–peroxidase complex technique. Immunostaining was performed using two different antibodies – an IgM mouse monoclonal antibody (clone RTJ1) (NCL-c-*erb*B3) obtained from Novocastra Lab, UK, and an IgG1 mouse monoclonal antibody (clone RTJ2) from Calbiochem, USA – which were raised against the synthetic peptide (49.3 kDa) from the cytoplasmic domain of the human c-*erb*B3 protein. Other primary antibodies used were oestrogen receptor (ID5, Dako), p53 (DO 7, Dako), c-*erb*B2 (polyclonal, Dako), cathepsin-D (polyclonal, Dako) and epidermal growth factor receptor (EGFR) (polyclonal, Oncogene Science, USA).

The sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol. After rinsing in distilled water, endogenous peroxidase was blocked with 3% hydrogen peroxide (in water) for 10 min at 37°C. The sections were washed briefly in phosphate-buffered saline (PBS) pH 7.6 for 5 min. The sections were incubated with the c-*erb*B3 primary antibodies (RTJ1 and RTJ2) (dilution 1:20) for 1.5 h at room temperature. After the incubation, the slides were washed in PBS for 15 min and incubated with biotinylated secondary antibody (LSAB/HRP, Duet, Dako) for 10 min at 37°C. After further washing for 5 min, the sections were incubated with streptavidin-conjugated peroxidase complex (LSAB/HRP, Duet, Dako) for another 10 min at 37°C. The tissue sections were washed again in PBS for 5 min. The colour reaction was developed by incubating the sections with the ABC (3-amino-9-ethylcarbazole) chromogen (Dako) and counterstained with Mayer's haematoxylin (Fluka, France). The slides were mounted in aqueous mounting media and examined under the microscope. Immunostaining was also performed for EGFR (dilution 1:15, 1 h), c-*erb*B2 (dilution 1:80, 1.5 h), cathepsin-D (dilution 1:20, 1 h), p53 and oestrogen receptor (dilution 1:20, 2 h). All the antibodies were diluted in 1% BSA (bovine serum albumin, Sigma, USA). For p53 and oestrogen receptor, we used microwave treatment to obtain optimal staining intensity. Initially, the sections were microwaved twice in 10 mM sodium citrate buffer (pH 6.0) for 5 min. The slides were cooled at room temperature for 20 min and washed in PBS before incubating with the primary antibody. The rest of the staining procedures were followed as above. Breast carcinoma tissues known to react with the marker were used as positive controls and incubated in each batch of staining. As for negative control, the primary antibody was omitted and replaced with PBS and negative control antibodies mouse IgG1 (clone DAK-G01, Dako) and IgM (clone DAK-G08, Dako). The antibodies were adjusted to the same concentration as the primary antibody.

Scoring and statistical analysis

The staining for c-*erb*B3 was cytoplasmic and was assessed according to the method described by Quinn et al (1994). Negative staining or equivocal staining was considered as less than normal expression, weak but definite positive staining was considered to represent normal expression and strong, granular staining indicated overexpression. However, for the purpose of statistical analysis, only the intensity of staining was considered. Strong membrane staining for c-*erb*B2 and epidermal growth factor receptor (EGFR) and strong cytoplasmic staining for cathepsin-D were considered to represent overexpression of the gene product. Tumours that exhibited strong positivity for c-*erb*B3, c-*erb*B2, EGFR and cathepsin-D were included in statistical analysis. For p53, the tumour scores were based on the method described by Isola et al (1992). Tumours were scored as strongly positive if more than 20% of the nuclei were stained. Immunostaining in more than 20% of the tumour cells was taken to represent p53 protein overexpression. If only a small proportion of the nuclei were stained (1–20%), the tumour was scored weakly positive. Weakly positive and negative tumours were not included in statistical analysis. Oestrogen receptor expression was considered positive when more than 10% of the tumour cells demonstrated positive nuclear staining and negative when less than 10% stained (Pertschuk et al, 1990). This method was used because it has been demonstrated to be more predictive of patients' prognosis.

The chi-squared analysis (contingency tables) was performed to assess the significance of association between expression of c-*erb*B3 and clinicopathological parameters and other tumour markers. Statistical Graphics System Version 5.0 was used to conduct the correlation tests. The level of significance used throughout the statistical test was 0.05 (5%).

RESULTS

In the present study, expression of c-*erb*B3 protein was detected using two monoclonal antibodies, RTJ1 and RTJ2. We observed identical staining for both the antibodies. In general, the staining was homogeneous in the majority of the tumour cells with mild

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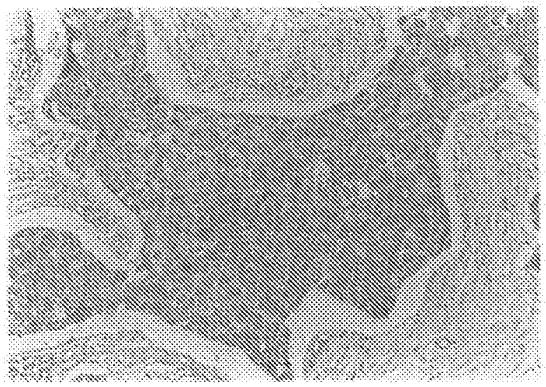


Figure 1 Immunohistochemical analysis of c-*erb*B3 expression detected by RTJ1 in paraffin-embedded invasive ductal carcinoma tissue. The intense staining for the c-*erb*B3 protein was observed as cytoplasmic staining in the malignant epithelial cells (magnification × 133)

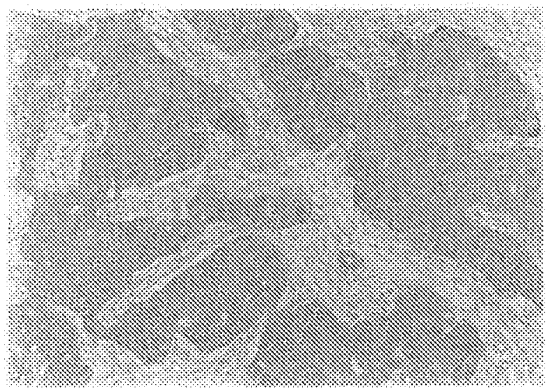


Figure 2 Expression of c-*erb*B3 protein was detected by RTJ2 immunohistochemically in paraffin-embedded invasive ductal carcinoma tissue. Strong staining for c-*erb*B3 protein was noted in the cytoplasm of the malignant cells (magnification × 66)

heterogeneous reactivity. The staining for c-*erb*B3 detected by both the antibodies was predominantly cytoplasmic, with none of the tumours exhibiting membrane immunoreactivity. The staining was finely granular throughout the cytoplasm of the malignant epithelial cells, which are shown in Figure 1 (RTJ1) and Figure 2 (RTJ2). Immunopositivity of c-*erb*B3 protein was found in 65% of the 104 primary breast carcinomas. Strong staining, which denotes overexpression, was seen in 54% (56/104) and weak staining in 11% (12/104) of the total breast carcinomas studied. No immunoreactivity was observed in non-malignant cells. Antibody reactivity was not observed in the stromal cells. No staining was noted in negative control tissue.

Immunopositivity of c-*erb*B3 was evaluated in comedo DCIS, invasive ductal carcinomas, carcinomas with combined lesions (in situ and invasive ductal component) and invasive lobular carcinomas. Our data indicated that six (67%) of the nine comedo DCIS were strongly positive for c-*erb*B3 protein. No weak staining was seen in the in situ carcinomas. Of the 84 invasive ductal carcinomas, 44 (52%) showed strong immunopositivity, whereas seven (8%) showed weak staining. Among the four invasive lobular carcinomas, one (25%) was strongly stained and three (75%) were weakly stained. Immunoreactivity of c-*erb*B3 protein was also observed in five (71%) of seven cases in which comedo DCIS was adjacent to the invasive ductal component. In these cases, both the in situ and the invasive component were strongly positive. No weak staining was observed.

Table 1 summarizes the relationship between c-*erb*B3 expression (detected by RTJ1 and RTJ2) and prognostic factors including

Table 1 Association between overexpression of c-*erb*B3 protein detected by RTJ1 and RTJ2 and clinicopathological parameters

Parameters	Total no. of cases ($n = 95$)	Positive cases for c-*erb*B3 (%)	χ^2
Oestrogen receptor (ER) status[a]			
ER+	27	10 (37)	$P = 0.06$
ER–	68	40 (59)	(NS)
Lymph node (N) status[a]			
N+	41	26 (63)	$P = 0.06$
N–	54	24 (44)	(NS)
Patient age (age at diagnosis)[a]			
<50 years	57	31 (54)	$P = 0.67$
≥50 years	38	19 (50)	(NS)
Histological grading			
Grade I	18	4 (22)	$P = 0.004$
Grade II	43	22 (51)	(S)
Grade III	34	24 (71)	

S, significance at $P < 0.05$; NS, not significant. [a]Nine DCIS cases were not included in statistical analysis.

Table 2 Association between overexpression of c-*erb*B3 protein detected by RTJ1 and RTJ2 and other tumour markers

Tumour markers	Total no. of cases ($n = 95$)	Presence (+)/ absence (–)	No. of positive cases for c-*erb*B3 (%)	χ^2
EGFR[a]	47	+	32 (68)	$P = 0.003$
	48	–	18 (38)	(S)
c-*erb*B2[a]	41	+	25 (63)	$P = 0.07$
	54	–	24 (44)	(NS)
p53[a]	31	+	14 (45)	$P = 0.31$
	64	–	36 (56)	(NS)
Cathepsin-D[a]	56	+	36 (64)	$P = 0.006$
	39	–	14 (36)	(S)

S, significance at $P < 0.05$; NS, not significant. [a]Nine DCIS cases were not included in statistical analysis.

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oestrogen receptor status, lymph node involvement, histological grade and patients' age. Nine comedo DCIS were analysed separately. We did not observe any differences between the antibodies. Overexpression of c-*erb*B3 was significantly associated with poorly differentiated tumours ($P = 0.004$). We demonstrated that 71% of the grade III tumours exhibited strong c-*erb*B3 positivity. No significant difference was observed with oestrogen receptor status ($P > 0.05$), lymph node involvement ($P > 0.05$) and patients' age ($P > 0.05$). Although the associations between c-*erb*B3 and oestrogen receptor or lymph node status were not supported statistically, we showed that a high percentage of oestrogen receptor-negative tumours (59%) and lymph node-positive tumours (63%) were c-*erb*B3 positive. Coexpression with other tumour markers, such as EGFR, c-*erb*B2, p53 and cathepsin-D, was analysed (Table 2). Our results showed a significant relationship between overexpression of c-*erb*B3 protein and EGFR ($P = 0.003$) and cathepsin-D ($P = 0.006$), but not with c-*erb*B2 ($P > 0.05$) or p53 ($P > 0.05$). We found that high percentage of tumours strongly positive for EGFR (68%) and cathepsin-D (64%) were also strongly positive for c-*erb*B3. Sixty three per cent of c-*erb*B2-positive tumours were strongly positive for c-*erb*B3, but the relationship was not supported statistically. All nine comedo DCIS obtained from patients below age 50 were negative for oestrogen receptor. Six of these were strongly positive for c-*erb*B3. Our data on comedo DCIS indicated that 67% (4/6) of EGFR-positive tumours, 67% (6/9) of c-*erb*B2-positive tumours, 75% (3/4) of p53-positive tumours and 60% (3/5) of cathepsin-D-positive tumours exhibited strong c-*erb*B3 positivity.

DISCUSSION

Our data showed that over half (54%) of 104 primary breast carcinomas of various histological types overexpressed c-*erb*B3 protein, which was detected by RTJ1 and RTJ2. Both the antibodies gave an identical staining pattern, which suggests the specificity of the antibodies. The staining was predominantly cytoplasmic, with none of the tumours demonstrating membrane staining. RTJ1, an IgM monoclonal antibody, was shown to give a better staining pattern than the polyclonal antibody when tested on formalin-fixed paraffin-embedded tissues or breast cancer cell lines using the immunohistochemical technique, although it was shown to detect other proteins of higher molecular weight in Western blots (Rajkumar et al, 1993). The superior performance of RTJ1 could be due to it being an IgM antibody with ten potential combining sites and a high avidity for multiple copies of the c-*erb*B3 protein in tissue sections (Rajkumar et al, 1993). RTJ2, an IgG1 monoclonal antibody directed against the same peptide (49.3 kDa) (Rajkumar et al, 1995), gave an identical immunohistochemical staining pattern with tissues which had earlier been stained with RTJ1 (Rajkumar et al, 1993) and rabbit polyclonal antibody (Prigent et al, 1992). Furthermore, RTJ2 has been shown to be specific for c-*erb*B3 protein as determined by Western blotting, immunoprecipitation and immunocytochemistry. Lemoine et al (1992*a*) showed good agreement between the intensity of signal on Northern blot analysis and the intensity of immunohistochemical staining with the 49.3-kDa polyclonal antibody using breast cancer and non-breast cancer cell lines. In addition, Rajkumar et al (1993) had shown that breast carcinoma cell lines and non-malignant breast carcinoma cell lines that had been previously shown to express high, intermediate and low levels of c-*erb*B3 mRNA (Lemoine et al, 1992*a*) demonstrated an identical staining reaction when stained with the polyclonal antibody (Lemoine et al, 1992*a*) and RTJ1. As RTJ1 and RTJ2 gave concordant results and identical staining patterns which were also indicated by the present study, the cytoplasmic staining of RTJ1 could not be due to non-specific reaction or cross-reaction. In a recent paper on a larger study with RTJ1, Travis et al (1996) reported that 15% of 346 primary breast cancer cases and 35% of 145 advanced breast cancer cases showed strong staining for c-*erb*B3. Previously, Lemoine et al (1992*a*) had reported increased expression of c-*erb*B3 in 22% of 195 primary infiltrating breast carcinomas. In another study by Quinn et al (1994), 29% of 97 primary breast cancer cases demonstrated c-*erb*B3 overexpression. Poller et al (1992), using polyclonal antibody and a smaller sample size, demonstrated that 13 of 14 primary breast carcinomas were positive for c-*erb*B3 immunoreactivity but failed to describe the staining intensity of the positive cells. This makes it difficult to interpret their data. Using RTJ1, these authors had shown that the staining was observed to be predominantly cytoplasmic. Prigent et al (1992) has suggested that there is a possibility that the majority of c-*erb*B3 protein at any one time is present in intracellular pools. Initially, it had been thought that the cytoplasmic immunoreactivity of c-*erb*B2 was non-specific (de Potter et al, 1989). However, Kumar et al (1991) had reported that only about 20% of the total c-*erb*B2 protein expressed in SKBR-3 breast cancer cells was located on the cell surface, the remainder being in other intracellular fractions. In addition, Coombs et al (1993) had shown that the cytoplasmic staining for c-*erb*B2 can be blocked by the immunizing peptide, suggesting that this represents expression of the c-*erb*B2 protein. This could also be one of the possibilities for c-*erb*B3. In contrast, Gasparini et al (1994), using the same antibody (RTJ1), has reported that 65% of the node-negative breast carcinomas showed staining of cell membranes, but 13% showed strong positivity and a much higher percentage (89%) showed cytoplasmic staining. In addition, the authors have indicated that there is a highly significant association between membrane and cytoplasmic staining. In common with other findings, the present data showed that these antibodies (RTJ1 and RTJ2) locate the c-*erb*B3 protein mainly in the cytoplasm, suggesting that cytoplasmic immunoreactivity may be significant. The high proportion of breast carcinomas with strongly expressed c-*erb*B3 indicates that the gene may have an important role in breast tumorigenesis.

Of the nine ductal carcinoma in situ of comedo subtype, six (67%) showed overexpression of c-*erb*B3 gene product. Interestingly, ductal carcinoma in situ has been shown to be a precursor of invasive carcinomas, because it is frequently present in tissues adjacent to breast cancer and is associated with the presence of invasive carcinoma in the same region of the same breast where the DCIS was found (Page et al, 1982, Betsill et al, 1987). Comedo DCIS, a subtype of DCIS, has been associated with a higher rate of proliferation, as determined by thymidine labelling index (TLI), compared with other subtypes of DCIS (Meyer, 1986). Furthermore, features such as comedo subtype have been demonstrated to be associated with local recurrence and progression to invasive breast cancer (Lagios et al, 1989; Lagios, 1990). This implies that comedo DCIS are biologically aggressive neoplasms. Based on these observations, we suggest that expression of c-*erb*B3 may be up-regulated during the preinvasive stages and could be an important pathogenic factor in early events of breast malignancies. In addition, we have demonstrated that five (71%) out of seven cases in which comedo DCIS were present adjacent to the invasive

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component were strongly stained for c-*erb*B3 protein in both the lesions. Similarly, Poller et al (1992) have shown that expression of c-*erb*B3 was detected in the invasive and in the in situ component present in the same tumour. This clearly indicates that expression of c-*erb*B3 is not only present in non-invasive stages, but also maintained in the invasive stages. We also noted that a high frequency of invasive ductal carcinomas (52%) showed elevated levels of c-*erb*B3 protein. The above information further strengthens our findings that c-*erb*B3 may be involved in the progression from preinvasive to invasive stage. The above observations have an important implication regarding the role of c-*erb*B3 in the initiation and progression of breast cancer. Elevated levels of c-*erb*B3 were seen in one (25%) invasive lobular carcinoma, but the role of c-*erb*B3 in this type of cancer cannot be determined because of an insufficient number of samples.

In addition, we have investigated the relationship between elevated levels of c-*erb*B3 protein and various clinicopathological parameters. Our data indicated that overexpression of c-*erb*B3 was significantly associated with high histological grade tumours. Expression of c-*erb*B3 was independent of oestrogen receptor status, nodal involvement and patients' age, although a high percentage of oestrogen receptor-negative tumours (59%) and lymph node-positive tumours (63%) were strongly positive for c-*erb*B3 protein. Lemoine et al (1992*a*) reported that overexpression of c-*erb*B3 was significantly correlated with the presence of lymph node metastases, but not with oestrogen receptor status and tumour grade. In a larger study, Travis et al (1996) were unable to demonstrate significant association between strong c-*erb*B3 positivity and oestrogen receptor status, histological grade, lymph node status or patients' age. Similar observation was noted in a smaller series of 97 malignant breast tumours by Quinn et al (1994). In lymph node-negative breast cancer patients, Gasparini et al (1994) could not show any association between c-*erb*B3 positivity and patients' age, histological grading or steroid hormone receptors. However, we were not able to compare our results with those of last study because the patient groups were different. Furthermore, Gasparini et al (1994) used membrane staining to interpret the data but did not determine the association between cytoplasmic staining and pathological or other clinical characteristics in order to observe the difference between membrane and cytoplasmic staining. It has been well documented that tumours that lack oestrogen receptors demonstrate lymph node involvement and poorly differentiated tumours were associated with poor prognosis. However, our results showed that expression of c-*erb*B3 protein was significantly associated with histological grade which has not been documented previously. These observations indicate c-*erb*B3, a new member of the type 1 growth factor receptor family, may have potential to be an indicator for poor prognosis.

Overexpression of c-*erb*B3 protein was significantly associated with EGFR and cathepsin-D, but independent of p53 and c-*erb*B2. Our data were in agreement with Lemoine et al (1992*a*) for p53 and c-*erb*B2, but not for EGFR. Travis et al (1996) and Quinn et al (1994) did not find any association between c-*erb*B3 and c-*erb*B2. Gasparini et al (1994) found a significant association with c-*erb*B2 in node-negative breast carcinoma patients, which is different from the group of patients in the present study. We have documented that a high percentage of EGFR-positive (67%), c-*erb*B2-positive (67%), p53-positive (75%) and cathepsin-D-positive (60%) DCIS were strongly positive for c-*erb*B3. Recent studies have shown that transfection of cells with c-*erb*B3 and c-*erb*B2 reconstitutes a higher affinity binding receptor, which is capable of generating a tyrosine phosphorylation signal in response to heregulin. Furthermore, in cells expressing c-*erb*B2 and c-*erb*B3, both proteins become tyrosine phosphorylated upon interaction with heregulin (Sliwkowski et al, 1994). This heregulin-stimulated phosphorylation of c-*erb*B3 is likely mediated by cross-phosphorylation of the c-*erb*B3 protein by the c-*erb*B2 receptor tyrosine kinase (Kim et al, 1994; Sliwkowski et al, 1994). It also has been shown that the binding of epidermal growth factor (EGF) to the EGFR results in the activation of its protein tyrosine kinase activity and the phosphorylation of the c-*erb*B3 protein on tyrosine residues (Kim et al, 1994). From various studies, it has been noted that overexpression of EGFR and cathepsin-D has been associated with poor prognosis (Lewis et al, 1990; Nicholson et al, 1990; Tandon et al, 1990; Winstanley et al, 1993), suggesting that coexpression of c-*erb*B3 protein and these tumour markers in the same tumours may be useful to predict the outcome or status of the disease.

Presence of high levels of c-*erb*B3 in non-invasive, non-invasive and invasive in the same lesions and invasive carcinomas may indicate that c-*erb*B3 could be involved in the progression of tumours from preinvasive to invasive stage. Furthermore, its association with established prognostic factors such as histological grade and with tumour markers including EGFR and cathepsin-D may suggest that c-*erb*B3 could have potential to be a prognostic indicator for breast cancer. However, other studies on a greater number of patients, with clinical follow-up, are needed to demonstrate the usefulness of this new marker.

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Novel antibody drug conjugates containing exatecan derivative-based cytotoxic payloads

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ABSTRACT

Trastuzumab conjugates consisting of exatecan derivatives were prepared and their biological activities and physicochemical properties were evaluated. The ADCs showed strong efficacy and a low aggregation rate. The exatecan derivatives were covalently connected via a peptidyl spacer (Gly-Gly-Phe-Gly), which is assumed to be stable in circulation, and were cleaved by lysosomal enzymes following ADC internalization into tumor tissue. These anti-HER2 ADCs exhibited a high potency, specifically against HER2-positive cancer cell lines in vitro. The ADCs, bearing exatecan derivatives which have more than two methylene chains, exhibited superior cytotoxicity. It was speculated that steric hindrance of the cleavable amide moiety could be involved in the drug release. The adequate alkyl lengths of exatecan derivatives (**13**, **14**, **15**) were from two to four in terms of aggregation rate. The ADC having a hydrophilic moiety showed good efficacy in a HER2-positive and Trastuzumab-resistant breast carcinoma cell model in mice.

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Antibody drug conjugates (ADCs) have been established over the past several years and are now one of the most successful and important strategies for treating patients with hematological malignancies and solid tumors. At present, only three ADCs have been launched; the first ADC (Mylotarg®, gemtuzumab ozogamicin, Pfizer) launched in 2001 for the treatment of patients with acute myelogenous leukemia (AML). However, Mylotarg® was withdrawn in 2010 due to both a lack of evidence confirming its clinical benefits and safety concerns. Since then, two ADCs, Adcetris® (brentuximab vedotin, Seattle Genetics) and Kadcyla® (trastuzumab emtansine, Genetech and Roche), have been launched. And currently, over 30 programs in clinical development have been carried out in pursuit of ADC drug candidates.[1]

Camptothecin (CPT) has been demonstrated to be effective against a broad spectrum of tumors. CPT's target is human DNA topoisomerase I (Topo I). CPT binds to complexes with Topo I and DNA, and thereby is stabilized. This stabilized complex causes apoptosis. Irinotecan hydrochloride (CPT-11, yakult) is a prodrug of a potent CPT analog (SN-38) used for the treatment of patients with various tumors. Exatecan methansulfonate (DX-8951f) is a water soluble CPT which exhibits a stronger Topo I inhibitory activity and antitumor activity than the other CPT analogs. Furthermore, exatecan is effective against P-glycoprotein (P-gp) mediated multi-drug resistant cells.[2]

ADCs are composed of a carrier monoclonal antibody (mAb) and cytotoxic drug payload. The ADCs discussed herein are ADCs composed of a mAb with exatecan derivatives via a peptidyl linker, as shown in Figure 1. It has been previously recognized that glycyl glycyl phenylalanyl glycyl (GGFG) is selectively cleaved by lysosomal enzymes (presumably cathepsins).[3] GGFG is known to release drugs into tumor tissue without releasing them into peripheral circulation.

Human epidermal growth factor receptor 2 (HER2) is a very effective therapeutic target for breast cancer patients. Trastuzumab (Herceptin®, Genetech and Roche) is an anti-HER2 antibody and is used for breast cancer HER2-positive patients in combination with the chemotherapy drugs, anthracycline and taxane. Trastuzumab is the carrier mAb of trastuzumab emtansine. Therefore, the tumor specificity of this mAb, trastuzumab, is suitable for being the ADC's carrier.

The synthesis of ADC (**1**) was carried out via the route shown in Scheme 1. Commercially available *tert*-butoxycarbonyl glycyl glycyl phenylalanyl glycine (BOC-GGFG-OH) was dissolved in dichloromethane. *N*-Hydroxysuccinimide (HO-Su) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (WSCI) was

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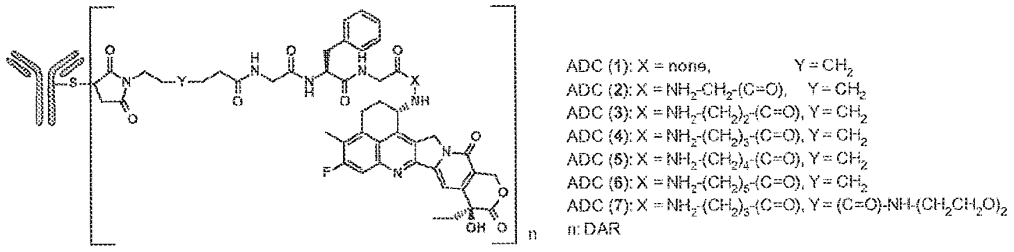
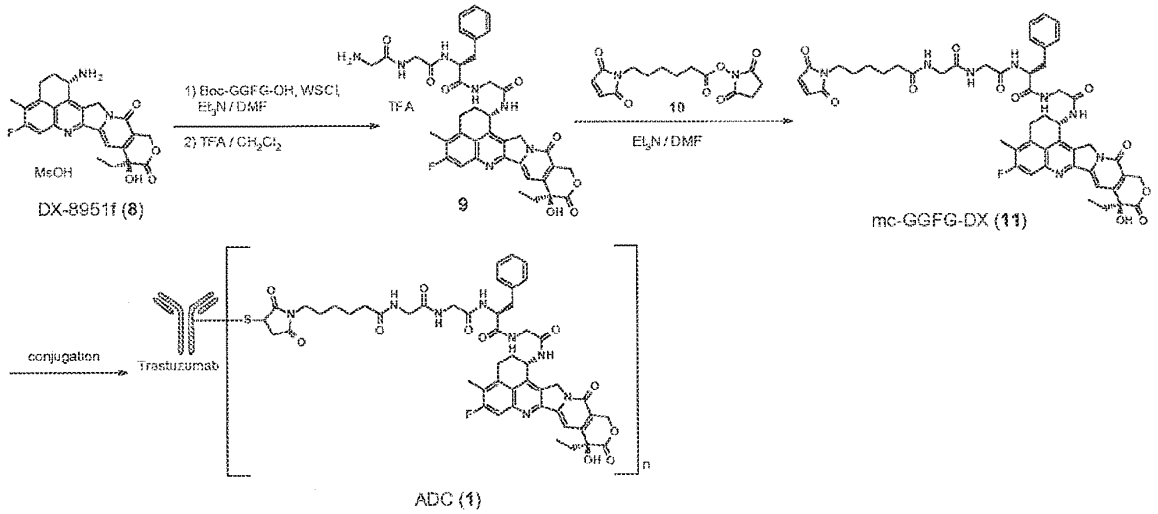


Figure 1. The structure of ADCs.



Scheme 1. The synthesis of ADC (1).

added. To this mixture, was added DX-8951f (**8**) and triethylamine solution in *N,N'*-dimethylformamide (DMF). The Boc-GGFG-exatecan was deprotected by trifluoroacetic acid (TFA), followed by condensation with 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (mc-OSu) in DMF to obtain the maleimide precursor drug linker (mc-GGFG-exatecan, **11**).[4]

The maleimide precursor drug linker (**11**) and mAb conjugation method used a conventional conjugated cysteine (Cys) strategy.[5] A disulfide group with hinges of mAb was reduced with tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl). And the precursor drug linker (**11**) was added to the reduced antibody. The drug to antibody ratio (DAR) was able to control an amount of the reducing agent, as shown in Table 1. The reaction mixture was purified using size exclusion chromatography (SEC). The DAR[5,6] and aggregation rate of the obtained product were measured.[7]

Exatecan derivatives were prepared by condensation and successive deprotection as shown in Scheme 2,[8] and these compounds were used as starting materials to obtain other ADCs (**2**)–(**7**) via similar synthetic routes of ADC (**1**).[4]

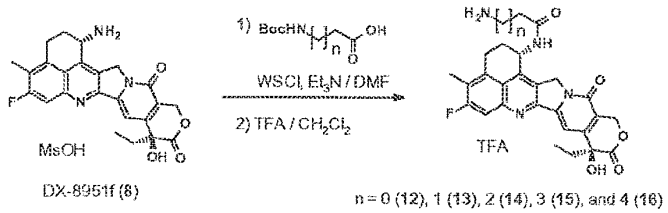
These prepared ADCs were shown to express in vitro cytotoxicities in a HER2-positive cell line, KPL-4, as shown in Table 1. GI_{50} was the concentration for 50% of maximal inhibition of cell proliferation. The KPL-4 cells used were a HER2-positive human breast cancer cell line. The KPL-4 was provided by Junichi Kurebayashi from Kawasaki Medical School in Japan. MCF7 cells used were a HER2-negative breast cancer cell line. The cell line was obtained from the European Collection of Cell Cultures (ECACC). None of the ADCs could kill the MCF7 cell line, GI_{50} > 100 nM. These results suggest that the anti-HER2 ADCs showed HER2 selective cytotoxicity. Though all of the ADCs (**1**)–(**7**) showed strong cytotoxicities in vitro, the ADCs (**1**) and (**2**) had a decreased cytotoxicity compared with that of other ADCs. It was speculated that degrading enzymes in tumor cells might not work efficiently because of the steric hindrance of the released drug moiety. The cleavable amide position (C-terminal amide of GGFG tetrapeptide) between the released drug and cleavable peptide linker was important for the affinity of the enzyme. Therefore, the cleavable amide position was changed away from the drug by adding some methylene chains between the drug and cleavable peptide. In particular, the drugs released from the ADCs (**3**)–(**6**) were changed to alkylamine derivatives with methylene alkyl chains from exatecan. These ADCs showed potent in vitro activity. However, the ADC (**6**) indicated more aggregates, possibly due to its hydrophobicity. The ADCs (**3**)–(**5**) had less aggregates than ADC (**6**). The alkyl length of exatecan derivatives influenced the aggregation rate. Having an alkyl length less than hexylene's is preferred from the viewpoint of an ADC's physicochemical properties. Therefore, an alkyl length from two to four seemed the best choice in this case. Moreover, a hydrophilic moiety could be added in the linker portion. ADC (**7a**) which has diethylene glycol showed less of an aggregation rate than ADC (**4**). Even a high drug load ADC (**7b**, DAR 6.2) had an acceptable low aggregation rate, and greater efficacy in vitro.

The in vivo efficacy of the ADC (**7b**) was compared with trastuzumab in mice bearing JIMT-1, a HER2-positive and

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Table 1
Drug linker structure, conjugation condition, and in vitro efficacy of ADCs

ADC entry	Drug linker structure		Conjugation condition			Characterization		In vitro
	X	Y	TCEP (equiv)	Drug linker (equiv)	Yield (%)	DAR	Aggregation rate (%)	KPL-4 GI50 (nM)
1	None	—$(CH_2)_3$—	4.6	9.2	55	3.4	26	0.33
2	—NH—CH_2—(C=O)—	—$(CH_2)_3$—	2.3	4.6	83	3.2	3	0.39
3	—NH—$(CH_2)_2$—(C=O)—	—$(CH_2)_3$—	4.6	9.2	62	3.8	2	0.07
4	—NH—$(CH_2)_3$—(C=O)—	—$(CH_2)_3$—	4.6	9.2	75	2.6	3	0.05
5	—NH—$(CH_2)_4$—(C=O)—	—$(CH_2)_3$—	4.6	9.2	59	3.4	4	0.07
6	—NH—$(CH_2)_5$—(C=O)—	—$(CH_2)_3$—	4.6	9.2	61	2.5	20	0.11
7a	—NH—$(CH_2)_3$—(C=O)—	—C(=O)—NH—$(CH_2$—CH_2—$O)_2$—	2.3	4.6	85	3.6	1	0.12
7b	—NH—$(CH_2)_3$—(C=O)—	—C(=O)—NH—$(CH_2$—CH_2—$O)_2$—	4.6	9.2	86	6.2	1	0.04



Scheme 2. The synthesis of exatecan derivatives.

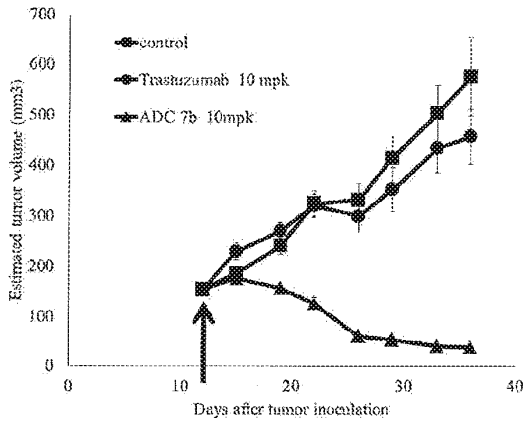


Figure 2. The in vitro efficacy of ADCs in JIMT-1 cells at an average starting tumor volume 150 mm^3 after 12 days.

trastuzumab-resistant breast carcinoma cell line. JIMT-1 was obtained from the Deutshe Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Mice were treated with a single i.v. injection of 10 mg/kg ADC (**7b**), or trastuzumab. Trastuzumab had no effect on tumor growth. In contrast, the mice treated with the ADC (**7b**) achieved tumor regressions, as shown in Figure 2.

These novel ADCs using exatecan derivatives having alkyl amine release the drug efficiently. Furthermore, adding a hydrophilic group to the C-terminus of the peptide moiety was effective in reducing aggregation rates. The ADC (**7b**), which has a maximum 8 DAR, showed an acceptable aggregation rate and high efficacy in vitro and in vivo. No change in mice body weight was observed by the ADC treatment indicating that the ADC was well tolerated.

Camptothecins are an especially compelling drug class for ADCs because they can be produced synthetically and modified to give analogs. However, aqueous insolubility of the camptothecins presents a significant challenge for ADCs. Design of camptothecin derivatives and implementation of a high polarity moiety is an ideal solution to overcome this. ADCs bearing exatecan derivatives of camptothecin analogs show a high potency for cancer therapy.

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4. Nuclear magnetic resonance (NMR) and liquid chromatography–mass (LC–MS) analysis. LC–MS ionization mode was electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). For precursor drug linker (**11**) of ADC (**1**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.87 (3H, t, J = 7.4 Hz), 1.15–1.21 (2H, m), 1.41–1.50 (4H, m), 1.80–1.90 (2H, m), 2.07–2.12 (4H, m), 2.17–2.23 (1H, m), 2.35–2.40 (1H, m), 2.41 (3H, s), 2.73–2.81 (1H, m), 2.98 (1H, dd, J = 13.7, 4.6 Hz), 3.15–3.20 (2H, m),3.53 (1H, dd, J = 16.6, 5.7 Hz), 3.62–3.77 (5H, m), 4.39–4.45 (1H, m), 5.22 (1H, d, J = 18.9 Hz),5.27 (1H, d, J = 18.9 Hz),5.39 (1H, d, J = 16.0 Hz), 5.44 (1H, d, J = 16.0 Hz), 5.55–5.60 (1H, m), 6.53 (1H, s), 6.98 (2H, s), 7.13–7.24 (5H, m), 7.32 (1H, s), 7.81 (1H, d, J = 10.3 Hz), 7.95–8.00 (1H, m), 8.05–8.09 (2H, m), 8.28–8.31 (1H, m), 8.41 (1H, d, J = 8.6 Hz). MS (APCI) m/z: 947 $(M+H)^+$. For precursor drug linker of ADC (**2**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.86 (3H, t, J = 7.2 Hz), 1.18–1.19 (2H, m), 1.45–1.48 (4H, m), 1.83–1.85 (2H, m), 2.12–2.17 (4H, m), 2.39 (3H, s), 2.68 (1H, dd, J = 24.4, 14.7 Hz), 2.83–2.87 (1H, m), 3.17–3.78 (12H, m), 4.42–4.45 (1H, m), 5.23 (2H, s), 5.41 (2H, s), 5.58–5.60 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.15–7.29 (6H, m), 7.76 (1H, d, J = 10.9 Hz), 7.97–8.00 (1H, m), 8.09–8.12 (3H, m), 8.25–8.28 (1H, m), 8.44 (1H, d, J = 8.2 Hz). MS (APCI) m/z: 1004 $(M+H)^+$. For precursor drug linker of ADC (**3**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.86 (3H, t, J = 7.2 Hz), 1.12–1.22 (2H, m), 1.39–1.51 (4H, m), 1.79–1.91 (2H, m), 2.02–2.20 (2H, m), 2.07 (2H, t, J = 7.4 Hz), 2.30–2.42 (4H, m), 2.40 (3H, s), 2.78 (1H, dd, J = 14.1, 9.4 Hz), 3.02 (1H, dd, J = 14.7, 4.9 Hz), 3.12–3.21 (2H, m), 3.26–3.42 (2H, m), 3.50–3.80 (6H, m), 4.40–4.51 (1H, m), 5.19 (1H, d, J = 19.6 Hz), 5.26 (1H, d, J = 19.2 Hz), 5.42 (2H, brs), 5.51–5.62 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.13–7.28 (5H, m), 7.31 (1H, s), 7.74–7.84 (2H, m), 8.01 (1H, t, J = 5.3 Hz), 8.06 (1H, t, J = 5.7 Hz), 8.14 (1H, d, J = 8.2 Hz), 8.25 (1H, t, J = 5.7 Hz), 8.53 (1H, d, J = 8.6 Hz). MS (ESI) m/z: 1018 $(M+H)^+$. For precursor drug linker of ADC (**4**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm:0.87 (3H, t, J = 7.3 Hz), 1.12–1.22 (2H, m), 1.40–1.51 (4H, m), 1.66–1.76 (2H, m), 1.80–1.91 (2H, m), 2.05–2.21 (6H, m), 2.39 (3H, s), 2.79 (1H, dd, J = 14.0, 9.8 Hz), 2.98–3.21 (5H, m), 3.55–3.77 (8H, m), 4.41–4.48 (1H, m), 5.15 (1H, d, J = 18.9 Hz), 5.24 (1H, d, J = 18.9 Hz), 5.40 (1H, d, J = 17.1 Hz), 5.44 (1H, d, J = 17.1 Hz), 5.54–5.60 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.20–7.27 (5H, m), 7.30 (1H, s), 7.70 (1H, t, J = 5.5 Hz), 7.80 (1H, d, J = 11.0 Hz), 8.03 (1H, t, J = 5.8 Hz), 8.08 (1H, t, J = 5.5 Hz), 8.14 (1H, d, J = 7.9 Hz), 8.25 (1H, t, J = 6.1 Hz), 8.46 (1H, d, J = 8.5 Hz). MS (APCI) m/z: 1032 $(M+H)^+$. For precursor drug linker of ADC (**5**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.87 (3H, t, J = 7.4 Hz), 1.13–1.21 (2H, m),

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1.36–1.52 (6H, m), 1.53–1.65 (2H, m), 1.79–1.92 (2H, m), 2.05–2.15 (4H, m), 2.19 (2H, s), 2.40 (3H, s), 2.79 (1H, dd, J = 13.7, 10.2 Hz), 2.98–3.10 (3H, m), 3.12–3.21 (2H, m), 3.29–3.37 (2H, m), 3.53–3.79 (6H, m), 4.41–4.50 (1H, m), 5.16 (1H, d, J = 18.8 Hz), 5.23 (1H, d, J = 18.8 Hz), 5.43 (2H, s), 5.52–5.60 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.12–7.28 (5H, m), 7.31 (1H, s), 7.63 (1H, t, J = 5.7 Hz), 7.80 (1H, d, J = 10.6 Hz), 8.02 (1H, t, J = 5.9 Hz), 8.08 (1H, t, J = 5.7 Hz), 8.12 (1H, d, J = 7.8 Hz), 8.24(1H, t, J = 5.7 Hz), 8.45 (1H, d, J = 8.6 Hz). MS (ESI) m/z: 1046 $(M+H)^+$. For precursor drug linker of ADC (**6**): ^{1}H NMR (400 MHz, CD_3OD) δ/ppm: 0.99 (3H, t, J = 7.4 Hz), 1.27 (2H, td, J = 11.6, 6.1 Hz), 1.38–1.44 (2H, m), 1.50–1.63 (6H, m), 1.65–1.80 (2H, m), 1.89–1.98 (2H, m), 2.17–2.25 (3H, m), 2.26–2.36 (3H, m), 2.40 (3H, s), 2.95 (1H, dd, J = 14.3, 9.2 Hz), 3.12 (1H, dd, J = 13.7, 5.7 Hz), 3.15–3.25 (4H, m), 3.44 (2H, t, J = 7.2 Hz), 3.65 (1H, d, J = 17.2 Hz), 3.76 (1H, d, J = 17.2 Hz), 3.79–3.86 (4H, m), 4.43 (1H, dd, J = 8.9, 6.0 Hz), 5.10 (1H, d, J = 18.9 Hz), 5.25 (1H, d, J = 18.9 Hz), 5.35 (1H, d, J = 16.6 Hz), 5.56 (1H, d, J = 16.0 Hz), 5.60–5.64 (1H, m), 6.76 (2H, s), 7.12–7.24 (6H, m), 7.58 (1H, s), 7.60 (1H, d, J = 10.9 Hz), 7.68 (1H, t, J = 5.7 Hz). MS (ESI) m/z: 1060 $(M+H)^+$. For precursor drug linker of ADC (**7**): ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.85 (3H, t, J = 7.4 Hz), 1.65–1.74 (2H, m), 1.77–1.90 (2H, m) 2.07–2.19 (4H, m), 2.30 (2H, t, J = 7.2 Hz), 2.33–2.36 (2H, m), 2.38 (3H, s), 2.76 (1H, dd, J = 13.7, 9.8 Hz), 2.96–3.18 (9H, m), 3.42–3.44 (4H, m), 3.53–3.76 (10H, m), 4.43 (1H, td, J = 8.6, 4.7 Hz), 5.14 (1H, d, J = 18.8 Hz), 5.23 (1H, d, J = 18.8 Hz), 5.38 (1H, d, J = 17.2 Hz), 5.42 (1H, d, J = 17.2 Hz), 5.52–5.58 (1H, m), 6.52 (1H, s), 6.98 (2H, s), 7.12–7.17 (1H, m), 7.18–7.25 (4H, m), 7.29 (1H, s), 7.69 (1H, t, J = 5.5 Hz), 7.78 (1H, d, J = 11.3 Hz), 7.98–8.03 (2H, m), 8.11 (1H, d, J = 7.8 Hz), 8.16 (1H, t, J = 5.7 Hz), 8.23 (1H, t, J = 5.9 Hz), 8.44(1H, d, J = 9.0 Hz). MS (APCI) m/z: 1149 $(M+H)^+$.

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6. DAR was calculated by UV absorption measurement of 280 nm and 370 nm.
7. Aggregation rate was measured by high performance liquid chromatography (HPLC). Column; Tosoh TSK gel G3000SWXL (300 × 7.8 mm). Mobile phase; 0.5 mL/min 0.03 M phosphate buffer (0.2 M arginine, pH 6.8). Sample; 10 μL (1 mg/mL) injection; ADC (**1**) in phosphate buffered saline (pH 7.5), ADC (**2**), (**7a**), and (**7b**) in acetate buffer red 5% sorbitol (pH 5.5), and ADC (**3**)–(**6**) in phosphate buffered saline (pH 6.0).
8. NMR and LC–MS analysis. Compound (**12**); ^{1}H NMR (400 MHz, CD_3OD) δ/ppm: 0.96 (3H, t, J = 7.0 Hz), 1.89–1.91 (2H, m), 2.14–2.16 (1H, m), 2.30 (3H, s), 2.40–2.42 (1H, m), 3.15–3.21 (2H, m), 3.79–3.86 (2H, m), 4.63–4.67 (1H, m), 5.00–5.05 (1H, m), 5.23 (1H, d, J = 16.0 Hz), 5.48 (1H, d, J = 16.0 Hz), 5.62–5.64 (1H, m), 7.40–7.45 (2H, m). MS (APCI) m/z: 493 $(M+H)^+$. Compound (**13**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.87 (3H, t, J = 7.2 Hz), 1.86 (2H, dquin, J = 14.6, 7.2 Hz), 2.06–2.27 (1H, m), 2.41 (3H, s), 2.46–2.57 (2H, m), 3.08 (2H, t, J = 6.8 Hz), 3.14–3.24 (2H, m), 5.22 (1H, d, J = 18.8 Hz), 5.29 (1H, d, J = 18.8 Hz), 5.43 (2H, s), 5.58 (1H, dt, J = 8.5, 4.5 Hz), 6.55 (1H, s), 7.32 (1H, s), 7.74 (3H, brs), 7.82 (1H, d, J = 11.0 Hz), 8.67 (1H, d, J = 8.6 Hz). MS (ESI) m/z: 507 $(M+H)^+$. Compound (**14**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.87 (3H, t, J = 7.2 Hz), 1.79–1.92 (4H, m), 2.10–2.17 (2H, m), 2.27 (2H, t, J = 7.0 Hz), 2.40 (3H, s), 2.80–2.86 (2H, m), 3.15–3.20 (2H, m), 5.15 (1H, d, J = 18.8 Hz), 5.26 (1H, d, J = 18.8 Hz), 5.42 (2H, s), 5.54–5.61 (1H, m), 6.55 (1H, s), 7.32 (1H, s), 7.72 (3H, brs), 7.82 (1H, d, J = 11.0 Hz), 8.54 (1H, d, J = 8.6 Hz). MS (APCI) m/z: 521 $(M+H)^+$. Compound (**15**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.88 (3H, t, J = 7.4 Hz), 1.52–1.71 (4H, m), 1.87 (2H, tt, J = 14.4, 6.9 Hz), 2.07–2.18 (2H, m), 2.22 (2H, t, J = 7.0 Hz), 2.40 (3H, s), 2.76–2.88 (2H, m), 3.13–3.22 (2H, m), 5.18 (1H, d, J = 18.8 Hz), 5.24 (1H, d, J = 18.8 Hz), 5.43 (2H, s), 5.53–5.61 (1H, m), 6.55 (1H, s),7.33 (1H, s), 7.65 (3H, brs), 7.81 (1H, d, J = 11.3 Hz), 8.49 (1H, d, J = 8.6 Hz). MS (ESI) m/z: 535 $(M+H)^+$. Compound (**16**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.88 (3H, t, J = 7.2 Hz), 1.31–1.41 (2H, m), 1.52–1.70 (4H, m), 1.80–1.94 (2H, m), 2.05–2.18 (2H, m), 2.21 (2H, t, J = 7.4 Hz), 2.40 (3H, s), 2.81 (2H, t, J = 7.4 Hz), 3.10–3.25 (2H, m), 3.33 (2H, brs), 5.18 (1H, d, J = 19.8 Hz), 5.22 (1H, d, J = 19.8 Hz), 5.41 (2H, d, J = 16.6 Hz), 5.45 (2H, d, J = 16.6 Hz), 5.53–5.60 (1H, m), 6.55 (1H, s), 7.32 (1H, s), 7.80 (1H, d, J = 10.9 Hz), 8.49 (1H, d, J = 9.2 Hz). MS (ESI) m/z: 549 $(M+H)^+$.

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ORIGINAL ARTICLE

TROP2 expression and its correlation with tumor proliferation and angiogenesis in human gliomas

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Abstract Trophoblast cell surface antigen 2 (TROP2) is a transmembrane glycoprotein which is associated with tumor development and progression in a variety of epithelial carcinomas, while its expression and role in gliomas have not been considered. The aim of the study was to investigate TROP2 expression in malignant gliomas with different World Health Organization (WHO) classification and its correlation with tumor proliferation and angiogenesis. Immunohistochemistry was used to determine TROP2 and Ki-67 expression and microvessel density (MVD) in tumor specimens and normal brain tissues from 69 glioma patients and the relationship between TROP2 and Ki-67 and MVD was investigated. Immunohistochemistry results showed that the TROP2 expression was found in 59 (85.5 %) of the 69 tumor specimens, but no expression in normal brain tissues. Furthermore, TROP2 expression is significantly higher in WHO grade III ($P = 0.025$) and WHO grade IV ($P = 0.011$) gliomas than in WHO grade II gliomas. TROP2 expression correlates with Ki-67 ($r = 0.676$, $P = 0.012$) and MVD ($r = 0.365$, $P = 0.035$), but not with gender or age in human gliomas. These results suggested that the TROP2 correlated with malignancy, proliferation and angiogenesis in human gliomas. This is the first study describing TROP2 expression in gliomas and its proliferation and angiogenesis-related characteristic may serve as a potential therapeutic target for glioma treatment.

Keywords Glioma · TROP2 · Ki-67 · MVD · Angiogenesis

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Introduction

Malignant glioma is the most common malignant primary brain tumor which is associated with extremely high morbidity and mortality [1]. The widely used World Health Organization (WHO) system classifies astrocytic tumors into I–IV prognostic grades according to their pathological features and malignant phenotypes [2]. Few patients with malignant glioma are candidates for curative resection because of its infiltrative nature. Despite aggressive treatments with chemotherapy and/or radiotherapy, the median survival time is only appropriately 1 year for patients with glioblastoma multiforme (GBM) and 2–5 years for patients with anaplastic astrocytoma (AA) [3]. Increasing evidence showed that the gene therapy was becoming an effective treatment for malignant glioma [4]. Therefore, there is a pressing need to explore novel therapeutic targets for glioma treatment.

Trophoblast cell surface antigen 2 (TROP2), also termed as GA733-1 or EGP-1, is a transmembrane glycoprotein which was originally identified on human trophoblast and choriocarcinoma cell lines [5]. TROP2-encoding gene is a member of tumor-associated calcium signal transducer (TACSTD) gene family which includes another member epithelial cell-adhesion molecule (EpCAM) encoding gene [6]. Subsequent research indicated that the TROP2 was highly expressed in various types of human malignant tumors and its high level was frequently associated with

poor prognosis [7–11]. Further evidence showed that ectopic expression of TROP2 was sufficient to increase anchorage-independent growth and tumorigenesis in colon cancer [10]. Moreover, TROP2 expression has been also known to confer invasive and metastatic potential to tumor cells [10–13]. Similar to the development of immunotherapeutic agents against EpCAM [14], exciting progress has been made based on TROP2 for immunotherapy for a variety of carcinomas [15–17]. In view of the restricted expression in normal tissues, TROP2 maybe a promising therapeutic target for cancer treatment.

Increasing evidence showed that the tumor proliferative activity and pathological angiogenesis correlated with recurrence interval and patient survival in astrocytic tumors [18–20]. As we know, Ki-67/MIB-1 labeling index (LI) and MVD are common markers for evaluating the proliferation and angiogenesis in tumors, respectively. The aim of our study was to investigate TROP2 expression in human glioma specimens. To assess the role of TROP2 in gliomas, we detected Ki-67 and CD34 expression and analyzed its correlation with tumor WHO classification, proliferation and angiogenesis.

Materials and methods

Patient selection and samples collection

We retrospectively investigated 69 patients with histologically confirmed glioma who received surgical resection in Provincial Hospital Affiliated to Shandong University. Normal brain-tissue samples were obtained from four brain contusion and laceration patients. Clinical and pathological information were retrieved from medical records. All the hematoxylin-eosin-stained sections were re-evaluated and classified by two pathologists according to the criteria of WHO histological classification [2]. Twenty-seven of the 69 glioma specimens were classified as diffuse astrocytoma (WHO grade II). Twenty-three and 19 tumor specimens belonged to anaplastic astrocytoma (WHO grade III) and glioblastoma multiforme (WHO grade IV), respectively. Demographic data and tumor characteristics were indicated in Table 1. The mean age of patients (female 38, male 31) was 44 years. None of the patients had received chemotherapy or radiotherapy prior to surgery. Written consent to use stored specimens was obtained from all living patients. The study was approved by the Research Ethics Committee of Provincial Hospital Affiliated to Shandong University.

Immunohistochemical staining

Tissue samples were obtained from the Department of Pathology, Provincial Hospital Affiliated to Shandong University. Representative formalin-fixed and paraffin-embedded blocks were selected and cut into 5-μm thick for staining. Primary antibodies used in the study were as follows: TROP2 (goat polyclonal, AF650, 1:50 dilution, R&D Systems Inc.), Ki-67 (mouse monoclonal, M7240, 1:100 dilution, DAKO), CD34 (BI-3C5) (mouse monoclonal, sc-19621, 1:100 dilution, Santa Cruz Biotechnology).

Table 1 Expression of TROP2, Ki-67 and MVD evaluated by immunohistochemistry in malignant gliomas with different WHO classification

WHO classification of malignant gliomas (number of samples)	Sex (male vs. female)	Mean age (years)	Ki-67 (*x*) %	MVD (*x*)	TROP2 (−/++/+++)
WHO grade II (27)	11/16	43	3.1	89.6	6/10/6/5
WHO grade III (23)	12/11	42	16.4	110.7	3/2/8/10
WHO grade IV (19)	8/11	47	18.2	131.4	1/2/7/9
Total (69)	31/38	44			

TROP2 trophoblast cell surface antigen, *MVD* microvessel density

Immunohistochemical staining was performed using the standard avidin–biotin method. Briefly, slides were deparaffinized and pretreated in citrate buffer (pH 6.0) using the microwave-induced epitope retrieval protocol. Endogenous peroxidase activity was quenched with 0.3 % hydrogen peroxide for 15 min at 37 °C, then the slides were incubated with primary antibodies working solution overnight at 4 °C. After several washes, the sections were incubated with biotinylated secondary antibodies for 30 min at 37 °C. Subsequently, the slides were incubated with peroxidase-conjugated-streptavidin complex reagent and developed with 3,3′-diaminobenzidine solution until desired staining was achieved (approximately 4–5 min). Slides were counterstained with hematoxylin or eosin, dehydrated, cleared and mounted. Sections of colon cancer tissue served as positive control during staining. Primary antibodies were substituted by phosphate-buffered solution during negative control staining.

Evaluation of immunohistochemical staining

Each slide was evaluated using light microscopy by two independent observers blinded to the clinicopathological background of patient samples. Sections upon which there were differing evaluations were re-evaluated simultaneously by observers using a double-headed microscope. TROP2 immunostaining for each sample was quantified as the product of a proportion score and an intensity score as described previously [8, 11]. The proportion of positive-stained tumor cells in lesions was scored as 0 (none), 1 (<10 %), 2 (10–50 %), 3 (51–80 %), or 4 (>80 %). The staining intensity was scored as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong), yielding a total score ranging

from 0 to 12: −, score 0; +, score 1,2,3; ++, score 4, 6, 8; +++, score 9, 12.

MIB-1 LI was calculated as the percentage of immunopositive glioma cells. One thousand neoplastic cells were counted in six separate areas for each specimen in which positive nuclear staining was evenly distributed [19]. Each CD34-positive endothelial cell or cluster of endothelial cells that was separate from adjacent microvessels was considered to be a countable microvessel. Vessel lumens were not required for structures to be counted as a microvessel [21]. Final MVD was recorded as the average count of six randomly selected vision fields (×200) with the highest vascular counts (hot-spot area) [22].

Quantitative real-time PCR

Total RNA was isolated from glioma tissues using TRIzol Reagent (Invitrogen) and cDNA was synthesized by SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed using SYBR Green Supermix (Bio-Rad) according to manufacturer's instructions and each sample in triplicate. TROP2 primers were used as following: sense: 5′-GCCTACTACTTCGAG AGGGACA-3′; anti-sense: 5′-CAGTTCCTTGATCTCCA CCTTC-3′. TROP2 expression is normalized to the β-actin level in every reaction system. β-actin primer sequences were as follows: sense: 5′-AGGGCTATGCTCTCCCT-CAC-3′; anti-sense: 5′-CTCTCAGCTGTGGTGGTGAA-3′

Statistical analysis

Statistical analyses were carried out with the software of SPSS version 17.0. Statistical differences among TROP2 expression in malignant gliomas with different WHO classification were evaluated by Fisher's exact test and non-parametric test. The Spearman correlation was applied to assess the correlation between TROP2 and Ki-67, MVD, gender and age. For all analyses, $P < 0.05$ was considered as statistically significant.

Results

TROP2 expression increased with malignancy in gliomas

TROP2 was mainly detected not only on the membrane of glioma cells but also in the cytoplasm. Immunohistochemistry results showed that the TROP2 protein presented in 85.5 % (59 of 69) of glioma samples but no expression in normal brain tissues; detailed information is described in Table 1. Based on the results, TROP2 protein level significantly correlated with the histopathologic grades of malignant gliomas. TROP2 expression was significantly higher in WHO grade III ($P = 0.025$) and WHO grade IV ($P = 0.011$) gliomas than in WHO grade II gliomas. However, there is no significant difference between WHO grades III and IV gliomas ($P = 0.924$). Representative immunohistochemical staining for TROP2 is shown in Fig. 1. We also randomly detected the TROP2 mRNA level in 30 glioma specimens with different WHO classification using quantitative real-time PCR. Consistent with its protein level, TROP2 mRNA is also significantly higher in WHO grade III ($P = 0.040$) and grade IV ($P = 0.034$) gliomas than in WHO grade II gliomas (Fig. 2).

TROP2 correlated with MIB-1 LI and MVD in gliomas

Results showed that the final MIB-1 LI was 3.1 in WHO grade II gliomas, and was 16.4 and 18.2 in WHO grades III and IV gliomas, respectively. Based on our immunohistochemical evaluation standard, MVD-detailed information of the samples was given in Table 1. Representative immunohistochemical staining for Ki-67 and CD34 is shown in Fig. 1. Furthermore, Spearman's rank correlation analysis indicated that the TROP2 expression was significantly correlated with Ki-67 ($P = 0.012$) and MVD ($P = 0.035$) as displayed in Table 2, but not with gender ($P = 0.340$) and age ($P = 0.432$).

Discussion

Malignant glioma is the most common malignant primary brain tumor with extremely high morbidity and mortality [3, 23]. Despite advances in surgical treatment with chemotherapy and radiotherapy, malignant glioma still remains a refractory disease because of its locally infiltrating nature and abnormal tumor vasculature [18]. With the development of gene therapy and targeting agents for human gliomas [4], identification of novel targets is of great importance for glioma therapy.

The present study provided evidence that the TROP2 was highly expressed in gliomas but not in normal brain tissues and TROP2 protein level also correlated with WHO grades of glioma. Spearman correlation analysis suggested that the TROP2 was closely correlated with the proliferative factor Ki-67, which is consistent with previous reports [13]. Interestingly, we found that the TROP2 was also associated with angiogenesis significantly in human gliomas. Although the number of patient samples in the study is limited, these results implied that TROP2 may play a vital role in tumorigenesis and angiogenesis in gliomas.

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Ex. 1002

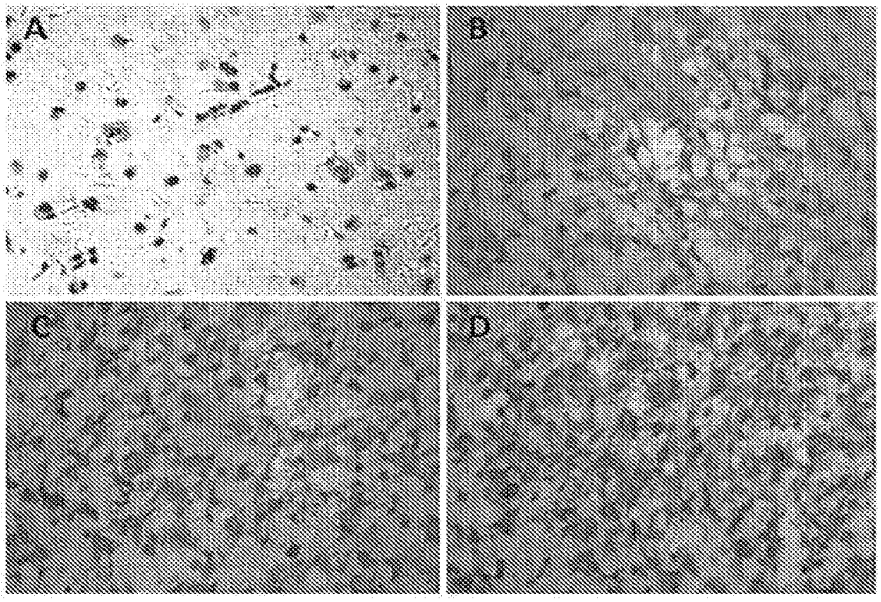


Fig. 1 Representative immunohistochemical staining for TROP2 in normal brain tissue and malignant gliomas with different WHO classification: Negative TROP2 expression in normal brain tissues (total score, 0) (a); WHO grade II glioma with low TROP2 expression (total score, 3) (b); WHO grade III glioma with high TROP2 expression (total score, 8) (c); WHO grade IV glioma with high TROP2 expression (total score, 12) (×400) (d)

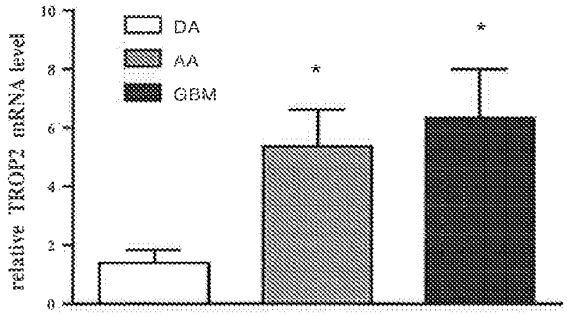


Fig. 2 Relative TROP2 mRNA level in 30 glioma specimens with different WHO classification was analysed with real-time PCR (10 DA, 10 AA, and 10 GBM). *DA* diffuse astrocytoma, *AA* anaplastic astrocytoma, *GBM* glioblastoma multiforme (*$P < 0.05$)

TROP2 protein is a type I transmembrane glycoprotein which is invariably upregulated in tumors compared with the corresponding normal tissues and predicts poor survival in a variety of cancer entities [7, 8, 11, 24]. Recent studies showed that overexpression of TROP2 protein was sufficient for tumor growth and growth rates were reduced after TROP2 knockdown in colon and breast cancer cell lines, which may be explained by higher proportion of S phase cells within TROP2 overexpressing cells [13]. High TROP2

Table 2 Correlation analysis of TROP2 expression with Ki-67, MVD, gender, and age

	TROP2	
	r	*P*
Ki-67	0.676	0.012
MVD	0.365	0.035
Gender	0.124	0.340
Age	0.241	0.432

Spearman rank correlation coefficient (r) is considered significant when $P < 0.05$

TROP2 trophoblast cell surface antigen 2, *MVD* microvessel density

L-cell tumors grew strikingly faster and showed more malignant phenotype than wild-type L cells tumors which also suggested that the TROP2 played a vital role in tumor progression [13]. mTROP2 expression led to increased tumor growth and liver metastasis in a murine pancreatic cancer model [12]. However, the molecular mechanism mediated by TROP2 in tumor development and progression still remains largely unknown. Previous studies showed that TROP2 upregulation increased the expression of cyclic AMP-responsive-element binding protein (CREB), NF-kB, STAT1 and other cancer regulatory effectors through cyclin D and MAPK pathway [12, 25]. In addition, the chimeric CYCLIN D1-TROP2 mRNA was found in human ovarian

cancer cells and could stimulate tumor growth in cooperation with activated RAS [26]. Recently, Witte et al. [27] showed that the TROP2 promoted tumorigenesis through driving stem-cell self-renewal via β catenin signaling in prostate cancer. To our knowledge, the relationship between TROP2 and angiogenesis has not been investigated systematically in human cancer.

Angiogenesis, the formation of new blood vessels, plays a critical role in glioma development and progression [28]. Clinical investigation also revealed that the patients with higher MVD showed poorer prognosis than the patients with lower MVD [29]. Several growth factors were reported to participate in glioma angiogenesis, such as vascular endothelial growth factor (VEGF) [30], epidermal growth factor (EGF) [31], and platelet-derived growth factor (PDGF) [32]. In addition, VEGF signaling inhibitors has been approved for use in patients with glioblastoma multiforme and other solid tumors [30, 33]. Furthermore, EpCAM and α-fetoprotein (AFP) positive specimens showed an association with MVD and VEGF in hepatocellular carcinoma (HCC) [34]. Our results demonstrated that the TROP2 was also associated with MVD in glioma, implying TROP2 may promote angiogenesis and may be a novel therapeutic target for glioma.

Recently, immunotherapy based on TROP2 has made great progress in different cancers. Uterine and ovarian carcinosarcomas overexpressing TROP2 showed sensitivity to hRS7 which was a humanized anti-TROP2 antibody [15, 16]. The hRS7 antibody labeled with ^{131}I-IMP-R4 treatment also decreased tumor volume in nude mice bearing human breast cancer xenografts [17]. Similarly, the anti-Trop-2 hRS7-CL2A-SN-38 antibody–drug conjugate (ADC) exhibited significant and specific antitumor effects against lung, colon, and pancreatic carcinomas [35]. In addition, the application of chimeric murine TROP2 virus-like particles (VLP) decreased tumor growth and altered tumor immune microenvironments in a murine pancreatic cancer model [12].

In conclusion, the present study indicated that high TROP2 expression was identified in gliomas and tended to increase with higher grades. Furthermore, our results showed that the TROP2 correlated with tumor proliferation potential and angiogenesis in gliomas. Since the limited expression in normal brain tissues, TROP2 may serve as a novel diagnostic and immunotherapeutic target for patients with glioma.

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Conflict of interest The authors declare that they have no conflict of interest.

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EXAMINER	
AKHOON, KAUSER M	

ART UNIT	PAPER NUMBER
1642	

NOTIFICATION DATE	DELIVERY MODE
07/21/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipdocketing@foley.com

Office Action Summary	Application No. 14/435,114	Applicant(s) MASUDA ET AL.	
	Examiner KAUSER AKHOON	Art Unit 1642	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *04/10/2015*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-48* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-48* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *07/30/2015, 08/21/2015, 08/25/2015, and 06/13/2016*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Claims 1-48 are currently pending.

Priority

Acknowledgment is made that this application is a 371 National Stage Entry of International Application PCT/JP13/006069, filed October 10, 2013, which claims priority to Japanese Patent Application No. 2012-225887, filed October 10, 2016.

Information Disclosure Statement

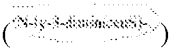
Applicant's Informational Disclosure Statements, filed on 07/30/2015, 08/21/2015, 08/25/2015, and 06/13/2016 have been considered. The signed and initialed PTO 1449s have been mailed with this action.

Specification

The disclosure is objected to because of the following informalities: the Sequence Listing was submitted electronically as a text file, thus the specification must be amended to contain an incorporation-by-reference of the material in the ASCII text file in a separate paragraph identifying the name of the ASCII text file, the date of creation, and the size of the ASCII text file in bytes. See MPEP 502.05 L and Fed. Register (Oct. 27, 2009, 74(206): 55,208-55,209)..

Appropriate correction is required.

Claim Objections

Claims 1, 7, 13 and 48 are objected for typographical error in spelling of Succinimid-3-yl which is spelled backwards ().

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

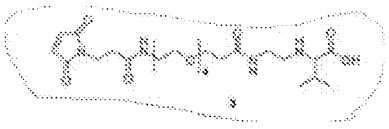
1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

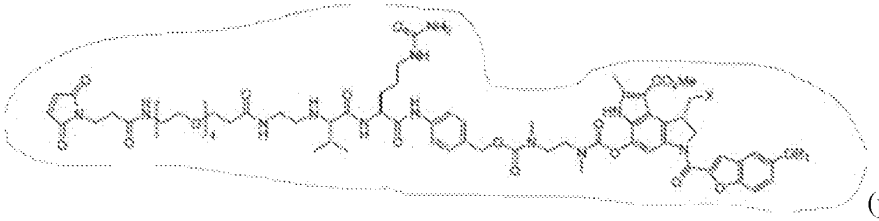
Claims 1-28 and 30-48 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Susaki et al. ("Susaki", US 6,835,807) in view of Boyd et al. ("Boyd", WO2005/112919A2).

Susaki teaches drug delivery systems (drug complexes) in which DX-8951 (exatecan, Formula I) is linked to a GGFG tetrapeptide. (Example 1, column 17, line 1 to column 19, lines 11). Susaki teaches the use of the drug complex to act as antineoplastic agents and anti-inflammatory agents to a tumorous site (column 28, lines 60--67). However, Susaki does not teach the drug complex as instantly claimed.

Boyd teaches drug complexes such as antibody drug conjugates with peptide linkers conjugates



(page 118) and teaches compounds such as



(page 120). Boyd teaches numerous chemical linkers that are attached to the drug and attaches to the antibody (page 39, line 15-page 53, line 22).

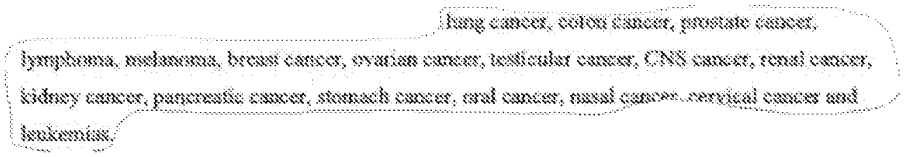
Therefore, it would have been obvious to one having ordinary skill in the art at the time of the instant invention to have prepared the Susaki exatecan compositions with the linkers taught by Boyd with a reasonable expectation of success. One having ordinary skill in the art

would have been motivated to combine the teaching of Susaki and Boyd because Boyd teaches antibody drug conjugates using a multitude of linker and peptide bonds to append the drug to the antibody.

Although Boyd and Susaki lists all of the linkers required by the claim, all of the possibilities identified in Boyd and Susaki are not found in any specific embodiment, one of ordinary skill in the art is able to "envisage" the claimed combination comprising the antibody drug conjugate from sufficiently limited or well delineated examples, (see MPEP 2131.02).

Regarding claims 26-28, Boyd also teaches antibodies such as CD20, CD22, PSMA, CD30 (pages 95-96).

Regarding claim 32, Boyd teaches examples of cancers or tumors such as:



(page 114, lines 7-13).

Thus, given the teachings of the prior art and level of a person of ordinary skill in the art at the time of Applicant's invention one would have had a reasonable expectation of success in practicing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references.

In the absence of any factual showing or unexpected results the presently claimed compositions are considered *prima facie* obvious over the prior art for the reasons stated above.

In light of the forgoing discussion, the subject matter defined by the claims would have been obvious within the meaning of 35 USC § 103(a).

Claims 1-48 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Susaki et al. ("Susaki", US 6,835,807) in view of Boyd et al. ("Boyd", WO2005/112919A2), as applied to claims 1-28 and 30-48 and further in view of Loo et al. ("Loo", Clin. Cancer Res. July 15, 2012, 18, 3834-3845).

Claim 1-28 and 30-48 have been shown to be obvious over Susaki and Boyd above. The teachings of Susaki and Boyd have been discussed at length above and the rational applies herein.

Although Boyd and Susaki teach antibodies, they do not explicitly teach anti-B7-H3 antibody as instantly claimed.

Loo teaches monoclonal antibody, anti-B7-H3 antibody, which has potent antitumor activity (Abstract).

Therefore, it would have been obvious to one having ordinary skill in the art at the time of the instant invention to have prepared the Susaki and Boyd antibody-exatecan conjugate compositions with anti-B7-H3 antibody taught by Loo with a reasonable expectation of success. One having ordinary skill in the art would have been motivated to combine the teaching of Susaki and Boyd with Loo because Loo teaches monoclonal antibody, anti-B7-H3 antibody, which has potent antitumor activity (Abstract).

Thus, given the teachings of the prior art and level of a person of ordinary skill in the art at the time of Applicant's invention one would have had a reasonable expectation of success in

practicing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references.

In the absence of any factual showing or unexpected results, the presently claimed compositions are considered *prima facie* obvious over the prior art for the reasons stated above. In light of the forgoing discussion, the subject matter defined by the claims would have been obvious within the meaning of 35 USC § 103(a).

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Application No. 14/436,458

Claims 1-48, are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-54 of copending Application No. 14/436,458.

Although the conflicting claims are not identical, they are not patentably distinct from each other because the composition encompasses the same drug, overlapping linker system and same antibodies, anticipates the conjugate of a drug and a targeting moiety claimed in the application currently under examination.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Application No. 15/180,203

Claims 1-48 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-18 of copending Application No. 15/180,203.

Although the conflicting claims are not identical, they are not patentably distinct from each other because the composition encompasses the same drug, overlapping linker system and same antibodies, anticipates the conjugate of a drug and a targeting moiety claimed in the application currently under examination.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to KAUSER M. AKHOON whose telephone number is (571) 270-1026. The examiner can normally be reached on M-F 8:30 AM-5:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Misook Yu can be reached on (571) 272-0839. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/K. A./
Examiner, Art Unit 1642

/PETER J REDDIG/
Primary Examiner, Art Unit 1642

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/436,458	04/23/2015	Hiroyuki NAITO	111119-0103	1083

22428 7590 07/19/2016

Foley & Lardner LLP
3000 K STREET N.W.
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WASHINGTON, DC 20007-5109

EXAMINER	
AKHOON, KAUSER M	

ART UNIT	PAPER NUMBER
1642	

NOTIFICATION DATE	DELIVERY MODE
07/19/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipdocketing@foley.com

Office Action Summary	Application No. 14/436,458	Applicant(s) NAITO ET AL.	
	Examiner KAUSER AKHOON	Art Unit 1642	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *04/23/2015*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-54* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-54* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *07/29/2015, 08/21/2015, 08/24/2015, 06/13/2016*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Claims 1-54 are currently pending.

Priority

Acknowledgment is made that this application is a 371 National Stage Entry of International Application PCT/JP13/006178, filed October 17, 2013, which claims priority to Japanese Patent Application No. 2012-231579, filed October 19, 2012.

Information Disclosure Statement

Applicant's Informational Disclosure Statements, filed on 07/29/2015, 08/21/2015, 08/24/2015, and 06/13/2016 have been considered. The signed and initialed PTO 1449s have been mailed with this action.

Specification

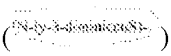
This application contains sequence disclosures that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 C.F.R. § 1.821(a)(1) and (a)(2). This application fails to comply with the requirements of 37 C.F.R. §§ 1.821-1.825. Claims 10-12, 26 in the specification contain sequences that are encompassed by the sequences rules and require sequence identifiers (SEQ ID numbers). Applicant is reminded to review the entire disclosure to ensure that the present application is in sequence compliance.

Additionally, if the Sequence Listing is submitted electronically as a text file, the specification must be amended to contain an incorporation-by-reference of the material in the ASCII text file in a separate paragraph identifying the name of the ASCII text file, the date of creation, and the size of the ASCII text file in bytes. See MPEP 502.05 L1.

Any questions regarding compliance with the sequence rules requirements specifically should be directed to the departments listed at the bottom of the Notice to Comply.

APPLICANT IS GIVEN THE TIME ALLOTED IN THIS OFFICE ACTION WITHIN WHICH TO COMPLY WITH THE SEQUENCE RULES, 37 C.R.F. §§ 1.821-1.825. Failure to comply with these requirements will result in ABANDONMENT of the application under 37 C.F.R. § 1.821(g). Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 C.F.R. § 1.136. In no case may an applicant extend the period for response beyond the six-month statutory period. Direct the response to the undersigned.

Claim Objections

Claims 1 and 39, are objected for typographical error in spelling of Succinimid-3-yl which is spelled backwards ().

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

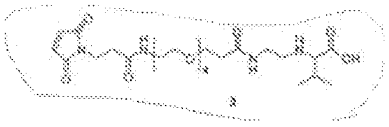
Claims 1-10, 13-25, 27-46 and 48-54 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Susaki et al. ("Susaki", US 6,835,807) in view of Boyd et al. ("Boyd", WO2005/112919A2).

Claims 11, 12, and 26 are free of prior art.

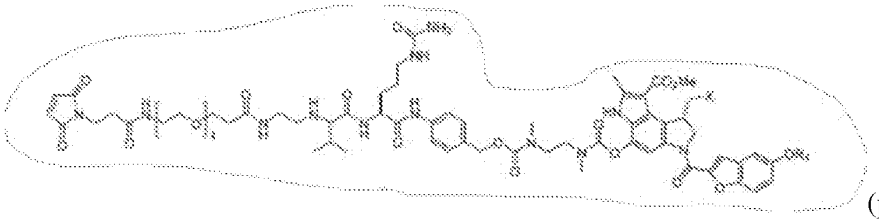
Susaki teaches drug delivery systems (drug complexes) in which DX-8951 (exatecan, Formula I) is linked to a GGFG tetrapeptide. (Example 1, column 17, line 1 to column 19, lines 11). Susaki teaches the use of the drug complex to act as antineoplastic agents and anti-

inflammatory agents to a tumorous site (column 28, lines 60--67). However, Susaki does not teach the drug linkers as instantly claimed.

Boyd teaches drug complexes such as antibody drug conjugates with peptide linkers conjugates



(page 118) and teaches compounds such as



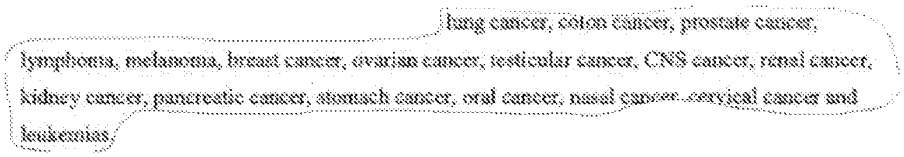
(page 120). Boyd teaches numerous chemical linkers that are attached to the drug and attaches to the antibody (page 39, line 15-page 53, line 22).

Therefore, it would have been obvious to one having ordinary skill in the art at the time of the instant invention to have prepared the Susaki exatecan compositions with the linkers taught by Boyd with a reasonable expectation of success. One having ordinary skill in the art would have been motivated to combine the teaching of Susaki and Boyd because Boyd teaches antibody drug conjugates using a multitude of linker and peptide bonds to append the drug to the antibody.

Although Boyd and Susaki lists all of the linkers required by the claim, all of the possibilities identified in Boyd and Susaki are not found in any specific embodiment, one of ordinary skill in the art is able to "envisage" the claimed combination comprising the antibody drug conjugate from sufficiently limited or well delineated examples, (see MPEP 2131.02).

Regarding claims 45-46, Boyd also teaches antibodies such as CD20, CD22, PSMA, CD30 (pages 95-96).

Regarding claim 50 and 52, Boyd teaches examples of cancers or tumors such as:



(page 114, lines 7-13).

Thus, given the teachings of the prior art and level of a person of ordinary skill in the art at the time of Applicant's invention one would have had a reasonable expectation of success in practicing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references.

In the absence of any factual showing or unexpected results the presently claimed compositions are considered *prima facie* obvious over the prior art for the reasons stated above. In light of the forgoing discussion, the subject matter defined by the claims would have been obvious within the meaning of 35 USC § 103(a).

Claims 1-10, 13-25 and 27-54 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Susaki et al. ("Susaki", US 6,835,807) in view of Boyd et al. ("Boyd", WO2005/112919A2), as applied to claims 1-10, 13-25, 27-46 and 48-54 and further in view of Loo et al. ("Loo", Clin. Cancer Res. July 15, 2012, 18, 3834-3845).

Claim 1-10, 13-25, 27-46 and 48-54 have been shown to be obvious over Susaki and Boyd above. The teachings of Susaki and Boyd have been discussed at length above and the rational applies herein.

Although Boyd and Susaki teach antibodies, they do not explicitly teach anti-B7-H3 antibody as instantly claimed.

Loo teaches monoclonal antibody, anti-B7-H3 antibody, which has potent antitumor activity (Abstract).

Therefore, it would have been obvious to one having ordinary skill in the art at the time of the instant invention to have prepared the Susaki and Boyd antibody-exatecan conjugate compositions with anti-B7-H3 antibody taught by Loo with a reasonable expectation of success. One having ordinary skill in the art would have been motivated to combine the teaching of Susaki and Boyd with Loo because Loo teaches monoclonal antibody, anti-B7-H3 antibody, which has potent antitumor activity (Abstract).

Thus, given the teachings of the prior art and level of a person of ordinary skill in the art at the time of Applicant's invention one would have had a reasonable expectation of success in practicing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references.

In the absence of any factual showing or unexpected results, the presently claimed compositions are considered *prima facie* obvious over the prior art for the reasons stated above. In light of the forgoing discussion, the subject matter defined by the claims would have been obvious within the meaning of 35 USC § 103(a).

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Application No. 14/435,114

Claims 1-54, are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-48 of copending Application No. 14/435,114.

Although the conflicting claims are not identical, they are not patentably distinct from each other because the composition encompasses the same drug, overlapping linker system and same antibodies, anticipates the conjugate of a drug and a targeting moiety claimed in the application currently under examination.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Application No. 15/180,203

Claims 1-54 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-18 of copending Application No. 15/180,203.

Although the conflicting claims are not identical, they are not patentably distinct from each other because the composition encompasses the same drug, overlapping linker system and same antibodies, anticipates the conjugate of a drug and a targeting moiety claimed in the application currently under examination.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to KAUSER M. AKHOON whose telephone number is (571) 270-1026. The examiner can normally be reached on M-F 8:30 AM-5:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Misook Yu can be reached on (571) 272-0839. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/K. A./
Examiner, Art Unit 1642

/PETER J REDDIG/
Primary Examiner, Art Unit 1642

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/180,203	06/13/2016	Takeshi Masuda	111119-0111	4222

22428 7590 07/25/2016

Foley & Lardner LLP
3000 K STREET N.W.
SUITE 600
WASHINGTON, DC 20007-5109

EXAMINER	
AKHOON, KAUSER M	

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ipdocketing@foley.com

Office Action Summary	Application No. 15/180,203	Applicant(s) MASUDA ET AL.	
	Examiner KAUSER AKHOON	Art Unit 1642	AIA (First Inventor to File) Status No

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- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *06/13/2016*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-18* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-18* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

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3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Claims 1-18 are currently pending.

Priority

Acknowledgment is made that this application is a continuation application of 14/435,114, filed April 10, 2015, which is a 371 National Stage Entry of International Application PCT/JP13/006069, filed October 10, 2013, which claims priority to Japanese Patent Application No. 2012-225887, filed October 10, 2016.

Information Disclosure Statement

Applicant's Informational Disclosure Statement, filed on 06/13/2016 has been considered. The signed and initialed PTO 1449 has been mailed with this action.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

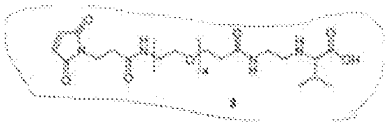
1. Determining the scope and contents of the prior art.
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3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

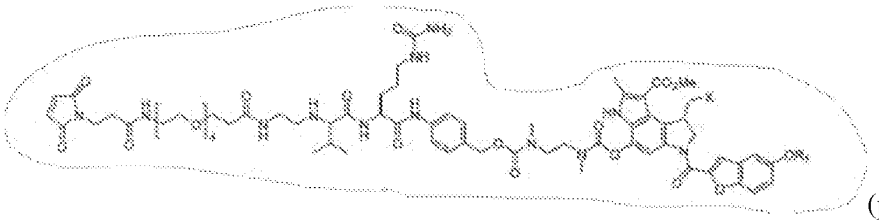
Claims 1-5, 7-13 and 15-18 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Susaki et al. ("Susaki", US 6,835,807) in view of Boyd et al. ("Boyd", WO2005/112919A2).

Susaki teaches drug delivery systems (drug complexes) in which DX-8951 (exatecan, Formula I) is linked to a GGFG tetrapeptide. (Example 1, column 17, line 1 to column 19, lines 11). Susaki teaches the use of the drug complex to act as antineoplastic agents and anti-inflammatory agents to a tumorous site (column 28, lines 60--67). However, Susaki does not teach the drug complex as instantly claimed.

Boyd teaches drug complexes such as antibody drug conjugates with peptide linkers conjugates



(page 118) and teaches compounds such as



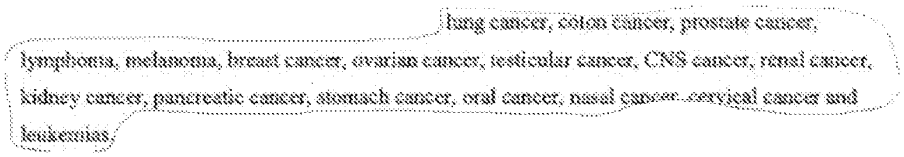
(page 120). Boyd teaches numerous chemical linkers that are attached to the drug and attaches to the antibody (page 39, line 15-page 53, line 22). Boyd further teaches attaching moiety of alkyl maleimide (page 35, lines 12-18) and further teaches that alkyl are n-pentyl groups (page 28, lines 6-13). Boyd also teaches aminobenzyl ether spacers (page 2, lines 1-7)

Therefore, it would have been obvious to one having ordinary skill in the art at the time of the instant invention to have prepared the Susaki exatecan compositions with the linkers taught by Boyd with a reasonable expectation of success. One having ordinary skill in the art would have been motivated to combine the teaching of Susaki and Boyd because Boyd teaches antibody drug conjugates using a multitude of linker and peptide bonds to append the drug to the antibody.

Although Boyd and Susaki lists all of the linkers required by the claim, all of the possibilities identified in Boyd and Susaki are not found in any specific embodiment, one of ordinary skill in the art is able to "envisage" the claimed combination comprising the antibody drug conjugate from sufficiently limited or well delineated examples, (see MPEP 2131.02).

Regarding claims 26-28, Boyd also teaches antibodies such as CD20, CD22, PSMA, CD30 (pages 95-96).

Regarding claim 32, Boyd teaches examples of cancers or tumors such as:



(page 114, lines 7-13).

Thus, given the teachings of the prior art and level of a person of ordinary skill in the art at the time of Applicant's invention one would have had a reasonable expectation of success in practicing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references.

In the absence of any factual showing or unexpected results the presently claimed compositions are considered *prima facie* obvious over the prior art for the reasons stated above. In light of the forgoing discussion, the subject matter defined by the claims would have been obvious within the meaning of 35 USC § 103(a).

Claims 1-18 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Susaki et al. ("Susaki", US 6,835,807) in view of Boyd et al. ("Boyd", WO2005/112919A2), as applied to claims 1-5, 7-13 and 15-18 and further in view of Loo et al. ("Loo", Clin. Cancer Res. July 15, 2012, 18, 3834-3845).

Claim 1-5, 7-13 and 15-18 have been shown to be obvious over Susaki and Boyd above. The teachings of Susaki and Boyd have been discussed at length above and the rational applies herein.

Although Boyd and Susaki teach antibodies, they do not explicitly teach anti-B7-H3 antibody as instantly claimed.

Loo teaches monoclonal antibody, anti-B7-H3 antibody, which has potent antitumor activity (Abstract).

Therefore, it would have been obvious to one having ordinary skill in the art at the time of the instant invention to have prepared the Susaki and Boyd antibody-exatecan conjugate compositions with anti-B7-H3 antibody taught by Loo with a reasonable expectation of success. One having ordinary skill in the art would have been motivated to combine the teaching of Susaki and Boyd with Loo because Loo teaches monoclonal antibody, anti-B7-H3 antibody, which has potent antitumor activity (Abstract).

Thus, given the teachings of the prior art and level of a person of ordinary skill in the art at the time of Applicant's invention one would have had a reasonable expectation of success in practicing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references.

In the absence of any factual showing or unexpected results, the presently claimed compositions are considered *prima facie* obvious over the prior art for the reasons stated above. In light of the forgoing discussion, the subject matter defined by the claims would have been obvious within the meaning of 35 USC § 103(a).

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Application No. 14/436,458

Claims 1-18, are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-54 of copending Application No. 14/436,458.

Although the conflicting claims are not identical, they are not patentably distinct from each other because the composition encompasses the same drug, overlapping linker system and same antibodies, anticipates the conjugate of a drug and a targeting moiety claimed in the application currently under examination.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Application No. 14/435,114

Claims 1-18 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-48 of copending Application No. 15/180,203.

Although the conflicting claims are not identical, they are not patentably distinct from each other because the composition encompasses the same drug, overlapping linker system and same antibodies, anticipates the conjugate of a drug and a targeting moiety claimed in the application currently under examination.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to KAUSER M. AKHOON whose telephone number is (571) 270-1026. The examiner can normally be reached on M-F 8:30 AM-5:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Misook Yu can be reached on (571) 272-0839. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/K. A./
Examiner, Art Unit 1642

/PETER J REDDIG/
Primary Examiner, Art Unit 1642

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/221,851	07/28/2016	Hiroyuki NAITO	111119-0105	3964

22428 7590 07/07/2017

Foley & Lardner LLP
3000 K STREET N.W.
SUITE 600
WASHINGTON, DC 20007-5109

EXAMINER	
DUFFY, PATRICIA ANN	

ART UNIT	PAPER NUMBER
1645	

NOTIFICATION DATE	DELIVERY MODE
07/07/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipdocketing@foley.com

Office Action Summary	Application No. 15/221,851	Applicant(s) NAITO et al.	
	Examiner Patricia Duffy	Art Unit 1645	AIA Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 4-13-2017 .

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____ .

2a)☐ This action is **FINAL**. 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☑ Claim(s) 1-11 is/are pending in the application.

5a) Of the above claim(s) 6 and 8 is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☑ Claim(s) 1-5,7 and 9-11 is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____ .

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____ .

4) ☐ Other: _____ .

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Election/Restrictions

Applicant's election of Group I claims, 1-5, 7 and 9-11 in the reply filed 4-13-2017. Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.01(a)).

Applicants request for rejoinder is noted, however at this time the product is not allowable and the method claims have not been rejoined. Claims 6 and 8 are withdrawn from consideration.

Information Disclosure Statement

The information disclosure statements have been considered. Initialled copies are enclosed.

Priority

It is noted that PCT/JP2015/000355, and Foreign priority applications filed 1/31/2014. 8, 22/2014 and 11/10/2014 are all in Japanese and no english translations have been filed. As such, the instant filing date 7-28-2016 is the priority date for prior art purposes.

Duplicate Claim Warning

Applicant is advised that should claim 4 be found allowable, claim 5 will be objected to under 37 CFR 1.75 as being a substantial duplicate thereof. When two claims in an application are duplicates or else are so close in content that they both cover the same thing, despite a slight difference in wording, it is proper after allowing one claim to object to the other as being a substantial duplicate of the allowed claim. See MPEP § 706.03(k).

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or

improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

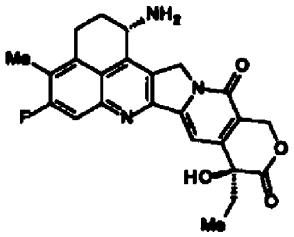
The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Please note that USSN 14/436,485, 14/435,114, 15/180,203, 15/187,179 have earlier effective filing dates.

Claim1-5, 7 and 9-11 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claim 39, 40-44, 48-50 of copending Application No. **14/436,485** in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31,

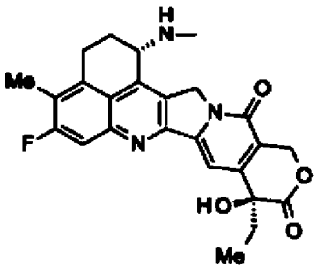
2007) and EP 1155702 A1 published 11-21-2001; of record. Although the claims at issue are not identical, they are not patentably distinct from each other because:

USSN 14/436,458 claim an antibody-drug conjugate comprising the antitumor compound



wherein the antitumor compound is conjugated to an antibody via a linker having a structure represented by the following formula:

And in claim 39:



wherein the nitrogen atom of the amino group at position 1 is the connecting position.

Claim 39 of the '458 application also teaches that the linker can be

-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-DGGF (SEQ ID NO. 34)-NH-CH_2-O-CH_2-C(=O)-(NH-DX),

. The '458 application also claims that the peptide in the linker (Lp) can be GGFG (see claim10). The '458 claims also teach that the antibody has the property of recognizing a target cell where the target cell is as tumor cell (see claim 44). The '485 claims also provide for a "drug containing the conjugate" and an antitumor drug and/or anticancer drug comprising the conjugate or salt thereof including breast cancer (see claims 48-50). Claims 40-42 teach that the number of antitumor compounds conjugated per antibody is in the range from 1-10, 1-8, or 3-8.. The claims differ from the instant claims by not reciting that the antibody can be the breast cancer antibody trastuzumab (comprising SEQ ID NO:1 and 2).

Chamberlain et al teach the amino acid sequence of tratsuzmab (SEQ ID NOS:11 and 12).

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US-11-932-151-12

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Qy         61 RFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPP 120
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Qy        121 SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Qy         61 ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS 120
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Qy        241 PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN 300
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Qy        301 STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE 360
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Qy        361 MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW 420
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Qy        421 QQGNVFSCSVMHEALHNHYTQKSLSLSPGK 450
              |||||||||||||||||||||||||||||
Db        421 QQGNVFSCSVMHEALHNHYTQKSLSLSPGK 450
```

EP1155702A1 teaches conjugates of cytotoxic drugs and saccharide polymeric carriers. WP 1155702A1 teach that the peptide Gly-Gly-Phe-Gly (GGFG) are suitable hydrolysable oligopeptide moieties (see paragraphs [0038], [0066], [0038], and [0111]).

As to claims 1-5, 7 and 9-11, it would have been prima facie obvious to one having ordinary skill in the art at the time the invention was filed to modify the antibody-exatecan conjugate of claim 39 having the linker

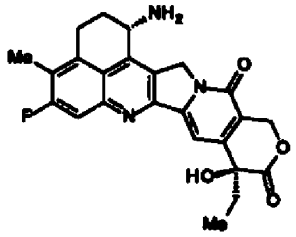
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-DGGF (SEQ ID NO. 34)-NH-CH_2-O-CH_2-C(=O)-(NH-DX),

of the '548 application by using the trastuzumab as the antibody and substituting the GGFG hydrolysable peptide of EP1155702A1 for the DGGF peptide in the linker of the trastuzumab-linker-exatecan conjugate because the '458 teaches that any antibody can be conjugated to treat breast cancer. The substitution of one hydrolysable peptide for another in the art is prima facie obvious as they function equivalently to release the linked drug at the appropriate site. It would further have been prima facie prima facie obvious to conjugate from 3-8 drug molecules per antibody according to the '458 claims and include the resulting conjugate or salt thereof in a pharmaceutical composition or a antitumor and/or anticancer drug containing the conjugate as modified in view of the identical claims in the '458 application.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Claims 1-5, 7 and 9-11 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 13, 14, 20, 21, 22-26 and 30-33 of copending Application No. **14/435,114** in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007). Although the claims at issue are not identical, they are not patentably distinct from each other because:

USSN 14/435,114 claims an antibody-drug conjugate comprising the antitumor compound



wherein the antitumor compound is conjugated to an antibody via a linker having a structure represented by the following formula:

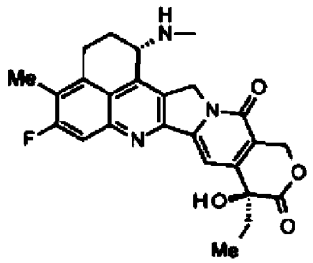
And in claim 13-14:

13. (Currently Amended) The antibody-drug conjugate according to any one of claims 1 to 7 wherein the structure of the linker is one structure selected from the following group:

-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)

wherein -(NH-DX) represents a group represented by the following formula:

[Formula 4]



wherein the nitrogen atom of the amino group at position 1 is the connecting position, and -GGFG- represents a peptide residue of -Gly-Gly-Phe-Gly-.

14. **(Previously Presented) The antibody-drug conjugate according to any one of claims 1 to 6, wherein the structure of the linker is one structure selected from the following group:**

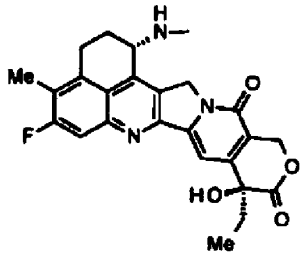
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)

-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)

-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)

wherein -(NH-DX) represents a group represented by the following formula:

[Formula 5]



wherein the nitrogen atom of the amino group at position 1 is the connecting position.

. Claims 20 and 21 recite the identical linker and conjugation site to exatrecan.

The claims also teach that the antibody has the property of recognizing a target cell where the target cell is as tumor cell (see claim 32). The '485 claims also provide for a "drug containing the conjugate" and an antitumor drug and/or anticancer drug comprising the conjugate or salt thereof including breast cancer (see claims 30-33). Claims 22-24 teach that the number of antitumor compounds conjugated per antibody is in the range from 1-10, 1-8, or 3-8.. The claims differ from the instant claims by not reciting that the antibody can be the breast cancer antibody trastuzumab (comprising SEQ ID NO:1 and 2).

Chamberlain et al teach the amino acid sequence of trastuzmab (SEQ ID NOS:11 and 12).

```
US-11-932-151-12

  Query Match             100.0%;  Score 1110;  DB 5;  Length 214;
  Best Local Similarity   100.0%;
  Matches  214;  Conservative    0;  Mismatches    0;  Indels    0;  Gaps    0;

Qy          1 DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPS 60
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Qy         61 RFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPP 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Qy        121 SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        121 SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT 180

Qy        181 LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC 214
              |||||||||||||||||||||||||||||||||
Db        181 LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC 214


  Query Match             100.0%;  Score 2412;  DB 5;  Length 450;
  Best Local Similarity   100.0%;
  Matches  450;  Conservative    0;  Mismatches    0;  Indels    0;  Gaps    0;

Qy          1 EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRY 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db          1 EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRY 60

Qy         61 ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db         61 ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS 120

Qy        121 ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        121 ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS 180

Qy        181 GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGG 240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        181 GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGG 240

Qy        241 PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        241 PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN 300

Qy        301 STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE 360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        301 STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE 360

Qy        361 MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW 420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        361 MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW 420

Qy        421 QQGNVFSCSVMHEALHNHYTQKSLSLSPGK 450
              |||||||||||||||||||||||||||||
Db        421 QQGNVFSCSVMHEALHNHYTQKSLSLSPGK 450
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As to claims 1-5, 7 and 9-11, it would have been prima facie obvious to one having ordinary skill in the art at the time the invention was filed to modify the antibody-exatecan conjugate of claims 13, 14, 20 and 21 having the linker

-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX) of the

'548 application by using the trastuzumab as the antibody because the '114 teaches that any antibody can be conjugated to the cytotoxic exatrecan including those treat breast cancer (claim 32) and trastuzumab was a well established antibody for treatment of breast cancer. It would further have been prima facie prima facie obvious to conjugate from 3-8 drug molecules per antibody according to the '458 claims and include the resulting conjugate or salt thereof in a pharmaceutical composition or a antitumor and/or anticancer drug containing the conjugate as modified in view of the identical claims in the '114 application.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Claims 1-5, 7 and 9-11 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-8 of copending **allowed but not yet issued Application No. 15/187,179** in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007). Although the claims at issue are not identical, they are not patentably distinct from each other because:

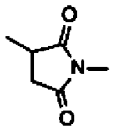
USSN **15/187,179** claims an antibody-drug conjugate comprising the antitumor compound

1. (Currently Amended) An antibody-drug conjugate, wherein a linker and an antitumor compound represented by the following formula and anti-TROP2 antibody are connected:

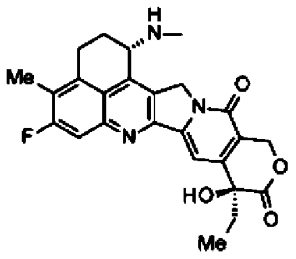
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)

wherein

-(Succinimid-3-yl-N)- has a structure represented by the following formula:



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, and (NH-DX) represents a group represented by the following formula:



wherein the nitrogen atom of the amino group at position 1 is the connecting position,

wherein the anti-TROP2 antibody comprises CDRH1 consisting of the amino acid sequence of SEQ ID NO: 23, CDRH2 consisting of the amino acid sequence of SEQ ID NO: 24 and CDRH3 consisting of the amino acid sequence of SEQ ID NO: 25 in its heavy chain variable region and CDRL1 consisting of the amino acid sequence of SEQ ID NO: 26, CDRL2 consisting of the amino acid sequence of SEQ ID NO: 27 and CDRL3 consisting of the amino acid sequence of SEQ ID NO: 28 in its light chain variable region.

2. (Original) The antibody-drug conjugate according to claim 1, wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 2 to 8.

3. (Original) The antibody-drug conjugate according to claim 1, wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 3 to 8.

4. (Original) A drug containing the antibody-drug conjugate according to claim 1 or a salt thereof.

5. (Original) An antitumor drug and/or anticancer drug containing the antibody-drug conjugate according to claim 1 or a salt thereof.

6. (Original) A method of treating cancer in an individual comprising administering to an individual with cancer the drug according to claim 5, wherein the cancer is lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, gastric cancer, cervical cancer, head and neck cancer, or esophageal cancer.

7. (Original) A pharmaceutical composition containing the antibody-drug conjugate according to claim 1 or a salt thereof as an active component, and a pharmaceutically acceptable formulation component.

8. (Original) A method of treating cancer in an individual comprising administering to an individual with cancer the pharmaceutical composition according to claim 7, wherein the cancer is lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, gastric cancer, cervical cancer, head and neck cancer, or esophageal cancer.

The claims differ by not using trastuzmab in order to treat breast cancer.

Chamberlain et al teach the amino acid sequence of trastuzmab (SEQ ID NOS:11 and 12). Which are identical to SEQ ID NO:1 and 2 recited in the claims.

As to claims 1-5, 7 and 9-11, it would have been prima facie obvious to one having ordinary skill in the art at the time the invention was filed to modify the antibody-exatecan conjugate of the '187 application by substituting the trastuzumab as the antibody for the anti-

TROP2 antibody because the '187 teaches that any antibody conjugate can be conjugated to the cytotoxic exatrecan including those treat breast cancer and trastuzumab was a well established antibody for treatment of breast cancer and as such it would have been prima facie obvious to one having ordinary skill in the art at the time of filing to substitute one breast cancer targeting antibody for another.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented. In view of the notice of allowance mailed 5-18-2017 in the '187 application indicating allowability of the claims therein, should the '203 application issue in the interim, this rejection will be converted to an actual double patenting rejection.

Claims 1-5, 7 and 9-11 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-8 of copending **allowed but not yet issued Application No. 15/180,203** in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007). Although the claims at issue are not identical, they are not patentably distinct from each other because:

USSN **15/180,203** claims an antibody-drug conjugate comprising the antitumor compound:

1. (Original) An antibody-drug conjugate, comprising

a linker that is connected to an antibody, represented by the following formula:

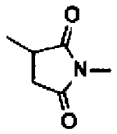
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-

and

an antitumor compound that is connected to the linker,

wherein

-(Succinimid-3-yl-N)- has a structure represented by the following formula:



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker containing this structure on the nitrogen atom at position 1, and

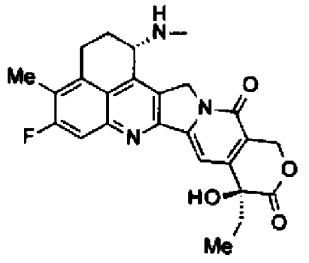
the antitumor compound is connected to the carbon atom of the carbonyl group within the -NH-CH_2-O-CH_2-C(=O)- moiety.

2. (Currently Amended) The antibody-drug conjugate according to claim 1, wherein the linker ~~liker~~ and the antitumor compound are in the following structure:

-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)

wherein

-(NH-DX) represents a group represented by the following formula:



wherein the nitrogen atom of the amino group at position 1 is the connecting position.

3. (Original) The antibody-drug conjugate according to claim 1, wherein an average of 2 to 8 linker and antitumor compound structures are conjugated to the antibody.

4. (Original) The antibody-drug conjugate according to claim 1, wherein an average of 3 to 8 linker and antitumor compound structures are conjugated to the antibody.

5. (Original) The antibody-drug conjugate according to claim 1, wherein the antibody is an anti-B7-H3 antibody, an anti-CD30 antibody, an anti-CD33 antibody, or an anti-CD70 antibody.

6. (Original) The antibody-drug conjugate according to claim 1, wherein the antibody is an anti-B7-H3 antibody.

7. (Original) A drug containing the antibody-drug conjugate according to claim 1, or a salt thereof.

8. (Original) An antitumor drug and/or anticancer drug containing the antibody-drug conjugate according to claim 1, or a salt thereof.

9. (Original) A method of treating cancer in an individual comprising administering to an

individual with cancer the drug according to claim 8, wherein the cancer is lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer.

10. (Original) A pharmaceutical composition containing the antibody-drug conjugate according to claim 1, or a salt thereof as an active component, and a pharmaceutically acceptable formulation component.

11. (Original) The antibody-drug conjugate according to claim 2, wherein an average of 2 to 8 linker and antitumor compound structures are conjugated to the antibody.

12. (Original) The antibody-drug conjugate according to claim 2, wherein an average of 3 to 8 linker and antitumor compound structures are conjugated to the antibody.

13. (Original) The antibody-drug conjugate according to claim 2, wherein the antibody is an anti-B7-H3 antibody, an anti-CD30 antibody, an anti-CD33 antibody, or an anti-CD70 antibody.

14. (Original) The antibody-drug conjugate according to claim 2, wherein the antibody is an anti-B7-H3 antibody.

15. (Original) A drug containing the antibody-drug conjugate according to claim 2, or a salt thereof.

16. (Original) An antitumor drug and/or anticancer drug containing the antibody-drug conjugate according to claim 2, or a salt thereof.

The claims differ by not using trastuzmab in order to treat breast cancer.

Chamberlain et al teach the amino acid sequence of trastuzmab (SEQ ID NOS:11 and 12). Which are identical to SEQ ID NO:1 and 2 recited in the claims.

As to claims 1-5, 7 and 9-11, it would have been prima facie obvious to one having ordinary skill in the art at the time the invention was filed to modify the antibody-exatecan conjugate of the '203 application by using trastuzumab as the antibody in the conjugate

because the '203 teaches that any antibody conjugate can be conjugated to the cytotoxic exatrecan including those treat breast cancer and trastuzumab was a well established antibody for treatment of breast cancer and as such it would have been prima facie obvious to one having ordinary skill in the art at the time of filing to substitute one breast cancer targeting antibody for another.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented. **In view of the notice of allowance mailed 5-23-2017 in the '203 application indicating allowability of the claims therein, should the '203 application issue in the interim, this rejection will be converted to an actual double patenting rejection.**

Claims 1-5, 7 and 9-11 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-16 of copending 15/285,156 in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007). Although the claims at issue are not identical, they are not patentably distinct from each other because:

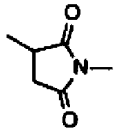
USSN 15/285,156 claims an antibody-drug conjugate comprising the antitumor compound:

1. (Currently Amended) An antibody-drug conjugate, wherein a linker and an antitumor compound represented by the following formula and anti-HER3 antibody are connected:

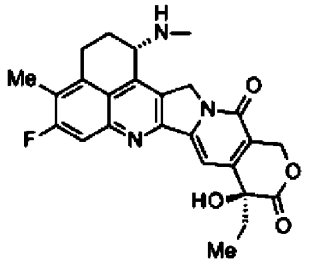
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG **(SEQ ID NO: 585)**-NH-CH_2-O-CH_2-C(=O)-(NH-DX)

wherein

-(Succinimid-3-yl-N)- has a structure represented by the following formula:



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, and (NH-DX) represents a group represented by the following formula:



wherein the nitrogen atom of the amino group at position 1 is the connecting position.

2. (Original) The antibody-drug conjugate according to claim 1, wherein an average number of units of the drug-linker structure conjugated per antibody is in a range of from 2 to 8.

3. (Original) The antibody-drug conjugate according to claim 1, wherein an average number of units of the drug-linker structure conjugated per antibody is in a range of from 3 to 8.

4. (Original) A drug containing the antibody-drug conjugate according to claim 1 or a salt thereof.

5. (Original) An antitumor drug and/or anticancer drug containing the antibody-drug conjugate according to claim 1 or a salt thereof.

6. (Original) A method of treating cancer in an individual comprising administering to an individual with cancer the drug according to claim 5, wherein the cancer is lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, gastric cancer, gastrointestinal stromal tumor, cervical cancer, head and neck cancer, esophageal cancer, epidermoid cancer, peritoneal cancer, adult glioblastoma multiforme, hepatic cancer, hepatocellular carcinoma, colon cancer, rectal cancer, colon and rectal cancer, endometrial cancer, uterus cancer, salivary cancer, renal cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anus carcinoma, or penis cancer.

7. (Original) A pharmaceutical composition containing the antibody-drug conjugate according to claim 1 or a salt thereof as an active component, and a pharmaceutically acceptable formulation component.

8. (Original) A method of treating cancer in an individual comprising administering to an individual with cancer the pharmaceutical composition according to claim 7, wherein the cancer is lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver

The claims differ by not using trastuzmab that binds HER2 in order to treat breast cancer.

Chamberlain et al teach the amino acid sequence of trastuzmab (SEQ ID NOS:11 and 12). Which are identical to SEQ ID NO:1 and 2 recited in the claims.

As to claims 1-5, 7 and 9-11, it would have been prima facie obvious to one having ordinary skill in the art at the time the invention was filed to modify the antibody-exatecan conjugate of the '156 application by substituting the anti-HER2 trastuzumab antibody for the anti-HER3 antibody in the conjugate because the '156 teaches that the HER3 antibody conjugate can be conjugated to the cytotoxic exatrecan to treat cancer including breast cancer and it would have been prima facie obvious at the time of invention to substitute the anti-breast cancer antibody, trastuzumab as it was a well established antibody for treatment of breast cancer and as such it would have been prima facie obvious to one having ordinary skill in the art at the time of filing to substitute one breast cancer targeting antibody for another.

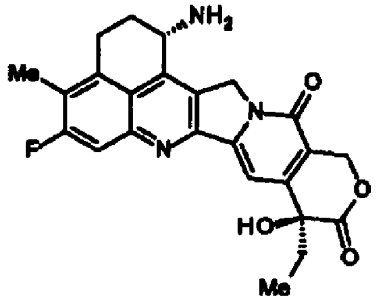
This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Claims 1-5, 7 and 9-11 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1, 2, 5, 18, 19 and 21-28 of copending 15/302,803 optionally in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007). Although the claims at issue are not identical, they are not patentably distinct from each other because:

The '803 application claims an antibody-drug conjugate where the drug is exatecan. Claim 1 recites the following formula:

1. (Original) An antibody-drug conjugate wherein an antitumor compound represented by the following formula:

[Formula 1]



is conjugated to an anti-HER2 antibody via a linker having a structure represented by the following formula:

$-L^1-L^2-L^P-NH-(CH_2)n^1-L^a-L^b-L^c-$ or $-L^1-L^2-L^P-$

wherein

the anti-HER2 antibody is connected to the terminal L^1, the antitumor compound is connected to the terminal L^c or L^P with the nitrogen atom of the amino group at position 1 as the connecting position,

wherein

n^1 represents an integer of 0 to 6,

L^1 represents -(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)-, -(Succinimid-3-yl-N)-CH[-$(CH_2)n^3$-COOH]-C(=O)-, -(Succinimid-3-yl-N)-CH_2CH_2-[N(-CH_2-COOH)]-CH_2-(C=O)-, -CH_2-C(=O)-NH-$(CH_2)n^4$-C(=O)-, -C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-, or -C(=O)-$(CH_2)n^5$-C(=O)-,

wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8, and n^5 represents an integer of 1 to 8,

L^2 represents -NH-$(CH_2CH_2-O)n^6$-CH_2CH_2-C(=O)-, -N[-$(CH_2CH_2-O)n^7$-CH_2CH_2-OH]-CH_2-C(=O)-, -NH-CH_2CH_2-[N(-CH_2-COOH)]-CH_2-(C=O)-, -NH-[CH(-CH_2-COOH)]-CH_2-(C=O)-, -NH-[CH(-CH_2-COOH)]-CH_2-O-CH_2-(C=O)-, -S-$(CH_2)n^8$-C(=O)-, or a single bond,

wherein n^6 represents an integer of 0 to 6, n^7 represents an integer of 1 to 4, and n^8 represents an integer of 1 to 6,

L^p represents a peptide residue consisting of 2 to 8 amino acids,

L^a represents -C(=O)-NH-, -NR^1-$(CH_2)n^9$-, -O-, or a single bond,

wherein n^9 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -$(CH_2)n^a$-COOH, or -$(CH_2)n^b$-OH, n^a represents an integer of 1 to 4, and n^b represents an integer of 1 to 6,

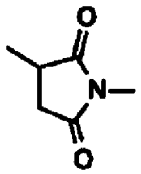
L^b represents -CR^2(-R^3)-, -O-, -NR^4-, or a single bond,

wherein R^2 and R^3 each independently represent a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -$(CH_2)n^c$-NH_2, -$(CH_2)n^d$-COOH, or -$(CH_2)n^e$-OH, R^4 represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^c represents an integer of 0 to 6, n^d represents an integer of 1 to 4, n^e represents an integer of 1 to 4, and when n^c is 0, R^2 and R^3 are not the same,

L^c represents -CH_2- or -C(=O)-,

-(Succinimid-3-yl-N)- has a structure represented by the following formula:

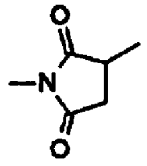
[Formula 2]



which is connected to the antibody at position 3 thereof and is connected to the methylene group in the linker structure containing this structure on the nitrogen atom at position 1,

-(N-ly-3-diminiccuS)- has a structure represented by the following formula:

[Formula 3]



which is connected to L^2 at position 3 thereof and is connected to the methylene group in the linker structure containing this structure on the nitrogen atom at position 1,

cyc.Hex(1,4) represents a 1,4-cyclohexylene group, and

when L^2 is -S-$(CH_2)n^8$-C(=O)-, L^1 is -C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-.

3. (Original) The antibody-drug conjugate according to claim 2, wherein L^c is -C(=O)-.

5. (Previously Presented) The antibody-drug conjugate according to claim 3, wherein -NH-$(CH_2)n^1$-L^a-L^b- in the linker is a structure selected from the following group:

-NH-CH_2-,

-NH-CH_2CH_2-,

-NH-$CH_2CH_2CH_2$-,

-NH-CH_2-O-CH_2-, and

-NH-CH_2CH_2-O-CH_2-.

7. (Previously Presented) The antibody-drug conjugate according to claim 1, wherein L^1 is -(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)-, and L^2 is a single bond or is -NH-$(CH_2CH_2$-O$)n^6$-CH_2CH_2-C(=O)-, -N[-$(CH_2CH_2$-O$)n^7$-CH_2CH_2-OH]-CH_2-C(=O)-, -NH-[CH(-CH_2-COOH)]-CH_2-(C=O)-, -NH-CH_2CH_2-[N(-CH_2-COOH)]-CH_2-(C=O)-, or -NH-[CH(-CH_2-COOH)]-CH_2-O-CH_2-(C=O)-.

19. (Currently Amended) The antibody-drug conjugate according to claim 2, wherein L^P is GGFG **(SEQ ID NO: 3)** or DGGFG **(SEQ ID NO: 10)**.

Claims 21-28 recite salts, pharmaceutical and drug compositions comprising the conjugate.

As to claims 1-5 and 7, it would have been prima facie obvious at the time the invention was filed to select the conjugate of claim or claim 2 of the '803 claims having L1 L^1 is -(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)-, and L^2 is a single bond 'according to claim 7 where n2=5, Lp is GGFG according to claim 18 or 19, LaLb is $-NH-CH_2-O-CH_2-$, according to claim 5, Lc C(=O)-. according to claim 3 and connected to the antibody at position 3 and the methylene group in the linker structure at position 1 according to claim 1 to arrive at the claimed conjugate according to claims 1-5 and 7 of the instant application as the selection of specifically called out functionally equivalent variations of a generic formula is prima facie obvious. All the structures of the claimed invention are set forth in the claims, but not found in any specific claimed embodiment, one of ordinary skill in the art is able to envisage the claimed combination from the sufficiently limited and well defined embodiments called out in the claims of the '803 application. The invention as a whole is prima facie obvious at the time of filing.

Chamberlain et al teach the amino acid sequence of trastuzmab (SEQ ID NOS:11 and 12). Which are identical to SEQ ID NO:1 and 2 recited in the claims (see alignments above).

As to claims 1-5, 7, and 9-11 it would have been prima facie obvious to the skilled artisan at the time of filing to substitute the anti-HER2 monoclonal antibody trastuzmab for the generic anti-HER2 antibody in the conjugate as modified above because the substitution of one functionally equivalent is prima facie obvious.

Claim Rejections - 35 USC § 103

It is noted that PCT/JP2015/000355, and Foreign priority applications filed 1/31/2014, 8/22/2014 and 11/10/2014 are all in Japanese and no english translations have been filed to establish priority. As such, the instant filing date 7-28-2016 is the priority date for prior art purposes.

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

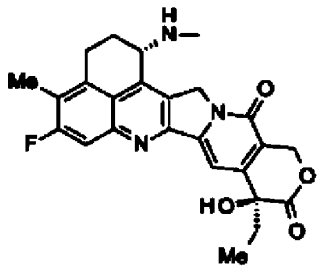
The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 1-5, 7 and 9-11 are rejected under 35 U.S.C. 103 as being unpatentable over WO 2014/061277 published 4-2-2014 hereinafter references as '277; English language equivalent of specification and claims provided from European family member in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007).

The '277 WIPO document teaches and claims;

Antibody drug conjugates of exatrecan. Exatrecan is the drug of the instant claims. See for example claim 39 of the '277 document.



wherein the nitrogen atom of the amino group at position 1 is the connecting position.

Claim 39 of the '458 application also teaches that the linker can be

-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-DGGF (SEQ ID NO. 34)-NH-CH_2-O-CH_2-C(=O)-(NH-DX),

. The '277 publication also indicates that the peptide in the linker (Lp) can be GGFG (see claim10). The '277 claims also teach that the antibody has the property of recognizing a target cell where the target cell is as tumor cell (see claim 44). The '277 claims also provide for a "drug containing the conjugate" and an antitumor drug and/or anticancer drug comprising the conjugate or salt thereof including breast cancer (see claims 48-50). Claims 40-42 teach that the number of antitumor compounds conjugated per antibody is in the range from 1-10, 1-8, or 3-8. The claims differ from the instant claims by not reciting that the antibody can be the breast

cancer anti-HER2 antibody and the anti-HER2 antibody trastuzumab (comprising SEQ ID NO:1 and 2) in particular. The '277 publication teaches that the antibody drug conjugate can contain a salt or hydrate thereof see paragraphs 76-78 of translation. The '277 publication teaches that the conjugates can be used to treat breast cancer at paragraph 78. The '277 publication differs by not teaching an anti-HER-2 antibody and trastuzmab in particular and does not exemplify the specifically claimed linker of claims 1-5, 7 and 9-11.

Chamberlain et al teach the amino acid sequence of the anti-HER2 antibody, trastuzmab (SEQ ID NOS:11 and 12) which are 100% identical as compared to SEQ ID NO:1 and 2 of the instant claims..

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US-11-932-151-12

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  Best Local Similarity   100.0%;
  Matches  214;  Conservative    0;  Mismatches    0;  Indels    0;  Gaps    0;

Qy          1 DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPS 60
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Qy         61 RFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPP 120
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              |||||||||||||||||||||||||||||||||
Db        181 LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC 214
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  Best Local Similarity   100.0%;
  Matches  450;  Conservative    0;  Mismatches    0;  Indels    0;  Gaps    0;

Qy          1 EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRY 60
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              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Qy        181 GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGG 240
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Qy        241 PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        241 PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN 300

Qy        301 STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE 360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        301 STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE 360

Qy        361 MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW 420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        361 MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW 420

Qy        421 QQGNVFSCSVMHEALHNHYTQKSLSLSPGK 450
              |||||||||||||||||||||||||||||
Db        421 QQGNVFSCSVMHEALHNHYTQKSLSLSPGK 450
```

As to claims 1-5, 7 and 9-11, it would have been prima facie obvious to one having ordinary skill in the art at the time the invention was filed to modify the antibody-exatecan conjugate of claim 39 having the linker

-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-DGGF (SEQ ID NO. 34)-NH-CH_2-O-CH_2-C(=O)-(NH-DX),

of the '277 publication by using the trastuzumab as the antibody for targeting breast cancer and substituting the GGFG hydrolysable peptide of claim 10 for DGGF in the linker of claim 39 of the '277 publication in the linker of the trastuzumab-linker-exatecan conjugate as modified above because the '277 publication teaches that antibody-linker-drug conjugates are useful for treating breast cancer and antibodies targeting breast cancer can be conjugated in order to treat breast cancer. The substitution of one disclosed hydrolysable peptide for another in the linker in this art is *prima facie* obvious as they function equivalently to release the linked drug at the

appropriate site according to the '277 disclosure. It would further have been prima facie prima facie obvious to conjugate from 3-8 exatecan drug molecules per antibody according to the '277 disclosure/claims and include the resulting conjugate or salt thereof in a pharmaceutical composition(s) or a antitumor and/or anticancer drug containing the conjugate as modified in view of the identical claims in the '277 publication as suitable for antibody-linker-drug conjugates.

Claims 1-5, 7 and 9-11 stand rejected. Claims 6 and 8 are withdrawn from consideration.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to PATRICIA DUFFY whose telephone number is (571)272-0855. The examiner can normally be reached on 7:30 am-4 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Nickol can be reached on 571-272-0835. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/PATRICIA DUFFY/
Primary Examiner, Art Unit 1645

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/821,662	11/22/2017	Toshinori AGATSUMA	111119-0119	2559

22428 7590 01/17/2018

Foley & Lardner LLP
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SUITE 600
WASHINGTON, DISTRICT OF COLUMBIA 20007-5109

EXAMINER	
AKHOON, KAUSER M	

ART UNIT	PAPER NUMBER
1642	

NOTIFICATION DATE	DELIVERY MODE
01/17/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipdocketing@foley.com

Office Action Summary	Application No. 15/821,662	Applicant(s) AGATSUMA et al.	
	Examiner KAUSER M AKHOON	Art Unit 1642	AIA Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 11/22/2017 .

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____ .

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-8 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-8 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 11/22/2017.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Claims 1-8 are currently pending.

Priority

Acknowledgment is made that this application is a continuation application of 15/187,179 (US patent 9,850,312), filed June 20, 2016, 371 National Stage Entry of International Application PCT/JP14/006421, filed December 24, 2014, which claims priority to Japanese Patent Application No. 2013-267548, filed December 25, 2013.

Information Disclosure Statement

Applicant's Informational Disclosure Statement, filed on 11/22/2017 has been considered. The signed and initialed PTO 1449 has been mailed with this action.

Claim Rejections - 35 USC § 103

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which

the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

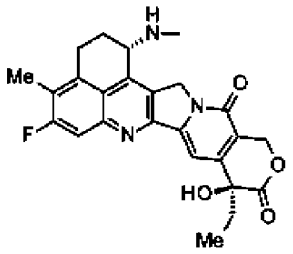
3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

Claims 1-8 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Susaki et al. ("Susaki", US 7,041,818, cited in IDS submitted 11/22/2017) in view of Govidan et al. ("Govidan", US2011/0293513, cited in IDS submitted 11/22/2017) and Flygare et al. ("Flygare", US2012/0121615, cited in IDS submitted 11/22/2017).

Susaki teaches drug delivery systems (drug complexes) in which DX-8951 (exatecan,



Formula I ((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)) is linked to a GGFG tetrapeptide. (Example 2, column 19, lines 50-60). Susaki teaches the drug bound by means of a spacer comprising GGFG tetrapeptide (column 6, lines 1-15). Susaki teaches that the tetrapeptide GGFG as having a high releasing rate (Table 1, column 10, line 8). Susaki teaches that the linker may be –O-$(CH_2)_n$-NH- where n is 1-16 (column 11, lines 48-52). Susaki teaches the use of the drug complex to treat liver cancer (claim 18, column 5, lines 1-8). However, Susaki does not teach the antibody drug conjugate with anti-TROP2 antibody and the linkers as instantly claimed.

Flygare teaches antibody drug conjugates using thiol reactive electrophilic functional group, maleimidocaproyl attached to an amino acid spacer attached to a drug moiety (maytansinoid) (page 16, paragraph [0144-0145] and Figure 1a).

Govidan teaches antibody drug conjugates having hRS7 which is an anti-TROP2 antibody with a drug (page16, paragraph [0102] and Table 2, page 35).

Therefore, it would have been obvious to one having ordinary skill in the art at the time of the instant invention to have prepared the antibody drug conjugate using the Susaki exatecan linked to GGFG tetrapeptide compositions with maleimidocaproyl taught by Flygare with the anti-TROP2 antibody of Govidan with a reasonable expectation of success. One having ordinary

skill in the art would have been motivated to combine the teaching of Susaki, Flygare and Govidan because Susaki teaches that the GGFG tetrapeptide has a high releasing rate (Table 1, column 10, line 8) and Flygare teaches that maleimidocaproyl group is thio reactive group for conjugating antibodies to the drug.

Although Flygare, Govidan and Susaki lists all of the linkers required by the claim, all of the possibilities identified in Flygare, Govidan and Susaki are not found in any specific embodiment, one of ordinary skill in the art is able to "envisage" the claimed combination comprising the antibody drug conjugate from sufficiently limited or well delineated examples, (see MPEP 2131.02). Thereby meeting the limitations of claims 1 and 4-5.

Regarding claims 2 and 3, Govidan teaches that hRS7 antibody conjugates typically 5 to 7 drugs per antibodies (Table 2, page 35, paragraph [0195]).

Regarding claims 6 and 8, Susaki teaches the use of the drug complex to treat liver cancer (claim 18, column 5, lines 1-8).

Regarding claim 7, Susaki teaches pharmaceutical preparations (column 15, lines 25-36).

Thus, given the teachings of the prior art and level of a person of ordinary skill in the art at the time of Applicant's invention one would have had a reasonable expectation of success in practicing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references.

In the absence of any factual showing or unexpected results the presently claimed compositions are considered *prima facie* obvious over the prior art for the reasons stated above. In light of the forgoing discussion, the subject matter defined by the claims would have been obvious within the meaning of 35 USC § 103(a).

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Application No. 14/435,114

Claims 1-8, are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 6, 8-14, 22-35 and 39-55 of copending Application No. 14/435,114 in view of Govidan et al. ("Govidan", US2011/0293513).

Copending claims composition encompasses the same drug, overlapping linker system and antibodies, however do not teach anti-TROP2 antibody. Govidan teaches antibody drug conjugates having hRS7 which is an anti-TROP2 antibody with a drug (page16, paragraph [0102] and Table 2, page 35).

Therefore, it would have been obvious to have prepared the copending antibody drug conjugate using the anti-TROP2 antibody of Govidan with a reasonable expectation of success.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Application No. 14/436,458, issued US 9,872,924

Claims 1-8, are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-47 of US Patent 9,872,924 in view of Govidan et al. ("Govidan", US2011/0293513).

Patented claims composition encompasses the same drug, overlapping linker system and antibodies, however do not teach anti-TROP2 antibody. Govidan teaches antibody drug conjugates having hRS7 which is an anti-TROP2 antibody with a drug (page16, paragraph [0102] and Table 2, page 35).

Therefore, it would have been obvious to have prepared the copending antibody drug conjugate using the anti-TROP2 antibody of Govidan with a reasonable expectation of success.

Application No. 15/180,203, issued US 9,808,537

Claims 1-8 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-18 of US Patent 9,808,537 in view of Govidan et al. ("Govidan", US2011/0293513).

Patented claims composition encompasses the same drug, overlapping linker system and antibodies, however do not teach anti-TROP2 antibody. Govidan teaches antibody drug conjugates having hRS7 which is an anti-TROP2 antibody with a drug (page16, paragraph [0102] and Table 2, page 35).

Therefore, it would have been obvious to have prepared the copending antibody drug conjugate using the anti-TROP2 antibody of Govidan with a reasonable expectation of success.

Application No. 15/187,179, issued US 9,850,312

Claims 1-8 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-18 of US Patent 9,850,312.

Patented claims are drawn to composition encompassing the same drug, overlapping same linker system and antibodies. Patented claims anticipate instant claims.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to KAUSER M AKHOON whose telephone number is (571)270-1026. The examiner can normally be reached on Monday-Friday IFP 8:00-:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Misook Yu can be reached on 571-272-0839. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/K. A./
Examiner, Art Unit 1642

/MARK HALVORSON/
Primary Examiner, Art Unit 1642

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NOTICE OF ALLOWANCE AND FEE(S) DUE

22428 7590 06/13/2018

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WASHINGTON, DISTRICT OF COLUMBIA 20007-5109
UNITED STATES OF AMERICA

EXAMINER	
DUFFY, PATRICIA ANN	
ART UNIT	PAPER NUMBER
1645	

DATE MAILED: 06/13/2018

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/221,851	07/28/2016	Hiroyuki NAITO	111119-0105	3964

TITLE OF INVENTION: ANTI-HER2 ANTIBODY-DRUG CONJUGATE

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	09/13/2018

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

22428 7590 06/13/2018
Foley & Lardner LLP
3000 K STREET N.W.
SUITE 600
WASHINGTON, DISTRICT OF COLUMBIA 20007-5109
UNITED STATES OF AMERICA

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/221,851	07/28/2016	Hiroyuki NAITO	111119-0105	3964

TITLE OF INVENTION: ANTI-HER2 ANTIBODY-DRUG CONJUGATE

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	09/13/2018

EXAMINER	ART UNIT	CLASS-SUBCLASS
DUFFY, PATRICIA ANN	1645	424-181100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/221,851	07/28/2016	Hiroyuki NAITO	111119-0105	3964

22428 7590 06/13/2018

Foley & Lardner LLP
3000 K STREET N.W.
SUITE 600
WASHINGTON, DISTRICT OF COLUMBIA 20007-5109
UNITED STATES OF AMERICA

EXAMINER	
DUFFY, PATRICIA ANN	
ART UNIT	PAPER NUMBER
1645	

DATE MAILED: 06/13/2018

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

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OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	**Application No.** 15/221,851	**Applicant(s)** NAITO et al.	
	Examiner Patricia Duffy	**Art Unit** 1645	**AIA Status** Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to 5-29-2018.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 1-4,7 and 9-11 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information , please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file areply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/PATRICIA DUFFY/
Primary Examiner, Art Unit 1645

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

22428 7590 11/02/2018

Foley & Lardner LLP
3000 K STREET N.W.
SUITE 600
WASHINGTON, DC 20007-5109

EXAMINER	
AKHOON, KAUSER M	
ART UNIT	PAPER NUMBER
1642	

DATE MAILED: 11/02/2018

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/821,662	11/22/2017	Toshinori AGATSUMA	111119-0119	2559

TITLE OF INVENTION: ANTI-TROP2 ANTIBODY-DRUG CONJUGATE

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	02/04/2019

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

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IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

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CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

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SUITE 600
WASHINGTON, DC 20007-5109

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/821,662	11/22/2017	Toshinori AGATSUMA	111119-0119	2559

TITLE OF INVENTION: ANTI-TROP2 ANTIBODY-DRUG CONJUGATE

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	02/04/2019

EXAMINER	ART UNIT	CLASS-SUBCLASS
AKHOON, KAUSER M	1642	424-181100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1_____
2_____
3_____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑Issue Fee ❑Publication Fee (if required) ❑Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

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Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/821,662	11/22/2017	Toshinori AGATSUMA	111119-0119	2559

22428 7590 11/02/2018

Foley & Lardner LLP
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WASHINGTON, DC 20007-5109

EXAMINER	
AKHOON, KAUSER M	
ART UNIT	PAPER NUMBER
1642	

DATE MAILED: 11/02/2018

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

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OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

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Notice of Allowability	**Application No.** 15/821,662	**Applicant(s)** AGATSUMA et al.	
	Examiner KAUSER M AKHOON	**Art Unit** 1642	**AIA Status** Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

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   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

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4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

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   1. ☐ Certified copies of the priority documents have been received.
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Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file areply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
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5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
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6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
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/K.M.A/ Examiner, Art Unit 1642	/MISOOK YU/ Supervisory Patent Examiner, Art Unit 1642

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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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Keywords DE-310 · Macromolecular prodrug
DX-8951 · Cellular uptake · Drug release · Drug delivery system

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

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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).

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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.

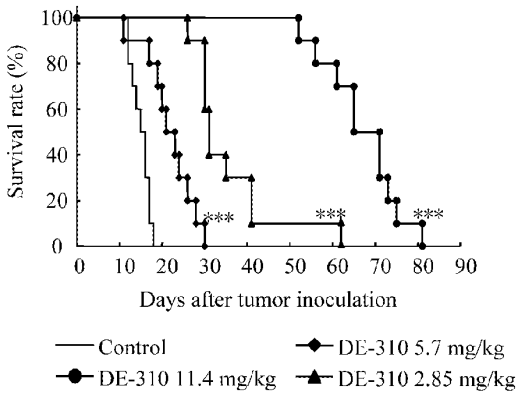


Fig. 1 In vivo life-span-increasing effects of DE-310 in Meth A-bearing mice ($n=10$). Mice inoculated i.p. with Meth A cells (1×10^6 cells) were treated i.v. with the doses of DE-310 shown in the figure beginning on day 1. Survival rate (%) = (number of mice that survived on day n/number of mice used)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

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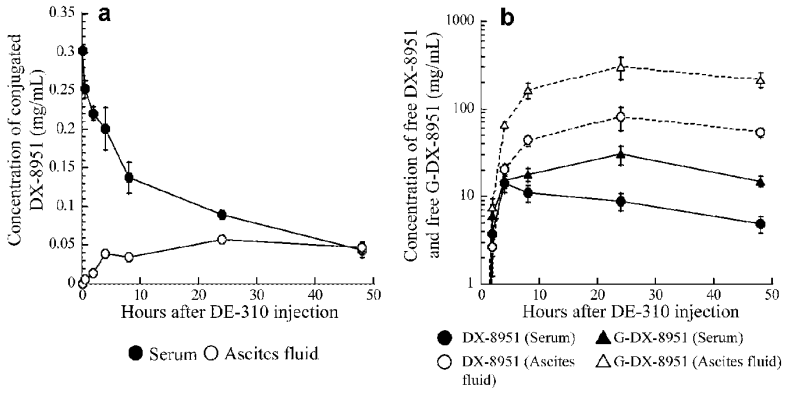


Fig. 2 The concentrations of conjugated DX-8951 (**a**), and free DX-8951 and free G-DX-8951 (**b**) after the i.v. injection of DE-310 into Meth A-bearing mice. Mice received DE-310 at 11.4 mg/kg (DX-8951 equivalents) on day 5 after Meth A cell injection. Each point represents the mean ± standard deviation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

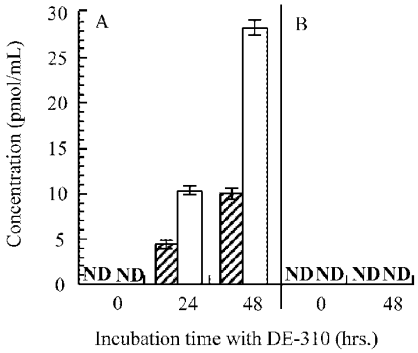


Fig. 3 The concentrations of free DX-8951 (*hatched bars*) and free G-DX-8951 (*open bars*) in samples from ascites cells of Meth A cells (**a**) and ascitic supernatant (**b**) incubated with 10 μg/ml of DE-310. The dose of DE-310 represents DX-8951 equivalents. Each column represents the mean ± standard 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

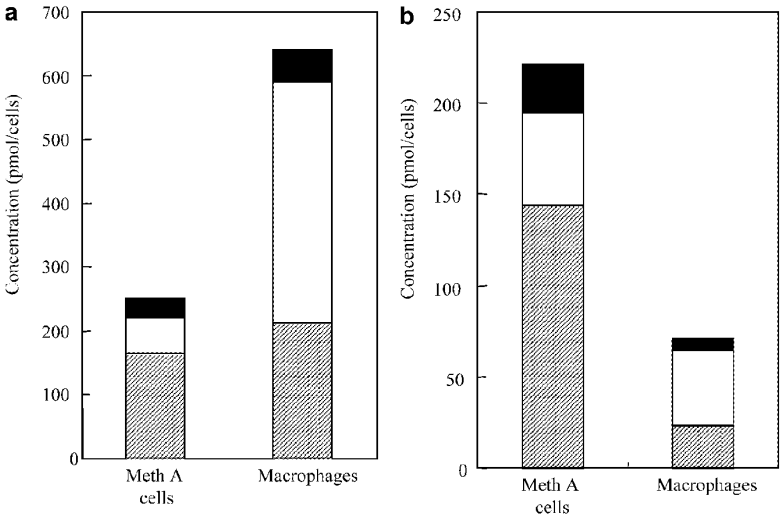


Fig. 4 The concentrations of free DX-8951, free G-DX-8951 and conjugated DX-8951 in Meth A cells and macrophages obtained from Meth A-bearing mice. The concentrations of free DX-8951 (*black*), free G-DX-8951 (*white*), and conjugated DX-8951 (*hatched*) were compared in the same number of cells (1×10^7 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

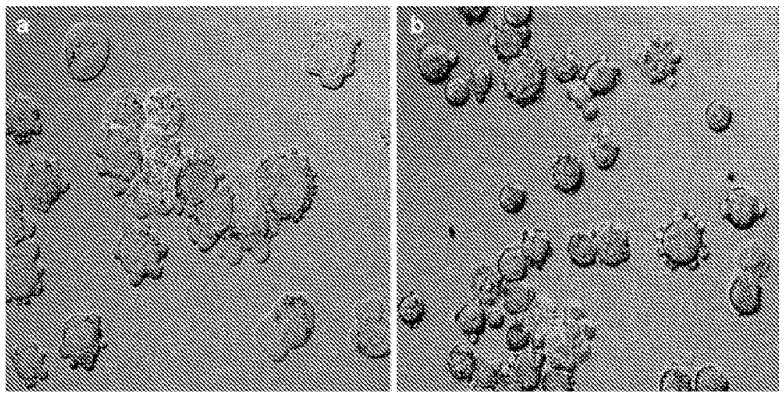


Fig. 5 CLSM images of ascites cells obtained from Meth A ascites-bearing mice treated i.p. with DE-310 (**a**) or CM-Dex-PA-DX-8951 (**b**). Composite images produced by projecting fluorescence derived from DX-8951 (*blue*) and FITC-labeled CD11b antibody for the membrane antigen of macrophages (*green*) 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

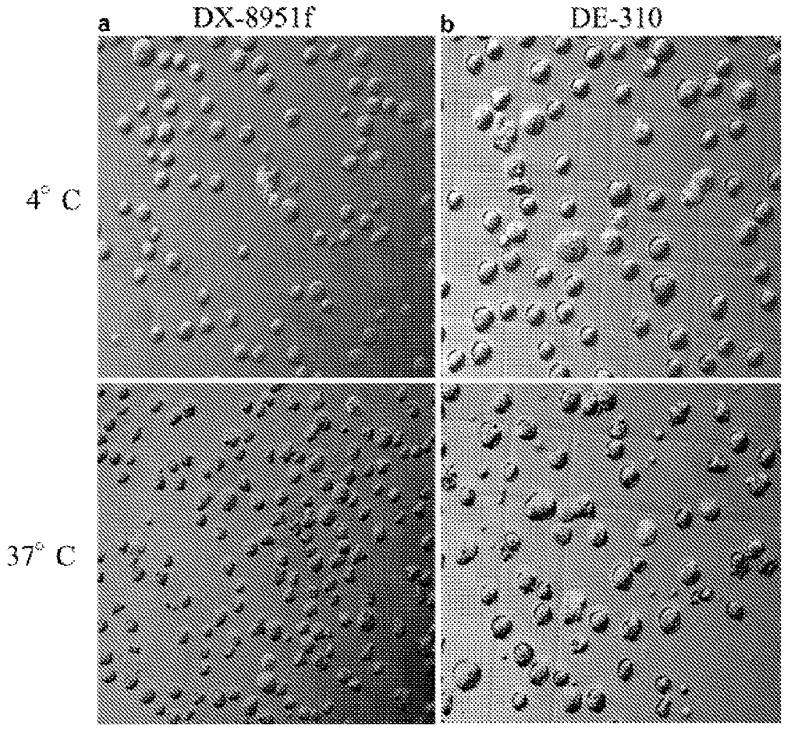


Fig. 6 CLSM images of Meth A ascites cells incubated in vitro with DX-8951f or DE-310. Composite images produced by projecting fluorescence derived from DX-8951 (*blue*) and FITC-labeled CD11b antibody (*green*) onto the differential interference image of ascites cells. Cells were incubated with DX-8951f (**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

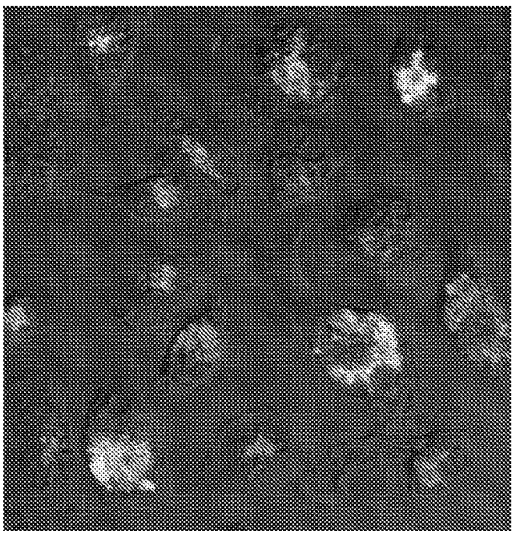


Fig. 7 CLSM images of macrophages incubated with DE-310 and a lysosome marker (Lysotracker Red DND-99). Composite images produced by projecting fluorescence derived from DX-8951 (*green*) and lysosome marker (*red*) onto the differential interference images of macrophages. Yellow color merged with DX-8951 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 semisynthesized 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

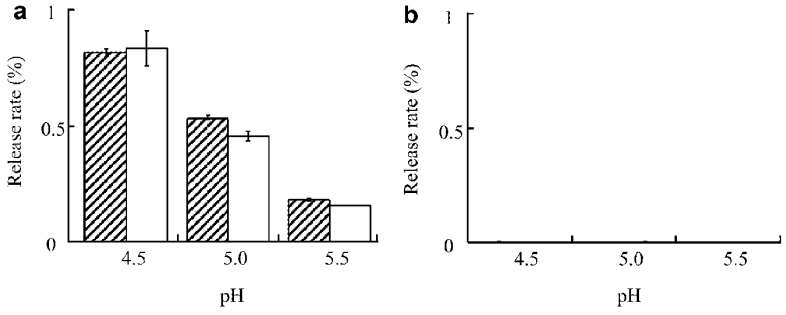


Fig. 8 Release rates of DX-8951 (*hatched bars*) and G-DX-8951 (*open bars*) from DE-310 in the presence of tissue homogenate at 40°C for 20 h without (**a**) or with E-64 (**b**) (final concentration 0.5 μ*M*)

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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.

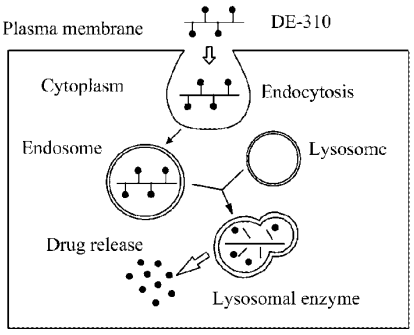


Fig. 9 Proposed drug-release mechanisms for DE-310 in cells

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特 許 協 力 条 約

PCT

国際調査報告

（法８条、法施行規則第40、41条）
〔ＰＣＴ18条、ＰＣＴ規則43、44〕

出願人又は代理人の書類記号 PD20-9018WO	今後の手続については、様式ＰＣＴ／ＩＳＡ／２２０及び下記５を参照すること。	
国際出願番号 ＰＣＴ／ＪＰ２０１７／０３６２１５	国際出願日 (日.月.年) ０５．１０．２０１７	優先日 (日.月.年) ０７．１０．２０１６
出願人（氏名又は名称） 第一三共株式会社		

国際調査機関が作成したこの国際調査報告を法施行規則第41条（ＰＣＴ18条）の規定に従い出願人に送付する。
この写しは国際事務局にも送付される。

この国際調査報告は、全部で 5 ページである。

☐ この国際調査報告に引用された先行技術文献の写しも添付されている。

1．国際調査報告の基礎
a．言語に関し、この国際調査は以下のものに基づき行った。
☑ 出願時の言語による国際出願
☐ 出願時の言語から国際調査のための言語である＿＿＿＿＿＿＿＿ 語に翻訳された、この国際出願の翻訳文（ＰＣＴ規則12.3(a)及び23.1(b)）

b．☐ この国際調査報告は、ＰＣＴ規則91の規定により国際調査機関が許可した又は国際調査機関に通知された明らかな誤りの訂正を考慮して作成した（ＰＣＴ規則43.6の2(a)）。

c．☑ この国際出願は、ヌクレオチド又はアミノ酸配列を含んでいる（第Ｉ欄参照）。

2．☐ 請求の範囲の一部の調査ができない（第Ⅱ欄参照）。

3．☐ 発明の単一性が欠如している（第Ⅲ欄参照）。

4．発明の名称は
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5．要約は
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☐ 第Ⅳ欄に示されているように、法施行規則第47条第１項（ＰＣＴ規則38.2）の規定により国際調査機関が作成した。出願人は、この国際調査報告の発送の日から１月以内にこの国際調査機関に意見を提出することができる。

6．図面に関して
a．要約とともに公表される図は、
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国際調査報告

国際出願番号 ＰＣＴ／ＪＰ２０１７／０３６２１５

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１．この国際出願で開示されたヌクレオチド又はアミノ酸配列に関して、以下の配列表に基づき国際調査を行った。

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☑　附属書C/ST.25テキストファイル形式
☐　紙形式又はイメージファイル形式

ｂ．☐　国際出願とともに、ＰＣＴ規則13の3.1(a)に基づき国際調査のためにのみ提出された、附属書C/ST.25テキストファイル形式の配列表

ｃ．☐　国際出願日後に、国際調査のためにのみ提出された配列表
☐　附属書C/ST.25テキストファイル形式（ＰＣＴ規則13の3.1(a)）
☐　紙形式又はイメージファイル形式（ＰＣＴ規則13の3.1(b)及びＰＣＴ実施細則第713号）

２．☐　さらに、複数の版の配列表又は配列表の写しが提出され、変更後の配列表又は追加の写しに記載された情報が、出願時における配列表と同一である旨、又は出願時における国際出願の開示の範囲を超えない旨の陳述書の提出があった。

３．補足意見：

国際調査報告

国際出願番号 PCT／JP2017／036215

A. 発明の属する分野の分類（国際特許分類（IPC））

Int.Cl. A61K39/395(2006.01)i, A61K47/68(2017.01)i, A61P35/00(2006.01)i

B. 調査を行った分野

調査を行った最小限資料（国際特許分類（IPC））

Int.Cl. A61K39/395, A61K47/68, A61P35/00

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報	1922－1996年
日本国公開実用新案公報	1971－2017年
日本国実用新案登録公報	1996－2017年
日本国登録実用新案公報	1994－2017年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

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C. 関連すると認められる文献

引用文献のカテゴリー＊	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
X	OGITANI, Y. et al., DS-8201a, A Novel HER2-Targeting ADC with a Novel DNA Topoisomerase I Inhibitor, Demonstrates a Promising Antitumor Efficacy with Differentiation from T-DM1, Clinical Cancer Research, 2016.03, Vol.22, p.5097-5108, ISSN 1078-0432, 特にAbstract, p.5098 左欄第2段落, p.5103 右欄第2段落-p.5105 左欄第1段落, p.5106 左欄第2-3段落, Figures 4,5	1-72

☑ C欄の続きにも文献が列挙されている。

☐ パテントファミリーに関する別紙を参照。

＊ 引用文献のカテゴリー
「A」特に関連のある文献ではなく、一般的技術水準を示すもの
「E」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「L」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「O」口頭による開示、使用、展示等に言及する文献
「P」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「T」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「X」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Y」特に関連のある文献であって、当該文献と他の1以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「&」同一パテントファミリー文献

国際調査を完了した日 02. 11. 2017	国際調査報告の発送日 21. 11. 2017
国際調査機関の名称及びあて先 日本国特許庁（ISA／JP） 郵便番号100－8915 東京都千代田区霞が関三丁目4番3号	特許庁審査官（権限のある職員） 高橋 樹理 4C 4498 電話番号 03－3581－1101 内線 3452

様式PCT／ISA／210（第2ページ）（2015年1月）

国際調査報告　　国際出願番号 PCT／JP2017／036215

C（続き）．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
X	WO 2015/115091 A1 (第一三共株式会社) 2015.08.06, 請求項 1-27, [0159], [0162], 評価例 1-14 & US 2016/0333112 A1, Claims 1-8, [0377], [0381], Evaluation Examples 1-14 & EP 3101032 A1 & CN 105829346 A & KR 10-2016-0113099 A	1-72
A	NAKADA, T. et al., Novel antibody drug conjugates containing exatecan derivative-based cytotoxic payloads, Bioorganic & Medicinal Chemistry Letters, 2016.02, Vol.26, p.1542-1545, ISSN 0960-894X	1-72
A	LOGANZO, F. et al., Tumor Cells Chronically Treated with a Trastuzumab-Maytansinoid Antibody-Drug Conjugate Develop Varied Resistance Mechanisms but Respond to Alternate Treatments, Molecular Cancer Therapeutics, 2015, Vol.14, No.4, p.952-963, ISSN 1535-7163	1-72
A	TAN, X. et al., Antibody-drug conjugates with modified linker-payloads overcome resistance to a trastuzumab-maytansinoid conjugate in multiple cultured tumor cell models, Cancer Research, 2014, Vol.74, No.19, Suppl., Abstract 1830, ISSN 1538-7445	1-72
A	KUMAZAWA, E. et al., Potent and broad antitumor effects of DX-8951f, a water-soluble camptothecin derivative, against various human tumors xenografted in nude mice, Cancer Chemotherapy and Pharmacology, 1998, Vol.42, p.210-220, ISSN 0344-5704	1-72
A	ESTEVA, F.J. et al., A Phase II Study of Intravenous Exatecan Mesylate (DX-8951f) Administered Daily for 5 Days Every 3 Weeks to Patients with Metastatic Breast Carcinoma, Cancer, 2003, Vol.98, No.5, p.900-907, ISSN 1097-0142	1-72
P,X	TAKEGAWA, N. et al., DS-8201a, a new HER2-targeting antibody-drug conjugate incorporating a novel DNA topoisomerase I inhibitor, overcomes HER2-positive gastric cancer T-DM1 resistance, Intenational Journal of Cancer, Vol.141, No.8, p.1682-1689 [オンライン], 2017.08.22 [検索日 2017.10.15], インターネット:<URL:http://onlinelibrary.wiley.com/wol1/doi/10.1002/ijc.30870/full>, ISSN 1097-0215, 特に Abstract	1-72

国際調査報告

国際出願番号 PCT／JP2017／036215

C（続き）． 関連すると認められる文献

引用文献のカテゴリー＊	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
P, X	TAMURA, K. et al., Single agent activity of DS-8201a, a HER2-targeting antibody-drug conjugate, in breast cancer patients previously treated with T-DM1: Phase 1 dose escalation, Annals of Oncology, 2016.10.13, Vol.27, Suppl.6, vi552-vi587, LBA17, ISSN 0923-7534, 全文	1-72

特 許 協 力 条 約

発信人　日本国特許庁（国際調査機関）

代理人
大野　聖二

様

あて名
〒100-0005
日本国東京都千代田区丸の内一丁目６番５号　丸の内北口ビル２１階　大野総合法律事務所

PCT

国際調査報告及び国際調査機関の見解書
又は国際調査報告を作成しない旨の決定
の送付の通知書
（施行規則第41条）
［PCT規則44.1］

	発送日 （日.月.年）　21. 11. 2017
出願人又は代理人 の書類記号　PD20-9018WO	今後の手続については、下記１及び４を参照。
国際出願番号 PCT／JP2017／036215	国際出願日 （日.月.年）　05. 10. 2017
出願人（氏名又は名称） 第一三共株式会社	

1．☑ 国際調査報告及び国際調査機関の見解書が作成されたこと、及びこの送付書とともに送付することを、出願人に通知する。
PCT19条の規定に基づく補正書及び説明書の提出
出願人は、国際出願の請求の範囲を補正することができる（PCT規則46参照）。
いつ　補正書の提出期間は、通常国際調査報告の送付の日から２月である。
どのように　好ましくはePCT経由で又は紙で　直接次の場所へ　The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: +41 22 338 82 70
詳細な手続については、PCT出願人の手引、国際段階段落９．００４－９．０１１を参照すること。

2．☐ 国際調査報告が作成されないこと、及び法第8条第2項（PCT17条(2)(a)）の規定による国際調査報告を作成しない旨の決定及び国際調査機関の見解書をこの送付書とともに送付することを、出願人に通知する。

3．☐ 法施行規則第44条（PCT規則40.2）に規定する追加手数料の納付に対する異議の申立てに関して、出願人に下記の点を通知する。
☐ 異議の申立てと当該異議についての決定を、その異議の申立てと当該異議についての決定の両方を指定官庁へ送付することを求める請求とともに、国際事務局へ送付した。
☐ 当該異議についての決定は、まだ行われていない。決定されしだい出願人に通知する。

4．今後の手続：　出願人は次の点に注意すること。
出願人は、国際調査機関の見解書に対する非公式コメントを、国際事務局に提出することができる。国際事務局は、国際予備審査報告が作成された場合又は作成される場合を除いて、このコメントの写しをすべての指定官庁に送付する。これらのコメントは、国際公開後公開される。
優先日から１８月経過後、国際出願は国際事務局によりすみやかに国際公開される。出願人が公開の中止又は延期を望むときは、国際出願又は優先権の主張の取下げの通知が、国際公開の事務的な準備が完了する前に国際事務局に到達しなければならない（PCT規則90の2.1及び90の2.3）。
いくつかの指定官庁については、出願人が国内段階の開始を優先日から３０月まで（官庁によってはさらに遅くまで）延期することを望むときは、優先日から１９月以内に、国際予備審査の請求書が提出されなければならない。そうでなければ、出願人はそれらの指定官庁に対して優先日から２０月以内に、国内段階の開始のための所定の手続を取らなければならない。その他の指定官庁については、１９月以内に国際予備審査の請求書が提出されない場合にも、３０月の（あるいはさらに遅い）期限が適用される。個々の指定官庁で適用される期限の詳細については、www.wipo.int/pct/en/texts/time_limits.html及びPCT出願人の手引、国内段階を参照。
出願人は、優先日から１９月以内に補充国際調査を提供する他の国際調査機関が補充国際調査を行うことを請求することができる（PCT規則45の2.1）。補充国際調査を請求するための手続は、PCT出願人の手引、国際段階段落８．００６―８．０３２に記載されている。

名称及びあて名 日本国特許庁（ISA／JP） 郵便番号１００－８９１５ 東京都千代田区霞が関三丁目４番３号	権限のある職員　4C　4498 特 許 庁 長 官 電話番号　０３－３５８１－１１０１　内線　３４５２

様式PCT／ISA／220　（２０１４年７月）

注　　意

＜今後の手続期間について＞

条約第１９条に規定する国際事務局に提出する請求の範囲の補正は、出願人への国際調査報告の送付の日から２ヶ月又は優先日から１６ヶ月のいずれか遅く満了する日までに行う必要があります（規則４６．１）。国際段階で当該手続をされる場合には、手続可能な期間にご注意ください。また、補充国際調査請求期間（※）、国際予備審査請求期間、条約２２条に規定する国内移行期限等、国際段階の手続の詳細については、以下をご参照ください。

ＰＣＴ国際出願制度の概要：
http://www.jpo.go.jp/seido/s_tokkyo/pdf/s_tokkyo/text.pdf

ＰＣＴ出願人の手引：
http://www.wipo.int/pct/ja/appguide/index.jsp

※同封の「国際調査報告及び国際調査機関の見解書又は国際調査報告を作成しない旨の決定の送付の通知書（様式 PCT/ISA/220（2014 年 7 月））」中、補充国際調査は「優先日から 19 月以内」に請求する旨の記載がありますが、PCT 規則の改正により 2017 年 7 月 1 日の時点で、補充国際調査の請求期限 19 ヶ月が経過していない国際出願については「優先日から 22 月」が請求期限となります。

＜文献の写しについて＞

出願人は、特許庁に国際調査報告に記載した引用文献の写しを請求することができます。特許庁に対して文献の写しの請求をすることができる期間は、国際出願日から７年です。

契約等の制限により、国際調査報告に記載した非特許文献の写しの一部又は全てが送付されない場合があります。
また、国際調査報告に記載した非特許文献の写しが、国際調査報告の送付から約1～2週間程度遅れて送付される場合もあります。

独立行政法人工業所有権情報・研修館（ＩＮＰＩＴ）では公報類の閲覧・複写および公報以外の文献の複写等を行うことができます。

〔ＩＮＰＩＴ担当〕
〒100-0013　東京都千代田区霞が関３丁目４番３号（特許庁庁舎２階）
独立行政法人工業所有権情報・研修館　知財情報部　閲覧担当
【公 報 類】　ＴＥＬ　03－3581－1101　内線3811、3812
【公報以外】　ＴＥＬ　03－3581－1101　内線3831、3832

特 許 協 力 条 約

発信人　日本国特許庁（国際調査機関）

代理人
大野　聖二
様

あて名
〒100-0005
日本国東京都千代田区丸の内一丁目６番５号　丸の内北口ビル２１階　大野総合法律事務所

ＰＣＴ
国際調査機関の見解書
（法施行規則第 40 条の 2）
〔ＰＣＴ規則 43 の 2.1〕

発送日 （日.月.年）　21. 11. 2017	
出願人又は代理人 の書類記号　P020-9018WO	今後の手続については、下記２を参照すること。

国際出願番号	国際出願日	優先日
ＰＣＴ／ＪＰ２０１７／０３６２１５	（日.月.年）　05. 10. 2017	（日.月.年）　07. 10. 2016

国際特許分類（ＩＰＣ）Int.Cl. A61K39/395(2006.01)i, A61K47/68(2017.01)i, A61P35/00(2006.01)i

出願人（氏名又は名称）
第一三共株式会社

１．この見解書は次の内容を含む。

- ☑ 第Ⅰ欄　見解の基礎
- ☐ 第Ⅱ欄　優先権
- ☐ 第Ⅲ欄　新規性、進歩性又は産業上の利用可能性についての見解の不作成
- ☐ 第Ⅳ欄　発明の単一性の欠如
- ☑ 第Ⅴ欄　ＰＣＴ規則 43 の 2.1(a)(i)に規定する新規性，進歩性又は産業上の利用可能性についての見解、それを裏付けるための文献及び説明
- ☐ 第Ⅵ欄　ある種の引用文献
- ☐ 第Ⅶ欄　国際出願の欠陥
- ☐ 第Ⅷ欄　国際出願についての意見

２．今後の手続
国際予備審査の請求がされた場合は、出願人がこの国際調査機関とは異なる国際予備審査機関を選択し、かつ、その国際予備審査機関がＰＣＴ規則 66.1 の 2(b)の規定に基づいて国際調査機関の見解書を国際予備審査機関の見解書とみなさない旨を国際事務局に通知していた場合を除いて、この見解書は国際予備審査機関の最初の見解書とみなされる。

この見解書が上記のように国際予備審査機関の見解書とみなされる場合、様式ＰＣＴ／ＩＳＡ／２２０を送付した日から３月又は優先日から２２月のうちいずれか遅く満了する期限が経過するまでに、出願人は国際予備審査機関に、適当な場合は補正書とともに、答弁書を提出することができる。

さらなる選択肢は，様式ＰＣＴ／ＩＳＡ／２２０を参照すること。

見解書を作成した日
０２．１１．２０１７

名称及びあて先	特許庁審査官（権限のある職員）	４Ｃ	４４９８
日本国特許庁（ＩＳＡ／ＪＰ） 郵便番号１００－８９１５ 東京都千代田区霞が関三丁目４番３号	高橋　樹理		
	電話番号　０３－３５８１－１１０１　内線　３４５２		

様式ＰＣＴ／ＩＳＡ／２３７（表紙）（２０１５年１月）

国際調査機関の見解書

国際出願番号 ＰＣＴ／ＪＰ２０１７／０３６２１５

第Ⅰ欄　見解の基礎

1．言語に関し、この見解書は以下のものに基づき作成した。

☑ 出願時の言語による国際出願

☐ 出願時の言語から国際調査のための言語である＿＿＿＿＿＿語に翻訳された、この国際出願の翻訳文（ＰＣＴ規則12.3(a)及び23.1(b)）

2．☐ この見解書は、ＰＣＴ規則91の規定により国際調査機関が許可した又は国際調査機関に通知された明らかな誤りの訂正を考慮して作成した（ＰＣＴ規則43の2.1(b)）。

3．この国際出願で開示されたヌクレオチド又はアミノ酸配列に関して、以下の配列表に基づき見解書を作成した。

a．☑ 出願時における国際出願の一部を構成する配列表
☑ 附属書C/ST.25テキストファイル形式
☐ 紙形式又はイメージファイル形式

b．☐ 国際出願とともに、ＰＣＴ規則13の3.1(a)に基づき国際調査のためにのみ提出された、附属書C/ST.25テキストファイル形式の配列表

c．☐ 国際出願日後に、国際調査のためにのみ提出された配列表
☐ 附属書C/ST.25テキストファイル形式（ＰＣＴ規則13の3.1(a)）
☐ 紙形式又はイメージファイル形式（ＰＣＴ規則13の3.1(b)及びＰＣＴ実施細則第713号）

4．☐ さらに、複数の版の配列表又は配列表の写しが提出され、変更後の配列表又は追加の写しに記載された情報が、出願時における配列表と同一である旨、又は出願時における国際出願の開示の範囲を超えない旨の陳述書の提出があった。

5．補足意見：

国際調査機関の見解書 | 国際出願番号 PCT/JP2017/036215

第V欄 新規性、進歩性又は産業上の利用可能性についてのPCT規則43の2.1(a)(i)に定める見解、それを裏付ける文献及び説明

1. 見解

新規性（N）	請求項	7-11, 16-21, 25-28, 33, 35, 43-47, 52-57, 61-64, 69, 71	有
	請求項	1-6, 12-15, 22-24, 29-32, 34, 36-42, 48-51, 58-60, 65-68, 70, 72	無
進歩性（IS）	請求項		有
	請求項	1-72	無
産業上の利用可能性（IA）	請求項	1-72	有
	請求項		無

2. 文献及び説明

文献1：OGITANI, Y. et al., DS-8201a, A Novel HER2-Targeting ADC with a Novel DNA Topoisomerase 1 Inhibitor, Demonstrates a Promising Antitumor Efficacy with Differentiation from T-DM1, Clinical Cancer Research, 2016.03, Vol.22, p.5097-5108, ISSN 1078-0432, 特にAbstract, p.5098 左欄第2段落, p.5103 右欄第2段落-p.5105 左欄第1段落, p.5106 左欄第2-3段落, Figures 4,5

文献2：WO 2015/115091 A1（第一三共株式会社）2015.08.06,
請求項1-27, [0159], [0162], 評価例1-14
& US 2016/0333112 A1, Claims 1-8, [0377], [0381], Evaluation Examples 1-14
& EP 3101032 A1 & CN 105829346 A & KR 10-2016-0113099 A

請求項1-6, 12-15, 22-24, 29-32, 34, 36-42, 48-51, 58-60, 65-68, 70, 72は国際調査報告で引用された文献1, 2より新規性及び進歩性を有しない。

文献1には、DS-8201a（本願請求項1に示される抗HER2抗体-薬物コンジュゲート）が、HER2ステータスがIHC3+/FISH+である、T-DM1（トラスツズマブエムタンシン）無効であった胃癌PDXモデルに対し抗腫瘍効果を発揮したこと、HER2 IHC2+/FISH-陽性である乳癌モデルやHER2 IHC1+/FISH-陰性のST565、ST313モデルでも抗腫瘍効果を示したことが記載されている（p.5103 右欄第2段落-p.5105 左欄第1段落, Figure 5）。文献1にはまた、DS-8201aはT-DM1に対する本来備わった耐性を克服する可能性を有することや、DS-8201aはHER2低発現の腫瘍に対しても有効であるためT-DM1に対する獲得耐性を有する腫瘍にも効果を発揮すると予測されることが記載されている（p.5106 左欄第2-3段落）。

文献2にも、トラスツズマブ及びトラスツズマブエムタンシンが腫瘍の増殖を抑制しない、HER2低発現である乳癌ST910腫瘍に対して、本願発明の抗HER2抗体-薬物コンジュゲートが増殖を抑制したこと等が記載されている（評価例9等）。

（補充欄へ続く）

国際調査機関の見解書　　国際出願番号 PCT／JP2017／036215

補充欄

いずれかの欄の大きさが足りない場合

第　V　欄の続き

請求項 7-36, 43-72 は国際調査報告で引用された文献 1, 2 により進歩性を有しない。

文献 1, 2 には、上述のとおり、本願発明の抗 HER2 抗体-薬物コンジュゲートが幅広い抗腫瘍活性を有し、トラスツズマブやトラスツズマブエムタンシンが無効の腫瘍にも効果を発揮することが記載されているから、当該コンジュゲートを、T-DM1 等の既存の抗癌薬では十分な効果が得られなかった患者に対し投与してみることは当業者が容易になし得たことである。

また、HER2 発現プロファイルが異なる癌や様々な部位の癌に適用してみること、用法・用量を好適化することも当業者の通常の創作能力の発揮に過ぎず、これらの事項により格別顕著な効果を奏するとも認められない。

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OFFICE OF SINGAPORE

In reply please quote our reference

Your reference:	9026SG184/MBR/cak
Our reference:	2016/19617461098W
Date:	22 April 2016
Writer's direct number:	63308615

SPRUSON & FERGUSON (ASIA) PTE LTD
P.O. BOX 1531
ROBINSON ROAD POST OFFICE
SINGAPORE 903031

Dear Sir/Madam,

Patent Application No.:	11201502887W
Title of invention:	ANTIBODY-DRUG CONJUGATE
Applicant(s):	DAIICHI SANKYO COMPANY, LIMITED

INVITATION TO RESPOND TO WRITTEN OPINION

We forward with this letter a copy of the Search Report and Written Opinion drawn up by the Examiner in connection with your request for a Search and Examination Report.

You are invited to respond to the opinion by submitting:

(a) Written submissions in response to the Examiner's opinion and/or
(b) An amendment of the specification of the application.

If you intend to respond, the response must be filed within **5 months** from the date of this letter. Otherwise, an Examination Report will be established based on the written opinion if no response is received by the end of the prescribed period.

Separately, you may be interested to know that the Intellectual Property Office of Singapore (IPOS) has established the ASEAN Patent Examination Co-operation (ASPEC) programme, a patent work sharing programmes with the ASEAN IP offices of Brunei Darussalam, Cambodia, Indonesia, Lao PDR, Malaysia, the Philippines, Thailand and Viet Nam. You may be eligible to benefit from ASPEC if you have filed a corresponding application and received the search and examination report from any other participating ASEAN IP Office.

To find out more about the procedures for requesting ASPEC at IPOS, please refer to our website at www.ipos.gov.sg [About IP > What is Intellectual Property and what are the different types? > What is a patent? > Applying for a patent].

If you have any further queries, please do not hesitate to contact the undersigned.

Thank you.

Mr. John Tan
for Registrar of Patents
Singapore

Encl:Search Report - 11201502887W-SRT-Search Report-[1]
Encl:Written Opinion - 11201502887W-WRO-Written Opinion-[1]
Encl:Cited Documents - 11201502887W-CDC-US6835807B1
Encl:Cited Documents - 11201502887W-CDC-US20030166513

IPOS
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OFFICE OF SINGAPORE

Search Report

Application No.
11201502887W

Application filing date	(Earliest) Priority Date	Examiner's Reference Number
10/10/2013	11/10/2012	IPOS/NKS

This Search Report issued under Section 29(5) of the *Patents Act* with effect from 14/02/2014 consists of a total of 3 pages.

☒ It is also accompanied by a copy of each prior art document cited in this report, except where otherwise indicated.

1. ☐ Certain claims where found **unsearchable** (*see Box I*)

2. ☐ **Unity of invention** is lacking (*see Box II*)

3. ☐ The application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the search was carried out on the basis of the sequence listing.

4. With regard to the **title**,

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established by this Office to read as follows:

5. With regard to the **abstract**,

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established by this Office as appears in Box **III**.

6. The figure of the **drawings** to be published with the abstract is NONE,

   ☐ as suggested by the applicant.

   ☐ because the applicant failed to suggest a figure.

   ☐ because this figure better characterizes the invention.

   ☐ because there are no drawings in the application.

   ☒ none of the figures.

INTELLECTUAL PROPERTY OFFICE OF SINGAPORE

Search Report

Application No. 11201502887W

A. CLASSIFICATION OF SUBJECT-MATTER

According to International Patent Classification (IPC)

A61K 39/395, C07K 16/28, A61P 35/00, A61K 31/4745, A61K 38/07

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K, C07K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the search (name of database and, where applicable, search terms used)

Database: STN, pat

Search terms: CAS # 171335-80-1, Exatecan, antibody conjugate, -gly-gly-phe-gly- and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim no.
Y A	US 6835807 B1 (SUSAKI H. ET AL) 28 December 2004 Example 1	1-19, 29-35 20-28, 36-39
Y	SHIOSE Y. ET AL, Relationship between drug release of DE-310, macromolecular prodrug of DX-8951f, and cathepsins activity in several tumors. *Biol. Pharm. Bull.*, 25 September 2007, Vol. 30, No. 12, pages 2365-2370 Fig. 1 Citation is not enclosed due to copyright restrictions.	1-19, 29-35
Y	BARGINEAR M.F. ET AL., Trastuzumab-DM1: a review of the novel immune-conjugate for HER2-overexpressing breast cancer. *The Open Breast Cancer Journal* , 31 December 2009, Vol. 1, pages 25-30 Page 25 2nd column last paragraph – page 26 1st column first paragraph; Fig. 3 Citation is not enclosed due to copyright restrictions.	1-19, 29-35

☒ Further documents are listed in the continuation of box C.

Special categories of cited documents:

A: document defining the general state of the art which is not considered to be of particular relevance

E: earlier application or patent but published on or after the filing date

L: document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O: document referring to an oral disclosure, use, exhibition or other means

P: document published prior to the filing date but later than the priority date claimed

T: later document published after the filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X: document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y: document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents; such combination being obvious to a person skilled in the art

&: document member of the same patent family

Date of submission of the request to the Intellectual Property Office of Singapore:	Date of actual completion of the search:
11/11/2015	01/03/2016
Intellectual Property Office of Singapore 51 Bras Basah Road #01-01 Manulife Centre Singapore 189554 Email: operation@iposinternational.com	**Authorized Officer** Ng Kay Siong (Dr)

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Search Report

Application No.
11201502887W

C. (Continuation) DOCUMENT CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim no.
Y	BECK A., The next generation of antibody-drug conjugates comes of age. *Discov Med*, 16 October 2010, Vol. 10, No. 53, pages 329-339 Figs. 1 and 2c Citation is not enclosed due to copyright restrictions.	1-19, 29-35
A	US 2003/0166513 A1 (IMURA A. ET AL.) 4 September 2003	

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OFFICE OF SINGAPORE

Written Opinion

Application No.
11201502887W

Application filing date	(Earliest) Priority Date	Examiner's Reference Number
10/10/2013	11/10/2012	IPOS/NKS

International Patent Classification (IPC) (as indicated in the search report)
A61K 39/395, C07K 16/28, A61P 35/00, A61K 31/4745, A61K 38/07

1. This first Written Opinion issued under Section 29(5) of the *Patents Act* with effect from 14/02/2014 consists of a total of 6 pages.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Unity of invention
V	☒	Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☒	Defects in the form or contents of the application
VII	☒	Clarity, Clear and Complete Disclosure, and Support
VIII	☐	Double patenting

3. The search report used was issued by the Intellectual Property Office of Singapore, and the date of completion is 01/03/2016.

4. If no reply is filed, the Examination Report will be established on the basis of this opinion.

Date of submission of the request to the Intellectual Property Office of Singapore:	Date of Written Opinion:
11/11/2015	24/03/2016
Intellectual Property Office of Singapore 51 Bras Basah Road #01-01 Manulife Centre Singapore 189554 E-mail address: operation@iposinternational.com	**Authorized Officer** Ng Kay Siong (Dr)

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OFFICE OF SINGAPORE

Written Opinion

Application No.
11201502887W

I. Basis of the opinion

1. This opinion has been drawn on the basis of:

☒ the **description**, (verified English translation)
page(s) 1-6, 10, 12-67, 69-414 as originally filed
page(s) 7, 8, 9, 9a-9q, 11, 68 filed as amendment sheet(s) dated 11/11/2015

☒ the **claim(s)**, (verified English translation)
no. 1-39 filed as amendment sheet(s) dated 11/11/2015

☒ the **drawing(s)**, (verified English translation)
page(s) 1-22 as originally filed

☒ the **sequence listing**, (verified English translation)
page(s) 1-33 as originally filed

2. ☐ This opinion has been established based on the exclusion of additional matter beyond the earlier application, as indicated in the supplemental box.

3. ☐ This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the supplemental box.

4. Additional observations, if necessary:

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OFFICE OF SINGAPORE

Written Opinion

Application No.
11201502887W

V. Reasoned statement with regard to novelty, inventive step or industrial applicability; Citation and explanation supporting such statement

Statement with regard to novelty, inventive step or industrial applicability

Novelty (N)	Claim(s)	1-39	YES
	Claim(s)	NONE	NO
Inventive Step (IS)	Claim(s)	NONE	YES
	Claim(s)	1-39	NO
Industrial applicability (IA)	Claim(s)	1-17, 19-39	YES
	Claim(s)	18	NO

1. Citations

The following citations are referred to in this opinion. Full bibliographic details are provided in the Search Report:

D1 – US 6835807 B1
D2 – SHIOSE Y. ET AL, 2007
D3 – BARGINEAR M.F. ET AL., 2009
D4 – BECK A., 2010
D5 – US 2003/0166513 A1

D5 is considered to be background art and will not be discussed further.

2. Novelty (Section 14 of the *Patents Act*)

None of D1-D5 discloses the subject matter of claims 1-39 and hence the aforementioned claims are considered to be novel.

3. Inventive Step (Section 15 of the *Patents Act*)

Claims 1-19 and 29-35

The inventive concept of claims 1-19 is directed to an antibody-drug conjugate wherein the anti-tumor compound is represented by a formula, which corresponds to the drug exatecan in the art, and the antibody and drug is conjugated by a linker which comprises a connecting group such as succinimid-3-yl-N or thioether group, and a tetrapeptide residue of GGFG; while the inventive concept of claims 29-35 is directed to a linker which comprises a connecting group such as succinimid-3-yl-N or thioether group, and a tetrapeptide residue of GGFG. From herein, the anti-tumor compound will be referred to as exatecan.

Both D1 and D2 disclose an antineoplastic agent DX-8951, also known as exatecan, conjugated to a drug carrier (i.e. carboxymethyl polyalcohol) via a Gly-Gly-Phe-Gly (GGFG) linker (see D1 Example 1 and D2 Fig. 1).

The difference between D1/D2 and the abovementioned inventive concepts lies in the specific choice of a linker which comprises thioether and a GGFG peptide, and the conjugation of exatecan to an antibody. Both D3 and D4 disclose a trastuzumab-DM1 drug conjugate (see D3 Fig. 3 and D4 Fig. 1) designed to focus on efficient delivery to HER2-expressing breast cancer cells, wherein DM1 is conjugated to trastuzumab via a linker having a structure represented by the following formula –L1-L2-LP-NH-(CH2)n1-La-Lb-Lc – wherein:
L1= -C(=))-cyc.Hex(1,4)-CH2-(N-ly-3-diminiccuS)-
L2= -S-(CH2)2-C(=O)-
LP= single peptide residue

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Written Opinion

Application No. 11201502887W

V. Reasoned statement with regard to novelty, inventive step or industrial applicability; Citation and explanation supporting such statement

-(CH2)n1- is non-existent, i.e. n1=0
La= single bond
Lb= single bond
LC= -C(=O)-.
In particular, D3 further discloses the method to obtain the antibody-drug conjugate which comprises the following steps: 1) N-succinimidyl-4-(maleimidomethyl)cyclohexanecarboxylate (SMCC) is first added to the peptide reside on the protein to produce a linker-modified antibody; 2) the thiol group in the drug is then reacted with the maleimide group of the linker to form the non-reducible thioether bond (see page 25 2nd column last paragraph – page 26 1st column first paragraph).

At the priority date, the person skilled in the art would be aware of the use of antibody-drug conjugates in breast cancer therapy. Therefore, the person skilled in the art, in wanting to provide an alternative drug compound for effective targeting of exacetan, would be motivated to conjugate exacetan to an antibody and would be directed to D3/D4, which discloses trastuzumab as a targeting agent in the DM1-trastuzumab conjugate for use in breast cancer therapy, wherein the conjugate contains a thioether linker, as discussed above. Therefore, starting from D1/D2, the person skilled in the art would be motivated to replace carboxymethyldextran polyalcohol with the targeting antibody trastuzumab, and by following the method in obtaining the antibody-drug conjugate as taught in D3, the person skilled in the art would obtain a trastuzumab-exacetan conjugate wherein exacetan is conjugated to trastuzumab via a linker which comprises thioether and a GGFG peptide and arrive at the current invention with no inventive effort. Furthermore, D4 discloses other alternative linker regions such as – (succinimid-3-yl-N)-$(CH_2)_5$-C(=O) – followed by a single bond (see D4 figure 2c) which is within the scope of L^1 and L^2, respectively, as defined in claims 1 and 3 of the current application and brentuximab, which is an anti-CD30 antibody, as an alternative antibody (see D4 figure 2c). Therefore, claims 1-19 and 29-35 cannot be conferred any inventiveness.

Claims 20-28 and 36-39
The additional features directed to drug-linker intermediates (claims 20-28) are also not inventive. The person skilled in the art would be aware of different ways of linking the drug to the antibody, such as first obtaining an antibody-linker intermediate and subsequently linking it to the drug, or alternatively, first obtaining a drug-linker intermediate and subsequently linking it to the antibody, all of which would allow the person skilled in the art to obtain the antibody-drug conjugate of the current application. The latter method would therefore be an obvious choice to the person skilled in the art seeking an alternative method to link a drug to an antibody. Therefore, the person skilled in the art would inevitably arrive at the drug-linker intermediates without any inventiveness. In the absence of any specific technical prejudice in the selection of the aforementioned features and any surprising effects demonstrated of the aforementioned features, an inventive step cannot be acknowledged for claims 20-28. Additionally, the additional features defined by claims 36-39, which are directed to derivatives of exatecan do not appear to be inventive as the selection of the different side groups connected to exatecan are considered arbitrary and do not appear to confer any specific technical advantage over exatecan.

Hence, all claims 1-35 are considered to lack an inventive step.

4. Industrial Applicability (Section 16 of the *Patents Act*)
The subject matter of claims 1-17, 19-39 is industrially applicable.

Claim 18 does not appear to comply with Section 16(2) of the *Patents Act* as the subject matter of the claim encompasses methods of treatment of the human or animal body by surgery or therapy practiced on the human or animal body and therefore is not taken to be capable of industrial application.

INTELLECTUAL PROPERTY OFFICE OF SINGAPORE

Written Opinion

Application No.
11201502887W

VI. Defects in the form or contents of the application
The following defects in the form or contents of the application have been noted: 1) Page 416 line 1 – There is a missing space between the terms “atom” and “L꜀” in the term “atomL꜀”.

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Written Opinion

Application No.
11201502887W

VII. Clarity, Clear and Complete Disclosure, and Support

The following observations on the clarity of the claim(s), on the question whether the disclosure is clear and complete, or on the question whether the claim(s) is (are) supported by the description, are made:

1. Clarity (Section 25(5)(b) of the *Patents Act*)
Claim 1 is unclear as n^4 is not defined. Hence the person skilled in the art will be unclear of the scope of the claim.

Claim 1 is unclear. The presence of the terms "formula X" in parenthesis, where X is a number as defined in the present claim is indefinite. There is no apparent meaning to what is a formula X as defined in the present claims and hence renders the scope of the formula encompassed within the formula X unclear. Similarly, claims 2-39 are also considered to be unclear in this regard.

2. Support (Section 25(5)(c) of the *Patents Act*)
Claim 18 is not fully supported by the description. Claim 18 is directed to the various types of cancers that the antitumor drug can be applied to. However, in the description, the anti-tumor tests (see test examples 11-16 in description of current application) were all performed using an antibody-drug conjugate comprising an anti-M30 antibody and exemplified to be effective only against melanoma and non-small cell lung cancer. Hence, given such a narrow exemplification provided in the description, it is unclear how the invention can be practised across such a wide range.

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If the above hyperlink does not work, please copy and paste http://goo.gl/1rRO7M into your browser to access the survey.

Thank you.

Clarke, Modet & Cº
COLOMBIA

May 9, 2017

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OHNO & PARTNERS
Attn: Shinsuke UMEDA, Ph.D.
Marunouchi Kitaguchi Bldg. 21F
1-6-5, Marunouchi, Chiyoda-ku,
Tokyo, 100-0005
JAPAN

EXTENSIBLE DEADLINE: AUGUST 1, 2017

Ref: COLOMBIA
Patent Application No. **NC2016/0000187** in the name of **DAIICHI SANKYO COMPANY LIMITED**
Y/Ref.: SHU/PD20-9008CO
O/Ref.: P-2016/12118

Dear Sirs:

Please be informed that the company **LABORATORIOS LEGRAND S.A.** has filed opposition against the application of the reference. The basic points of this are described below:

1. Opposition filed by LABORATORIOS LEGRAND S.A.

1.1 Clarity and conciseness (Art. 30 of Decision 486/00)

The opponent states that:

- Claims 1 to 16 do not specifically define the technical characteristics of the antibody-drug conjugate. According to the opponent, the main claims indicates different drug-linker structures which comprises an undetermined amount of compounds.

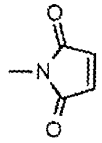
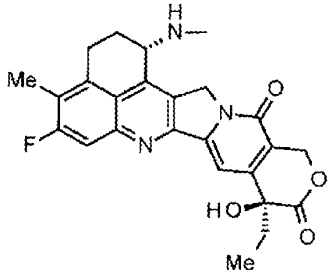
- Although the claims include formulas and sequences of the antibody, all the possible molecules for which a protection is sought are not divulged. Besides, the molecules are defined in terms of substitutions and results to be

achieved which are not be accepted as technical characteristics of the invention.

- Claim 1 mentions as essential and technical characteristics the compound of formula I, the drug-linker structures and the antibodies (such as trastuzumab). However, as indicated in section 1.3, the state of the art anticipates this type of conjugates, as well as the antitumor agents, antibodies such as trastuzumab, and the **succinimid** part of the linker.

- Claim 1 lacks clarity and conciseness since it is not clear the preamble and the characterizing part, so that t claims protection for subject matter which is widely known in the state of the art, such as anti-HER2 antibodies (that may be trastuzumab) and /or camptothecin derivatives.

- Claims 11 to 16 lack clarity since they refer to the reducing condition in the method:

> *"A method for preparing anti-HER2 antibody-drug conjugate, wherein the anti-HER2 antibody is treated in a reducing condition and thereafter reacted with a compound selected from the compound group shown below:*
> *(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2-O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX),*
> *(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX), or*
> *(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX),*
> *wherein (maleimid-N-yl)- is a group represented by the following formula:*
> *[Formula 4]*
>
> 
>
> *wherein the nitrogen atom is a connecting position,*
> *-(NH-DX) is a group represented by the following formula:*
> *[Formula 5]*
>
> 
>
> *wherein the nitrogen atom of the amino group at position 1 is a connecting position, and*

wherein the anti-HER2 antibody comprises a heavy chain and a light chain selected from the group;
a heavy chain consisting of an amino acid sequence consisting of positions 1 to 449 of SEQ ID NO: 1 and a light chain consisting of an amino acid sequence consisting of positions 1 to 214 of SEQ ID NO: 2, and
a heavy chain consisting of the amino acid sequence of SEQ ID NO: 1 and a light chain consisting of the amino acid sequence of SEQ ID NO: 2"

In addition, the sequences may be selected among different segments of the amino acid sequences SEQ ID NO: 1 and 2.

- Regarding claim 9, the opponent indicates the description of the medicament is too general refers to several compositions comprising antibody-drug conjugates.

1.2 Unity of Invention (Art. 25 of Decision 486/00)

According to the examiner, the method disclosed in claims 11 to 16 refer to regions which are not related to the structure or function of the conjugates disclosed in claims 1 to 11. In this way, the application lacks unity of invention.

1.3 Inventive step (Art. 18 of Decision 486/00)

The opponent is of the opinion that current application lacks of inventive step in view of the following documents:

No.	Document	Affected requirement
Govindan et al	US 2016/0287722 A1	Inventive step

According to the description, the objective technical problem which the present invention solves is decreasing the toxicity of the cancer treatment with an antitumor by means of a conjugate between an antitumor compound, a linker and an anti-HER2 antibody (which may be trastuzumab). However, this had been already disclosed in the state of the art, for instance, in *Govindan et al.*

"*[0010] In one embodiment, the invention relates to an immunoconjugate comprising:*
[0011] (a) a targeting moiety;
[0012] (b) a chemotherapeutic moiety; and
[0013] (c) a linker binding to the targeting moiety via a thiol group, and to the chemotherapeutic moiety via an intracellularly-cleavable moiety other than a hydrazone. In another embodiment, the invention relates to an immunoconjugate comprising:
[0014] (a) targeting moiety;
[0015] (b) a chemotherapeutic moiety; and
[0016] (c) a linker binding to the targeting moiety via a thiol group, and to the chemotherapeutic moiety via an intracellularly-cleavable moiety other