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Preclinical report

Antitumor activity, optimum administration method and pharmacokinetics of 13,14-dihydro-15-deoxy-Δ^7-prostaglandin A_1 methyl ester (TEI-9826) integrated in lipid microspheres (Lipo TEI-9826)

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13,14-Dihydro-15-deoxy-Δ^7-prostaglandin A_1 methyl ester (TEI-9826), an antitumor prostaglandin analog, is a candidate for clinical trial. In the present study, we examined its biological stability *in vitro*, antitumor activity *in vitro* and *in vivo*, and pharmacokinetics. Although TEI-9826 was rapidly hydrolyzed to the carboxylic acid form (TOK-4528), TOK-4528 as well as Δ^{12}-prostaglandin J_2 (PGJ_2) were found to be stable in rat, mouse and human serum *in vitro*. TEI-9826 exhibited nearly identical or greater potential antitumor activity compared to Δ^{12}-PGJ_2 and Δ^7-PGA_1 *in vitro* against Colon26 tumor cells. Further evaluation of TEI-9826 using the 38 human cancer cell lines panel and COMPARE analysis suggested that its mode of action is quite different from other anticancer agents that are currently used. TEI-9826 was integrated into lipid microspheres (Lipo TEI-9826) for dosing. Growth inhibition by Lipo TEI-9826 against Colon26 tumor inoculated s.c. in mice depended on administration route, i.e. at 80 mg/kg, no growth suppressive effect was observed for daily bolus i.v., but significant growth suppressive effect was observed for daily i.p., daily s.c. every other day s.c. and 4 times a day continuous (5 min) i.v. These tumor growth-suppressive effects were cytostatic and the tumor started to regrow at the end or a few days after the end of administration. The pharmacokinetic study suggested that maintaining the blood level of TEI-9826 and/or TOK-4528 was essential for their antitumor effects. These results show that continuous i.v. infusion might be the most suitable administration method of Lipo TEI-9826 for clinical trial. [© 2001 Lippincott Williams & Wilkins.]

Key words: Antitumor effect, cyclopentenone PG, emulsion, lipid microspheres, pharmacokinetics, prostaglandin, prostaglandin J_2.

Introduction

Δ^7-Prostaglandin A_1 (Δ^7-PGA_1) and Δ^{12}-prostaglandin J_2 (Δ^{12}-PGJ_2) are representative compounds of antitumor prostaglandins which have a cross-conjugated dienone unit.[1–5] Such PG species are actively incorporated into tumor cells and transfer into the nuclei.[6–8] G_1 arrest is induced at approximately the IC_{50} concentration, and G_2/M arrest and/or apoptosis are induced at approximately the IC_{90} concentration.[5,9,10] Recently, the antitumor mechanisms of these PGs have been clarified.

Gorospe *et al.* demonstrated that PGA_2 induce p21 expression independently of p53.[11,12] Tanikawa *et al.*

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reported that Δ^7-PGA_1 and its analogs also induce p21 expression.[13,14] Ohtani *et al.* reported that Δ^{12}-PGJ_2 activates the gadd45 promoter independently of p53.[15] These studies suggest that antitumor PG might mimic p53 in cells lacking p53 and thereby exhibit antitumor effects. Activation of peroxisome proliferator-activated receptor-γ (PPARγ) by antitumor PG might be also related to the antitumor mechanism. Kliewer *et al.*[16] and Forman *et al.*[17] demonstrated that 15-deoxy-$\Delta^{12,14}$-PGJ_2, a metabolite of Δ^{12}-PGJ_2, is a ligand for PPARγ and induces adipogenesis. Altiok *et al.* reported that E2F expression is suppressed by activation of PPARγ, resulting in cell arrest.[18] These reports suggest that G_1 arrest by antitumor PG depends on PPARγ activation. Furthermore, Bishop *et al.* reported that 15-deoxy-$\Delta^{12,14}$-PGJ_2 and PGA_2 cause endothelial cell apoptosis by activation of PPARγ, suggesting that inhibition of angiogenesis is one antitumor mechanism of PGs.[19] Another mechanism, reported by Suzuki *et al.*, is inhibition of topoisomerases by Δ^{12}-PGJ_2.[20] Thus, antitumor PG may be good candidate for clinical trial.

Although we expect the development of Δ^{12}-PGJ_2 or its analogs as anticancer agents, mass synthesis methods, which can produce sufficient drugs for preclinical and clinical study, have not yet been established. Conversely, mass synthesis of Δ^7-PGA_1 and its analogs has been established by Noyori and Suzuki,[21–24] and sufficient supply for preclinical and clinical study is possible. Many analogs of Δ^7-PGA_1 have been synthesized and their anticancer effects *in vitro* and *in vivo* have been studied.[25] One of these analogs, TEI-9038, racemic methyl esters of Δ^7-PGA_1 and its three stereoisomers, exhibited similar or stronger anticancer effects than Δ^7-PGA_1 *in vitro*.[26] TEI-9038 has been selected as a candidate for clinical trial and a preclinical study, including its chronic toxicity, has been carried out.[26–28] In the preclinical study, oil in water (O/W)-type lipid emulsion (lipid microspheres) containing TEI-9038 dissolved in the oil phase was selected as the dosage form (Lipo TEI-9038) because TEI-9038 is a liquid oil at low temperature (4°C) and is clearly miscible with purified soy bean oil, the oil phase of the lipid microsphere.[26,29] However, Lipo TEI-9038 exhibited only marginal anticancer effects *in vivo*, contrary to expectations. One of the reasons for such a marginal activity *in vivo* is that Δ^7-PGA_1 was rapidly converted to Δ^7-PGC1, which exhibits no anticancer effects, in rat and mouse serum used in an *in vivo* model.[30,31] Investigation of the stability of Δ^7-PGA_1 analogs in rat and mouse serum *in vitro* has revealed that the stability of 13,14-dihydro-15-deoxy-Δ^7-PGA_1 (TOK-4528) is identical to Δ^{12}-PGJ_2.[32]

The IC_{50} of TOK-4528 against Colon26 tumor cells *in vitro* is nearly identical to that of Δ^{12}-PGJ_2 and Δ^7-PGA_1. For the present study, we selected the methyl ester of TOK-4528 (TEI-9826) because TOK-4528 itself is a highly lipophilic crystalline powder and preparation of a dosage form for parenteral injection is considered difficult. Conversely, TEI-9826 is an oily liquid even at −20°C and is miscible with soy bean oil at any proportion; therefore, integration into a lipid microsphere is relatively simple. Furthermore, in the synthetic procedure, TEI-9826 is synthesized first and then TOK-4528 is produced by hydrolysis of TEI-9826. Thus, for large-scale synthesis, TEI-9826 has a marked advantage. Sasaki *et al.* reported that Lipo TEI-9826 exhibited only minimal cross-resistance to cisplatin *in vitro* and that it significantly inhibited the growth of a cisplatin-resistant tumor cell ($A2980_{CDDP}$ human ovarian cancer) *in vivo*.[33] They suggested that Lipo TEI-9826 might be capable of overcoming cisplatin resistance. In the present study, we describe the stability and antitumor activity *in vitro* of TEI-9826, and the antitumor activity, the optimum administration method and pharmacokinetics of Lipo TEI-9826 *in vivo*.

Materials and methods

Chemicals

Δ^7-PGA_1 and TEI-9826 were kindly supplied by Teijin (Tokyo, Japan). TOK-4528 was kindly supplied from Dr M Suzuki, one of the authors. These PGs were synthesized according to the methods of Noyori and Suzuki methods.[21–24] Δ^{12}-PGJ_2 was kindly supplied by Ono Pharmaceutical (Kyoto, Japan). Figure 1 shows the chemical structures of these PGs. Purified egg lecithin (PL 100E) was kindly supplied by QP (Tokyo, Japan) of clinical grade. Fetal bovine serum and 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Nacalai Tesque (Kyoto, Japan). RPMI 1640 (powder) was purchased from Nissui (Tokyo, Japan). Dispase was purchased from Takara Shuzo (Tokyo, Japan). Kanamycin was a product of Meiji Seika (Tokyo, Japan).

Cell line

Colon26 carcinoma cells were maintained in monolayer culture using RPMI 1640 medium containing 10% fetal bovine serum and 100 μg/ml Kanamycin at 37°C in a 5% CO_2-humidified incubator. Single-cell suspensions were prepared by treating the monolayer of Colon26 cells with Dispase, and these cell suspensions

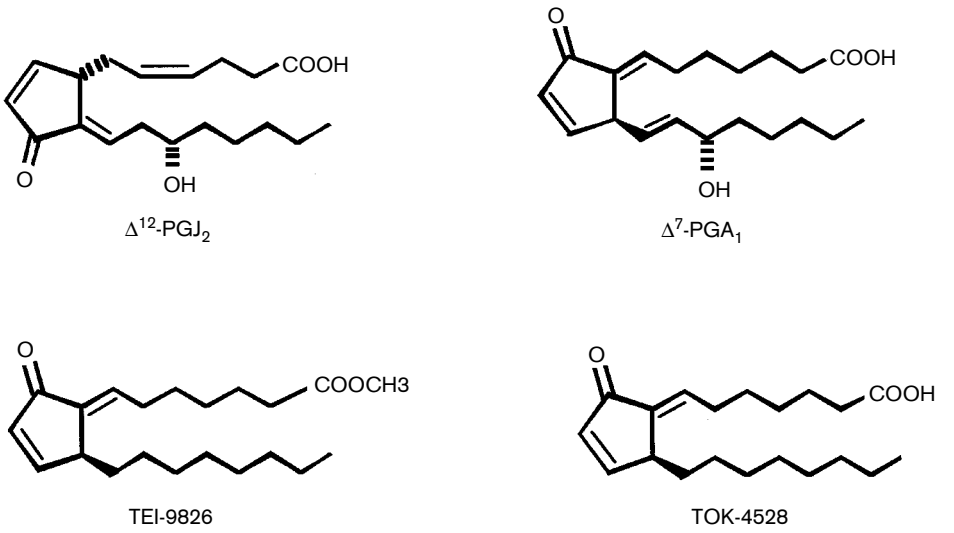

Figure 1. Chemical structure of PGs examined in the study.

were used for subculture, study *in vitro* and inoculation into mouse *in vivo*.

Animals

Male CDF1 mice (4 weeks old) were obtained from Charles River Japan (Kanagawa, Japan). Male Wistar rats (7 weeks old) were obtained from Japan SLC (Shizuoka, Japan). These animals were bred for at least 7 days in an animal room lit for 12 h at 25°C prior to initiating experiments.

Stability of Δ^7-PGA$_1$, Δ^{12}-PGJ$_2$, TEI-9826 and TOK-4528 in serum *in vitro*

Freshly prepared serum from male CDF1 mice, male Wistar rats and humans was used in the experiments. Each PG was dissolved in ethanol (1 mg/ml, 80 μl) and introduced into serum (1520 μl) previously warmed at 37°C (final ethanol concentration was 5%) and incubated at 37°C. Aliquots (100 μl) were periodically withdrawn and vigorously mixed with acetonitrile (200 μl) containing 2-ethylhexyl-*p*-hydoxybenzoate (internal standard for HPLC analysis). The mixture was then centrifuged at 10 000 r.p.m. for 1 min and the supernatant frozen at −20°C until analysis. The concentration of each PG in supernatant was determined by HPLC. HPLC conditions were as follow;

Pump: Shimadzu LC-6A
Auto-injector: Tosoh AS-8020
Column oven: Tosoh CO-8020
Detector: Shimadzu SPD-10A
Integrator: Shimadzu C-R6A
Column: Licrospher 100 RP18 (5 μm, 250 × 4 mm, Merck)
Column temperature: 40°C
Mobile phase: 0.02 M acetic acid buffer (pH 5.0):methanol
1:2 for TEI-9826
19:24 for TOK-4528, Δ^{12}-PGJ$_2$ and Δ^7-PGA$_1$
Flow rate: 1.0 ml/min
Detector wavelength: UV 248 nm

In vitro antitumor activity of PGs against Colon26 carcinoma

Colon26 cells were seeded into each well of a 96-well microplate ($1x10^4$ cells/well). After 24 h, freshly prepared culture medium containing each PG was added to each well (day 0). Culture medium without PG was used as control. Growth profile was evaluated by MTT assay according to the Mosmann's method[34] on days 0, 1 and 2. Briefly, 25 μl of MTT solution (5 mg/ml in PBS, pH 7.0) was added into each well on days 0, 1 and 2 and the culture was maintained for 4 h. Then, 125 μl culture medium was discarded and 150 μl isopropyl alchol–HCl (0.04 N) was added to dissolve formazan generated from MTT. The absorbance of each well was measured by a microplate reader using a test wavelength of 560 nm and reference wavelength of 655 nm. The growth rate was calculated as the ratio of absorbance at days 1 and 2 to the absorbance at day 0, and growth inhibition rate was calculated by the following equation:

$$\text{Growth rate } (\%) = \frac{\text{ABS (day 1 or 2)} - \text{ABS (day 0)}}{\text{ABS (day 0)}} \times 100$$

$$\text{Growth inhibition } (\%) = \frac{\text{growth rate (control)} - \text{growth rate (treated)}}{\text{growth rate (control)}} \times 100$$

A dose–response curve was obtained based on the inhibition rate at day 2 and IC_{50} was determined from the curve.

Colony-forming efficacy after exposure to TEI-9826

Five hundred Colon26 cells in 50 μl culture medium were seeded into a 10-cm culture dish containing 5 ml culture medium, and cultured for 24 h. Culture medium was then withdrawn and freshly prepared culture medium containing each PG at several concentrations was introduced, and cells were cultured for several exposure periods. Culture medium without PG was used as control. After exposure, the culture medium was withdrawn and cells were washed three times with RPMI 1640 medium without fetal bovine serum. Immediately after wash, freshly prepared culture medium without PG was added and cells were cultured for 7 days. Colony numbers were counted and colony forming efficacy was calculated by the following equation:

$$\text{Colony forming efficacy} = \frac{\text{Colony numbers (treated)}}{\text{Colony numbers (control)}} \times 100$$

Antitumor activity and spectrum in a human cancer cell line panel using 38 human cancer strains *in vitro*

We used a cell line panel system established by Yamori *et al.*,[35,36] based on the system of the National Cancer Institute.[37–39] The 38 human cancer cell lines used in the present study are listed in Table 1. Each cell type was seeded into a 96-well microplate ($1–2 \times 10^4$ cells/well) and cultured overnight. The cells were exposed to drugs for 48 h. After 48 h, cell growth was determined by sulforhodamine B assay and 50% growth inhibition concentration (GI_{50}) was determined. The antitumor spectrum pattern (mean graph which we call a fingerprint) was made up by the value of [log(mean GI_{50}) – log (each GI_{50})]. The data were compared with those of more than 200 antitumor compounds including currently used anticancer drugs using the COMPARE computer algorithm.[36,38]

Table 1. GI_{50}s of PGs against 38 human tumor strains *in vitro*

Strain	Cell line	GI_{50} (μM)	
		Δ^7-PGA$_1$	TEI-9826
Br	HBC-4	2.6	3.0
	BSY-1	2.4	2.1
	HBC-5	3.7	2.6
	MCF-7	4.5	3.4
	MDA-MB-231	4.2	2.5
CNS	U251	4.2	4.1
	SF-268	4.2	4.4
	SF-295	11.0	3.8
	SF-539	2.7	2.5
	SNB-75	15.0	10.0
	SNB-78	11.0	4.3
Co	HCC2998	3.5	9.6
	KM-12	2.3	2.2
	HT-29	7.6	3.5
	WiDr	7.5	4.2
	HCT-15	5.3	1.9
	HCT-116	1.8	1.7
Lu	NCI-H23	4.6	2.1
	NCI-H226	15.0	2.9
	NCI-H522	1.7	2.0
	NCI-H460	17.0	5.3
	A549	21.0	13.0
	DMS273	2.5	1.9
	DMS114	5.4	2.6
Me	LOX-IMVI	2.6	2.0
Ov	OVCAR-3	5.2	2.4
	OVCAR-4	10.0	4.0
	OVCAR-5	11.0	3.1
	OVCAR-8	3.6	3.0
	SK-OV-3	11.0	7.0
Re	RXF-631L	13.0	7.3
	ACHIN	4.2	2.6
St	St-4	4.6	4.5
	MKN1	4.7	2.5
	MKN7	3.1	2.3
	MKN28	3.3	2.5
	MKN45	3.6	3.6
	MKN74	2.5	2.3
Mean		6.4 μM (2.2 μg/ml)	3.8 μM (1.3 μg/ml)

Cells were plated at proper density in a 96-well microplate and cultured overnight. The cells were exposed to drugs for 48 h and cell growth was determined by sulforhodamine B assay. The concentration necessary for 50% growth inhibition (GI_{50}) was calculated and the mean value was obtained.

Preparation of lipid microspheres containing TEI-9826 (Lipo TEI-9826)

We selected lipid microsphere as the formulation for PGs,[26,29,40,41] due to the highly lipophilic nature of the compounds. TEI-9826 is an oily liquid even at −20°C and is freely miscible with purified soy bean oil, an oil component of fatty lipid emulsion used clinically. The preparation of Lipo TEI-9826 is, therefore, relatively easy by the ordinary preparation procedure for fatty lipid emulsions using a high-pressure homogenizer. We used a Microfluidizer (model 110Y) as homogenizer because it was suitable for laboratory-scale preparation and easy temperature control (at the present time, we are using a new type of homogenizer, a controlled high-pressure process homogenizer, DeBEE2000 or mini DeBEE; Bee International, Nesher, Israel). Purified egg yolk lecithin (1.2 g) was well dispersed in injection-grade water (85.8 g) containing glycerin (2.5 g). The mixture oil (clear oily liquid) of TEI-9826 (0.5 g) and purified soy bean oil (9.5 g) was introduced into the lecithin dispersion solution (total volume was 100 ml) and emulsified using the Microfluidizer at low pressure (4 000 p.s.i.) to prepare a rough emulsion. Then the roughly prepared emulsion was finely emulsified using the Microfluidizer at high pressure (20 000 p.s.i.). Oil droplet size was measured by a dynamic light scattering particle sizer (Zeta-Plus; Brookhaven Instrument, New York, NY) (mean value = 180 nm). TEI-9826 content in the formulation was 5.0 mg/ml.

Antitumor activity of Lipo TEI-9826 against Colon26 tumor *in vivo*

Colon26 tumor cells (1×10^6 cells in 0.1 ml) were inoculated into the right inguinal flank of 5-week-old CDF1 mice (day 0) bred in an animal room lit for 12 h at 25°C over the period of the experiment. At day 10 or day 14, mice with well-formed solid tumors were divided into groups with approximately equal mean tumor size and variation, and the administration of Lipo TEI-9826 or control emulsion was initiated. The following administration routes and schedules were used: once a day bolus i.v., once a day i.p., twice a week s.c., every other day s.c. and 4 times a day continuous i.v. (5 min for an injection). The injection site for s.c. administration was opposite the tumor site (left inguinal flank). Administration periods depended on the study and are indicated in the Results. Tumor size and mouse body weight were measured almost every day. Tumor volume was calculated using the following equation:

$$\text{Tumor volume} = \frac{1}{2}ab^2$$

where a is the longest length and b is the shortet length.

Statistical analysis

Repeated measurement analysis of variance was performed for the results of *in vivo* antitumor activity. If the main group effect or the group × time interaction was significant, the *ad hoc t*-test was applied to compare the non-treatment group and treated group at each time point. The significant level was set at 0.05.

Pharmacokinetic study of Lipo TEI-9826 (80 mg/kg dose) in rat using several administration routes

Male Wistar rats (8 weeks old) were anesthetized by i.p. injection of sodium pentobarbital and restrained in a supine position on a board that maintained the surface temperature at 37°C. A polyethylene tube was inserted in to the carotid artery for blood collection. Lipo TEI-9826 was administered by bolus i.v. injection into the right femoral vein, single i.p. injection and single s.c. injection into the right inguinal flank at 80 mg/kg. For continuous i.v. infusion, Lipo TEI-9826 was infused into the right femoral vein through a polyethylene tube inserted into the vein in advance using a syringe-type infusion pump. The total dose was 80 mg/kg, and the infusion rate was 80 mg/kg/h (60 min infusion), 40 mg/kg/h (120 min infusion) and 20 mg/kg/h (240 min infusion). Whole blood was periodically collected from the carotid artery through the inserted tube and immediately 0.3 ml of whole blood was vigorously mixed with 0.6 ml acetonitrile containing 2-ethylhexyl-*p*-hydoxybenzoate (internal standard for HPLC analysis). The mixture was then centrifuged at 10 000 r.p.m. for 1 min and the supernatant was frozen until analysis. The concentrations of TEI-9826 and TOK-4528 in the supernatant were determined separately by injection of supernatant into HPLC. HPLC conditions were as described above.

Results

Stability of Δ^7-PGA_1, Δ^{12}-PGJ_2, TEI-9826 and TOK-4528 in serum *in vitro*

TEI-9826 was rapidly hydrolyzed to its carboxylic acid form (TOK-4528) ($t_{\frac{1}{2}} < 30$ s) in rat and mouse serum.

Conversely, the stability of TOK-4528 was similar to that of Δ^{12}-PGJ_2 in rat and mouse sera. Compared to TOK-4528, Δ^7-PGA_1 was rapidly converted to Δ^7-PGC1 ($t_{\frac{1}{2}} < 60$ s).[31] Figure 2(A and B) shows the stability of these PGs in rat serum. In human serum, however, the elimination of TEI-9826 and Δ^7-PGA_1 was much slower than in rat or mouse serum (Figure 2C), and the converted compounds differed from those in rat and mouse serum (identification of the converted compounds is in progress). TOK-4528 and Δ^{12}-PGJ_2 were equally stable in human, rat and mouse serum (Figure 2C).

In vitro antitumor activity of PGs against Colon26 carcinoma

Figure 3 shows the dose–response curve of each PG against Colon26 tumor *in vitro*. All PGs exhibited a dose-dependent antitumor effect. Δ^7-PGA_1 and Δ^{12}-PGJ_2 exhibited similar antitumor effects ($IC_{50} = 1.21$ and 1.60 μg/ml, respectively). Although the antitumor effects of TEI-9826 and TOK-4528 were weaker than Δ^7-PGA_1 and Δ^{12}-PGJ_2, the antitumor effects of TEI-9826 and TOK-4528 were nearly identical ($IC_{50} = 2.78$ and 2.73 μg/ml, respectively).

Colony-forming efficacy after exposure to TEI-9826 *in vitro*

Figure 4 shows the colony-forming efficacy of Colon26 cells at several TEI-9826 concentrations and exposure times. The logarithmic plot of efficacy versus concentration exhibited two-phase profiles at the exposure period of 3–72 h: the phase at low concentrations, which had a gentle slope, and the phase at high concentration, which had a steep slope. According to Ozawa *et al.*,[42] pharmacokinetic cell-killing kinetics of anticancer drugs are classified into two types, wherein

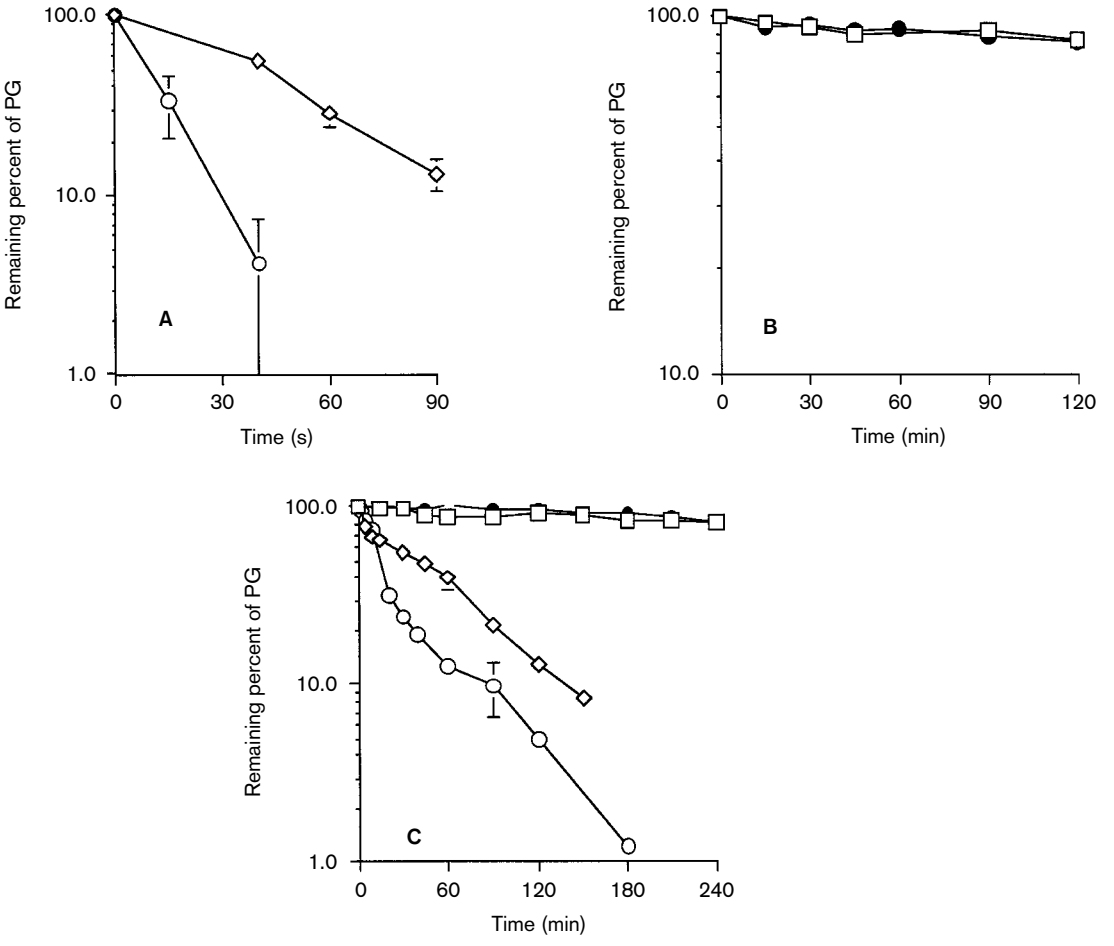

Figure 2. Stability of PGs in rat and human serum. Ethanol solution of each PG was added into prewarmed serum and the concentration was periodically determined by HPLC. Time scale is second order in (A), and minute order in (B) and (C). ○, TEI-9826; ●, TOK-4528; ◇, Δ^7-PGA_1; □, Δ^{12}-PGJ_2. (A and B) Rat serum. (C) Human serum. Data are expressed as mean ± SEM ($n = 3$).

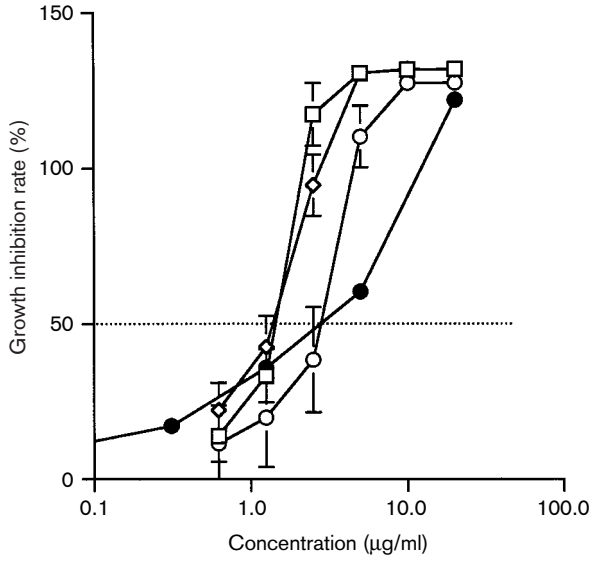

Figure 3. Dose–response curves of PGs against Colon26 tumor *in vitro* calculated from the growth rate at day 2. Colon26 cells were seeded into a 96-well microplate and precultured for 24 h. Freshly prepared medium containing PG was added, and growth rate at days 1 and 2 calculated from the MTT assay data. Growth inhibition rate was calculated from the data at day 2. ○, TEI-9826; ●, TOK-4528; ◇, Δ^7-PGA_1; □, Δ^{12}-PGJ_2. Data are expressed as mean ± SEM (n = 3).

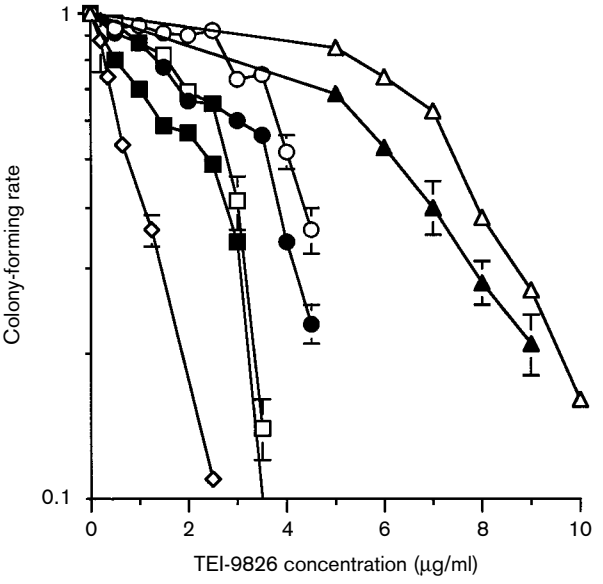

Figure 4. Colony-forming rate of Colon26 tumor cells after several concentrations and time exposure to TEI-9826. Five hundred Colon26 cells were seeded and TEi-9826 added at several concentrations. After several exposure time, cells were washed and cultured for 7 days. The colony numbers were determined and forming rate to relative control was calculated. △, 3 h; ▲, 6 h; ○, 12 h; ●, 24 h; □, 48 h, ■, 72 h; ◇, continuous. Data are expressed as mean ± SEM (n = 4–5).

the logarithmic plot of colony-forming efficacy versus concentration exhibits a linear relationship or a concave relationship. In the present study, the logarithmic plot of colony-forming efficacy versus concentration exhibited a convex curve and, thus, TEI-9826 could not be simply classfied as a conventional type of anticancer drugs.

Antitumor activity and spectrum in a human cancer cell line panel using 38 human cancer strains *in vitro*

Table 1 shows the GI_{50}s of Δ^7-PGA_1 and TEI-9826 against 38 human cancer cell types and the mean GI_{50} of all cell types. GI_{50}s of Δ^7-PGA_1 against 31 cell types were under 10.0 μM, and mean GI_{50} was 6.4 μM (2.2 μg/ml). GI_{50}s of TEI-9826 were under 10.0 μM against all cell types and mean GI_{50} was 3.8 μM (1.3 μg/ml). TEI-9826 exhibited lower GI_{50}s than Δ^7-PGA_1 against 33 out of 38 cell types. Figure 5 shows the mean graphs of Δ^7-PGA_1 and TEI-9826. The fingerprints of Δ^7-PGA_1 and TEI-9826 were compared with those of over 200 standard compounds using the COMPARE program, and Pearson's correlation coefficients (r) were calculated. The r value between Δ^7-PGA_1 and TEI-9826 was 0.690. The r values between Δ^7-PGA_1 or TEI-9826 and other compounds were all less than 0.5, except for Lactacystin (r = 0.62).

Antitumor activity of Lipo TEI-9826 against Colon26 tumor *in vivo*

Figure 6(A) shows the tumor growth profiles for the groups with no treatment, once a day bolus i.v. at 80 mg/kg of Lipo TEI-9826 and once a day i.p. at 80 mg/kg of Lipo TEI-9826 over 9 days. Although tumor growth-suppressing tendency was observed for i.v. injection of Lipo TEI-9826, the effect over the experimental period compared with the no-treatment group was not significant. Conversely, a significant tumor-suppressive effect was observed for i.p. injection during administration period.

Figure 6(B) shows the tumor growth profiles for the no-treatment, twice a week i.p. injection and twice a week s.c. injection at 80 mg/kg groups. We initially confirmed that tumor growth was clearly suppressed by once a day s.c. injection and the suppressive effect continued for 3 days after the last dose. Therefore, we compared the effects of twice a week s.c. and i.p. injection. Subcutaneous injection of Lipo TEI-9826 remarkably inhibited tumor growth for 3 days after an injection. Conversely, i.p. injection seemed to inhibit tumor growth only for 1 day after each injection.

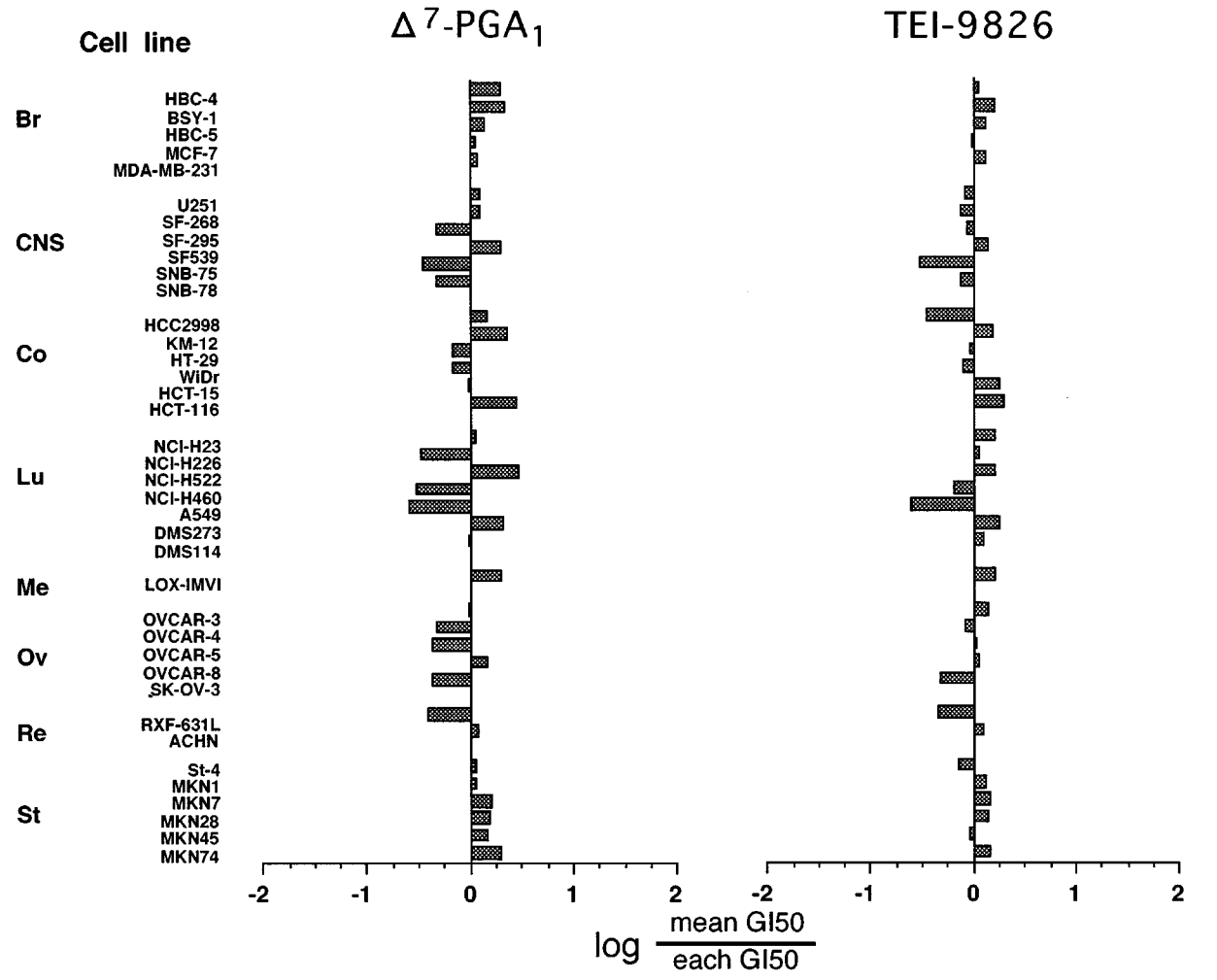

Figure 5. Mean graph of Δ^7-PGA_1 and TEI-9826 based on GI_{50}. Each bar shows the value of log(mean GI_{50}/each GI_{50}). The bar extending to the right, sensitivity to drugs; the bar extending to the left, resistance to drugs. The mean graphs of Δ^7-PGA_1 and TEI-9826 were compared with more than 200 other compounds using the COMPARE computer algorithm, and Pearson's correlation coefficient (*r*) calculated. If the *r* value between A and B is over 0.5, they are suggested to have the same antitumor mechanism.

Figure 6(C) shows the tumor growth profiles for the no-treatment, once a day bolus i.v. injection and 4 times a day continuous i.v. injection (5 min for an injection) groups. Frequent and continuous i.v. injections of Lipo TEI-9826 exhibited significant tumor-suppressive effects during the administration period compared with once a day bolus i.v. injection.

Figure 6(D) shows the tumor growth profiles for the no-treatment, every other day i.p. and every other day s.c. injection over 18 days (10 injections) groups. Although i.p. injection of Lipo TEI-9826 exhibited significant growth-suppressive effects compared with the no-treatment group, the effect was weaker than s.c. injection. The every other day s.c. injection of Lipo TEI-9826 remarkably suppressed tumor growth during the administration period. However, the tumor resumed growth after the treatment using this dose and schedule was completed.

Pharmacokinetic study of Lipo TEI-9826 (80 mg/kg dose) in rat using several administration routes

Figure 7 shows the whole blood concentration of TEI-9826 and TOK-4528 after bolus i.v., single i.p. and single s.c. injection at 80 mg/kg. Although whole blood TOK-4528 concentration at 5 min after bolus i.v. injection was high (average 65 μg/ml), it rapidly decreased and could not be detected after 2 h. The whole blood concentration of TEI-9826 at 5 min after

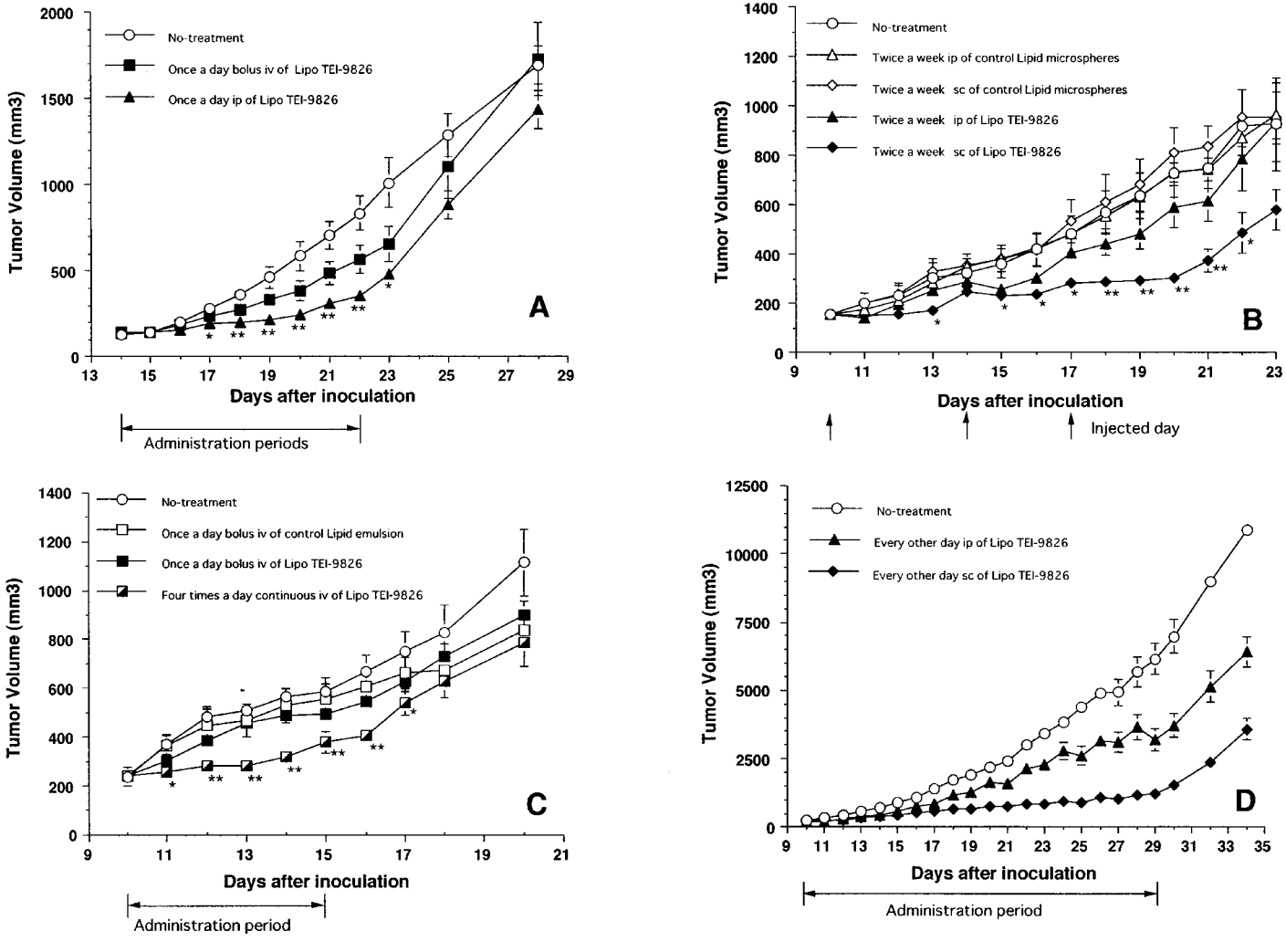

Figure 6. Antitumor activity of Lipo TEI-9826 at a dose of 80 mg/kg against Colon26 tumor s.c. inoculated in CDF1 mice at various administration routes and schedules. Colon26 tumor cells were inoculated into the right inguinal flank of mice (day 0). At day 14 (A) or day 10 (B–D), administration of Lipo TEI-9826 or control lipid microspheres was started. Administration route, schedule and period are shown. (A) Comparison of once a day bolus i.v. and once a day i.p., $n=6$. (B) Comparison of intermittent i.p. and intermittent s.c., $n=4$–6. (C) Comparison of once a day bolus i.v. and 4 times a day continous (5 min for an injection) i.v., $n=5$–6. (D) Comparison of every other day i.p. and every other day s.c. for a long time, $n=4$–5. Data are expressed as mean $\pm$ SEM. The main group effects and the group $\times$ time interactions in (A)–(D) were all significant. $^{*}p<0.05$; $^{**}p<0.01$ versus no-treatment group. In (D), there was a significant difference ($p<0.01$) between the s.c. group and no-treatment group from day 13 to 34, and between the i.p. group and no-treatment group from day 18 to 32.

bolus i.v. injection was 1.45 μg/ml and was also eliminated within 2 h. Whole blood TOK-4528 concentration appeared to be sustained after single i.p. injection; TOK-4528 could be detected until 3 h and, although TEI-9826 concentration was low, it was also detected until 3 h. A concentration around 1.0 μg/ml was sustained for over 90 min. Conversely, the whole blood TOK-4528 concentration was remarkably sustained after single s.c. injection compared with i.v. and i.p. injection. Although the initial profiles for the s.c.

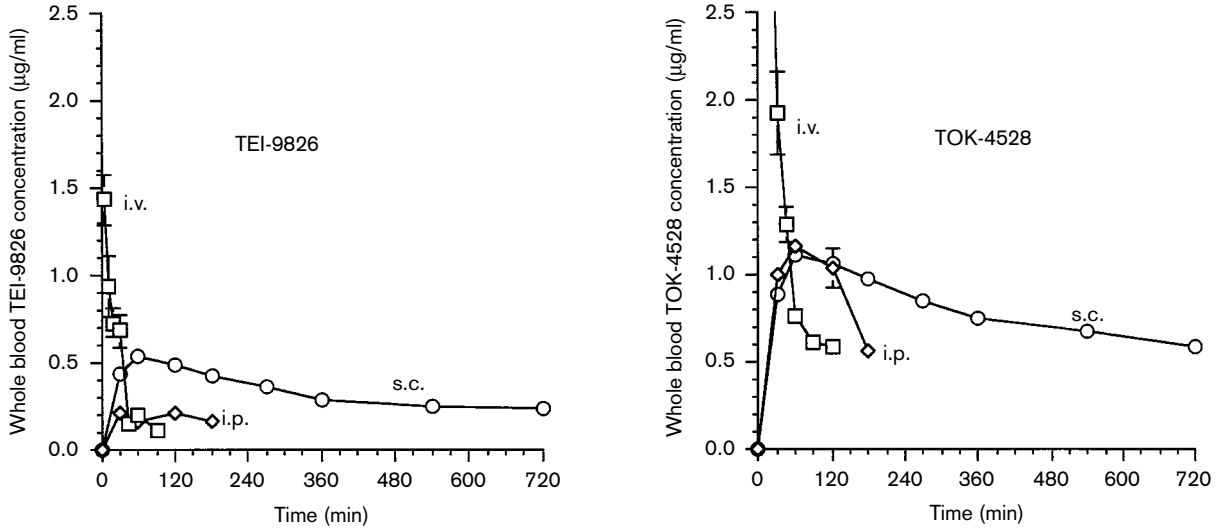

Figure 7. Whole blood concentration of TEI-9826 and TOK-4528 after administration of Lipo TEI-9826 (80 mg/kg) at bolus i.v., i.p. and s.c. in Wistar rats. Whole blood was collected from the carotid artery through an inserted polyethylene tube and immediately mixed with acetonitrile containing internal standard for HPLC. TEI-9826 and TEI-9826 in supernatant were determined by HPLC. The detection limit concentration was 0.1 μg/ml for TEI-9826 and 0.4 μg/ml for TOK-4528 as the concentration in whole blood. Data are expressed as mean ± SEM (n=3).

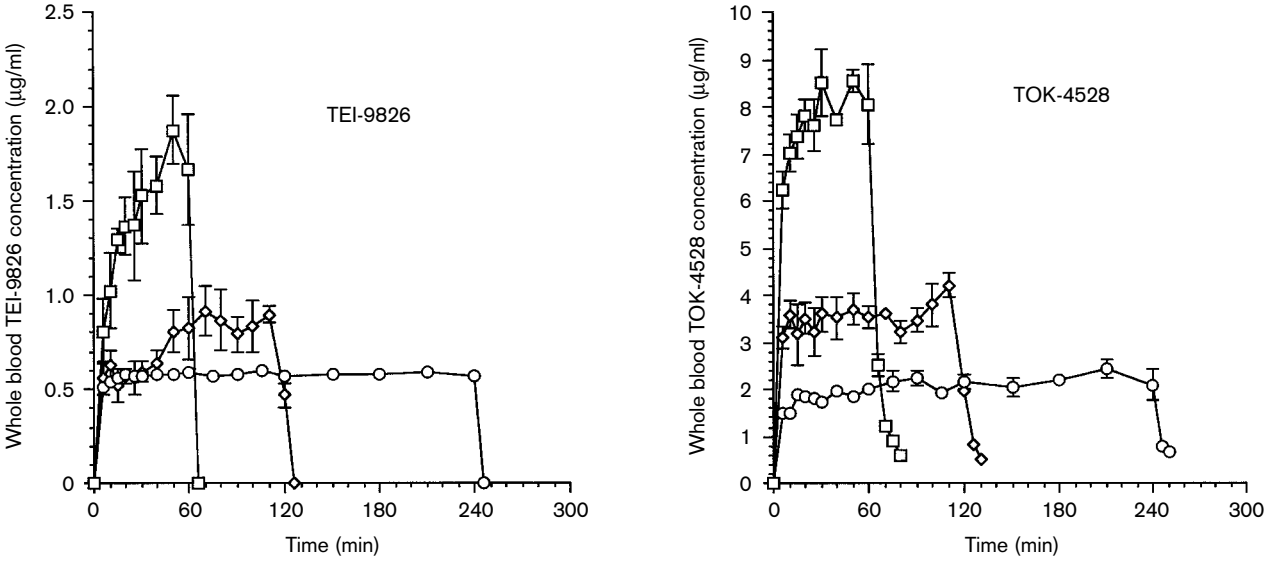

Figure 8. Whole blood concentration of TOK-4528 and TOK-4528 at constant i.v. infusion of Lipo TEI-9826 (80 mg/kg) at three infusion rates. Lipo TEI-9826 was infused using a syringe-type infusion pump into the right femoral vein through an inserted polyethylene tube and whole blood was collected from the carotid artery through an inserted polyethylene tube. Collected whole blood was immediately mixed with acetonitrile containing internal standard for HPLC. TEI-9826 and TEI-9826 in supernatant were determined by HPLC. The detection limit concentration was 0.1 μg/ml for TEI-9826 and 0.4 μg/ml for TOK-4528 as the concentration in whole blood. □, 80 mg/kg/h, 60 min; ◇, 40 mg/kg/h, 120 min; ○, 20 mg/kg/h, 240 min. Data are expressed as mean ± SEM (n=3).

injection was similar to the i.p. injection, TOK-4528 and TEI-9826 could be detected after 12 h. We conducted a constant infusion study in order to estimate pharmacokinetic parameters. Figure 8 shows the whole blood concentration profiles of TOK-4528 and TEI-9826 at three infusion rates. In the TOK-4528 concentration–time profile, steady state was reached within 10 min at the 20 and 40 mg/kg/h infusion rates, and within 30 min at the 80 mg/kg/h infusion rate. The steady state was maintained during the infusion period, and TOK-4528 and TEI-9826 were rapidly eliminated after the end of infusion.

Table 2 shows the calculated pharmacokinetic parameters for Lipo TEI-9826. Although the area under the concentration–time curve (AUC) for bolus i.v. and single s.c. was nearly identical, AUC for single i.p. was significantly smaller. The AUCs for the three constant infusion rates, bolus i.v. and single s.c. were nearly identical. The steady-state concentration (C_{ss}) was in proportion to infusion rate ($r = 0.998$). The half-life ($t_{\frac{1}{2}}$) was very short and no significant differences were observed among groups.

Discussion

Hydrolysis of TEI-9826 to TOK-4528 by esterase was very rapid ($t_{\frac{1}{2}} < 30$ s) in rat and mouse serum *in vitro*. This hydrolysis rate did not change when Lipo TEI-9826 was used instead of TEI-9826 ethanol solution (data not shown), suggesting that TEI-9826 was rapidly released from oil droplets in the lipid microsphere and also rapidly hydrolyzed to TOK-4528. Conversely, due to the low esterase activity in human serum compared to rat and mouse serum, the elimination half-time of TEI-9826 in human serum *in vitro* was 30 min and the converted compound was not TOK-4528. Identification of the compound is currently in progress.

Although the growth inhibitory effects of TEI-9826 and TOK-4528 against Colon26 tumor cells *in vitro* were weaker than those of Δ^7-PGA_1 and Δ^{12}-PGJ_2, the average GI_{50} of TEI-9826 against 38 human tumor strains was lower than that of Δ^7-PGA_1, and each GI_{50} against 33 strains out of 38 human tumor strains was lower than that of Δ^7-PGA_1. Thus TEI-9826 possesses a similar or greater potential for growth inhibitory effects than Δ^7-PGA_1 *in vitro*. COMPARE analysis using a panel of 38 human cancer cell lines suggested that Δ^7-PGA_1 and 13,14-dihydro-15-deoxy-Δ^7-PGA_1 (TEI-9826) share a similar mode of action which is different from those of other antitumor compounds.

In order to clarify the cytotoxic kinetics of TEI-9826 *in vitro*, we conducted a colony-forming assay and the results was analyzed based on Sugiyama's method.[42] However, the logarithmic plot of colony-forming efficacy versus concentration exhibited a two-phase profile and we could not classify TEI-9826 following Sugiyma's classification, which is based on the assumption that a logarithmic plot of colony-forming efficacy via concentration exhibits a linear relation. The effect of antitumor PG on the cell cycle depends on its concentration; at the IC_{50} level cells are arrested at the G_1 phase, and above the IC_{50} level cells are arrested at the G_2/M phase and irreversible cell death begins.[5] The two-phase profiles observed in the colony-forming assay in the present study might result from the concentration-dependent effect of TEI-9826.

The antitumor effect of Lipo TEI-9826 on Colon26 tumor-bearing mice *in vivo* was studied under various injection routes. Contrary to the weak anticancer effect of the once a day bolus i.v. injection, significant growth suppression was observed after i.p. injection and s.c. injection. The injection route-dependent anticancer effect might not be due to the intrinsic characteristics of TEI-9826, but rather to the volume of Lipo TEI-9826 injected. Specifically, the injection volume of Lipo TEI-9826 at 80 mg/kg was as high as 0.4 ml/mouse. Because of this large volume, absorption of emulsion and/or TEI-9826 release from emulsion into the body was sustained at i.p. and s.c. injection, and therefore the blood concentration of TEI-9826 and/or TOK-4528 was sustained, resulting in a significant anticancer effect. This is especially true for s.c. injection, as Lipo TEI-9826 might remain at the

Table 2. Pharmacokinetic parameters of TOK-4528

	Dose (mg/kg)	C_{ss} (μg/ml)	$AUC_{0-\infty}$ (μg/ml·min)	CL_t (ml/min/kg)	V_d (l/kg)	k_{el} (min^{-1})	$t_{1/2}$ (min)
Bolus i.v.	80		529.8±78.7	171.7±27.8	1.93±0.55	0.19	7.57±1.41
i.p.	80		161.5±10.2				
s.c.	80		520.6±32.7				
60 min continuous i.v. infusion	80	7.65±0.31	514.8±12.0	174±6.75	0.96±0.12	0.19±0.02	3.76±0.37
120 min continuous i.v. infusion	80	3.43±0.23	435.8±25.1	197±14.0	1.59	0.11	6.1
240 min continuous i.v. infusion	80	1.96±0.05	507.6±18.1	170±0.68	1.42±0.68	0.10±0.06	5.73±2.60

Calculated from the data of figures 7 and 8; $n = 3$–5, mean±SEM.

injection site for a long time and, thus, the growth-suppressive effect of Lipo TEI-9826 might be greater than for i.p. injection. For bolus i.v. injection, because Lipo TEI-9826 immediately mixed with blood and TEI-9826 was rapidly released, blood concentration might not be sustained, resulting in only weak effect. According to this hypothesis, the anticancer effect might be observed for continuous i.v. injection or frequent i.v. injection. Therefore, the effects were compared between single bolus i.v. injection and 4 times a day continuous injection (5 min per injection). The results indicate that, although the tumor growth profiles of the no-treatment and single bolus i.v. injection groups were nearly identical, significant growth-suppressive effect was observed for the frequent and continuous i.v. injection groups.

A special feature of the effects of Lipo TEI-9826 on tumor growth *in vivo* was that tumor growth was suppressed only during the administration period and tumor growth resumed after administration was completed. In order to confirm this result, tumor growth profiles were examined after long-term administration (every other day s.c. and i.p. injection for 20 days at 80 mg/kg). Even under this administration schedule, tumor growth was suppressed only during the administration period. These results suggest that Lipo TEI-9826 possesses a cytostatic effect at this dose (80 mg/kg) *in vivo*. As previously mentioned,[5] antitumor PG exhibited G_1 arrest at the GI_{50} concentration *in vitro*, suggesting that Colon26 tumor might be arrested at the G_1 phase in this *in vivo* treatment study.

In order to examine the pharmacokinetics of Lipo TEI-9826, whole blood concentrations of TEI-9826 and TOK-4528 were determined in rats. Under s.c. administration, for which the growth-suppressive effect was the greatest, TEI-9826 and TOK-4528 were detectable for longer than 12 h. Because AUC were identical for s.c. administration and bolus i.v. injection, and because a growth-suppressive effect was not observed for bolus i.v. injection, the effect of Lipo TEI-9826 might not depend on AUC, but rather duration of blood concentration. The AUC for i.p. injection less than one-third of s.c. administration was most likely as a result of hepatic metabolism after absorption into the portal vein before penetration into systemic circulation. Under s.c. injection, peak blood concentration of TEI-9826 was 0.6 μg/ml and a concentration over 0.3 μg/ml was maintained over 12 h. Peak blood concentration of TOK-4528 was 1.2 μg/ml and a concentration over 0.6 μg/ml was maintained over 12 h. These concentrations were roughly equal to the IC_{50} under continuous exposure conditions in the *in vitro* colony-forming assay. These results suggest that concentrations of Lipo TEI-9826 must be maintained at a certain level over a long period for antitumor effects to be observed and that the most suitable administration method of Lipo TEI-9826 is continuous i.v. infusion. In the constant rate i.v. infusion study, the steady-state TOK-4528 and TEI-9826 concentrations were proportional to the infusion rate, and no significant difference was observed among the three infusion rates for AUC, V_d, and K_{el}. These results show the linear disposition of Lipo TEI-9826 at a dose of 80 mg/kg and, therefore, it is possible to predict the concentration from the infusion rate.

In conclusion, the present study demonstrates the antitumor activity of TEI-9826 and suggests that TEI-9826 possess a new antitumor mechanism which is different from those of other clinically used antitumor drugs. Lipo TEI-9826 exhibited a growth-suppressive effect *in vivo* and continuous i.v. infusion is suggested to be the optimum administration route of Lipo TEI-9826. Control and prediction of the disposition of Lipo TEI-9826 by continuous i.v. infusion might be easy because Lipo TEI-9826 exhibits linearity. These results warrant the clinical trial of Lipo TEI-9826 by continuous i.v. infusion.

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