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FK317: a novel substituted dihydrobenzoxazine with potent antitumor activity which does not induce vascular leak syndrome

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Abstract *Purpose*: FK973, a substituted dihydrobenzoxazine, is an antitumor antibiotic which has shown high therapeutic efficacy in a phase I study, but its development has been abandoned because of the side effect of vascular leak syndrome (VLS) in the clinical study. This study was performed to investigate whether or not FK317, a new benzmethoxy derivative of FK973, retains the antitumor activity of FK973 without the side effect of VLS. *Methods*: VLS was evaluated by the volume of pleural effusion in rats. Cytotoxic activities were determined by a tetrazolium-based colorimetric assay (MTT assay) against murine (B16, P388) and human (HeLa S3, KB) tumor cell lines. Antitumor activities against murine ascitic leukemia (P388, L1210), murine solid tumors (reticulum cell sarcoma M5076, Colon 38 carcinoma) and human xenografts (mammary carcinoma MX-1, lung carcinoma LX-1) were examined. *Results*: FK973 (1.8 mg/kg) given i.v. to rats induced pleural effusion, one of the elements of VLS, 36 days after the first dosing, but did not 28 days after dosing. This model reflects clinical VLS delayed-type effusion with high protein concentrations. In contrast, FK317 (1.0–3.2 mg/kg) did not induce pleural effusion at all. FK317 had stronger cytotoxic effects against in vitro cultured B16, P388, HeLa S3 and KB tumor cell lines, and in in vivo experiments, FK317 showed equivalent antitumor activity against P388, M5076 and MX-1, and more potent antitumor activity against L1210, Colon 38 and LX-1 compared with FK973. *Conclusion*: These results suggest that FK317 retains the antitumor activity of FK973 and does not induce VLS, and FK317 is a drug with high clinical potential for treating tumors in humans.

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Introduction

The potent antineoplastic agent, FK973 [11-acetyl-8-carbamoyloxymethyl-4-formyl-14-oxa-1,11-diazatetracyclo ($7.4.1.0^{2,7}.0^{10,12}$) tetradeca-2,4,6-trien-6,9-diyl diacetate], was obtained by chemical modification of a novel antibiotic FR900482 isolated from the fermentation products of *Streptomyces sandaensis* No. 6897 [6, 7, 13]. The antitumor activity of FK973 is higher than or equivalent to that of mitomycin C (MMC), adriamycin and cisplatin against murine tumors and human xenografts in mice, and its hematotoxic and myelosuppressive effects are weaker than those of MMC in mice [14]. FK973 has also been shown to have high therapeutic efficacy in clinical studies. The development of this compound, however, was abandoned because of its side effect of vascular leak syndrome (VLS) which is characterized by pericardial and pleural effusion, ascites and subcutaneous edema [8, 10]. The mechanism of VLS has not been well elucidated, but it has been encountered in many clinical trials using high doses of interleukin-2 (IL-2) [9, 11, 12, 16], recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) [2, 4] or immunotoxins [1, 19].

During attempts to synthesize new FK973 derivatives which retain the antitumor activity of FK973 without the side effect of VLS, FK317 [11-acetyl-8-carbamoyloxymethyl-4-formyl-6-methoxy-14-oxa-1,11-diazatetracyclo($7.4.1.0^{2,7}.0^{10,12}$) tetradeca-2,4,6,-trien-9-yl acetate] was selected. In the present study, FK317 showed equivalent or more potent antitumor activity than FK973 and MMC, and did not induce pleural effusion in rats. These results indicate that FK317 would probably show a similar high therapeutic efficacy without the side effect of VLS in clinical studies.

Materials and methods

Drugs

FK317 and FK973 were prepared in the Fujisawa Research Laboratories. The chemical structures of FK317 and FK973 are shown in Fig. 1. MMC was purchased from Kyowa Hakko Kogyo Co., Tokyo, Japan. FK317 and FK973 were dissolved in and diluted with 10% polyoxyethylenehydrogenated caster oil 60 in saline (HCO60 solution). MMC was dissolved in and diluted with saline. The solutions were given intraperitoneally (i.p.) or intravenously (i.v.) at a volume of 10 ml/kg body weight. Saline or 10% HCO60 solution was given to the control animals.

In the vitro culture experiments, all the drugs were dissolved in or diluted with culture medium supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, Utah).

Animals

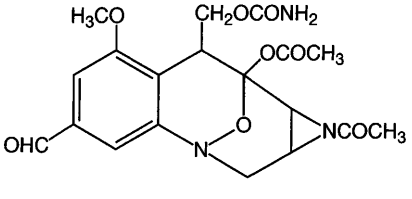
Male Sprague-Dawley (SD) rats were purchased from SLC Japan, Hamamatsu, Japan. Female BDF_1, CDF_1, $B_6C_3F_1$, DBA/2 and C57BL/6 mice were purchased from Charles River Japan, Atsugi, Japan. Male BALB/cnu/nu mice were purchased from CLEA Japan, Tokyo, Japan. The experiments in this study were performed in accordance with the guidelines for animal experiments of Fujisawa Pharmaceutical Co. Ltd.

Evaluation of VLS in rat model

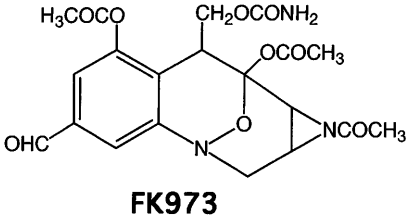
VLS in rats evaluated according to a modification of the method of Siegall et al. [15]. Drugs were given i.v. to 7-week-old male SD rats twice a week for 2 weeks. The rats were killed by exposure to carbonic acid gas and the hydrothorax fluid was collected by placing the rats on their back, carefully opening the chest wall, and aspirating the fluid using a 2.5-ml syringe with an oesophageal intubation tube.

In vitro cytotoxicity

The MTT [3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, Mo.) assay was used to measure cytotoxicity. P388 cells were maintained and treated in suspension in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin (Flow Laboratories, North Ryde, Australia), and 50 μ*M* 2-mercaptoethanol (Sigma). B16 cells were maintained and treated in suspension in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. HeLa S3 and KB cells were maintained and treated in suspension in DMEM medium (Sigma) supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. The cell were grown in an atmosphere of 5% CO_2/95% air at 37 °C. The cells were incubated with drugs in 96-well microplates for 48 h (P388 and B16 cells) or 72 h (HeLa S3 and KB cells). After the addition of MTT (10 μl/well, 5 mg/ml in phosphate-buffered saline), the plates were incubated for 4 h. The absorbance was measured at 580 nm using a Titertek Twinreader (Titertek, McLean, Va.).



FK317



FK973

Fig. 1 Chemical structures of FK317 and FK973

Evaluation of antitumor effect in vivo

L1210 and P388 leukemia cells were maintained i.p. by serial passage in DBA/2 mice. Colon 38 carcinoma cells were maintained s.c. by serial passage in C57BL/6 mice. M5076 reticulum cell sarcoma cells were maintained i. p.by serial passage in C57BL/6 mice. Human tumor cells (LX-1 small cell lung carcinoma, MX-1 mammary adenocarcinoma) were maintained s.c. by serial passage in BALB/c nu/nu mice.

P388 cells (1×10^6) or L1210 cells (1×10^5) were inoculated i.p. into CDF_1 mice on day 0. In the experiments with P388 cells, 12 and 6 mice were used in the control and drug-treated groups, respectively. In the test with L1210, 20 and 10 mice were used in the control and drug-treated groups, respectively. Drug efficacy was assessed in terms of the median survival time (MST) of the treated group expressed as a percentage of that of the control group.

In the experiments on murine solid tumors, fragments ($2 \times 2 \times 2$ mm) of Colon 38 or M5076 cells (1×10^6) were inoculated s.c. into the left flank to female BDF_1 or $B_6C_3F_1$ mice on day 0, respectively. In all the experiments, 10 mice were used per group. Tumor weight was calculated using the following formula: tumor weight (mg) $= L \times W^2/2$ where L and W represent the length (mm) and the width (mm) of the tumor mass, respectively, measured using calipers. Antitumor activity was determined by comparing the mean tumor weight of each group (T) with that of the respective control (C) and is expressed as a percentage ($T/C \times 100$).

Tumor masses in BALB/c nu/nu mice were excised, cut into fragments ($3 \times 3 \times 3$ mm) of MX-1 or LX-1, and transplanted s.c. into the right flank of other BALB/c nu/nu mice. Six mice were used per group. The initial and final tumor volume were calculated on the first injection day (just before injection) and last evaluation day, respectively. The changes in mean tumor weight are shown for each group of mice. Change in mean tumor weight = mean tumor weight (final)/mean tumor weight (initial). The antitumor activity was estimated 10 days after the last administration and determined by comparing the mean tumor weight of the test group (T) with that of control group (C). Growth inhibition (%) was determined from the expression (1 – change in mean tumor weight test group/change in mean tumor weight control group) × 100.

Results

Effects of FK317 and FK973 in a rat VLS model

We found that four i.v. injections of FK973 to rats induced pleural effusion more than 1 month after the first injection. FK973 (1.8 mg/kg) was given i.v. to SD rats twice a week for 2 weeks, and the volume of pleural effusion was measured. The average volume of the

Table 1 Effect of FK973 on hydrothorax in rats. FK973 was administered i.v. Fluid accumulated in the thoracic cavity was collected on the indicated days after the first administration of the drug. Three and 10 rats were used in the control and drug-treated groups, respectively. Values are means ± SE. Numbers in parentheses are number of survivors/number of rats tested

	Hydrothorax fluid (ml)		
	Day 28	Day 36	Day 44
Control	0 ± 0 (3/3)	0 ± 0 (3/3)	0 ± 0 (3/3)
FK9731 1.8 mg/kg	0.6 ± 0.1 (10/10)	3.3 ± 1.9 (8/10)	6.2 ± 1.4 (5/10)

pleural effusion is shown in Table 1. Five out of ten rats died as a result of the toxicity of FK973 by 44 days after the first dosing. The pleural effusion on day 36 contained a high concentration of protein (average 56.3 mg/ml). This phenomenon was thought to be a similar syndrome to the VLS induced by FK973 in clinical trials. Next, we examined whether FK317 (1.0–3.2 mg/kg) would cause pleural effusion 35 days after the first dosing in this rat model. As shown in Fig. 2, large amounts of clear fluid in the thoracic cavity (hydrothorax) of rats were observed at a dose of 1.8 mg/kg of FK973. On the other hand, FK317 at doses of 1.0, 1.8 and 3.2 mg/kg did not induce pleural effusion at all. However, 7 of 15 rats died as a result of myelosuppressive toxicity in the FK317 (3.2 mg/kg) groups. The results suggests that FK317 would hardly induce VLS in clinical use.

Cytotoxic activity

The cytotoxicity of FK317, FK973 and MMC were examined in various murine (B16, P388) and human (HeLa S3, KB) tumor cell lines, and the results are shown in Table 2. The cytotoxic activities of FK317 against murine and human cell lines were approximately threefold and six- to ninefold stronger than those of FK973, respectively. FK317 showed the strongest cytotoxic effect of all, especially against human tumor cell lines.

Table 2 Cytotoxicity of FK317, FK973 and MMC against various tumor cell lines. Each value is the average of three determinations (IC_{50} concentration producing 50% inhibition of cell growth)

Drug	IC_{50} (nM)			
	B16	P388	HeLa S3	KB
FK317	1100	11	3.4	8.4
FK973	3200	30	19	77
MMC	5800	90	110	500

Antitumor activities against murine ascitic tumors

We tested the antitumor effects of FK317 against two kinds of murine ascitic tumors in comparison with the effects of FK973 and MMC. Murine ascitic tumors were inoculated i.p. into mice, and drugs were injected i.p.

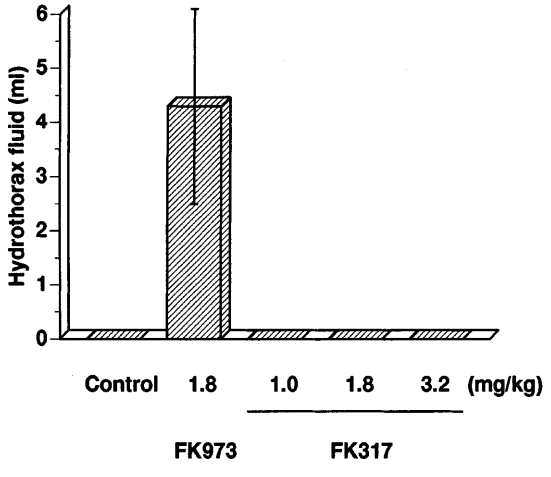


Fig. 2 Effects of FK317 and FK973 on hydrothorax in rats. FK317 or FK973 was administered i.v. Fluid accumulated in the thoracic cavity was collected 35 days after the first administration of the drugs. Ten and 13–15 rats were used in the control and drug-treated groups, respectively

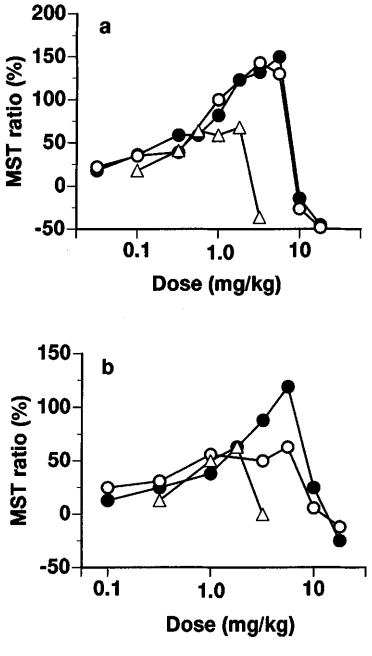


Fig. 3a,b Comparative antitumor activities of FK317 (●), FK973 (○) and MMC (△) in murine ascitic tumors. P388 (**a**) and L1210 (**b**) tumor cells were inoculated i.p. into mice (day 0) and the drugs were given i.p. once a day for 5 days (day 1–5, P388) or for 9 days (day 1–9, L1210) (*MST* median survival time)

once a day for 5 consecutive days from day 1 (P388) or 9 consecutive days from day 1 (L1210). As shown in Fig. 3a, FK317, FK973 and MMC dose-dependently prolonged the life of mice bearing P388. The highest MST ratio (%) values of FK317, FK973 and MMC were 250%, 243% and 168%, respectively. The antitumor activity of FK317 against P388 was almost equal to that of FK973. In addition, both FK317 and FK973 were active at doses of 0.032 to 5.6 mg/kg. FK317 was decidedly superior to MMC in both its antitumor effect and the width of its effective dose range. Against L1210, these drugs also showed antitumor effects in mice (Fig. 3b). The highest MST ratio (%) values of FK317, FK973 and MMC were 219%, 163% and 163%, respectively. FK317 had the strongest and widest antitumor effect compared with FK973 and MMC. The antitumor activity of FK317 was greater or equivalent to that of FK973, and greater than that of MMC against P388 and L1210.

Antitumor activities against murine solid tumors

To compare the antitumor effects of the drugs against murine solid tumors, M5076 and Colon 38 were implanted s.c. into mice on day 0. The drugs were administrated i.v. every 3 days from day 1 for a total of four doses. The results are shown in Fig. 4. FK317, at doses of 3.2 and 5.6 mg/kg, inhibited the growth of M5076 with T/C values of 68% and 79%, respectively. This efficacy of FK317 was approximately equivalent to that of FK973 and MMC. In the tests on Colon 38, the tumor weights were markedly decreased by treatment with FK317 at doses of 3.2 to 10 mg/kg, with T/C values of more than 90%. Although the maximum percent tumor growth inhibition of FK317 was almost the same as those of FK973 and MMC, the effective dose range of FK317 was wider. The antitumor activity of FK317 was greater or equivalent to that of FK973 and MMC against M5076 and Colon 38.

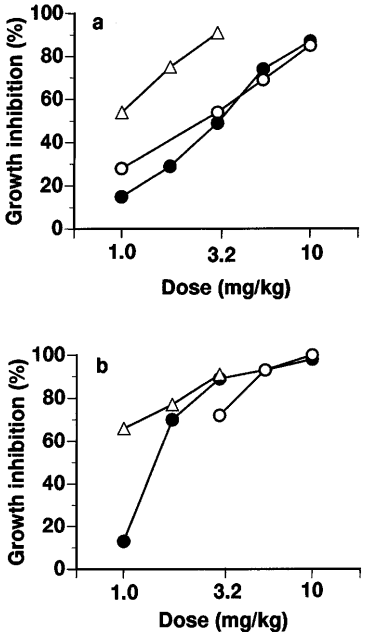


Fig. 4a,b Comparative antitumor activities of FK317 (●), FK973 (○) and MMC (△) in murine solid tumors. M5076 (**a**) and Colon 38 (**b**) tumor cells were inoculated s.c. into mice on day 0. The drugs were given i.v. to the mice on days 1, 4, 7 and 10. Tumor weights were measured on day 21

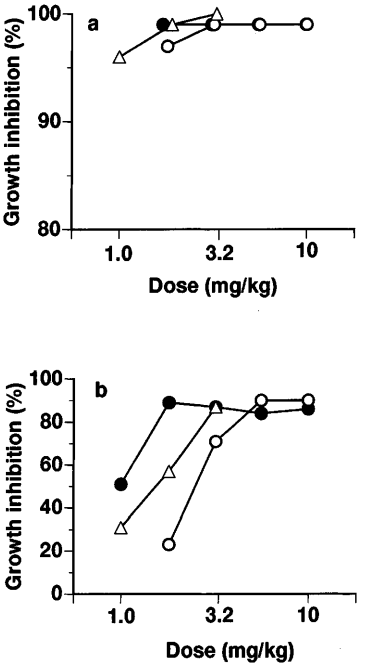


Fig. 5a,b Comparative antitumor activities of FK317 (●), FK973 (○) and MMC (△) in human tumor xenografts. MX-1 (**a**) and LX-1 (**b**) tumor cells were inoculated s.c. into mice (day 0) and the drugs were given i.v. three times at 4-days intervals beginning on day 10. Tumor weights were measured on day 21

Antitumor activity against human tumor xenografts

The antitumor effects of FK317 were evaluated against human MX- 1 mammary carcinoma and LX-1 small cell lung carcinoma xenografts implanted s.c. into BALB/c nude mice and compared with those of FK973 and MMC (Fig. 5). When the estimated tumor weight in the mice had grown to between 100 and 300 mg, the drugs were given i.v. to the mice every 4 days for a total of three doses. All drugs in doses of more than 1 mg/kg showed curative activity against MX-1 with a tumor growth inhibition of ≥96%. Against LX-1, FK317 exhibited wide curative activity at doses ranging from 1.8 to 10 mg/kg. The efficacy of FK317 was almost the same as that of FK973 and MMC, but FK317 showed the widest effective dose range. These drugs had a strong antitumor activity against MX-1, and FK317 was more potent than FK973 and MMC against LX-1.

Discussion

FK317 is a novel derivative of an active antitumor antibiotic, FK973. FK973 has been shown to have high therapeutic efficacy in a phase I study, but its development has been abandoned because it induces VLS characterized by pericardial and pleural effusions, ascities, and subcutaneous edema [8, 10]. VLS was first observed a little after 1 month from the first i.v. administration of FK973, and the pericardial fluid collected from the patients was considered to be exudate because of its very high protein concentration. This delayed side effect was not foreseen from the results of animal studies [8, 10]. It has been reported that the toxic effects of IL-2 [9, 11, 12, 16], rhGM-CSF [2, 4] and immunotoxins [1, 19] can also be described as VLS. Siegall et al. [15] established the VLS model in rats as seen in human clinical trials utilizing targeted immunotoxins. In this model, VLS is observed 24 h after administration of immunotoxin. We also produced an FK973-induced VLS model in rats. Intravenous injection of FK973 (1.8 mg/kg) into rats twice a week for 2 weeks induced pleural effusion more than 1 month after the first injection. VLS induced by FK973 was observed 36 days, but not 28 days, after the first dosing. This model is thought to reflect well clinical VLS delayed-type effusion with high protein concentration.

VLS induced by FK973 might be caused by inflammatory effects because the thoracic fluid contains high concentrations of protein, and C-reactive protein and erythrocyte sedimentation rates of the patients were high in the clinical study [8, 10]. However, the precise mechanism of FK973-induced VLS is unclear. Some direct and indirect effects on the integrity of the vascular endothelial cells have been postulated as mechanisms in VLS. Cytokines such as tumor necrosis factor (TNF), arachidonic acid metabolites, oxygen free radicals and the complement system might be included in the cause of endothelial damage. TNF is known to be produced or released by IL-2. However, undetectable or low levels of TNF have been observed in patients treated with FK973 [10].

The reason why FK317 does not induce pleural effusion in rats is not clear. FK317 as well as FK973 were considered to induce endothelial damage in vitro. The disappearance rate of FK317 in several organs was also faster than FK973 in mice and rats (data not shown). FK973 produced skin inflammation/edema at the site of injection, but FK317 did not to any significant extent (data not shown). In addition, the benzmethoxy group of FK317 is very stable in the blood, and the benzacetoxy group of FK973 is easily hydrolyzed in the blood (data not shown). These differences might explain why FK317 does not induce pleural effusion in rats.

The in vitro antitumor activity of FK317 was greater than that of FK973 or MMC against all the tumors tested. In particular, the cytotoxic effect of FK317 against human tumor cell lines was much stronger. In vivo, FK317 showed equivalent antitumor activities against P388, M5076 and MX-1, and more potent antitumor activities against L1210, Colon 38 and LX-1 than FK973, and its activity was superior to MMC. The effects of chemotherapeutic agents against murine and human tumors, which were used in this study, have been reported to be positively correlated [3, 5, 17, 18]. MMC has been widely used in the treatment of various human solid tumors. FK973 produced a partial response in colorectal cancer, pancreatic cancer, malignant lymphoma, synovial sarcoma, and histiocytoma in the phase I study [8, 10]. FK317 showed more potent or equivalent antitumor activity against the same types of tumor in mice. These findings suggest that FK317 may show antitumor effects at least against these tumors.

In conclusion, FK317 is a derivative which retains the antitumor activity of FK973 and does not induce VLS. These results suggest a very promising potential for FK317 in cancer chemotherapy.

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