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# FK317, a Novel Substituted Dihydrobenzoxazine, Exhibits Potent Antitumor Activity against Human Tumor Xenografts in Nude Mice

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**The antitumor effects of FK317, a novel substituted dihydrobenzoxazine, were evaluated using human tumor xenografts (small cell lung cancer, non-small cell lung cancer, stomach cancer, colon cancer, pancreatic cancer, breast cancer, cervical cancer and ovarian cancer). Tumor growth-inhibitory effects and the effective dose-range of FK317 were much stronger and broader, respectively, than those of reference drugs such as mitomycin C, adriamycin, cisplatin, taxol and irinotecan. Furthermore, the body weight decrease and myelosuppression in FK317-treated mice were less than in the animals given any of the reference drugs. To explain this tumor selectivity, the distribution of FK317 was investigated after dosing tumor-bearing mice with the $^{14}$C-labelled compound. The concentration of FK317 in tumor tissues was relatively low, and long tumor retention was not observed. However, thin-layer chromatographic separation revealed that the radioactivity in the tumor resided mainly in strongly cytotoxic metabolites, while that in other tissues resided mainly in non-cytotoxic metabolites. These results suggest that FK317 shows strong antitumor activity without side effects, and one reason for this is its specific metabolite pattern. FK317 is now undergoing phase I clinical trials.**

Key words: FK317 — Antitumor effect — Human tumor xenograft — Selective toxicity — Mitomycin C

A 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 the novel antibiotic FR900482, which was isolated from the fermentation products of *Streptomyces sandaensis* No. 6897.[1–3] This compound has a unique chemical structure including a hydroxylamine function, whose hydroxyl group forms an intramolecular hemiketal moiety. The antitumor activity of FK973 is equivalent to, or more potent than, those of mitomycin C (MMC), adriamycin (ADR) and cisplatin (CDDP) against murine tumors and human xenografts in mice, and its hematotoxic and myelosuppressive effects are weaker than those of MMC in mice.[4] FK973 showed good efficacy in clinical studies, but its development was terminated because of a vascular leak syndrome (VLS) side effect which was characterized by pericardial and pleural effusion, ascites and subcutaneous edema.[5,6]

Various FK973 derivatives were synthesized in an attempt to isolate the antitumor activity of FK973 from the VLS side effect, and 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 for further examination. FK317 showed similar antitumor activity to FK973, but did not induce pleural effusion in rats.[7] FK317 contains an aziridine ring and a carbamoyl moiety, which are also present in MMC. MMC is a "bioreductive alkylating agent," which causes damage to DNA after reductive activation of the prodrug to form a DNA-reactive species.[8,9] FK317 also forms DNA-DNA interstrand and DNA-protein cross-links in the cell. However, FK317 can not form DNA cross-links in a cell-free system. We found that FK317 requires two activation steps to convert it to a DNA reactive species. The first step is deacetylation. FK317 has two acetyl groups, one of which masks the aziridine ring, which is an important moiety for formation of cross-links. Deacetyl metabolites of FK317 are activated to DNA-reactive species by reduction in the cell.[10] Thus, FK317 is a new type of bioreductive alkylating agent with unique activation characteristics, and is expected to have strong antitumor activity and low toxicity.

This paper describes the activity of FK317 against non-sensitive human carcinomas xenografted in athymic mice and demonstrates the selectivity of FK317.

## MATERIALS AND METHODS

**Chemicals** FK317, the metabolites of FK317 (M1–M7), and [$^{14}$C]FK317 (332 MBq/mmol) were prepared in the

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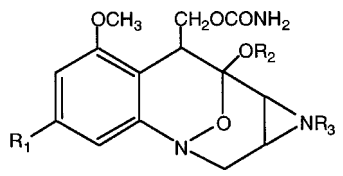
Fujisawa Research Laboratories. The chemical structures of FK317 and its metabolites are shown in Fig. 1. MMC and ADR were purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo. CDDP was purchased from Sigma Chemical Co., St. Louis, MO. Irinotecan hydrochloride (CPT-11) was purchased from Yakult Honsha Co., Ltd., Tokyo. Taxol was purchased from Bristol-Myers Squibb Co., New York, NY.




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| FK317 | CHO | $COCH_3$ | $COCH_3$ |
| M1 | CHO | H | $COCH_3$ |
| M7 | CHO | H | H |
| M2 | COOH | $COCH_3$ | $COCH_3$ |
| M3 | COOH | H | $COCH_3$ |
| M4 | COOH | H | H |
| M6 | $CH_2OH$ | H | $COCH_3$ |
| M5 | $CH_2OH$ | H | H |
| [$^{14}$C]FK317 | $^{14}$CHO | $COCH_3$ | $COCH_3$ |

Fig. 1. Chemical structures of FK317 and its metabolites.

Table I. Antitumor Activity of FK317 and Reference Drugs on Human Tumor Xenografts in Nude Mice (SCLC and NSCLC [Adenocarcinoma of Lung])

| Drug | Dose (mg/kg) | Growth inhibition (%) | | | |
|---|---|---|---|---|---|
| | | LX-1 | Lu-134 | LC-17 | PC-9 |
| FK317 | 1.0 | 78 | 27 | | |
| | 1.8 | 98 | 56 | 77 | 31 |
| | 3.2 | 99 | 92 | 94 | 41 |
| | 5.6 | 98 | 99 | 97 | 75 |
| | 10 | TOX[a)] | TOX | | |
| MMC | 1.0 | 23 | 27 | 55 | 23 |
| | 1.8 | 47 | 70 | 85 | 34 |
| | 3.2 | 90 | 99 | 95 | 62 |
| ADR | 1.8 | −1 | | | |
| | 3.2 | 24 | | 33 | 23 |
| | 5.6 | 36 | | 42 | 44 |
| | 10 | TOX | | | |
| CDDP | 1.8 | 15 | | | |
| | 3.2 | 38 | | 12 | 4 |
| | 5.6 | 71 | | 56 | 38 |
| | 10 | TOX | | | |

Tumor cells were inoculated s.c. into nude mice. Drugs were administered i.v. to mice when tumor weight had grown to between 100 and 300 $mm^3$. Antitumor activity was evaluated around 10 days after the last administration. Mice were used in groups of six.
*a*) TOX, a survival rate of <65% on the evaluation day was taken to indicate toxicity.

**Animals and tumor cells** Female mice of the $BDF_1$ strain were purchased from Charles River Japan Inc., Yokohama, and male mice of the BALB/c *nu*/*nu* strain were purchased from CLEA Japan Inc., Tokyo. Human tumors were maintained s.c. by serial passage in BALB/c *nu*/*nu* mice: LX-1 and Lu-134 (small cell lung cancer), LC-17 and PC-9 (non-small cell lung cancer (NSCLC) [adenocarcinoma]), LC-6 and Lu-99 (NSCLC [large cell carcinoma]), QG-56 (NSCLC [squamous cell carcinoma]), SC-6, NS-8 and MKN-45 (stomach cancer), COL-5 (colon cancer), and OC-5, OC-9, OC-10 and OC-11 (ovarian cancer). LX-1 and MX-1 were supplied by Dr. T. Tashiro (Cancer Chemotherapy Center, Japan Foundation for Cancer Research, Tokyo). PC-9 and MKN-45 were

Table II. Antitumor Activity of FK317 and Reference Drugs on Human Tumor Xenografts in Nude Mice (NSCLC [Large Cell and Squamous Cell Carcinoma of Lung])

| Drug | Dose (mg/kg) | Growth inhibition (%) | | |
|---|---|---|---|---|
| | | LC-6 | Lu-99 | QG-56 |
| FK317 | 1.0 | 98 | 80 | 46 |
| | 1.8 | 100 | 82 | 56 |
| | 3.2 | 100 | 82 | 74 |
| | 5.6 | 100 | 88 | 91 |
| | 10 | TOX[a)] | | |
| MMC | 1.0 | 77 | 61 | 40 |
| | 1.8 | 97 | 73 | 52 |
| | 3.2 | 99 | 82 | 72 |
| | 10 | TOX | | |
| ADR | 1.8 | | | 4 |
| | 3.2 | | 36 | 42 |
| | 5.6 | | 60 | 48 |
| | 10 | | | TOX |
| CDDP | 1.8 | | | 27 |
| | 3.2 | | 39 | 30 |
| | 5.6 | | 55 | 62 |
| | 10 | | | TOX |
| CPT-11 | 56 | | | 71 |
| | 100 | | | 88 |
| Taxol | 10 | | | 80 |
| | 18 | | | 97 |

Tumor cells were inoculated s.c. into nude mice. Drugs were administered i.v. to mice when tumor weight had grown to between 100 and 300 $mm^3$. Antitumor activity was evaluated around 10 days after the last administration. Mice were used in groups of six or five.
*a*) TOX, a survival rate of <65% on the evaluation day was taken to indicate toxicity.

obtained from Immuno Biological Laboratories Co., Ltd., Fujioka. The others were obtained from the Central Institute for Experimental Animals, Kawasaki.

**Evaluation of antitumor effect *in vivo*** Fragments (3×3×3 mm) of human solid tumor were s.c. implanted into the right flank of BALB/c *nu*/*nu* mice. When the esti-

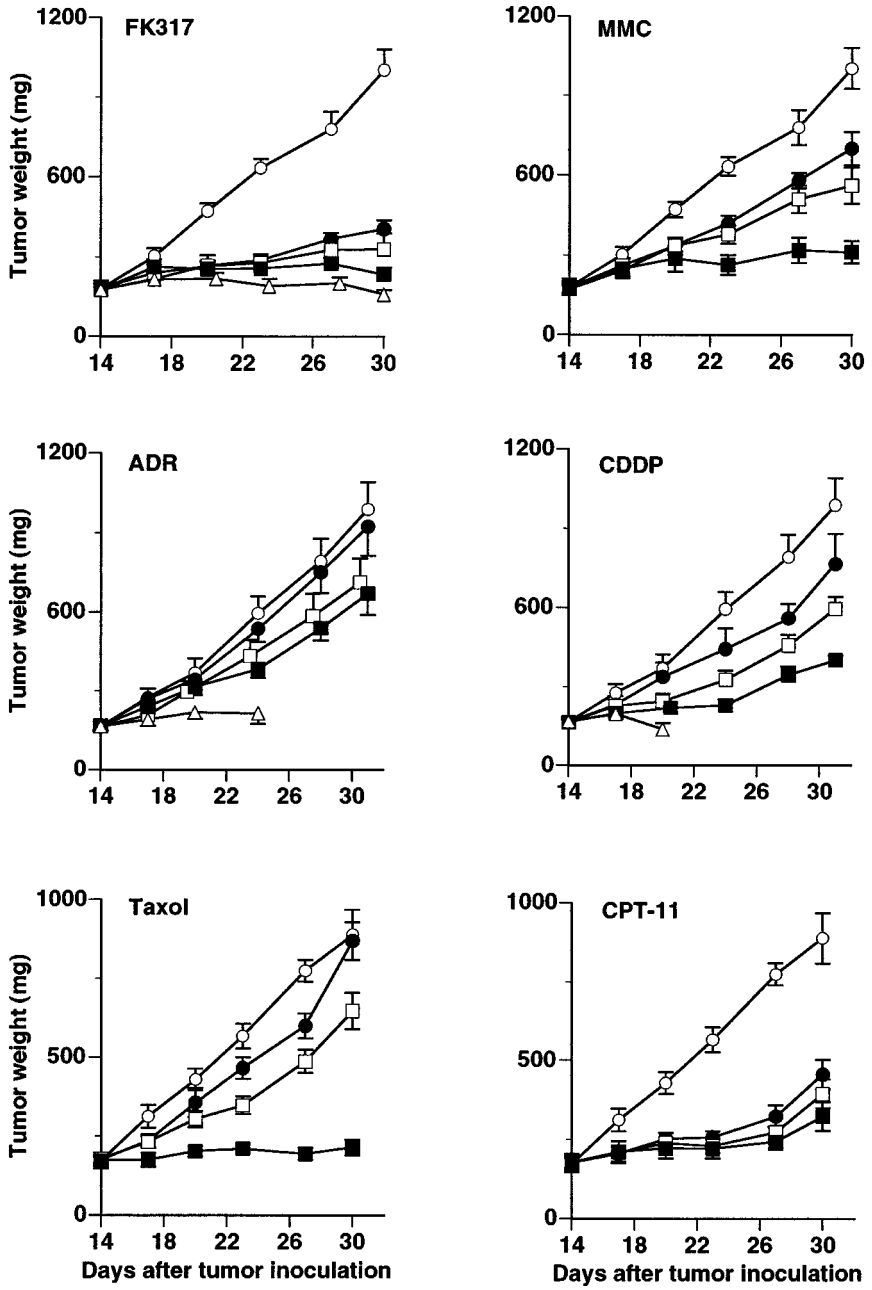



Fig. 2. Changes in the weight of COL-5 (human colon cancer) after treatment with FK317, MMC, ADR, CDDP, taxol and CPT-11. Tumor cells were inoculated s.c. into nude mice. The drugs were given i.v. to mice 3 times at 3-day intervals, beginning 14 days after tumor inoculation. Mice were used in groups of six. The results are expressed as mean tumor weight±SE. ○ control. FK317: ● 1.0 mg/kg, □ 1.8 mg/kg, ■ 3.2 mg/kg, △ 5.6 mg/kg. MMC: ● 1.0 mg/kg, □ 1.8 mg/kg, ■ 3.2 mg/kg. ADR: ● 1.8 mg/kg, □ 3.2 mg/kg, ■ 5.6 mg/kg, △ 10 mg/kg. CDDP: ● 1.8 mg/kg, □ 3.2 mg/kg, ■ 5.6 mg/kg, △ 10 mg/kg. Taxol: ● 5.6 mg/kg, □ 10 mg/kg, ■ 18 mg/kg. CPT-11: ● 32 mg/kg, □ 56 mg/kg, ■ 100 mg/kg.

mated tumor weight in the mice had reached 100 to 300 mg, the animals were divided into experimental groups of five or six and treated i.v. with a test drug every 3 days for a total of three doses (q3d×3). The tumor weight was calculated from the following formula: tumor weight (mg)=$L \times W^2/2$ where $L$ and $W$ represent the length and the width of the tumor mass, respectively. The criteria for activity were based on regression and/or percent tumor growth inhibition of ≥80%.

**Effects on CFU-C (colony-forming units in culture) and CFU-S (colony-forming units in spleen)** The drugs were given i.v. to mice. The mice were killed by cervical dislocation. Both ends of the femurs were cut off aseptically. Bone marrow cells (BMC) were then flushed out with a syringe with a 26-gauge needle into Hanks' balanced solution. The BMC were counted with an automatic blood analyzer (Sysmex E-4000, Toa Medical Electronics Co., Kobe) and pooled from 5 mice.

CFU-C were measured according to a modification of the method of Pike and Robinson.[11] Briefly, $5 \times 10^4$ BMC in 1 ml of α-minimum essential medium (Flow Laboratories, McLean, VA) supplemented with 0.7% methylcellulose, 16% horse serum (Flow Laboratories) and penicillin (40 units/ml)-streptomycin (40 μg/ml) were plated in 35 mm diameter culture dishes, and 16% L-cell-conditioned medium was used as a source of colony-stimulating factor. Triplicate cultures were made of each cell suspension, and incubated at 37°C in 5% $CO_2$ in humidified air. The colonies were grown for 7 days and counted.

CFU-S were assayed according to a modification of the method of Till and McCulloch.[12] Briefly, BMC ($1 \times 10^5$ cells) were given i.v. to recipient mice that had been irradiated (750 R) with X-rays (Hitachi X-ray Irradiator MBR-1520R, Hitachi Medico Co., Ltd., Tokyo). On day 7, the spleen of the recipient mice was removed and fixed with Bouin's solution, and the spleen colonies were counted under a dissection microscope.

**Plasma and tissue sample preparation** [$^{14}$C]FK317 (1 mg/kg) was administered by a single i.v. bolus injection to LX-1 tumor-bearing mice ($n$=3). Blood and tissues were obtained at 5 min and 2, 6 and 24 h after administration of the drug. Blood was collected from the carotid artery into 1.5 ml heparinized microtubes. The tubes were immediately centrifuged at 3000 rpm for 10 min and the plasma supernatant was separated. The brains, lungs, livers, kidneys and tumors (LX-1) were collected, weighed and disrupted in a Potter homogenizer after addition of 1% acetic acid. Bone marrow was obtained from the femurs. Radioactivity in all the tissues was measured.

FK317 and its metabolites were extracted from the plasma, lung, liver, kidney and tumor by adding acetonitrile; samples were then centrifuged at 10,000 rpm for 3 min, and supernatants were collected and dried under nitrogen flow. The dry residues were reconstituted in 50%

Table III. Antitumor Activity of FK317 and Reference Drugs on Human Tumor Xenografts in Nude Mice (Stomach Cancer)

| Drug | Dose (mg/kg) | Growth inhibition (%) | | |
|---|---|---|---|---|
| | | SC-6 | NS-8 | MKN-45 |
| FK317 | 1.0 | 62 | 36 | 54 |
| | 1.8 | 84 | 72 | 73 |
| | 3.2 | 97 | 95 | 81 |
| | 5.6 | 99 | 98 | 86 |
| | 10 | 99 | TOX | |
| | 18 | TOX[a] | | |
| MMC | 1.0 | 71 | 12 | 29 |
| | 1.8 | 95 | 28 | 53 |
| | 3.2 | 99 | 42 | 80 |
| | 10 | TOX | TOX | |
| ADR | 1.8 | | 8 | |
| | 3.2 | | 27 | 21 |
| | 5.6 | | 49 | 41 |
| | 10 | | TOX | |
| CDDP | 1.8 | | 35 | |
| | 3.2 | | 69 | 6 |
| | 5.6 | | 93 | 48 |
| | 10 | | TOX | |

Tumor cells were inoculated s.c. into nude mice. Drugs were administered i.v. to mice when tumor weight had grown to between 100 and 300 mm$^3$. Antitumor activity was evaluated around 10 days after the last administration. Mice were used in groups of six or five.

*a*) TOX, a survival rate of <65% on the evaluation day was taken to indicate toxicity.

Table IV. Antitumor Activity of FK317 and Reference Drugs on Human Tumor Xenografts in Nude Mice (Ovarian Cancer)

| Drug | Dose (mg/kg) | Growth inhibition (%) | | | |
|---|---|---|---|---|---|
| | | OC-5 | OC-9 | OC-10 | OC-11 |
| FK317 | 1.8 | 82 | | | |
| | 3.2 | 90 | 78 | 64 | 82 |
| | 5.6 | 93 | 83 | 77 | 88 |
| MMC | 1.8 | 51 | | | |
| | 3.2 | 77 | 66 | 50 | 59 |
| CPT-11 | 56 | 61 | | | |
| | 100 | 81 | | | |
| Taxol | 10 | 35 | | | |
| | 18 | 86 | | | |

Tumor cells were inoculated s.c. into nude mice. Drugs were administered i.v. to mice when tumor weight had grown to between 100 and 300 mm$^3$. Antitumor activity was evaluated around 10 days after the last administration. Mice were used in groups of five or eight.

methanol in water. Thin-layer chromatography (TLC) was conducted using precoated Silica gel $60F_{254}$ glass plates (0.25 mm, Merck, Darmstadt, Germany) and an ethyl acetate/formic acid/water (8:1:1, v/v) solvent system. Radioactive spots on the TLC plates were measured using a BAS-2000 (Fuji Film, Tokyo).

***In vitro* cytotoxicity** HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and penicillin (50 units/ml)-streptomycin (50 $\mu$g/ml). Growth inhibition experiments were carried out in 96-well flat-bottomed microplates, and the amount of viable cells at the end of the incubation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, essentially as described by Mosmann.[13] Thus, $1\times10^4$ cells/well in 100 $\mu$l were plated and the drug or the medium alone as a control was added. The cells were cultured for 72 h and after addition of MTT (10 $\mu$l/well, 5 mg/ml in phosphate-buffered saline), the plates were incubated for a further 4 h. The medium was removed and the blue dye formed was dissolved in 150 $\mu$l of 0.04 *N* HCl in isopropanol. The absorbance was measured at 580 nm using a Titertek Twinreader (Titertek, McLean, VA).

## RESULTS

**Antitumor effects on human tumor xenografts** Experiments were performed to evaluate the antitumor effects of FK317 against 18 kinds of human tumors implanted s.c. in BALB/c *nu/nu* mice. After the tumor weight reached about 100 to 300 mg, the drug was given to mice three times at 3-day intervals. The antitumor activity was estimated around 10 days after the final injection. The toxic dose levels of FK317, MMC, ADR, CDDP, taxol and CPT-11 are 10 mg/kg, 5.6 mg/kg, 10 mg/kg, 10 mg/kg, 32 mg/kg and 180 mg/kg, respectively.

FK317 exhibited marked antitumor activity against all 7 kinds of lung cancers tested. The results are shown in

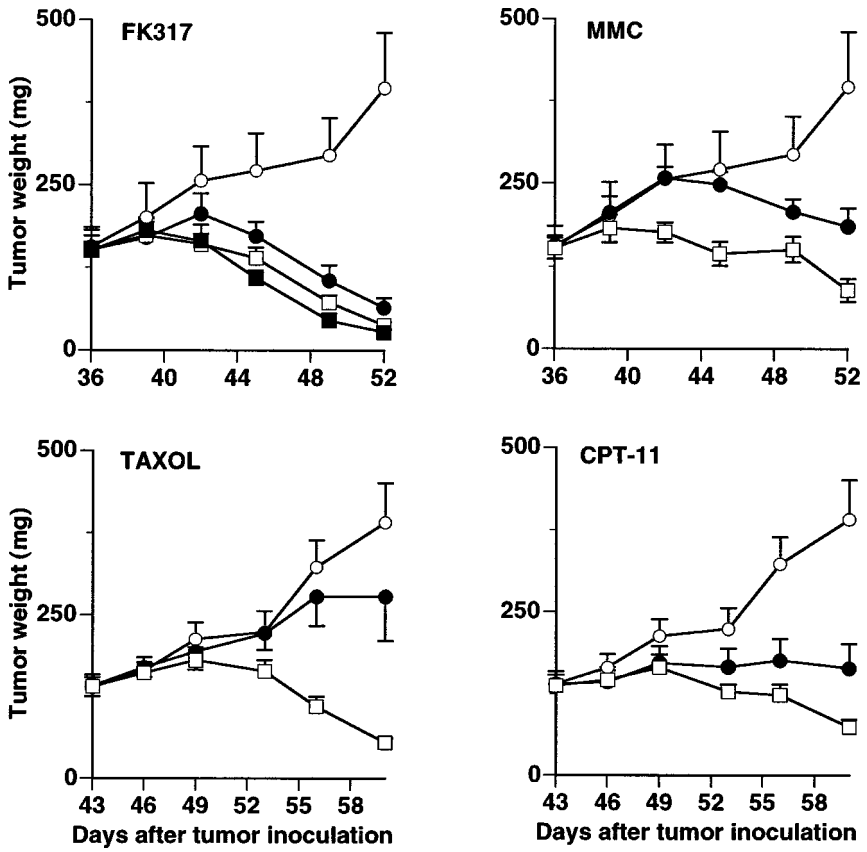



Fig. 3. Changes in the weight of OC-5 (human ovarian cancer) after treatment with FK317, MMC, taxol and CPT-11. Tumor cells were inoculated s.c. into nude mice. The drugs were given i.v. to mice 3 times at 3-day intervals, beginning 36 or 43 days after tumor inoculation. Mice were used in groups of six. The results are expressed as mean tumor weight±SE. ○ control. FK317: ● 1.8 mg/kg, □ 3.2 mg/kg, ■ 5.6 mg/kg. MMC: ● 1.8 mg/kg, □ 3.2 mg/kg. Taxol: ● 10 mg/kg, □ 18 mg/kg. CPT-11: ● 56 mg/kg, □ 100 mg/kg.

Tables I and II, and Fig. 2. FK317 induced marked regression of s.c. tumors of LX-1, Lu-134, LC-17, LC-6 and Lu-99 at various doses tested, and especially over a 5-fold dose range (1.0 to 5.6 mg/kg) in the tests on LC-6 and Lu-99. MMC was also effective on these lung cancers, but its effective dose range was quite narrow when compared with that of FK317. Neither ADR nor CDDP exhibited antitumor activity at all. Against QG-56, FK317 at a dose of 5.6 mg/kg showed strong antitumor activity, which was approximately equal to that of CPT-11 or taxol. MMC, ADR and CDDP were ineffective. Although FK317 failed to show growth inhibition of more than 80% against PC-9, which is a slow-growing tumor, FK317 at a dose of 5.6 mg/kg did cause regression of this tumor.

Against human stomach cancer, SC-6, NS-8 and MKN-45, FK317 exhibited antitumor activity at multiple doses tested, and tumor regression was observed in all cases (Table III). MMC also exhibited antitumor activity against SC-6 and MKN-45, but its effective dose range was narrower than that of FK317, and it had no antitumor effect on NS-8. CDDP at a dose of 5.6 mg/kg showed antitumor activity against NS-8, but ADR was ineffective against all the stomach cancers tested.

The results of the tests on ovarian cancer (OC-5, OC-9, OC-10 and OC-11) are shown in Table IV and Fig. 3. Although the growth rates of these tumors were very slow, FK317 showed tumor growth inhibition of more than 80% against OC-5, OC-9 and OC-11, and tumor regression activity was observed at various dose against all the ovarian cancers tested. MMC, CPT-11 and taxol showed activities against OC-5 at doses of 3.2, 100 and 18 mg/kg, respectively, whereas the effective dose range of FK317 was the widest of all.

FK317 also demonstrated good inhibitory activity in four additional human tumor xenograft models (COL-5, PAN-3, MX-1 and UCC-6). The results are shown in Table V. FK317 at a dose of 5.6 mg/kg showed good antitumor effects on COL-5 and UCC-6, on which MMC was ineffective. COL-5 was not sensitive to CPT-11 or taxol, and none of the reference drugs (MMC, ADR and CDDP) was effective against UCC-6.

Table VI shows the maximum reduction in body weight observed in the studies using human lung cancer (LC-17 and PC-9) and stomach cancer (MKN-45). The reduction induced by FK317 was obviously smaller than that induced by the reference drugs, and scarcely any reduc-

Table V. Antitumor Activity of FK317 and Reference Drugs on Human Tumor Xenografts in Nude Mice (Colon, Pancreatic, Breast and Cervical Cancer)

| Drug | Dose (mg/kg) | Growth inhibition (%) | | | |
|---|---|---|---|---|---|
| | | COL-5 | PAN-3 | MX-1 | UCC-6 |
| FK317 | 1.0 | 60 | | 100 | 18 |
| | 1.8 | 69 | 23 | 100 | 40 |
| | 3.2 | 77 | 68 | 100 | 77 |
| | 5.6 | 84 | 92 | 100 | 95 |
| | 10 | | | | TOX |
| MMC | 1.0 | 30 | 0 | 100 | 28 |
| | 1.8 | 45 | 35 | 100 | 33 |
| | 3.2 | 66 | 84 | 100 | 57 |
| | 10 | | | | |
| ADR | 1.8 | 7 | –7 | 25 | 16 |
| | 3.2 | 29 | –14 | 36 | 23 |
| | 5.6 | 32 | 29 | 77 | 31 |
| | 10 | TOX[a)] | TOX | TOX | TOX |
| CDDP | 1.8 | 24 | –1 | 100 | 0 |
| | 3.2 | 39 | –1 | 100 | 10 |
| | 5.6 | 59 | 28 | 100 | 46 |
| | 10 | TOX | TOX | TOX | TOX |
| CPT-11 | 32 | 51 | | | |
| | 56 | 58 | | | |
| | 100 | 66 | | | |
| Taxol | 5.6 | 8 | | | |
| | 10 | 31 | | | |
| | 18 | 77 | | | |

Tumor cells were inoculated s.c. into nude mice. Drugs were administered i.v. to mice when tumor weight had grown to between 100 and 300 $mm^3$. Antitumor activity was evaluated around 10 days after the last administration. Mice were used in groups of six.
*a*) TOX, a survival rate of <65% on the evaluation day was taken to indicate toxicity.

Table VI. Effects on Body Weight

| Drug | Dose (mg/kg) | Maximal reduction in body weight (g) | | |
|---|---|---|---|---|
| | | LC-17 | PC-9 | MKN-45 |
| FK317 | 1.0 | | | 2.1 (29) |
| | 1.8 | 0.9 (38) | 0.9 (34) | 2.5 (29) |
| | 3.2 | 0.7 (28) | 0.8 (41) | 2.2 (29) |
| | 5.6 | 3.8 (31) | 1.9 (31) | 2.9 (29) |
| MMC | 1.0 | 0.8 (28) | 1.6 (34) | 2.3 (32) |
| | 1.8 | 1.8 (35) | 2.5 (37) | 3.4 (32) |
| | 3.2 | 5.3 (31) | 4.9 (31) | 4.4 (29) |
| ADR | 3.2 | 1.7 (30) | 0.5 (31) | 3.1 (28) |
| | 5.6 | 2.6 (34) | 4.2 (34) | 4.8 (28) |
| CDDP | 3.2 | 2.9 (30) | 0.5 (27, 31) | 3.7 (28) |
| | 5.6 | 6.2 (30) | 6.2 (31) | 7.1 (31) |

Maximal reduction in body weight: The body weights of mice were measured twice a week, and differences from the values before the drug administration were calculated. The reduction in the body weight when the difference between the value in the control group and that in the drug-treated group was maximal is shown in the table. ( ): days after tumor inoculation.

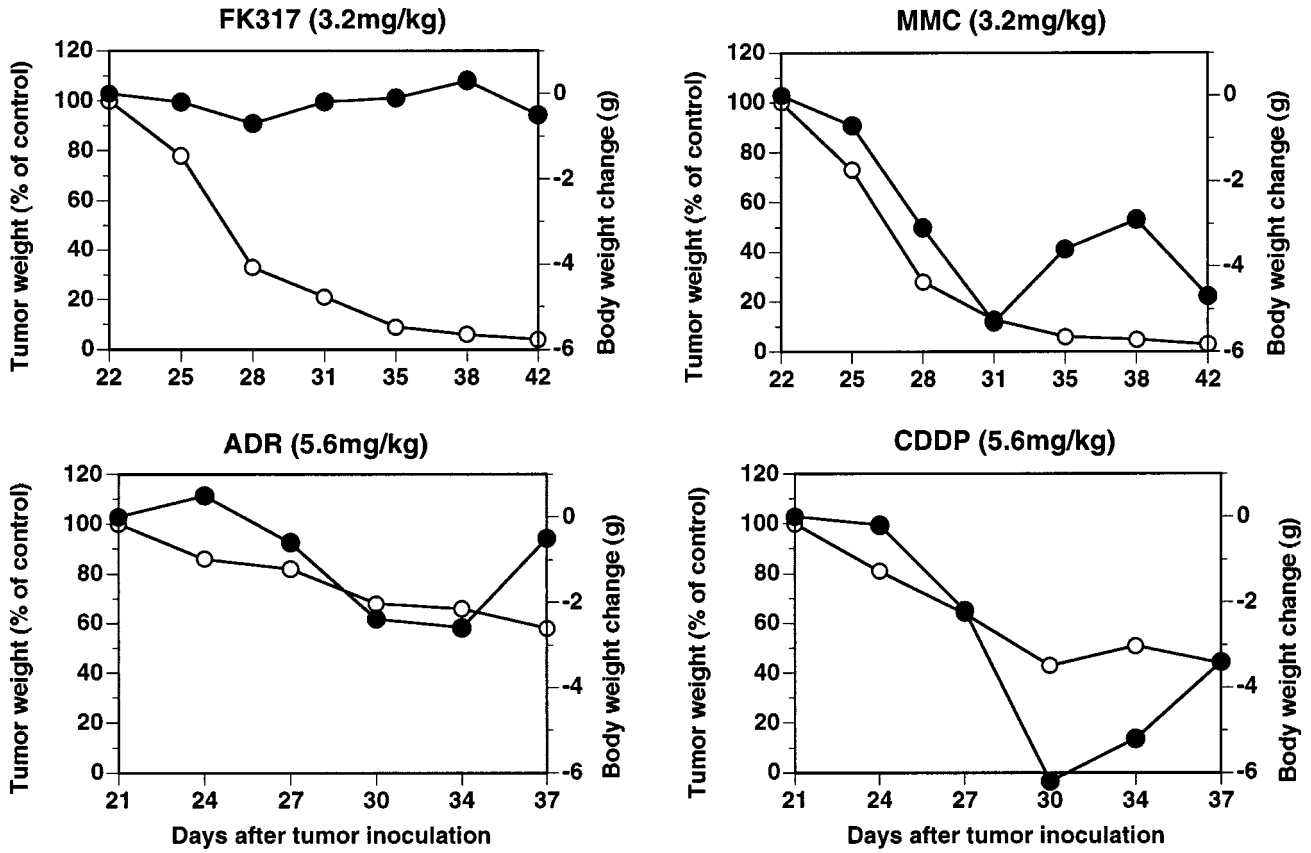



Fig. 4. Tumor growth-inhibitory effect and body weight change. Tumor cells (human lung cancer LC-17) were inoculated s.c. into nude mice. The drugs were given i.v. to mice 3 times at 3-day intervals, beginning 21 or 22 days after tumor inoculation. The results are expressed as percentage of control in tumor weight and the difference from control in body weight change. Mice were used in groups of six. ○ tumor weight, ● body weight change.

tion was observed at a dose of 3.2 mg/kg of FK317, which showed strong antitumor activity against LC-17 (Fig. 4). On the other hand, both tumor growth-inhibitory effect and body weight reduction were observed with 3.2 mg/kg of MMC. ADR and CDDP induced body weight loss, and had no antitumor effect.

**Effects on CFU-C and CFU-S** Fig. 5 shows the time course of percent change in the CFU-C and CFU-S of BMC after a single i.v. injection of FK317 or MMC. FK317 and MMC dose-dependently decreased the number of CFU-C and CFU-S, with the maximum decrease on the day after injection. When compared at the same dose (3.2 mg/kg), the inhibitory effect of FK317 was much weaker than that of MMC. However, the number of CFU-C and CFU-S in the mice treated with 10 and 18 mg/kg of FK317 gradually returned to the control level on day 7 or 14, and the recovery was faster than that in the MMC-treated animals.

**Tissue distribution of FK317** FK317 exhibited strong antitumor effects and good selectivity against human tumor-bearing mice. To explain this good selectivity, the plasma and tissue levels of FK317 were investigated after dosing tumor-bearing mice with [$^{14}$C]FK317. The data represent mean values for three animals and were calculated as nanogram equivalents of FK317 per milliliter or gram. Since these values were based on total drug-related radioactivity, they represent the sum of both FK317 and its metabolites in the samples. The distribution of radioactivity varied widely among the tissues sampled and tumors (Table VII). The plasma concentration observed at the first sampling time (5 min after administration) was 878 ng eq/ml, after which the radioactivity diminished rapidly and at 2 h was approximately 20% of the concentration measured at the initial sampling time. At 6 h the plasma concentration was 60 ng eq/ml, which represented about 7% of the concentration measured at 5 min. The

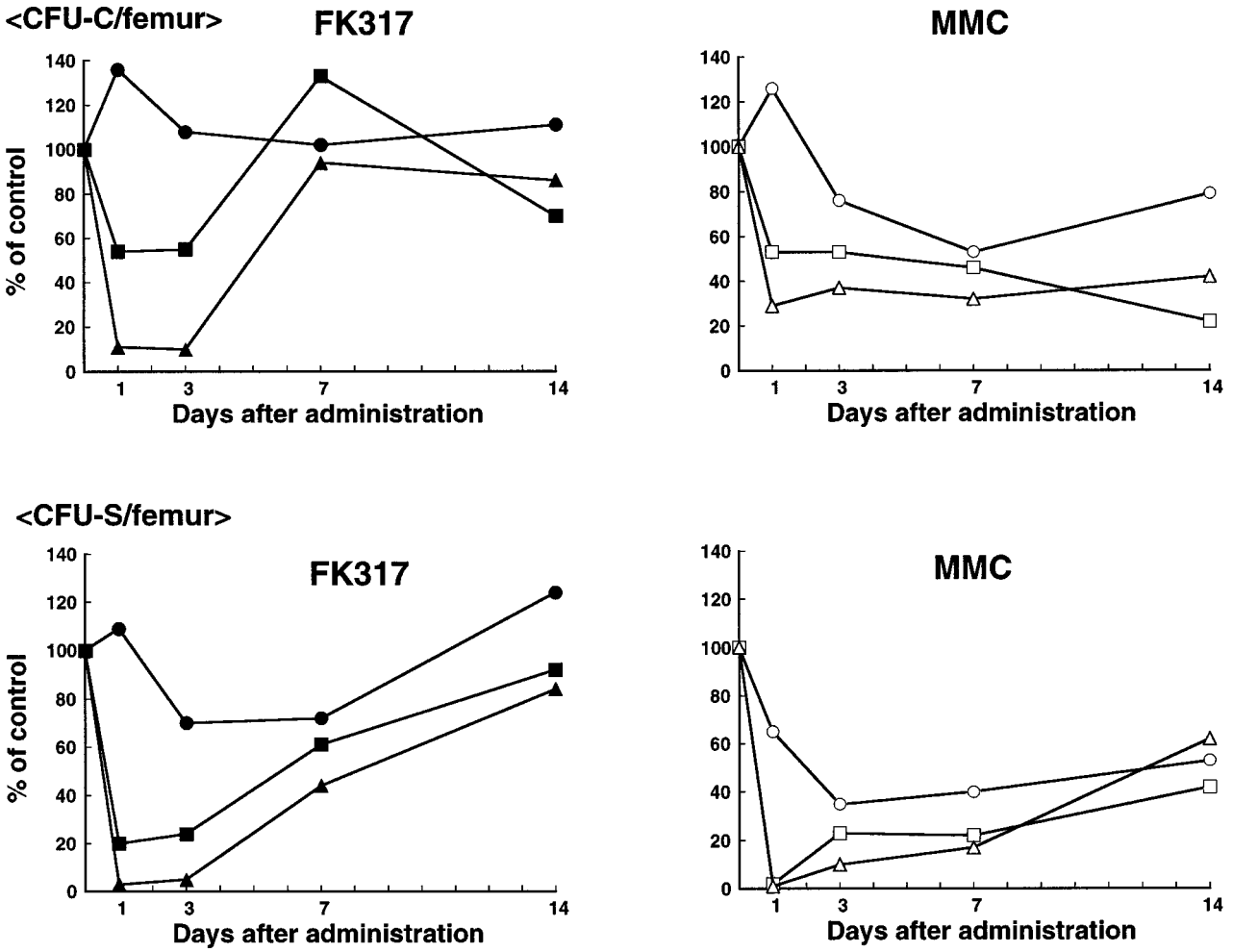



Fig. 5. Effects on the number of CFU-C and CFU-S of bone marrow cell per femur. The drugs were given i.v. to mice on day 0. The numbers of CFU-C and CFU-S of bone marrow cells per femur were examined at various times after treatment. Mice were used in groups of five for each experiment. FK317: ● 3.2 mg/kg, ■ 10 mg/kg, ▲ 18 mg/kg. MMC: ○ 1.0 mg/kg, □ 3.2 mg/kg, △ 5.6 mg/kg.

greatest accumulations were found in the liver and kidney, where the concentrations of radioactivity in these tissues ranged from 2513 to 3383 ng eq/g and were at least 3-fold higher than that of the simultaneous plasma sample. However, the lowest concentrations were found in the tumor and bone marrow. These concentrations were approximately one-third of that in the plasma at the same time, and rapidly decreased. No radioactivity was detectable in the brain.

**Metabolic pattern of FK317 in tissues and tumor** As we could not explain the strong antitumor effects and good selectivity of FK317 in terms of the tissue distribution of FK317, we next examined the metabolic patterns of FK317 in tumor and normal tissues (Fig. 6). Because FK317 has two acetyl groups and a 4-formyl moiety in its chemical structure, FK317 is thought to be metabolized by deacetylation, oxidation and/or reduction in tissues (Fig. 7). FK317 and its metabolites were extracted from plasma, lung, liver, kidney and tumor, and separated into 4-aldehyde, carboxylic acid and alcohol derivatives by TLC. The metabolic pattern in the tumor was quite different from those in normal tissues. The main metabolites in the tumor were 4-alcohol derivatives. On the other hand, in the plasma, lung, liver and kidney, 4-carboxylic acid derivatives were the main metabolites.

**Cytotoxic activities of FK317 and its metabolites** Since the metabolic patterns for FK317 in tumor and normal tissues were different, we tested the cytotoxic effects of FK317 and its metabolites *in vitro*. As shown in Table VIII, the 4-aldehyde (FK317, M1 and M7) and alcohol

Table VII. Tissue Distribution of Radioactivity after i.v. Administration of 1 mg/kg of [$^{14}$C]FK317 to LX-1-bearing Mouse

| Organ | Radioactivity (ng eq/ml or g) | | | |
|---|---|---|---|---|
| | 5 min | 2 h | 6 h | 24 h |
| Plasma | 878[a] (1.0[b]) | 186 (1.0) | 60 (1.0) | n.d.[c] |
| Blood | 2647 (3.0) | 1724 (9.3) | 944 (15.8) | 123 |
| Brain | n.d. | n.d. | n.d. | n.d. |
| Lung | 1222 (1.4) | 370 (2.0) | 148 (2.5) | n.d. |
| Liver | 3383 (3.9) | 1083 (5.9) | 142 (2.4) | n.d. |
| Kidney | 2513 (2.9) | 463 (2.5) | 157 (2.7) | n.d. |
| Bone marrow | 297 (0.3) | 44 (0.2) | n.d. | n.d. |
| Tumor | 256 (0.3) | 84 (0.5) | n.d. | n.d. |

*a*) Mean value ($n$=3).
*b*) Tissue/plasma ratio.
*c*) Not detectable.

derivatives (M5 and M6) exhibited strong antitumor activities against HeLa S3 cells. On the other hand, the carboxylic acid derivatives (M2, M3 and M4) did not have strong cytotoxic activities.

## DISCUSSION

Since human tumor xenografts are among the best models for predicting drug efficacy in clinical settings,[14–16] the activity of FK317 against many human tumors xenografted in athymic mice was examined and compared with those of clinically used antitumor agents, MMC, ADR, CDDP, taxol and CPT-11. FK317 could produce regression and/or ≥80% tumor growth inhibition of almost all tumor xenografts examined, except PC-9 and

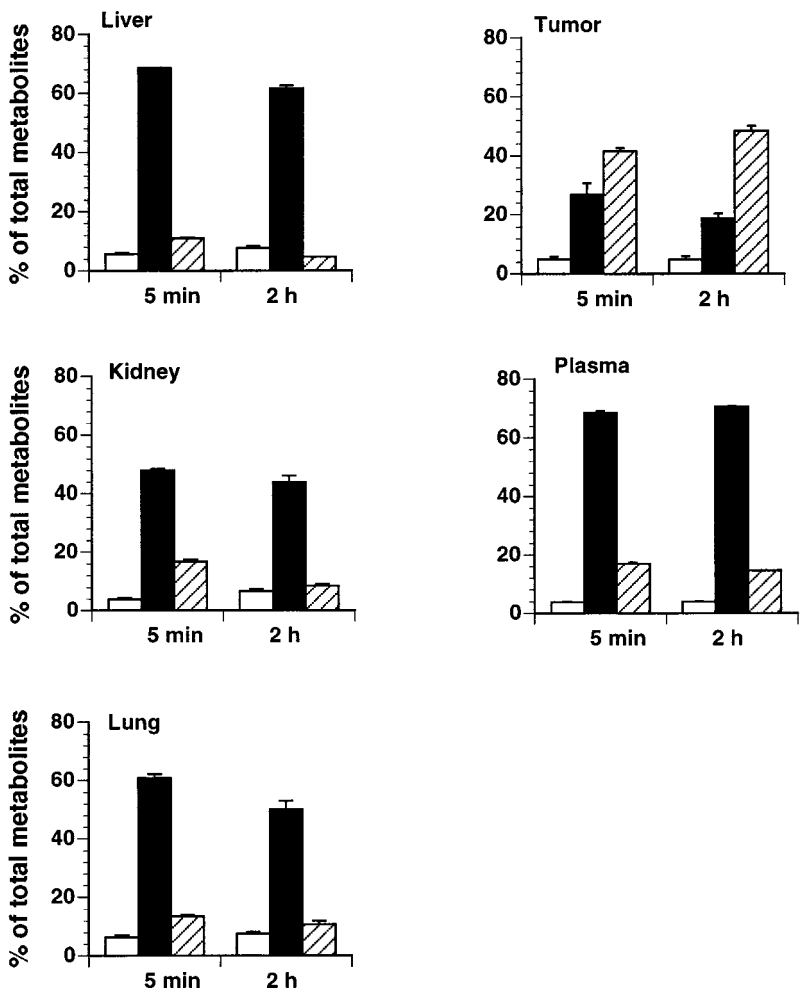



Fig. 6. Proportions of metabolites in tissues after i.v. administration of [$^{14}$C]FK317 to tumor-bearing mice. Tumor cells (human lung cancer LX-1) were inoculated s.c. into nude mice. When the estimated tumor weight in the mice had grown to between 100 and 300 mg, [$^{14}$C]FK317 at a dose of 1 mg/kg was given i.v. to the mice. □ aldehyde metabolites (FK317+M1+M7), ■ carboxylic metabolites (M2+M3+M4), ▧ alcohol metabolites (M5+M6).

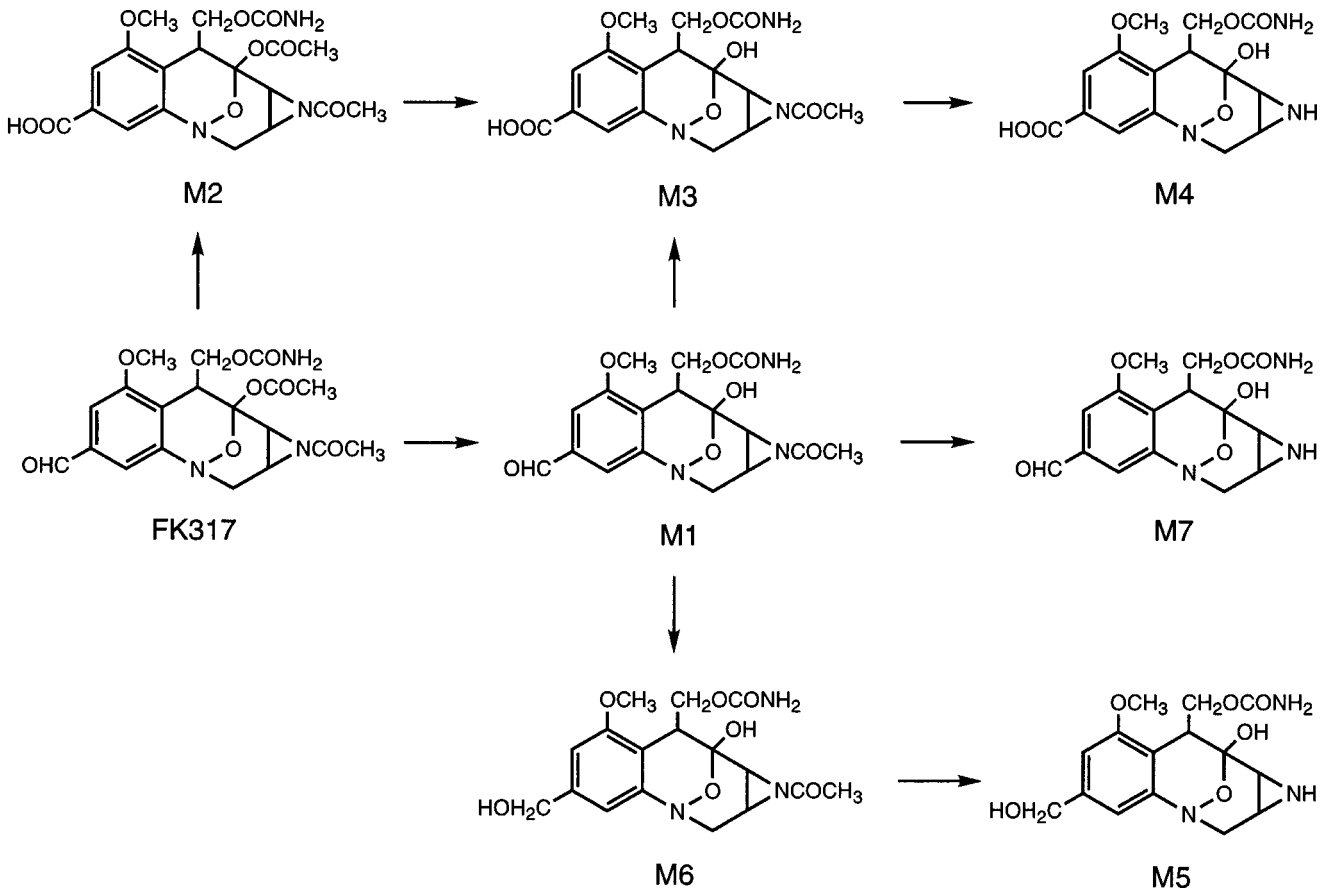



Fig. 7. Proposed metabolic pathways of FK317.

Table VIII. Cytotoxicity of FK317 and Its Metabolites towards HeLa S3 Cells

| Metabolite | $IC_{50}$[a] (*M*) |
|---|---|
| FK317 | $<10^{-9}$ |
| M1 | $<10^{-9}$ |
| M7 | $1.5\times10^{-9}$ |
| M2 | $7.7\times10^{-6}$ |
| M3 | $>10^{-5}$ |
| M4 | $>10^{-5}$ |
| M6 | $6.7\times10^{-9}$ |
| M5 | $3.5\times10^{-8}$ |

HeLa S3 cells were exposed for 72 h to FK317 or its metabolites. Cytotoxicity was determined by MTT assay.
*a*) Concentration of each agent causing 50% inhibition of cell growth.

OC-10. In addition, FK317 demonstrated curative activity (≥95% tumor growth inhibition) against LX-1, Lu-134, LC-17, LC-6, SC-6, NS-8, MX-1 and UCC-6, while MMC, ADR and CDDP did not display strong antitumor effects. Furthermore, the antitumor activity of FK317 is characterized by its wide effective dose range. FK317 exhibited broad curative activity at doses ranging from 1.8 to 5.6 mg/kg against LX-1, from 3.2 to 5.6 mg/kg against Lu-134, from 3.2 to 5.6 mg/kg against LC-17, from 1.0 to 5.6 mg/kg against LC-6, from 3.2 to 5.6 mg/kg against SC-6, from 3.2 to 5.6 mg/kg against NS-8, and from 1.0 to 5.6 mg/kg against MX-1. These data indicate that the antitumor activity of FK317 showed much stronger efficacy and a wider therapeutic window as compared with MMC, ADR, CDDP, taxol or CPT-11, all of which are widely used in clinics. It has been reported that the response rates of human tumor xenografts to these drugs correlate well with results in the clinic.[14–16] Thus, we consider that FK317 is likely to show good activity against many kinds of tumors, especially cervical, stomach and lung cancers; the clinical effect of FK317 might be strong-er than those of MMC, ADR, CDDP, taxol and CPT-11.

Many antitumor agents used in cancer chemotherapy possess either cytotoxic or cytocidal activity. However,

since they do not have selective toxicity, they injure not only tumor cells, but also the normal cells of cancer patients. MMC, ADR and CDDP have been widely used in the treatment of various human solid tumors, but their strong toxicities, mainly gastrointestinal toxicity and myelosuppression, sometimes hamper their clinical application.[17–20] The toxicity of FK317, estimated in terms of body weight change in mice, was the lowest among the drugs used. FK317 at a dose of 3.2 mg/kg showed a curative antitumor effect against LC-17 without inducing loss of body weight. Furthermore, the myelosuppressive effect of FK317 was much weaker than that of MMC at the same dose, although FK317 showed antitumor activity equal to (LC-17 tumor) or stronger (other tumors) than that of MMC at a dose of 3.2 mg/kg. To explain the good selectivity of FK317, we examined the tissue distribution of [$^{14}$C]FK317 in tumor-bearing mice. Docetaxel possesses a wide spectrum of efficacy against human tumor xenografts in mice because of its long retention in the tumor.[21] With [$^{14}$C]FK317, the highest tissue concentrations were observed in the liver and kidney, and contrary to our expectation, the concentration of [$^{14}$C]FK317 in the tumor was low (almost one-third of that detected in the plasma at 5 min), nor was the drug retained over a long period in the tumor. Moreover, the concentration of [$^{14}$C]FK317 in bone marrow was also as low as that in the tumor. Furthermore, FK317 has no selective cytotoxic effect against tumor cells *in vitro* (data not shown). These results were difficult to explain, but nevertheless indicated good selectivity of FK317.

Since this good selectivity could be due to a different metabolic pattern for FK317 in the tumor, we measured the metabolites of FK317 in tumor and normal tissues. Since FK317 has a 4-formyl moiety in its chemical structure, it is expected to be metabolized by oxidation or reduction in the tissue. Interestingly, we found that the metabolic pattern in tumors was quite different from that in normal tissues. The main metabolites in tumors were 4-alcohol derivatives, which show strong cytotoxicity. In plasma, lung, liver and kidney, the main metabolites were 4-carboxylic acid derivatives, which had no cytotoxic activity at all. Although alcohol derivatives were found in plasma, lung, liver and kidney, decrease of these metabolites was faster in normal tissues than in the tumor. We could not examine the metabolic pattern in BMC, because the mass was very small, but non-toxic metabolites may also be formed in BMC. The low myelosuppressive effect of FK317 might be explained by the short retention and the distribution of less toxic metabolites in BMC. Although there may be microenvironmental differences between tumor and normal tissues, the precise mechanism of the different metabolic patterns of FK317 remains unclear. In the tumor, FK317 is metabolized to DNA-reactive species, which produce DNA-DNA interstrand and DNA-protein cross-links that lead to the death of the tumor cell.[10] Reduction of FK317 is an important step in the reaction with DNA, suggesting that FK317 is a bioreductive alkylating agent. Bioreductive alkylating agents such as MMC have been viewed as a means of targeting hypoxic tumors, since such tumors have greater propensity for reductive metabolism.[22–24] However, MMC causes severe damage to normal cells because it is activated by the same mechanism in such cells.[25, 26] Antitumor activity or detoxification of MMC depends on the target molecules.[27] That is, MMC is inactivated when it reacts with molecules which are not essential to proliferation, but shows cytotoxic effects when it reacts with molecules which are necessary for cell division, such as DNA. However, the action of FK317 does not depend on the target molecules, because it has different metabolic pathways in the tumor and normal tissues. FK317 was oxidized to non-toxic metabolites in aerobic tissues such as liver, kidney and lung. Thus, its tissue-specific metabolic pattern may be very important for its selectivity. There have been no previous reports of anticancer drugs that are selectively activated in tumors and inactivated in normal tissues. Furthermore, FK317 has very remarkable antitumor effects against human multidrug-resistant tumor xenografts *in vivo*, but not *in vitro*. This antitumor activity of FK317 against multidrug-resistant tumors may be due to the deacetyl metabolites, which are readily formed in the blood.[28] Since these metabolites do not bind P-glycoprotein, they can show strong cytotoxic effects against multidrug-resistant cells *in vivo*. FK317 thus appears to be a member of a new class of cancer chemotherapeutic agents due to its favorable metabolite pattern (deacetylation, reduction and oxidation).

In conclusion, FK317 showed remarkable antitumor effects against human tumor xenografts without causing severe body weight loss or myelosuppression, since it is selectively metabolized to cytotoxic species in tumors, and is catabolized in normal tissues. These results suggest that FK317 has great potential for cancer chemotherapy.

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