A New Antitumor Antibiotic, FK973: Its Metabolism in the Blood and the Antitumor Effects of Its Metabolites on Experimental Models

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Abstract—Our previous studies showed that a new, substituted dihydrobenzoxazine, 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), which is a triacetylated derivative of the fermentation product FR900482 of Streptomyces sandaensis No. 6897, had potent antitumor effects on experimental tumors in vivo and in vitro. In the present study, we investigated the metabolism of FK973 in the blood of human and animals and the antitumor effects of its metabolites. After the incubation of FK973 in the blood (hemolysate) or serum of humans, dogs, rats and mice, it was rapidly metabolized to two diacetates and a monoacetate, and slowly to FR900482. FK973 and all its deacetylated metabolites showed strong cytotoxicity on in vitro cultured murine L1210 leukemia cells, and the cytotoxicity of FK973 was the most potent. In the in vivo experiments, FK973 and its metabolites prolonged the life of mice bearing ascitic P388 leukemia, and it potently inhibited the growth of murine B16 melanoma and Colon 38 adenocarcinoma implanted subcutaneously in mice. FK973 was the most effective compound. Thus, these results suggest that the antitumor effects of FK973 are stronger than those of its deacetylated metabolites produced in the blood of humans and animals.

FK973, a new substituted dihydrobenzoxazine, (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), is a triacetylated derivative of the antitumor antibiotic FR900482 (1-3), which was isolated from the fermentation product of Streptomyces sandaensis No. 6897. In our previous studies, FK973 and FR900482 exhibited potent antitumor effects on various kinds of animal and human tumor models (4, 5), and it also showed strong cytotoxicity on in vitro cultured tumor cell lines (2, 4). FK973 was much more active than FR900482 and is now under development as an antitumor drug. By alkaline elution analysis, we found that FK973 and FR900482 formed concentration- and time-dependent interstrand DNA-DNA and DNA-protein cross-links, but did not break the DNA in the cells (6, 7).

FK973 has three different acetyl groups in its chemical structure. These groups seem to be unstable in the blood, because many drugs with acetyl group(s), such as aspirin (8), heroin (9), thymoxamine (10) and diltiazem (11), are substrates for esterase in the blood. It is likely that the acetyl groups of FK973 are also hydrolyzed in the blood by esterase. Although the antitumor effects of the deacetylated metabolites of FK973 have not been examined, such a study would provide further information on the mechanism of the antitumor activity of FK973.

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Materials and Methods

Chemicals: FK973 and its deacetylated derivatives, FR67042, FR66874, FR66980 and FR900482, were prepared in the Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. The chemical structures of these compounds are shown in Fig. 1. For the in vitro cytotoxicity tests, the compounds were dissolved in phosphate-buffered saline and diluted with culture medium; and for the in vivo tests, they were dissolved in and diluted with 10% polyoxyethylated (60 mol) hydrogenated castor oil in saline solution. The solvent was given to the control animals.

Animals: Male beagle dogs were purchased from Hazleton Research Animals, VA, U.S.A. Male Sprague-Dawley rats and male ICR mice were from CLEA Japan, Inc., Kawasaki, Japan. Female mice of the CDF1 (BALB/c×DBA/2), BDF1 (C57BL/6×DBA/2), C57BL/6 and DBA/2 strains were from Charles River Japan, Inc., Atsugi, Japan.

Tumors: P388 was maintained i.p. by serial passage in DBA/2 mice. 1316 and Colon 38 were maintained s.c. by serial passage in C57BL/6 mice.

Blood preparation (hemolysate) and serum preparation: Fresh heparinized or non-heparinized blood was obtained from male volunteers, dogs, rats and mice. To obtain the blood preparation, the heparinized blood was diluted with 2 volumes of 0.02 M phosphate buffer (pH 7.4) and incubated at 4°C for 30 min to hemolyze. The resultant suspension was centrifuged at 1700 g for 10 min to sediment the membranes, and the supernatant was used as the blood preparation. To obtain the serum, the non-heparinized blood was allowed to stand at 4°C for about 30 min and centrifuged at 1700 g at 4°C for 10 min, and the supernatant was used as serum.

Incubation conditions and high-performance liquid chromatography (HPLC) analysis: To determine the metabolites of FK973, 30 μl of the compound solution was incubated with 30 μl of the blood preparation with shaking for 20 min at 37°C. The reaction was stopped by the addition of 10 μl of 10% acetic acid, and the mixture was centrifuged for 1 min. Ten μl of the supernatant was analyzed by HPLC using a Varian 5000 liquid chromatograph equipped with a UV detector (UV 240 nm) and fitted with a column (15 cm×4-mm i.d.) packed with TSK GEL LS-410 ODS SIL (5 μm particle). The eluant was a linear gradient of acetonitrile (0–30%)/0.02 M phosphate buffer (pH 2.5) in a 30-min period, and the flow rate was 1 ml/min. The retention times of authentic FK973, FR67042, FR66974, FR66980 and FR900482 were 27, 24, 21, 17 and 7.4 min, respectively. For the quantitative analysis, the calibration curves were obtained by plotting the peak height against various concentrations of each compound, and their amounts were determined from the respective calibration curves.

To measure the in vitro half-lives of the metabolites of FK973, the authentic compound (3 μg) was incubated in 30 μl of blood preparation or serum with shaking at 37°C for various periods. HPLC analysis was then performed as described above. The half-lives were determined by plotting the amount of each compound remaining at given times.

Cell culture and cytotoxicity test: L1210 cells were incubated in suspension in RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.) and penicillin (50 units/ml)-streptomycin (50 μg/ml) (Flow Laboratories, North Ryde, Australia) at 37°C in a humidified atmosphere of 95% air/5% CO₂. To assess cytotoxicity, L1210 cells (2×10⁴) were exposed to a drug in 200 μl of medium in a 96-well plate at 37°C for 48 hr, and the viable cells were counted by the
trypan blue dye exclusion method. The mean of viable cells from triplicate experiments was expressed as a percentage of the control.

Evaluation of antitumor effects against ascitic tumors: P388 cells \((1 \times 10^6)\) were inoculated i.p. in CDF1 mice. The drugs were given i.p. to mice once a day for 5 days (Days 1–5). Twenty and 10 mice were used in the control and treated groups, respectively. Compound efficacy against the ascitic tumor was assessed as a percentage of the MST (mean survival time) of the treated group (T) to that of the control group (C).

\[
\text{MST ratio} (\%) = \frac{\text{MST of } T}{\text{MST of } C} \times 100
\]

Evaluation of antitumor effects against solid tumors: B16 cells \((5 \times 10^5)\) or fragments \((2 \times 2 \times 2 \text{ mm})\) of Colon 80 were implanted s.c. into the left flank of BDF1 mice. In both experiments, 20 and 10 mice were used in the control and drug treated groups, respectively. The drugs were given i.v. once a day on Days 1, 4, 7 and 10. Tumor weight, as derived from caliper measurements of the length and width of tumors, was calculated by the formula:

\[
\text{Tumor weight (mg)} = \frac{1}{2} \times a \times b^2
\]

where \(a\) represents the length (mm) and \(b\) represents the width (mm) of the tumor.

Compound efficacy against murine solid tumors was based on the percentage of mean tumor weight of the treated group (T) to that of the control group (C).

\[
\text{Tumor growth inhibition (\%)} = \left(1 - \frac{\text{Mean tumor weight, } T}{\text{Mean tumor weight, } C}\right) \times 100
\]

Results

Metabolism of FK973 in the blood of mice, dogs and humans: Figure 2 shows the HPLC pattern of the supernatant after the incubation of the compound for 20 min in the blood preparation of the mouse. FK973 underwent a rapid metabolism in the blood. The metabolites of FK973 were FR67042, FR66974 and FR66980, which were identified by direct comparison of the HPLC retention times of the metabolites with those of the authentic compounds and by the observation of a single homogeneous peak in the HPLC pattern when mixed with the authentic compounds. No other metabolite was detected after the incubation of FK973 in the blood preparation. There was no background peak at the retention times of FK973 or its metabolites.

After the incubation of FK973 in the blood preparation of mice, dogs or humans for 20 min, the amounts of its metabolites were measured by HPLC. As shown in Table 1, more than 85% of the FK973 was metabolized.
to FR67042 and FR66980 in the human blood preparation, but about 70% remained unchanged in the dog blood preparation. FR66974 was detected only in the mouse blood preparation, but not in those of humans or dogs. Thus, the hydrolysis of FK973 was best accomplished in the human blood preparation, followed in order by those in the mouse and dog blood preparations.

In vitro half-lives of FR67042, FR66974 and FR66980: Table 2 shows the in vitro half-lives of FR67042, FR66974 and FR66980 incubated in the blood preparation or serum at 37°C. FR67042 was quickly hydrolyzed to FR66980 in the blood preparations of all the species used, with half-lives of a only few min and had a half-life of 13.3 min in the mouse serum. However, it was very stable in the sera of rats, dogs and humans. FR66974 was hydrolyzed to FR66980 in the blood preparations and sera of mice, rats, dogs and humans, with half-lives of 2–32 min. FR66980 was mainly stable in the blood preparations and sera of all the species used, and it was converted to FR900482 only after incubation for a long period.

Cytotoxicity against in vitro cultured leukemia cells: The cytotoxic effects of FK973 and its metabolites, FR67042, FR66974, FR66980 and FR900482, were examined on in vitro cultured L1210 cells. Figure 3 shows that all the authentic compounds concen-
Antitumor effects on murine ascitic tumors in mice: The antitumor effects of FK973 and its metabolites were examined on P388 ascitic leukemia cells in mice. The tumor cells were inoculated i.p. to mice on Day 0, and the authentic compounds were given i.p. to mice once a day on Days 1, 4, 7 and 10, and the tumor weights were measured on Days 7, 11, 14, 18 and 21. As shown in Fig. 4, FK973 and FR66980 dose-dependently inhibited the growth of B16 melanoma. FR-67042, FR66974 and FR900482 also were active against the melanoma (data not shown), and FR67042 was toxic only in a dose of 18 mg/kg. To compare their efficacies, the tumor growth inhibition (%) was calculated and plotted as shown in Fig. 5. The efficacies of the antitumor effects were in the order: FK973 > FR67042 > FR66974 > FR900482 > FR66980.

Against Colon 38, FK973 markedly inhibited the tumor growth, but in a dose of 18 mg/kg, it induced toxicity. FR66980 had antitumor effects only in doses of 10 and 18 mg/kg (Fig. 6). FK973 was much more active than FR66980 on the tumor in mice.

Discussion

In this study, FR973 was shown to be metabolized to two diacetates (FR67042 and FR66974) and a monoacetate (FR66980) after incubation in the mouse blood preparation for 20 min. In the dog and human blood preparations, FK973 was changed to FR-67042 and FR66980, but not to FR66974. From the results, it seems that the phenolic-acetyl group of FK973 is first hydrolyzed, followed by the alcoholic-acetyl group in the dog and human blood preparations, but both groups are hydrolyzed in the mouse blood preparation. Finally, FK973 is metabolized to FR66980 in the blood or sera of all the species tested. The results suggest that FK973 is unstable in the blood preparations of mice, dogs and humans, and that the hydrolytic rate of the acetyl groups depends upon the species used. The hydrolysis of the alcoholic-acetyl group of FR67042 was fast in the mouse, rat, dog and human blood preparations, and in the serum of mice, but not in the sera of rats, dogs and humans. The phenolic-acetyl group in FR66974 was easily hydrolyzed in the blood preparations and sera of all the species tested, whereas FR66980 was comparatively stable in the blood prep-
Table 3. Antitumor effects of FK973 and its deacetylated metabolites on ascitic P388 leukemia

| Drug  | Dose (mg/kg) | T/C (%) | Activity² |
|-------|--------------|---------|-----------|
| FK973 | 0.032        | 130     | +         |
|       | 0.1          | 130     | +         |
|       | 0.32         | 150     | +         |
|       | 1.0          | 180     | ++        |
|       | 3.2          | 200     | ++        |
|       | 10           | 95      | −         |
| FR67042| 0.1          | 140     | +         |
|       | 0.32         | 160     | +         |
|       | 1.0          | 185     | ++        |
|       | 3.2          | 285     | ++        |
|       | 10           | 95      | −         |
| FR66974| 0.1          | 130     | +         |
|       | 0.32         | 130     | +         |
|       | 1.0          | 155     | +         |
|       | 3.2          | 185     | ++        |
|       | 10           | 165     | +         |
| FR66980| 0.032        | 110     | −         |
|       | 0.1          | 120     | +         |
|       | 0.32         | 125     | +         |
|       | 1.0          | 130     | +         |
|       | 3.2          | 170     | +         |
|       | 10           | 230     | ++        |

Tumor cells were inoculated i.p. to CDF₁ mice on Day 0 and the authentic compound was given i.p. to mice on Days 1–5. MST was measured. ¹The median survival time of the control group was 10 days. ²Criteria: +, T/C ≥ 120; ++, T/C ≥ 175.

Fig. 4. Antitumor effects of FK973 and FR66980 on B16 melanoma in mice. The tumor cells (5 × 10⁶) were implanted s.c. to BDF₁ mice on Day 0; and the authentic compound was given i.v. to mice on Days 1, 4, 7 and 10. Ten mice (20 mice in the control group) were used per group. A: FK973, 1.0 (○), 3.2 (△) and 10 mg/kg (▲); B: FR66980, 3.2 (●), 10 (△) and 18 mg/kg (▲). The control (○) was given the solvent. Values, mean ± S.E.
Preparations and sera of all the species tested, and was slowly changed to FR900482. Lockridge et al. have shown that human serum cholinesterase converts heroin (diacetylmorphine) to 6-acetylmorphine in vitro (9). Elbaum and Nagel have reported the catalytic activity of human hemoglobins toward the hydrolysis of p-nitrophenylacetate (12). Their findings indicate that controlling factors in the hydrolysis of the acetyl groups of FK973 are: blood preparation or serum, animal species, and the position of the acetyl groups.

Fig. 5. Relative inhibitory effects of FK973 and its deacetylated metabolites on the tumor growth of B16 melanoma in mice. The tumor cells ($5 \times 10^5$) were implanted s.c. to BDF, mice on Day 0, and the authentic compound was given i.v. to mice on Days 1, 4, 7 and 10. Tumor weights were measured on Day 18. Ten mice (20 mice in the control group) were used per group. ○, FK973; △, FR67042; ◻, FR66974; ■, FR66980; ●, FR900482. Values, mean±S.E.

Fig. 6. Antitumor effects of FK973 and FR66980 on Colon 38 adenocarcinoma in mice. A tumor (2×2×2 mm) was implanted s.c. to BDF, mice on Day 0, and the authentic compound was given i.v. to mice on Days 1, 4, 7 and 10. Ten mice (19 mice in the control group) were used per group. A: FK973, 0.32 (●), 1.0 (△), 3.2 (▲), 10 (◻) and 18 mg/kg (■); B: FR66980, 0.32 (●), 1.0 (△), 3.2 (▲), 10 (◻) and 18 mg/kg (■). The control (〇) was given the solvent. Values, mean±S.E.
in the chemical structure. When FK973 is given i.v. to humans and animals, it may be deacetylated in their blood and then further metabolized in their liver, kidney, etc. The metabolism of FK973 in the liver seems to produce no active compounds, although the metabolites of FK973 are active after incubation of FK973 in blood of humans and animals. The study of the detailed metabolism of FK973 in a living body is underway in our laboratories.

Against in vitro and in vivo tumor cells, FK973 and its deacetylated metabolites were active in all the tests. FK973 and FR67042 were more active than any other metabolites of FK973 against in vitro cultured tumor cells, and FK973 was the strongest of all the compounds on in vivo solid tumor, when the compounds were given i.v. to mice. The results suggest that FK973 itself is mainly incorporated in tumor cells, although FK973 was unstable and metabolized to FR67042, FR66974 and FR66980 in mouse blood. FK973 seems to be more lipophilic than its deacetylated metabolites, because FK973 has three acetyl groups that may facilitate its incorporation in the cells. FK973 is incorporated in the cells and gradually forms the DNA-DNA and DNA-protein cross-links and thereby induces the antitumor effects on tumor cells (6). FR900482 was also found to form these cross-links (7). Accordingly, FR67042, FR66974 and FR66980 may also induce the formation of these cross-links in tumor cells.

FK973, when given i.p., also had a potent antitumor effect on P388 leukemia inoculated i.p. into mice. In this experiment, there was no blood in the peritoneal cavities of mice with leukemia. Since the peritoneal fluid of mice has many kinds of enzymes, FK973 may be metabolized in the fluid. However, we do not know what metabolites were produced from FK973, FR67042, FR66974, FR66980 and FR900482 in the peritoneal fluid of the mouse.

In summary, our findings show that FK973 has more potent antitumor activities than any of its metabolites.

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