Different Effects of FK317 on Multidrug-resistant Tumor in vivo and in vitro

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FK317, a novel substituted dihydrobenzoxazine, was examined for antitumor effects on multidrug-resistant (MDR) tumor cells in vitro and in vivo. In nude mice, FK317 markedly inhibited the growth of s.c. implanted KB-V1 vinblastine (VLB)-resistant human epidermal carcinoma KB cells, as well as the parent cells (KB-3-1). However, KB-V1 showed much greater resistance to FK317 than to VLB and adriamycin (ADM) in the in vitro study. This resistance was reversed by the addition of verapamil, whereby intracellular accumulation of FK317 in the KB-V1 cells was also decreased. After incubation of FK317 in human and mouse blood, it was shown to be rapidly metabolized to a monodeacetylated form, and slowly metabolized further to a dideacetylated form. With the removal of the acetyl groups from FK317, resistance indexes in KB-V1 and SBC-3/ADM, ADM-resistant human lung carcinoma, decreased. In addition, photolabeling of P-glycoprotein with [3H]azidopine in KB-V1 plasma membrane was completely inhibited by FK317, but not by the deacetylated metabolites. These results indicate that FK317 is metabolized to deacetylated forms, which do not bind to P-glycoprotein and are incorporated into MDR cells, causing cytotoxic effects.

Key words: FK317 — Antitumor effect — Deacetyl metabolite — MDR — P-Glycoprotein

It is widely accepted that drug resistance is a major obstacle to cancer chemotherapy. Cross-resistance of cancer cells to many types of drugs which show little structural similarity is termed multidrug resistance (MDR).1, 2) Over the past several years, evidence has accumulated to show the association between the development of MDR and the appearance of a P-glycoprotein in resistant cells.3, 4) The human gene coding for the P-glycoprotein, which exhibits similarity to certain bacterial transport proteins and has ATPase activity, has been sequenced.5–9) P-Glycoprotein appears to act as an efflux pump for functionally and structurally unrelated drugs such as the vinca alkaloids and anthracyclins, which are very important in the treatment of many cancers, and is responsible for decreasing the drug levels and cytotoxicity in MDR tumor cells.10–12) Therefore, there is clearly a need for chemosensitizers which can overcome MDR, or new antitumor agents which are effective on MDR tumors.

FK973, which is a triacetyl derivative of FR900482 isolated from the fermentation products of Streptomyces sandoensis No.6897 in the Fujisawa Research Laboratories (Osaka), forms DNA-DNA interstrand and DNA-protein cross-links after being metabolically activated, shows strong cytotoxicity against tumor cells, and has strong antitumor activities against a wide variety of animal tumor models and human xenografts.13, 14) FK973 showed high therapeutic efficacy in clinical studies.15, 16) However, it was not developed for clinical use because of its propensity to induce capillary leak syndrome. In attempts to discover new FK973 derivatives which retain the antitumor activity without the capillary leak syndrome side effect, the novel analog FK317 (11-acetyl-8-carbamoylmethyl-4-formyl-6-methoxy-14-oxa-1,11-diaza-tetra-cyclo[7.4.1.02, 7.010, 2]tetradeca-2,4,6-trien-9-yl-acetate) was identified. FK317 showed strong antitumor activities against a wide variety of animal tumor models and human xenografts, but did not induce pleural effusion in rats.17) In particular, FK317 potently inhibited the tumor growth of human lung cancer LX-1, which is highly resistant to many antitumor agents, and expresses P-glycoprotein in the membrane.17, 18) These findings prompted us to examine the antitumor activity of FK317 against MDR tumors, and in the present study we found a difference in antitumor effects against MDR tumors under in vitro and in vivo conditions. In this paper, we show that this difference may be explained by the generation of deacetylated metabolites of FK317 which do not bind to P-glycoprotein.

MATERIALS AND METHODS

Chemicals The sources of materials used in this work were as follows: FK317, FR70496, FR157471, FK506 and [4-formyl-14C]FK317 (332 MBq/mmol) were prepared in Fujisawa Research Laboratories. The chemical structures of FK317 and its derivatives are shown in Fig. 1. Mitomycin C (MMC) and adriamycin (ADM) were purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo.
blastoine (VLB) was purchased from Shionogi & Co., Ltd., Osaka. Verapamil hydrochloride was purchased from Nacalai Tesque Inc., Kyoto. [3H]Azidopine (1.48 TBq/mmol) was purchased from Amersham Japan Ltd., Tokyo. FK317, FR70496 and FR157471 were generously supplied by Dr. I. Pastan (National Cancer Institute, NIH, Bethesda, MD). SBC-3 and SBC-3/ADM: human small cell lung carcinoma cell line (SBC-3) and its ADM-resistant variant (SBC-3/ADM) were generously supplied by Dr. I. Kimura (Second Department of Medicine, Okayama University Medical School, Okayama). Male mice of the BALB/c nu/nu strain were purchased from CLEA Japan Inc., Tokyo.

**Evaluation of antitumor effect in vivo** When the estimated tumor weight in mice had grown to between 100 and 300 mg, the animals were divided into experimental groups of 6 and were treated i.v. with a test drug every 3 days for a total of three doses (q3d). The tumor weight was calculated from the following formula: tumor weight (mg) = L × W^2/2 where \(L\) and \(W\) represent the length and width of the tumor mass, respectively. The antitumor activity was determined by comparing the mean tumor weight of the test group (\(T\)) with that of the control group (\(C\)).

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\text{Growth inhibition (1−T/C) (%) = } \left\{ 1−\text{change in mean tumor weight (T)/change in mean tumor weight (C)} \right\} \times 100
\]

**In vitro cytotoxicity** Growth inhibition experiments were carried out in 96-well flat-bottomed microwells, and the number of viable cells at the end of the incubation determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, essentially as described by Mosmann.\(^{21}\) Thus, 5 × 10^4−4 × 10^5 cells/well in 100 µl were plated and the drug or the medium alone as a control was added. The cells were cultured for 3 days. After addition of MTT (10 µl/well, 5 mg/ml in phosphate-buffered saline (PBS)), the plates were incubated for 4 h. The medium was removed and the blue dye formed was dissolved in 150 µl of 0.04 N HCl in isopropanol. The absorbance was measured at 580 nm using a Titertek Twinreader (Titertek, McLean, VA).

**Fig. 1. Chemical structures of FK317, FR70496 and FR157471.**
Photoaffinity labeling of plasma membrane  Analysis of the binding of P-glycoprotein was carried out as described by Sato et al.25 The plasma membranes (50 µg of protein) were photolabeled in 40 mM Tris-HCl buffer (pH 7.2), containing 4% dimethyl sulfoxide and 200 nM [3H]azidopine in a final volume of 50 µl. Photolabeled membranes were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using gradient
RESULTS

Antitumor activities of FK317 against MDR tumors in vivo We tested the antitumor effects of FK317 against human MDR (KB-V1) tumor and the parent sensitive tumor (KB-3-1), and compared them with those of VLB and ADM in vivo. As shown in Fig. 2, all drugs exhibited strong antitumor activities against KB-3-1. Both FK317 and VLB exhibited broad curative activities at doses ranging from 1.8 to 5.6 mg/kg and 3.2 mg/kg, respectively. VLB at a dose of 5.6 mg/kg showed toxicity. ADM at a dose of 5.6 mg/kg inhibited the growth of KB-3-1 with a 1−T/C value of 96%. Moreover, FK317 exhibited very strong antitumor activity against the KB-V1 tumor as well. FK317 at a dose of 5.6 mg/kg inhibited the growth of KB-V1 with a 1−T/C value of 90%. VLB and ADM showed no activity against KB-V1 at a dose of 3.2 mg/kg or 5.6 mg/kg, respectively. Thus, we demonstrated that FK317 has strong antitumor activity against MDR tumors in vivo.

Cytotoxic activity of FK317 against MDR cells The in vitro sensitivities of MDR cells to FK317, VLB, ADM and MMC were examined (Table I). The IC_{50} values of FK317 against sensitive KB-3-1 and resistant KB-V1 cell lines were 6.6×10^{-10} and 4.7×10^{-6} M, respectively, indicating an approximate 7100-fold resistance. KB-V1 showed resistance to VLB (1000-fold) and ADM (880-fold), and only weak resistance to MMC (23-fold). In addition, verapamil dose-dependently reversed the resistance of KB-V1 and was capable of completely restoring the sensitivity of KB-V1 cells to VLB at a concentration of 10 μM. However, a low degree of resistance to FK317 persisted even in the presence of verapamil (10 μM) (Table II).

Table I. Sensitivity of KB-3-1 and KB-V1 Cells to FK317, VLB and ADM

| Drug   | IC_{50} (M) | Resistance index a) |
|--------|-------------|---------------------|
|        | KB-3-1      | KB-V1               |
| FK317  | 6.6×10^{-10}| 4.7×10^{-6}         | 7100                |
| VLB    | 3.6×10^{-10}| 3.6×10^{-7}         | 1000                |
| ADM    | 6.6×10^{-9} | 5.8×10^{-6}         | 880                 |

KB-3-1 or KB-V1 cells were cultured with each drug for 72 h and growth-inhibitory activity was determined.

a) Ratio of IC_{50} value for KB-V1 cells to that for KB-3-1 cells.

Table II. Effects of Verapamil on the Cytotoxicities of FK317 and VLB against KB-3-1 and KB-V1 Cells

| IC_{50} (nM) | VLB | FK317 |
|--------------|-----|-------|
|              | KB-3-1 | KB-V1 | RI a) | KB-3-1 | KB-V1 | RI |
| Verapamil 0 (M) | 3.9×10^{-9} | 8.1×10^{-7} | 210 | 2.1×10^{-8} | 2.4×10^{-3} | 1100 |
| 1×10^{-6} | 2.6×10^{-9} | 4.0×10^{-7} | 150 | 2.9×10^{-9} | 9.8×10^{-6} | 340 |
| 3.2×10^{-6} | 2.2×10^{-9} | 4.7×10^{-8} | 21 | 2.6×10^{-9} | 3.3×10^{-6} | 130 |
| 1×10^{-5} | 1.7×10^{-9} | 5.0×10^{-8} | 2.9 | 1.4×10^{-9} | 1.2×10^{-6} | 86 |

Cells were cultured and treated with each drug for 72 h and growth-inhibitory activity was determined. Verapamil was added simultaneously with FK317.

a) Resistance index: the ratio of IC_{50} value for resistant cells to that for sensitive cells.
Intracellular accumulation of FK317 in MDR cells

Since the MDR phenotype is characterized by a defect in the accumulation of MDR cytotoxics, we examined the intracellular accumulation of FK317 in the KB-3-1 and KB-V1 cell lines. Accumulation of $[^{14}C]$FK317 in KB-3-1 cells increased and approached a plateau after 2 h, at a concentration of approximately 2000 pmol of $[^{14}C]$FK317 in $10^8$ cells (Fig. 3), while only 20 pmol of $[^{14}C]$FK317 was accumulated in the KB-V1 cells. Thus, only about 1/100 of the amount of FK317 found in the KB-3-1 cells was accumulated in KB-V1 cells.

Stability of FK317 in blood

FK317 had strong antitumor effects against MDR tumors in vivo, although MDR cells showed cross-resistance to FK317 in vitro. A possible explanation of this phenomenon would be the formation of metabolites in the blood. FK317 was indeed metabolized into deacetylated forms in the blood. Table III shows the in vitro half-lives of FK317 and monodeacetylated FK317, FR70496 incubated in the blood preparation at 37°C. FK317 was quickly hydrolyzed to FR70496 in both human and mouse blood preparations with half-lives of only a few minutes. FR70496 was then slowly hydrolyzed to dideacetylated FK317, FR157471.

Cytotoxic effects of deacetyl metabolites of FK317 against MDR cells

Since it was found that FK317 was metabolized to deacetyl metabolites in blood, we examined the cytotoxic effects of FK317 and its metabolites against MDR cells in vitro. The IC$_{50}$ values of FK317, FR70496 and FR157471 towards KB-3-1 were $6.6 \times 10^{-10}$, $4.0 \times 10^{-9}$ and $5.2 \times 10^{-8} \text{M}$, and those towards KB-V1 were $4.7 \times 10^{-6}$, $5.4 \times 10^{-7}$ and $2.0 \times 10^{-7} \text{M}$, indicating approximately 7100-, 140- and 39-fold resistance, respectively (Table IV). Furthermore, the decrease in the resistance index with the metabolites was also observed in SBC-3 and SBC-3/ADM cells, and SBC-3/ADM showed little resistance to FR157471 (Table IV).

Inhibition of $[^{3}H]$azidopine photolabeling of P-glycoprotein by FK317

In order to study the mechanism of the cytotoxic effects of the metabolites of FK317 against MDR cells, we studied the binding of FK317 and its metabolites to P-glycoprotein. Fig. 4 shows that FK317 at 25 $\mu$M partially, and at 250 $\mu$M almost completely, inhibited $[^{3}H]$azidopine binding. However, FR70496 and

| Drug            | Half-life (min) |
|-----------------|-----------------|
| Mouse          | Human       |
| FK317→FR70496  | 2             | 6             |
| FR70496→FR157471| 108           | 72            |

The authentic compound (3 $\mu$g) was incubated with 30 $\mu$l of blood preparation with shaking at 37°C. HPLC analysis was then performed.

Table IV. Sensitivity of MDR Variants and the Parent Cells to FK317 and Its Deacetyl Metabolites

| Drug            | IC$_{50}$ (nM) | RI$^a$ | IC$_{50}$ (nM) | RI      |
|-----------------|---------------|-------|---------------|--------|
|                 | KB-3-1        | KB-V1 | SBC-3         | SBC-3/ADM |       |
| FK317           | 6.6$\times$10$^{-10}$ | 4.7$\times$10$^{-6}$ | 7100         | 8.9$\times$10$^{-9}$ | 3.8$\times$10$^{-4}$ | 43     |
| FR70496         | 4.0$\times$10$^{-9}$  | 5.4$\times$10$^{-7}$ | 140          | 1.1$\times$10$^{-7}$ | 1.1$\times$10$^{-6}$ | 10     |
| FR157471        | 5.2$\times$10$^{-9}$  | 2.0$\times$10$^{-7}$ | 38           | 1.6$\times$10$^{-7}$ | 6.1$\times$10$^{-7}$ | 3.8    |

Cells were cultured and treated with each drug for 72 h and growth-inhibitory activity was determined.

$^a$) Resistance index: the ratio of IC$_{50}$ value for resistant cells to that for sensitive cells.
FR157471, which are deacetyl metabolites of FK317, did not inhibit the binding of [\(^3\)H]azidopine. Verapamil and FK506 at 100 \(\mu\)M almost completely inhibited the binding of [\(^3\)H]azidopine to P-glycoprotein. These results indicate that FK317 directly interacted with P-glycoprotein, as did FK506 and verapamil, but FR70496 and FR157471 did not.

DISCUSSION

FK317, a novel substituted dihydrobenzoxazine, is a very attractive candidate as an antitumor agent, because it has a novel structure and shows strong antitumor effects on many refractory human tumor xenografts in nude mice. For example, s.c.-implanted human lung carcinoma LX-1 was almost completely eliminated by treatment with FK317.\(^{17}\) Although expression of P-glycoprotein was observed in LX-1, and ADM and vincristine did not show any antitumor effects at all against LX-1, the precise mechanism of resistance of LX-1 is unclear.\(^{18}\) Therefore, we examined the antitumor effect of FK317 on KB-V1, which shows resistance to colchicine, ADM and VLB.\(^{19}\) Several studies using these human KB cell lines have revealed that in vitro mechanisms of resistance are correlated with P-glycoprotein/MDR1 overexpression.\(^{19, 23}\) P-Glycoprotein works as an efflux pump to remove a variety of structurally dissimilar anticancer drugs from MDR cells.\(^{10–12}\) We report herein that FK317 has strong antitumor effects against KB-V1 tumors, whereas VLB and ADM are not effective in vivo. The lack of cross-resistance properties of FK317 in vitro may be clinically relevant for the treatment of human tumors that express the MDR phenotype.

On the other hand, in an in vitro study KB-V1 showed very high cross-resistance to FK317 and the resistance rate was much higher than that of VLB, which was used for drug selection of KB-V1. The cellular accumulation of FK317 in KB-V1 was very low, and the resistance to FK317 was reversed by verapamil. These results indicate that FK317 may be transported by P-glycoprotein and that the affinity of FK317 to P-glycoprotein may be stronger than that of VLB. It is very interesting that FK317 showed excellent antitumor effects on KB-V1 in vivo, while showing very high cross-resistance in vitro.

We focused on the formation of metabolites of FK317 in the blood. FK317, which has two acetyl groups in its chemical structure, was shown to be metabolized to a monoacetate (FR70496) after incubation for several minutes in human and mouse blood preparations. Additionally, the monoacetate was slowly hydrolyzed further to the deacacetate (FR157471). Further study in mice after a single i.v. dose of FK317 has shown that FK317 is rapidly deacetylated in vivo (data not shown). Heroin (diacetylmorphine) is converted to 6-acetylmorphine by serum cholinesterase in vitro,\(^{24}\) and human hemoglobin catalyzes the hydrolysis of \(p\)-nitrophenyl acetate.\(^{25}\) Which factor is responsible for the hydrolysis of FK317 in blood is not yet clear, but these findings indicate that FK317 is rapidly hydrolyzed to FR70496 and then to FR157471 in blood.

The cytotoxic effects of FR70496 and FR157471 towards KB-V1 or SBC-3/ADM were stronger than that of FK317. Surprisingly, the cytotoxic activities of FR157471 towards SBC-3 and SBC-3/ADM were approximately the same. In contrast, KB-V1 showed some resistance to FR157471 compared with KB-3-1. Furthermore, verapamil did not completely restore the sensitivity of KB-V1 to FK317, while the resistance to VLB was almost completely reversed. Why does FR157471 show only slight resistance to KB-V1 and why is the resistance of KB-V1 to FK317 not completely overcome by verapamil, a well-known chemosensitizer? KB-V1 also showed weak resistance to MMC. It has been reported that MDR cell lines showed moderate cross-resistance to MMC.\(^{26, 27}\) The mechanisms in these MDR cells are related not only to overexpression of P-glycoprotein, but also to decreased activity of DT-diaphorase, an MMC-activating enzyme, and to increased glutathione S-transferase \(\pi\) (GST \(\pi\)) activity. FK317 requires reductive activation for interaction with DNA, being similar to MMC. Although the mechanism by which FK317 is activated is unclear, the activity of activating enzyme or detoxification of FK317 in KB-V1 might be decreased or increased, respectively. In contrast, FR157471 did not show cross-resistance to SBC-3/ADM. It has been reported that the mechanisms of resistance of SBC-3/ADM are high expression of P-glycoprotein, increased GST \(\pi\) activity and decreased topoisomerase II activity.\(^{29}\) These data suggest that DT-diaphorase is a factor in the activation of FK317 for interaction with DNA. If this is the case, DT-diaphorase in the KB-V1 cells may be decreased.

For the purpose of characterizing the action of these high-potency deacetyl metabolites of FK317 against MDR cell lines, the binding affinity of FK317, FR70496 and FR157471 to P-glycoprotein was investigated using photolabeled [\(^3\)H]azidopine as the substrate. FR70496 and FR157471 did not inhibit [\(^3\)H]azidopine photolabeling of P-glycoprotein, whereas FK317 inhibited it entirely. These results suggest that FR70496 and FR157471 are not transported by P-glycoprotein, but are incorporated into MDR cells, inducing cell death. The inhibitory effect of FK506 is more stronger than that of verapamil. Thus, FK506 might have higher affinity than verapamil to P-glycoprotein.

It is well known that structurally unrelated hydrophobic cytotoxic drugs are substrates of P-glycoprotein and that a wide variety of lipophilic agents can reverse MDR, presumably by interacting with cytotoxic drug-binding sites on P-glycoprotein.\(^{10–12}\) Therefore, P-glycoprotein is
thought to have a unique substrate-binding site(s) which can recognize a wide variety of compounds. However, little is known about the FK317-binding sites of P-glycoprotein. The most important determinant of the ability to be transported is the relative hydrophobicity, and drugs that are substrates for the transporter must have partition coefficients (octanol/water) of approximately 1 or greater. As FK317 has two acetyl groups in its chemical structure, FK317 is more hydrophobic than FR70496 and FR157471, which are deacetyl metabolites. The conversion of FK317 to less hydrophobic metabolites may thus account for the decreased interaction with P-glycoprotein.

In conclusion, FK317 has remarkable antitumor effects against human MDR tumor xenografts in vivo, but not in vitro. This antitumor activity of FK317 against MDR tumors may be due to the generation of deacetyl metabolites in the blood. Since these metabolites do not bind P-glycoprotein, they can show strong cytotoxic effects against MDR cells in vivo. These results suggest that FK317 may have strong clinical antitumor effects against P-glycoprotein-overexpressing MDR tumors.

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Antitumor Activity of FK317 against MDR Tumors

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