Jpn. J. Cancer Res. **89**, 1047–1054, October 1998

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

Yoshinori Naoe,[1] Masamichi Inami, Shoji Takagaki, Sanae Matsumoto, Ikuo Kawamura, Fusako Nishigaki, Susumu Tsujimoto, Toshitaka Manda and Kyoichi Shimomura

Department of Pharmacology, Pharmacological Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-1-6 Kashima, Yodogawa-ku, Osaka 532-8514

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 [^{3}H]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

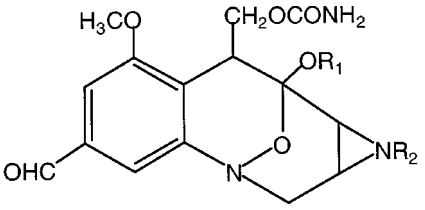
It is widely accepted that drug resistance is a major obstacle to cancer chemotherapy. Cross-resistance of cancer cells to many types of drugs which show little structural similarity is termed multidrug resistance (MDR).[1, 2] Over the past several years, evidence has accumulated to show the association 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 sandaensis* 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-diazatetra-cyclo-[7.4.1.0^{2,7}.0^{10,2}]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 rat.[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-^{14}C]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. Vin-

[1] To whom requests for reprints should be addressed.



	R_1	R_2
FK317	$COCH_3$	$COCH_3$
FR70496	H	$COCH_3$
FR157471	H	H

Fig. 1. Chemical structures of FK317, FR70496 and FR157471.

blastine (VLB) was purchased from Shionogi & Co., Ltd., Osaka. Verapamil hydrochloride was purchased from Nacalai Tesque Inc., Kyoto. [^{3}H]Azidopine (1.48 TBq/mmol) was purchased from Amersham Japan Ltd., Tokyo.

Tumor cells and animals KB-3-1 and KB-V1: human epidermal carcinoma cell line (KB-3-1) and its VLB-resistant subline (KB-V1) were generously supplied by Dr. I. Pastan (National Cancer Institute, NIH, Bethesda, MD).[19] 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).[20] 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×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 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).

Growth inhibition $(1-T/C)$ (%) =
{1−change in mean tumor weight (T)/change in mean tumor weight (C)}×100

***In vitro* cytotoxicity** Growth inhibition experiments were carried out in 96-well flat-bottomed microplates, 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, 5×10^3–4×10^4 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)

Cellular accumulation of drug The cells (1.0×10^6/ml) were seeded in 1 ml of culture medium/well in 24-well plates. After 24 h incubation, culture medium was removed and cells were incubated with 15 μ*M* [^{14}C]FK317 at 37°C. After incubation, the cells were washed five times with ice-cold PBS containing the drug (0.1 mg/ml) and lysed in 0.25 ml of 0.2 *N* NaOH.

Blood (hemolysate) preparation Fresh heparinized blood was obtained from male volunteers and mice. 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 it. The resultant suspension was centrifuged at 1700*g* for 10 min to precipitate the membranes, and the supernatant suspension was used as the blood preparation.

Incubation conditions and high-performance liquid chromatography (HPLC) analysis To measure the *in vitro* half-lives of the metabolites of FK317, the authentic compound (3 μg) was incubated in 30 μl of blood preparation with shaking at 37°C for various periods. The reaction was stopped by the addition of 10 μl of 10% acetic acid, and the mixture was centrifuged for 1 min. Ten microliters of the supernatant was analyzed by HPLC using a Varian 5000 liquid chromatograph (Varian, CA) 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 μ*M* particle) (Tosoh, Tokyo). Elution was carried out in a linear gradient of acetonitrile (0–30%)/0.02 *M* phosphate buffer (pH 2.5) over a 35-min period, and the flow rate was 1 ml/min. The retention times of authentic FK317, FR70496 and FR157471 were 31.7, 25.6 and 18.4 min, respectively. The half-lives were determined by plotting the amount of each compound remaining at given times.

Plasma membrane preparation Cells were suspended in 10 m*M* Tris-HCl (pH 7.4), 10 m*M* NaCl, 1.5 m*M* $MgCl_2$ and 0.02 m*M* phenylmethylsulfonyl fluoride at a cell density of 4×10^7 cells/ml, and then homogenized with 20 strokes using a Dounce homogenizer. Cell homogenate was centrifuged at 1000*g* for 10 min. The supernatant was overlaid on 35% sucrose solution and centrifuged for 60 min at 18,000*g*. The membrane fraction at the interface was removed and resuspended in 10 m*M* Tris-HCl (pH 7.4) and 250 m*M* sucrose (buffer A). The sample was then centrifuged for 60 min at 100,000*g*. Pellets were resuspended in buffer A and stored at −80°C until used.

Protein concentration was determined by the use of a BIO-Rad protein kit (BIO-Rad, CA) with bovine serum albumin as a standard.

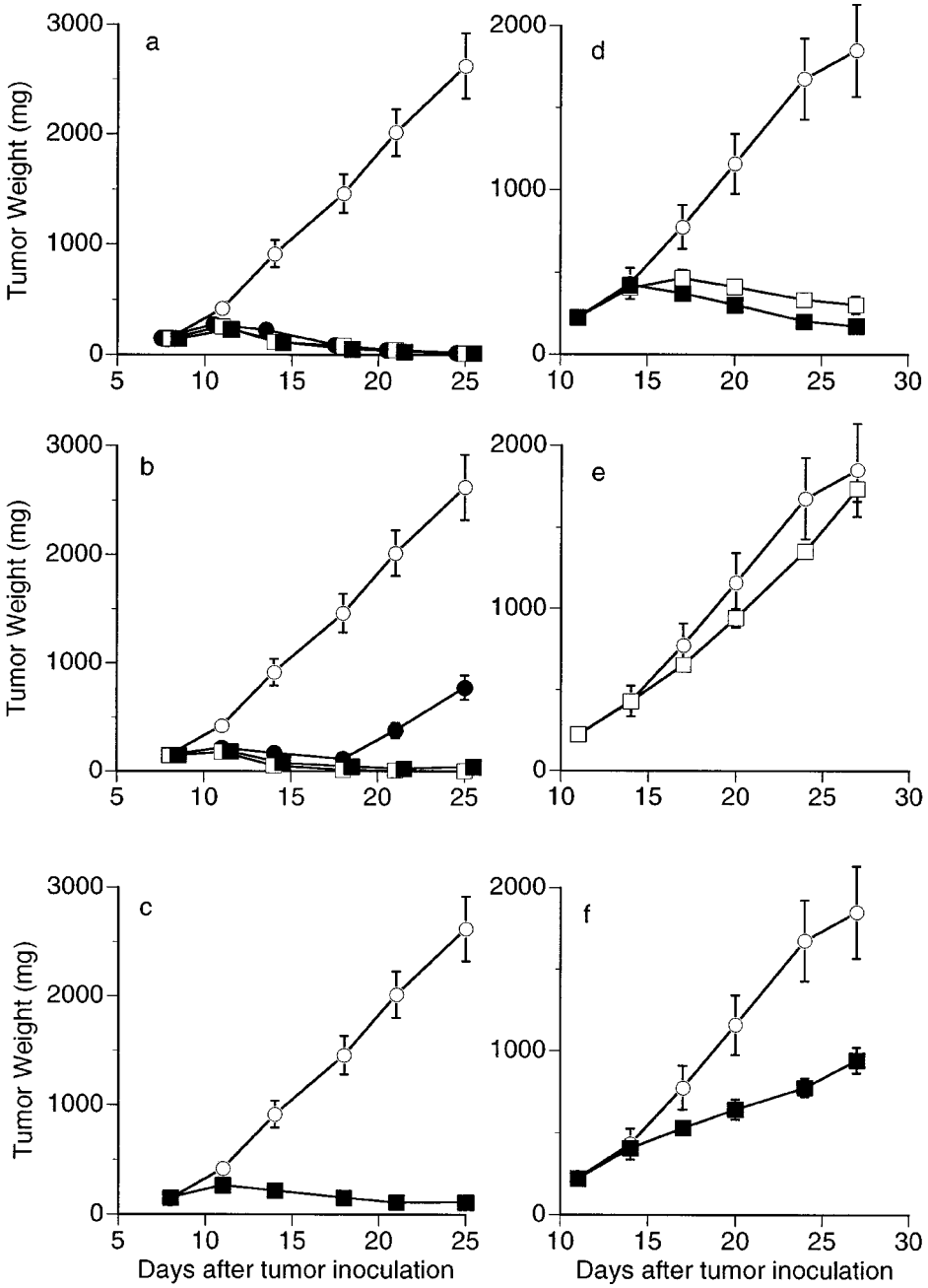


Fig. 2. Comparative antitumor activities of FK317 (a, d), VLB (b, e) and ADM (c, f) in KB-3-1 (a, b, c) and KB-V1 (d, e, f). Tumor cells were inoculated s.c. into nude mice. The drugs were administered i.v. to mice 3 times at 3-day intervals when the tumor weight had reached between 100 and 300 mg. Mice were used in groups of six. ○ control, ● 1.8 mg/kg, □ 3.2 mg/kg, ■ 5.6 mg/kg.

Photoaffinity labeling of plasma membrane Analysis of the binding of P-glycoprotein was carried out as described by Sato *et al.*[22] The plasma membranes (50 μg of protein) were photolabeled in 40 m*M* Tris-HCl buffer (pH 7.2), containing 4% dimethyl sulfoxide and 200 n*M* [^{3}H]azidopine in a final volume of 50 μl. Photolabeled membranes were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using gradient

gels (4–20%). A total of 12 μg of protein was loaded onto each lane. The gel was fixed, treated with the fluorographic reagent Amplify (Amersham Japan, Tokyo), dried and then exposed for 10 days at −70°C using Kodak XAR-5 film (Kodak, NY).

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*.

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.

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 μ*M*. However, a low degree of resistance to FK317 persisted even in the presence of verapamil (10 μ*M*) (Table II).

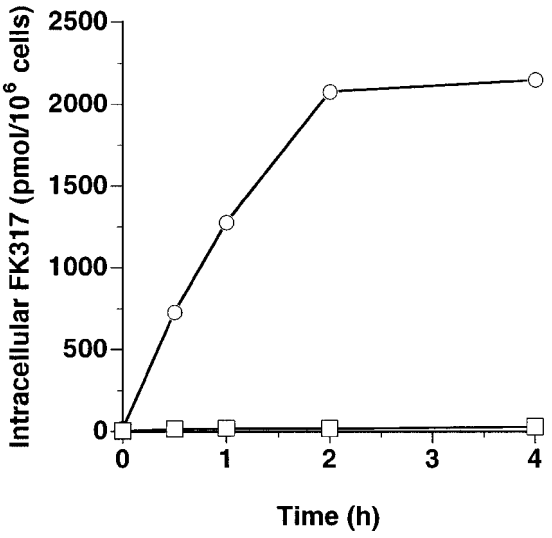


Fig. 3. Uptake of [^{14}C]FK317 in KB-3-1 (○) and KB-V1 (□) cells. Cells were incubated at 37°C with 15 μ*M* [^{14}C]FK317 (332 Mbq/mmol). At various intervals, the amount of intracellular [^{14}C]FK317 was determined.

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

	IC_{50} (n*M*)					
	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^{-5}	1100
1×10^{-6}	2.6×10^{-9}	4.0×10^{-7}	150	2.9×10^{-8}	9.8×10^{-6}	340
3.2×10^{-6}	2.2×10^{-9}	4.7×10^{-8}	21	2.6×10^{-8}	3.3×10^{-6}	130
1×10^{-5}	1.7×10^{-9}	5.0×10^{-9}	2.9	1.4×10^{-8}	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 10^6 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 6.6×10^{-10}, 4.0×10^{-9} and 5.2×10^{-9}*M*, and those towards KB-V1 were 4.7×10^{-6}, 5.4×10^{-7} and 2.0×10^{-7} *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 μM partially, and at 250 μM almost completely, inhibited [^{3}H]azidopine binding. However, FR70496 and

Table III. Half-lives of Deacetylation of FK317 and FR70496 in Mouse and Human Blood Preparations

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

The authentic compound (3 μg) was incubated with 30 μl of blood preparation with shaking at 37°C. HPLC analysis was then performed.

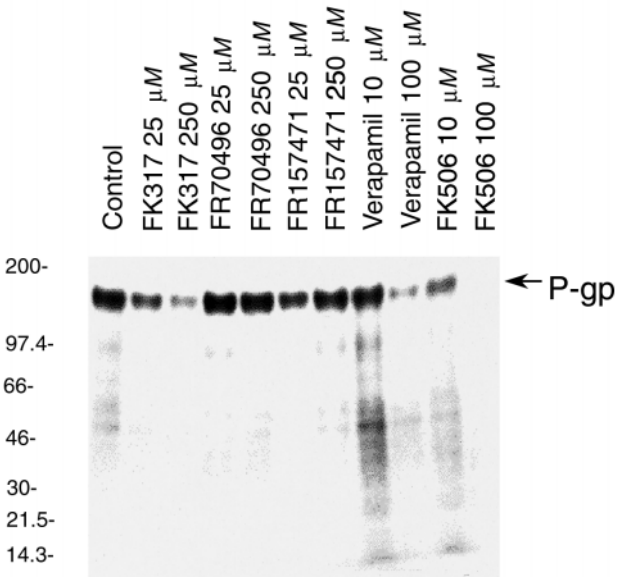


Fig. 4. Inhibition of [^{3}H]azidopine photolabeling of P-glycoprotein. KB-V1 membrane vesicles (50 μg of protein) were incubated with 200 n*M* [^{3}H]azidopine in the absence or presence of FK317, FR70496, FR157471, FK506 and verapamil at the indicated concentrations. After solubilization, photolabeled protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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

Drug	IC_{50} (n*M*)		RI[a)]	IC_{50} (n*M*)		RI
	KB-3-1	KB-V1		SBC-3	SBC-3/ADM	
FK317	6.6×10^{-10}	4.7×10^{-6}	7100	8.9×10^{-8}	3.8×10^{-6}	43
FR70496	4.0×10^{-9}	5.4×10^{-7}	140	1.1×10^{-7}	1.1×10^{-6}	10
FR157471	5.2×10^{-9}	2.0×10^{-7}	38	1.6×10^{-7}	6.1×10^{-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 μM 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 vivo* 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 deacetate (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 π (GST π) 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 π activity and decreased topoisomerase II activity.[20] 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.[28] 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.

ACKNOWLEDGMENTS

We thank Dr. David Barrett, Medical Chemistry Research Laboratories, for a critical reading of the manuscript.

(Received June 3, 1998/Revised July 14, 1998/Accepted July 22, 1998)

REFERENCES

1) Biedler, J. L. Genetic aspects of multidrug resistance. *Cancer*, **70**, 1799–1809 (1992).
2) Safa, A. R. Photoaffinity labeling of P-glycoprotein in multidrug-resistant cells. *Cancer Invest.*, **11**, 46–56 (1993).
3) Juliano, R. L. and Ling, V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta*, **455**, 152–162 (1976).
4) Kartner, N., Riordan, J. R. and Ling, V. Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. *Science*, **221**, 1285–1288 (1983).
5) Roninson, I. B., Chin, J. E., Choi, K., Gros, P., Housman, D. E., Fojo, A., Shen, D.-W., Gottesman, M. M. and Pastan, I. Isolation of human mdr DNA sequences amplified in multidrug resistant KB carcinoma cells. *Proc. Natl. Acad. Sci. USA*, **83**, 4538–4542 (1986).
6) Chen, C., Chin, J. E., Ueda, K., Clark, D. R., Pastan, I., Gottesman, M. M. and Roninson, I. B. Internal duplication and homology with bacterial transport protein in the *mdr*1 (p-glycoprotein) gene from multidrug-resistant human cells. *Cell*, **47**, 381–389 (1986).
7) Gros, P., Croop, J. and Housman, D. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. *Cell*, **47**, 371–380 (1986).
8) Gerlach, J. H., Endicott, J. H., Juranka, P. F., Henderson, G., Sarangi, F., Deuchars, K. L. and Ling, V. Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. *Nature*, **324**, 485–489 (1986).
9) Hamada, H. and Tsuruo, T. Purification of the 170- to 180-kilodalton membrane glycoprotein associated with multidrug resistance: the 170- to 180-kilodalton membrane glycoprotein is an ATPase. *J. Biol. Chem.*, **263**, 1454–1458 (1988).
10) Dano, K. Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. *Biochim. Biophys. Acta*, **323**, 466–483 (1988).
11) Inaba, M., Kobayashi, H., Sakurai, Y. and Johnson, R. K. Active efflux of daunomycin and adriamycin in sensitive and resistant sublines of P388 leukemia. *Cancer Res.*, **39**, 2200–2203 (1979).
12) Gros, P., Neriah, Y. B., Croop, J. M. and Housman, D. E. Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature*, **323**, 728–731 (1986).
13) Shimomura, K., Manda, T., Mukumoto, S., Masuda, K., Nakamura, T., Mizota, T., Matsumoto, S., Nishigaki, F., Oku, T., Mori, J. and Shibayama, F. Antitumor activity and hematotoxicity of a new, substituted dihydrobenzoxazine, FK973, in mice. *Cancer Res.*, **48**, 1166–1172 (1988).
14) Masuda, K., Nakamura, T., Mizota, T., Mori, J. and Shimomura, K. Interstrand DNA-DNA and DNA-protein cross-links by a new antitumor antibiotic, FK973, in L1210 cells. *Cancer Res.*, **48**, 5172–5177 (1988).
15) Majima, H., Hasegawa, K., Fukuoka, M., Furuse, K., Wakui, A., Furue, H., Masaoka, T., Hattori, T., Taguchi, T., Ogura, T. and Niitani, H. Phase I clinical and pharmacokinetic study of FK973. *Proc. Am. Soc. Clin. Oncol.*, **9**, 78 (1990).
16) Pazdur, R., Ho, D. H., Daugherty, K., Bradner, W. T., Krakoff, I. H. and Raber, M. N. Phase I trial of FK973: description of a delayed vascular leak syndrome. *Invest. New Drugs*, **9**, 337–382 (1991).
17) Naoe, Y., Inami, M., Matsumoto, S., Nishigaki, F., Tsujimoto, S., Kawamura, I., Miyayasu, K., Manda, T. and Shimomura, K. FK317; a novel substituted dihydrobenzoxazine with potent antitumor activity which does not induce vascular leak syndrome. *Cancer Chemother. Pharmacol.*, **42**, 31–36 (1998).
18) Ueyama, Y. Applications for research on cancer treatment methods using cancer characteristics. *In* "The Nude Mouse and Anticancer Drug Evaluation," ed. T. Nakamura, Y. Sakurai and M. Inaba, pp.197–203 (1996). Central Institute for Experimental Animals Press, Kawasaki.
19) Shen, D.-W., Cardarelli, C., Hwang, J., Cornwell, M., Richert, N., Ishii, S., Pastan, I. and Gottesman, M. M.

Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins. *J. Biol. Chem.*, **261**, 7762–7770 (1986).
20) Kiura, K., Ohnoshi, T., Tabata, M., Shibayama, T. and Kimura, I. Establishment of an adriamycin-resistant subline of human small cell lung cancer showing multifactorial mechanisms of resistance. *Acta Med. Okayama*, **47**, 191–197 (1993).
21) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55–63 (1983).
22) Sato, W., Fukazawa, N., Suzuki, T., Yusa, K. and Tsuruo, T. Circumvention of multidrug resistance by a newly synthesized quinoline derivative, MS-073. *Cancer Res.*, **51**, 2420–2424 (1991).
23) Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M. and Pastan, I. Partial purification and reconstitution of the human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis. *Proc. Natl. Acad. Sci. USA*, **89**, 8472–8476 (1992).
24) Lockridge, O., Mottershaw, J. N., Eckerson, H. W. and La Du, B. N. Hydrolysis of diacetylmorphine (heroin) by human serum cholinesterase. *J. Pharmacol. Exp. Ther.*, **215**, 1–8 (1980).
25) Elbaum, D. and Nagel, R. L. Esterase activity of hemoglobin. *J. Biol. Chem.*, **256**, 2280–2283 (1981).
26) Mizuno, K., Furuhashi, Y., Maeda, O., Iwata, M., Misawa, T., Kawai, M., Kano, T. and Tomoda, Y. Mitomycin C cross-resistance induced by adriamycin in human ovarian cancer cell *in vitro*. *Cancer Chemother. Pharmacol.*, **26**, 333–339 (1990).
27) Wakusawa, S., Nakamura, S. and Miyamoto, K. Establishment by adriamycin exposure of multidrug-resistant rat ascites hepatoma AH130 cells showing low DT-diaphorase activity and high cross resistance to mitomycins. *Jpn. J. Cancer Res.*, **88**, 88–96 (1997).
28) Zamora, J. M., Pearce, H. L. and Beck, W. T. Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Mol. Pharmacol.*, **33**, 454–462 (1988).