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Cytotoxic Mechanisms of FK317, a New Class of Bioreductive Agent with Potent Antitumor Activity

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FK317 is a member of a new class of bioreductive agents that exhibit strong cytotoxicity against various human cancer cells. The effect of FK317 was found to be stronger than that of mitomycin C (MMC), adriamycin (ADR) or cisplatin (CDDP). Alkaline elution analysis indicated that FK317 formed interstrand DNA-DNA and DNA-protein cross-links in cells. On the other hand, no DNA single-strand breaks were observed in the cells treated with FK317. In a cell-free system the deacetylated metabolites produced cross-linked DNA under reductive conditions, though FK317 itself did not form DNA-DNA cross-links. In order to elucidate the metabolic activation mechanisms, we established an FK317-resistant subline from human non-small cell lung cancer cells (Lu99) by stepwise and brief exposure (1 h) to FK317. The resistant subline (Lu99/317) showed cross-resistance to MMC and carboquone (CQ), but not to ADR or CDDP. DT-diaphorase, which is one of the activation enzymes of MMC and CQ, was deficient in Lu99/317 cells as determined by enzyme activity assay. However, the levels of NADPH:cytochrome P450 reductase, which is another activation enzyme for MMC and CQ, were comparable in resistant and parent cell lines. Treatment of the cells with dicumarol, an inhibitor of DT-diaphorase, reduced the cytotoxicity of FK317 to Lu99 cells, but not to Lu99/317 cells. These results indicate that deacetylation of FK317 is necessary for its reductive activation, and deacetylated FK317 is reduced by DT-diaphorase to form an active metabolite, which produces DNA-DNA interstrand and DNA-protein cross-links that lead to cell death.

Key words: FK317 — Antitumor effect — Bioreductive agent — DT-diaphorase — Deacetyl metabolite

FK317 is a new analog of FK973, which we previously showed to exhibit strong antitumor activities in a wide variety of animal tumor models and human xenografts, and has strong cytotoxic effects against *in vitro*-cultured tumor cell lines.[1)] FK973 has also been shown to have high therapeutic efficacy in clinical studies. However we were unable to develop this compound because it induces vascular leak syndrome (VLS) as a major side effect.[2, 3)] In attempts to synthesize new FK973 derivatives which retain the antitumor activity, without the VLS side effect, 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 identified. This agent has a unique bicyclic hydroxylamine hemiketal ring system, and its antitumor activity is more potent than, or equivalent to, that of mitomycin C (MMC), adriamycin (ADR), cisplatin (CDDP), taxol or irinotecan hydrochloride against murine tumors and human xenografts in mice. Moreover, its hematotoxic and myelosuppressive effects were weaker than those of MMC in mice. These preclinical data suggested that FK317 might represent a major advance in the treatment of cancer.

The mechanisms by which FK317 induces these potent antitumor effects have not yet been defined, but if more clearly understood, would offer valuable information for its clinical application. The chemical structure of FK317 contains reactive moieties such as carbamoyl and aziridine groups, which MMC also possesses, suggesting that FK317 and MMC may have a common mechanism of action. MMC causes damage to DNA following reductive activation to DNA-reactive species.[4)] The most conspicuous type of DNA damage caused by activated MMC is the formation of DNA-DNA interstrand cross-links; these occur both in cell-free systems and in drug-treated cells.[5, 6)] In general, DNA-DNA cross-links represent highly lethal lesions, presumably because cross-links which are unrepaired at a replication fork irreversibly halt the progress of the fork, thereby causing cell death.

In this study, we compared the action of FK317 against DNA with that of MMC, and further investigated the mechanism of FK317 cytotoxicity using a resistant cell line that we have established.

MATERIALS AND METHODS

Chemicals FK317, FR70496, FR157471 and FR160516 were prepared in the Fujisawa Research Laboratories. The

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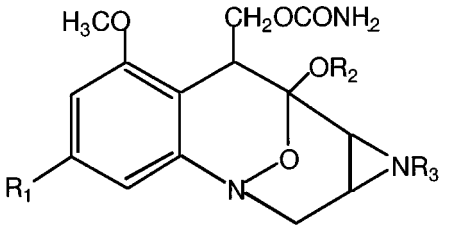
chemical structures of FK317 and its derivatives are shown in Fig. 1. MMC and ADR were purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo. CDDP, dicumarol and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co., St. Louis, MO. Carboquone (CQ) was purchased from Sankyo Co., Ltd., Tokyo. [Methyl-^{14}C]thymidine (2.2 GBq/mmol) was purchased from NEN Research Products, Boston, MA. Sodium hydrosulfite and pBR322 were purchased from Nacalai Tesque Inc., Kyoto, and Toyobo, Tokyo, respectively.

Tumor cells The human non-small cell lung cancer cell line (A549), and the colon cancer cell lines (SW-480 and SW-620) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) and penicillin (50 units/ml)-streptomycin (50 μg/ml). Human non-small cell lung cancer cell lines (PC-1, PC-9, PC-10, Lu99 and Lu65), small cell lung cancer cell lines (ADH and NCI-H69) and colon cancer cell lines (COLO201 and COLO205) were cultured in RPMI-1640 medium supplemented with 10% FCS and penicillin (50 units/ml)-streptomycin (50 μg/ml). Mouse leukemia L1210 cells were grown in RPMI-1640 supplemented with 10% FCS and penicillin (50 units/ml)-streptomycin (50 μg/ml).

***In vitro* cytotoxicity** Growth inhibition experiments using human tumor cell lines were carried out in 96-well flat-bottomed microplates, and the amount of viable cells at the end of the incubation was determined by MTT assay, essentially as described by Mosmann.[7] Thus, 5×10^3–4×10^4 cells/well in 100 μl were plated, and drug or the medium alone (control) was added. The cells were then cultured for 72 h. 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).



	R_1	R_2	R_3
FK317	CHO	$COCH_3$	$COCH_3$
FR70496	CHO	H	$COCH_3$
FR157471	CHO	H	H
FR160516	CH_2OH	H	H

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

L1210 cells (2×10^6) were incubated with drugs for 1 h in 2 ml of the cultured medium, washed twice with PBS by centrifugation, and resuspended in the medium. The cells (1×10^4) were seeded onto a 96-well plate in 100 μl of the medium. After incubation for 48 h, the viable cells were counted by the trypan blue dye exclusion method.

Alkaline elution assay Alkaline elution was performed according to the method of Kohn *et al*.[8] Cellular DNAs were radioactively labeled in L1210 cells by incubation with [^{14}C]thymidine (3.7 kBq/ml) for 20 h at 37°C. The cells were exposed to drug for 1 h at 37°C, washed twice with PBS by centrifugation, and resuspended in PBS. The cells were irradiated with X-rays (400 rads) on ice immediately before alkaline elution was carried out. Cells (5×10^5) were collected on a polycarbonate filter (pore size: 2.0 μm, 25 mm diameter, Nucleopore Corp., Pleasanton, CA), lysed by pouring 5 ml of 2% SDS/ 25 m*M* EDTA (pH 9.7) onto the filter, and treated with proteinase K (0.5 mg/ml, 20 units/mg, Boehringer Mannheim GmbH, Mannheim, Germany) for 1 h at room temperature in the dark to digest all DNA-bound proteins. The filter was washed by passing 3 ml of 25 m*M* EDTA solution (pH 10.0) through the filter under gravity. An elution solution (pH 12.1) containing tetrapropylammonium hydroxide (Sigma), 20 m*M* EDTA and 0.1% SDS was pumped through the filter at a flow rate of 2 ml/h in the dark. The fractions were collected at 1 h intervals for 15 h.

pBR322 DNA cross-linking assay Analysis of cross-linking of pBR322 DNA was carried out as described by Williams and Scott.[9] The substrate was pBR322 plasmid DNA that had been linearized by digestion with *Hin*dIII. Reactions (8 μl) were run in 10 m*M* Tris-HCl/1 m*M* EDTA (pH 8.0). Reactions of this substrate with drugs (4 m*M*) in the presence and absence of $Na_2S_2O_4$ were conducted for 1 h at 37°C. Upon completion of this incubation, each reaction mixture was added to 15 μl of alkaline loading buffer (50 m*M* NaOH/1 m*M* EDTA/2.5 g Ficoll/ 0.25 g bromocresol green in 100 ml H_2O) and placed on a 1.2% alkaline agarose gel (33 m*M* NaOH/2 m*M* EDTA). Electrophoresis was carried out in recirculating 33 m*M* NaOH/2 m*M* EDTA buffer at 40 V/80 mA for 10 h. After the electrophoresis was complete, the gel was neutralized with 30 m*M* Tris-HCl (pH 7.5) and stained with ethidium bromide.

Isolation of FK317-resistant cell line An FK317-resistant subline was developed by 1 h exposure of Lu99 cells to progressively increasing concentrations of FK317 (ranging from 2 to 320 n*M*) over a period of 3 months. Following drug exposure, cells were washed with PBS and subcultured in fresh, drug-free medium until the

growth of the surviving cells had reached confluence, after which the drug-treatment cycle was resumed. Lu99/317 cells did not lose their resistant phenotype for at least 9 months in FK317-free medium.

Assay of enzyme activity DT-diaphorase activity was assayed according to the method of Ernster,[10] as modified by Benson *et al*.[11] NADPH:cytochrome P-450 reductase activity was measured according to the method of Vermillion and Coon.[12]

RESULTS

Cytotoxicity towards *in vitro*-cultured human tumor cells The cytotoxicity of FK317 towards various human tumor cell lines, human non-small cell lung cancer (A549, PC-1, PC-9, PC-10, Lu99 and Lu65), small cell lung cancer (ADH and NCI-H69), and colon cancer (COLO201, COLO205, SW-480 and SW-620) cell lines, was examined and the results are shown in Table I. The potency of FK317 was stronger than that of MMC, ADR or CDDP. The concentration of FK317 required to reduce cell survival by 50% (IC_{50}) ranged from 1.9 to 1400 n*M*.

Formation of DNA cross-links in L1210 cells MMC has aziridine and carbamoyl moieties in its chemical structure, and forms DNA-DNA interstrand cross-links in cells. Since FK317 also possesses the same functionalities, the formation of DNA cross-links was examined using the alkaline elution method. L1210 cells were incubated with the drug for 1 h, and alkaline elution was performed. Fig. 2 shows that the retention of ^{14}C-labeled DNA on the filter increased dose-dependently in the cells treated with FK317 and MMC, suggesting that both of these drugs form DNA-DNA interstrand cross-links, and that the effect of FK317 is stronger than that of MMC. Furthermore, FK317 formed DNA-protein cross-links dose-dependently and more potently than MMC (data not shown). On the other hand, FK317 had no single-strand DNA-breaking activity in the cells (data not shown). The cytotoxic activity of FK317 to L1210 cells exposed for 1 h was also studied and compared with that of MMC. The IC_{50} values of FK317 and MMC were approximately 0.5 and 1.0 μM, respectively.

Table I. Cytotoxicity of FK317 against Various Human Tumor Cell Lines

Cell lines	IC_{50}[a] (n*M*)			
	FK317	MMC	ADR	CDDP
Non-small cell lung cancer				
PC-1	720[b]	970	590	11000
PC-9	300	1100	1700	8300
PC-10	10	270	160	1000
Lu65	10	900	320	790
Lu99	1.9	7.5	290	920
Small cell lung cancer				
ADH	28	10	570	790
NCI-H69	51	1900	1500	2900
Colon cancer				
COLO201	28	790	430	17000
COLO205	28	980	160	17000
SW-480	1400	1400	600	17000
SW-620	35	2100	480	6200

Cells seeded in a 96-well microplate were incubated with the drugs for 72 h.
The IC_{50} value was determined by MTT assay.
a) Concentration of each drug producing 50% inhibition of cell growth.
b) All values are the average of three determinations.

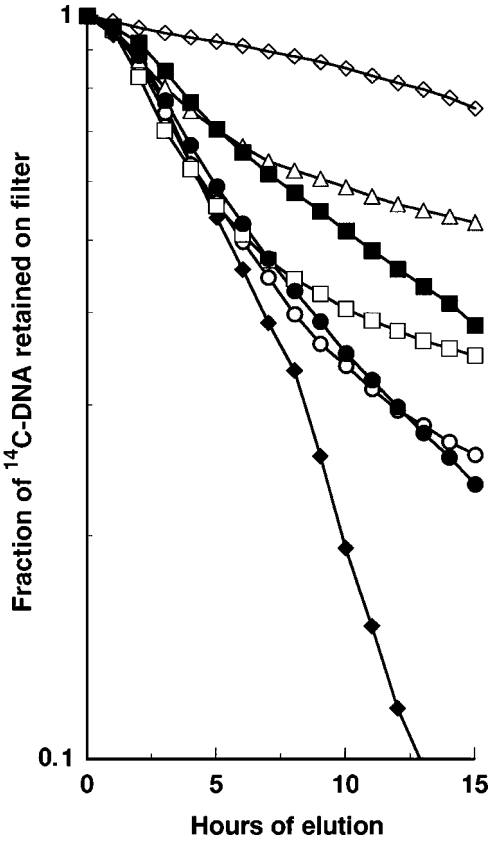


Fig. 2. DNA-DNA interstrand cross-link formation in L1210 cells. Cellular DNA was radioactively labeled in the cells by incubation with [^{14}C]thymidine for 20 h at 37°C. The cells were exposed to a drug for 1 h at 37°C and irradiated with 400 rads of X-ray on ice. The cells were then collected on a filter, lysed with a solution containing sodium dodecyl sulfate and treated with proteinase K. DNA was eluted with alkaline solution (pH 12.1). ◇ no drug (no X-rays), ◆ no drug (400 R), ○ FK317 0.1 μM (400 R), □ FK317 1 μM (400 R), △ FK317 10 μM (400 R), ● MMC 10 μM (400 R), ■ MMC 32 μM (400 R).

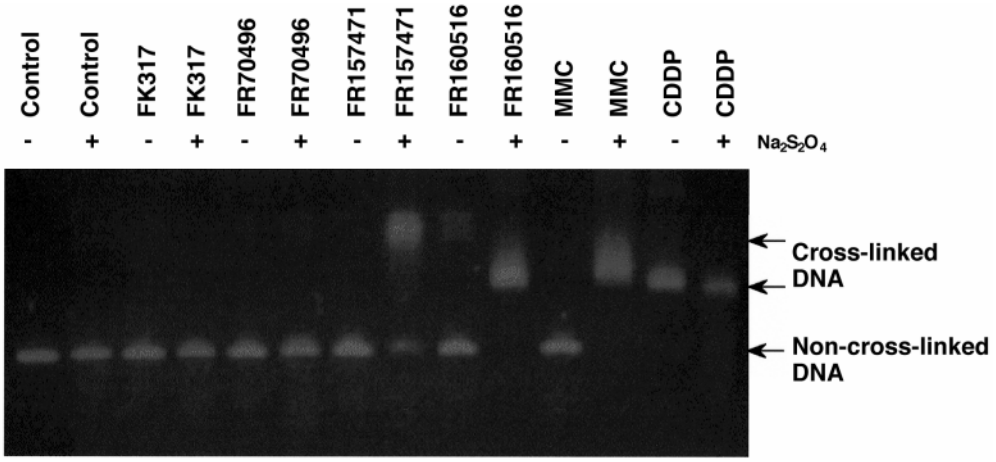


Fig. 3. DNA-DNA interstrand cross-link formation under reductive conditions. Linearized pBR322 was incubated with a drug in Tris-EDTA buffer (pH 8.0) for 1 h at 37°C in the presence or absence of 10 m*M* $Na_2S_2O_4$. The reaction mixtures were placed on alkaline agarose gel, and electrophoresis was carried out. After electrophoresis was complete, the gel was neutralized and stained with ethidium bromide.

Table II. Cytotoxicity of Various Autitumor Drugs against Lu99 and Lu99/317 Cells and Enzyme Activity of Those Cells

	IC_{50} (n*M*)					nmol/min/mg protein	
	FK317	MMC	ADR	CDDP	CQ	DT-diaphorase	NADPH:cytochrome P-450 reductase
Lu99	4.2	23	15	710	0.009	1400	27
Lu99/317	170	300	23	2400	110	ND[b)]	85
Resistance index[a)]	40	13	1.5	3.4	12000		

Lu99 and Lu99/317 seeded in a 96-well microplate were incubated with the drugs for 72 h.
The IC_{50} value was determined by MTT assay.
Activity of DT-diaphorase or NADPH:cytochrome P-450 reductase was measured using DCPIP or cytochrome *c* as the electron acceptor, respectively.
a) Ratio of IC_{50} value for Lu99/317 cell to that for Lu99 cells.
b) Not detectable (<5 nmol/min/mg protein).

Table III. Effect of Dicumarol on the Cytotoxicity of FK317 in Lu99 and Lu99/317 Cells

Drug	IC_{50}[a)] (n*M*)		Resistance index[a)]
	Lu99	Lu99/317	
None	7.2	430	60
10 μM Dicumarol	160	390	2.4

Cells seeded in a 96-well microplate were incubated with the drugs for 72 h.
The IC_{50} value was determined by MTT assay.
a) Ratio of IC_{50} value for Lu99/317 cells to that for Lu99 cells.

Formation of DNA-DNA interstrand cross-links under reductive conditions As it has been reported that MMC requires reductive conditions to produce DNA cross-links in a cell-free system, we examined the reactivity of FK317 with DNA in a cell-free system and found that FK317 did not form DNA-DNA interstrand cross-links under these conditions (Fig. 3). Furthermore, FK317 did not produce DNA-DNA interstrand cross-links even in the presence of a reducing agent, $Na_2S_2O_4$ (10 m*M*). However, FR70496, which is mono-deacetylated FK317, and FR157471, a di-deacetylated metabolite of FK317, formed DNA-DNA interstrand cross-links in the presence of $Na_2S_2O_4$. FR160516, a reduction product of FR157471, formed DNA-DNA interstrand cross-links even in the absence of $Na_2S_2O_4$. Cisplatin produced DNA-DNA interstrand cross-links without metabolic activation.

Sensitivity of the Lu99/317 subline to other anticancer drugs Table II shows the cytotoxic effects of various anticancer drugs towards Lu99 and its resistant subline, Lu99/317. Lu99/317 showed about 40-fold resistance to FK317. It is noteworthy that Lu99/317 cells showed 40-, 13-, and

12000-fold resistance to FK317, MMC and CQ, respectively, but did not show cross-resistance to ADR or CDDP.

Enzyme activities of Lu99 and Lu99/317 cells To investigate the mechanisms of resistance to FK317, we determined the activities of DT-diaphorase and NADPH: cytochrome P-450 reductase, as shown in Table II. The activity of DT-diaphorase in Lu99 cells was 1400 nmol/min/mg protein, whereas DT-diaphorase activity was not detected in Lu99/317 cells. On the other hand, NADPH: cytochrome P-450 reductase activities in Lu99 and Lu99/317 cells were almost the same. Thus, DT-diaphorase was deficient in Lu99/317 cells, and this may be relevant to the resistance to FK317.

Effect of dicumarol on the cytotoxicity towards Lu99 and Lu99/317 cells To examine further the role of DT-diaphorase in the activation mechanisms of FK317, the effect of dicumarol on the cytotoxicity of FK317 was investigated (Table III). Co-incubation of the cells with dicumarol (10 μM), which is an inhibitor of DT-diaphorase, significantly lowered the sensitivity of Lu99 cells to FK317. In contrast, the cytotoxicity of FK317 towards Lu99/317 cells was not affected by treatment with dicumarol.

DISCUSSION

FK317 is an attractive new antitumor agent that has a novel structure and displays strong antitumor effects *in vitro* and *in vivo*.[13] In this study, we examined the mechanisms by which FK317 induces cytotoxic effects on various cancer cell lines *in vitro*. FK317 showed strong cytotoxic effects against human non-small cell lung cancer, small cell lung cancer and colon cancer cell lines. In general, the cytotoxicity of FK317 was stronger than that of MMC, ADR or CDDP, which are frequently used in the clinic. In particular, the cytotoxicities of FK317 towards Lu65, NCI-H69, COLO205 and SW-620 were 90-, 37-, 35- and 60-fold stronger than those of MMC, respectively. Moreover, the range of IC_{50} values for FK317 was the greatest among the drugs examined (1.9–1400 nM). These findings indicate that certain factors in tumor cells may be required for the cytotoxic activity of FK317.

FK317 has an aziridine ring and a carbamoyl moiety in its chemical structure, like MMC. Since an aziridine ring and a carbamoyl moiety are both important in the reaction of MMC with DNA,[5, 14] we studied the effects of FK317 on DNA using the alkaline elution method. After cells were exposed to a drug for 1 h, we detected concentration-dependent DNA-DNA interstrand and DNA-protein cross-links formed by both FK317 and MMC. The effects of FK317 were much stronger than those of MMC, and may reflect the differences in the cytotoxic activities. The cytotoxic effects of FK317 are derived mainly from mechanisms related to its formation of DNA-DNA interstrand and DNA-protein cross-links, but not DNA single-strand breaks, although the involvement of other mechanisms remains to be clarified.

Since MMC requires reductive activation for interaction with DNA, we examined whether FK317 also required a similar activation. However, contrary to our expectation, FK317 did not form DNA-DNA interstrand cross-links, even in the presence of a reducing agent. FK317 has two acetyl groups, in addition to its aziridine moiety and also a hemi-ketal ring, that may augment its antitumor activity. When FR70496 and FR157471, which are deacetylated analogs of FK317, were examined in a cell-free system, they produced DNA-DNA interstrand cross-links in the presence of a reducing agent. Surprisingly, FR160516, a reduction product of FR157471, formed DNA-DNA interstrand cross-links even in the absence of a reducing agent. These results suggest that deacetylation of FK317 is necessary for its reductive activation, and that the deacetyl metabolites of FK317 require reductive activation in order to interact with DNA. FK317 is a new class of bioreductive agent, because its activity is masked by the acetyl groups. Deacetylation is required for the antitumor activity of FK317, but not for that of MMC; however, this difference can not completely explain why FK317 shows stronger antitumor activity, with lower side effects than MMC. Quantification of acetyl esterase and reductase activity in the tumor and also in normal tissue will be of value in clinical investigations to predict the antitumor effects and the organ-specific adverse effects of FK317.

In order to investigate further the mechanisms of FK317, we established an FK317-resistant variant (Lu99/317) of Lu99 human non-small cell lung cancer. Lu99/317 cells showed cross-resistance to MMC and CQ, but not to ADR or CDDP. Both MMC and CQ have a common quinone moiety and require reductive activation. These results suggest that the decrease in activity of MMC or CQ activation enzymes may be related to the mechanism of FK317-resistance in Lu99/317. Activation of MMC and CQ to afford an alkylating species has been demonstrated with one-electron reducing enzymes such as NADPH:cytochrome P-450 reductase (EC 1.6.2.4)[15–17] and xanthine oxidase (EC 1.2.3.2),[15] and by two-electron reducing enzymes such as DT-diaphorase (EC 1.6.99.2).[17, 18] The activity of DT-diaphorase in Lu99, which is very sensitive to FK317, was very high, and this enzyme was deficient in Lu99/317 cells. The activity of NADPH:cytochrome P-450 reductase was comparable in both cell lines. The activity of xanthine oxidase in Lu99 cell was not detectable (data not shown). The role of DT-diaphorase in the sensitivity of MMC has been discussed in human normal and tumor cells.[19–21] In accordance with these reports, our results indicate a strong likelihood that

the deficiency of DT-diaphorase is involved in the mechanism of FK317-resistance in Lu99/317 cells. Furthermore dicumarol, which is an inhibitor of DT-diaphorase, clearly reduced the sensitivity of Lu99 cells to FK317. In contrast, the cytotoxicity of FK317 against Lu99/317 cells was not affected by treatment with dicumarol. These results indicate that DT-diaphorase is a key enzyme which activates FR157471, a deacetyl metabolite of FK317, to yield a DNA-reactive species, though the possibility of involvement of other reductive enzymes remains to be clarified. DT-diaphorase activity is found to be increased in human tumors of the lung, liver, colon and breast.[22] In this study, FK317 showed strong cytotoxic effects against human non-small cell lung cancer and colon cancer cell lines. From these results, FK317 should show strong clinical antitumor effects against tumors in which DT-diaphorase activity is high.

The major structural differences between MMC and FK317 are the presence of a quinone in MMC, and the hydroxylamine hemi-ketal of FK317. Although DT-diaphorase is well known for catalyzing two-electron reduction of quinones to quinols without the formation of semiquinone radicals,[18] this enzyme is also reported to reduce such non-quinone compounds as dinitrophenyl aziridine CB 1954,[23, 24] suggesting that FK317 can also be reduced by DT-diaphorase. Which part of the chemical structure of FK317 is reduced by the enzyme? FR157471 may be activated to a DNA-reactive species by reduction of the formyl groups, since FR160516 formed DNA-DNA interstrand cross-links without reduction. Furthermore, it may be important to cleave the N-O bond of the unique hydroxylamine hemi-ketal of FR157471 or FR160516 by reductive activation. Goto and Fukuyama proposed that FR900482, the parent compound of FK317, undergoes reductive activation *in vivo*.[25] This proposal holds that reduction of the N-O bond initiates a reaction cascade which ultimately yields a mitosene-like nucleus presumed to cross-link DNA.[26] This suggests that the formyl group and N-O bond of hydroxylamine hemi-ketal are targets for reduction by DT-diaphorase.

In conclusion, the present results indicate that FK317 has strong antitumor activities against human tumor cell lines, and that it is deacetylated and activated to DNA reactive species by a reductase, DT-diaphorase, which produces DNA-DNA interstrand and DNA-protein cross-links that lead to the death of the cell.

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