BIOLOGICAL ACTIVITIES OF NEW ANTHRACYCLINES CONTAINING FLUORINE, FAD104 AND ITS METABOLITES

SETSUKO KUNIMOTO, KEIKO KOMURO, CHISATO NOSAKA, TSUTOMU TSUCHIYA[†], SHUNZO FUKATSU[††] and TOMIO TAKEUCHI

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan
[†] Institute of Bioorganic Chemistry,
1614 Ida, Nakahara-ku, Kawasaki 211, Japan
[††] Meiji Seika Kaisha Ltd.,
760 Morookachou, Kouhoku-ku, Yokohama 222, Japan

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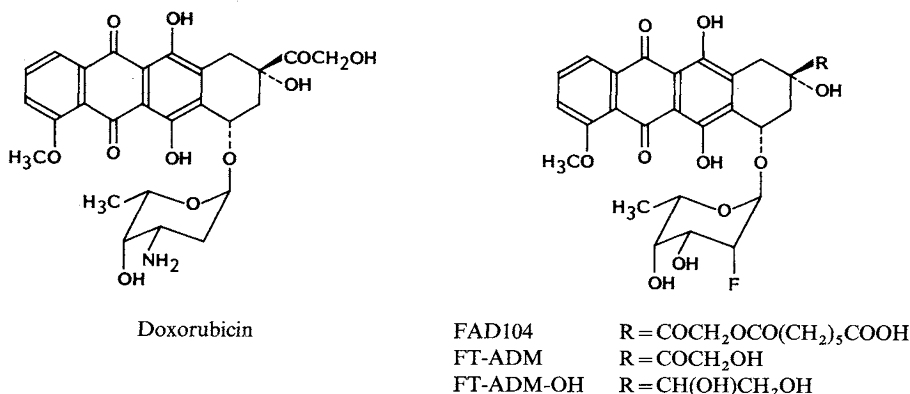
From various new anthracyclines containing fluorine in their sugar moieties, 7-*O*-(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)adriamycinone-14-hemipimerate (FAD104) has been selected for clinical investigation, because of its excellent antitumor activity and solubility. In this paper, the mechanism whereby FAD104 exhibits good antitumor activity *in vivo* was studied through experiments *in vitro* in comparision with doxorubicin (DOX). FAD104 had weaker activity than DOX in DNA single and double strand scission in P388 cells and in binding to calf thymus DNA. FAD104 was taken up by P388 cells faster than DOX. The ester linkage in FAD104 was gradually hydrolyzed at neutral pH. FAD104 was metabolized to 7-*O*-(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)adriamycinone (FT-ADM) when incubated with mouse or human serum. In mouse serum the esterase activity was about 100 times higher than in human serum. The product of nonenzymatic and enzymatic esterolysis, FT-ADM, was rapidly taken up by P388 cells and accumulated, reaching at a 9.7-fold higher level than DOX. Thus FAD104 was less active than DOX in itself, but it showed much higher activity through conversion into FT-ADM, due to the action of esterase or to spontaneous and gradual hydrolysis at physiological pH. 7-*O*-(2,6-Dideoxy-2-fluoro-α-L-talopyranosyl)adriamycinol (FT-ADM-OH), another metabolite found in mouse serum had the least biological activity among the fluorine-containing anthracycline glycosides, but its activity was still higher than DOX.

An anthracycline antibiotic, doxorubicin (DOX) has been clinically important in cancer chemotherapy. It has a wide spectrum of activity against human tumors such as stomach, breast, ovary and bladder cancers, and acute leukemia. Its utility, however, is limited due to irreversible cardiotoxicity and other undesirable side effects. Natural and acquired resistance to muliple drugs including DOX is an important clinical problem. Efforts to make new analogs have been continued by many research groups with the objective of obtaining compounds with superior activity and less cardiotoxicity, and to overcome multi-drug resistance. We previously discovered aclarubicin (aclacinomycin A) from culture filtrates of *Streptomyces galilaeus* in 1975[1], which had lower general and cardiac toxicities than DOX and was effective against multi-drug resistant cells. It has been used clinically to treat acute myeloid leukemia since 1981. In 1979, pirarubicin (4′-*O*-tetrahydropyranyladriamycin) was synthesized[2] and has been used since 1988 against solid tumors as a drug with less cardiotoxicity and side effects such as nausea, vomitting and hair loss than DOX. In previous papers[3~7], we reported syntheses of new fluorine-containing anthracycline glycosides. The electrophilic functional atom, fluorine, was introduced at the C-2 position of the sugar. Amongst these analogs, 7-*O*-(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)adriamycinone (FT-ADM) and its 14-*O*-acyl derivatives had superior antitumor activity and weaker toxicity than DOX. Out of their group 7-*O*-(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)adriamycinone-14-hemipimerate (FAD104) was selected for

Fig. 1. Structure of doxorubicin and fluorine-containing anthracyclines.



FAD104	$R = COCH_2OCO(CH_2)_5COOH$
FT-ADM	$R = COCH_2OH$
FT-ADM-OH	$R = CH(OH)CH_2OH$

clinical investigations because of its excellent antitumor activity and solubility: It has good therapeutic activity against leukemia L1210 over a wide dose range, curing many mice, while it showed a good solubility at neutral pH necessary for intravenous administration. In this paper, we report its cytotoxic effect, antitumor activity, DNA strand scission, inhibition of nucleic acid synthesis, DNA binding, uptake and retention by cultured cells. FT-ADM and 7-*O*-(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)adriamycinol (FT-ADM-OH) are both observed in the plasma of mice, rats (Meiji Seika Kaisha; personal communication) and rabbits (H. Fujita; personal communication) after administration of FAD104. FAD104 is predicted to be metabolized to FT-ADM by cellular and/or serum carboxylesterase like *N*-(trifluoroacetyl)adriamycin 14-*O*-hemiester derivatives[8] and subsequently to FT-ADM-OH. We, therefore studied the hydrolysis of FAD104 by serum esterase and by spontaneous hydrolysis without enzyme, and the biological activities of the products, FT-ADM and FT-ADM-OH, together.

Materials and Methods

Materials

DOX was supplied by the National Cancer Institute, Bethesda, Md., U.S.A. FAD104 and FT-ADM were synthesized by us[3~5]. FT-ADM-OH was synthesized by Meiji Seika Kaisha Ltd., Japan. [*Methyl*-^{3}H]thymidine (54 Ci/mmol) and [5-^{3}H]uridine (30 Ci/mmol) were products of Amersham International plc, Buckinghamshire, England. Calf thymus DNA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Company, St. Louis, U.S.A.

Mice

Female CDF_1 (Balb/c × DBA/2) mice, 4 to 6 weeks of age, were purchased from Charles River Japan Inc. and kept under standard laboratory conditions.

Cell Cultures

Mouse lymphoblastoma L5178Y and leukemia L1210 cells were cultured in RPMI1640 medium supplemented with 10% horse serum and calf serum, respectively. P388 and DOX-resistant cell lines obtained from Dr. M. Inaba, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, were maintained through serial ip transplantation in DBA mice. Ascitic cells obtained after 7 days after transplantation in CDF_1 mice were used in cytotoxicity tests and in experiments to study drug uptake or efflux. P388 and P388/ADR cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 10 μM 2-mercaptoethanol. Cytotoxicity against murine tumor cells was determined by

counting cell number using a Coulter counter or the automated microculture tetrazolium assay with MTT (MTT assay)[9] after 48 hours of drug treatment. MTT assay was as follows: Rapidly growing cells (5×10^4 cells/ml, 100 μl) were inoculated into wells of 96-well microtiter plates. To appropriate wells, 5 μl of drug solution at different concentrations was added. The plate was incubated for 48 hours at 37°C in a CO_2 incubator, to each microculture well MTT (5 mg/ml, 10 μl) was added and incubated for 2 hours at 37°C. To each well 50 μl of 10% sodium dodecyl sulfate was added to solubilize the MTT-formazan product. After overnight incubation optical density was measured on a Titertek Multiskan MCC plate reader, using a reference wavelength of 690 nm and a test wavelength of 570 nm.

Antitumor Activity

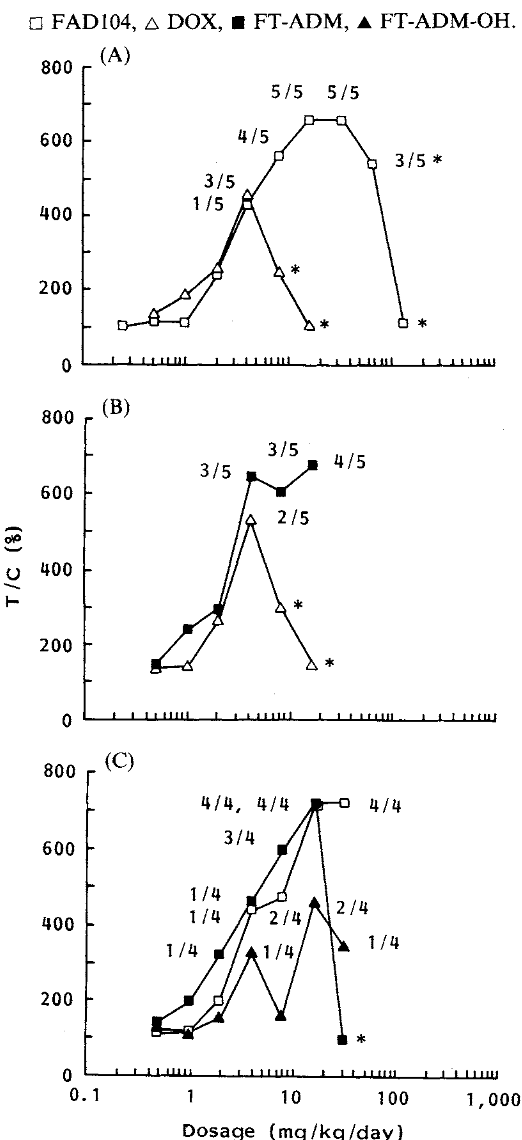
L1210 Ascitic lymphatic leukemia was maintained by serial ip passage in CDF_1 mice. For evaluation of antitumor activity 10^5 cells (0.25 ml) were inoculated ip in CDF_1 mice. Drugs were dissolved in saline, 0.05 M sodium or potassium phosphate buffer (pH 7.4) containing 5% DMSO as shown in the legend of Fig. 2. After tumor inoculation drugs were administered ip three times on days 1, 5 and 9. Antitumor activity was evaluated by mean survival days of 4 or 5 mice and expressed in terms of T/C (mean survival days of treated/mean survival days of control) percentage or the number of survivors at 60 days after tumor inoculation.

DNA Strand Scission

Drug-induced DNA single- and double-strand breaks were measured with the alkaline and the neutral DNA filter elution methods, respectively, as described by Pommier *et al.*[10].

DNA single strand breaks were measured as follows: P388 Cells grown in suspension culture were diluted to 5×10^4 cells/ml (10 ml in each flask, 2 flasks) with fresh medium and incubated. On the next day [^{3}H]thymidine (1 μCi) or [^{14}C]thymidine (0.25 μCi) was added to each flask to radioactively label cellular DNA. After 1 day of culture unincorporated radioactivity was removed by centrifugation and washing. P388 Cells (10^6/ml, 1 ml) labeled with [^{14}C]thymidine were treated with drug for 1 hour at 37°C. Cells were washed twice with a cold fresh medium. P388 Cells labeled with [^{3}H]thymidine were irradiated with 150R of X-ray on ice, and used as an internal standard. Cells (5×10^5) treated with drug were mixed with the same amount of the X-ray irradiated internal standard cells, and deposited onto a polycarbonate membrane filter (25 mm diameter, 2 μm pore size, Nucleo-

Fig. 2. Activity of anthracyclines against L1210 leukemia.



L1210 Cells (10^5) were inoculated ip into CDF_1 mice. The drugs were administered ip on days 1, 5 and 9. (A) FAD104 was dissolved in 0.05 M KH_2PO_4-NaOH buffer, pH 7.4. DOX was dissolved in saline. (B) FT-ADM was dissolved in saline containing 5% DMSO. (C) all the drugs were dissolved in 0.05 M Na phosphate buffer, pH 7.4 containing 5% DMSO. The numbers on the symbols represent 60 days survivors/total in group. The marks * mean that toxicity appeared as decreased body weight.

pore, Pleasanton, CA, U.S.A.) in a Swinnex 25 filter holder (Millipore Co., Bedford, MA, U.S.A.), washed with cold phosphate-buffered saline (pH 7.2) without Ca^{++} and Mg^{++}(PBS) and lysed for 1 hour in 5 ml of a solution containing 0.1 M glycine, 0.025 M Na_2EDTA and 2% SDS, and 0.5 mg/ml proteinase K, pH 9.6. The elution of fragmented DNA was performed in the dark with 40 ml of a solution containing 0.02 M EDTA, 0.1% SDS plus tetrapropylammonium hydroxide, pH 12.2, at a flow rate of 0.035 ~ 0.045 ml/minute using a multichannel peristaltic pump (model IPS-16, Ismatic Co., Swiss). Fractions were collected at 1-hour intervals and each fraction was neutralized with 0.3 ~ 0.75% acetic acid, mixed with 3.5 ml of Atomlite scintillation cocktail (New England Nuclear, Boston, U.S.A.), and processed for radioactive counting. Radioactivity remaining on the filter after elution was determined upon treatment with 0.4 ml of 1 N HCl for 1 hour at 65°C and subsequent addition of 1 ml of 1N NaOH and Atomlite. Resulting ^{14}C and ^{3}H dpm of the eluted fractions were expressed as fraction of the total dpm resulting from the sum of dpm in the eluted fractions and the extract from the filter for each treatment. The fraction of ^{14}C DNA retained on the filter was plotted vs. ^{3}H DNA internal standard retained on the filter.

In determination of DNA double strand breaks, 2.5×10^5 cells labeled with [^{14}C]thymidine and treated with drug were processed in the same way as for single-strand breaks except that the pH of eluting solution was 9.6. Internal standard cells were not used, because this method was known to be influenced by total cell number. The ^{14}C retained on the filter was plotted vs. the time of elution.

Inhibition of Nucleic Acid Synthesis

Nucleic acid synthesis was determined using a modified method of MATSUZAWA *et al.*[11]. L5178Y Cells in exponential phase of growth were suspended in RPMI1640 medium - 10 mM HEPES, pH 7.2. After preincubation of cells (5.5×10^5/ml, 85 μl) with various concentrations of drugs (5 μl) at 37°C for 30 minutes or without preincubation, [*methyl*-^{3}H]thymidine (0.1 μCi, 10 μl) or [5-^{3}H]uridine (3 μCi, 10 μl) was added and the mixture was incubated for 1 hour. The reaction was stopped by the addition of 1 ml of cold 5% TCA. After standing in an ice bath for 15 minutes, the acid-insoluble fraction was collected using an Eppendorf microcentrifuge. The precipitate was washed with 1 ml of cold TCA twice, and solubilized by treatment with 0.3 ml of 0.5 N KOH at 60°C for 30 minutes. Radioactivity was measured using 1.5 ml of PCS scintillation cocktail (Amersham Co., U.S.A.).

Drug Uptake and Efflux

Drug uptake and efflux were determined by fluorometry as previously described[12]. P388 Cells were harvested from ascitic fluid, washed by centrifugation repeatedly, and suspended in RPMI1640 medium - 10 mM HEPES, pH 7.2. For uptake experiments cell suspensions (2×10^6/ml, 0.4 ml in an Eppendorf tube) were incubated with 0.1 ml of drug solution (21.25 μM or 2.125 μM) in the same medium at 37°C. Uptake was terminated by centrifugation at $12,000 \times g$ for 30 seconds in an Eppendorf microcentrifuge. After washing with 1 ml of cold PBS the cells were disrupted by ultrasonication in 1 ml of PBS. Drugs were extracted from 0.8 ml portions of the sonicated cell suspensions by the addition of 2 ml of 40% TCA and 0.3 ml of 3.3% bovine serum albumin and overnight incubation. The incubated mixtures were centrifuged and fluorescence intensity at 598 nm was determined using a Hitachi MPF-4 spectrofluorometer with an excitation wavelength of 500 nm. When drug uptake at low drug concentration was examined, bovine serum albumin was not added to avoid nonspecific adsorption of the anthracyclines to the protein.

For efflux experiments P388 cells (2.6×10^6/ml, 10 ml) were preloaded by incubation for 15 minutes at 37°C with PBS - 10 mM NaN_3 containing DOX (70 μg/ml), FAD104 (75 μg/ml), FT-ADM (8 μg/ml) or FT-ADM-OH (15 μg/ml). Cells were centrifuged, suspended in 12 ml of cold fresh RPMI1640 medium - 10 mM HEPES, pH 7.0, and incubated at 37°C. Time-dependent increases of concentration of effluxed drug from the cells into the medium was determined using fluorescence after TCA extraction. Preloaded concentrations of DOX, FAD104, FT-ADM and FT-ADM-OH were 0.36, 0.17, 0.16 and 0.23 μg/10^6 cells, respectively.

Hydrolysis of Ester Bond of FAD104

Determination of FAD104 and FT-ADM after enzymatic or nonenzymatic treatment was done using

HPLC. The incubation mixture (0.5 ml) contained FAD104 and mouse, horse, or human serum, or purified hog liver esterase in various buffer solutions. After incubation the pH was adjusted to less than 2 with 1 N HCl. Drugs were extracted by shaking with 1.5 ml ethyl acetate. The ethyl acetate layer was separated and dried in an evaporator then dissolved in 0.5 ml Britton-Robinson buffer, pH 3.0 and methyl alcohol mixture (1 : 2) containing 25 μg/ml DOX as an internal standard for HPLC. The HPLC system was composed of SP8700 solvent delivery system, S8750 organizer as the injector (Spectra-Physics, Co., U.S.A.), Y-2100 incubator and Y-1000 UV detector (Senshu Scientific Co., Japan) with Chromatocorder 11 (System Instruments, Co., Japan) as the recorder. Samples were eluted with a mixture of 0.05 M sodium acetate buffer, pH 5.6 and acetonitrile (65 : 35) at 30°C at a flow rate of 1.0 ml/minute using a column of Inertsil ODS 5 μm, 4.6 × 250 mm (Gasukuro Kogyo Inc., Japan). Retention times of DOX, FAD104, and FT-ADM were approximately 6.4, 17.9 and 11.7 minutes, respectively. In these experiments FT-ADM-OH was not detected.

Results

Antitumor Activity

The antitumor activity of FAD104 and its metabolites, FT-ADM and FT-ADM-OH against L1210 is shown in Fig. 2. FT-ADM and FT-ADM-OH are sparingly soluble in water or buffer, hence they were administered as solutions in phosphate buffer pH 7.4 containing 5% DMSO as shown in the legend of Fig. 2. Many mice administered with FAD104 and FT-ADM survived for 60 days. FAD104 and FT-ADM had much superior antitumor activity to DOX. FT-ADM-OH had inferior activity to FAD104 or FT-ADM, but it did not kill as many mice in the 60-day period as DOX. In their model FAD104 showed the best antitumor activity and the least toxicity among the drugs tested.

Cytotoxicity

Growth inhibition of L1210, L5178Y, P388 and DOX-resistant P388 (P388/ADR) cells by DOX, FAD104, FT-ADM and FT-ADM-OH is shown in Table 1. FAD104 and FT-ADM showed stronger cytotoxicities against L1210, L5178Y and P388 cells than DOX. FT-ADM-OH showed about the same cytotoxicity as DOX. All the compounds showed less activity on P388/ADR cells than on other cells. However, FAD104 and FT-ADM inhibited the growth of P388/ADR cells at about a 10 times lower concentration than DOX.

Table 1. Cytotoxic activities of DOX, FAD104, FT-ADM and FT-ADM-OH.

	IC_{50} (μM)			
	L1210	L5178Y	P388	P388/ADR
DOX	0.31	0.15	0.060	2.75 (46)
FAD104	0.019	0.031	0.021	0.27 (13)
FT-ADM	0.0079	0.0092	0.0028	0.25 (89)
FT-ADM-OH	0.25	0.34	0.11	5.65 (50)

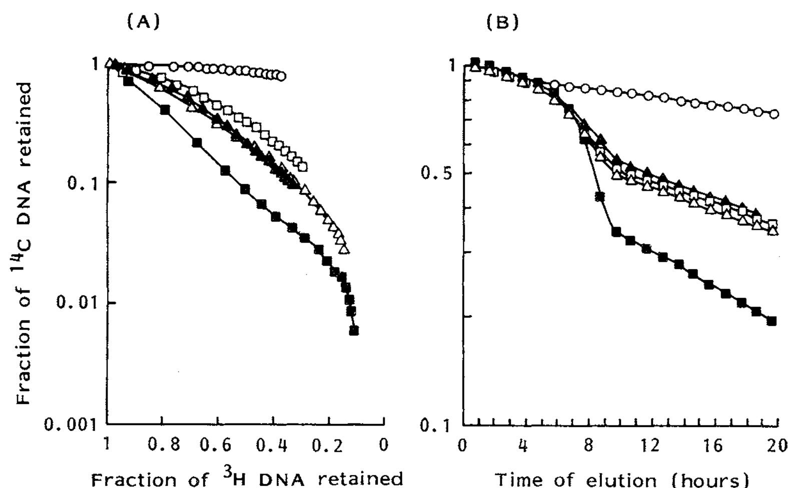
Cytotoxic activities of drugs are shown in drug concentrations inhibiting 50% growth of various cell lines. In brackets in the column of P388/ADR relative resistance was expressed in ratio of IC_{50} values for resistant P388 cells to that for sensitive P388 cells.

DNA Strand Scission

Anthracycline-induced strand scission was studied using the filter elution method. P388 Cells were incubated with 2.8 μM of drug for 1 hour, washed twice with cold medium and analyzed for DNA single- and double-strand scissions at pH 12.2 and 9.6, respectively. As shown in Fig. 3, DNA single-strand (A) and double-strand (B) breaks were observed for the three fluorine-containing compounds and DOX. FT-ADM gave the greatest damage to DNA. The effect of FAD104 was smaller than that of DOX on single-strand breaks and equal to DOX on double-strand breaks, although its cytotoxic effect was stronger than DOX. FT-ADM-OH was similar to DOX in both single and double strand DNA scission.

Fig. 3. DNA strand scission in P388 cells treated with anthracyclines.

○ Control, △ DOX, □ FAD104, ■ FT-ADM, ▲ FT-ADM-OH.



DNA single- and double-strand breaks were measured by filter elution method at pH 12.2 (A) and pH 9.6 (B), respectively, in the presence of protease K. See "Materials and Methods" for details.

Table 2. Inhibition of nucleic acid synthesis in L5178Y cells by DOX and fluorine-containing anthracyclines.

	IC_{50} (μM)		Ratio of IC_{50}
	DNA	RNA	DNA/RNA
DOX	26	35	0.74
FAD104	3.1	3.4	0.91
FT-ADM	1.4	1.1	1.27
FT-ADM-OH	10	11	0.90

Table 3. Binding of DOX and fluorine-containing anthracyclines to calf thymus DNA.

	K_{app} (M^{-1})	n_{app} (mol/nucleotide)
DOX	8.60×10^6	0.155
FAD104	0.33×10^6	0.201
FT-ADM	3.06×10^6	0.150
FT-ADM-OH	0.86×10^6	0.220

Inhibition of Nucleic Acid Synthesis

As shown in Table 2 all the fluorine-containing anthracyclines inhibited DNA and RNA synthesis in intact L5178Y cells. No selectivity in inhibition of RNA synthesis over DNA synthesis was observed with all the agents including DOX. Introduction of the fluorine group did not give selective inhibition of DNA or RNA synthesis. The order of potencies of nucleic acid synthesis inhibition was FT-ADM, FAD104, FT-ADM-OH and DOX.

DNA-Binding

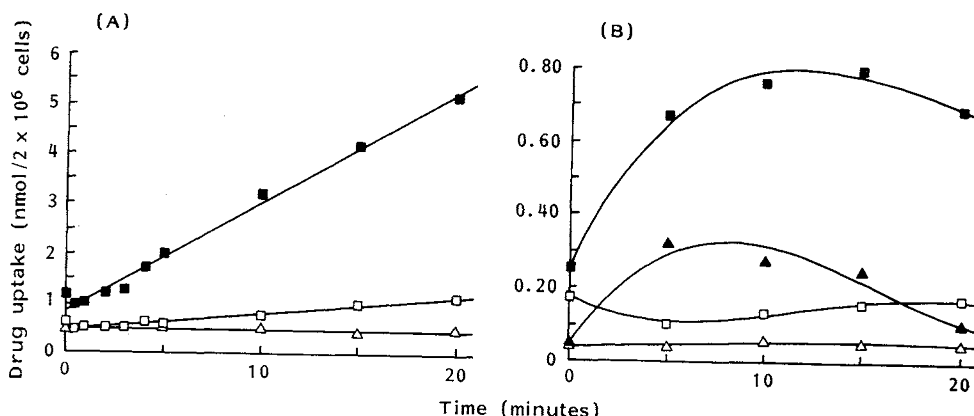
The calf thymus DNA-binding characteristics of the agents were studied using the fluorescence titration technique. Table 3 lists apparent association constant (K_{app}) and apparent number of binding sites (n_{app}). These values were obtained from a Scatchard analysis of the interaction of these agents with calf thymus DNA. DOX had the strongest affinity for DNA. The K_{app} of FAD104 and FT-ADM-OH were markedly lower than that of DOX. Some differences were seen in the values of n_{app} among these drugs, but the accuracy of this analysis in determining the size of the binding site has not been established.

Drug Uptake and Efflux

Uptake of FAD104, FT-ADM and FT-ADM-OH by P388 cells in RPMI1640 medium containing

Fig. 4. Drug uptake by P388 cells.

△ DOX, □ FAD104, ■ FT-ADM, ▲ FT-ADM-OH.

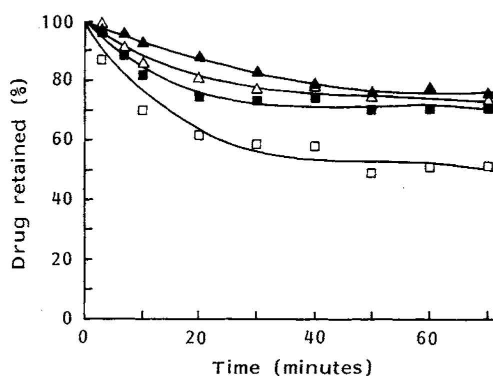


Drug uptake was determined by incubating P388 cell suspension (2×10^6/ml, 0.4 ml) with drug solution (21.25 μM in (A) or 2.125 μM in (B), 0.1 ml) in RPMI1640 - 10 mM HEPES, pH 7.2. Drug taken up into the cells was measured by fluorescence after extraction with TCA.

10 mM HEPES, pH 7.2, were compared with the uptake of DOX. As shown in Fig. 4(A), where the initial drug concentration was 17 μM, FAD104 was taken up by P388 cells slightly faster than DOX. FT-ADM was much more rapidly incorporated than either DOX or FAD104. After 20 minutes FAD104 and FT-ADM were accumulated 2.25 and 9.73-fold, respectively, more than DOX. FT-ADM-OH was taken up into P388 cells much more slowly than FT-ADM as shown in Fig. 4(B), where the initial concentration of all the drugs was 1.7 μM, or one tenth of that used in the experiment shown in Fig. 3(A). This lowering of drug concentration was necessitated because of the low solubility of FT-ADM-OH.

Fig. 5. Drug efflux from P388 cells.

△ DOX, □ FAD104, ■ FT-ADM, ▲ FT-ADM-OH.



P388 Cells were preloaded with drug by incubation in PBS containing a drug and 10 mM NaN_3. Efflux was started by resuspending in RPMI1640 medium without washing. Preloaded drug concentrations were 0.36, 0.17, 0.16, and 0.23 $\mu g/10^6$ cells with DOX, FAD104, FT-ADM, and FT-ADM-OH, respectively. Drug released into medium was determined and drug retention was calculated.

Efflux kinetics of these agents from P388 cells was shown in Fig. 5. Initial rates of efflux of FT-ADM, FT-ADM-OH and DOX were different from each other, but the proportion of drugs retained at equilibria were similar. However, FAD104 was more rapidly released from the cells and its retention was lower at equilibrium than DOX.

Hydrolysis of Ester Bond of FAD104

As described above, FAD104 had the strongest antitumor activity, but it had the least activity in the DNA-binding assay. DNA strand scission activity of FAD104 was also lower than DOX in spite of its faster uptake. The intracellular accumulation of FAD104 does not seem sufficient to account for its strong antitumor activity. On the other hand, FT-ADM showed the strongest cytotoxicity and the fastest uptake

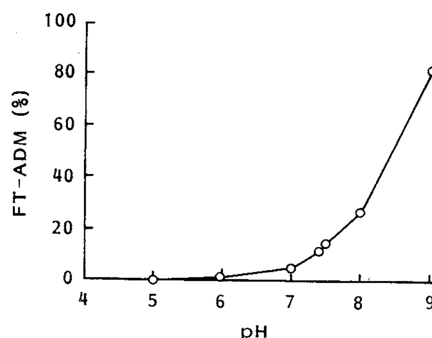
Table 4. Generation of FT-ADM from FAD104 in serum.

Serum	μl	pH	Incubation time	FT-ADM (%)	Conversion by esterase
Mouse	10	7.4	15 minutes	9.55	9.12
	50			24.2	23.77
Human	10			0.25	−0.18
	50			0.45	0.02
None				0.43	
Mouse	10	7.4	1 hour	30.9	22.7
Human	100			10.8	2.6
Horse	100			65.4	57.2
None				8.2	
Mouse	0.25	5.7	3 hours	6.0	2.0
	0.5			8.9	4.9
	1			12.8	8.8
	1.5			19.0	15.0
	2			17.8	13.8
Human	200			17.9	13.9
None				4.0	

Conversion by esterase was calculated by subtracting FT-ADM (%) without serum from that with serum.

among the fluorine-containing anthracyclines. HPLC analysis of serum of animals after administration of FAD104 showed the formation of FT-ADM and FT-ADM-OH[6,7]. For this reason spontaneous and enzymatic hydrolysis of the ester bond of FAD104 was determined. As shown in Fig. 6, FAD104 was gradually hydrolyzed at a pH range between 6.5 and 8.0. At pH 9.0 it was rapidly hydrolyzed to FT-ADM as was expected for an ester. A nonspecific esterase exists in plasma and cells. The conversion of FAD104 to FT-ADM by esterase in serum was tested at pH 7.4 and 5.7 with a long incubation time in order to distinguish this enzyme action from alkaline hydrolysis. As shown in Table 4, FAD104 was rapidly hydrolyzed by mouse serum but only slowly hydrolyzed by human serum. Horse serum had an intermediate activity. The result obtained at pH 5.7 showed that esterase activity hydrolyzing the carboxylic acid ester of FAD104 was about 100 times more active in mouse serum than in human serum.

Fig. 6. Hydrolysis of carboxylic acid ester of FAD104.



FAD104 (100 μg/ml) was dissolved in various buffers. Acetate buffer, sodium phosphate buffer, and Tris-HCl buffer were used at 0.02 M at pH 5, pH 6~8, or pH 9, respectively. After incubation at 37°C for 1.5 hours reaction was stopped by addition of 1 N HCl to adjust to pH 2 and analyzed as described in "Materials and Methods".

Discussion

In this report we describe the antitumor activity and biochemical properties of the newly developed fluorine-containing anthracycline glycoside, FAD104, and its metabolites, FT-ADM and FT-ADM-OH. FAD104 had the highest antitumor activity against mouse L1210 leukemia[5,6]. FT-ADM had nearly equal activity to FAD104 but had higher toxicity as shown in Fig. 2. FT-ADM-OH showed weaker antitumor

activity than FAD104 or FT-ADM. Some biochemical properties of these compounds were tested to elucidate the mechanism of antitumor activity. Cytotoxic activity was the strongest for FT-ADM. FAD104 showed a much stronger cytotoxic activity than DOX, but weaker than FT-ADM. FT-ADM-OH had a similar cytotoxic activity to DOX. It is of interest to note that FAD104 was more active against multi-drug resistant P388 cells (P388/ADR) than DOX and the IC_{50} of FAD104 was less than one tenth of DOX as shown in Table 1. It appears reasonable to expect that FAD104 will be active against clinical tumors which are not so severely resistant to DOX, because FAD104 can be administered in 10 times higher amount of DOX because of its less acute toxicity. TSURUO *et al.*[13] reported that FAD104 did not show antitumor activity against P388/ADR leukemia, but was effective against P388/VCR which was also multi-drug resistant, but its resistance was lower than shown by P388/ADR.

It appears that breakdown of the ester bond of FAD104 due to a metabolic activity or to the instability of the bond has an important role in exerting the *in vivo* antitumor activity of the drug. FAD104 itself has biological activity as shown in DNA-binding ability (Table 3), but FT-ADM has the stronger activity in DNA strand scission (Fig. 3), inhibition of nucleic acid synthesis (Table 2) and intercalation to double stranded DNA (Table 3) than FAD104. The former two activities might be caused mostly by higher intracellular accumulation of FT-ADM but the last activity uses a cell free system that does not involve drug incorporation and metabolism. The conversion of FAD104 to FT-ADM progressed both spontaneously and enzymatically as shown in Fig. 6 and Table 4. Different species of animals had different enzyme activities in hydrolyzing the ester bond of FAD104. Mouse serum had approximately 100 times higher activity than human serum, and horse serum had an intermediate activity. Such diversity in enzyme activities in sera and organs is thought to influence drug distributions and effects of the drug in different organs. As the uptake of FAD104 into cells occurs at a slower rate than that of FT-ADM, and the FAD104 taken up is released by the cells rapidly, it appears that FAD104 may remain in the bloodstream for a long time, and FT-ADM is incorporated rapidly into target tissues as it is gradually formed in the bloodstream, hence a strong antitumor activity is maintained. A clinical investigation of FAD104 is now in progress as ME2303 in Japan. Further studies such as studies on *in vitro* biochemical activity, pharmacokinetics, *in vivo* metabolism, *etc.* are needed to elucidate the biological mechanisms responsible for the strong antitumor activity of FAD104. These studies are currently in progress in our laboratory.

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