EFFECTS OF AN ANTITUMOR AGENT, ASCOFURANONE, ON THE MACROMOLECULAR SYNTHESES OF INTACT CELLS

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Ascofuranone (AF) has antitumor protective property on experimental tumors. We examined the action of AF on lymphoma L5178Y to explore the mechanism of the antitumor activity.

AF completely prevented the growth of L5178Y at 25 μg/ml cytostatically. The compound exhibited general inhibitory effects on the macromolecular syntheses. Among them, protein synthesis was most severely inhibited by AF and to the same extent as by cycloheximide. AF, however, did not affect protein synthesis by cell-free system even at 2 mg/ml.

Although AF inhibited the incorporation of [^{14}C]acetate into total acid precipitable products only slightly, the synthetic pattern of simple lipids from [^{14}C]acetate was significantly changed. Especially, the incorporation of [^{14}C]acetate into squalene was almost completely blocked at 25 μg/ml. The incorporation of [^{14}C]acetate into triglyceride was inhibited and that into cholesterol was enhanced. Concerning the diglycerides, the incorporation of [^{14}C]acetate was enhanced and that of [^{3}H]glycerol was inhibited. The incorporation of [^{3}H]glycerol and [^{3}H]mevalonate into the intact cell was significantly inhibited as compared with [^{14}C]acetate. As those effects were not observed with cycloheximide, they were suggested to be characteristic of AF.

AF inhibited hypotonic hemolysis. In contrast, hemolysis by deoxycholate was stimulated.

Possible mechanism of the antitumor activity of AF is discussed.

Ascofuranone (AF) is an isoprenoid antibiotic produced by a phytopathogenic fungus, *Ascochyta visiae*. It has been reported that AF affected lipid metabolism in rats by reducing serum lipid.[1,2,3]

Recently, we found that AF was also effective on murine experimental tumors.[4] The antitumor activity of AF was characteristic in that AF was effective by pretreatment as well as posttreatment. This phenomenon indicates that the antitumor activity is host-mediated. In fact, AF treatment of normal mice enlarged the solid lymphoid organs.

To study the antitumor activity of AF, we examined the *in vitro* action of AF on leukemia L5178Y because L5178Y has the properties of a malignant tumor cell and a leukocyte.

Materials and Methods

Antibiotics

AF used in this study was micronized crystalline powder with purity above 99%. Cycloheximide (CX) was obtained from Namai Kagaku Co. These agents were dissolved in dimethyl sulfoxide (DMSO). In following experiments, final concentration of DMSO was adjusted to 1% (v/v) for this concentration of DMSO did not affect the growth of L5178Y in this condition.

Cell Culture

Mouse leukemia cell line, L5178Y, was kindly supplied from Dr. H. SUZUKI, Institute of Applied

Microbiology, the University of Tokyo. FISCHER's medium (GIBCO) supplemented with 10% donor horse serum (Flow Laboratories) was used for the culture.

Effect of AF on Macromolecular Syntheses

Radioactive precursors were added to the cell suspension of L5178Y (4×10^5 cells/ml) and the suspension was incubated at 37°C for the given period. The reaction was terminated by the addition of ice-cold Ca free phosphate buffered saline and immediately centrifuged at 1,000 rpm for 5 minutes. Then, the cells were resuspended in Ca free phosphate buffered saline and 1 volume of 10% trichloroacetic acid (TCA) was added to the suspension. The precipitate was collected on a Whatman GF/C glass microfiber filter. The filter was successively washed with 5% TCA and 1% acetic acid. The filter was dried and the radioactivity was counted.

Cell-free Protein Synthesis[5]

The liver taken from a mouse starved for 18 hours was homogenized with the mixture of 0.25 M sucrose, 50 mM KCl, 5 mM magnesium acetate and 50 mM Tris-HCl buffer (pH 7.6) using a Teflon homogenizer (1,000 rpm, 10 strokes), and dithiothreitol was added to the homogenate at a final concentration of 1 mM. After centrifuging the homogenate at $30,000\times g$ for 20 minutes, the supernatant (S-30) was used for an assay. The protein content in the S-30 was determined by LOWRY's method[6] using bovine serum albumin as a standard. The reaction mixture (100 μl) containing 50 mM KCl, 5 mM ATP, 1 mM GTP, 5 mM phosphoenolpyruvate, 2×10^{-5} M leucine-free Casamino Acids, 2 μCi [^{3}H]-leucine, 1 μg pyruvate kinase, the given amount of S-30, 1 mM dithiothreitol and 0.25 M sucrose was incubated at 37°C for 40 minutes in the presence or absence of AF or a comparative control agent, CX. The reaction was stopped by the addition of 2 ml or 10% TCA and the mixture was kept at 4°C for an additional 10 minutes. Then, the mixture was centrifuged at 3,000 rpm for 5 minutes and the supernatant was discarded. The precipitate was redissolved in 0.5 ml of 1 N NaOH and again precipitated by adding 2 ml of 10% TCA. This procedure was repeated three times and finally, washed precipitate was transferred on a Whatman GF/C glass microfiber filter and the radioactivity was counted.

Extraction of Lipids and Thin-layer Chromatography (TLC)

Labeled cells were collected and suspended in Ca free phosphate buffered saline, followed by the extraction of the whole lipids with BLIGH-DYER method.[7] From the medium, lipids were extracted with FOLCH's method.[8] Extracted lipids were dissolved in 20 μl of chloroform and spotted on thin-layer sheets (Merck; Art 5735). After the migration, dried sheets exposed to X-ray film for 7~15 days. Spots were detected with iodine vapor or sulfic acid.

Identification was performed by co-migration with authentic samples in various solvent systems. Squalene, cholesterol ester, cholesterol, 1,3-diglyceride, 1,2-diglyceride, triglyceride, monoglyceride, and phospholipids were identified but the other spots remained to be identified.

The Assay of the Hemolysis[9]

The adult human peripheral blood was collected. After two washes, cells were suspended in the isotonic buffer (0.2% glucose, 0.8% NaCl, 0.12% Na_2HPO_4, 0.016% $NaH_2PO_4\cdot H_2O$) at $5\sim8\times10^8$ cells/ml. 100 μl of the suspension was added to the hypotonic buffer (0.2% glucose, 0.36% NaCl, 0.046% Na_2HPO_4, 0.0063% $NaH_2PO_4\cdot H_2O$) and incubated for 30 minutes at 37°C (hypotonic hemolysis) or added to the isotonic buffer containing 0.04% deoxycholate and incubated for 60 minutes at 37°C (deoxycholate hemolysis). After the centrifugation at 1,500 rpm for 5 minutes, the absorption of the supernatant was measured at 583 nm. The maximum lysis was performed by the addition to 1 mM phosphate buffer (0.014% Na_2HPO_4, 0.002% $NaH_2PO_4\cdot H_2O$) instead of each buffer. The hemolysis was assessed by % hemolysis.

$$\%\,\text{Hemolysis}=\frac{\text{Absorption of the experimental lysis}}{\text{Absorption of the maximal lysis}}\times100$$

Results

Growth Inhibition

AF inhibited the growth of L5178Y as shown in Fig. 1. The inhibitory effect was observed at

Fig. 1. Cell growth of L5178Y in the presence of AF.

L5178Y cells were cultured in FISCHER's medium supplemented with donor horse serum in the presence of 0 (●), 6.3 (△), 12.5 (□) and 25 (○) μg/ml of AF.

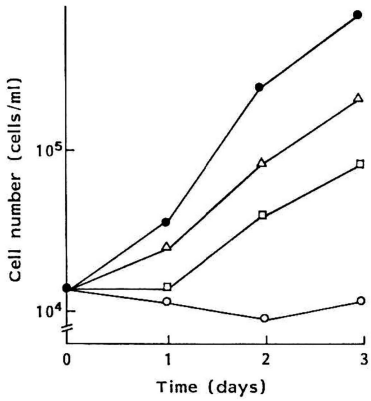


Fig. 2. Cytostatic growth inhibition of AF.

L5178Y cells were collected and cultured with 0 (●), 6.3 (△), 12.5 (□) and 25 (○) μg/ml of AF for 8 hours. Cells were washed and resuspended in the fresh medium. The cell number was counted at the given time.

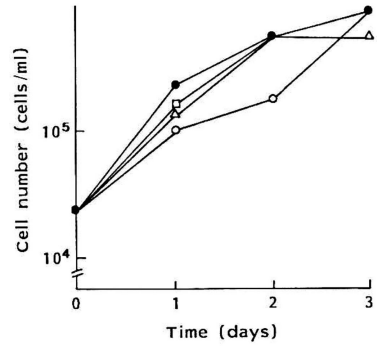


Fig. 3. Effect of AF on macromolecular syntheses of L5178Y cells.

L5178Y cells (2×10^5 cells/ml) were cultured in the presence of 0 (●), 6.3 (△), 12.5 (□) and 25 (○) μg/ml of AF. Concentration of radio-labeled precursors added to the tubes was as follows; [^{3}H]-thymidine 0.06 μCi/ml in a, [^{3}H]uridine 0.23 μCi/ml in b, [^{3}H]leucine 0.11 μCi/ml in c, [^{14}C]acetate 0.03 μCi/ml in d and [^{3}H]glucosamine 0.85 μCi/ml in e.

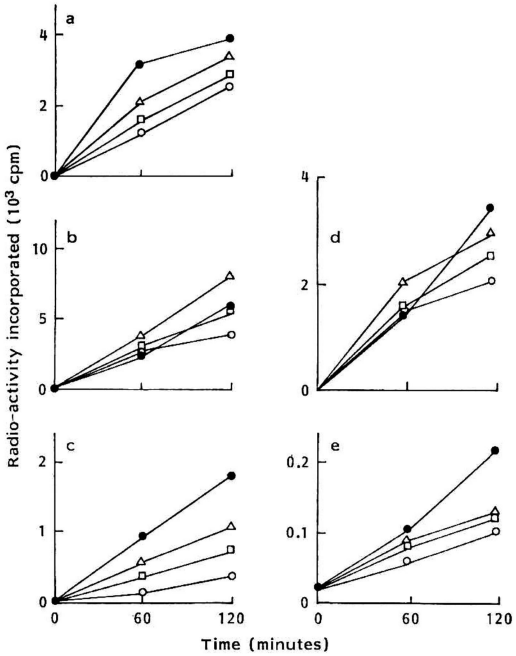


Table 1. Effect of AF and CX on protein synthesis in cell-free extract of mouse liver homogenate.

Experiment No.	Agent added (μg/ml)		Radioactivity incorporated (cpm/tube)	Inhibition (%)
1	None		150± 30	—
	AF	5	130±123	13
		25	141± 22	6
		125	145± 9	3
	CX	0.01	130± 13	8
		1.0	111± 15	26
		100	79± 9	47
2	None		131± 9	—
	AF	20	131± 19	0
		200	114± 20	13
		2,000	112± 12	15
	CX	10	64± 24	51
		100	36± 2	73
		1,000	−4± 8	100

Incorporation of [^{3}H]leucine into TCA insoluble fraction was determined after incubation with 1.22 mg protein (experiment 1) or 0.5 mg protein (experiment 2) of S-30 fraction from mouse liver homogenate. The mean±SD of triplicate assay are presented.

concentration higher than 5 μg/ml and 25 μg/ml of AF completely suppressed the cell growth. Viability of the cells evaluated by dye exclusion test with trypan blue was approximately 100% even after the treatment for 48 hours at 25 μg/ml. As the cells washed and resuspended in the fresh medium after AF treatment began to proliferate

after a certain lag period, the effect of AF was cytostatic (Fig. 2).

Macromolecular Syntheses

To study the effect of AF on macromolecular syntheses, the incorporation of radioactive precursors into acid precipitable products of intact cells was investigated (Fig. 3). AF inhibited the incorporation of all the precursors examined. The inhibitory effect was relatively strong on the incorporation of [^{3}H]leucine and [^{3}H]glucosamine and relatively weak on that of [^{14}C]acetate. As direct inhibition of protein synthesis was expected from these results, the effect of AF on protein synthesis in a cell-free system using mouse liver S-30 fraction was examined (Table 1). AF, however, did not inhibit the incorporation of [^{3}H]leucine into acid precipitable products even at 2 mg/ml. In contrast, cycloheximide (CX) completely inhibited it at 1 mg/ml. Although it is possible that the inhibitory effect in intact cells is attributed to metabolite of AF, AF itself would not directly attack the enzymic system of protein synthesis.

Modulation of Lipid Metabolism

AF has been reported to affect lipid metabolism in rats.[1,2,3] Although AF inhibited the incorporation of [^{14}C]acetate only slightly, we examined the synthetic pattern of lipids in AF treated cells. Lipids were extracted from the cells incubated with AF and [^{14}C]acetate for 2 hours. Fig. 4 represents the synthetic pattern of simple lipids (Fig. 4, b) and phospholipids (Fig. 4, a) in cells. Although AF showed little effect on the synthetic pattern of phospholipids from [^{14}C]acetate, it dramatically affected that of simple lipids. Most profound change was found on the upper spot, where the incorporation of [^{14}C]acetate was almost completely inhibited at 25 μg/ml. Although two substances, squalene and cholesterol ester, belonged to this spot, the former contributed to the majority of the radioactivity incorporated.

Fig. 4. Modulation of cellular lipid metabolism by AF.

1×10^{6} cells/ml of L5178Y were incubated with 1 μCi/ml [^{14}C]acetate for 2 hours. Cells were collected and the lipids were extracted. The lipids were migrated on TLC and detected by the autoradiography.

a; Phospholipids (chloroform-methanol-acetate-water, 25:15:4:2). b; Simple lipids (hexane-ether-acetate, 80:20:1).

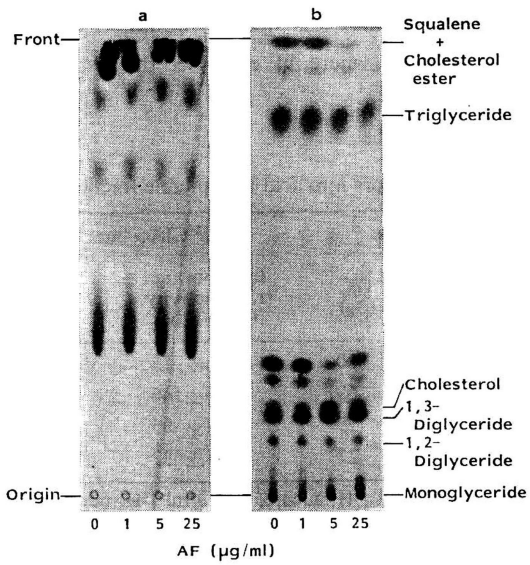
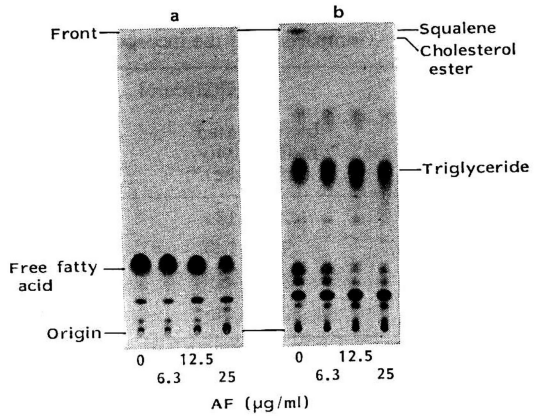


Fig. 5. Comparison of lipids in cells and medium cultured with or without AF.

Experiments were performed as described in the legend of Fig. 4. The lipids were extracted from a; medium or b; cells.

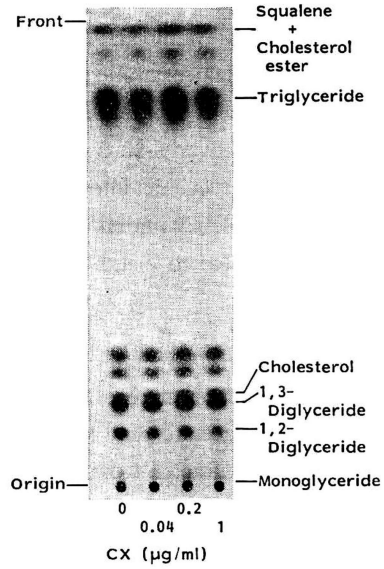


Squalene and cholesterol ester were clearly separated by the migration of shorter period (Fig. 5, b). The inhibition by AF of the incorporation of [^{14}C]acetate into both cholesterol ester and squalene was again observed. The radioactivity in the squalene observed in control lane completely disappeared after 2 hours of chase and AF did not block this disappearance (data not shown). In the medium, the majority of [^{14}C]acetate was incorporated into free fatty acids, where the incorporation was significantly inhibited by AF (Fig. 5, a).

To study the possibility that these changes in synthetic pattern of lipids might be attributed to the secondary effect of the inhibition of protein synthesis, the same analysis was performed with CX (Fig. 6). CX did not affect synthetic pattern of lipids. This suggests that the above effects of lipid synthesis was characteristic of AF.

Fig. 6. Synthetic pattern of cellular simple lipids in the presence of CX.

Cells of L5178Y were incubated in the presence of CX and lipids were extracted and fractionated as described in Fig. 4.



Lipogenesis from [^{3}H]Glycerol and [^{3}H]Mevalonate

To study directly the biosynthesis of cholesterol and glycerides, the incorporation of [^{3}H]glycerol and [^{3}H]mevalonate into the intact cell was examined (Table 2). It was found that the incorporation of these two precursors was significantly inhibited as compared with that of [^{14}C]acetate. Synthetic pattern of lipids from [^{3}H]glycerol and [^{14}C]acetate was compared by double label experiment and the results are presented in Table 3. [^{3}H]Glycerol was incorporated into triglycerides, diglycerides, monoglycerides and phospholipids. Radioactivity shown around the spot of cholesterol corresponded to 1,3-diglycerides. The incorporation of [^{3}H]glycerol into triglycerides was strongly inhibited. Interestingly, the reverse effect was observed in 1,3- and 1,2-diglycerides, where AF enhanced the incorporation of [^{14}C]acetate but inhibited that of [^{3}H]glycerol.

Table 2. Comparison of the incorporation of various lipid precursors into acid precipitable fraction.

AF (μg/ml)	[^{3}H]Glycerol		[^{3}H]Mevalonate		[^{14}C]Acetate	
	Incorporated radioactivity (cpm/tube)	T/C (%)	Incorporated radioactivity (cpm/tube)	T/C (%)	Incorporated radioactivity (cpm/tube)	T/C (%)
0	1,237±134	100	182±20	100	1,096± 55	100
6.3	941±249	76	125± 6	69	846±146	79
12.5	339± 29	27	139±59	79	925± 65	87
25	297± 45	24	49± 9	27	794± 32	74

4×10^{5} of L5178Y cells were incubated for 2 hours with 0.2 μCi/ml [^{3}H]leucine, 1 μCi/ml [^{3}H]mevalonate or 0.5 μCi/ml [^{14}C]acetate in the presence or absence of AF. Radioactivity incorporated into the TCA insoluble fraction was counted. Figures were shown as mean±SD of triplicate samples.

Table 3. Comparison of the incorporation of [^{3}H]glycerol and [^{14}C]acetate into each spot of lipid component.

Fraction	AF (25 μg/ml)	[^{14}C]Acetate		[^{3}H]Glycerol	
		Radioactivity incorporated (cpm/spot)	T/C (%)	Radioactivity incorporated (cpm/spot)	T/C (%)
Cell					
Cholesterol ester+squalene	−	1,615	100	0	—
	+	187	11	0	—
Triglyceride	−	4,476	100	6,059	100
	+	3,712	69	1,167	12
Cholesterol	−	209	100	117	100
	+	437	208	0	0
1,3-Diglyceride	−	1,640	100	391	100
	+	3,259	199	129	37
1,2-Diglyceride	−	279	100	274	100
	+	447	161	68	25
Medium					
Free fatty acid	−	2,201	100	0	—
	+	987	45	0	—

L5178Y were incubated with [^{14}C]acetate (1 μCi/ml) and [^{3}H]glycerol (2 μCi/ml) in the presence of AF and extracted lipids were migrated on TLC as in Fig. 4, b. Radioactivity of autoradiographically detected spots was counted. Figures were shown as mean of the duplicate samples.

Table 4. Protective property of AF on hypotonic hemolysis.

AF (μg/ml)	Hemolysis (%)	T/C (%)
0	81.6± 8.1	100
2.5	61.0± 2.0	75
5	43.4±16.1	53
10	24.5±11.2	30
20	14.5± 1.9	12
40	22.3±14.9	27

After 60 minutes of preincubation with AF in an isotonic buffer, human erythrocytes were transferred to a hypotonic buffer. The rate of hemolysis was calculated as described in methods. Figures are shown as mean±SD of triplicate samples.

Table 5. Effect of AF on hemolysis with deoxycholate.

AF (μg/ml)	Hemolysis (%)	T/C (%)
0	15± 3.8	100
2.5	15± 1.4	100
5	12± 2.0	88
10	33±22.1	220
20	59±46.2	394
40	100± 2.0	667

Human erythrocytes were incubated with AF for 60 minutes in an isotonic buffer containing 0.04% deoxycholate. Absorption at 583 nm of the supernatant was measured. Figures were shown as mean± SD of triplicate samples.

Effects on Hemolysis

The modulation of lipid metabolism would lead to the alteration of the membrane properties because lipid is one of the major components of the plasma membrane. AF affected the lytic property of human erythrocytes. Hypotonic hemolysis was inhibited (Table 4). In contrast, hemolysis with deoxycholate was stimulated (Table 5) by AF; both suggest alteration of the membrane. The modulation of membrane properties has been reported to affect the activity of enzymes on the membrane.[10] AF, however, only negligibly affected the activity of two typical membrane enzymes, Na-K ATPase and alkaline phosphatase (data not shown).

Discussion

AF inhibited the growth of L5178Y cytostatically. Macromolecular syntheses, especially protein synthesis, were also inhibited. Cell-free protein synthesis, however, was not inhibited by AF, suggesting that AF has no direct effect on the enzymes system of protein synthesis. As most of the previously reported antitumor agents have their primary target on the macromolecular synthesis, especially DNA synthesis,[11] the action of AF would be somewhat different from these agents. Although AF did not inhibit the incorporation of [^{14}C]acetate into acid precipitable products, the synthetic pattern of lipids from [^{14}C]acetate was significantly affected. As lipid is one of the major components of the membrane, the modulation of lipid metabolism would alter the properties of the membrane. In fact, hemolysis was significantly affected by AF treatment. It is possible that the inhibition of the incorporation of [^{3}H]leucine, [^{3}H]mevalonate and [^{3}H]glycerol by AF is resulted from the inhibition of transport of these compounds, because the alteration of membrane properties would also affect the activity of the transport systems.[12]

It has been reported that the activity of effectors and the sensitivity of targets in the immune system are modulated by the lipid components in the plasma membrane.[13~16] The modulation of lipid metabolism elicited by AF might contribute to the host-mediated antitumor property to a considerable extent. The effect of AF on immune system will be reported elsewhere.

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