THE ACTION MECHANISM OF BREFELDIN A

I. GROWTH RECOVERY OF *CANDIDA ALBICANS* BY LIPIDS FROM THE ACTION OF BREFELDIN A

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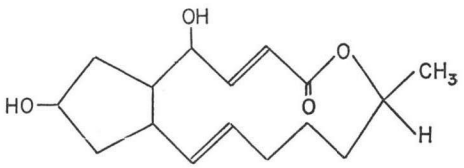
Brefeldin A is an antifungal antibiotic and a fatty acid derivative biosynthesized from palmitate (C_{16}). The action mechanism of the antibiotic was studied using *Candida albicans* IAM 4888, the most sensitive strain tested. The antifungal activity was reversed to some extent by a variety of fatty acids (C_{10}~C_{14}), fatty alcohols (C_{10}~C_{14}) and isoprenoids such as ergosterol and geraniol. However, amino acids and nucleosides were ineffective in reversing the antifungal activity. The results suggest that the antibiotic growth inhibition is due to the inhibition or disturbance of lipid metabolism.

Brefeldin A (cyanein, decumbin) is an antifungal antibiotic produced by some fungi[1,2,3,4]. The antibiotic has been characterized as cytostatic for HeLa cell[5,6], antimitotic[7] and antinematocidal agent[8]. SUZUKI and co-workers[9] found that the antibiotic inhibits the growth of lucerne and rape seedlings. We also found that it showed antiviral activity in the agar-diffusion plaque-inhibition assay method[10,11].

Action mechanism of brefeldin A has been studied by BETINA *et al.*[5,12] using HeLa cells, the Syrian hamster BHK 21/13 fibroblasts or *E. coli* protoplast. The antibiotic (100 μg/ml) stimulated glycolysis, but had no effect on the permeability of HeLa cells. It also affected protein and nucleic acid syntheses, and the former was much more inhibited than the latter. However, the primary site of action of brefeldin A still remains obscure.

Brefeldin A and prostaglandins have similar structure (Fig. 1) and are biosynthesized from palmitate[13] and arachidonic acid[14,15], respectively. This similarity stimulated our interest and prompted us to study the mode of action of brefeldin A. The effect of lipids on the action of the antibiotic was studied, and it was found that the antifungal activity can be reversed by divers lipids such as higher fatty acids, higher fatty alcohols, steroids and isoprenoids, as reported in this paper.

Fig. 1. Structure of brefeldin A.



Materials and Methods

The test organism and media. Sensitivity of various microorganisms to brefeldin A was tested. *Candida albicans* IAM 4888 was found to be most sensitive and was used in this study. SABOURAUD medium or the modified ROSE and NICKERSON's medium[16] was used as a semi-synthetic or synthetic medium, respectively. *C. albicans* was grown for 20 hours at 30°C with

shaking, and 0.5 ml of the culture was added to 9.5 ml of fresh SABOURAUD medium. The growth was followed by measuring optical density at 550 mμ ($OD_{550m\mu}$).

Growth recovery test in disc-agar plate method: Agar plates were prepared in Petri dishes of 9-cm diameter by pouring 10 ml of either SABOURAUD medium or the synthetic medium containing 1.5 % agar, 2×10^{-5} M brefeldin A and 5 % culture of *C. albicans* in logarithmic phase of growth. Compounds to be tested for growth recovery action were dissolved in methanol or water, and paper discs (8 mm) impregnated with the test materials placed on the agar surface. The Petri dishes were incubated at 30°C for 3 days, and diameters of growth-recovery zones and growth-inhibitory inner zones were measured with a caliper. The extent of growth recovery was recorded as no growth −, scant growth ±, poor growth +, moderate growth ++ and good growth +++.

Growth recovery test in liquid culture method: The test materials were added to 9.5 ml of SABOURAUD medium containing 0.5 ml of culture and 2×10^{-5} M brefeldin A, and *C. albicans* growth was followed turbidometrically.

Chemicals: Brefeldin A was kindly given by Dr. TAKASHI MORI (Chugai Pharmaceutical Co.). Ergosterol, lanosterol and cholesterol was obtained from Nakarai Chemical Co. Propionic, capric, lauric, palmitic, stearic and oleic acids were obtained from Junsei Pure Chemicals

Table 1. Reversal of brefeldin A activity by fatty acids.

Paper-disc (8 mm) soaked in 1 % fatty acid methanolic solutions. The numbers in parentheses represent inner growth inhibitory zones, and the extent of growth recovery were recorded as no growth −, scant growth ±, poor growth +, moderate growth ++, and good growth +++. No growth recovery zone was represented 0 mm

Subatance		Growth recovery zone			
		SABOURAUD medium		Synthetic medium	
		Diameter (mm)	Extent	Diameter (mm)	Extent
Acetic acid	C_2	12	±	15	±
Propionic acid	C_3	12	±	18	±
Butyric acid	C_4	12	±	13	±
Valeric acid	C_5	12	±	16	±
Caproic acid	C_6	14	+	15 (10)	±
Heptylic acid	C_7	20	+	40 (35)	±
Caprylic acid	C_8	26 (14)	+	50 (42)	±
Pelargonic acid	C_9	26 (15)	+	50 (43)	±
Capric acid	C_{10}	24 (12)	++	30 (27)	++
Undecylic acid	C_{11}	21 (9)	++	28 (12)	++
Lauric acid	C_{12}	17	+++	27 (9)	+++
Tridecylic acid	C_{13}	12	+++	12	+++
Myristic acid	C_{14}	9	++	9	++
Pentadecylic acid	C_{15}	0	−	0	−
Palmitic acid	C_{16}	0	−	0	−
Stearic acid	C_{18}	0	−	0	−
Arachidic acid	C_{20}	0	−	0	−
Lignoceric acid	C_{24}	0	−	0	−
Palmitoleic acid	$C_{16:1}$	10	+	12	+
Oleic acid	$C_{18:1}$	10	+	13	+
Linoleic acid	$C_{18:2}$	0	−	13	+
Linolenic acid	$C_{18:3}$	12	+	18	+
Iso-stearic acid	C_{18}	13	+	13	+

Table 2. Reversal of brefeldin A activity by fatty alcohols, isoprenoids, miscellaneous lipids and surfactantis in the disc-agar plate method.

The experimental conditions were the same as those in the Table 1

Substance		Growth recovery zone			
		SABOURAUD medium		Synthetic medium	
		Diameter (mm)	Extent	Diameter (mm)	Extent
Butyl alcohol	C_2	0	−		
Hexyl alcohol	C_6	0	−		
Octyl alcohol	C_8	0	−		
Decyl alcohol	C_{10}	45	++		
Lauryl alcohol	C_{12}	40	+++		
Myristyl alcohol	C_{14}	10	++		
Cetyl alcohol	C_{16}	0	−		
Mevalonic acid		0	−	0	−
Geraniol		90	++	90	++
Farnesol		27	+		
Squalene		0	−	0	−
Lanosterol		16	++	23	++
Ergosterol		25	+++	30	+++
Cholesterol		0	−	0	−
β-Carothine		12	+	25	+
Lecithine (bovine)		0	−	0	−
Lecithine (soybean)		0	−	20	+
Phosphatidylethanolamine		10	+		
Phosphatidylserine		10	+		
Rape seed oil		0	−	0	−
Span-20 (laurate ester)		18	++	19	++
Span-40 (palmitate ester)		0	−	0	−
Span-60 (stearate ester)		0	−	0	−
Span-80 (oleate ester)		0	−	0	−

Table 3. Reversal of brefeldin A activity by vitamins

Paper-disc soaked in 1 % vitamins aqueous and methanolic solution, and other experimentals condition were the same those in the Tables 1 and 2

Substance	Growth recovery zone			
	SABOURAUD medium		Synthetic medium	
	Diameter (mm)	Extent	Diameter (mm)	Extent
Nicotinic acid	0	−	0	−
Pantothenic acid	0	−	0	−
Folic acid	10	+	10	+
α-Tocopherol	0	−	0	−
Thiamine	15	+	20	+
Riboflavine	12	+	17	+
Vitamin D_2	10	+	12	+
Vitamin A	14	++	17	++

Co. Butyric, valeric, pelargonic, arachidic, lignoceric, linoleic, and iso-stearic acids, and octyl, decyl and cetyl alcohols, squalene, geraniol, phosphatidylethanolamine, phosphatidylserine and lecithine (soybean) were obtained from Tokyo Kasei Kogyo Co. Myristyl alcohol, terpineol, polypeptone was obtained from Wako Pure Chemical Industries Co. Mevalonic acid and lecithin (bovine) were obtained from Nutritional Biochemicals Co. Farnesol was purified from the commercial product of Tokyo Kasei Kogyo Co. Paper-discs were obtained from Toyo Roshi Co.

Results

Growth Recovery Test in the Disc-agar Plate Method

The disc-agar plate method was applied first to find growth-recovery substances with anti-frefeldin A growth-stimulating activity.

The effect of fatty acids on the growth-inhibitory property of brefeldin A is shown in Table 1. The antifungal activity was slightly reversed by $C_2 \sim C_9$ fatty acids (acetic, butyric, valeric, caproic, heptylic, caprylic and pelargonic acids), and reversed to some extent by $C_{10} \sim C_{14}$ fatty acids (capric, undecylic, lauric, tridecylic and myristic acids). Lauric acid was the most effective among fatty acids tested. However, the growth-inhibition was not revesed by $C_{16} \sim C_{24}$ straight-chain fatty acids (palmitic, stearic, arachidic and lignoceric acids). The inhibition was also slightly reversed by unsaturated or branched chain fatty acids such as palmitoleic acid ($C_{16:1}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$) and iso-stearic acid (C_{18}).

Many of the fatty acids having growth-recovery properties showed more or less inhibitory activity against test organism, and growth-recovery zones were formed outside of growth-inhibitory inner zones. It was generally observed that both growth-recovery and inhibitory zones were larger with the synthetic medium than with SABOURAUD medium.

The effect of higher fatty alcohols, isoprenoids, miscellaneous lipids and surfactants is shown in Table 2. Decyl alcohol (C_{10}) and lauryl alcohol (C_{12}) as well as lauric acid significantly reversed the inhibitory effect of brefeldin A. The inhibition was markedly reversed by geraniol, ergosterol and lanosterol, and slightly reversed by farnesol and β-carotene. However, cholesterol, squalene and mevalonic acid had no effect. Phosphatidylethanolamine and phosphatidylserine had poor reversing effects and soybean lecithine also had reversing effect only with the synthetic medium. A surfactant, Span-20 (polyoxyethylene sorbitan laurate) is effective on the growth-recovery, whereas neither their analogs, Span-40 (palmitate ester), Span-60 (stearate ester) nor Span-80 (oleate ester), exerted such activity.

The effect of vitamins is shown in Table 3. Thiamine, riboflavin, folic acid, vitamin A and vitamin D_2 had a slight recovering effect.

Growth recovery was not shown by amino acids (serine, threonine, isoleucine, valine, lysine, arginine and glutamic acid) and nucleosides (uridine, tymidine, adenosine, guanosine and cytidine).

Growth Recovery Test in the Liquid Culture Method

Straight-chain fatty acids ($C_{10} \sim C_{14}$) showed growth-recovering effect in the presence of brefeldin A (Fig. 2). Therefore, the results obtained in the tube culture method are in good accordance with that in the disc-agar plate method (Table 1).

Dose-response relationship was examined using the potent antibiotic antagonist substance,

Fig. 2. Growth recovery by straight-chain fatty acids in the liquid culture method.

Zero point one ml of methanolic solution of fatty acids were added to 10 ml of SABOURAUD medium containing brefelding A 2×10^{-5} M. The tubes were shaken at 30°C for 20 hours. Control (no brefeldin A). Caprylic acid (C_8). Capric acid (C_{10}). Lauric acid (C_{12}). Myristic acid (C_{14}). Palmitic acid (C_{16}).

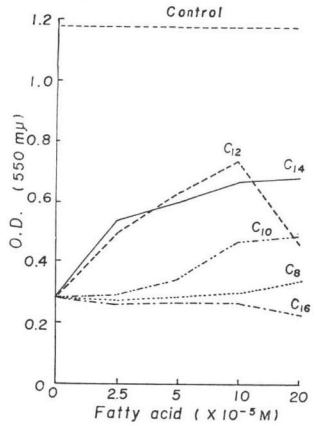


Fig. 3. Growth recovery by lauric acid.

The concentration of brefeldin A was 1.4×10^{-5} M in this experiment, and other experimental conditions were the same as those in Fig. 2. Control (no brefeldin A). Lauric acid 0, 0.5×10^{-5} M, 5×10^{-5} M, 15×10^{-5} M.

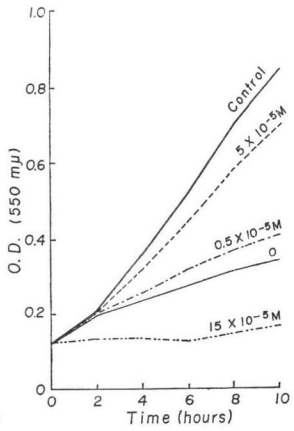


Fig. 4. Growth recovery by lauric acid on varying amount of brefeldin A.

Brefeldin A concentration of the curve (1), (2), (3), (4) and (5) was 0, 1, 2, 3 and 4×10^{-5} M respectively, while lauric acid concentration was increased from 0.25 to 2.5×10^{-5} M.

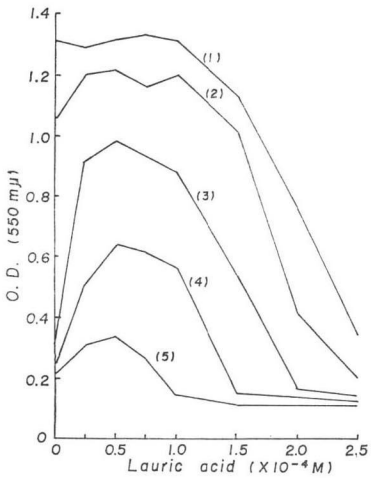
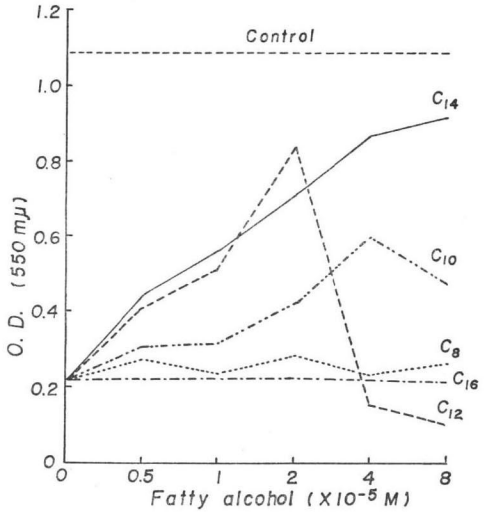


Fig. 5. Growth recovery by fatty alcohols in the liquid culture method.

The experimental conditions were the same as those in Fig. 2. Control (no brefeldin A). Octyl alcohol (C_8). Decyl alcohol (C_{10}). Lauryl alcohol (C_{12}). Myristyl alcohol (C_{14}). Cetyl alcohol (C_{16}).



lauric acid (Fig. 3). The extent of the growth increased as the concetration of lauric acid increased up to 5×10^{-5} M. At a higher concentration of lauric acid (15×10^{-5} M), lauric acid alone affected a little the growth, but growth was strongly inhibited in the presence of both lauric acid and brefeldin A.

Fig. 6. Growth recovery by isoprenoids in the liquid culture method.

The experimental conditions were the same as those in Fig. 2. Control (no brefeldin A). Geraniol (Ger). Ergosterol (Erg). Farnesol (Far). Mevalonic acid (MVA). Squalene (Squ). Cholesterol (Cho).

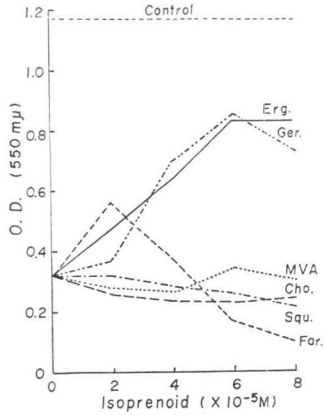
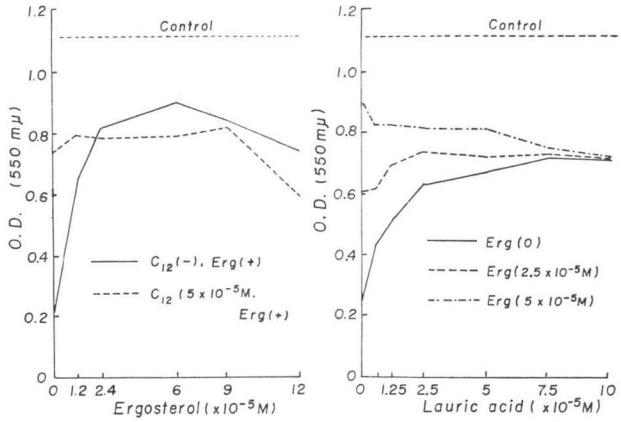


Fig. 7. Synergistic growth recovery by lauric acid and ergosterol.

(a) Ergosterol added to the growth recovery system with lauric acid.

(b) Lauric acid added to the growth recovery system with ergosterol.



The effect of lauric acid on the growth of *C. albicans* was examined varying the concentration of brefeldin A (Fig. 4). The extent of growth-recovery reduced as the concentration of the antibiotic increased, but the optimal concentration of lauric acid was constant 0.5×10^{-4} M at any levels of the antibiotic. This observation suggests that the effect is not caused by direct interaction between lauric acid and the antibiotic as polyene antibiotics do with sterols[17,18].

As shown in Fig. 5, the inhibition was reversed by $C_{10}\sim C_{14}$ fatty alcohols, and myristyl alcohol (C_{14}) was the most potent substance. Ergosterol and geraniol had strong reversal properties, and the optimal concentration was 6×10^{-5} M (Fig. 6), and farnesol restored only slightly the growth at 2×10^{-5} M. Although, cholesterol, squalene and mevalonic acid did not restored the growth at all.

The synergistic effect of some potent growth recovery substances was examined. As shown in Fig. 7 (a), (b), addition of varying concentration of ergosterol to lauric acid-containing test system, or the reverse, had no effect, *i.e.*, synergistic effect was not observed.

Discussion

Although BETINA *et al.* reported that brefeldin A affects the synthesis of protein, RNA and DNA in mammalian cells and bacterial protoplast[5,12], the primary action site are still remain to be resolved. They also reported that the antibiotic induced some morphological changes in pathogenic fungi[19].

Growth-recovery test is a useful method for studies on mode of action of antibiotics, and there are number of instances that growth-recovering substances indicated the primary site of attack by antibiotics. Brefeldin A is a fatty acid derivative biosynthesized from palmitate (C_{16}) in *Penicillium cyaneum*, and the effect of various lipids and other compound on the action of brefeldin A was examined.

The antifungal activity of brefeldin A was reversed by various lipids, especially by lauric acid, ergosterol and geraniol (Tables 1, 2). It is very difficult at present to indicate the primary

site of action of the antibiotic, but the results presented in this paper suggest that the antibiotic action has something to do with lipid metabolism.

Several lipids are known to remove inhibitory activity of some antibiotics, and action of polyene antibiotics[17,18], pyrrolnitrin[20] and mycobacillin[21] can be neutralized by various lipids such as cholesterol and phospholipids, because of their formation of inactive antibiotic-lipid complex. The effect of lipids on brefeldin A action differs from these instances, because optimal concentration of lipids for recovery of action of brefeldin A is constant (Fig. 3), whereas, that for polyene antibiotic, pyrrolnitrin and other varies in accordance with the concentration of antibiotics.

Recently, cerulenin was found to inhibit the initial step of the biosynthesis of sterols and fatty acids from acetyl-CoA, and its antimicrobial action is reversed by ergocarciferol, retinol, thiamine, pantothenic acid, lauric acid and oleic acid[16,22]. The action mechanism of brefeldin A may be different from that of cerulenin, because acetic acid incorporation into the lipid fraction of whole cell was not inhibited by brefeldin A[23].

YOSHIYAMA *et al.* found that the higher fatty acids reversed the impaired cell division of UV-irradiated *Escherichia coli*, and the maximal activity was just below the concentration causing complete suppression of cell growth[24]. A simlarity exists here in that the optimal concentration of lipids for recovery of brefeldin A action was lower several times than the concentration causing inhibition of growth.

Lipid analyses of *C. albicans* grown in the presence of brefeldin A showed significant differences in comparison with the control[23], and it was suggested that the growth inhibition by brefeldin A is due to inhibition or disturbance of lipid metabolism. Effect of brefeldin A on lipid metabolism will be presented in a subsequent paper.

Acknowledgment

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