Spermidine-Analogous Triamines Suppressed the Growth of *Candida albicans*

Eizo Takahashi,^{*,a} Seiji Oono,^a Shigeo Yamamoto,^b Sakae Arimoto,^a Tomoe Negishi,^a and Keinosuke Okamoto^a

^aLaboratory of Protein Function, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University; and ^bDepartment of Molecular Biopharmaceutical Sciences, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University; 1-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530, Japan.

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We examined the antifungal activity of various synthetic triamines on several fungi. Among various triamines having a general structure $\text{H}_2\text{N}(\text{CH}_2)_a\text{NH}(\text{CH}_2)_b\text{NH}_2$ ($a=2-5$, $b=3-8$), some triamines ($a=4$ or 5) showed inhibitory effect on the growth of *Candida albicans* and *C. tropicalis*. Determination of the minimum inhibitory concentrations (MICs) of these triamines on *C. albicans* showed that triamine 4–8 ($a=4$, $b=8$) and triamine 5–8 had strong antifungal activity. Further analysis revealed that the antifungal effect of triamine 4–8 was fungistatic and the antifungal effect was diminished by the addition of spermidine, a physiological triamine, to the medium. These results suggested that triamine 4–8 is antagonistic to spermidine and the antifungal activity is due to the suppression of the action of intrinsic polyamines. On the agar medium, *C. albicans* formed microcolonies even in the presence of triamine 4–8 by long cultivation. We then observed the form of *C. albicans* using microscope and found that the cells cultivated with triamine 4–8 were round, similar to the yeast form, while most of the cells on the agar medium without triamine 4–8 were hyphal form. Subsequently, we investigated the synergistic effect of two compounds with triamine 4–8, cyclohexylamine and *dl*- α -difluoromethylornithine which are inhibitors of enzymes involving in the biosynthesis of physiological polyamines such as spermidine. The results showed that the antifungal activity of triamine 4–8 increased by the addition of these enzyme inhibitors.

Key words *Candida albicans*; antifungal activity; triamine

Polyamines are polyvalent cations ubiquitously present in all living cells including both prokaryotes and eukaryotes.^{1–4)} It is well known that polyamines are involved in a variety of biological processes including cell proliferation, cell death, gene regulation, translation and so on.^{1–4)} Polyamines in the cells interact with the nucleic acid, especially ribonucleic acid, and consequently affect the stability of RNA. Furthermore, it has been reported that polyamines are utilized for the hypusine modification of eukaryotic transcription initiation factor 5A (eIF5A) and this modification accelerates protein synthesis.^{4–7)}

In living cells, putrescine, spermidine and spermine are major functional polyamines. It has been reported that the concentration of intracellular polyamines is controlled strictly by regulation of the biosynthetic and metabolic pathways of polyamines.^{2,4,8)} In addition, organisms can take up extracellular polyamines and excrete intracellular polyamines through polyamine transporters in order to adjust the concentration of polyamines.⁹⁾

As described above, polyamines play multiple roles in many biological processes in the cell. It has been thought that alteration of the level of these polyamines in the cells causes various diseases⁴⁾; therefore, these polyamines have become a target in drug development. It has been demonstrated that various polyamine analogues and inhibitors of the metabolism of polyamines possess anticancer and antimicrobial properties and so on.^{10–14)} Previously, it has been reported that, in *Saccharomyces cerevisiae*, which is one of best-characterized organisms for the function of polyamines, trivalent cationic spermidine is essential for hypusine biosynthesis and cell

growth, but tetravalent spermine is not.^{7,15)} Accordingly, in the present study, we examined whether a series of synthetic spermidine analogues, which had different lengths of aliphatic moiety, exhibited antifungal activity on several fungi.

MATERIALS AND METHODS

Strains and Medium *Candida (C.) albicans* FH01, *C. tropicalis* NA01, *C. glabrata* TIMM1062, *Aspergillus fumigatus* FH01, and *A. nidulans* IAM2006 were usually cultivated in nutrient broth medium (NB) (Eiken Chemical Co., Ltd., Tokyo, Japan) at 37°C. *S. cerevisiae* AH22 and INVSc1 were cultivated in yeast extract-peptone dextrose (YPD) at 37°C.

Bacteria strains used were *Streptococcus mutans* 3125001, *Enterococcus faecalis* ATCC51299, *Bacillus subtilis* ISW1214, *Escherichia coli* HB101, *Vibrio parahaemolyticus* RIMD2210633, and *Aeromonas sobria* 288. These strains except *V. parahaemolyticus* were usually cultivated in NB. For cultivation of *V. parahaemolyticus*, NB which was added NaCl to reach 3% was used.

Reagents The spermidine analogous triamines used in this study (Fig. 1) were synthesized and purified according to the procedure described by Koumoto *et al.*¹⁶⁾ Synthetic triamines having a general structure $\text{H}_2\text{N}(\text{CH}_2)_a\text{NH}(\text{CH}_2)_b\text{NH}_2$ ($a=2-5$, $b=3-8$) were abbreviated as triamine $a-b$ in this study. Spermine tetrahydrochloride and putrescine dihydrochloride were purchased from Sigma (St. Louis, MO, U.S.A.), spermidine trihydrochloride was from Nacalai Tesque Inc. (Kyoto, Japan), and amphotericin B, cyclohexylammonium chloride (CHA), and *dl*- α -difluoromethylornithine hydrochloride (DFMO) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

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*To whom correspondence should be addressed. e-mail: takahashi@pharm.okayama-u.ac.jp

Fig. 1. The Structure of Triamines Used in This Study

The triamine of $\text{H}_2\text{N}(\text{CH}_2)_a\text{NH}(\text{CH}_2)_b\text{NH}_2$ is abbreviated to triamine a - b . $a=2$ -5 and $b=3$ -8. Spermidine is represented as triamine 3-4 according to this abbreviation rule.

Evaluation of Antifungal and Antibacterial Activities Using Disk Diffusion Method To test the susceptibility of *C. albicans*, *C. tropicalis*, *C. glabrata*, *A. fumigatus*, and *A. nidulans*, five-times diluted Sabouraud dextrose agar (Eiken Chemical Co., Ltd.) supplemented with 1.5% agar was prepared. When *S. cerevisiae* was tested, five-times diluted YPD medium supplemented with 1.5% agar was used. After autoclaving, these agar media were poured into 90-mm Petri dishes (about 20 mL/dish). After solidification, 100 μL overnight culture in NB or YPD described above was spread on the surface of each agar, respectively. A sterile 8-mm paper disc was placed on the agar, and 10 μL solution of test compound dissolved in sterile MilliQ water was applied to each paper disc. After cultivation for 24 h at 37°C, the diameters of the inhibitory zones were measured.

The susceptibility of bacteria was also evaluated by the disk method. The medium used for the test of *E. coli*, *V. parahaemolyticus* and *A. sobria* was Mueller-Hinton agar (Nissui Seiyaku, Tokyo, Japan) (in the case of *V. parahaemolyticus*, NaCl was added to the medium to reach 3%), and that used for the test of *S. mutans*, *E. faecalis* and *B. subtilis* was Brain Heart Infusion Agar (Becton, Dickinson and Company, Sparks, MD, U.S.A.). Susceptibility was judged by the generation of inhibitory zone around the disk after cultivation at 37°C for 24 h.

Monitoring of Cell Growth by the Turbidimetric Method and Determination of Minimum Inhibitory Concentration (MIC) To determine the MIC of test compounds on *C. albicans*, the broth dilution method was carried out. Two-fold serially diluted solution of test compounds were added to 2 mL of the sterile yeast nitrogen base (Becton, Dickinson and Company) supplemented with 2% glucose and 10 mM K_2HPO_4 in a test tube (volume of test tube is 10 mL). Then, a portion of the pre-culture of *C. albicans* (100 μL containing approximately 6×10^3 colony-forming unit (CFU) of *C. albicans*) was inoculated into these media, and optical density (O.D.)₅₃₀ of the cultures was measured after cultivation for 48 h at 37°C with rotation (45 revolutions per minute (rpm)). When the O.D.₅₃₀ of the culture to which the test compound was added did not increase, it was considered that fungus growth was completely inhibited. The minimum concentration of test compound to inhibit fungus growth was determined as the MIC.

For the continuous observation of cell growth, the O.D.₅₃₀ of the culture was measured at the cultivation period indicated (Fig. 4). When the O.D.₅₃₀ was over 1.0, the culture was diluted appropriately using the same medium and the O.D.₅₃₀ of the diluted culture was measured.

Survival of *C. albicans* in the Medium Containing Triamine To elucidate the fungicidal effect of triamine 4-8 on *C. albicans*, the survival of *C. albicans* exposed to triamine 4-8 was examined by the plating method. After *C. albicans* was cultivated in medium with and without triamine 4-8 for 48 h at 37°C, 100 μL of each culture was spread onto the agar

plate composed of yeast nitrogen base supplemented with 2% glucose, 10 mM K_2HPO_4 and 1.5% agar. After cultivation for 48 h at 37°C, the generating colonies were counted. When the number of colonies emerged was too many to count, the culture was diluted appropriately using the medium. The ability to form colony of the diluted solution was estimated by the same manner.

Effects of CHA and DFMO on the Growth of *C. albicans* in the Medium Containing Triamine *C. albicans* FH01 was cultured in the liquid medium of yeast nitrogen base supplemented with 2% glucose and 10 mM K_2HPO_4 at 37°C for 24 h with rotation (45 rpm). Then a portion of the culture (100 μL , approximately 6×10^3 CFU) was inoculated into each of 6 tubes containing 2 mL of the same medium and following compound; (a) 1 mM DFMO, (b) 1 mM CHA, (c) 1 μM triamine 4-8, (d) 1 mM DFMO and 1 μM triamine 4-8, (e) 1 mM CHA and 1 μM triamine 4-8, (f) no addition. These were cultured at 37°C with rotation (45 rpm) and O.D.₅₃₀ of these cultures was measured at times indicated in Fig. 4.

Microscopic Observation of Colonies on the Agar Medium *C. albicans* was cultivated in a yeast nitrogen base supplemented with 2% glucose, 10 mM K_2HPO_4 until O.D.₅₃₀ reached 0.4. After cultivation, a portion of the culture was spread onto the same medium agar plate supplemented with 1.5% agar. Likewise, another portion was spread onto the medium agar containing the test compound. After cultivation for 72 h at 37°C, the microcolonies which formed on the plates with and without test compound were directly observed by phase-contrast microscope. In addition, we picked up the colonies using sterile toothpicks and suspended them in sterile saline, and the suspension was spread onto a glass slide. The samples were observed by phase-contrast microscope to examine the morphology of *C. albicans*.

Statistical Analysis The experiments were carried out in triplicate, and the data were analysed by using the Student's *t*-test and *p*-value was estimated. A *p*-value of <0.05 was considered significant. The data was represented as an arithmetic mean $\pm$ S.D.

RESULTS

Antifungal Activity of Various Triamines on Several Fungi

We assessed the effect of various triamines on the growth of several fungi. Growth inhibition was evaluated by the appearance of an inhibitory zone around the disk in which 0.2 μmol triamines were soaked. As shown in Table 1, triamines 4-X or 5-X, whose structures include the amino-butyl moiety ($a=4$) or aminopentyl moiety ($a=5$), formed a comparative size of inhibitory zone on the agar medium onto which *C. albicans* and *C. tropicalis* were spread, but triamine 5-5 did not form. The sizes of the inhibitory zone by these triamines on *C. albicans* were generally larger than those on *C. tropicalis*. Two triamines, 5-5 and 5-8, also created an inhibitory zone on *A. nidulans*, while other triamines did not. The effect of these triamines was not observed on the growth of *C. glabrata*, *A. fumigatus* and *S. cerevisiae*. Similar experiments showed that triamines 2-X and 3-X did not influence the growth of any fungi (Table 1).

Likewise, we tested the effect of these triamines on the growth of Gram-positive bacteria (*Streptococcus mutans*, *Enterococcus faecalis*, *Bacillus subtilis*) and Gram-negative bac-

Table 1. Antifungal Activity of Triamines on Several Fungi

Triamines (a-b)	Inhibitory zone (mm)					
	<i>C. albicans</i>	<i>C. glabrata</i>	<i>A. fumigatus</i>	<i>A. nidulans</i>	<i>S. cerevisiae</i>	
	FH01	TIMM1062	FH01	IAM2006	AH22	INVSc1
2-3	—	—	—	—	—	—
2-4	—	—	—	—	—	—
2-5	—	—	—	—	—	—
2-6	—	—	—	—	—	—
2-7	—	—	—	—	—	—
2-8	—	—	—	—	—	—
3-3	—	—	—	—	—	—
3-4	—	—	—	—	—	—
3-5	—	—	—	—	—	—
3-6	—	—	—	—	—	—
3-7	—	—	—	—	—	—
3-8	—	—	—	—	—	—
4-4	13	—	—	—	—	—
4-5	23	17	—	—	—	—
4-6	24	21	—	—	—	—
4-7	25	23	—	—	—	—
4-8	22	17	—	—	—	—
5-5	—	—	—	14	—	—
5-6	10	—	—	—	—	—
5-7	17	22	—	—	—	—
5-8	20	20	—	14	—	—

*: — means that no inhibitory zone occurred.

teria (*Escherichia coli*, *Vibrio parahaemolyticus*, *Aeromonas sobria*). No triamines tested in this study exhibited any effect on the growth of bacteria (data not shown).

MICs of Various Triamines on *C. albicans* Triamines 4-X and 5-X exhibited inhibitory effects on the growth of *C. albicans*. To assess the antifungal activity of these triamines, we determined their MIC on *C. albicans*, as described in Materials and Methods. In the determination of the effect of triamines on the growth of *Candida*, we used the medium which does not contain amino acids. Because some amino acids, especially arginine, are involved in the synthesis of physiological polyamines. To remove the participation of these amino acids in the suppression of growth of fungi by synthetic triamines, yeast nitrogen base medium supplemented with 2% glucose and 10 mM K₂HPO₄ was used. This medium is developed for susceptibility testing of fungi. Amino acids contained in the medium are restricted to three kinds (L-histidine monohydrochloride 10 mg/L, LD-methionine 20 mg/L, and LD-tryptophan 20 mg/L). As the medium using for CLSI M27-A3 which is internationally accepted protocol for drug susceptibility contains many kinds of amino acids, we did not use CLSI M27-A3 in this experiment.

As shown in Table 2, the MICs of triamine 4-X and 5-X were in the range of 0.24–1.9 μ M, except for triamine 4-4 and 4-5. Even 1 mM of triamines 4-4 or 4-5 in the medium did not suppress the growth of *C. albicans*. Similarly, MIC of spermidine (a=3, b=4), which did not affect the growth of *C. albicans*, was determined to be over 1 mM in this condition (data not shown). Among these triamines 4-X and 5-X, MICs of triamine 4-8 and 5-8 were small and were determined to be 0.98 μ M and 0.24 μ M, respectively. In the same manner, the MIC of amphotericin B was evaluated to be 7.8 μ M. Ampho-

Table 2. MICs of Triamines on *C. albicans*

Chemicals	MIC (μ M)
Triamines (a-b) 4-4	>1000*
4-5	>1000
4-6	1.9
4-7	1.9
4-8	0.98
5-5	1.9
5-6	1.9
5-7	1.9
5-8	0.24
Amphotericin B	7.8

*: >1000 of MIC means no inhibitory effect.

tericin B is a polyene antifungal drug and is demonstrated to kill *Candida* spp.. Amphotericin B is used with high frequency not only in treating various systemic fungal infections but also in tissue culture to prevent cell cultures from contaminating with fungi. Therefore, we used amphotericin B as positive control. These results indicated that the triamines might be more effective than amphotericin B in antifungal activity to *C. albicans*.

Effect of Physiological Polyamines on the Antifungal Activity of Triamine 4-8 Triamine 4-8 was evaluated to be one of the triamines possessing effective antifungal activity on *C. albicans* with MIC of 0.98 μ M in this study. To elucidate the mode of action of triamines, we examined the effect of major physiological polyamines, putrescine, spermidine and spermine, on the antifungal activity of triamine 4-8 (Fig. 2a). To examine the effect of physiological polyamines on the antifungal activity of triamine 4-8, we added these physiological

Fig. 2. The Structure of Putrescine, Spermidine, Spermine (a) and the Effects of These Polyamines on the Growth of *C. albicans* in the Presence of Triamine 4-8 (b)

The stripped bar represents the O.D.₅₃₀ obtained by incubation without neither triamine 4-8 nor polyamines. When *C. albicans* was cultivated in medium containing triamine 4-8 at the concentration of MIC (0.98 μM) for 48 h, it could not proliferate and O.D.₅₃₀ of the culture did not increase (lane of 1 μM triamine 4-8 without polyamine (0 μM)). Each bar represents the O.D.₅₃₀ of the sample cultivated in the presence of triamine 4-8 at the concentration of MIC and physiological polyamines indicated. White bar, gray bar and black bar show the addition of putrescine, spermidine and spermine, respectively. When the value of O.D.₅₃₀ of the culture was over 1.0, the culture was appropriately diluted with the medium and the O.D.₅₃₀ of the diluted sample was measured. The experiments were performed in triplicate, and the arithmetic means ± S.D. were plotted. Asterisks indicate statistically significant differences versus the control value obtained by incubation without physiological polyamines (*, *p* < 0.05, **, *p* < 0.01).

Table 3. Alteration of MIC of Triamine 4-8 on *C. albicans* by the Addition of Different Concentrations of Spermidine

	Triamine 4-8 only	Spermidine concentration added (μM)			
		1	10	40	80
MIC of triamine 4-8 (μM)	0.98	3.9	15.6	125	250

polyamines to the medium containing triamine 4-8 at the concentration of MIC (1 μM). These physiological polyamines was added to the medium to become 0.1 μM, 1 μM, 10 μM and 100 μM (Fig. 2b). *C. albicans* was cultured in these media at 37°C for 48 h.

After cultivation, we measured the O.D.₅₃₀ of the culture in order to monitor the growth of *C. albicans*. As shown in Fig. 2b, *C. albicans* could not grow in the medium containing 1 μM triamine 4-8 for 48 h, while *C. albicans* could grow in the medium without triamine 4-8. When spermidine was added to the medium containing triamine 4-8, the O.D.₅₃₀ of the culture was increased in a spermidine dose-dependent manner (Fig. 2b). The addition of 100 μM spermidine neutralized the antifungal activity of 1 μM triamine 4-8, and the growth of *C. albicans* in this medium was comparable to that in the medium without triamine 4-8. Likewise, the O.D.₅₃₀ of the culture containing triamine 4-8 was increased slightly by the addition of putrescine in a dose-dependent manner; however, the addition of spermine did not counteract the antifungal activity of triamine 4-8. These results indicated that triamine 4-8 antagonized the action of physiological polyamines, especially spermidine. It is thought that the spermidine added provided enough amount of spermidine to *C. albicans* to diminish the action of triamine 4-8.

Subsequently, we evaluated the change of MIC of triamine 4-8 in the presence of spermidine. As shown in Table 3, a higher amount of triamine 4-8 was required to inhibit the growth of *C. albicans* in the presence of spermidine. Finally, in the presence of 80 μM spermidine, MIC of triamine 4-8 became 250 μM, which is 250 times higher than that in the

absence of spermidine.

Morphological Change of *C. albicans* Cell on Agar Medium Containing Triamine 4-8 *C. albicans* formed the colonies on agar medium without triamine by cultivation at 37°C for 48 h, but could not on agar medium containing 10 μM triamine 4-8; however, as shown in liquid medium, the addition of 100 μM spermidine to agar medium with triamine 4-8 allowed *C. albicans* to proliferate and form a colony on this agar medium.

From other experiments we found that very small colonies were formed on agar medium containing triamine 4-8 without spermidine by continuous cultivation for an additional 24 h (total 72 h). Subsequently, we observed differences between the colonies yielded on these agar media with and without triamine 4-8. Microscopic observation demonstrated that the peripheries of colonies on the agar medium without triamine 4-8 were fuzzy, while those on agar medium with triamine 4-8 were smooth (Figs. 3a, b). Further, we picked up the colonies using a sterile toothpick, suspended them in sterile saline, spread them on a glass slide, and observed them under a highly magnified field. These results showed that the majority of *C. albicans* cells had a different shape between the conditions with and without triamine 4-8. As shown in Fig. 3d, the *C. albicans* cells grown without triamine 4-8 were elongated, suggesting that most of the cells had a hyphal form, which is considered to be the active form for proliferation. In contrast, as shown in Fig. 3e, the *C. albicans* cells grown with triamine 4-8 were round, suggesting that most of the cells in this condition had a yeast form. These results indicated that the mode of antifungal activity of triamine 4-8 was fungistatic. *C. albi-*

Fig. 3. Photomicrographs of Microcolonies of *C. albicans* Cultivated on Agar Medium Containing Triamine 4–8

C. albicans was cultivated on agar medium at 37°C for 72 h (a, d). Figures (b, e) show *C. albicans* cultured on medium containing 10 μ M triamine 4–8, and (c, f) show *C. albicans* cultured on medium containing both 10 μ M triamine 4–8 and 100 μ M spermidine. The colonies on the agar medium were observed directly by phase-contrast microscope (a–c). These colonies were then picked up and suspended in saline, and the suspension was spread on a glass slide. Figures (d–f) were obtained from the observation of these samples.

Fig. 4. Synergistic Inhibitory Effect of Triamine 4–8 and Either DFMO or CHA on the Growth of *C. albicans*

C. albicans was cultured in a sterile yeast nitrogen base supplemented with 2% glucose and 10 mM K_2HPO_4 . The growth of *C. albicans* was measured by absorbance at 530 nm ($O.D._{530}$) at the time indicated in the figure. Symbols in the figures show the compound added to each culture, ○: without any compound, ●: with triamine 4–8 (1 μ M), ◇: with DFMO (1 mM), ◆: with DFMO (1 mM) and triamine 4–8 (1 μ M), △: with CHA (1 mM), ▲: with CHA (1 mM) and triamine 4–8 (1 μ M). The experiments were performed in triplicate, and the arithmetic means $\pm$ S.D. were plotted. Asterisks indicate statistically significant differences between two values of $O.D._{530}$ at the time indicated. (*: $p < 0.05$, **: $p < 0.01$).

C. albicans could not actively proliferate in the presence of triamine 4–8, because triamine 4–8 prohibited the morphological transition from the yeast form to hyphal form.

The feature of colonies grown on agar medium containing both triamine 4–8 and spermidine was similar to that on agar medium without triamine 4–8 (Figs. 3c, f). We presume from these results that spermidine lowers the action of triamine 4–8 in cell and that the morphology of the cells in the presence of spermidine is restored to the original hyphal form.

Mode of Action of Triamine 4–8 MIC of triamine 4–8 on *C. albicans* FH01 is ca. 1 μ M (Table 2). MIC was determined by the incubation for 48 h. However, as shown by the line with black circle of Fig. 4 (growth in the medium containing 1 μ M triamine 4–8), further incubation (incubation for 72 h) allows the *Candida* to grow. This shows triamine 4–8 suppresses the growth of *C. albicans* but does not kill it, demonstrating that triamine 4–8 functions fungistatically but not

fungicidally.

Consequently, in order to examine the survival of *C. albicans* in the presence of triamine 4–8, we checked the ability of colony formation of *C. albicans* which was incubated in the liquid medium containing triamine 4–8 for 48 h. After cultivation for 48 h, the culture sample derived from the incubation with 1 μ M triamine 4–8 formed 1.7×10^4 CFU/mL, while the culture in liquid medium without triamine 4–8, in which *C. albicans* could grow, formed 0.7×10^8 CFU/mL. The number of *C. albicans* inoculated into 2 mL of medium was 6×10^3 CFU as described in Materials and Methods. The result supported the above hypothesis that triamine 4–8 inhibited the growth of *C. albicans*, but did not function fungicidally.

Synergistic Effect of the Inhibitors of Polyamine Biosynthesis on the Action of Triamine 4–8 Previous data suggested that the mechanism of antifungal activity of triamines is to antagonize the action of physiological polyamines, espe-

cially spermidine; therefore, we thought that the deficiency of physiological polyamines in the cell might enhance the effect of the antifungal activity of triamines. We therefore investigated whether DFMO, an inhibitor of ornithine decarboxylase, and CHA, an inhibitor of spermidine synthase, promoted the antifungal activity of triamine 4–8. Each enzyme is involved in the biosynthesis of intrinsic polyamines in the cell.^{17–21)}

We cultured *C. albicans* in liquid medium containing triamine 4–8 and the inhibitor (DFMO or CHA), and measured the cell growth to examine the synergistic effect of the inhibitor. Inoculation and cultivation of the cell were performed as described in Materials and Methods.

As shown in Fig. 4, about 24 h after initiation of the culture, the O.D.₅₃₀ of the culture without any compound began to increase. The O.D.₅₃₀ of the culture with 1 μ M triamine 4–8 did not increase until 48 h, and then slightly increased at 72 h and surpassed 1.0 by continuous cultivation. The addition of 1 mM CHA did not significantly affect the growth of *C. albicans*, while the addition of 1 mM DFMO slightly delayed the growth. The result indicates that these inhibitors of the biosynthesis of polyamines did not greatly affect the growth of *C. albicans* under this condition (Fig. 4).

When *C. albicans* was cultivated in medium containing both 1 μ M triamine 4–8 and 1 mM DFMO, *C. albicans* did not grow during the observation period (120 h) (Fig. 4a). With the combination of 1 μ M triamine 4–8 and a lower concentration of DFMO, the period of growth inhibition by triamine 4–8 was prolonged in a DFMO dose-dependent manner (data not shown). Similarly, the addition of 1 mM CHA also provided the prolonged period of growth inhibition by triamine 4–8 (Fig. 4b). These results suggested that the antifungal activity of triamine 4–8 was synergistically strengthened by inhibition of the biosynthesis of physiological polyamines.

DISCUSSION

In this study, we found that triamine 4-X and 5-X, whose structure contained the aminobutyl moiety or aminopentyl moiety, possessed antifungal activity to *C. albicans* and *C. tropicalis*, while triamine 2-X and 3-X, such as triamine 2–4, 2–5, 3–4 (spermidine), and 3–5, did not. In triamine 4-X and 5-X (X=4–8), triamines possessing a longer aliphatic chain showed stronger antifungal activity, as shown in Tables 1 and 2. The size of the inhibitory zone which was created by the disc diffusion method by triamines possessing a longer aliphatic chain tended to be larger, and the MICs of the larger triamines on *C. albicans* tended to be smaller. These results suggested that not only the aminobutyl moiety or aminopentyl moiety but also the length of the latter aliphatic moiety of triamines is involved in the antifungal activity of triamines.

On the other hand, these triamine 4-X and 5-X did not significantly inhibit the growth of other fungi, including *C. glabrata*, *A. fumigatus*, *A. nidulans* and *S. cerevisiae*. It remains unclear why these fungi are insensitive to triamine 4-X and 5-X and two strains of *Candida*, *C. albicans* and *C. tropicalis*, are sensitive to these triamines. Among the *Candida* species tested, *C. glabrata* did not respond to the antifungal activity of triamines, while triamines inhibited the growth of *C. albicans* and *C. tropicalis* effectively. Interestingly, it has been reported that *C. glabrata* is closer to *S. cerevisiae* than *C. albicans* and *C. tropicalis* in dedrogram analysis accord-

ing to the sequence of the 18S ribosomal RNA (rRNA) gene, although *C. albicans* is close to *C. tropicalis*.²²⁾ Hence, the reaction of *C. glabrata* to the antifungal activity of triamines may be similar to that of *S. cerevisiae*. As shown in the result, the fungistatic action of triamines to two strains of *Candida* (*C. albicans* and *C. tropicalis*) might be due to the suppression of activity of spermidine in these fungi. To clarify why two strains are specifically sensitive to triamines and why other fungi are insensitive, the role of spermidine in fungi must be clarified. The penetration of these triamines into fungi must be also examined. Further studies are required to clarify the specific action of these triamines to two strains of *Candida*.

Similarly, we tested the effect of triamines on the growth of prokaryotes, both Gram-positive and Gram-negative bacteria, including *S. mutans*, *E. faecalis*, *B. subtilis*, *E. coli*, *V. parahaemolyticus*, and *A. sobria*; however, inhibition of the growth of these bacteria by the triamines used in this experiment did not occur. Koumoto *et al.* showed that a polyamine auxotroph of *E. coli* can utilize spermidine-analogous triamines instead of physiological polyamines,¹⁶⁾ and Chattopadhyay *et al.* reported that polyamines are not required for the aerobic growth of *E. coli*.²³⁾ Consequently, we considered that the bacteria can beneficially utilize spermidine-analogous triamines for their growth, while the growth of some fungi was inhibited by some triamines.

C. albicans ubiquitously inhabits the oral cavity, gastrointestinal tract, skin and so on, causing opportunistic infection, and is one of the causative agents of nosocomial infectious disease.²⁴⁾ Hence, spermidine-analogous triamines may be useful for the treatment of pathogenic *C. albicans* infection. It is necessary to define the spectrum of antifungal activity of triamines for further analysis. In addition, the toxicity of triamine to human beings must be clarified to use the triamine as a drug. As the preliminary experiment, we exposed Chinese hamster ovary cells (CHO cells) to triamine 4–8 and observed the morphological changes of the cells. The concentration of triamine 4–8 was 100 times as much as MIC and the cells were observed for 24 h. The injury of the cells was not observed (data not shown). This suggests that the cytotoxicity of triamine 4–8 to human beings is low. Further studies on the safety of triamine itself and of combined treatment of triamine with CHA and/or DFMO may contribute to the development of antifungal agent.

In this study, we analyzed the mechanism of the antifungal activity of triamines and showed the relationship between synthetic triamine 4–8 and intrinsic polyamines. The inhibitory effect of triamine 4–8 on the growth of *C. albicans* was weakened by the addition of spermidine, and MIC of triamine 4–8 increased depending on the amount of spermidine added (Table 3). Putrescine partially recovered the growth of *C. albicans*, which was suppressed by triamine 4–8, but spermine did not (Fig. 2b). These results indicated that the antifungal activity of triamine 4–8 was due to an antagonistic effect against spermidine, and spermidine might play an important role in the physiology in *C. albicans*, as in *S. cerevisiae*.⁷⁾

Subsequently, we revealed the synergistic effect of the inhibitor of the biosynthesis of intrinsic polyamines with triamine 4–8. We found that co-exposure to both triamine 4–8 and either CHA or DFMO showed continuous inhibition on the growth of *C. albicans* compared to the treatment of triamine 4–8 only, although these inhibitors alone did not affect

to the growth of *C. albicans* (Fig. 4). These results suggested that the decrease of concentration of intracellular polyamines enhanced the effect of triamine 4–8.

In this study, we found by microscopic observation that *C. albicans* treated with triamine 4–8 took only yeast form, although most cells in the condition without triamine 4–8 took the hyphal form. *C. albicans* is a dimorphic fungus and the network to regulate the morphological transition has been studied.^{24–27} It has been reported that polyamines play an important role in the hyphae formation of *C. albicans*.^{28,29} Herero *et al.* reported that *odc*-deficient *C. albicans*, which could not convert from L-ornithine to putrescine and lacked the ability to synthesize polyamines, grew in the yeast form and did not transform to the hyphal form.²⁹ This report supported our hypothesis, which is that synthetic triamines antagonize the function of polyamines and hence induce phenomena similar to those induced by the deficiency of intracellular polyamines. It has been reported for *C. albicans* that morphological transition is involved in its pathogenicity.^{24,30} It is expected that treatment with triamine 4–8 will result not only in the growth inhibition of *C. albicans* but also attenuation of its pathogenicity by prevention of morphological transition.

To date, various properties of polyamine analogues have been studied. Jakus *et al.* reported that 1,7-diaminoheptane and 1,8-diaminooctane inhibited deoxyhypusine synthase.³¹ Part of the chemical structure of triamine 4–8 is identical to 1,8-diaminooctane, which suggested that one of the target molecules of triamine 4–8 might be deoxyhypusine synthase. Further study is required to elucidate the details of the mechanism of the antifungal activity of triamines.

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