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Structural and Functional Relationship among Diamines in Terms of Inhibition of Cell Growth

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Following the report that agmatine has an anti-proliferative effect on cell growth through induction of antizyme [Satriano *et al.* (1998) *J. Biol. Chem.* 273, 15313–15316], we examined the effects of 16 different diamines on cell growth. Many diamines had little or no effect on cell growth, but agmatine and 1,6-hexanediamine had anti-proliferative effects, with agmatine having the strongest effect. Inhibition of cell growth occurred after 2 days, and inhibitory effects paralleled the degree of antizyme induction. Decreased spermine levels indicated that induction of spermidine/spermine N^1-acetyltransferase was also involved in the inhibition of cell growth by agmatine and 1,6-hexanediamine. The frameshift efficiency (ratio of antizyme synthesis with or without frameshift) measured in a rabbit reticulocyte cell-free system was also increased by 1,3-propanediamine and *cis*-1,4-cyclohexanediamine in addition to agmatine and 1,6-hexanediamine. However, the intracellular levels of 1,3-propanediamine and *cis*-1,4-cyclohexanediamine were low when these compounds were added to the cell-culture medium. Other diamines had no effect on cell growth or frameshift efficiency. The results suggest that the presence of two amino-groups separated by an appropriate distance is important for the enhancement of frameshifting by diamines.

Key words: antizyme, cell growth, diamine, frameshift, spermidine/spermine N^1-acetyltransferase.

The cellular content of polyamines, which play important roles in cell proliferation and differentiation, is regulated by biosynthesis, degradation, and transport (*1–3*). In the polyamine biosynthetic pathway, ornithine decarboxylase (ODC), the first and rate-limiting enzyme, catalyzes the decarboxylation of L-ornithine, leading to formation of putrescine. Spermidine synthase catalyzes the production of spermidine from putrescine, and spermine synthase subsequently catalyzes the production of spermine from spermidine, both enzymes adding an aminopropyl group to the precursor. The donor of the aminopropyl moiety is decarboxylated *S*-adenosylmethionine, which is produced from *S*-adenosylmethionine by *S*-adenosylmethionine decarboxylase, another rate-limiting enzyme of formation of spermidine and spermine. The levels of ODC and polyamine uptake activity in animal cells are influenced by intracellular polyamine levels (*4*, *5*). Such regulation of ODC and polyamine transport activity by polyamines is mainly accomplished through antizyme (*6*, *7*).

Antizyme is known to be induced by polyamines and to inhibit the activity of ODC by forming an antizyme-ODC complex, leading to the rapid degradation of ODC by 26S proteasome without ubiquitination (*8–10*). Thus, antizyme has an important role in the regulation of polyamine biosynthesis by negative feedback. Furthermore, antizyme negatively regulates polyamine transport (*11–13*). Accumulation of excess intracellular polyamines caused by the overproduction of ODC can be restored to normal levels by transfection of the antizyme gene (*12*), and this involves inhibition of polyamine uptake and stimulation of polyamine excretion by antizyme (*13*).

Antizyme gene expression requires programmed ribosomal frameshifting, and polyamines increase the frameshift efficiency (*14*). It has been reported that agmatine suppresses cell proliferation by frameshift induction of antizyme (*15*). Therefore, an inducer of antizyme might be applicable as an anti-cancer drug. In this study, we examined the effects of 16 different diamines on cell growth, and found that only agmatine and 1,6-hexanediamine have an anti-proliferative effect through antizyme induction. We also discuss the structural requirements of diamines for antizyme induction.

MATERIALS AND METHODS

Preparation of Putrescine Analogues—Figure 1 shows the structures of putrescine analogues used. 1,3-Propanediamine·2HCl [1], *N*-(3-aminopropyl)-*n*-propylamine·2HCl [2], *N*-(3-aminopropyl)-*n*-butylamine·2HCl [3], *N*-ethylputrescine [4], *trans*-1,4-diamino-2-butene·2HCl [5], *cis*-1,4-diamino-2-butene·2HCl [6], *trans*-1,4-cyclohexanediamine·2HCl [8], *cis*-1,4-cyclohexanediamine·2HCl [9], and 4-aminomethylpiperidine·2HCl [10] were synthesized and crystallized as described previously (*16*, *17*).

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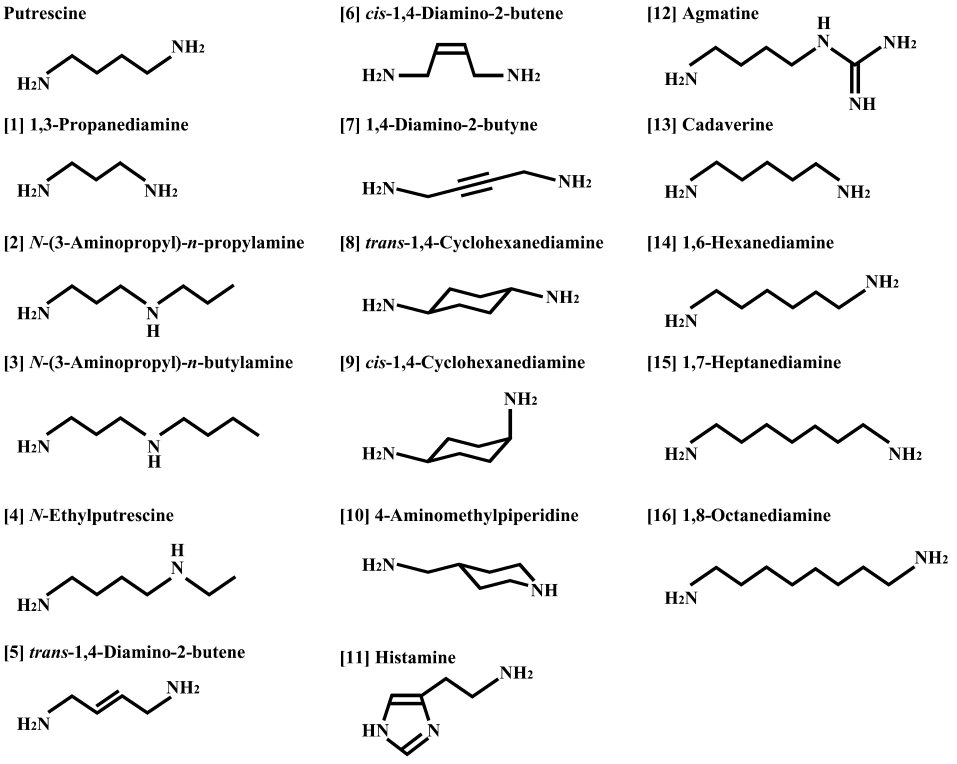

Fig. 1. **Structures of putrescine analogues.**

1,4-Diamino-2-butyne·2HCl [7] was synthesized from 1,4-dichlorobutyne by the method used for putrescine synthesis (*18*). Purity of these diamines was estimated to be more than 98% based on elemental analysis. Histamine·2HCl [11], agmatine sulfate [12], and cadaverine (1,5-pentanediamine)·2HCl [13] were purchased from Wako Chemicals. 1,6-Hexanediamine [14] and 1,7-heptanediamine [15] obtained from Wako Chemicals and 1,8-octanediamine [16] obtained from Aldrich were used after neutralization with hydrochloric acid.

Culture of ODC-Overproducing FM3A (EXOD-1) Cells—Mouse ODC-overproducing FM3A (EXOD-1) cells (*19*) were used for this study because the level of antizyme was more easily detected than with the parent FM3A cells. Cells (5×10^4/ml) were cultured in ES medium (Nissui Pharmaceutical) supplemented with 50 U/ml streptomycin, 100 U/ml penicillin G, and 2% heat-inactivated fetal calf serum at 37°C in an atmosphere of 5% CO_2. When the effect of putrescine analogues on cell growth was examined, 1 mM aminoguanidine, an inhibitor of Cu^{2+}-dependent amine oxidase (*20*), was added to the medium. The viable cell number was counted in the presence of 0.25% trypan blue. EXOD-1 cells were maintained in the above medium containing 20 mM α-difluoromethylornithine (DFMO), an inhibitor of ODC, until the commencement of these experiments.

Measurement of Polyamines and Putrescine Analogues—EXOD-1 cells (6×10^6) cultured for 24 h in the presence or absence of putrescine or putrescine analogues were homogenized with 0.3 ml of 0.2 M $HClO_4$, and the supernatant obtained by centrifugation at 12,000 × *g* for 10 min was used for the measurement of polyamines and putrescine analogues. Amines were analyzed with a Toyo Soda HPLC system as described previously (*21*). The amounts of putrescine analogues were determined from a calibration curve made with four different concentrations of putrescine analogues.

Assay of Spermidine/Spermine N^1-Acetyltransferase (SSAT)—EXOD-1 cells (5×10^7) were suspended in 0.8 ml of the buffer containing 10 mM Tris-HCl, pH 7.5, 20% glycerol, 1 mM dithiothreitol and 20 μM 6-amino-2-naphthyl-4-guanidinobenzoate dihydrochloride (FUT-175), an inhibitor of serine protease (*22*). FUT-175 was a kind gift of Torii Pharmaceutical Co. The suspension was frozen, thawed, and homogenized with a Teflon homogenizer. The homogenate was centrifuged for 10 min at 12,000 × *g*. The supernatant was dialyzed against the above buffer and used for the assay for SSAT, which was performed as described previously (*23*).

Western Blot Analysis of ODC and Antizyme—The 12,000 × *g* supernatant obtained above was centrifuged for 90 min at 100,000 × *g*. The 100,000 × *g* supernatants containing 1 and 20 μg protein were used for Western blot analysis of ODC and antizyme, respectively. Antibodies against ODC and antizyme were prepared as described in the references 24 and 25, respectively. Western blotting was performed as described (*26*), using a Proto Blot Western blot AP system (Promega).

Assay for Antizyme Synthesis—In vitro protein synthesis was performed using a rabbit reticulocyte cell-free system as described previously (*27*) with some modifications. The reaction mixture (0.02 ml) contained 30 mM Hepes-KOH buffer, pH 7.6, 10 μM hemin, 1.5 mM magnesium acetate, 0.1 mM spermidine, 100 mM potassium acetate, 2 mM dithiothreitol, 0.5 mM glucose 6-phosphate, 1.0 mM ATP, 0.4 mM GTP, 8 mM creatine phosphate, 3 μg of creatine kinase, 0.015 A_{260} unit of antizyme mRNA, 0.4 MBq [^{35}S]methionine with 1 μM methionine, 30 μM concentrations of each of the 19 other common amino acids and 10 μl of Sephadex G-25 filtered cell

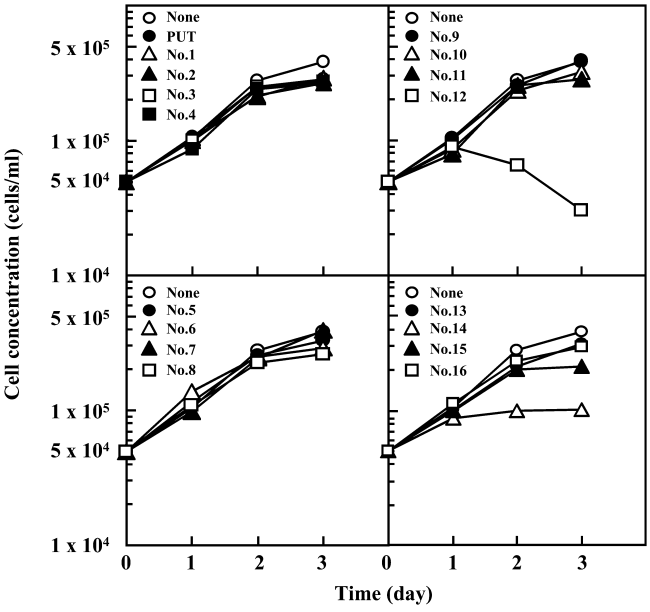

Fig. 2. **Effect of putrescine analogues on cell growth of EXOD-1 cells.** Cells were cultured in the presence of 0.25 mM putrescine analogues and 1 mM aminoguanidine. Each value is the average of three determinations. Standard error was within ± 10% of each point.

lysate (0.85 mg protein). After incubation at 33°C for 60 min, 5 μl of 20 mM methionine and 75 μl of the buffer containing 10 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1% Triton X-100, and 0.1% sodium dodecyl sulfate were added to the reaction mixture. The amount of [^{35}S]methionine-labeled antizyme synthesized was determined by the method of Philipson *et al.* (*28*) using an antibody against antizyme and PANSORBIN cells (Calbiochem). Radioactivity of labeled antizyme was quantified using a Fujix Bas 2000II imaging analyzer. Antizyme mRNAs used were synthesized by T7 RNA polymerase using *Eco*RI digested pGEM4Z-AZ and pGEM4Z-AZ (ΔT205) (*14*) as a template and an AmpliScribe T7 High Yield transcription kit (Epicentre Technologies). Antizyme was synthesized from these AZ and AZ (ΔT205) mRNAs with or without frameshifting, respectively.

RESULTS

Effecst of Putrescine and Putrescine Analogues on Cell Growth—Putrescine analogues were examined to determine if they inhibit cell growth like agmatine. For this purpose, ODC-overproducing FM3A (EXOD-1) cells were used because it is important to measure the level of antizyme precisely. As shown in Fig. 2, only 1,6-hexanediamine and agmatine inhibited cell growth when cells were cultured in the presence of 0.25 mM analogue. Agmatine was the strongest inhibitor of cell growth. The other 14 analogues shown in Fig. 1 did not affect cell growth. Essentially the same results were obtained with FM3A cells, from which EXOD-1 cells were isolated (data not shown).

Mechanism of the Inhibition of Cell Growth by Agmatine and 1,6-Hexanediamine—The levels of polyamines and putrescine analogues were determined in EXOD-1 cells cultured with putrescine analogues. As shown in Table 1, significant amounts of each analogue accumulated in cells after culture with that analogue. When agmatine and 1,6-hexanediamine accumulated in cells, the levels of putrescine, spermidine and spermine decreased greatly. Thus, we next measured the levels of antizyme and ODC in the cells. As shown in Fig. 3, induction of antizyme was high and the level of ODC was low in cells treated with agmatine and 1,6-hexanediamine. The other 14 putrescine analogues did not significantly affect the level of ODC.

Table 1. **Levels of polyamines and putrescine analogues in EXOD-1 cells.**

Polyamine content (nmol/mg protein)	Putrescine analogue			
	Putrescine	Spermidine	Spermine	Analogue
None	5.16	11.6	14.6	
Putrescine	68.4	12.4	8.74	
1. 1,3-Propanediamine	4.67	12.0	13.8	13.9
2. *N*-(3-Aminopropyl)-*n*-propylamine	2.56	14.2	11.7	51.4
3. *N*-(3-Aminopropyl)-*n*-butylamine	2.42	13.8	12.5	18.3
4. *N*-Ethylputrescine	N.D.	12.1	16.1	60.4
5. *trans*-1,4-Diamino-2-butene	2.96	9.88	12.6	56.4
6. *cis*-1,4-Diamino-2-butene	2.40	10.4	16.5	15.6
7. 1,4-Diamino-2-butyne	2.28	10.3	17.3	6.06
8. *trans*-1,4-Cyclohexanediamine	N.D.	8.71	14.2	58.5
9. *cis*-1,4-Cyclohexanediamine	2.78	9.25	12.4	8.78
10. 4-Aminomethylpiperidine	N.D.	12.9	12.4	53.6
11. Histamine	2.36	8.21	13.8	7.60
12. Agmatine	0.56	6.31	8.58	55.3
13. Cadaverine (1,5-Pentanediamine)	2.93	9.18	13.0	56.9
14. 1,6-Hexanediamine	0.89	7.56	10.5	87.5
15. 1,7-Heptanediamine	1.63	8.50	12.0	40.3
16. 1,8-Octanediamine	1.87	8.24	15.4	25.9

Polyamine and putrescine analogues were measured as described in "MATERIALS AND METHODS." Each value is the average of two determinations. N.D., not determined, because the peak position overlapped that of the putrescine analogue.

Table 2. **SSAT activity in EXOD-1 cells treated with putrescine analogues.**

Diamine	SSAT activity (pmol/min/mg protein)
None	<1.0
2. *N*-(3-Aminopropyl)-*n*-propylamine	5.2 ± 0.63
12. Agmatine	112.8 ± 5.23
14. 1,6-Hexanediamine	30.0 ± 0.60
15. 1,7-Heptanediamine	13.5 ± 0.15

SSAT activity was measured as described in "MATERIALS AND METHODS." Values are means ± SE of triplicate determinations.

Agmatine has been reported to induce SSAT, thus enhancing conversion of spermine to spermidine and of spermidine to putrescine (*29*, *30*). In cells treated with agmatine or 1,6-hexanediamine, the level of spermine decreased together with those of putrescine and spermidine (Table 1). Thus, SSAT activity in these cells was measured. As shown in Table 2, agmatine strongly induced SSAT activity, and 1,6-hexanediamine induced it significantly. In their ability to stimulate protein synthesis, 1 mol of spermine is equivalent to 4 mol of spermidine and 100 mol of putrescine (*27*). It has been also reported that induction of SSAT causes programmed cell death (*31*). So, induction of SSAT is probably responsible for the inhibition of cell growth together with induction of antizyme, which mainly causes the decrease in putrescine and spermidine.

In a previous study of MCT (mouse kidney proximal tubule) cells, ODC activity and putrescine uptake were inhibited by 90% and 85%, respectively, by 1 mM agmatine within 2 h (*15*). However, under our experimental conditions, inhibition of cell growth started at 24 h in the presence of 0.25 mM agmatine (Fig. 2). Inhibition of cell growth by 0.25 mM agmatine was not observed at 12 h (data not shown). Thus, the timing of antizyme induction and decrease in the level of ODC in EXOD-1 cells was investigated. As shown in Fig. 4, significant induction of antizyme was observed at 10 h and further induction of antizyme at 24 h. A small decrease in the level of ODC was observed at 10 h and dramatic decrease in ODC was seen at 24 h. These results indicate that the decrease in

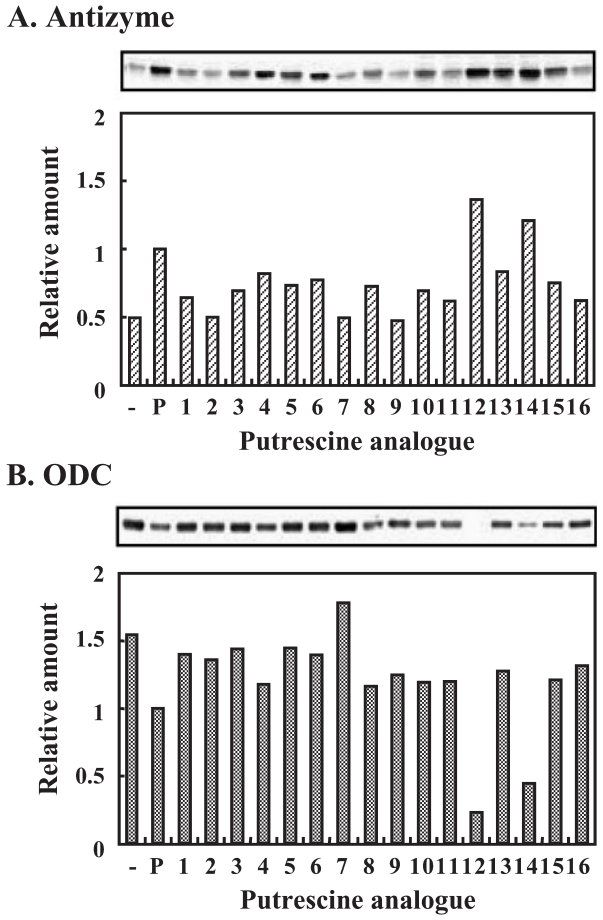

Fig. 3. **Effect of putrescine analogues on the level of antizyme (A) and ODC (B) in EXOD-1 cells.** Cells were cultured in the presence of 0.25 mM putrescine analogues and 1 mM aminoguanidine for 24 h. Western blot analysis of antizyme and ODC was performed as described in "MATERIALS AND METHODS." The numbers on the horizontal axis are those of the analogues shown in Fig. 1. Experiments were repeated twice with reproducible results.

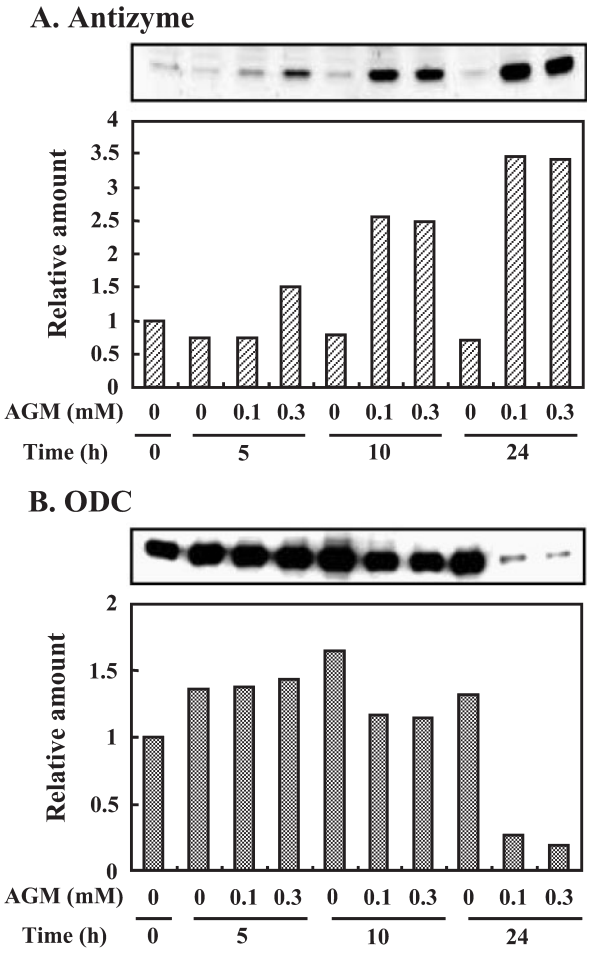

Fig. 4. **Levels of antizyme (A) and ODC (B) in EXOD-1 cells treated with agmatine.** Cells were cultured in the presence of various concentrations of agmatine for the times shown in the figure. Western blot analysis of antizyme and ODC was performed as described in "MATERIALS AND METHODS." Experiments were repeated twice with reproducible results.

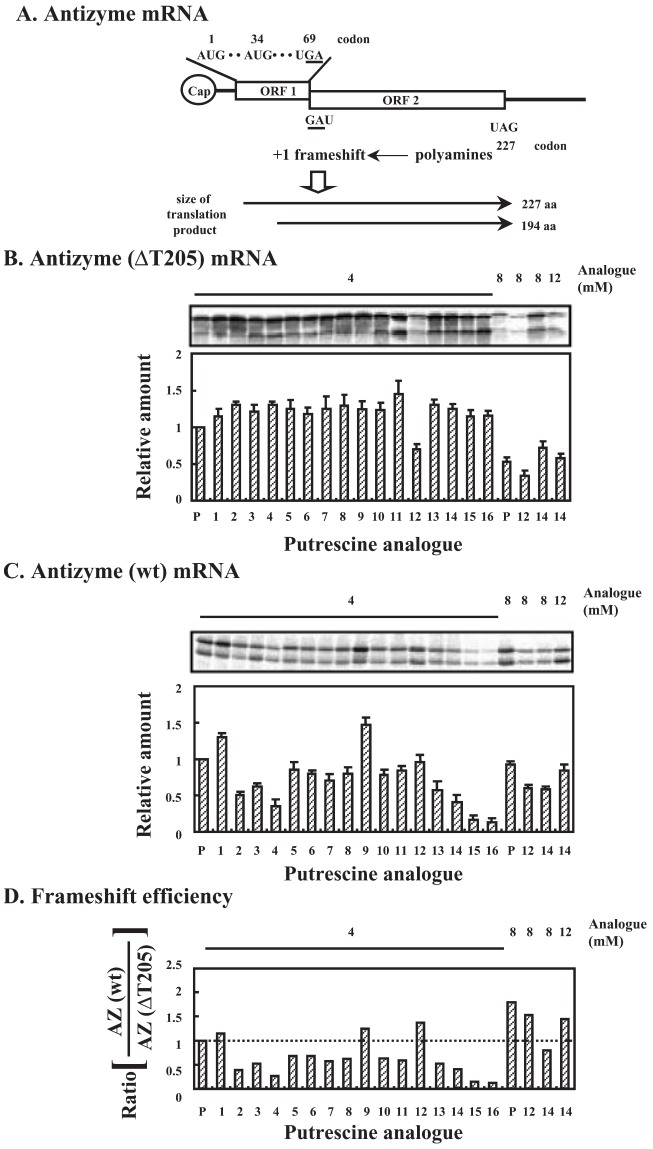

Fig. 5. **Effect of putrescine analogues on frameshift of antizyme mRNA in a rabbit reticulocyte cell-free system.** (A) Schematic of antizyme mRNA and its translational product. (B) Antizyme synthesis without frameshift was performed using AZ (ΔT205) mRNA. The amount of antizyme synthesized in the presence of 4, 8 or 12 mM putrescine or putrescine analogues was estimated by fluorography and expressed relative to the amount synthesized in the presence of 4 mM putrescine. (C) Antizyme synthesis with frameshift was performed using wild-type AZ mRNA. (D) Frameshift efficiency was determined as the ratio of C to B. The numbers on the horizontal axis are those of the analogues shown in Fig. 1. Data are means ± SE of triplicate determinations.

the level of ODC parallels the decrease in putrescine and spermidine and the inhibition of cell growth.

Efficiency of Antizyme Induction of Putrescine Analogues in a Cell-Free Protein Synthetic System—Antizyme is synthesized by frameshifting, and the frameshift is stimulated by polyamines (*14*). We thus determined whether putrescine analogues alter frameshift efficiency by comparing antizyme synthesis from wild-type and ΔT205 antizyme mRNA in a rabbit reticulocyte cell-free

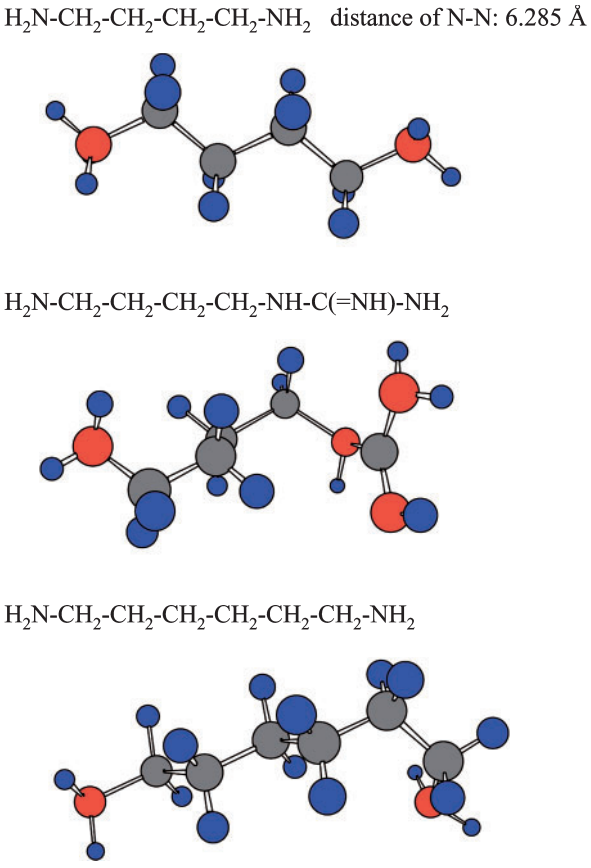

Fig. 6. **Stereochemical structure of putrescine, agmatine and 1,6-hexanediamine.** Stereochemical structure was calculated using MOPAC (AM1). Gray circles, carbon atoms; red circles, nitrogen atoms; blue circles, hydrogen atoms.

system. The wild-type mRNA requires a frameshift to synthesize antizyme (Fig. 5C), whereas the ΔT205 mRNA does not require a frameshift (Fig. 5B). The frameshift efficiency was estimated as the ratio of antizyme synthesized from wild-type mRNA to that from ΔT205 mRNA (Fig. 5D). Two initiation codon AUGs are present in antizyme mRNA, so two kinds of antizyme, consisting of 227 and 194 amino acid residues, are synthesized (Fig. 5A). Antizyme synthesis was estimated from the total amount of both kinds of antizyme.

An increase in frameshift efficiency by analogues at 4 mM compared with putrescine was observed with 1,3-propanediamine [1], *cis*-1,4-cyclohexanediamine [9], and agmatine [12], but not with 1,6-hexanediamine [14]. These results are not what one would expect based on data from the cell culture system, in which compounds [1] and [9] were inactive and compounds [12] and [14] reduced cell growth. For 1,3-propanediamine and *cis*-1,4-cyclohexanediamine, the amounts accumulated in cells were very low (Table 1), suggesting that these two analogues are not taken up well, which explains their lack of effect in the cell culture system. On the contrary, large amounts of 1,6-hexanediamine accumulated in cells (Table 1). Thus, effect of 8 and 12 mM 1,6-hexanediamine on the frameshift was examined, and the frameshift efficiency was found to increase with both concentrations

(Fig. 5D). The results suggest that, like putrescine, 1,6-hexanediamine is efficiently taken up by cells and causes the frameshift of antizyme mRNA.

DISCUSSION

We have previously shown that antizyme possesses antitumor activity *in vivo,* using nude mice inoculated with H-*ras* transformed NIH3T3 cells (*32*). In this study, we looked for diamines that inhibit cell growth through antizyme induction, because the diamine agmatine is known to inhibit cell growth by this mechanism (*15*). Our results indicate that agmatine is the strongest inducer of antizyme among 16 putrescine analogues tested.

There may be a stricter structural requirement for antizyme frameshift than stimulation of protein synthesis, because *N*-(3-aminopropyl)-*n*-propylamine [2], *N*-(3-aminopropyl)-*n*-butylamine [3], *N*-ethylputrescine [4] and cadaverine [13] did not influence the frameshift (Fig. 5C) but stimulated antizyme synthesis without the frameshift (Fig. 5B). In the case of agmatine, the degree of stimulation of antizyme synthesis from ΔT205 antizyme mRNA was less than that with putrescine (Fig. 5B), suggesting that it does not efficiently stimulate general protein synthesis. This was confirmed with globin synthesis (data not shown). Thus, the frameshift efficiency of agmatine was higher than that of putrescine (Fig. 5D). This is also the case in *Escherichia coli*, since agmatine stimulated the growth of a polyamine-requiring mutant (*33*) to a lesser degree than putrescine (data not shown). The degree of stimulation of protein synthesis by 1,6-hexanediamine at 12 mM was also less than that of putrescine at 4 mM, and the frameshift efficiency became more than 1.0. Therefore, agmatine and high concentrations of 1,6-hexanediamine inhibit cell growth through induction of antizyme.

It should be noted that six methyl (or amino) groups are present between the two amino groups in agmatine and 1,6-hexanediamine. The frameshift effects of 1,3-propanediamine and *cis*-1,4-cyclohexanediamine *in vitro* suggest that a distance of four methyl groups or shorter between the two amino groups has maximal effect on the frameshift. If agmatine and 1,6-hexanediamine are in a non-extended form, the distance between the two amino groups of these compounds may become close to that of putrescine (Fig. 6). Such a bent form may not stimulate protein synthesis effectively. The bent form of agmatine is slightly more stable than the linear form, and the bent form of 1,6-hexandiamine is slightly less stable than the linear form. Thus, the presence of two amines separated by an appropriate distance is important for the enhancement of frameshifting by diamines (Fig. 6).

It has been also reported that agmatine induces SSAT (*29*, *30*). We confirmed the induction of SSAT by agmatine. 1,6-Hexanediamine also induced SSAT significantly. So, the induction of SSAT, together with the induction of antizyme, is probably involved in the inhibition of cell growth through conversion of spermine to spermidine and spermidine to putrescine.

Inhibition of protein synthesis by agmatine or 1,6-hexanediamine may contribute to the inhibition of cell growth together with the decrease in polyamine content caused by the induction of antizyme and SSAT, because a large amount of agmatine or 1,6-hexanediamine accumulated in cells after 24 h. The concentration of agmatine and 1,6-hexanedimanine was 11.0 and 17.5 mM, respectively, assuming that 1 mg of cellular protein corresponds to 5 μl of cell volume (*34*), and these concentrations would inhibit general protein synthesis judging from the data obtained using AZ (ΔT205) mRNA.

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