Studies of non-metabolizable polyamines that support growth of SV-3T3 cells depleted of natural polyamines by exposure to α -difluoromethylornithine

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A number of synthetic polyamine derivatives that included five achiral *gem*-dimethylspermidines and two analogous tetramethylated spermines were tested for their abilities to serve as substrates for enzymes metabolizing polyamines and for their capacities to substitute for the natural polyamines in cell growth. It was found that none of the compounds were effective substrates for spermine synthase, and only one, namely 8,8-dimethylspermidine, was a substrate for spermidine/spermine *N*¹-acetyltransferase. However, all of the spermidine derivatives and 1,1,12,12-tetramethylspermine were able to support the growth of SV-3T3 cells in which endogenous polyamine synthesis was prevented by the addition of α -difluoromethylornithine. These results suggest that either spermidine or spermine can support cell growth without the need for metabolic interconversion. In contrast with the result with 1,1,12,12-tetramethylspermine, 3,3,10,10-tetramethylspermine did not restore growth of polyamine-depleted SV-3T3 cells. Comparison of the properties of these derivatives may prove valuable in understanding the physiological role of polyamines.

INTRODUCTION

Naturally occurring polyamines play many metabolic roles in cellular physiology and are required for the normal growth and development of mammalian cells (Jänne *et al.*, 1983; Tabor & Tabor, 1984; Pegg, 1986). The enzymes of polyamine biosynthesis and interconversion work together to adjust levels of these compounds by complex regulatory processes involving changes in the rate of enzyme synthesis, turnover and possibly post-translational modification (Kay & Lindsay, 1973; Morris & Fillingame, 1974; Clark & Fuller, 1975; Canellakis & Theoharides, 1976; Kallio *et al.*, 1977; McCann *et al.*, 1979; Atmar & Kuehn, 1981; Russell, 1981). However, specific functions of individual polyamines have been difficult to evaluate. Several experiments have shown that cells that are unable to synthesize putrescine or spermidine, either because they lack the enzyme ornithine decarboxylase (ODC) or because they have been treated with an inhibitor of ODC, reach a quiescent (i.e. non-growing) state as putrescine and spermidine levels become depleted. Cell growth does not resume unless essential polyamines are provided (Steglich & Scheffler, 1982; Pegg & McCann, 1982; Porter & Bergeron, 1983; Pohjanpelto *et al.*, 1985; Pegg, 1984, 1986).

The importance of the tetramine spermine in mammalian cell growth remains somewhat ambiguous. ODC inhibitors like α -difluoromethylornithine (DFMO) and *E*-2-(fluoromethyl)dehydro-ornithine (Mamont *et al.*, 1986) do not greatly affect spermine content (Pegg, 1984, 1986). It has been suggested that low residual levels of this polyamine may help keep spermidine depleted cells in a viable, though non-growing state (Pegg *et al.*, 1982;

Casero *et al.*, 1984; McGovern *et al.*, 1986). However, when exogenous spermine is added to DFMO-treated cells, a normal rate of growth is restored (Mamont *et al.*, 1980; Porter & Bergeron, 1983; Pegg, 1984). Under these conditions, only a small amount of spermidine (at most 10% of normal levels) is produced from spermine by the well-known pathway involving *N*-acetylation and oxidation. If all growth-related functions of polyamines are satisfied by spermine, as these experiments imply, it is unclear why growth is arrested in DFMO-treated cells that still contain significant amounts of spermine. It is also intriguing to ask why mammalian cells biosynthesize spermine at all, since spermidine can restore full growth to depleted cells, and selective depletion of spermine using inhibitors of spermine synthase does not diminish the growth of cultured fibroblasts (Pegg *et al.*, 1986). One very simple explanation might be that depleted cells, when supplemented with either spermidine or spermine, derive the missing polyamine by biosynthesis or interconversion pathways, and perhaps some minimal levels of each are required for mammalian cell growth.

In order to differentiate the roles of individual polyamines, we have designed a new family of spermidine and spermine analogues that fulfill all the requirements for cell growth without being susceptible to the enzymes that interconvert the parent polyamines. Those of us at Cornell University (Nagarajan & Ganem, 1986) recently reported the synthesis of five achiral *gem*-dimethylspermidines (compounds 1–5, Fig. 1) and two analogous tetramethylated spermines (compounds 6–7, Fig. 1) as stable hydrochloride salts. These ‘non-metabolizable’ polyamines were specifically composed to resist or inhibit the action of flavin-dependent polyamine oxidase (PAO), and thus to serve as useful probes of individual polyamine

Abbreviations used: AdoMet, *S*-adenosylmethionine; ODC, L-ornithine decarboxylase; DFMO, α -DL-difluoromethylornithine; PAO, polyamine oxidase; SAT, spermidine/spermine *N*¹-acetyltransferase.

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Fig. 1. Structures of the polyamine derivatives 1–7

- 1, 1,1-Dimethylspermidine; 2, 2,2-dimethylspermidine;
3, 3,3-dimethylspermidine; 4, 5,5-dimethylspermidine;
5, 8,8-dimethylspermidine; 6, 1,1,12,12-tetramethylspermine;
7, 3,3,10,10-tetramethylspermine.

function. We also tested compounds 1–7 for their effects on two other key enzymes involved in polyamine interconversion: spermine synthase (Raina *et al.*, 1983), which catalyses the transfer of an aminopropyl group from decarboxylated *S*-adenosylmethionine (AdoMet) to spermidine, and spermidine/spermine *N*¹-acetyltransferase (SAT) (Della Ragione & Pegg, 1982). SAT catalyses terminal *N*-acetylation of spermidine and spermine, which is an essential intermediate step in the oxidative pathway to converting them into putrescine and spermidine respectively. The effects of compounds 1–7 on the growth of cultured mammalian cells (SV40-virus-transformed mouse fibroblasts), in both the absence and presence of DFMO, were also examined. In the latter experiments, several of these compounds added at 10 μ M were able to override the normal growth inhibition brought about by polyamine depletion. Results with compound 6 provide clear evidence that exogenous spermine does not need to be converted into spermidine in order to support growth.

MATERIALS AND METHODS

Polyamines

The compounds tested are shown in Fig. 1 and were prepared as recently described (Nagarajan & Ganem, 1986).

Other materials

DFMO was generously provided by Merrell–Dow Research Institute, Cincinnati, OH, U.S.A. Radiochemicals were obtained from du Pont–New England Nuclear Products, Boston, MA, U.S.A. Biochemical reagents and polyamines were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Assay of rat prostate spermine synthase

Rat prostate spermine synthase was isolated as previously described (Raina *et al.*, 1983; Pegg *et al.*, 1986). The activity of this aminopropyltransferase was determined by measuring the production of 5'-methyl-[³⁵S]thioadenosine from decarboxylated Ado[³⁵S]Met (Hibasami & Pegg, 1978). Activity was measured using spermidine as a control and in the presence of compounds 1–7. The labelled product was separated on small columns of cellulose phosphate as described by Raina *et al.* (1983). The standard assay medium contained 5 μ M-³⁵S-labelled decarboxylated AdoMet (0.1–0.25 μ Ci), 0.5 mM-spermidine, 50 mM-sodium phosphate buffer, pH 7.2, and 0.1 mM-dithiothreitol in a total volume of 0.2 ml. Sufficient enzyme was added to ensure that about 7000 c.p.m. of radioactivity was incorporated into product during a 30 min incubation at 37 °C with spermidine as acceptor.

Assay of rat liver SAT

SAT was purified from livers of rats treated with carbon tetrachloride (Della Ragione & Pegg, 1982). Activity was assayed as described by Della Ragione & Pegg (1983) using either spermidine or the compounds 1–7 as acceptors. The standard assay medium used contained 8 μ M-acetyl-CoA in a total volume of 0.1 ml.

Cell culture and polyamine analysis

SV-3T3 cells were maintained and transferred as described by Pegg (1984). They were plated at a density of 0.2×10^6 per dish in Dulbecco's modified Eagle's medium in the presence of 3% (v/v) horse serum/2% (v/v) fetal-calf serum and 1 mM-aminoguanidine. In some experiments, 5 mM-DFMO was added at the time of plating. Compounds 1–7 were added at the time and concentration shown in the legends.

Intracellular polyamine concentrations were determined by a published method involving extractions of the cell pellets with 10% (w/v) HClO₄, separation of the polyamines by ion-pair reversed-phase h.p.l.c. and detection and quantification after post-column derivatization with *o*-phthalaldehyde (Seiler & Knödgen, 1985).

RESULTS

Table 1 summarizes experiments to test whether compounds 1–7 can be acetylated by SAT at three different concentrations. SAT acetylates spermidine exclusively at the *N*¹ position (Della Ragione & Pegg, 1982, 1983). Only spermidine analogue 5, whose *gem*-

Table 1. Ability of compounds 1–7 to act as substrates for spermidine/spermine-*N*¹-acetyltransferase

The compounds shown were tested as substrates in standard assay at 0.1 mM-, 1 mM- and 10 mM- concentrations. Results are expressed as the percentage of the activity found when 10 mM-spermidine was used as substrate. Putrescine which is known not to be a substrate for SAT (Della Ragione & Pegg, 1983) was used as a negative control.

Putative substrate	[Substrate] (mM)...	Activity (%)		
		0.1	1	10
Spermidine		17	66	100
1		2	3	6
2		2	3	9
3		2	2	2
4		2	1	2
5		15	64	116
6		2	2	< 1
7		1	1	< 1
Putrescine		2	2	4

dimethyl substituents are most removed from the locus of acetylation, functioned as a substrate for this enzyme. Acetylation of 5 by SAT proceeded as efficiently as with the normal substrate, spermidine.

Compounds 1–7 were tested both as potential inhibitors of spermidine synthase and as substrates for this reaction (Table 2). All of the compounds proved to be only weak inhibitors of spermidine synthase when this was examined with spermidine as substrate (Table 2). They were therefore tested as substrates for the aminopropyl-transfer reaction catalysed by this enzyme. Significant production of 5'-methylthioadenosine occurred only when the compounds were added at 5 mM concentration, and even this value was less than 15% of the rate obtained with spermidine. Since the K_m value of spermidine for the spermine synthase reaction is in the range of 60–100 μ M for the rat prostate enzyme (Pajula *et al.*, 1979; Pegg, 1983), we conclude that none of the methylated polyamines is a good substrate at physiologically relevant concentrations. These data are in agreement with earlier observations that no amine acceptor other than spermidine served as an efficient substrate for mammalian spermine synthase (Hibasami & Pegg, 1978; Pajula *et al.*, 1979; Pegg, 1983). Even when presented with polyamines 1–5 containing the *bona fide* spermidine framework, the enzyme appears to be extremely sensitive to steric hindrance.

The *gem*-dimethyl polyamine derivatives did not affect the growth of SV-3T3 transformed mouse fibroblasts when added to the culture medium. A representative experiment using compounds 1–3 for up to 6 days is shown in Table 3. It can be seen that there was no effect on the cell number when a 10 μ M concentration of the compounds or spermidine were added at 4 h after plating and the cells were counted at 72 h. The cells were then replated in the presence of the compounds, and there was no effect on growth for a further 72 h, although the presence of compounds 1–3 or spermidine may have diminished the plating efficiency.

In order to determine whether compounds 1–7 could

Table 2. Ability of compounds 1–7 to act as inhibitors and substrates for spermine synthase**(a) Effect of compounds as inhibitors of spermine synthase**

The compounds were tested as inhibitors, added at the concentration shown, with 0.1 mM-spermidine as substrate in standard assay.

Inhibitor	Concn. (mM)...	Inhibition (%)	
		1	5
None		0	0
1		25	78
2		49	87
3		16	65
4		21	64
5		24	83
6		46	91
7		18	74

(b) Effect of compounds as substrates of spermine synthase

The compounds were tested as substrates in standard assay at 0.05, 0.5 and 5 mM concentrations. Results are expressed as the percentage of the activity found when 5 mM-spermidine was used as substrate.

Putative substrate	[Substrate] (mM)...	Activity (%)		
		0.05	0.5	5
Spermidine		45	95	100
1		< 1	2	7
2		< 1	2	7
3		< 1	3	14
4		< 1	4	10
5		< 1	< 1	13
6		< 1	< 1	4
7		< 1	< 1	5

support growth of cells deprived of physiological polyamines, they were added to SV-3T3 cells treated with the ODC inhibitor DFMO. As previously reported (Mamont *et al.*, 1980; Pegg & McCann, 1982; Pegg, 1986), the depletion of spermidine brought about by this inhibitor led to great decrease in the rate of cell growth. This growth arrest was reversed by all of the compounds except 7 (Table 4). Exogenous spermidine, which was used as a control, seemed to give slightly better results, particularly when only 1 μ M concentrations were compared, but a strong resumption of growth was produced by 10 μ M concentrations of compounds 1–6. These experiments indicate that the presence of *gem*-dimethyl substituents does not prevent analogues 1–6 from performing all the functions of polyamines needed for cell growth.

In order to examine the extent of uptake of the *gem*-dimethyl polyamines and their effect on the cellular spermine content, DFMO-treated cells were grown in the presence of compounds 1, 2, 3 and 5 for 72 h, and their intracellular polyamine concentrations were determined (Table 5). The compounds accumulated to intracellular concentrations which were about the same as the normal

Table 3. Effect of compounds 1–3 on growth of SV-3T3 cells

The cells were plated at a density of 0.2×10^6 per dish in 35 mm-diameter dishes in the presence of 3% (v/v) horse serum/2% (v/v) fetal-bovine serum and Dulbecco's modified Eagle medium. After 6 h, the compounds shown were added at $10 \mu\text{M}$. The cell number was determined at 72 h and then the cells were re-plated at the original starting density (0.2×10^6 /dish) in the continued presence of the compound, and the cell numbers were determined as shown. Results are means $\pm$ S.D. for at least four estimations.

Compound	Time (h)...	$10^{-6} \times \text{Cell no.}$			
		72	96	120	144
Control		3.0 ± 0.09	0.49 ± 0.02	1.70 ± 0.09	2.93 ± 0.08
$10 \mu\text{M}$ -1		2.96 ± 0.11	0.32 ± 0.01	1.35 ± 0.07	2.88 ± 0.17
$10 \mu\text{M}$ -2		3.12 ± 0.13	0.26 ± 0.01	1.09 ± 0.03	2.62 ± 0.10
$10 \mu\text{M}$ -3		3.28 ± 0.02	0.45 ± 0.03	1.66 ± 0.05	2.85 ± 0.05
$10 \mu\text{M}$ -Spermidine		2.89 ± 0.14	0.24 ± 0.02	1.12 ± 0.07	2.69 ± 0.08

Table 4. Effect of compounds 1–7 on growth of SV-3T3 cells in the presence of DFMO

The cells were plated at a density of 0.2×10^6 per dish in 35 mm-diameter dishes in the presence of 3% (v/v) horse serum/2% (v/v) fetal-bovine serum and Dulbecco's modified Eagle medium with or without 5 mM-DFMO. After 72 h, the control cell number was $2.05 \pm 0.13 \times 10^6$ per dish. When cells were plated in the presence of 5 mM DFMO the cell number was $0.58 (\pm 0.04) \times 10^6$ per dish. The compounds shown were added at $1 \mu\text{M}$ or $10 \mu\text{M}$ as indicated to dishes of the cells grown in DFMO, and the cell number counted at the times shown. Results shown are means $\pm$ S.D. for at least four estimations. Abbreviation: N.M., not measured.

Treatment	Time (h)...	$10^{-6} \times \text{Cell no.}$			
		24	48		96
	Concn. (μM)...	10	10	1	10
DFMO (alone)		0.68 ± 0.04	0.80 ± 0.05	0.80 ± 0.05	0.79 ± 0.05
+1		1.19 ± 0.09	1.79 ± 0.31	1.19 ± 0.04	2.04 ± 0.31
+2		1.14 ± 0.07	2.34 ± 0.18	1.15 ± 0.13	2.52 ± 0.32
+3		N.M.	1.75 ± 0.29	1.13 ± 0.15	2.34 ± 0.24
+4		1.05 ± 0.04	1.75 ± 0.03	0.99 ± 0.05	2.34 ± 0.29
+5		1.31 ± 0.13	2.07 ± 0.21	1.16 ± 0.03	2.66 ± 0.32
+6		1.32 ± 0.11	2.03 ± 0.08	1.17 ± 0.16	2.44 ± 0.16
+7		0.77 ± 0.06	0.94 ± 0.03	0.75 ± 0.02	0.96 ± 0.13
+Spermidine		1.49 ± 0.08	2.73 ± 0.25	1.70 ± 0.11	2.87 ± 0.24

Table 5. Effect of compounds 1, 2, 3 and 5 on polyamine content of SV-3T3 cells in the presence of DFMO

The cells were plated at 0.24×10^6 cells per dish in 60 mm-diameter dishes and grown for 72 h in the presence of 5 mM-DFMO, 3% (v/v) horse serum/2% (v/v) fetal-bovine serum and Dulbecco's modified Eagle medium. The drugs shown were then added at $10 \mu\text{M}$. Only the control cells (not treated with DFMO) contained putrescine (4.1 nmol/mg of protein). Results shown are means $\pm$ S.D. for at least four estimations.

Addition	$10^{-6} \times \text{Cell no.}$	Concn. (nmol/mg of protein)		
		Spermidine	Compound	Spermine
None	1.6 ± 0.1	1.3 ± 0.4	*	14.8 ± 3.1
$10 \mu\text{M}$ -1	4.8 ± 0.2	0.9 ± 0.2	23.3 ± 1.4	4.1 ± 0.2
$10 \mu\text{M}$ -2	4.6 ± 0.4	0.3 ± 0.1	20.4 ± 0.5	2.8 ± 0.3
$10 \mu\text{M}$ -3	4.6 ± 0.2	0.6 ± 0.2	19.1 ± 2.5	3.7 ± 0.4
$10 \mu\text{M}$ -5	5.1 ± 0.1	0.3 ± 0.1	16.6 ± 2.4	2.6 ± 0.2
$10 \mu\text{M}$ -Spermidine	5.1 ± 0.3	11.2 ± 2.3	*	11.5 ± 1.5
Control (not treated with DFMO)	5.2 ± 0.3	24.3 ± 1.0	*	9.5 ± 1.2

* No synthetic analogues were present in these cells.

cellular spermidine content. The cellular spermine content, which was not diminished by DFMO as previously reported (Mamont *et al.*, 1980; Pegg & McCann, 1982; Pegg, 1986), was depleted by the addition of the compounds, particularly **2** and **5**. This diminution probably reflects both the competitive inhibition of spermine synthase and the growth of the cells, which increases the amount of cellular protein over a period during which endogenous polyamine synthesis is blocked by DFMO. There was no indication that compounds **1**, **2**, **3** and **5** were metabolized to other polyamines, since no other polyamine peaks were found in the h.p.l.c. chromatograms from these cells. This is consistent with the results in Tables 1 and 2.

DISCUSSION

In contrast with the variety of inhibitors now available for studying polyamine biosynthesis, few if any structural analogues have been designed as probes for the reactions which utilize polyamines. *N*-Acetylation, oxidative cleavage and glutaminase-catalysed protein binding are all of considerable importance in polyamine metabolism. Acetylation can be catalysed either by a nuclear enzyme, which is probably also a histone acetylase, or by the cytosolic protein SAT. These enzymes exhibit different substrate specificity (Della Ragione & Pegg, 1983, 1984) and respond differently to inhibitors (Erwin *et al.*, 1984). The significance of acetylation by the nuclear enzyme is unclear, since the only known fate of its product, *N*⁸-acetylspermidine, is deacetylation (Blankenship & Marchant, 1984; Della Ragione & Pegg, 1984). However, SAT may play an important role in regulating polyamine levels and in mediating hormonally induced changes in polyamine content (Della Ragione & Pegg, 1984; Seiler, 1987). For example, SAT is stimulated by $1\alpha,25$ -dihydroxyvitamin D₃ as an early event in the vitamin D-induced biosynthesis of putrescine from higher polyamines through the action of polyamine oxidase. The resulting elevated levels of putrescine in vitamin D target tissues may be related to cell growth and/or differentiation (Shinki *et al.*, 1985, 1986). Detailed studies of the specificity, kinetics and active site of SAT have been carried out on homogeneous preparations of SAT (Della Ragione & Pegg, 1983, 1984). The enzyme has no effect on histones and only acetylates primary amine groups when present as R-NH-[CH₂]₃-NH₂. It therefore converts spermidine exclusively into *N*¹-acetylspermidine, does not acetylate *sym*-homospermidine and readily accepts *sym*-norspermidine as a substrate. Consistent with these results, SAT acetylated **5**, which contains the required aminopropyl group, but had no effect on spermidine analogues **1–3** and spermine analogues **6–7**, which contain branched aminopropyl appendages. However, the enzyme also failed to process **4**, which does possess the requisite structural feature for acetylation. This finding suggests that, besides being able to discriminate among primary amine groups in polyamines, SAT is also quite sensitive to steric effects in candidate substrates.

Unlike bacteria and many lower eukaryotes, mammalian cells produce a separate polyamine aminopropyltransferase (spermine synthase) that converts spermidine into spermine. Although the specific function of spermine remains unknown, it is clearly not essential for life. Some organisms like *Escherichia coli* or *Trypanosoma brucei brucei* do not normally produce

spermine, but will do so by using endogenous spermidine synthase under exceptional circumstances (Pegg & McCann, 1982; Tabor & Tabor, 1984; Pegg, 1986). The design of new methods to regulate levels of specific polyamines would be of value in determining what part spermine plays in mammalian cell growth and differentiation. Several 5'-substituted adenosine derivatives have been reported which are inhibitors of spermidine spermine synthase. Although these aminopropyltransferase inhibitors drastically diminish the production of the corresponding polyamine, they have little effect in lowering total net polyamine (spermidine + spermine) content (Pegg *et al.*, 1982, 1986).

A number of halogenated, hydroxylated and methylated putrescines have recently been studied *in vitro* and *in vivo* as potential substrates for spermidine synthase (Sarhan *et al.*, 1987a). Interestingly, 1-methylputrescine was a poor substrate for this transferase, whereas 1,4-dimethylbutane-1,4-diamine formed none of the corresponding spermidine derivatives. Metabolism of 1-methylputrescine *in vivo* led only to the formation of 8-methylspermidine and none of the corresponding spermine, presumably because the second aminopropylation was sterically hindered by the nearby methyl group. However substitution at C-2 of putrescine was relatively well tolerated: 2-methylputrescine was as good as putrescine itself in spermidine synthase assays. Our present findings that spermidine analogues **1–5** are not substrates for mammalian spermine synthase (Table 2) are consistent with this structure-activity profile. Apparently one, but not two (*geminal*), branch-points can be tolerated by the polyamine aminopropyltransferases. Only 2,2-difluoroputrescine was metabolized by chick embryos to the expected difluorospermidine and spermine analogues (Sarhan *et al.*, 1987b). The ability of substituted putrescine derivatives to support growth has been examined in DFMO-treated chick embryos (Sarhan *et al.*, 1987a). Both 1-methyl- and 1,4-dimethyl-putrescine restored growth (judged by weight gain) as well as did putrescine itself, but were less effective in ensuring long-term survival. It was concluded that only those diamines capable of forming significant amounts of spermidine and spermine analogues could help chick embryos survive.

Consistent with these findings, spermidine analogues **1–5** successfully revitalized quiescent DFMO-treated polyamine-depleted SV-3T3 cells. Since these compounds were not substrates for SAT or spermine synthase, our results further demonstrated that the biosynthesis of spermine was not required to support growth in this system. However, similar results with spermine analogue **6**, which cannot be converted into the spermidine derivative, provides clear evidence that spermine need not be degraded to spermidine for cell growth to resume in DFMO-treated cells.

Taken as a whole, these results imply that either spermidine or spermine alone is capable of supporting the growth of cultured SV-3T3 fibroblasts. This growth-promoting function can be fulfilled by the *gem*-dimethyl derivatives of spermidine and spermine shown in Fig. 1, except for **7**. Why quiescent cells fail to grow in the presence of exogenous **7** is not clear at this time. It remains to be determined how structural differences between isomers **6** and **7** affect their function as polyamines.

It should be noted that the physiological effects of the

gem-dimethyl polyamines as demonstrated in our experiments differ greatly from those of the *N*-substituted derivatives. Those derivatives, particularly the bis(ethyl) compounds, are unable to support cell growth and can exert profound inhibitory effects on polyamine biosynthesis by the negative regulation of ODC and AdoMet decarboxylase (Porter *et al.*, 1985, 1986; 1987*a,b*). The depletion of natural polyamines brought about by these compounds therefore leads to a cessation of cell growth.

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