

Inhibition of cell growth through inactivation of eukaryotic translation initiation factor 5A (eIF5A) by deoxyspergualin

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The mechanism of inhibition of cell growth by deoxyspergualin was studied using mouse mammary carcinoma FM3A cells. Results of studies using deoxyspergualin analogues showed that both the guanidinoheptanate amide and glyoxyspermidine moieties of deoxyspergualin were necessary to cause inhibition of cell growth. When deoxyspergualin was added to the medium, there was a strong inhibition of cell growth and formation of active eukaryotic translation initiation factor 5A (eIF5A) at the third day of culture. There was also a marked decrease in cellular putrescine content and a small decrease in spermidine content. Accumulation of decapped mRNA, which is typically associated

with eIF5A deficiency in yeast, was also observed. The inhibition of cell growth and the formation of active eIF5A was not reversed by addition of spermidine. The activity of deoxyhypusine synthase, the first enzyme in the formation of active eIF5A, was inhibited by deoxyspergualin in a cell-free system. These results, taken together, indicate that inhibition of active eIF5A formation is strongly involved in the inhibition of cell growth by deoxyspergualin.

Key words: spermidine, hypusine, FM3A cells.

INTRODUCTION

Deoxyspergualin, a synthetic derivative of spergualin produced by *Bacillus laterosporus*, and its analogues were first developed as anti-proliferative reagents [1–3] and have been used as immunosuppressants [4–6]. The immunosuppressant effect of deoxyspergualin is thought to involve binding to heat-shock cognate 70 ('Hsc70'), a member of the heat-shock protein 70 ('Hsp70') family of heat shock proteins, which disturbs the nuclear transport of nuclear factor κ B ('NF- κ B'), an important transcriptional factor for the immune response [7–9]. Since nuclear factor κ B is related to the expression of many genes, it is expected that the disturbance of its nuclear transport by deoxyspergualin is involved in the anti-proliferative effects of the deoxyspergualin.

It has been reported that the spermidine moiety in deoxyspergualin is absolutely required for its anti-proliferative activity [10], and that deoxyspergualin inhibits cell growth through inhibition of polyamine biosynthesis [11]. However, in a previous study [11], relatively high concentrations of deoxyspergualin were used to show inhibition of polyamine biosynthesis. It is possible that high concentrations of deoxyspergualin cause non-specific effects in addition to its main effect. Therefore we re-examined the effect of deoxyspergualin on polyamine biosynthesis and active eukaryotic translation initiation factor 5A (eIF5A) formation using relatively low concentrations of deoxyspergualin.

Active eIF5A contains hypusine [*N*^ε-(4-amino-2-hydroxybutyl)lysine], which is derived from spermidine, and is essential for cell growth in eukaryotes, although the function of active eIF5A is still unclear [12–14]. Hypusine biosynthesis involves two enzyme-catalysed steps. In the first step, deoxyhypusine

synthase (DHS) catalyses the NAD-dependent transfer of the aminobutyl moiety of spermidine to the ϵ -amino group of a specific lysine residue of the eIF5A precursor protein to form the intermediate deoxyhypusine [15]. In the second step, mediated by deoxyhypusine hydroxylase, the conversion of the deoxyhypusine residue to the hypusine residue completes active eIF5A formation [15]. Our results strongly suggest that deoxyspergualin inhibits active eIF5A formation and subsequently inhibits cell growth.

MATERIALS AND METHODS

Cell culture and assays for DNA, RNA and protein synthesis

Established mouse FM3A mammary carcinoma cells (1×10^4 /ml) (Japan Health Science Foundation, Tokyo, Japan) were cultured in ES medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 50 units/ml streptomycin, 100 units/ml penicillin G and 2% (v/v) heat-inactivated fetal-calf serum at 37 °C in an atmosphere of 5% CO₂, as described by Ayusawa et al. [16]. When DNA, RNA and protein synthesis were measured, the cells were cultured for 96 h as described, collected by centrifugation, and then 3 ml cell suspensions (5×10^5 /ml) were prepared by counting the cell number. To the cell suspension, 555 kBq [³H]thymidine (248 GBq/mmol), 185 kBq [³H]uridine (1110 GBq/mmol) or 555 kBq [³H]leucine (1480 GBq/mmol) was added and incubated for 1 h. The reaction was terminated by the addition of non-labelled thymidine, uridine or leucine at a final concentration of 1 mM. The cells were lysed and treated with ice-cold 5% (v/v) trichloroacetic acid (TCA). The radioactivity of the precipitate obtained following treatment with TCA was measured as described by Seyfried and Morris [17].

Abbreviations used: AdoMetDC, S-adenosylmethionine decarboxylase; DHS, deoxyhypusine synthase; eIF5A, eukaryotic translation initiation factor 5A; FUT-175, 6-amino-2-naphthyl-4-guanidinobenzoate; GC₇, *N*¹-guanyl-1,7-diaminoheptane; N-01, 11,15-dideoxyspergualin; N-02, 11,15-dideoxy-11R-hydroxymethylspergualin; N-30, 1-amino-16-(4'-guanidine-phenyl)-11R-hydroxymethyl-4,9,11-triazahexadecan-10,13-dione; N-353, 1-amino-11R-hydroxymethyl-16-phenyl-4,9,11-triazahexadecan-10,13-dione; ODC, ornithine decarboxylase; SSAT, spermidine/spermine *N*¹-acetyltransferase; TCA, trichloroacetic acid.

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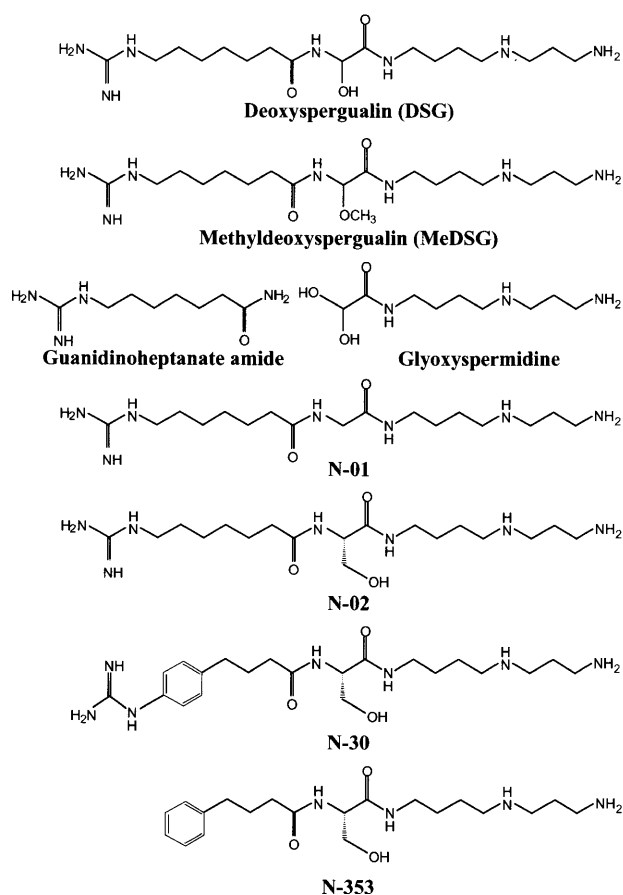


Figure 1 Structure of deoxyspergualin and its analogues

Measurement of mRNA synthesis and the amount of capped mRNA

A 10 ml cell suspension (5×10^5 /ml) was incubated at 37 °C for 1 h in the presence of 1.85 MBq [3 H]uridine (1110 GBq/mmol). Termination of the incorporation of label was achieved by addition of non-labelled uridine at a final concentration of 1 mM. mRNA was isolated using a Quick Prep Total RNA Extraction Kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) and an mRNA Purification Kit (Amersham Biosciences). Ice-cold 5% (v/v) TCA was added and the radioactivity of the precipitate obtained in the mRNA fraction was measured in a liquid-scintillation counter [17]. The amount of capped mRNA was measured similarly by labelling mRNA with 1.54 MBq [methyl- 3 H]S-adenosylmethionine (2764 GBq/mmol).

Chemicals

Deoxyspergualin, methyldeoxyspergualin, guanidinoheptanate amide, glyoxyspermidine, N-01 (11,15-dideoxyspergualin), N-02 (11,15-dideoxy-11R-hydroxymethylspergualin), N-30 [1-amino-16-(4'-guanidine-phenyl)-11R-hydroxymethyl-4,9,11-triazahexadecan-10,13-dione] and N-353 (1-amino-11R-hydroxymethyl-16-phenyl-4,9,11-triazahexadecan-10,13-dione) were synthesized as described by Maeda et al. [18]. The structure of these compounds is shown in Figure 1.

Measurement of polyamines, glyoxyspermidine and methyldeoxyspergualin

FM3A cells (6×10^6 cells) were harvested and extracted with 0.3 ml of 5% (v/v) TCA. The supernatant, obtained following centrifugation, was used for the assay of polyamines and methyldeoxyspergualin. Polyamines (putrescine, spermidine and spermine) were measured by using a TOSOH HPLC system (Tosoh Co., Tokyo, Japan) as described previously [19]. Methyldeoxyspergualin was measured using the same system as for polyamines except that the buffer used for elution contained 3 M NaCl instead of 2 M NaCl. The retention time for methyldeoxyspergualin was 16–18 min. For measurement of glyoxyspermidine in cells, amino acids were removed before the analysis by cellulose phosphate column chromatography, because glyoxyspermidine content in cells was low. The 5% TCA supernatant was neutralized with 6 M KOH and applied to a cellulose phosphate column (1 ml) equilibrated previously with 0.1 M borate/0.025 M NaCl (pH 8.0). Amino acids were eluted with 10 ml of the same solution, and glyoxyspermidine was eluted with 5 ml of 0.2 M borate/0.8 M NaCl (pH 8.0). The retention time for glyoxyspermidine was 5 min.

Assays for ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (AdoMetDC) and spermidine/spermine N^1 -acetyltransferase (SSAT)

FM3A cells (2×10^8 cells) were suspended in 0.8 ml of buffer A [10 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol, 10% (v/v) glycerol, 1 mM EDTA and 20 μ M 6-amino-2-naphthyl-4-guanidinobenzoate (FUT-175, a protease inhibitor [20]). FUT-175 was kindly supplied by Torii Pharmaceutical Co. (Tokyo, Japan). Cells were frozen, thawed, and homogenized with a Teflon homogenizer. The homogenate was centrifuged at 12000 g for 10 min at 4 °C. The supernatant was dialysed against buffer A and used for the enzyme assays. Assays of ODC, AdoMetDC and SSAT were performed as described previously [21,22], with some modifications [23]. Protein concentration was determined by the method of Lowry et al. [24].

Northern-blot analysis of ODC and AdoMetDC mRNA

Total RNA was isolated from 2.5×10^7 cells using the Quick Prep Total RNA Extraction Kit (Amersham Biosciences). Northern-blot analysis of ODC and AdoMetDC mRNA was performed as described previously [25,26], using 10 μ g of total RNA and 32 P-labelled ODC and AdoMetDC cDNA probes.

Purification of eIF5A and preparation of an antibody against eIF5A

The plasmid pBKS(–)-5A, encoding human eIF5A, was kindly supplied by Dr J. W. B. Hershey (School of Medicine, University of California, Davis, CA, U.S.A.) [27]. The eIF5A cDNA was amplified from pBKS(–)-5A by PCR using the nucleotides 5'-GAAGCCTCTTCATATGGCAGATG-3' and 5'-AGAGACTGCAGGTTTCAGAGGATC-3', containing *Nde*I and *Pst*I restriction sites respectively. The *Nde*I–*Pst*I fragment of eIF5A cDNA was inserted into the *Nde*I–*Pst*I site of pT7-7 [28], and the plasmid was transformed into *Escherichia coli* BL21 (DE3). The *E. coli* cells BL21 (DE3)/pT7-7eIF5A were grown at 37 °C in Luria–Bertani broth containing 100 μ g/ml ampicillin. At $A_{600} = 0.5$, eIF5A was induced with 1 mM isopropyl β -D-thiogalactoside for 3 h. Purification of eIF5A was performed by the method of Kim et al. [29]. Since hypusine formation does not occur in *E. coli*, purified eIF5A is termed precursor eIF5A. An antibody against eIF5A was prepared by injecting 1 mg of precursor

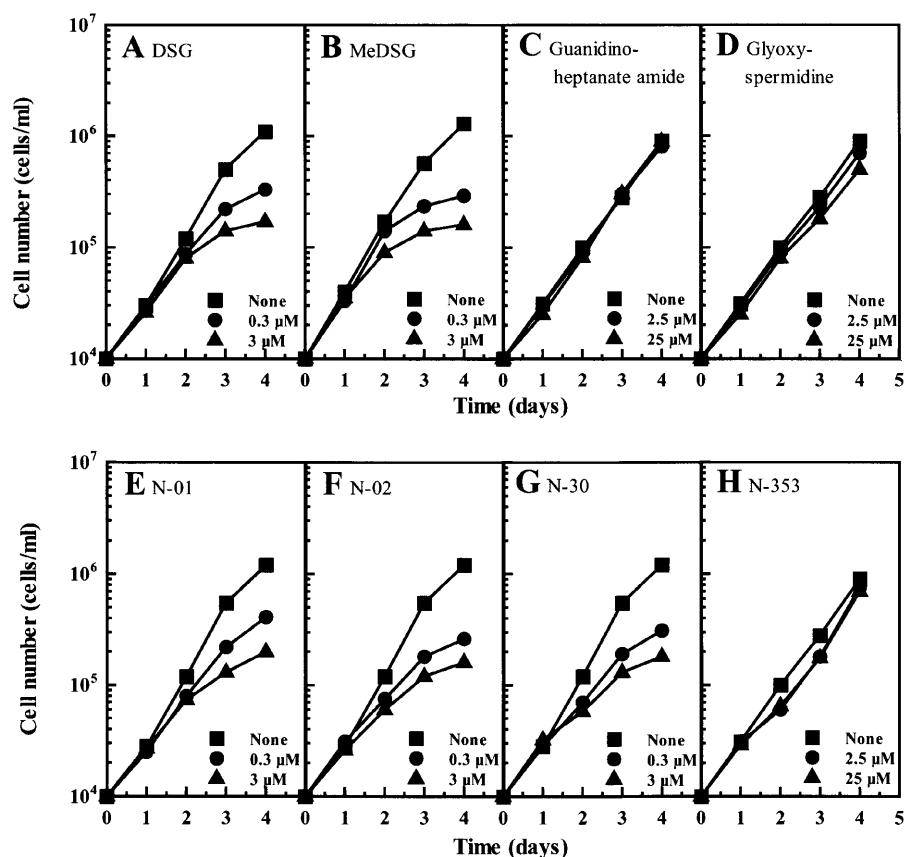


Figure 2 Effects of deoxyspergualin and its analogues on cell growth

Cells were treated in the absence or presence of various concentrations of deoxyspergualin (DSG) (A), methyldeoxyspergualin (MeDSG) (B), guanidinoheptanate amide (C), glyoxyspermidine (D), N-01 (E), N-02 (F), N-30 (G) and N-353 (H), and cell growth determined over time. Each value is the mean of triplicate determinations. S.D. was within $\pm 10\%$ for each point.

eIF5A emulsified with Freund's complete adjuvant subcutaneously into the back of a rabbit, as described previously [30].

Measurement of the amount of eIF5A and the level of hypusine in eIF5A

Cell lysate was prepared as described previously [31], and the supernatant was obtained by centrifugation at 100 000 *g* for 1 h at 4 °C. The amount of eIF5A in the 100 000 *g* supernatant (30 μ g of protein) was determined by Western-blot analysis using the ProtBlot Western Blot AP system (Promega, Madison, WI, U.S.A.) [32]. To measure the level of hypusine in eIF5A, FM3A cells were cultured with 925 kBq [3 H]spermidine (0.07 μ M) in the presence of 1 mM aminoguanidine, an inhibitor of amine oxidase in serum [33], for 24 h at the designated time. Then, SDS/PAGE and fluorography were performed as described by Laemmli [34] and Laskey and Mills [35] respectively, using 100 μ g of protein of the 100 000 *g* supernatant. Furthermore, hypusine in eIF5A was measured directly by reversed-phase HPLC with a fluorescence detection of *o*-phthalaldehyde using 1 mg of protein from the 100 000 *g* supernatant, as described previously [36].

Measurement of eIF5A synthesis

A 10 ml cell suspension (6×10^5 /ml) in methionine-free ES medium (Nissui Pharmaceutical Co.) was cultured with 11.1 MBq [35 S]methionine (25.5 nM) for 2 h at 37 °C. Cells were harvested, and the cell lysate was prepared as described previously

[31]. The amount of eIF5A synthesized was determined by immunoprecipitation using 1×10^7 c.p.m. of [35 S]methionine-labelled total protein and 6 μ l of anti-(eIF5A) serum, gel electrophoresis and fluorography [37]. Radioactivity of the labelled eIF5A protein was quantified using a Fujix BAS 2000II image analyser.

Assay for DHS

The plasmid pT7-7hDHS, encoding human DHS [38], was kindly supplied by Dr M. Miyazaki (School of Science, Nagoya University, Nagoya, Japan). DHS was induced with 1 mM isopropyl β -D-thiogalactoside in *E. coli* BL21 (DE3)/pT7-7hDHS as described above. Cell lysate was prepared from the cells by sonication using a buffer B [50 mM Tris/HCl (pH 7.0), 1 mM dithiothreitol, 10% (v/v) glycerol and 10 μ M FUT-175], and used as the source of DHS (DHS lysate). The reaction mixture (0.05 ml) for the assay of DHS contained 50 mM Tris/HCl (pH 8.0), 2 mM dithiothreitol, 10% (v/v) glycerol, 50 μ M NAD $^+$, 20 μ M FUT-175, 20 μ M precursor eIF5A synthesized in *E. coli*, 3 μ M [3 H]spermidine (98.7 GBq/mmol) and 1.3 μ g of protein from the DHS lysate. The reaction mixture, except [3 H]spermidine, was preincubated at 37 °C for 1 h, and further incubation of the reaction mixture with [3 H]spermidine was carried out at 37 °C for 1 h. After incubation, ice-cold 5% (v/v) TCA was added and the radioactivity in a sample (40 μ l) of the resulting precipitate was measured in a liquid-scintillation counter.

RESULTS

Anti-proliferative effects of deoxyspergualin and its analogues

The anti-proliferative effects of deoxyspergualin and a number of structural analogues (Figure 1) were studied. Anti-proliferative effects were observed with micromolar concentrations of deoxyspergualin, methyldeoxyspergualin, N-01, N-02 and N-30, but not with guanidinoheptanate amide, glyoxyspermidine and N-353 (Figure 2). Thus both the guanidinoheptanate amide and

glyoxyspermidine moieties of deoxyspergualin are necessary for its anti-proliferative effects. The degree of inhibition was very similar among the five active compounds. Addition of 1 mM aminoguanidine, an inhibitor of serum amine oxidase [33], did not influence the anti-proliferative effects (results not shown), indicating that the effects are due to the deoxyspergualin analogues themselves and not to their degradation products. Since methyldeoxyspergualin is more stable than deoxyspergualin [39], methyldeoxyspergualin was used in subsequent experiments. The

Table 1 Polyamine and methyldeoxyspergualin contents in FM3A cells

Cells were cultured at the designated day, and polyamine and methyldeoxyspergualin contents were measured as described in the Materials and methods section. Results are means \pm S.D. of triplicate experiments. Statistical differences were assessed using an unpaired Student's *t* test. ***P* < 0.01 compared with the control experiments.

Culture	Time (days)	Polyamines (nmol/mg of protein)			Methyldeoxyspergualin (nmol/mg of protein)
		Putrescine	Spermidine	Spermine	
Control	1	9.52 \pm 2.40	24.6 \pm 4.04	12.5 \pm 1.42	–
	2	10.80 \pm 1.66	22.7 \pm 3.55	9.57 \pm 0.59	–
	3	9.58 \pm 0.84	21.7 \pm 1.34	10.0 \pm 1.04	–
	4	9.23 \pm 0.69	18.9 \pm 0.78	10.0 \pm 0.80	–
Methyldeoxyspergualin (3 μ M)	1	8.30 \pm 0.20	22.8 \pm 0.31	10.6 \pm 1.58	0.76 \pm 0.40
	2	7.85 \pm 0.84	19.1 \pm 1.35	10.5 \pm 0.23	0.68 \pm 0.12
	3	6.37 \pm 0.74**	15.8 \pm 1.46	11.4 \pm 0.57	0.57 \pm 0.07
	4	2.68 \pm 0.27**	15.5 \pm 0.81	11.7 \pm 0.46	0.55 \pm 0.09

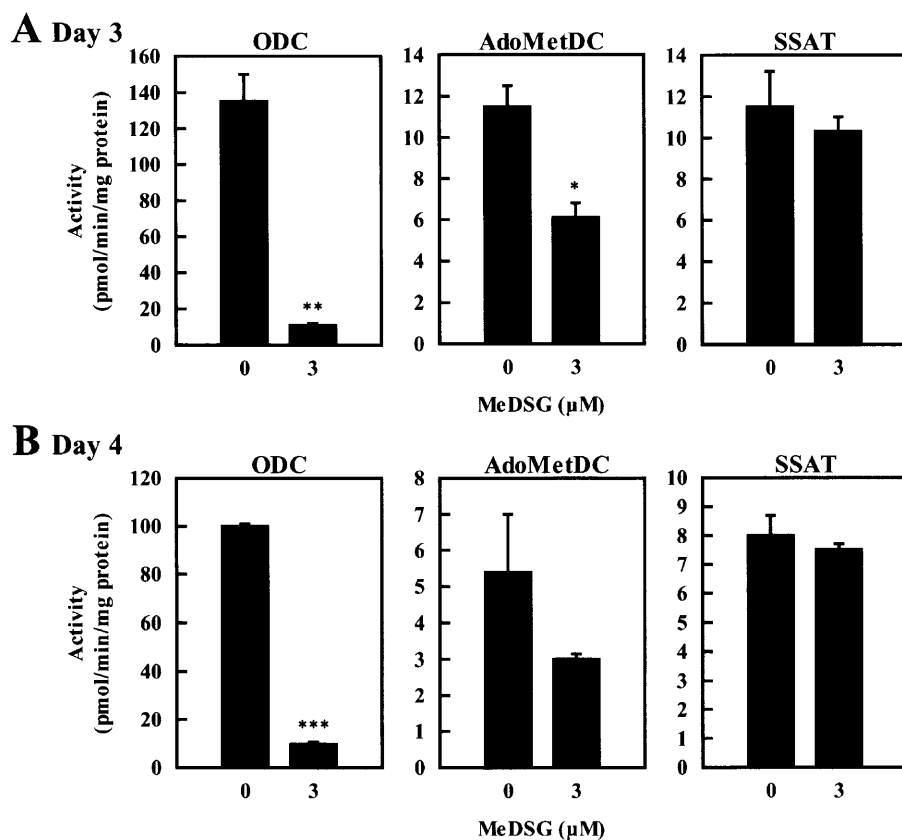


Figure 3 Effect of methyldeoxyspergualin on ODC, AdoMetDC and SSAT

Cells were cultured for 3 (A) or 4 (B) days in the absence (0) or presence of 3 μ M methyldeoxyspergualin. After incubation, cells were lysed and ODC, AdoMetDC and SSAT were assayed, as described in the Materials and methods section. Values are means \pm S.D. of triplicate determinations. Statistical differences were assessed using an unpaired Student's *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with the control experiments.

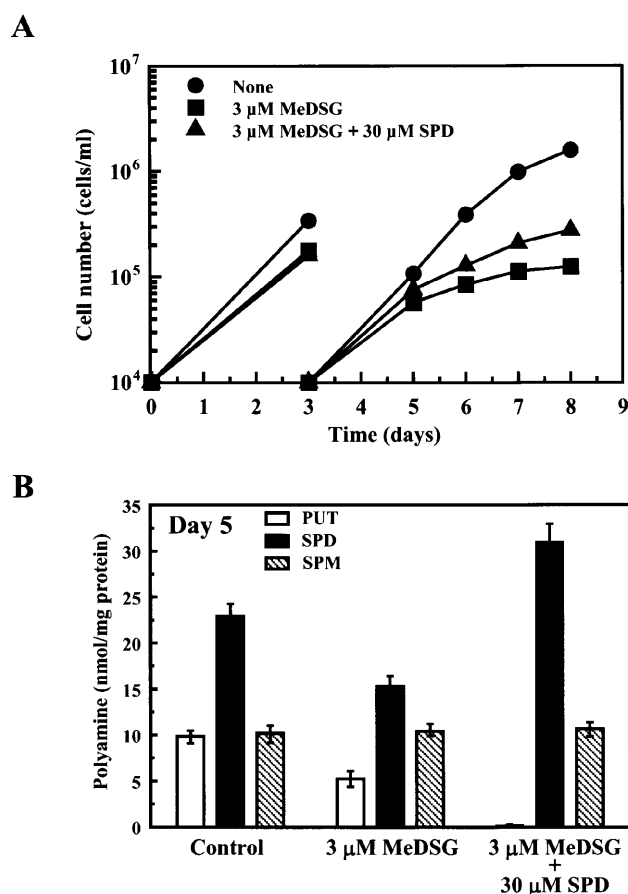


Figure 4 Effect of spermidine on cell growth and polyamine content of methyldeoxyspergualin-treated cells

(A) Cells were cultured in the absence (●) and presence of 3 μ M methyldeoxyspergualin (■) or 3 μ M methyldeoxyspergualin and 30 μ M spermidine (SPD) (▲) for 3 days, and then harvested, washed and recultured for a further 5 days under the same conditions. Cell number was determined. Each value is the mean of triplicate determinations. S.D. was within $\pm 10\%$ for each point. (B) Polyamine content of putrescine (PUT; white bars), spermidine (SPD; black bars) and spermine (SPM; hatched bars) in FM3A cells was measured 2 days after the initial 3 days of treatment (i.e. after 5 days in culture). Values are means \pm S.D. of triplicate determinations.

degree of inhibition was greatest at days 3–4 of culture with deoxyspergualin and methyldeoxyspergualin, confirming previous results [40].

A spermidine moiety is included in methyldeoxyspergualin. Thus the effects of methyldeoxyspergualin on polyamine content were examined. As shown in Table 1, significant amounts of methyldeoxyspergualin accumulated in the cells, and putrescine and spermidine contents started to decrease at the third day of culture. The decrease in putrescine and spermidine content correlated with the inhibition of cell growth by deoxyspergualin. In light of this, we also measured the activities of enzymes involved in the synthesis and metabolism of polyamines. Although the activities of ODC and AdoMetDC decreased at days 3–4 of culture, SSAT activity did not significantly change (Figure 3). The results indicate that the decrease in putrescine and spermidine content was due to the decrease in ODC and AdoMetDC activities. The level of ODC mRNA greatly decreased in cells treated with methyldeoxyspergualin, but the level of AdoMetDC mRNA did not significantly change (results not

shown). Thus the decrease in AdoMetDC activity may occur at the post-transcriptional level.

Because methyldeoxyspergualin reduces putrescine and spermidine levels, experiments were carried out to determine whether cell growth could be recovered by the addition of exogenous spermidine. After incubation of FM3A cells with methyldeoxyspergualin (3 μ M) or methyldeoxyspergualin (3 μ M) plus spermidine (30 μ M) for 3 days, the cells were harvested, washed and recultured under the same conditions. As shown in Figure 4(A), cell growth was not significantly recovered by spermidine. Under these conditions, the content of methyldeoxyspergualin in cells treated in the absence of spermidine at day 4 (0.65 ± 0.14 nmol/mg of protein; mean \pm S.D.) was similar to that for cells treated with methyldeoxyspergualin and spermidine (0.59 ± 0.12 nmol/mg of protein; mean \pm S.D.), although the content of spermidine was higher in the latter cells (approx. 32 nmol/mg of protein) than in the former cells (approx. 15 nmol/mg of protein) (Figure 4B). This contrasts with results using cells treated with α -difluoromethylornithine, an inhibitor of ODC, in which the addition of polyamines to the medium led to a complete recovery of cell growth [41,42]. The results suggest that the anti-proliferative effects of methyldeoxyspergualin are not simply due to a decrease in the polyamine content of cells.

Inhibition of active eIF5A formation by methyldeoxyspergualin

Active eIF5A is the only known protein that has a polyamine derivative (hypusine) and is essential for cell growth [12–14]. The active eIF5A is formed through reactions involving two enzymes, DHS and deoxyhypusine hydroxylase [15]. Thus the effects of deoxyspergualin and its analogues on DHS were examined in a cell-free system using human DHS expressed in *E. coli*. Although the optimal pH of DHS is 9.3 [14], experiments were carried out at pH 8.0, relatively close to the physiological pH. As a positive control we used 1,3-diaminopropane, an established inhibitor of DHS [14]. As shown in Figure 5 (left-hand panel), deoxyspergualin, methyldeoxyspergualin, N-01, N-02 and N-30, which exhibit anti-proliferative effects, strongly inhibited the activity of DHS. A similar degree of inhibition was seen with glyoxyspermidine, although it did not significantly inhibit cell growth (see Figure 2). This was probably due to the very slow uptake of glyoxyspermidine into cells, as the content of glyoxyspermidine in cells treated with 25 μ M was less than 0.01 nmol/mg of protein at the fourth day. The results suggest that methyldeoxyspergualin may function as an inhibitor of DHS even after it is degraded to guanidinoheptanate amide and glyoxyspermidine in cells.

Since the concentration of deoxyspergualin necessary to inhibit DHS was much higher than that accumulated in FM3A cells (see Table 1), the effect of preincubation of deoxyspergualin or glyoxyspermidine with DHS and eIF5A was tested (Figure 5, right-hand panel). The degree of inhibition by deoxyspergualin or glyoxyspermidine greatly increased with the prolongation of preincubation. Increase in the degree of inhibition mainly occurred with the preincubation of deoxyspergualin or glyoxyspermidine with DHS, but not with eIF5A (results not shown). These results strongly suggest that the accumulated deoxyspergualin in cells is enough to cause inhibition of cell growth from the third day of the culture.

Next, the amount of eIF5A and hypusine in eIF5A was measured by Western blotting of eIF5A and labelling of eIF5A with [3 H]spermidine for 24 h respectively. As shown in Figure 6(A), the amount of eIF5A did not significantly change in cells treated with methyldeoxyspergualin for 4 days. However, the level of hypusine in eIF5A was slightly decreased at the third day

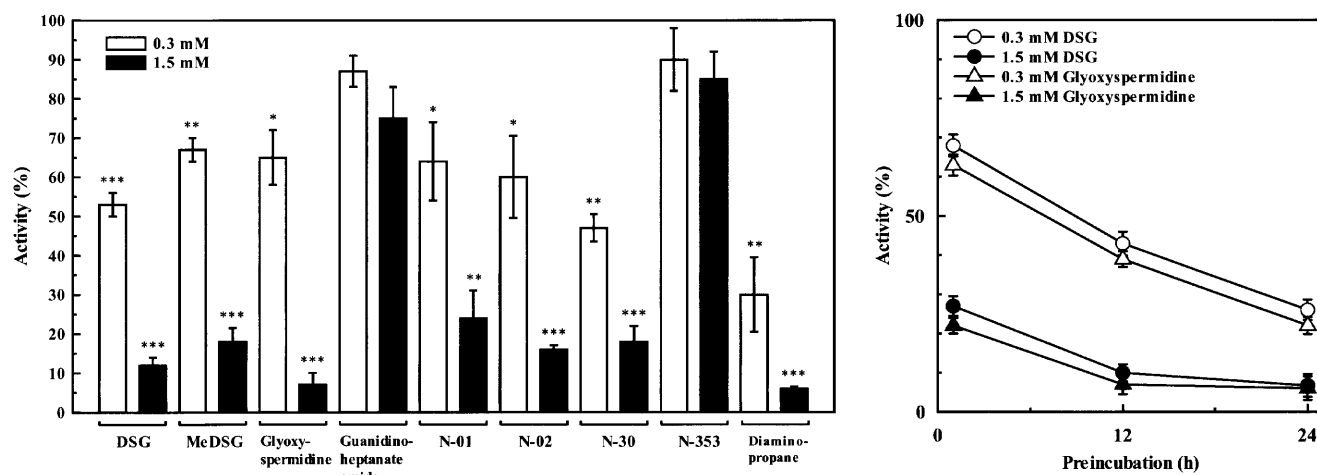


Figure 5 Effects of deoxyspergualin and its analogues on DHS

Left-hand panel: activity of DHS was measured under standard conditions in the presence (\square , 0.3 mM; and \blacksquare , 1.5 mM) of deoxyspergualin and its analogues, as described in the Materials and methods section. Control activity in the absence of deoxyspergualin was 181.6 pmol/min per mg of protein. Right-hand panel: preincubation of deoxyspergualin (DSG) or glyoxyspermidine with DHS and eIF5A was performed for the designated times shown in the Figure, and the activity of DHS was measured, as described in the Materials and methods section. Control activity in the absence of deoxyspergualin decreased with the progression of preincubation, such that the activity after 24 h preincubation was 68.3 pmol/min per mg of protein. Values are means \pm S.D. of triplicate determinations expressed as a percentage of the untreated control. Statistical differences were assessed using an unpaired Student's *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with the control experiments.

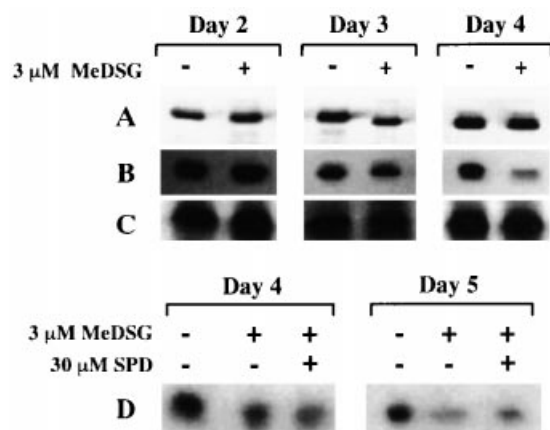


Figure 6 Effects of methyldeoxyspergualin on eIF5A (A) and hypusine in eIF5A (B and D) levels and eIF5A synthesis (C)

Cells were treated in the absence (–) or presence (+) of methyldeoxyspergualin (MeDSG; 3 μ M) alone (A–C) or in combination with spermidine (SPD; 30 μ M) (D) for the indicated times. The level of eIF5A (A) was determined by Western-blot analysis, and that of hypusine in eIF5A (B and D) by the incorporation of [3 H]spermidine into eIF5A, as described in the Materials and methods section. Synthesis of eIF5A (C) was measured by immunoprecipitation of [35 S]methionine-labelled protein with antiserum against eIF5A, as described in Materials and methods section.

of culture and greatly decreased at the fourth day of culture (Figure 6B). Synthesis of eIF5A was measured by immunoprecipitation of [35 S]methionine-labelled total protein for 2 h (Figure 6C), and it was not influenced by the treatment of cells with methyldeoxyspergualin for 4 days. The results indicate that formation of active eIF5A is inhibited by methyldeoxyspergualin. When cells were treated with 3 μ M methyldeoxyspergualin and 30 μ M spermidine, cell growth was not significantly recovered

Table 2 Hypusine contents in FM3A cells

Cells were cultured on the designated day, and hypusine was measured as described in the Materials and methods section. Each value is the mean for duplicate determinations.

Culture	Time (days)	Hypusine (pmol/mg of protein)
Control	3	26.5
	4	25.3
Methyldeoxyspergualin (3 μ M)	3	20.5
	4	10.2

(see Figure 4). Under these conditions, formation of active eIF5A was not significantly recovered, although spermidine was accumulated in cells (Figure 6D).

It is possible that the incorporation of [3 H]spermidine into eIF5A may not reflect the level of hypusine in eIF5A if the activity of spermidine uptake was to be lower in the cells treated with methyldeoxyspergualin. Thus the level of hypusine in eIF5A was measured directly after hydrolysis of eIF5A. As shown in Table 2, the decrease in hypusine in eIF5A of cells cultured with methyldeoxyspergualin was parallel with the decrease in the [3 H]spermidine in eIF5A (see Figure 6B).

It has been reported that a temperature-sensitive eIF5A mutant in yeast accumulates uncapped mRNA [43]. In methyldeoxyspergualin-treated cells, RNA synthesis did not significantly change, although DNA and protein synthesis were inhibited (Table 3). Isolation of mRNA was then performed by an oligo(dT)-cellulose column. Although the amount of mRNA was significantly increased by treatment of cells with methyldeoxyspergualin, the amount of capped mRNA was greatly decreased (Table 3). The results indicate that inhibition of active eIF5A formation in mammalian cells by methyldeoxyspergualin also causes the accumulation of uncapped mRNA.

Table 3 Effect of methyldeoxyspergualin on macromolecular synthesis and the level of capped mRNA

Cells were cultured in the absence and presence of methyldeoxyspergualin for 4 days. DNA, RNA and protein synthetic activities were measured as described in the Materials and methods section. Synthesis of mRNA was measured by isolating mRNA using oligo(dT)-cellulose column, and the amount of capped mRNA by incorporation of [methyl-³H]S-adenosylmethionine into mRNA as described in the Materials and methods section. Results are means \pm S.D. of triplicate determinations. Statistical differences were assessed using an unpaired Student's *t* test. **P* < 0.05; ***P* < 0.01 compared with the control experiments.

Macromolecular synthesis or level of capped mRNA	Control	Methyldeoxyspergualin (3 μ M)	Percentage of control
DNA synthesis (c.p.m./ μ g of protein)	63.2 \pm 5.15	38.1 \pm 3.82**	60.0
RNA synthesis (c.p.m./ μ g of protein)	144.3 \pm 8.15	149.5 \pm 9.76	104.0
Protein synthesis (c.p.m./ μ g of protein)	98.2 \pm 6.54	55.3 \pm 5.42**	56.3
mRNA synthesis (c.p.m./ μ g of mRNA)	10240 \pm 1320	14030 \pm 1710*	137.0
Capped mRNA (c.p.m./ μ g of mRNA)	163 \pm 8.17	45.6 \pm 5.13**	28.0

DISCUSSION

In the present study, we have tried to clarify the mechanism of the anti-proliferative effects of methyldeoxyspergualin. A strong inhibition of cell growth occurred after 3 days in culture, in parallel with a decrease in the level of active eIF5A. The decrease in the level of eIF5A was due to inhibition of DHS by methyldeoxyspergualin. However, the decrease in the level of eIF5A is small at the third day of culture. Thus a decrease in putrescine and spermidine content is also involved in inhibition of cell growth at the third day of culture. Furthermore, the decrease in spermidine content in cells probably facilitates the inhibition of active eIF5A formation by methyldeoxyspergualin, since spermidine competes with methyldeoxyspergualin for the binding site on DHS (results not shown). On the fourth day of culture, the severe decrease in the level of active eIF5A probably becomes the major reason for the inhibition of cell growth by methyldeoxyspergualin, since the addition of spermidine to the medium together with methyldeoxyspergualin did not prevent the inhibition of cell growth.

The present study is the first to show that there is a correlation between the decrease in active eIF5A and the accumulation of uncapped mRNA in mammalian cells, although it has already been reported that a temperature-sensitive eIF5A mutant in yeast accumulates uncapped mRNA [43]. It has been reported that *N*¹-guanyl-1,7-diaminoheptane (GC₇) is a potent inhibitor of DHS [14], and that it inhibits cell growth without causing a decrease in polyamine content [44]. GC₇ also greatly decreased the amount of capped mRNA (results not shown). The use of methyldeoxyspergualin or GC₇ may be helpful in clarifying how eIF5A is involved in cell proliferation and the degradation of decapped mRNA. There is also a report that polyamine depletion causes stabilization of some kinds of mRNA [45]. It was suggested that the phenomenon may be related to the decrease in active eIF5A. Thus it is of interest to know whether eIF5A influences the degradation of all species of decapped mRNA or not.

As studied in a cell-free system, the concentration of methyldeoxyspergualin required to cause inhibition of DHS was relatively high (approx. 0.2–1 mM) (Figure 5, left-hand panel). However, the cellular concentration of accumulated methyldeoxyspergualin was low (approx. 15–150 μ M) (Table 1) if the concentration is calculated assuming a level of 5.5 μ l of cell volume/mg of protein [21]. To resolve the apparent discrepancy, we preincubated deoxyspergualin or its degraded product, glyoxyspermidine, with DHS and found that the concentration necessary for the inhibition of DHS gradually decreased with the progress of preincubation (Figure 5, right-hand panel). These results strongly suggest that a low concentration of deoxyspergualin causes the inhibition of DHS after incubation for

several days, that is, at the third day of culture. It is also noted that the formation of active eIF5A was not recovered even if spermidine accumulated in cells through its addition to the medium (Figure 4). Thus it is proposed that deoxyspergualin or glyoxyspermidine may react with amino acid residues located in the active site of DHS to form covalent linkages. In this case, deoxyspergualin may function as an inhibitor of DHS after degradation into glyoxyspermidine and guanidinoheptanate amide. Experiments are now in progress to clarify this point.

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