Antiproliferative Effects of Inhibitors of Deoxyhypusine Synthase

INHIBITION OF GROWTH OF CHINESE HAMSTER OVARY CELLS BY GUANYL DIAMINES*

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Certain guanyl diamines are effective inhibitors of deoxyhypusine synthase (Jakus, J., Wolff, E. C., Park, M. H., and Folk, J. E. (1993) J. Biol. Chem. 268, 13151–13159), the first enzyme involved in the biosynthesis of the unusual amino acid hypusine (N*-4-amino-2-hydroxybutyl)lysine). Evidence that hypusine is implicated in cell growth prompted this study of the cellular effects of these inhibitors. In Chinese hamster ovary (CHO) cells, inhibition of hypusine biosynthesis followed by progressive arrest in cellular proliferation was observed with both N*-mono- and N*,N*-bisguanyl derivatives of 1,6-diaminoheptane, 1,7-diaminoheptane, and 1,8-diaminooctane. Cells treated with these compounds showed no significant change in polyamine distribution, suggesting that the observed growth inhibition is not mediated through an interference with polyamine metabolism. N*-guanyl-1,7-diaminoheptane, the most potent inhibitor of deoxyhypusine synthase both in vitro and in cells, exhibited the highest antiproliferative activity toward CHO cells. No early cytotoxic effects were observed with this inhibitor, and its antiproliferative activity appeared to be reversible. Transport studies showed that N*-guanyl-1,7-diaminoheptane is actively taken up by the polyamine transport system. Mutant CHO cells defective in polyamine transport were found to be resistant to growth inhibition by this compound. The findings suggest that the antiproliferative effect of N*-guanyl-1,7-diaminoheptane is exerted intracellularly through inhibition of hypusine synthesis.

Hypusine (N*-4-amino-2-hydroxybutyl)lysine is a unique amino acid that is formed posttranslationally in only one protein, eukaryotic translation initiation factor 5A (eIF-5A, older nomenclature eIF-4D) (Ref. 1 and, for a review, see Ref. 2). Its biosynthesis, which occurs by modification of a single lysyl residue, involves two enzymes (3). Deoxyhypusine synthase (4–6) catalyzes the transfer of the 4-amino-5-butyrolactone moiety of the polyamine spermidine to the e-amino group of the lysine to form the intermediate, deoxyhypusine. Deoxyhypusine hydroxylase (7) then mediates the hydroxylation of this intermediate to complete hypusine synthesis and eIF-5A maturation. Normally, hypusine biosynthesis occurs efficiently, and no accumulation of either unmodified eIF-5A precursor (8, 9) or the deoxyhypusine-containing intermediate (3) is detectable.

Hypusine has been shown to be essential for the activity of eIF-5A in stimulating methionyl puromycin synthesis, an in vitro model assay for translation initiation (10, 11). However, the precise physiological function of eIF-5A in eukaryotic protein synthesis, or in other cellular metabolism, is as yet unknown (2, 12). The marked increase in hypusine synthesis observed following mitogen treatment of human peripheral blood lymphocytes (13), together with a general correlation noted between hypusine synthesis and cell proliferation in other mammalian cells (2, 14–17), suggested an important role for hypusine in cellular proliferation. Recently, a requirement for hypusine in yeast was implied by a complete loss of growth potential following inactivation of both eIF-5A genes in this organism (18). Expression of at least one of the two eIF-5A genes to produce an eIF-5A precursor protein (12, 18, 19) and its subsequent modification to form hypusine (18) are required for yeast growth. Additional support for the involvement of hypusine in cell growth derives from studies in which cellular spermidine, the polyamine substrate for hypusine formation, was depleted by the use of inhibitors of polyamine biosynthesis (20, 21).

Targeted inhibition of the enzymes responsible for hypusine biosynthesis offers a promising means for controlling production of this amino acid and thus for further defining its specific cellular role. In addition, selective inhibitors should provide a mechanism for regulation of cell proliferation, if indeed hypusine is essential for this process (22). We recently reported (23) several effective inhibitors of deoxyhypusine synthase. Among these are mono- and bisguanyl diamines structurally related to spermidine, which, in preliminary studies, were found to be effective deterrents to hypusine formation in cells. The most potent of these compounds is N*-guanyl-1,7-diaminoheptane (GC7), the K value of which, 10 nm, is approximately 400-fold lower than the K of spermidine. Here we report the effects of mono- and bisguanyl derivatives of several diamines on hypusine formation, protein synthesis, polyamine metabolism, and growth in CHO cells with special focus on GC7.

EXPERIMENTAL PROCEDURES

Materials

[1,8-3H]Spermidine HCl (15 Ci/mmol) and [4,5-3H]Lysine (94 Ci/mmol) were purchased from DuPont NEN. N*,N*-bisguanyl-1,8-diaminoheptane (GC8), N*,N*-bisguanyl-1,7-diaminoheptane (GC7), N*-guanyl-1,8-diaminooctane (GC9), and N*-guanyl-1,7-diaminoheptane (GC6) were synthesized as described previously (22).

* A preliminary account of a portion of this work has been presented at the ASBMB meeting in Washington D.C. on May 22, 1994 (Park, M. H., Wolff, E. C., Lee, Y. B., and Folk, J. E. (1994) FASEB J. 8, 1310 (Abstr. 305). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: eIF-5A, eukaryotic translation initiation factor 5A; CHO, Chinese hamster ovary; Z, benzoyloxy carbonyl; MGBG, methylglyoxal bis(guanyl-hydrazone); GCN, N*-guanyl-1,8-diaminooctane; GC7, N*-guanyl-1,7-diaminoheptane; GC8, N*-guanyl-1,8-diaminooctane; GC9, N*,N*-bisguanyl-1,8-diaminooctane; GC6, N*,N*-bisguanyl-1,7-diaminoheptane; GC7, N*,N*-bisguanyl-1,7-diaminoheptane; and GC9, N*,N*-bisguanyl-1,8-diaminooctane.

1 A. Shirahata, personal communication.
The MGBG-resistant mutant of CHO cells (CHO-MG) that is defective in polyamine transport (24) and the corresponding wild type CHO cells (CHO-P5) were kindly provided by Dr. Wayne F. Flintoff, University of Toronto, Toronto, Canada.

N'-Guanyl-1,6-diaminoheptane (GCN) was prepared through the following series of reactions: N,N'-bisbenzoxycarbonyl-1,6-diaminoheptane, m.p. 125 °C, was obtained in 90% yield by the method described for preparation of the bis-Z derivative of cadaverine (25). Attempts to convert this monomer to N'-guanyl-1,6-diaminoheptane by the published procedure using HBr in glacial acetic acid (25) were unsuccessful. Mono-N,Z-1,6-diaminoheptane-0.5 HSO\textsubscript{4}, m.p. 150 °C dec., was used as the short term hydrolysis (5 min at 30 lb/in\textsuperscript{2}) of the bis-Z compound in 100 ml of a 1:1 (v/v) mixture of methanol and dichloromethane containing 1.4 equivalents of concentrated H\textsubscript{2}SO\textsubscript{4}, using 0.5 g of 10% palladium on charcoal as catalyst. The compound was obtained in 17% yield after two recrystallizations from water/methanol. Its conversion to N'-Z,N'-guanyl-1,6-diaminoheptane-0.5 HSO\textsubscript{4} was accomplished in 34% yield by guanylation in aqueous methanol using O-methylsourea as the solvent and triethylamine as the base. Removal of the Z group by catalytic hydrolysis in methanol containing 0.5 equivalent of H\textsubscript{2}SO\textsubscript{4} gave N'-guanyl-1,6-diaminoheptane-0.5 H\textsubscript{2}SO\textsubscript{4} m.p. 256 °C dec., in 48% yield from water/methanol/acetic acid, CIMS 199 (M \textsuperscript{+} + 1).

\[ \text{C}_{17}\text{H}_{39}\text{N}_{3}\text{H}_2\text{SO}_4\text{H}_2\text{O} \]

Calculated: C 30.64 H 8.08 N 20.42
Found: C 30.54 H 8.14 N 20.29

N'-Guanylated-1,7-diamino-7'H-heptane (\[^{14}\text{H}GC\]), (18 Ci/mmol) was prepared by vapor-phase HCl hydrolysis of its N'-acetyl derivative formed by custom catalytic tritiation (Amersham Corp.) of 7-timidinohoeptanotirile in a mixture of glacial acetic acid and acetic anhydride at 1 atm with platinum oxide catalyst. The 7-timidinohoeptanotirile, m.p. 140–140.5 °C, was produced from 7-aminohoeptanotirile (20) by guanylation with O-methylsourea hydrogen sulfate and triethylamine. The product after its separation by ion exchange chromatography (28) was dissolved in water, acidified with acetic acid, dried in vacuo, and crystallized as the acetate salt from water/methanol/acetone, CIMS 189 (M \textsuperscript{+} + 1).

\[ \text{C}_{18}\text{H}_{38}\text{N}_{2}\text{C}_2\text{H}_4\text{O}_2 \]

Calculated: C 52.61 H 8.83 N 24.54
Found: C 52.43 H 8.87 N 24.46

\[^{14}\text{H}GC\] was purified by silica gel chromatography as outlined (27), except that only water was used to wash radioactively impurities from the column before elution of the labeled product. Upon ion exchange chromatography (28), together with unlabeled carrier N'-guanyl-1,7-diaminoheptane in a buffer mixture containing nine parts of sodium citrate buffer (3.0 N Na\textsubscript{2}), pH 5.55, and one part 2-propanol, 98% of the eluted radioactivity was found to coelu e with the carrier compound.

Methods

Cell Culture—CHO cells were cultured in the modification of Eagle's medium supplemented with 10% fetal bovine serum, penicillin (50 IU/ml), streptomycin (50 mg/ml), and 1 mm aminoguanidine. Aminoguanidine was included because in preliminary experiments it was found that the deoxyhypusine synthase inhibitor GCN was rapidly degraded in certain cell culture media, especially those containing calf serum, as measured fluorometrically after separation by ion exchange chromatography. This loss of GCN, which occurred in media without cells as rapidly as in those with cells, was prevented by inclusion in the media of 1 mm aminoguanidine, a well known inhibitor of amine oxidases (29), suggesting that GCN is degraded by an amine oxidase. This activity was much lower in media containing fetal serum but was not negligible. Therefore in all of the experiments described in this paper, aminoguanidine was added to the culture medium.

Exponentially growing cells were incubated with the test compounds as described in the figure legends. Cell viability was tested by the trypan blue exclusion method.

Determination of Hypusine and Protein Synthesis—A radiolabeled precursor, \[^{14}\text{H}llysine or \[^{14}\text{H}\]lysine, was added to the cells 10–20 min after the addition of the compound being tested. After incubation for the indicated times, the cells were washed twice with phosphate-buffered saline and harvested, and the cellular macromolecules were precipitated with 5% trichloroacetic acid. In the case of the samples labeled with \[^{14}\text{H}llysine, the trichloroacetic acid precipitates were washed twice with 5% trichloroacetic acid containing 1 mm each of nonlabeled putrescine, spermidine, and spermine and then hydrolyzed with 6 N HCl for 18 h at 108 °C. The amount of radiolabeled hypusine or deoxyhypusine in the hydrolysate was determined after ion exchange chromatographic separation (28, 30). For the samples labeled with \[^{14}\text{H}llysine, the trichloroacetic acid precipitates were washed once with 5% trichloroacetic acid and then dissolved in 1 ml of 0.1 N NaOH. A portion of this solution was used for the determination of radioactivity.

Determination of Polyamine Pools—Untreated cells, or cells treated with a guanilamine, were washed, harvested, and extracted with 5% trichloroacetic acid. The basic amine compounds in the trichloroacetic acid supernatant solution were resolved by ion exchange chromatography according to a published procedure (28) and quantitated fluorometrically after postcolumn derivitization with o-phthalaldehyde.

Measurement of the Transport of Spermidine and GCN—\[^{14}\text{H}llysine or \[^{14}\text{H}GHC\], was added to dishes of cells with densities near confluence but still in exponential growth, either in the presence of or in the absence of unlabeled GCN, or of unlabeled spermidine, respectively. The cells were then incubated at 37 or 4 °C, for periods up to 2 h. After the incubation period, the cells were washed twice with ice-cold phosphate-buffered saline containing 1 mm each of nonlabeled polyamines putrescine, spermidine, and spermine. The washed cells were dissolved in 1 ml of 0.1 N NaOH, and a portion of this solution was used for the determination of radioactivity.

Determination of GCN—This compound was measured either fluorometrically or by measurement of the radioactivity of the labeled compound after its separation by ion exchange chromatography (28). Elution of GCN was significantly later than spermine in a buffer mixture containing nine parts of sodium citrate buffer (3.0 N Na\textsubscript{2}), pH 5.55, and one part 2-propanol.

RESULTS

Effects of Guanil Diamines on the Synthesis of Hypusine and Protein in CHO Cells—The effects of each member of a series of guanil diamines on cellular synthesis of hypusine and protein are shown graphically in Fig. 1, A and B, respectively. For comparative purposes, the \( K_i \) values of these guanil diamines, as measures of their efficacies as in vitro inhibitors of deoxyhypusine synthase, are given in Fig. 1C. Hypusine synthesis in cells (Fig. 1A) was followed by measuring the incorporation of radioactivity from \[^{14}\text{H}llysine into \[^{14}\text{H}\]hypusine in the eIF-5A protein. The specific radioactivities of the cellular spermidine pools after the 18-h incubation were not significantly different in cells treated with the guanil diamines at the concentrations tested, compared with controls. Because deoxyhypusine is efficiently converted to hypusine in cells by the action of deoxyhypusine hydroxylase (3), and since no accumulation of labeled deoxyhypusine was observed in the cells in these experiments, the reduction in \[^{14}\text{H}l hypusine formed (Fig. 1A) was taken as a measure of the degree of inhibition of deoxyhypusine synthase. Incorporation of \[^{14}\text{H}llysine into total protein, measured in parallel cultures (Fig. 1B), serves as a measure of general protein synthetic activity and, in addition, provides an index of eIF-5A precursor protein synthesis, as discussed below.

It is evident from Fig. 1A that the inhibition of hypusine formation in CHO cells by the guanil diamines is concentration-dependent within the 1–10 mm range tested. The monoguanil deriva tives proved to be more inhibitory than their bisguanil counterparts, and the compound in each group with the seven-member carbon chain was the most effective inhibitor.

Inhibition of hypusine synthesis in cells was most pronounced with \( GCN \), the strongest inhibitor of deoxyhypusine synthase in vitro, as reflected by its \( K_i \) value (Fig. 1C). A 1 mm level of \( GCN \) caused more than 97% inhibition of hypusine production in CHO cells in 18 h. In order to achieve this degree of inhibition, the effect of \( GCN \) on deoxyhypusine synthase must have been exerted almost immediately after its addition to the cells. Only

3 It should be pointed out that the data shown in Fig. 1A illustrate the effects of the guanil diamines on synthesis of hypusine but do not accurately reflect the hypusine content of the cells.

4 Lower concentrations of \( GCN \) were also effective with 90% inhibition at 0.3 mm, 71% at 0.1 mm, and 21% at 0.03 mm.
Antiproliferative Effects of Guanyl Diamines

Polyamine Levels in Cells Treated with Guanyl Diamines—In view of the structural similarities of the guanyl diamines to polyamines, it was considered that these compounds might affect enzymes (in addition to deoxyhypusine synthase) that are involved in polyamine biosynthesis or metabolism. However, no pronounced changes in the polyamine patterns of CHO cells were seen after treatment with the guanyl diamines, as shown in Table I. Some elevations in the levels of putrescine were observed in treated cells, but relatively small differences were found in the amounts of spermidine and spermine in treated and untreated cells.

Table I

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Putrescine (nmol/mg protein)</th>
<th>Spermidine (nmol/mg protein)</th>
<th>Spermine (nmol/mg protein)</th>
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</thead>
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<tr>
<td>None</td>
<td>0.6</td>
<td>8.6</td>
<td>4.5</td>
</tr>
<tr>
<td>GC,</td>
<td>0.8</td>
<td>7.0</td>
<td>5.6</td>
</tr>
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<td>7.5</td>
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<td>4.3</td>
</tr>
<tr>
<td>GC,</td>
<td>1.5</td>
<td>8.3</td>
<td>4.4</td>
</tr>
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</table>

Fig. 1. The inhibition of hypusine formation (A) and of total protein synthesis (B) in CHO cells by guanyl diamines; comparison with the efficiency of these guanyl diamines as in vitro inhibitors of deoxyhypusine synthase (C). CHO cells were cultured as described under "Experimental Procedures." When the cells reached a density of 1 x 10^6 cells/60-mm dish, the guanyl diamine was added to two dishes at the indicated concentration. After 10-20 min, [1-^3H]lysine (15 µCi/dish) was added to one of the dishes (A), and [3H]lysine (15 µCi/dish) was added to the other dish (B). After 18 h of incubation, the amounts of radioactivity in protein-bound hypusine and total protein were measured as described under "Experimental Procedures." The K_i values for the inhibition of deoxyhypusine synthase activity in vitro (C) were reported previously (23), except for the K_i value for GC,.

As noted in Fig. 1A, the individual guanyl diamines exhibited markedly different degrees of inhibition of hypusine formation in these cells. This is as expected on the basis of their K_i values for inhibition of deoxyhypusine synthase in vitro (Fig. 1C). For example, GC, and GC, which have K_i values significantly lower than the K_m for spermidine (4 µM), are strong inhibitors of cellular hypusine formation, whereas GC, and GC,G, with K_i values much greater than the K_m for spermidine, are much less effective. In the case of the latter inhibitors, the modest reduction in hypusine formation may be largely due to the reduced amount of eIF-5A precursor protein synthesized (compare panels A and B of Fig. 1). With the stronger inhibitors, GC, GC, and GC,G, however, the effect seen offers a clear indication of efficient inhibition of deoxyhypusine synthase in cells.

Inhibition of Proliferation of CHO Cells by Guanyl Diamines—Each of the compounds tested inhibited the growth of CHO cells in a concentration- and time-dependent manner (Fig. 2). Consistent with their effects on cellular hypusine synthase, the monoguanyl compounds were more effective in inhibiting growth than the corresponding bisguanyl compounds. GC, the most effective inhibitor of deoxyhypusine synthase both in vitro and in cells, exhibited the greatest antiproliferative activity.

In the 24-h period after the addition of the inhibitor, the cell numbers continued to increase in all cases, albeit at different rates. Upon continued incubation (48-72 h), further inhibition of growth was observed, depending on the compound and the concentration. Whereas untreated cells approached maximum density (35 x 10^6 cells/60-mm dish) by 72 h in this period (48-72 h), total arrest of growth occurred at concentrations as low as 1 and 3 µM for GC, and GP, respectively. Levels ≥10 µM were required for total growth suppression by other compounds (GC, GC, GC, and GC,G). In the concentration range between 1 and 10 µM, there appeared to be no early toxic effects of these compounds, and a large portion of the treated cells remained viable for 3–4 days in culture.

In Fig. 3 is shown a more detailed examination of the time course of growth inhibition by the most effective of the compounds, GC, at levels of 0.2–3 µM. The IC_50 for growth inhibition at 48 h after GC addition was estimated as approximately 0.3 µM. At a concentration of 1 µM, cytostasis was observed after the initial doubling period. When the culture medium was replaced with a GC,-free medium at 96 h, cell growth was resumed and continued until the cells reached confluence, show-
FIG. 2. The inhibition of growth of CHO cells by guanyl diamines. CHO cells were cultured as described under "Experimental Procedures." The guanyl diamine was added at 1, 3, and 10 μM levels to the media at 24 h (vertical arrow) when the cells had reached a density of ~3 × 10⁵ cells/60-mm dish. After an additional 24 and 48 h of incubation, the cells were collected after trypsinization. Cell numbers (expressed on a logarithmic scale) were determined by the use of a Coulter counter.

FIG. 3. The concentration dependence of inhibition of growth of CHO cells by GC₆. Cells were cultured as described under "Experimental Procedures." The indicated concentration of GC₆ was added to the cells at 24 h, at a density of ~2 × 10⁶ cells/60-mm dish. Cell numbers (expressed on a logarithmic scale) were determined as in Fig. 2. Two sets of cells were treated with GC₆ at 1 μM at 24 h. In one set, the medium was changed to a medium free of both GC₆ and aminoguanidine at 96 h as indicated by the dashed arrow and dashed line. In the other set, the medium was not changed. Incubation was continued for 72 additional hours, and cell numbers were determined during this time.

Antiproliferative Effects of Guanyl Diamines

In Fig. 3) than in a GC₆-free medium containing 1 mM aminoguanidine (data not shown).

Cellular Transport and Intracellular Stability of GC₆—With the use of radiolabeled GC₆ and spermidine, we were able to show that GC₆ is transported efficiently and actively into CHO cells by way of the polyamine transport system (Fig. 4). The affinity of this transport system for GC₆ appears to be lower than for spermidine, judging from the fact that inhibition of GC₆ uptake by spermidine (Fig. 4A) is greater than the reverse (Fig. 4B). Consistent with this mechanism for GC₆ transport are the results shown in Fig. 4, C and D, in which CHO cells defective in polyamine transport were tested for GC₆ and spermidine uptake. The cells used, a mutant strain of CHO cells (24), are resistant to MGBG, a cytotoxic inhibitor of S-adenosylmethionine decarboxylase known to be transported by the polyamine transporter. This mutant, CHO-MG, was reported to be deficient in the transport of polyamines, but not in general transport of unrelated materials (33). As expected, CHO-MG proved to be defective in the uptake of GC₆ (Fig. 4D) and, in fact, was able to grow normally in the presence of a high level (10 μM) of GC₆ (data not shown). It seems likely, based on this finding, that transport of GC₆ into cells is required for its effects.

When CHO cells were incubated with [³H]GC₆ (15 μCi) for 20 h and the cells were collected, washed, and extracted with 5% trichloroacetic acid, virtually all the radioactivity in the extract was found to chromatograph at the position of GC₆. Thus, GC₆ appears not to undergo any significant metabolic conversion but rather to remain largely intact in these cells. The release of [³H]GC₆ from CHO cells preloaded with this compound (0.2–1 μM, 2–20 h) was also followed. After a 20-h chase in a medium free of GC₆ and aminoguanidine, approximately 50% of the radioactivity was found in the medium, indicating that the efflux of GC₆, like that of polyamines, is slow. The amount of radioactivity exported was even less if the chase medium contained 1 mM aminoguanidine. These findings are consistent with the delayed recovery of growth rates described above.
The finding reported here that the deoxyhypusine synthase essentially abolishing production of deoxyhypusine at concentrations as low as 1 μM. Its efficient uptake by way of the polyamine transporter, together with its extremely high affinity for deoxyhypusine synthase, probably accounts for the effectiveness of this inhibitor. Because the radioactivity associated with cells at 20 h after exposure to [3H]GC, is entirely in the form of GC, it seems clear that the intracellular effects of this compound are caused by GC, and not by its potential metabolites. Furthermore, the relatively minor effects of 1 μM GC, on DNA and protein synthesis, up to 3 h after its addition, suggest that this guanyldiamine does not interfere directly with macromolecular synthetases but rather that there is a gradual cellular change that leads to suppression of proliferation. It is reasonable to assume that at the time GC, is added to cells there exists sufficient pre-formed mature eIF-5A to sustain cell growth for a protracted period of time since the half-life of eIF-5A in mammalian cells appears to be relatively long (8, 17, 34). Hence the lag time for suppression of cell growth of 18–24 h seen in Figs. 2 and 3 may represent the time needed for reduction of eIF-5A below the threshold level through normal intracellular degradation and dilution by growth. Since the precise function of eIF-5A is unknown, it is, of course, impossible to delineate the sequence of cellular events that leads to growth arrest by GC,. Even though the effects of GC, on processes other than hypusine synthesis cannot be excluded, the correlation seen here between inhibition of hypusine synthesis and cell growth is consistent with deoxyhypusine synthase as the primary cellular target of GC,.

In contrast to GC, and GC, which are the most potent inhibitors of deoxyhypusine synthase, growth suppression by GC, GC,GC, and GC,GC may be due to other than inhibition of hypusine synthesis. These latter compounds all displayed strong inhibition of growth at 10 μM without a commensurate reduction in synthesis of hypusine. Intervention in other cellular processes in addition to hypusine synthesis probably contributes to the arrest of growth by these compounds.

An important aspect of the growth inhibition by the guanyldiamines reported here is its lack of dependence on depletion of spermidine or the other polyamines. It is well established that certain polyamine antimetabolites (31, 32), for example α-di-fluoromethylornithine, MGBG, and bisethylnospermine (35), halt cell proliferation through depletion of the polyamine spermidine. Although recent studies suggest that the growth arrest following spermidine depletion is a consequence of hypusine deprivation (20, 21), it is likely that depletion of spermidine also leads to disturbance of a number of other cellular processes. If this disruption in cell proliferation by polyamine antimetabolites is primarily due to the lack of hypusine or eIF-5A, selective inhibition of deoxyhypusine synthase should accomplish the same end without alteration in other cell functions that accompany depletion of spermidine. In this regard, the inhibitors that specifically target deoxyhypusine synthase, but not other polyamine biosynthetic or metabolic enzymes, may have advantages over the polyamine antimetabolites.

The information given in this report is limited to studies carried out with CHO cells. However, growth inhibition by guanyldiamines was observed with other mammalian cells, including a number of human cancer cell lines. Sensitivity to the guanyldiamines varied with the cell line. Although for the differences in sensitivity is not yet fully understood, it could be related to different rates of cellular uptake and efflux, to different intracellular target(s) and/or to differences in the intracellular metabolism of the compounds. These factors may form the basis of a selective inhibition and open the possibility for practical application of these compounds as chemotherapeutic agents against those tumor cells with, for example, a highly efficient polyamine transport system.

FIG. 4. Cellular transport of GC, in CHO cells and CHO-MG cells. CHO cells were cultured as described under "Experimental Procedures." To cells, growing at a density of ~2 x 10³ cells/60-mm dish were added the following: 15 μCi of [3H]spermidine (0.25 μM) together with (●) or without (□) 5 μM unlabeled GC, (A); 22 μCi of [3H]GC, (0.3 μM) together with (■) or without (▲) 5 μM unlabeled spermidine (B), and incubation was continued. Radioactivity in the cells was measured as outlined under "Experimental Procedures"; the amounts indicated are the total radioactivity (in dpm) incorporated per 2 x 10³ cells. Spd, spermidine. In C and D the experiments described in A and B, respectively, were repeated, except in each case two sets of cells, wild type (CHO-P5) and CHO-MG were used; unlabeled spermidine and unlabeled GC, were added at 50 μM; incubations were at 4 or 37 °C for 20 min.

DISCUSSION

The present study, undertaken with the notion that hypusine may play a vital role in cell proliferation, was designed to determine if in vitro inhibitors of deoxyhypusine synthase could be used in cells to prohibit both hypusine formation and growth and, if so, to establish to what extent the two events could be related. The findings confirm the effectiveness of certain guanyldiamine inhibitors, particularly GC, in cells and provide substantial support for a critical role of hypusine (and its precursor, the polyamine spermidine) in eukaryotic cell proliferation.

Experiments in which cells were depleted of either eIF-5A precursor protein (12, 18, 19) or spermidine (20, 21), while supplying information consistent with a strategic role of hypusine, did not eliminate the possibility that the precursor protein, or the polyamine, per se, functions in a vital manner. The finding reported here that the deoxyhypusine synthase inhibitor GC, curtails mammalian cell growth without depleting cellular levels of spermidine or of the eIF-5A precursor protein adds support to our contention that hypusine plays a key role in cellular proliferation in eukaryotes (22).

GC, is a remarkable inhibitor of cellular hypusine formation, essentially abolishing production of deoxyhypusine at concentrations as low as 1 μM. Its efficient uptake by way of the polyamine transporter, together with its extremely high affinity for deoxyhypusine synthase, probably accounts for the effectiveness of this inhibitor. Because the radioactivity associated with cells at 20 h after exposure to [3H]GC, is entirely in the form of GC, it seems clear that the intracellular effects of this compound are caused by GC, and not by its potential metabolites. Furthermore, the relatively minor effects of 1 μM GC, on DNA and protein synthesis, up to 3 h after its addition, suggest that this guanyldiamine does not interfere directly with macromolecular synthetases but rather that there is a gradual cellular change that leads to suppression of proliferation. It is reasonable to assume that at the time GC, is added to cells there exists sufficient pre-formed mature eIF-5A to sustain cell growth for a protracted period of time since the half-life of eIF-5A in mammalian cells appears to be relatively long (8, 17, 34). Hence the lag time for suppression of cell growth of 18–24 h seen in Figs. 2 and 3 may represent the time needed for reduction of eIF-5A below the threshold level through normal intracellular degradation and dilution by growth. Since the precise function of eIF-5A is unknown, it is, of course, impossible to delineate the sequence of cellular events that leads to growth arrest by GC,. Even though the effects of GC, on processes other than hypusine synthesis cannot be excluded, the correlation seen here between inhibition of hypusine synthesis and cell growth is consistent with deoxyhypusine synthase as the primary cellular target of GC,.

In contrast to GC, and GC, which are the most potent inhibitors of deoxyhypusine synthase, growth suppression by GC, GC,GC, and GC,GC may be due to other than inhibition of hypusine synthesis. These latter compounds all displayed strong inhibition of growth at 10 μM without a commensurate reduction in synthesis of hypusine. Intervention in other cellular processes in addition to hypusine synthesis probably contributes to the arrest of growth by these compounds.

An important aspect of the growth inhibition by the guanyldiamines reported here is its lack of dependence on depletion of spermidine or the other polyamines. It is well established that certain polyamine antimetabolites (31, 32), for example α-di-fluoromethylornithine, MGBG, and bisethylnospermine (35), halt cell proliferation through depletion of the polyamine spermidine. Although recent studies suggest that the growth arrest following spermidine depletion is a consequence of hypusine deprivation (20, 21), it is likely that depletion of spermidine also leads to disturbance of a number of other cellular processes. If this disruption in cell proliferation by polyamine antimetabolites is primarily due to the lack of hypusine or eIF-5A, selective inhibition of deoxyhypusine synthase should accomplish the same end without alteration in other cell functions that accompany depletion of spermidine. In this regard, the inhibitors that specifically target deoxyhypusine synthase, but not other polyamine biosynthetic or metabolic enzymes, may have advantages over the polyamine antimetabolites.

The information given in this report is limited to studies carried out with CHO cells. However, growth inhibition by guanyldiamines was observed with other mammalian cells, including a number of human cancer cell lines. Sensitivity to the guanyldiamines varied with the cell line. Although for the differences in sensitivity is not yet fully understood, it could be related to different rates of cellular uptake and efflux, to different intracellular target(s) and/or to differences in the intracellular metabolism of the compounds. These factors may form the basis of a selective inhibition and open the possibility for practical application of these compounds as chemotherapeutic agents against those tumor cells with, for example, a highly efficient polyamine transport system.
REFERENCES