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Effect of Polyamine Analogues on Hypusine Content in JURKAT T-Cells

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The availability of synthetic hypusine and deoxyhypusine has made it possible to develop analytical methods which allow for the measurement of these compounds in various tissues. The methods involve dansylation of extracts from the pellet remaining after perchloric acid precipitation of cell or tissue homogenates, followed by high-performance liquid chromatography. To demonstrate the utility of this approach, the impact of four polyamine analogues, N^1,N^{11}-diethylnorspermine (DENSPM), N^1,N^{14}-diethylhomospermine (DEHSPM), 1,6,12-triazadodecane [(4,5) triamine], and 1,7,13-triazatridecane [(5,5) triamine], on hypusine levels in a human T-cell line (JURKAT) is evaluated. All four analogues are active in controlling cell growth and compete well with spermidine for the polyamine transport apparatus. After 144 h of exposure to JURKAT cells, DENSPM reduces putrescine to below detectable limits and spermidine to 10% of the level in control cells. The other three analogues diminish both putrescine and spermidine to below detectable limits. The effectiveness with which the compounds lower spermine levels is DENSPM > DEHSPM > (4,5) triamine > (5,5) triamine. The analogues decrease the activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase in a similar fashion. Of the four polyamines, DENSPM and DEHSPM are potent at lowering intracellular hypusine levels after 144 h: $59 \pm 9\%$ and $73 \pm 12\%$ of control levels, respectively. The other two analogues have marginal effects.

Introduction

Eukaryotic initiation factor 5A (eIF-5A), a 17-kDa protein, was originally thought to be an essential factor in protein synthesis. This conclusion was based on the observation that eIF-5A stimulated methionyl-puromycin synthesis, a model reaction for formation of the first peptide bond during protein assembly.[1] However, when *Saccharomyces cerevisiae* was genetically manipulated such that eIF-5A protein is rendered unavailable, overall protein synthesis, rather than ceasing completely, was reduced by about 30%.[2] The implication is that eIF-5A may be associated with translation of a subgroup of mRNAs and not with global protein synthesis. The identification of the specific family of mRNAs has not been established.[2]

Studies suggest that eIF-5A is highly conserved among many eukaryotic species from yeast to higher mammals, an observation consistent with its importance from an evolutionary perspective.[3,4] What is most striking is the extreme conservation of the 12-amino acid region surrounding the hypusine residue, L-Ser-L-Thr-L-Ser-L-Lys-L-Thr-Gly-**Hpu**-L-His-Gly-L-His-L-Ala-L-Lys.[5]

Hypusine [(2*S*,9*R*)-2,11-diamino-9-hydroxy-7-azaundecanoic acid, Hpu] assembly involves the posttranslational modification of “immature” eIF-5A.[6] An aminobutyl group is first removed from spermidine; deoxyhypusine synthase catalyzes the attachment of the aminobutyl fragment to Lys-50 of the human protein.[7,8] This is followed by hydroxylation of C-9 in the (*R*)-configuration, catalyzed by deoxyhypusine hydroxylase.[6]

While the precise function (or functions) of hypusine in cellular biochemistry remains to be elucidated, from an operational perspective two issues are clear: eIF-5A is critical to mitotic processes,[9] and the hypusination of Lys-50 of eIF-5A is required for the protein’s activity.[10]

The protein eIF-5A has now been shown to be essential for the replication of human immunodeficiency virus (HIV). During viral replication, eIF-5A serves as a transactivating factor.[11] The eIF-5A molecule binds to a complex formed between the Rev response element (RRE) in the stem−loop IIB of the viral mRNA and Rev, a viral protein that serves as a nuclear export signal.[12] Once eIF-5A binds to Rev-RRE, the now active eIF-5A−Rev-RRE complex is able to be exported from the nucleus; viral replication ensues. In experiments in which antisense nucleotides were used to prevent eIF-5A synthesis, viral replication was inhibited.[11] It has also been demonstrated in gel shift experiments that the hypusine-containing fragments were required for this binding of eIF-5A to Rev-RRE.[13] Rev has domains which direct both nuclear import and nuclear export.[13,14] Certain eIF-5A mutants, while capable of being transported into the nucleus and binding to Rev-RRE, actually prevent nuclear export and, thus, viral replication.[13,14]

Two observations render inhibition of eIF-5A deoxyhypusination or hypusination an interesting target in therapeutic strategies for anticancer and antiviral drug development: eIF-5A is required for both mitotic events and HIV viral replication, and “immature” eIF-5A must be deoxyhypusinated or hypusinated for activity. However, implicit in the development of such therapeutic

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strategies is the ability to monitor deoxyhypusine and hypusine levels as a function of various cellular challenges. Without a reliable analytical method it would be impossible to accurately assess how changes in these two essential eIF-5A fragments impact viral replication. In the current study, we describe the development of such an analytical method and its application in evaluating how polyamine analogues affect hypusine and deoxyhypusine levels in JURKAT cells. The development of the analytical methodology was made possible by the availability of deoxyhypusine and hypusine as standards; these were accessed through the synthetic methods described in the companion paper.

Analytics

Previous methods of following the levels of deoxyhypusine and hypusine have generally involved exposing cells to spermidine that was tritium-labeled in the aminobutyl fragment. Incorporation of the radiolabel into deoxyhypusine or hypusine made it possible to identify hypusinated eIF-5A as it was biosynthesized.[6] While effective, these methods are labor-intensive and do not lend themselves to the determination of total deoxyhypusine or hypusine levels in tissue samples. Another method has been described in which protein hydrolysates were reacted with 4-(dimethylamino)-azobenzene-4′-sulfonyl chloride (DABSYL-Cl).[15] The authors were able to demonstrate that their precolumn derivatization of hypusine made it possible to quantitate hypusine using reverse-phase C_{18} HPLC. The methodology described generally works well, although the authors point out that the predabsylation column step, an Amberlite removal of "excess" amino acids, limits the sensitivity of this method. Furthermore, the applicability of the assay to the measurement of deoxyhypusine was not addressed.

On the basis of our previous experience with dansylation as an analytical method for quantitating natural polyamines, polyamine analogues, and their corresponding metabolites in tissue,[16-18] we extended this method to both hypusine and deoxyhypusine. The procedure developed first required homogenizing tissues, followed by a perchloric acid precipitation of protein; polyamine analysis can be performed on the supernatant. The protein pellet was hydrolyzed in 6 N HCl; the basic amino acid fraction, including hypusine and deoxyhypusine, was quantitatively separated from other amino acids on cellulose phosphate, a strong cation-exchange resin. A sample of the hypusine-containing amino acid solution was reacted with dansyl chloride in acetone in the presence of 1,6-diaminohexane (as an internal standard) and excess solid sodium carbonate at 70 °C. The dansylation reaction was terminated by addition of excess proline. A preliminary cleanup of the mixture was performed by loading the solution onto an SPE column. After loading, the sample was washed with acetonitrile–water, followed by elution with methanol.

Initially, when the method was tested using pure synthetic hypusine and/or deoxyhypusine, the methanol cut was run immediately on HPLC utilizing a methanol/acetonitrile/water gradient. Under these conditions, we observed three peaks derived from the reaction of dansyl chloride with either hypusine or deoxyhypusine, depicted as peaks A, B, and C for hypusine and peaks a, b, and c for deoxyhypusine in Figure 1; DHX in the figure represents the 1,6-diaminohexane internal standard. In each case the central peak (B or b) was the largest; however, over time this peak appears to convert to peak A (or a), the signal with the shortest retention time. After remaining in methanol for 12 h peak A (in the case of hypusine) increases from 5% to 85%; peak B decreases from 80% to 10%; peak C decreases from 13% to 5% of the total peak area (A + B + C). The scenario is very similar with deoxyhypusine, i.e., peak a increases in size from 5% of the total peak area (a + b + c) to 90%, while peak b decreases from 80% to 5% of total area, and peak c decreases from 20% to 5%. A time-course study revealed that after 15 h peaks A and a in both cases are stable as well as easily quantified; this stability was sustained for the remainder of the 48-h analysis (Figure 2). There is a linear relationship between the fluorescence signal and the concentration of both hypusine and deoxyhypusine synthetic standards. Protein samples were 'spiked' with known amounts of synthetic standard at different stages in the analytical procedure. Samples spiked before the hydrolysis step showed a recovery of 74 ± 12.5% of the added standards. Samples spiked at any stage after the

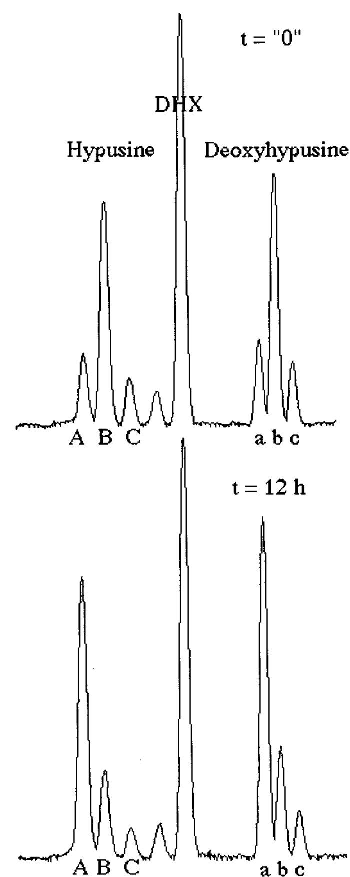

Figure 1. Chromatographs of dansylated hypusine and deoxyhypusine. The upper panel represents the peaks found when the dansylated product is run on HPLC immediately (time = 0); the lower panel represents the products found when the HPLC is performed 12 h after dansylation. The uppercase letters represent the peaks obtained from hypusine; the lowercase letters represent the peaks obtained from deoxyhypusine. DHX, 1,6-diaminohexane internal standard.

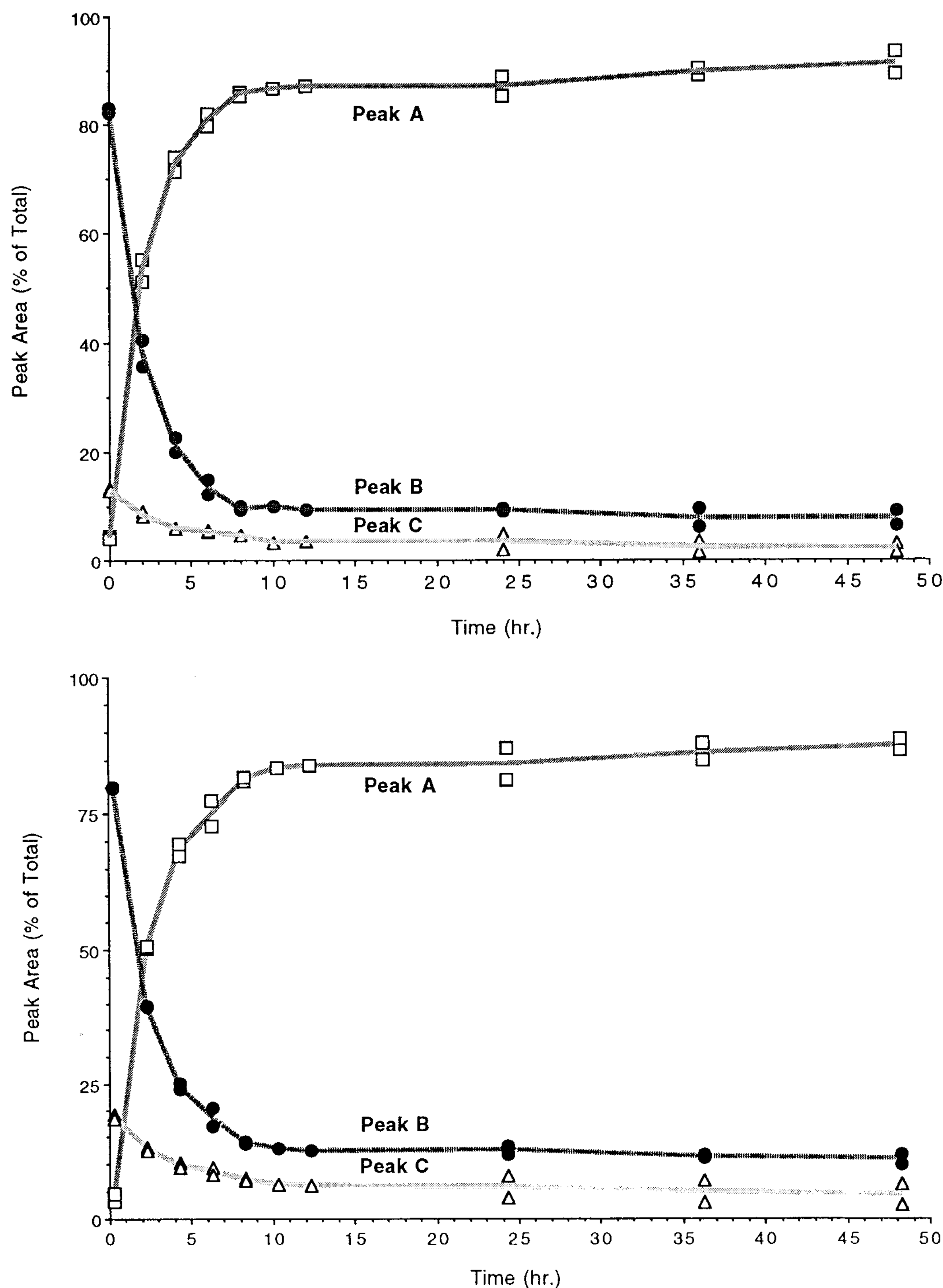

Figure 2. Time course analysis of dansylated hypusine (top) and deoxyhypusine (bottom) from 0 to 48 h. Peaks A, B, and C in the top and bottom panels correspond, respectively, to peaks A−C and a−c in Figure 1.

hydrolysis step yielded a greater than 95% recovery of added standard.

Liquid chromatography−electrospray tandem mass spectrometry of the dansylated polyamines, hypusine, and deoxyhypusine revealed that peaks A and B in the case of hypusine (and peaks a and b for deoxyhypusine) differed by 32 mass units. When the same procedure was run substituting ethanol for methanol, peaks A and B (and a and b) gave a difference of 46 mass units. This is consistent (e.g., in the case of deoxyhypusine) with structures **2** and **3** (Figure 3) which are distinguished by conjugate addition of either methanol or ethanol, respectively, to the α,β-unsaturated carboxylic acid of disulfonamide **1**. While speculative at best, compound **1** could arise under the reaction conditions employed in the assay, i.e., high temperature, strong base, and excess dansyl chloride, from a series of dansyl sulfinate eliminations to produce an imine followed by cyclization and redansylation.

Biological Evaluations

Because of the interest in the role that eIF-5A plays in the replication of HIV and because the virus infects T-cells, we chose to examine hypusine biosynthesis in JURKAT clone E6-1, an acute T-cell leukemia line. This line is often used as a model in following HIV infection

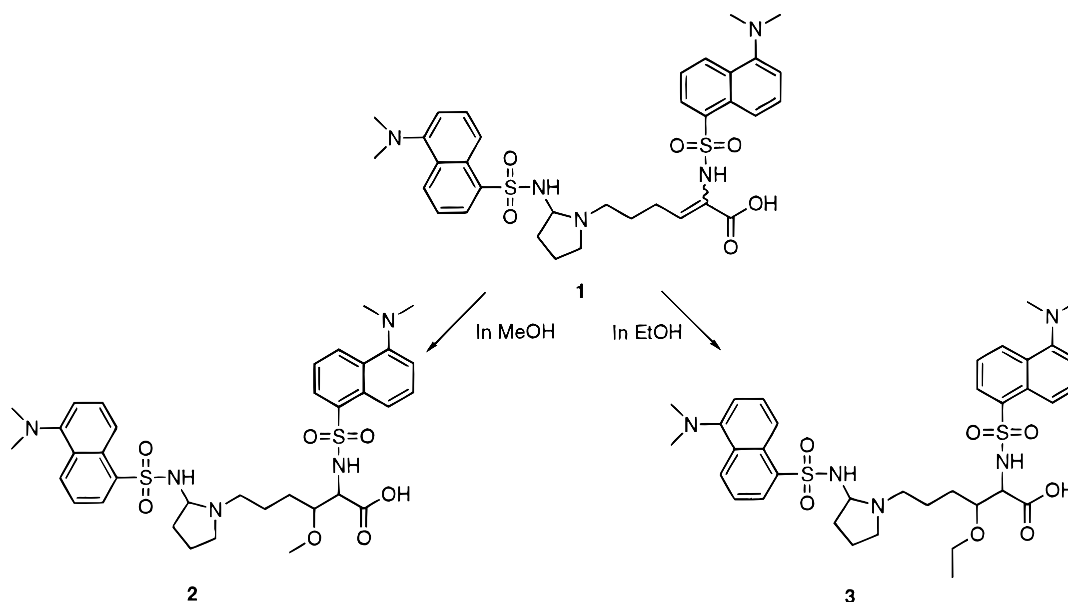

Figure 3. Potential side product of deoxyhypusine from the dansylation procedure (**1**). It is possible that this compound, upon addition of methanol or ethanol, would result in compounds **2** and **3**, respectively.

parameters in vitro. Of course, JURKAT cells are rapidly dividing and likely to have constitutively high levels of eIF-5A. In contrast, resting T-cells contain much lower concentrations of eIF-5A;[19] any extension of our findings to resting T-cells must be made with caution.

Four polyamine analogues were evaluated in the JURKAT cell line—two tetraamines [N^1,N^{11}-diethylnorspermine (DENSPM) and N^1,N^{14}-diethylhomospermine (DEHSPM)] and two triamines (1,6,12-triazadodecane [(4,5) triamine] and 1,7,13-triazatridecane [(5,5) triamine]). The (4,5) and (5,5) triamines were assessed because of their profound impact on spermidine pools in L1210 cells[16] and the homology between the two molecules. Recall that the aminobutyl fragment of hypusine is derived from spermidine. Therefore, the (4,5) triamine could provide an aminobutyl fragment in hypusine biosynthesis; the (5,5) triamine could not. We have measured the growth-inhibiting properties of these analogues in these cells (Table 1), the ability of the analogues to compete with spermidine for the polyamine transport apparatus (Table 1), their effects on native polyamine pools and hypusine biosynthesis (Table 2), and the activities of ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC) (Table 3). The influence of DENSPM and DEHSPM on JURKAT cell growth and metabolism is most interesting because we have substantial data in a number of cell lines regarding the IC_{50}, impact on polyamine pools, effect on the activity of enzymes involved in polyamine metabolism (ODC, AdoMetDC, and spermidine/spermine N^1-acetyltransferase), and metabolic fate of these compounds.[20-24] We have also documented the metabolic, pharmacokinetic, and toxicity profiles of DENSPM and DEHSPM in dogs and rodents.[17,18] These two tetraamines are currently in clinical trials as well, DENSPM as an antineoplastic and DEHSPM for treatment of AIDS-related diarrhea.

Antiproliferative Activity: IC_{50}. At 144 h, DENSPM, DEHSPM, and (5,5) triamine were all similar in their ability to inhibit the growth of JURKAT cells with IC_{50} values of 0.65, 0.2, and 1.2 μM, respectively. However, the (4,5) triamine had an IC_{50} value of 70 μM, Table 1.

Table 1. Effect of Polyamine Analogues on the Polyamine Transport in and the Growth of JURKAT Cells

compound	IC_{50} (μM, 144 h)[a]	K_i, μM[b]
DENSPM	0.65 (2.0)[c]	14.4 (17)
DEHSPM	0.2 (0.07)	8.8 (2.0)
(4,5) triamine	70 (0.18)	2.8 (1.4)
(5,5) triamine	1.2 (0.4)	13.8 (14)

[a] IC_{50} was estimated from growth curves for JURKAT cells grown in the presence of nine different concentrations of drug spanning 4 log units: 0, 0.03, 0.1, 0.3, 1.0, 3, 10, 30, and 100 μM. IC_{50} data are presented as the mean of at least two experiments with variation from the mean typically 10−25% for the 144-h IC_{50} values. [b] K_i determinations were made by following analogue inhibition of spermidine transport. All polyamine analogues exhibited simple substrate-competitive inhibition of [^{3}H]SPD transport by JURKAT cells. Each experiment examined the rate of uptake of [^{3}H]SPD at six different SPD concentrations in the absence of inhibitor (analogue) and at three different inhibitor concentrations (50, 25, and 10 μM). Values reported in the table represent the mean of at least two or three such experiments with a variation typically less than 10%. [c] The 96-h IC_{50} values and K_i results for L1210 cells are shown in parentheses following the value obtained in JURKAT cells.[16,25]

This behavior is somewhat different than that observed in L1210 cells.[16,25] While it is not completely legitimate to directly compare the results obtained in JURKAT cells to those obtained in L1210 cells because of the differences in doubling times (~12 h for L1210, ~23 h for JURKAT), the trends are still notable. In L1210 cells after 96 h of exposure as in JURKAT cells after 144 h, DEHSPM was more active than DENSPM with IC_{50} values of 0.07 and 2.0 μM, respectively.[25] The difference is thus more pronounced in L1210 cells than in JURKAT cells. Both triamines displayed similar activity in L1210 cells with a 0.18 μM IC_{50} value for the (4,5) triamine and a 0.4 μM value for the (5,5) triamine.

Competitive Uptake in JURKAT Cells: K_i. At the level of polyamine transport, the tetraamines also behaved somewhat differently in JURKAT cells than in L1210 cells (Table 1). In L1210 cells, DEHSPM was

Table 2. Effect of Polyamine Analogues on Native Polyamine and Hypusine Levels in JURKAT Cells

compound	concn (μM)	analogue[a]	PUT[b]	SPD[b]	SPM[b]	Hpu[b]
DENSPM	2 (10)[c]	1.72 (1.6)	0 (30)	9.2 ± 2.1 (14)	5.5 ± 1.5 (31)	58.9 ± 8.6
DEHSPM	2 (10)	2.29 (3.0)	0 (0)	0 (0)	13.4 ± 2.0 (61)	73.2 ± 12.1
(4,5) triamine	5 (100)	2.13 (3.0)	0 (0)	0 (0)	25.2 ± 4.4 (53)	80.2 ± 12.1
(5,5) triamine	5 (100)	2.73 (2.6)	0 (0)	1.1 ± 1.6 (0)	38.8 ± 7.8 (33)	86.4 ± 5.5
DENSPM-L[d]	2	1.48	0	3.8 ± 1.1	13.4 ± 1.6	56.8 ± 14.9

[a] Following treatment with the concentration of the compound indicated, nmol of intracellular analogue/10^6 cells. The results shown are the means of 2−5 determinations, each performed in triplicate. [b] Following treatment with the concentration of the compound indicated, percent of putrescine (PUT), spermidine (SPD), and spermine (SPM) or hypusine (Hpu) levels in control cells after 144 h. The results shown are the means ± standard deviations of 2−5 determinations, each being performed in triplicate. Typical control values in pmol/10^6 JURKAT cells are PUT = 49, SPD = 1812, SPM = 1976, Hpu = 1548.Under the experimental conditions, treated cell growth was typically 26%, 33%, 58%, and 38% of that of untreated cells for DENSPM, DEHSPM, (4,5) triamine, and (5,5) triamine, respectively. [c] The values in parentheses represent the concentration used and the results obtained in L1210 cells over a 48-h treatment period.[16,25] [d] This row shows the values obtained for polyamine pools and hypusine levels in L1210 cells treated with DENSPM for 72 h, equivalent to the same number of doubling times as in 144 h in JURKAT cells. Typical control values in pmol/10^6 L1210 cells are PUT = 105, SPD = 2337, SPM = 469, Hpu = 1089. Analogue amount is expressed as nmol/10^6 cells.

Table 3. Effect of Polyamine Analogues on Polyamine Biosynthetic Enzymes in JURKAT Cells

compound	ODC[a]	AdoMetDC[a]
DENSPM	16.9 ± 2.7 (10)[b]	51.4 ± 7.3 (42)
DEHSPM	29.8 ± 10.8 (7)	61.6 ± 0.5 (41)
(4,5) triamine	34.7 ± 8.2 (19)	79.3 ± 1.6 (57)
(5,5) triamine	53.3 ± 8.2 (16)	84.9 ± 2.3 (88)

[a] Expressed as percent of control (untreated) cells. In the ODC assay, the cells were exposed to 1 μM drug for 4 h before evaluating enzyme activity; in the AdoMetDC assay, the cells were incubated with the same concentration of drug for 6 h. Each experiment included a positive 'control' (DEHSPM) in L1210 cells which had a known, reproducible impact on enzyme activities in this cell line (mean ± SD): 1 μM DEHSPM reduced ODC to 6.7 ± 2.6% of untreated control; 1 μM DEHSPM lowered AdoMetDC to 40.7 ± 6.2% of untreated control. [b] The values in parentheses are those obtained under the same reaction conditions in L1210 cells.[16,25]

8-fold more effective at competing with labeled spermidine for the polyamine transport apparatus than was DENSPM with K_i values of 2 and 17 μM, respectively.[25] In contrast, the K_i values observed in JURKAT cells were relatively close to each other, 9 and 14 μM, respectively (Table 1).

The ability of the triamines to compete with spermidine for the polyamine transport apparatus was strikingly similar between the two cell lines. The (4,5) triamine had a K_i of 1.4 μM in L1210 cells;[16] the observed K_i value in JURKAT cells was 2.8 μM (Table 1). The (5,5) triamine was a less effective competitor in both cell lines with a K_i of 14 μM in L1210 cells[16] and a K_i of 13.8 μM in JURKAT cells (Table 1).

Impact of Analogues on Polyamine Pools in JURKAT Cells. In parallel with the IC_{50} and K_i findings, a similar trend was present regarding the effect of DENSPM and DEHSPM on polyamine pools in JURKAT cells (Table 2) as in L1210 cells. In L1210 cells, after 48 h (4 doubling times) of exposure to 10 μM DENSPM, putrescine, spermidine, and spermine were reduced to 30%, 14%, and 31%, respectively, of control values; the analogue accumulated to 1.6 nmol/10^6 cells. DEHSPM was more effective at decreasing putrescine and spermidine but not spermine, amassing to 3 nmol/10^6 cells and lowering the pools to 0%, 0%, and 61% of controls.[25] In JURKAT cells, after 144 h of exposure (6 doubling times) to 2 μM DENSPM, the intracellular analogue level reached 1.7 nmol/10^6 cells; the putrescine, spermidine, and spermine were 0%, 9%, and 6% of control values, respectively (Table 2). A 144-h treatment of JURKAT cells with 2 μM DEHSPM resulted in drug levels of 2.3 nmol/10^6 cells and diminished putrescine, spermidine, and spermine to 0%, 0%, and 13% of the values in untreated JURKAT cells.

The effects of the (4,5) and (5,5) triamines on polyamine pools in both L1210 and JURKAT cells were quite profound. In L1210 cells, the cells were exposed to a 100 μM concentration of triamines, either (4,5) or (5,5).[16] In 48 h, this dose of either drug lowered putrescine and spermidine to 0% with a decline of spermine to 53% of the control value with (4,5) triamine and to 33% of the control value in the case of (5,5) triamine. Under the conditions of this experiment, the (4,5) and (5,5) triamines accumulated to levels of 3 and 2.6 nmol/10^6 cells, respectively. In JURKAT cells, treatment with 5 μM (4,5) triamine resulted in the decrease of putrescine and spermidine to 0% after 144 h; spermine levels were 25% of control. This analogue reached levels of 2.1 nmol/10^6 cells (Table 2). Treatment of JURKAT cells with 5 μM (5,5) triamine also diminished putrescine and spermidine to or near 0% with spermine remaining at 39% of control values, and the analogue level was 2.7 nmol/10^6 cells.

Consequences of Analogue Treatment on Hypusine Levels in JURKAT Cells. At 144 h of treatment with the polyamine analogues evaluated, only DENSPM markedly curtailed the level of hypusine in JURKAT cells (Table 2). After this exposure time, 2 μM DENSPM lowered hypusine to 59% of control. Treatment of JURKAT cells with 2 μM DEHSPM did not have as notable an effect, reducing hypusine to 73% of control levels. Neither 5 μM (4,5) triamine nor (5,5) triamine had an appreciable impact on hypusine amounts, with levels falling to 80% and 86% of those in untreated cells, respectively. What is more interesting is the fact that at 144 h DEHSPM and (4,5) triamine depleted spermidine to below detectable limits (i.e., <15 pmol/10^6 cells). On this basis alone, DEHSPM and (4,5) triamine would be expected to be even more effective than DENSPM at reducing hypusine. At 216 h, all four of the polyamine analogues exhibited a rather substantial impact on hypusine levels. Hypusine was attenuated by DENSPM to 43%, DEHSPM to 45%, (4,5) triamine to 51%, and (5,5) triamine to 56% of controls after this longer exposure time.

Finally, we chose to examine the effect of DENSPM on L1210 cell hypusine levels. For purposes of comparison, L1210 cells were exposed to 2 μM DENSPM for 72 h. L1210 cells undergo the same number of cell doublings during this time period as JURKAT cells do

in 144 h. It is not too surprising, based on the fact that DENSPM impacts on polyamine pools and enzymes in L1210 cells much the same way it does in JURKAT cells, that hypusine was diminished to about the same extent in both cell lines, 57 ± 15% in L1210 and 59% in JURKAT (Table 2).

Impact of Analogues on ODC and AdoMetDC Activities. All four polyamine analogues lowered ODC activity in JURKAT cells (Table 3) after a 4-h exposure of the cells to 1 μM polyamine analogue; however, the response was not as great as that seen in L1210 cells. The most effective compound in JURKAT cells was DENSPM with a decrease in activity to 17% of control values; the least was (5,5) triamine, which diminished ODC activity to 53% of that seen in untreated cells. In contrast, in L1210 cells there was less of a difference observed among the analogues. DEHSPM was the most effective at depleting ODC activity, to 7% of control, followed by DENSPM (10%), (5,5) triamine (16%), and (4,5) triamine (19%).[16,25] After 6 h of treatment with 1 μM polyamine analogue, all four compounds again reduced AdoMetDC activity. The effects, however, were not as profound as those seen with ODC. DENSPM was the most effective at lowering AdoMetDC with the depletion in JURKAT cells to 51% of that in untreated cells; (5,5) triamine again showed the least impact on AdoMetDC activity, a reduction to 85% of that in untreated cells. In this instance, the difference between JURKAT cells and L1210 cells was not as significant. In L1210 cells, DEHSPM reduced AdoMetDC activity the most, to 41% of controls; the order of effectiveness for the remaining analogues was DENSPM (42%), (4,5) triamine (57%), and (5,5) triamine (88%).[16,25]

Metabolites. In an earlier study in vivo, we demonstrated that DENSPM and DEHSPM were dealkylated in various tissues.[17,18] Additionally, in L1210 cells,[16] we were able to show that DENSPM was converted to N^1-monoethylnorspermine and subsequently to either norspermine via a second deethylation or to N^1-monoethylnorspermidine via deaminopropylation. Finally, N^1-monoethylnorspermidine can be catabolized to either norspermidine or N^1-methyl-1,3-diaminopropane. With DEHSPM in L1210 cells, we did not observe any catabolism.[16] In the current series of experiments in JURKAT cells, DENSPM catabolism was barely detectable; no metabolic products assignable to DEHSPM were found. When we analyzed the levels of metabolites of the (4,5) and (5,5) triamines, we found that cadaverine in treated JURKAT cells was "questionably" detectable.

Discussion

Dansylation of synthetic hypusine and deoxyhypusine proved to be an excellent method for quantitating these amino acids in tissues. Dansylation methodologies therefore make it possible to evaluate polyamine analogue uptake in cells, determine cellular polyamine pools, and follow the impact of polyamine analogues on hypusine levels, all in the same biological sample.

Of the four analogues studied—DENSPM, DEHSPM, (4,5) triamine, and (5,5) triamine—DENSPM was the most effective at suppressing hypusine content in JURKAT cells. The suppression was, as expected, time-dependent in each case. The longer the JURKAT cells were treated with the polyamine analogues, the lower the hypusine level became. Recall that eIF-5A has a rather protracted half-life. These two observations are consistent because even though the analogues suppress the formation of spermidine, a requisite substrate for deoxyhypusine synthase, time is required for the hypusinated eIF-5A already present in the cells to turn over. In addition, because of the very low K_m (10^{-9} M) any available spermidine that would arise, for example, from the degradation of spermine, would be likely utilized by this enzyme, further extending the time required for an impact of the analogues on hypusine synthesis to be observed.

JURKAT cells were chosen simply because they are of human T-cell origin and have served as models in other HIV studies. A comparison of the polyamine metabolic properties of JURKAT cells with a murine leukemia cell line, L1210 cells, revealed a number of interesting similarities. At the level of growth inhibition, the compounds were all similar in behavior except for (4,5) triamine which was much less active in JURKAT cells. Except for DEHSPM, the K_i values for all four analogues were quite similar in the two cell lines. DEHSPM was about six times more effective at competing for the polyamine transport apparatus in L1210 cells than in JURKAT cells. The analogues were all substantially more potent in L1210 cells than in JURKAT cells at reducing ODC activity. While the difference between L1210 and JURKAT cells was not as profound with AdoMetDC, the analogues except for (5,5) triamine still exhibited a greater impact on enzyme activity in L1210 cells. The four analogues were, at similar intracellular concentrations, about as useful in JURKAT and L1210 cells at suppression of putrescine and spermidine; however, the analogues were generally more effective at decreasing spermine in JURKAT cells. Of course, this must be taken with the caveat that JURKAT cells have a doubling time of about 23 h, whereas L1210 cells have a 12-h doubling time.

Whereas certain polyamine analogues appear to affect intracellular hypusine concentrations, the mechanistic details of hypusine suppression remain to be clarified. Among the possible explanations are (1) inhibition of growth of JURKAT T-cells, in effect returning them to a more quiescent state akin to nonleukemic, resting T-cells;[19] (2) increased degradation of eIF-5A; or (3) decreased rate of hypusine formation due to deprivation of spermidine, the aminobutyl donor, by polyamine analogues.

Experimental Section

Chemical reagents were purchased from Aldrich, Fluka, or Sigma Chemical Co. and used without further purification. Cellgro RPMI-1640 medium was obtained from Fisher Scientific (Pittsburgh, PA); α-MEM was purchased from Life Technologies, Inc. (Gaithersburg, MD). Fetal bovine serum, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 3-(N-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma. Cell numbers were determined by electronic particle analysis (Coulter counter model Z_F, Coulter Electronics, Hialeah, FL). JURKAT clone E6-1 (ATCC TIB-152) and Chinese hamster ovary (CHO) (ATCC 1781 Pro5) cells were obtained from the American Type Culture Collection (Rockville, MD). The solid-phase extraction columns (SPE; 3 mL, 500 mg) were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ).

[^{3}H]Spermidine for uptake determinations and acetyl coenzyme A (acetyl-*1*-^{14}*C*) were purchased from NEN Life Science (Boston, MA). L-[*Carboxyl*-^{14}C]ornithine and *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine for enzyme assays were obtained from Amersham Corp. (Arlington Heights, IL).

Cell Culture and IC$_{50}$ Determination. JURKAT cells were maintained in logarithmic growth in RPMI-1640 medium containing 10% fetal calf serum, 7.5% (w/w) sodium bicarbonate, 2 mM L-glutamine, and 1 mM aminoguanidine. CHO cells were cultured in α-MEM medium supplemented with 10% fetal calf serum and 1 mM aminoguanidine. The IC$_{50}$, the concentration of compound which reduces cell growth to 50% of untreated control cell growth, was determined in JURKAT cells after 144 h of exposure to the polyamine analogues as detailed elsewhere.[25]

Uptake Determinations. DENSPM, DEHSPM, (4,5) triamine, and (5,5) triamine were studied for their ability to compete with [^{3}H]spermidine for uptake into JURKAT cells.[25] Lineweaver–Burke plots indicated simple competitive inhibition with respect to spermidine.

Polyamine Pool Analysis. JURKAT cells in logarithmic growth were treated with polyamine analogue for 144 h at the concentrations indicated in Table 2 (2 μM for the tetraamines, 5 μM for the triamines). The cells were washed twice with ice-cold RPMI-1640, and the pellet was treated with 1.2 N $HClO_4$ (1 mL/10^7 cells). Polyamine contents of the perchloric acid extracts were quantitated by reversed-phase HPLC of the DANSYL derivatives as previously described.[20] Briefly, separation was on a C$_{18}$ support, elution was with a gradient of 63% aqueous acetonitrile and 93% aqueous methanol, and detection was with fluorescence.

Hypusine and Deoxyhypusine Analysis. The cell pellet remaining after perchloric acid extraction of polyamines was washed with 1.2 N perchloric acid (2 × 1 mL). The washed pellet was resuspended in 6 M HCl (1 mL) and transferred to a heavy glass reaction tube (Ace Glass Co., Vineland, NJ). The tube was rinsed with additional 6 M HCl (1 mL), and the rinse was added to the reaction tube. The reaction tube was flushed with Ar and placed in a 110 °C oil bath for approximately 14 h. The hydrolysate was cooled and evaporated to dryness on a vacuum centrifuge without heat (Savant SC210A). The residue was resuspended in water (1 mL); sonication was used if required. About 5 μL of 5 M potassium acetate was added to neutralize the remaining HCl. The sample was transferred to a cellulose phosphate column (1.4 mL of bed volume activated with 3 M HCl and washed with water until the rinse was neutral). The column was eluted successively with water (1 mL) to remove nonbasic amino acids, 3 M HCl (2.5 mL), and water (5 mL); the latter two fractions were collected together. The acidic fractions containing hypusine and basic amino acids were combined and evaporated to dryness. The residue was dissolved in 0.6 N HCl (300 μL) and dansylated in the following manner. To a 50-μL sample, 120 mg of solid anhydrous Na_2CO_3, 400 μL of a 10 mg/mL solution of dansyl chloride in dry acetone, and 30 μL of 1.25×10^{-4} M 1,6-diaminohexane in 0.6 N $HClO_4$ were added. After mixing, the vial (protected from light from this point forward) was placed in a water bath at 70 °C for 20 min and then cooled to room temperature. Excess dansyl chloride was reacted with 100 μL of a solution of L-proline (250 mg/mL in water), and the mixture was incubated at room temperature for 10 min. Five hundred microliters of 25% aqueous acetonitrile was added to the sample, the solution was vortexed, and this plus two additional 500-μL rinses of 25% acetonitrile were applied to a C$_{18}$ SPE column (washed with 3 column volumes of methanol and 3 column volumes of 25% aqueous acetonitrile). After application of vacuum, the columns were washed with 25% aqueous acetonitrile (2 × 2.5 mL). The sample was eluted with methanol (3 mL); HPLC analysis as performed for the dansylated polyamines (above) injected 20 μL of this eluate.

Enzyme Assays. ODC and AdoMetDC activities were determined as $^{14}CO_2$ released from [^{14}C]carboxyl-labeled L-ornithine[26] or *S*-adenosyl-L-methionine,[27] respectively. In the ODC assay, the cells were exposed to 1 μM of the drug for 4 h before evaluating enzyme activity; in the AdoMetDC assay, the cells were incubated with the same concentration of drug for 6 h. Included in each assay were untreated JURKAT cells as negative controls as well as cells treated with DEHSPM, a drug having a known reproducible effect on each enzyme in L1210 cells, as positive controls.

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