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Effect of N^8-Acetylspermidine Deacetylase Inhibition on the Growth of L1210 Cells

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ABSTRACT. A selective inhibitor of N^8-acetylspermidine deacetylase has been employed to study the role of N^8-acetylspermidine deacetylation in the regulation of L1210 cell growth. This inhibitor, 7-[*N*-(3-aminopropyl) *a*mino] *h*eptan-2-one (APAH), was found to stimulate the growth of L1210 cells at concentrations between 10 μM and 0.5 mM. Maximum stimulation was seen at 100 μM, resulting in significantly increased rates of cell division and maximum cell density. N^8-Acetylspermidine levels in L1210 cells were shown to increase significantly after the APAH treatment as would be expected for deacetylase inhibition. The effects of deacetylase inhibition were mimicked by addition of N^8-acetylspermidine to the culture medium at concentrations greater than 1 mM as indicated by a subsequent increase in rate of cell growth and maximum cell density. The magnitudes of the increases in growth observed were not large, but this might be expected in cells that are already in a rapid growth phase. Other exogenously added polyamines including N^1-acetylspermidine, spermidine, putrescine, and spermine did not stimulate cell growth. These data suggest that stimulation of cell growth occurs as a consequence of N^8-acetylspermidine accumulation and N^8-acetylspermidine deacetylase inhibition. BIOCHEM PHARMACOL **57**;10:1095–1103, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. polyamine; N^8-acetylspermidine; N^8-acetylspermidine deacetylase; APAH; cell growth; L1210 cells

Spermidine is a member of a group of ubiquitous compounds known as the polyamines, which are found in virtually all living organisms from bacteria to humans. These polyamines appear to play a vital role in a variety of cellular processes involving nucleic acids [1, 2]; however, the specific biological functions of spermidine and related compounds remain to be elucidated. Spermidine contains three nitrogens joined by a 3-carbon and a 4-carbon aliphatic chain. It has been shown to undergo two different enzyme-catalyzed *N*-acetylation reactions in mammalian tissues [3], with acetylation on the nitrogen adjacent to the 3-carbon chain to yield N^1-acetylspermidine receiving the most attention [4]. N^1-Acetylspermidine can be further oxidatively deaminated to yield putrescine by polyamine oxidase, providing a key step in the polyamine metabolic cycle.

The present study is focused on the less well characterized metabolism of spermidine, involving acetylation on the terminal nitrogen adjacent to the 4-carbon chain to produce N^8-acetylspermidine. The reaction is catalyzed by spermidine N^8-acetyltransferase [5]. It is a nuclear enzyme activity associated with chromatin and possibly the same enzyme(s) responsible for histone acetylation [6]. N^8-Acetylspermidine is quickly deacetylated back to spermidine by the cytosolic deacetylase [7], the focus of this study.

Given the apparent rapid turnover of N^8-acetylspermidine, the significance of this metabolite and the pathway are not fully understood. N^8-Acetylspermidine has been shown to induce differentiation of HL60 cells [8]. It has been suggested that the N^8-acetylation pathway serves only as a storage form of spermidine for rapid releases [9]. Since either N^1- or N^8-acetylation of spermidine decreases its ability to stabilize DNA [10], it has been proposed that the acetylation of spermidine in the cell nucleus may influence its interaction with DNA and thereby affect processes such as DNA replication or transcription and cell proliferation [10, 11]. Moreover, the unique nuclear localization of N^8-acetyltransferase would suggest that N^8-acetylation might have a greater chance than N^1-acetylation of influencing DNA stability and transcription and cell proliferation.

To test the latter hypothesis, we have developed means of altering the levels of N^8-acetylspermidine in tissues or cultured cells through synthesis of several N^8-acetylspermidine deacetylase inhibitors [12]. *In vitro* studies with these synthetic compounds have confirmed their inhibitory effects and have led to selection of a particular inhibitor for this study. Bioisosteric replacement of the 8-nitrogen of N^8-acetylspermidine with a carbon resulted in a potent

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inhibitor, APAH* [12]. Initial *in vitro* studies have shown that it is not only a potent inhibitor, with a K_i of 0.18 μM, but also a selective inhibitor. When mice were administered 200 mg/kg of APAH, i.p., N^8-acetylspermidine was raised to detectable levels in the liver and spleen. N^8-Acetylspermidine also was increased in HeLa cells after the treatment with APAH [13]. The availability of this inhibitor has enabled us to determine in the present study the effects of inhibition of N^8-acetylspermidine deacetylation and of the resultant elevated levels of intracellular N^8-acetylspermidine on growth and polyamine content of L1210 cells in culture.

MATERIALS AND METHODS

Materials

Putrescine, N^1-acetylspermidine, N^8-acetylspermidine, spermidine, spermine, DAH, and aminoguanidine bicarbonate were purchased from the Sigma Chemical Co. RPMI 1640 medium, penicillin/streptomycin, and horse serum were obtained from GIBCO. APAH was synthesized as described previously [12].

Cell Culture

L1210 cells were obtained from the American Type Culture Collection (ATCC CCL 219) and grown in 25-cm^2 plastic tissue culture flasks as suspension cultures in RPMI 1640 medium, supplemented with 10% (v/v) horse serum, 100 μg/mL of streptomycin, and 100 IU/mL of penicillin. Cultures were maintained at 37° in a CO_2 incubator (VWR model 1710) under humidified air containing 5% CO_2. For the experiments in which APAH or polyamines were added, aminoguanidine (1 mM) was added to the medium to inhibit the amine oxidases present in the serum.

Treatment of Cell Cultures with APAH or Other Polyamines

L1210 cells were seeded at a density of approximately 5×10^4 cells/mL into 25-cm^2 flasks, and allowed to grow for 1 day before the treatment. All compounds were dissolved in sterile water as 100-fold concentrated stock solutions, filtered through 0.22-μm sterile filters, and added directly into the culture medium to yield desired concentrations. The control cultures received equal volumes of sterile water.

Evaluation of Cell Cultures

Cell numbers were counted in a hemocytometer. Cell viability was assessed by trypan blue dye exclusion (0.4% trypan blue stain in 0.85% saline). The effects of the exogenous compounds on the L1210 cell culture were evaluated based on the growth curves, mPDT, the slope of the log growth phase, and cell density (cell number/mL of suspension culture). Cell density was expressed as "% of control" by the following formula:

$$\text{Cell density (\% of control)} = \left\{\frac{(\text{Cell density}_{\text{treated cells}}) - (\text{Initial inoculum})}{(\text{Cell density}_{\text{untreated cells}}) - (\text{Initial inoculum})}\right\} \times 100\%$$

mPDT was calculated from the exponential growth phase as the following:

$$\text{mPDT} = (\Delta T \times \log 2)/\log (C_t/C_o)$$

where C_o is the initial cell density; C_t is the cell density at time t; ΔT is the time between the time t and the initial point.

Determination of Polyamine Contents in L1210 Cells by HPLC

Numerous methods have been developed for the separation and detection of polyamines in biological samples (see Ref. 14 for a recent review). HPLC and gas chromatography are most commonly used. For this study, cellular polyamine levels were measured by HPLC as previously reported [13], with small modifications. Briefly, aliquots of L1210 cell suspensions containing approximately 10^7 cells were pelleted by centrifugation (700 *g*, 10 min) and washed twice with 1 mL of ice-cold PBS (pH 7.4). PBS supernatant was removed carefully, and the remaining cell pellets were extracted with 0.5 mL of 0.6 M perchloric acid containing 1 μM DAH as an internal standard to control for extraction efficiency. The acid-insoluble material was removed by centrifugation at 700 *g* for 10 min (4°), and the supernatant was frozen at −80° until analysis by HPLC. Each supernatant was assayed by HPLC at least twice.

Polyamines were separated by reversed-phase HPLC (Spectra-Physics) with a μBondapak C18 column. The elution gradient consisted of two buffers: A and B [15]. Buffer A was 0.1 M sodium acetate adjusted to pH 4.50 with acetic acid and contained 10 nM octane sulfonate. Buffer B consisted of two portions. Portion 1 maintained the same pH and octane sulfonate content as buffer A, with an increase of sodium acetate concentration to 0.2 M. Before use, portion 1 was mixed with portion 2 (HPLC grade acetonitrile) at a ratio of 10:3. We developed a gradient that allowed completion of each individual assay within 35 min. The gradient was started with 80% buffer A and 20% buffer B, and changed at a rate of 4% per min over 20 min to a final composition of 100% B and 0% A. This composition was maintained for 10 min. Then, over the next 5 min, buffer B was decreased linearly to 20% while buffer A was increased from 0 to 80% so that the HPLC system was ready for the next sample.

Post-column derivatization (in-line) of polyamines with *o*-phthalaldehyde reagent delivered by a mini-pump al-

* *Abbreviations*: APAH, 7-[*N*-(3-aminopropyl) amino] heptan-2-one; DAH, 1,7-diaminoheptane; and mPDT, mean population doubling times.

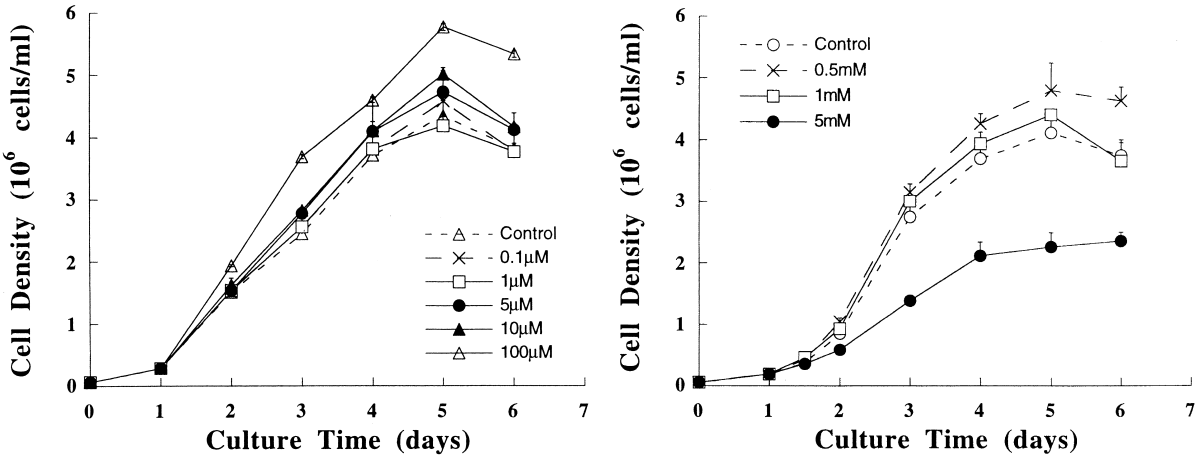

FIG. 1. Growth of cultured murine L1210 cells after treatment with APAH at concentrations between 0.1 and 100 μM (left panel), or 0.5 and 5 mM (right panel). Cells were treated with various concentrations of APAH on day 1 and allowed to grow for 5 more days during the experiment. Each point represents the mean ± SD of duplicate counts of at least three cell culture flasks.

lowed the rapid detection of these compounds in a flow cell-equipped fluorometer (Perkin-Elmer 204A) [13]. The excitation wavelength was set at 345 nm, and emission was measured at 455 nm. Quantitation was achieved using polyamine standards with DAH as an internal standard to control for variations among individual samples.

Statistical Analysis

Student's *t*-test (for single test group) [16] and Dunnett's *t*-test (for multiple comparisons) [17] were used for statistical analysis when applicable. Cell growth curves were compared using regression analysis [16].

RESULTS

Effect of APAH on the Growth of L1210 Cells

L1210 cells were seeded at an initial density of 5×10^4 cells/mL. After a brief lag phase, cells quickly grew into an exponential (log) phase and reached the plateau phase in 3–4 days with maximum cell density around 4×10^6 cells/mL of medium (Fig. 1, left panel). At least 3 flasks were treated and analyzed for each of the APAH concentrations, with additional flasks cultured for 100 μM APAH (9 flasks) and for the control (15 flasks). Addition of APAH at 5 and 10 μM produced an upward shift of the growth curves ($P < 0.05$, with regression analysis of slopes) and increased the maximum cell density (Table 1). APAH at 10 μM significantly increased the maximum cell density by 16% ($P < 0.05$). The greatest effect was seen with 100 μM APAH, which raised both growth rate and maximum cell density. The growth curve was shifted upward by as much as 28% at maximum cell density ($P < 0.01$), and mPDT was shortened by 12% ($P < 0.01$). Cell densities were increased at all time points examined ($P < 0.01$).

Higher concentrations of APAH, at 0.5 and 1 mM, also significantly increased the maximum cell density by 17% ($P < 0.05$) and 14% ($P < 0.05$), respectively. APAH at 0.5 mM reduced mPDT by 12% ($P < 0.05$) (Table 1).

At concentrations of 5 mM APAH or greater, growth was retarded, as mPDT was increased by 32% ($P < 0.01$) and the maximum density was decreased by 43% ($P < 0.01$) (Fig. 1, right panel). The growth curve was significantly depressed compared with that of the control ($P < 0.0005$). In another experiment, when the cells were treated with 10 mM APAH, L1210 cells were killed rapidly, and few viable cells were seen after 24 hr of incubation (data not shown), indicating the toxicity of this compound at concentrations above 5 mM.

HPLC Determination of Intracellular Polyamines Including APAH

Using an HPLC technique with the modified gradient, major forms of polyamines and their metabolites were separated and detected. Putrescine was eluted at 15.2 min

TABLE 1. Effect of APAH on the mean population doubling time (mPDT) and maximum cell density of cultured L1210 cells

APAH (μM)	mPDT (hr)	Maximum cell density (% of control)
0 (control)	11.1 ± 0.2	100 ± 7
0.1	11.0 ± 0.4	106 ± 10
1	11.1 ± 0.9	97 ± 4
5	11.0 ± 0.5	109 ± 9
10	10.7 ± 0.7	116 ± 9*
100	9.7 ± 0.6†	128 ± 8†
500	9.8 ± 0.3*	117 ± 11*
1000	10.5 ± 0.8	114 ± 8*
5000	14.6 ± 1.0†	57 ± 4†

Data are means ± SD ($N \geq 3$) for all measuremetns except for 0 APAH where N = 15 and for 100 μM where N = 9. The starting cell density was 6×10^4 cells/mL; in untreated cells, the maximum cell density was 4×10^6 cells/mL.

*,†Significance was measured using Dunnett's *t*-test [17]: *$P < 0.05$, and †$P < 0.01$.

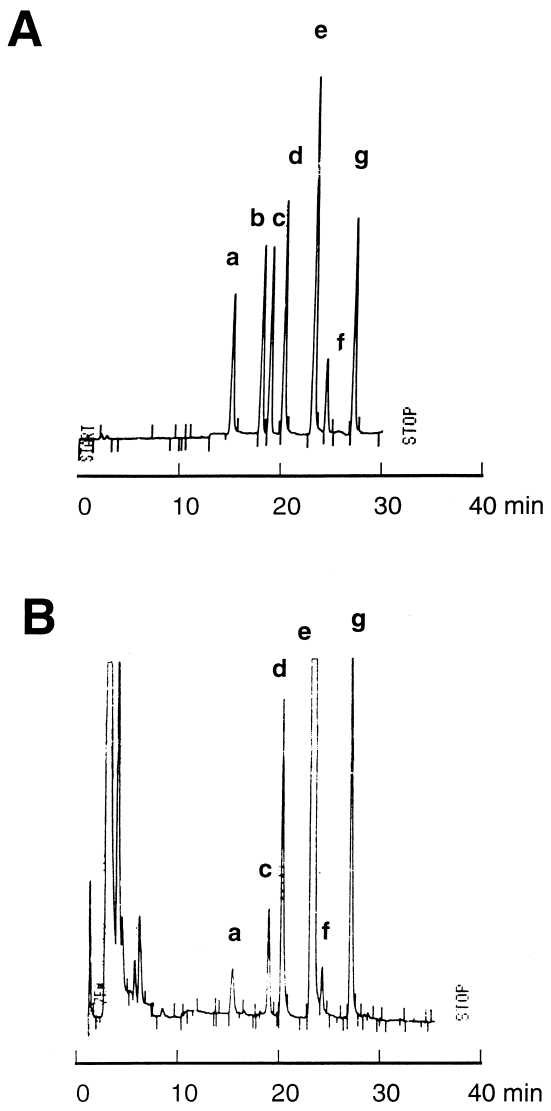

FIG. 2. Representative HPLC analyses of polyamines in a mixture of polyamine standards (A) and L1210 cells treated with APAH (B). Key: (a) putrescine; (b) N^1-acetylspermidine; (c) N^8-acetylspermidine; (d) DAH; (e) spermidine; (f) APAH; and (g) spermine.

(Fig. 2A), followed by N^1-acetylspermidine and N^8-acetylspermidine at 18.0 and 18.9 min, respectively. The resolution factor for N^1-acetylspermidine and N^8-acetylspermidine was 2.7, which is considered to be sufficiently separated [18]. The peak at 23.2 min was identified as spermidine, which was followed by APAH at 24.5 min. Spermine had the longest elution time at 27.2 min. These conditions resulted in completion of one assay in about 35 min, which was nearly 25 min shorter than previous assay time [13], and still produced adequate resolution for N^1-acetylspermidine and N^8-acetylspermidine and for spermidine and APAH, two pairs of closely related compounds. DAH is a relatively stable compound and was used as an internal standard to assess the extraction recovery rate of other polyamines from the cell samples. It was detected at 20.3 min, and was well separated from other polyamines. The extraction recovery rate of DAH was 92.3 ± 8.4%. The reproducibility of HPLC assays, as determined by variations between duplicated HPLC runs, fell within 6% for all of the above polyamines. The detection limits (lowest concentration to achieve a signal-to-noise ratio of not less than 2:1) were at or below 4 pmol/20 μL for the polyamines studied. Good linearity (regression coefficient between 0.9970 and 1.000) was observed for each amine at the concentrations studied (4–1000 pmol/20 μL).

Effect of APAH on the Intracellular Polyamine Pool

In control L1210 cells, the spermidine level was about 4 times as much as that of spermine, both of which were much higher than that of putrescine. N^8-Acetylspermidine was not detectable in control cells, whereas N^1-acetylspermidine was measurable in some of these samples at 1 pmol/10^6 cells.

Intracellular APAH was detected 24 hr after it was added into cultures at concentrations above 10 μM, and increased in a concentration-dependent manner (Table 2). Addition of APAH at a 100 μM concentration yielded 188 pmol of

TABLE 2. Intracellular polyamine levels after 24-hr treatment with APAH

	Polyamine levels (pmol/10^6 cells)					
Treatment	**PUT**	**N^1-AcSpd**	**N^8-AcSpd**	**SPD**	**APAH**	**SPM**
Control	45	1	UD	2208	UD	485
0.1 μM APAH	85	1	UD	926	UD	217
1 μM APAH	84	3	8	1285	UD	340
5 μM APAH	83	4	7	987	UD	327
10 μM APAH	73	3	4	946	1.3	326
100 μM APAH	55	8	11	1047	188	359
0.5 mM APAH	65	4	20	2105	611	414
1 mM APAH	133	3	16	1810	1312	443
5 mM APAH	46	5	22	1201	4828	798

Values are means of at least two assays with samples taken from 3 or more flasks at each APAH concentration.

Abbreviations: UD, not detectable; PUT, putrescine; N^1-AcSpd, N^1-acetylspermidine; N^8-AcSpd, N^8-acetylspermidine; SPD, spermidine; and SPM, spermine.

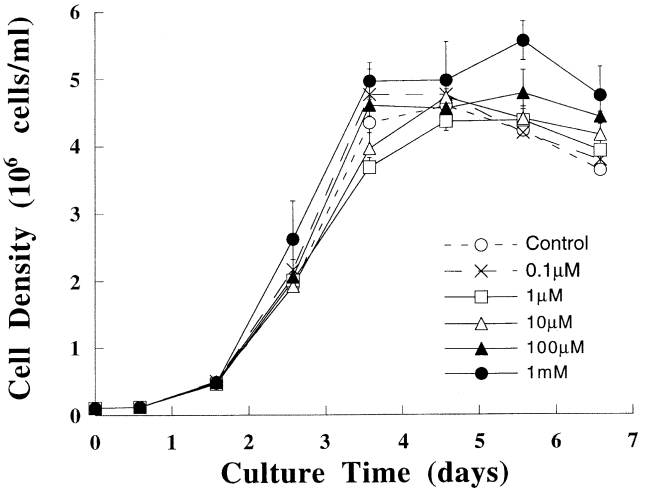

FIG. 3. Growth of cultured murine L1210 cells after treatment with N^8-acetylspermidine at concentrations between 0.1 μM and 1 mM. Cells were treated with various concentrations of N^8-acetylspermidine 14 hr after inoculum (day 0.6), and allowed to grow for more than 6 days during the experiment. Each point represents the mean ± SD of duplicate counts of at least three cell culture flasks.

APAH/10^6 cells, which was 144 times higher than the level after 10 μM APAH treatment. The highest level was measured at 4828 pmol/10^6 cells after addition of 5 mM APAH to the cell culture. Intracellular N^8-acetylspermidine was detected readily in the presence of APAH at 1 μM or higher, indicative of N^8-acetylspermidine deacetylation inhibition. N^8-Acetylspermidine was measured at 11 pmol/10^6 cells after treatment with 100 μM APAH and increased to about 20 pmol/10^6 cells with higher APAH concentrations. Although no APAH was detected in cells treated with 1 or 5 μM APAH, N^8-acetylspermidine was measurable in both cases, suggesting that a very low concentration of APAH is able to inhibit N^8-acetylspermidine deacetylase in L1210 cells. N^1-Acetylspermidine was increased slightly after the APAH treatment. Treatment with APAH had variable effects on the levels of putrescine, spermidine, and spermine. Both spermidine and spermine levels appear to be depressed particularly at 0.1 to 100 μM APAH. Further studies are needed to confirm the changes in these two polyamines and to investigate possible mechanisms.

Effect of Exogenous N^8-Acetylspermidine on the Growth of L1210 Cells

To examine whether stimulation of growth by APAH is mediated through the elevated enzyme substrate N^8-acetylspermidine, we investigated the effect on cell growth of the addition of N^8-acetylspermidine directly to the culture medium. Indeed, N^8-acetylspermidine at 1 mM increased maximum cell density by 28% ($P < 0.01$) after 48 hr of incubation and reduced mPDT by 8% ($P < 0.01$) (Fig. 3). Similar effects were seen after 5 mM N^8-acetylspermidine, which lowered mPDT by 5% ($P < 0.05$) and increased maximum cell density by 19% ($P < 0.05$) (Fig. 3). No significant changes were observed after treatment with N^8-acetylspermidine below 1 mM. Intracellular N^8-acetylspermidine was only detected after treatments with 1 and 5 mM N^8-acetylspermidine, at 52 and 672 pmol/10^6 cells, respectively, 12 hr after treatment (Table 3), and these levels were maintained for at least another 12 hr (data not shown).

Effect of Exogenous N^1-Acetylspermidine, Spermine, and Putrescine on the Growth of L1210 Cells

Three closely related polyamines, N^1-acetylspermidine, spermidine, and putrescine, share, among other similarities, the polycationic property of N^8-acetylspermidine and APAH. They, therefore, deserve careful examination with

TABLE 3. Intracellular polyamine levels after 12-hr treatment with APAH (100 μM) and/or N^8-acetylspermidine (1 μM to 5 mM)

	Polyamines (pmol/10^6 cells)					
Treatment	**PUT**	**N^1-AcSpd**	**N^8-AcSpd**	**SPD**	**APAH**	**SPM**
Control	231	UD	UD	3828	—	2302
N^8-AcSpd (1 μM)	106	UD	UD	3124	—	1752
N^8-AcSpd (10 μM)	362	UD	UD	2944	—	1934
N^8-AcSpd (100 μM)	53	UD	UD	3494	—	2515
N^8-AcSpd (1 mM)	38	UD	52	1520	—	2313
N^8-AcSpd (5 mM)	24	UD	672	2074	—	2838
N^8-AcSpd (1 μM) + APAH (0.1 mM)	249	UD	11	2237	137	1562
N^8-AcSpd (10 μM) + APAH (0.1 mM)	125	UD	9	2682	51	1795
N^8-AcSpd (100 μM) + APAH (0.1 mM)	77	17	168	1098	22	1640
N^8-AcSpd (1 mM) + APAH (0.1 mM)	73	3	101	2420	31	2414
N^8-AcSpd (5 mM) + APAH (0.1 mM)	57	UD	229	1127	25	2204

Values are the means of at least two assays with samples taken from 3 or more flasks at each APAH/AcSPD concentration.

Abbreviations: PUT, putrescine; N^1-AcSPD, N^1-acetylspermidine; N^8-AcSPD, N^8-acetylspermidine; SPD, spermidine; SPM, spermine; "—", not meaningful; and UD, under detectable level.

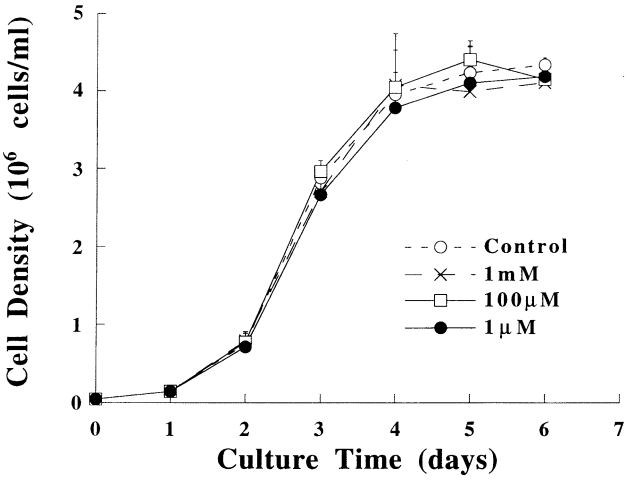

FIG. 4. Growth of cultured murine L1210 cells after treatment with N^1-acetylspermidine at concentrations between 1 μM and 1 mM. Cells were treated with N^1-acetylspermidine on day 1, and allowed to grow for 5 more days during the experiment. Each point represents the mean ± SD of duplicate counts of at least three cell culture flasks.

regard to effects on growth of L1210 cells in comparison to N^8-acetylspermidine. Each of these polyamines was tested over concentrations ranging from 1 μM to 1 mM under the same conditions used for N^8-acetylspermidine; however, none produced any significant changes in the cell growth rate, mPDT, or maximum cell density (only the curve of N^1-acetylspermidine is shown in Fig. 4).

Comparing the Effect of APAH vs N^8-Acetylspermidine

The effects of APAH (100 μM) were compared with those of N^8-acetylspermidine (1 and 5 mM). The overall effects of N^8-acetylspermidine and APAH were very similar in terms of increases in growth rate and maximum cell density (Fig. 5). There was no significant difference among the growth curves of N^8-acetylspermidine (1 or 5 mM) and APAH (100 μM), which were all significantly increased compared with that of the control ($P < 0.05$ at least).

Effect of Co-treatment with APAH and N^8-Acetylspermidine on L1210 Cell Growth

L1210 cells were also treated with both APAH (100 μM) and N^8-acetylspermidine (1 μM to 1 mM) to test additive effects of these two compounds (Table 4, and Fig. 5). While the combinations studied all enhanced cell growth to or above stimulation levels produced by individual compounds alone, these differences were not statistically significant. Co-treatment with 100 μM APAH and 1 mM N^8-acetylspermidine increased the maximum cell density by 26.75% ($P < 0.01$ compared with control), which was 8% (not significant) higher than 100 μM APAH or 1 mM N^8-acetylspermidine alone (Fig. 5). It also reduced mPDT by 1.1 hr ($P < 0.01$ compared with control), but this reduction was not significant compared with treatment of 10^{-4} M APAH (0.84 hr reduction) or 10^{-3} M N^8-acetylspermidine (0.74 hr reduction).

Effect of Co-treatment with APAH and N^8-Acetylspermidine on L1210 Cellular Polyamine Content

Intracellular polyamines were analyzed after 12- and 24-hr incubation with APAH and/or N^8-acetylspermidine. APAH was recovered inside cells after 12 hr of incubation with APAH (Table 3), and the levels more than doubled at 24 hr (data not shown). Intracellular APAH concentrations were decreased in the presence of N^8-acetylspermidine. As the N^8-acetylspermidine levels increased, APAH levels dropped from 254 pmol/10^6 cells without N^8-acetylspermidine to 50 pmol/10^6 cells with the addition of 1 mM N^8-acetylspermidine, suggesting that N^8-acetylspermidine may inhibit uptake of APAH into L1210 cells. N^8-Acetylspermidine levels were increased dramatically in the presence of APAH, further supporting evidence for inhibition of N^8-acetylspermidine deacetylase by this compound. N^8-Acetylspermidine levels after treatment with 1 mM N^8-acetylspermidine were 4–6 times higher when APAH was present. Even in the absence of added N^8-acetylspermidine, its levels were raised from undetectable to 11 pmol/10^6 cells after treatment with 100 μM APAH. There were only small increases in N^1-acetylspermidine levels in some samples.

Intracellular spermidine levels were decreased in the presence of APAH. Interestingly, putrescine levels were decreased when N^8-acetylspermidine was added. This sug-

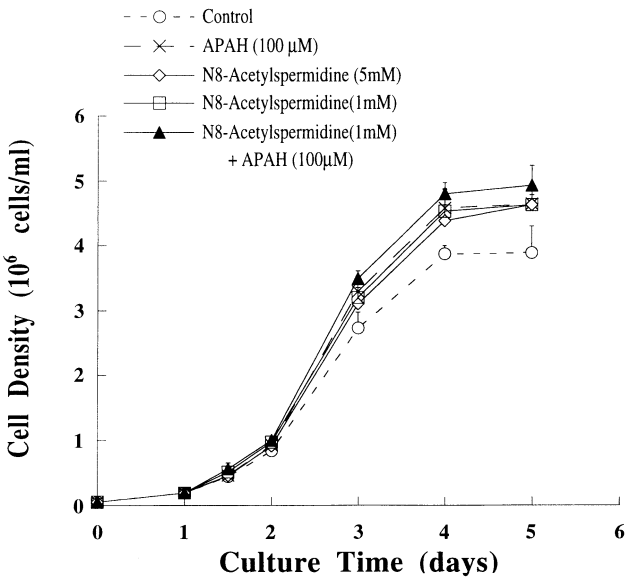

FIG. 5. Growth of cultured murine L1210 cells after treatment with N^8-acetylspermidine at 1 or 5 mM and/or APAH at 100 μM. Cells were treated with these compounds on day 1, and allowed to grow for 5 days during the experiment. Each point represents the mean ± SD of duplicate counts of at least three cell culture flasks.

TABLE 4. Effect of APAH and N^8-acetylspermidine on the mean population doubling time (mPDT) and maximum cell density of cultured L1210 cells

Treatment	mPDT (hr)	Maximum cell density (% of control)
Control	12.6 ± 0.4	100 ± 3
APAH (0.1 mM)	11.7 ± 0.4*	120 ± 4
APAH (0.1 mM) + N^8-AcSpd (1 μM)	11.6 ± 0.4†	121 ± 5*
APAH (0.1 mM) + N^8-AcSpd (10 μM)	12.0 ± 0.2	123 ± 2*
APAH (0.1 mM) + N^8-AcSpd (100 μM)	11.6 ± 0.3†	121 ± 2*
APAH (0.1 mM) + N^8-AcSpd (1 mM)	11.5 ± 0.2†	127 ± 3†
N^8-AcSpd (1 mM)	11.8 ± 0.2*	119 ± 16*

Data are means ± SD (N ≥ 3). The starting cell density was 5×10^4 cells/mL; in untreated cells, maximum cell density was 4×10^6 cells/ml.

*,†Significance was measured using Dunnett's *t*-test [17]: *$P < 0.05$, and †$P < 0.01$.

gests that N^8-acetylspermidine can compete for polyamine transporter [19]. Spermine levels also were increased slightly after 12 hr of incubation with high concentrations of N^8-acetylspermidine, but did not seem to be affected by APAH. Cellular spermine levels fell 2- to 3-fold 12 hr later regardless of the addition of N^8-acetylspermidine or APAH (measured in 24-hr samples; data not shown, but comparable to levels shown in Table 2).

DISCUSSION

In this study, addition of APAH or N^8-acetylspermidine to cultured L1210 cells was found to stimulate cell growth and increase maximum cell density. This stimulation seems to correlate with an increase in cellular content of N^8-acetylspermidine as a result of either inhibition of its deacetylation by APAH or accumulation of the compound following addition to the medium.

The increases observed in this study in cell growth rates and maximum cell density are small, but they are consistent in a variety of experiments and experimental conditions. A significant increase in either of these parameters is somewhat surprising in that it is observed in comparison with control cells that are already undergoing rapid cell division (log phase) to a plateau phase of maximum cell density. Although small, these effects should be reported and studied, since even small changes may be of biologic and pharmacological importance.

N^8-Acetylspermidine is apparently unique in its ability to produce this effect on L1210 cell growth in comparison with other polyamines. Early studies suggested that the physiological effect of polyamines resulted from their polycationic nature through binding to RNA and DNA. However, the effects observed for N^8-acetylspermidine and APAH are not simply due to their cationic nature and charges. Experiments with addition of N^1-acetylspermidine, putrescine, or spermidine, which all have multiple positive charges, failed to yield similar stimulation effects.

HPLC determination of intracellular polyamines revealed that APAH was readily taken up into cells. Accumulation of N^8-acetylspermidine in cells exposed to APAH confirmed inhibition of N^8-acetylspermidine deacetylation, as previously shown *in vitro* [8, 9]. It was further found to be a potent inhibitor in L1210 cells, as evidenced by the detection of N^8-acetylspermidine after the addition of as little as 1 μM APAH.

In some samples, N^1-acetylspermidine was increased slightly after the treatment with APAH. It has also been shown to increase slightly after the administration of APAH to mice [13]. However, in both cases, N^1-acetylspermidine levels did not depend on APAH concentration, arguing against significant inhibition of N^1-acetylspermidine deacetylation by APAH. Other indirect causes, such as induction of spermidine N^1-acetyltransferase [20] or inhibition on polyamine oxidase, may attribute to this result. Polyamine oxidase converts N^1-acetylspermidine to putrescine. N^8-Acetylspermidine is a known noncompetitive inhibitor of polyamine oxidase with a K_i of 11 μM, whereas the K_m for N^1-acetylspermidine is 14 μM [21]. In experiments in which high concentrations of APAH and/or N^8-acetylspermidine were added to the cultures, N^1-acetylspermidine was not increased significantly. Selective inhibition of N^8-acetylspermidine deacetylase was also seen in rat hepatoma cells after administration of a similar inhibitor, 7-amino-2-heptanone, with increases in N^8-acetylspermidine but no accumulation of acetylputrescine or N^1-acetylspermidine [22].

Although cellular APAH concentrations were directly correlated with the APAH levels added to the medium, initial effects occurred at concentrations below our limit of detection in cells. Cellular APAH was not detected until greater than 10 μM APAH was present in the medium; however, 5 μM APAH produced stimulation of growth and measurable N^8-acetylspermidine levels. Moreover, cellular APAH levels did not seem to be saturable, but the effect of APAH, on both cell growth and N^8-acetylspermidine accumulation, peaked at 100–500 μM. On the other hand, not only was N^8-acetylspermidine detectable after treatment with APAH, but its intracellular level resulting from APAH treatment also appeared to correlate well with the stimulatory effect on growth. For example, after treatment with 5 μM APAH, APAH was not detectable in L1210 cells, whereas N^8-acetylspermidine was measurable and the cell cultures started to show the stimulation effect of

APAH. Approximately 5 pmol/10^6 cells of intracellular N^8-acetylspermidine, which accumulated after treatment with APAH, stimulated the growth of L1210 cells. N^8-Acetylspermidine accumulation and inhibition of N^8-acetylspermidine deacetylation seemed to be saturable, because at very high APAH concentrations, a 50-fold increase in APAH level resulted in only a 2-fold increase in N^8-acetylspermidine. These results suggest that the effects of APAH on L1210 cell growth are closely related to N^8-acetylspermidine levels or inhibition of N^8-acetylspermidine deacetylation.

We further investigated whether addition of the enzyme substrate N^8-acetylspermidine would produce effects similar to those of APAH. Indeed, N^8-acetylspermidine behaved much like APAH in stimulating cell growth, but with lower potency. Only high concentrations of N^8-acetylspermidine (above 1 mM) were effective.

Unlike what happened after treatment with APAH alone, cellular N^8-acetylspermidine levels were not as closely correlated with the growth stimulation effect as a result of addition of exogenous N^8-acetylspermidine with or without the concurrent addition of APAH. Therefore, simply increasing cellular levels of N^8-acetylspermidine may not be as effective in enhancing cell growth as is inhibition of deacetylation by APAH of the N^8-acetylspermidine generated in the cell nucleus. One explanation that might be considered would be the possibility that APAH or the accumulating N^8-acetylspermidine might block histone deacetylation, but in previous studies using HeLa cells, we found that histone deacetylation was not blocked by APAH at comparable concentrations [13]. The possibility still remains that there might be an interaction with the histone deacetylation process.

Since histone acetylation has been implicated in the regulation of replication and transcription [23, 24], we have proposed that polyamine acetylation may play a similar role through affecting DNA structure and function [5, 25]. In other words, polyamine acetylation–deacetylation levels may influence binding and interaction of polyamines with nucleic acids [5, 25]. Spermidine is one of the most active polyamines in interacting and stabilizing DNA [25, 26]. Acetylation of spermidine decreases its capability of binding to DNA, possibly because of reduced charges and/or altered 3-dimensional structure [10, 27]. Both N^1-acetylspermidine and N^8-acetylspermidine are less capable of binding to and stabilizing DNA against thermal denaturation than is spermidine [25, 26, 28]. Considering the fact that spermidine N^8-acetyltransferase is the only enzyme [5] in the polyamine metabolic pathway localized in the cell nucleus, N^8-acetylation of spermidine seems to be a better candidate than N^1-acetylation for the role of regulating spermidine binding to DNA and cell proliferation.

The present study indicates that shifting the acetylation/deacetylation reaction balance towards the N^8-acetylation of spermidine with an inhibitor of N^8-acetylspermidine deacetylase or by direct addition of N^8-acetylspermidine can indeed enhance L1210 cell growth. This is consistent with the hypothesis that N^8-acetylation and the resultant decrease in polyamine binding to DNA should facilitate replication, transcription and related processes resulting in increased rates of cell growth.

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