ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1989, p. 828–834
0066-4804/89/060828-07$02.00/0

Vol. 33, No. 6

Relationship between Intracellular Concentration of *S*-Adenosylhomocysteine and Inhibition of Vaccinia Virus Replication and Inhibition of Murine L-929 Cell Growth

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Received 3 November 1988/Accepted 16 March 1989

9-(*trans*-2′,*trans*-3′-Dihydroxycyclopent-4′-enyl)-adenine (compound 1) and -3-deazaadenine (compound 2), which are specific inhibitors of *S*-adenosylhomocysteine (AdoHcy) hydrolase, were reported earlier by our laboratory (M. Hasobe, J. G. McKee, D. R. Borcherding, and R. T. Borchardt, Antimicrob. Agents Chemother. 31:1849–1851, 1987) to have anti-vaccinia virus activity with reduced murine L-929 cell toxicity compared with the prototype compound neplanocin A. In this study, we showed that the antiviral and cytotoxic effects of compounds 1 and 2 can be related to intracellular concentrations of AdoHcy, which are elevated in cells treated with these inhibitors of AdoHcy hydrolase. For example, concentrations of analogs 1 and 2 that produce 50% inhibition of vaccinia virus replication caused only slight elevations in intracellular levels of AdoHcy (from 50 [controls] to 100 to 125 [drug-treated cells] pmol/mg of protein) and elevations in the ratios of AdoHcy/*S*-adenosylmethionine (from 0.05 to 0.1 [controls] to 0.15 to 0.19 [drug-treated cells]). In contrast to the extreme susceptibility of virus replication to slight elevations in intracellular AdoHcy, cell viability was quite tolerant to higher levels of this metabolite. For example, concentrations of analogs 1 and 2 that produced 50% inhibition of L-929 cell replication caused significant increases in intracellular levels of AdoHcy (to 825 to 950 pmol/mg of protein) and elevations in AdoHcy/*S*-adenosylmethionine ratios (approximately 1.3). These data make it possible to assign a therapeutic index of 7 to 8 to these compounds on the basis of the comparison of intracellular levels of AdoHcy that caused 50% inhibition of vaccinia virus replication with those that caused 50% inhibition of L-929 cell replication.

S-Adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1) has become an attractive target for the design of antiviral agents (7, 19). This cellular enzyme catalyzes the hydrolysis of AdoHcy to adenosine and homocysteine, thus maintaining low cellular concentrations of this product inhibitor of *S*-adenosylmethionine (AdoMet)-dependent methylations (31). Inhibition of cellular AdoHcy hydrolase, however, results in intracellular accumulation of AdoHcy, causing a significant increase in the intracellular AdoHcy/AdoMet ratio and subsequent inhibition of virus replication (3, 18). The inhibition of viral AdoMet-dependent mRNA methylations caused by elevated cellular levels of AdoHcy has been proposed by us to be the molecular mechanism by which AdoHcy hydrolase inhibitors, such as neplanocin A and adenosine dialdehyde, inhibit vaccinia virus replication in murine L-929 cells (17–19). Ransohoff et al. (28) have reported direct evidence showing that inhibition of influenza virus replication in CHO cells by neplanocin A is due at least in part to impaired recognition of undermethylated cellular mRNA cap structures by the influenza virus polymerase complex.

Neplanocin A, like many other AdoHcy hydrolase inhibitors, also exhibits considerable cellular toxicity, which limits its utility as an antiviral agent (6). The cytotoxic effects of neplanocin A are mediated at least in part through its phosphorylation by adenosine kinase and subsequent conversion of neplanocin A triphosphate to *S*-neplanocylmethionine (9, 10, 14, 15, 20, 21). Recently, our laboratory described the synthesis (4) and biological activity (12, 13, 19, 25) of 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-adenine (Fig. 1, compound 1) and -3-deazaadenine (Fig. 1, compound 2), which are neplanocin A analogs that retain inhibitory activity toward AdoHcy hydrolase but are devoid of substrate activity for adenosine kinase. These carbocyclic nucleosides inhibit vaccinia virus replication in mouse L-929 cells, but their cytotoxicity is reduced compared with that of neplanocin A (12). These data confirm earlier studies by De Clercq and Cools (8) which showed that AdoHcy hydrolase is the molecular target which mediates the antiviral effects of neplanocin A. These data also confirmed earlier work by Glazer et al. (9, 11) with 3-deazaneplanocin A which showed that transformation by cellular adenosine kinase mediates in part the cytotoxic properties of neplanocin A.

However, since synthetic analogs 1 and 2, which are pure inhibitors of AdoHcy hydrolase, still exhibit some toxicity in murine L-929 cells (12), we were interested in determining whether a relationship existed between this cellular toxicity and the elevated intracellular concentrations of AdoHcy. In this study, we showed that the antiviral and cytotoxic effects of compounds 1 and 2 can be related to elevations in the intracellular concentrations of AdoHcy and that it is possible to define a therapeutic index for these analogs on the basis of the intracellular levels of AdoHcy needed to inhibit virus replication and the intracellular levels of AdoHcy needed to produce cellular toxicity.

MATERIALS AND METHODS

Materials. The following standard chemicals and supplies were purchased from commercial suppliers: Waymouth 752/1 (low-calcium) medium (for spinner culture), Waymouth MB 752/1 medium (for dish culture), and bovine calf serum (Hazleton Research Products, Denver, Pa.); 24-well (13-mm

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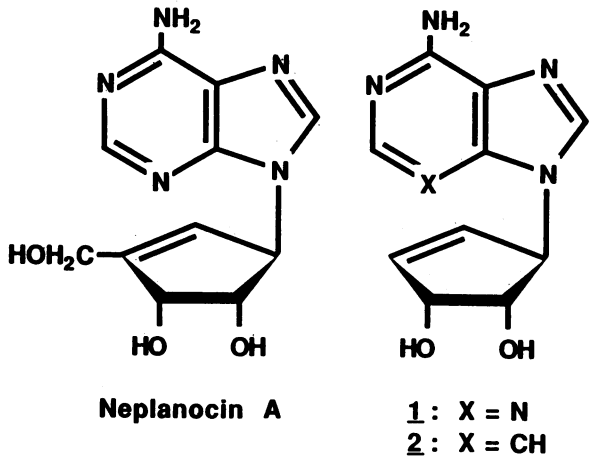

FIG. 1. Structures of neplanocin A and analogs 1 and 2.

diameter) or 6-well (30-mm diameter) multiculture dishes (Costar, Cambridge, Mass.); cell culture dishes (60-mm diameter; Corning Glass Works, Corning, N.Y.); monobasic sodium phosphate (high-pressure liquid chromatography [HPLC] grade), 1-heptanesulfonic acid sodium salt (HPLC grade; Fisher Scientific Co., Pittsburgh, Pa.); Zorbax C-8 reversed-phase HPLC column (Du Pont Co., Wilmington, Del.); guanidine thiocyanate (Eastman Kodak Co., Rochester, N.Y.); oligo(dT)-cellulose (Pharmacia Biotechnology Products, Piscataway, N.J.); [*methyl*-^{3}H]methionine (80 Ci/mmol; Amersham Corp., Arlington Heights, Ill.); [^{14}C]uridine (517.5 mCi/mmol; Dupont, NEN Research Products, Boston, Mass.); 1,1,2-trichlorotrifluoroethane (Aldrich Chemical Co., Inc., Milwaukee, Wis.); tri-*n*-octylamine (Sigma Chemical Co., St. Louis, Mo.); ammonium dihydrogen phosphate (HPLC grade; Fisher Scientific); and a Partisil-10 SAX column (4.6 by 250 mm; Whatman, Inc., Clifton, N.J.). Neplanocin A was kindly donated by Toyo Jozo Co., Shizuoka, Japan. Compounds 1 and 2 were synthesized as described earlier by us (4).

Cell culture. Stock cultures of clone 929 mouse cells, strain L (Earle), were grown in suspension at 37°C in Waymouth 752/1 medium supplemented with 4% bovine calf serum and 70 μg of gentamicin per ml as described previously (3, 16). Experimental cultures of L-929 cells plated in tissue culture dishes or multiculture plates were grown in Waymouth MB 752/1 medium containing 2% calf serum.

Determination of intracellular levels of AdoHcy and AdoMet. Cultures of mouse L-929 cells (approximately 3 × 10^6/60-mm-diameter dish) were incubated at 37°C with Waymouth MB 752/1 medium containing 2% calf serum with or without compound 1 or 2. Cells were removed from the culture dishes at various times by trypsin treatment and lysed in 100 μl of 0.25 N perchloric acid by vigorous vortexing and rapid freezing on dry ice-acetone. The samples were stored at −70°C before analysis. Under these conditions of storage, AdoMet and AdoHcy were stable indefinitely. In preparation for HPLC analysis, the samples were rapidly thawed and the cell debris was removed by centrifugation in an Eppendorf microcentrifuge (12,400 rpm, 1 min). The supernatant (100 μl) was injected into a Perkin-Elmer series 3 HPLC equipped with a Zorbax C-8 reverse-phase column (25 cm by 4.6 mm). AdoHcy and AdoMet were separated by a two-step gradient program at a flow rate of 1.0 ml/min. Solvent A was acetonitrile, and solvent B was 50 mM sodium phosphate (pH 3.2)–10 mM heptanesulfonic acid. The program was 5 to 20% solvent A for 15 min and 20 to 25% solvent A for 10 min. Quantitation was by A_{254}.

Anti-vaccinia virus assay. Experimental cultures for the anti-vaccinia virus assay were plated in 24-well multiculture plates and grown in Waymouth MB 752/1 medium containing 2% calf serum. Nearly confluent cell monolayers were infected with vaccinia virus at about 200 PFU per well. After a 60-min virus adsorption period, the viral inoculum was diluted by addition of culture medium and then completely removed by aspiration. Immediately after infection, each well containing a cell monolayer was overlaid with medium (400 μl) containing 0.1% methylcellulose and 2% calf serum to which was added medium (100 μl) containing test compounds in serial dilutions in increments of 0.5 order of magnitude. After incubation for 48 h at 37°C, cultures were washed with phosphate-buffered saline and stained with 0.1% crystal violet. The plaque number was then counted under a microscope. The experiments were conducted in duplicate, and the data are expressed as IC_{50}s (concentrations needed to produce 50% inhibition of plaque formation).

Murine L-929 cell toxicity assay. The effects of analogs 1 and 2 on cell growth were determined by trypan blue staining of L-929 cells grown in various concentrations of the test compounds. L-929 cells were plated at 4.5 × 10^5 per dish (30-mm diameter) and grown in Waymouth MB 752/1 medium containing 2% calf serum. After 6 h of incubation, the cells were fed with fresh medium with or without the test compounds. After incubation for 72 h, the cells were dispersed by trypsin treatment and suspended in phosphate-buffered saline containing 0.04% trypan blue. Viable cells were counted with a hemacytometer. The experiments were conducted in triplicate, and the data are expressed as ID_{50}s (concentrations needed to produce 50% inhibition of cell growth).

Cytoplasmic poly(A)$^+$ mRNA methylation in vaccinia virus-infected L-929 cells. L-929 cells in spinner culture (6 × 10^5 cells per ml in 550 ml) were grown in Waymouth 752/1 medium lacking methionine and containing 2% dialyzed calf serum with or without analog 1. After incubation for 18 h in suspension at 37°C, the cells were precipitated by centrifugation, the supernatant was aspirated, and the concentrated cells (100 ml per bottle) treated with analog 1 were infected with vaccinia virus (multiplicity of infections, 15) and then treated with cycloheximide (final concentration, 100 μg/ml). After 1 h of incubation, adenosine (20 μM) and guanosine (10 μM) were simultaneously added, and 1 h later the cells were pulse-labeled with [*methyl*-^{3}H]methionine (10 μCi/ml) and [^{14}C]uridine (0.5 μCi/ml). After 3 h of labeling, the infected cells were harvested by centrifugation and washed with phosphate-buffered saline. The total cytoplasmic RNA was then prepared by the guanidine isothiocyanate method (at pH 9) (22). The total cytoplasmic RNA was fractionated to poly(A)$^+$ mRNA by oligo(dT)-cellulose affinity chromatography (22).

Separation and quantitation of rNTPs by HPLC. Cultures of L-929 cells (approximately 6 × 10^6/60-mm-diameter dish) were incubated at 37°C with Waymouth MB 752/1 medium containing 2% calf serum with or without 10 μM compound 1 or 2 or neplanocin A. After incubation for 6 h, the cells were washed with cold phosphate-buffered saline, harvested into a microcentrifuge tube with trypsin treatment, and then centrifuged at 14,000 rpm for 1 min in an Eppendorf microcentrifuge. The cell pellet was suspended in 120 μl of cold trichloroacetic acid solution (final concentration, 0.3 M) and vortexed vigorously for 15 min at 4°C. After centrifugation (14,000 rpm for 1 min), the acid-soluble fraction was sepa-

rated and treated with cold Freon (1,1,2-trichlorotrifluoroethane) containing 0.5 M tri-*n*-octylamine for a few minutes at 4°C with gentle vortexing (30). The mixture was then centrifuged (14,000 rpm for 1 min), and the aqueous fraction was separated and analyzed with the HPLC system described above under Determination of intracellular levels of AdoHcy and AdoMet, except for a Partisil-10 SAX column (4.6 mm by 25 cm). Ribonucleoside 5'-triphosphates (rNTPs; CTP, UTP, ATP, and GTP) were separated by elution with 0.4 M $NH_4H_2PO_4$ (pH 3.30)–CH_3CN (10:1) at a flow rate of 2 ml/min and quantitated at 254 nm (30).

RESULTS

Intracellular AdoHcy levels and AdoHcy/AdoMet ratios in murine L-929 cells treated with analogs 1 and 2. Treatment of murine L-929 cells with analog 1 caused concentration-dependent accumulation of intracellular AdoHcy (Fig. 2A). The time-dependent profile for accumulation of AdoHcy in cells treated with analog 1 was characterized by an accumulation phase (up to about 12 h), followed by a plateau phase (from 12 to 72 h) (Fig. 2A). In contrast, the intracellular content of AdoMet in L-929 cells treated with analog 1 increased in the first 2 h, after which it was somewhat variable before stabilizing at 72 h (Fig. 2B). Thus, the profile of the AdoHcy/AdoMet ratios, which is shown in Fig. 2C, exhibited an accumulation phase (up to 24 h) followed by a plateau phase (from 24 to 72 h). Data similar to those shown in Fig. 2 for analog 1 were also obtained with analog 2 (data not shown). Increases in both AdoHcy levels and AdoHcy/AdoMet ratios were observed in cells treated for 24 h with analog 1 and 2 concentrations as low as 0.3 and 1 μM, respectively (Fig. 3).

Relationship between elevated intracellular AdoHcy levels (and AdoHcy/AdoMet ratios) and anti-vaccinia virus activity and inhibition of cell growth. The anti-vaccinia virus activities of compounds 1 and 2 were evaluated by measuring the inhibition of virus plaque formation during 48 h of exposure to the drug. The IC_{50}s of compounds 1 and 2 were determined to be 0.28 and 0.95 μM, respectively (Fig. 3).

The effects of analogs 1 and 2 on L-929 cell growth were evaluated by determination of ID_{50}s after exposure to the drugs for 72 h. The ID_{50}s of compounds 1 and 2 were determined to be 17 and 56 μM, respectively (Fig. 4). Thus, the antiviral indexes (ID_{50}/IC_{50}) were calculated to be 61 and 59 for analogs 1 and 2, respectively.

The IC_{50}s caused only a slight elevation in the AdoHcy/AdoMet ratios (Fig. 3; Table 1) compared with the ID_{50}s (Fig. 4; Table 1). At the IC_{50}s of compounds 1 and 2, the intracellular AdoHcy levels were approximately 100 to 125 pmol/mg of protein and the AdoHcy/AdoMet ratios were approximately 0.15 to 0.19 (Table 1), whereas at the ID_{50}s of compounds 1 and 2 the intracellular levels of AdoHcy were approximately 825 to 950 pmol/mg of protein and the AdoHcy/AdoMet ratios were approximately 1.3 (Table 1). Thus, cytotoxicity was observed at cellular concentrations of AdoHcy seven to eight times the concentration needed to inhibit virus replication.

Effects of analog 1 on methylation of poly(A)$^+$ RNA isolated from drug-treated, virus-infected L-929 cells. To determine whether analog 1 at concentrations (0.03 to 3 μM) which produce 20 to 90% inhibition of virus replication causes inhibition of the methylation of poly(A)$^+$ RNA, drug-treated, virus-infected cells were pulse-labeled with [*methyl*-^{3}H]methionine and [^{14}C]uridine, the total cytoplasmic RNA was isolated by the guanidine isothiocyanate method with an

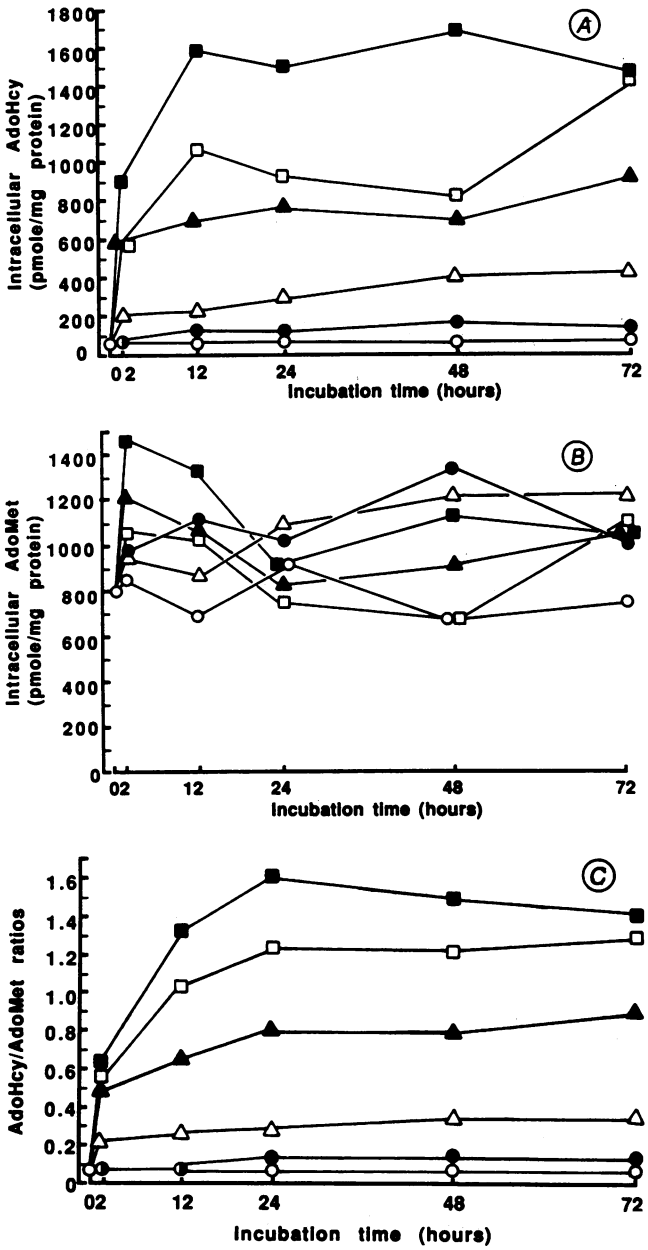

FIG. 2. Intracellular AdoHcy and AdoMet contents and AdoHcy/AdoMet ratios in murine L-929 cells treated with various concentrations of analog 1. Cultures of mouse L-929 cells (approximately 3 × 10^6/60-mm dish) were incubated with Waymouth MB752/1 medium containing 2% calf serum with or without compound 1. Symbols: ○, 0 μM; ●, 0.1 μM; △, 1 μM; ▲, 3.2 μM; □, 10 μM; ■, 32 μM. At various times, the cells were harvested by trypsin treatment and lysed with 0.25 N perchloric acid and the AdoHcy (A) and AdoMet (B) levels were determined by HPLC as described in Materials and Methods. The AdoHcy/AdoMet ratios calculated from the data shown in panels A and B are presented in panel C. The data are averages of duplicate assays.

RNase inhibitor (vanadyl nucleoside complex) (22), and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography (22). Treatment of virus-infected cells with analog 1 suppressed the methylation of poly(A)$^+$ RNA (Table 2, experiment 1). The ^{3}H/^{14}C ratios in poly(A)$^+$ RNA isolated from cells treated with analog 1 at 0.03 to 3.0 μM were 26 to

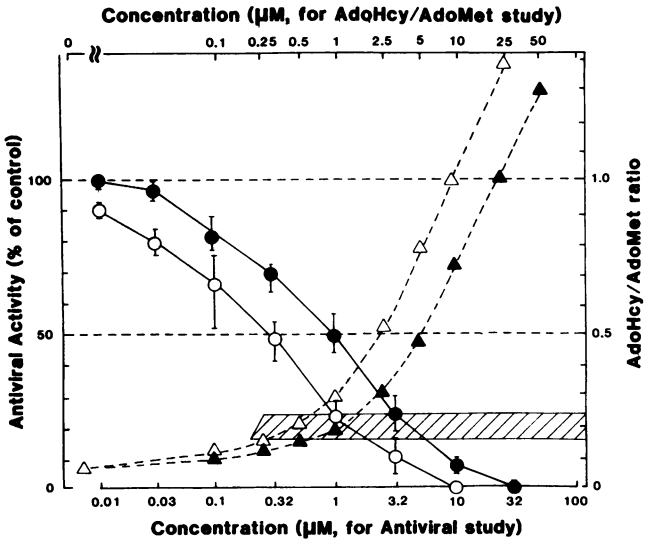

FIG. 3. Relationship between anti-vaccinia virus activity and AdoHcy/AdoMet ratios in murine L-929 cells treated with various concentrations of analogs 1 and 2. Nearly confluent cell monolayers were infected with vaccinia virus at about 200 PFU per well as described in Materials and Methods. After a 60-min virus adsorption period, the viral inoculum was removed and the cells were overlaid with medium containing 0.1% methylcellulose with or without analog 1 (○) or 2 (●). After 48 h of incubation, the cultures were washed and stained and the plaques were counted as described in Materials and Methods. The data are averages of three separate assays done in duplicate. AdoHcy/AdoMet ratios (△, analog 1; ▲, analog 2) were determined after 24 h as described in the legend to Fig. 2.

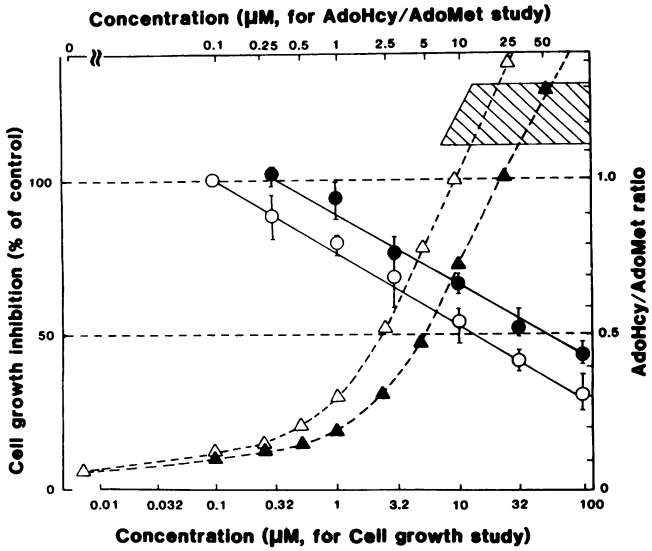

FIG. 4. Relationship between L-929 cell toxicity and AdoHcy/AdoMet ratios in murine L-929 cells treated with various concentrations of analogs 1 and 2. L-929 cells were plated at 4.5×10^5 per dish (30-mm diameter) and grown in Waymouth MB 752/1 medium containing 2% calf serum. After 6 h of incubation, the cells were fed with fresh medium without or with various concentrations of analog 1 (○) or 2 (●). After incubation for 72 h, the cells were harvested and viable cells were determined by trypan blue staining. The data are averages of experiments done in triplicate. AdoHcy/AdoMet ratios (△, analog 1; ▲, analog 2) were determined after 24 h as described in the legend to Fig. 2.

37% lower than those of poly(A)$^+$ RNA isolated from non-drug-treated, virus-infected cells (control samples).

It should be noted that the extent of incorporation of [*methyl*-^{3}H]methionine and [^{14}C]uridine into poly(A)$^+$ RNA and the yield of poly(A)$^+$ RNA by the guanidine isothiocyanate isolation method and the oligo(dT)-cellulose chromatography method varied from experiment to experiment. The reproducibility of the data observed in experiment 1 (Table 2) was verified by repeating the experiments with slightly different concentrations of analog 1 (Table 2, experiment 2). Again a reduction of 26 to 28% was observed in the ^{3}H/^{14}C ratios in poly(A)$^+$ RNA isolated from cells treated with analog 1.

Effects of analogs 1 and 2 and neplanocin A on rNTP metabolism. After 6 h of incubation with or without 10 μM compound 1 or 2 or neplanocin A, cellular rNTPs (CTP, UTP, ATP, and GTP) were analyzed. The rNTP pools in L-929 cells were not disturbed by treatment with compound 1 or 2 (Table 3). With neplanocin A treatment, however, all rNTPs were decreased as follows relative to the control values: CTP, 47%; UTP, 48%; ATP, 62%; GTP, 81%. An ATP analog attributed to neplanocin A triphosphate was detected at a concentration of approximately 573 pmol/10^6 cells.

DISCUSSION

AdoHcy hydrolase plays an important role in regulating cellular levels of AdoHcy and subsequently regulating critical cellular AdoMet-dependent transmethylations (2, 27, 31). Since AdoMet-dependent transmethylations are also critical in the processing of viral mRNA (1) and thus virus replication, modulating the cellular levels of AdoHcy by inhibition of cellular AdoHcy hydrolase has become an attractive approach to the design of antiviral agents (7, 19, 24). AdoHcy hydrolase inhibitors have been shown to be broad-spectrum antiviral agents possessing activity against poxviruses (e.g., vaccinia virus), negative-strand RNA viruses

TABLE 1. Relationships among IC_{50}s, ID_{50}s, and changes in AdoHcy/AdoMet ratios in cells treated with analogs 1 and 2

Analog	At IC_{50}			At ID_{50}			Antiviral index (ID_{50}/IC_{50})	Selectivity	
	IC_{50} (μM)[a]	AdoHcy content (pmol/mg of protein)[a]	AdoHcy/AdoMet ratio[a]	ID_{50} (μM)[b]	AdoHcy content (pmol/mg of protein)[b]	AdoHcy/AdoMet ratio[b]		AdoHcy content at ID_{50} minus AdoHcy content at IC_{50}	AdoHcy/AdoMet ratio at ID_{50} minus AdoHcy/AdoMet ratio at IC_{50}
1	0.28	100	0.15	17	825	1.34	61	725	1.19
2	0.95	125	0.19	56	950	1.30	59	825	1.11

[a] Data taken from Fig. 3.
[b] Data taken from Fig. 4.

TABLE 2. Effects of analog 1 on RNA methylation in vaccinia virus-infected L-929 cells[a]

Sample and concn (μM) of analog 1 (expt no.)	[^{3}H]methionine dpm	[^{14}C]uridine dpm	^{3}H/^{14}C ratio	% Inhibition of *methyl*-^{3}H incorporation
Poly(A)$^+$RNA				
0 (1)	10,498	5,893	1.78	0
0 (2)	27,262	10,937	2.49	0
0.03 (1)	7,936	6,027	1.32	26
0.05 (2)	11,877	6,345	1.87	25
0.03 (1)	14,781	11,571	1.28	28
0.5 (2)	32,514	18,177	1.79	28
3 (1)	12,065	10,722	1.13	37
Total cytoplasmic RNA				
0 (1)	29,168	10,258	2.84	0
0.03 (1)	17,100	6,353	2.69	5
0.3 (1)	14,544	6,283	2.31	19
3 (1)	11,074	5,415	2.05	28

[a] L-929 cells in spinner culture with or without analog 1 were incubated for 18 h as described in Materials and Methods. The cells were infected with vaccinia virus (multiplicity of infection, 15) and then treated with cycloheximide. After 1 h of incubation, adenosine and guanosine were simultaneously added and 1 h later the cells were pulse-labeled with [*methyl*-^{3}H]methionine (10 μCi/ml) and [^{14}C]uridine (0.5 μCi/ml). After 3 h of labeling the infected cells were harvested and total cRNA and poly(A)$^+$ RNA were isolated as described in Materials and Methods. Small samples of RNA (100 μl) containing greater than 5,000 dpm of ^{14}C radioactivity which were obtained by the guanidine thiocyanate method and the oilgo(dT)-cellulose affinity chromatography method were applied to DE-81 paper disks and washed with 5% Na_2HPO_4 five times (5 ml per disk) and with distilled water twice, and the radioactivity was counted. To ensure reproducibility, experiment 2 was performed on another day by the same method but with different concentrations of analog 1.

(e.g., the paramyxoviruses parainfluenza virus and measles virus and the rhabdoviruses rabies virus and vesicular stomatitis virus), and double-stranded RNA viruses (reovirus and rotavirus) (7). In contrast, herpes viruses and positive-strand RNA viruses (the picornaviruses enterovirus and rhinovirus and togavirus) are virtually resistant to the compounds (7).

For viruses that replicate within the cytoplasm (e.g., vaccinia virus), it has been proposed that inhibition of AdoHcy hydrolase and the subsequent elevation in intracellular AdoHcy cause inhibition of viral mRNA methyltransferases which are responsible for methylating the terminal guanosine residue (m^7G) and the penultimate nucleoside (Nm) in the mRNA cap (3, 18). This undermethylation is thought to lead to inefficient association of viral mRNA with ribosomes and, thus, reduced viral protein synthesis (3, 18). AdoHcy inhibitors also exhibit antiviral activity against influenza virus, a negative-strand RNA virus which replicates its genome within infected cell nuclei (7). In influenza virus, the viral mRNA is methylated by cellular enzymes rather than viral enzymes (26). Ransohoff et al. (28) have recently shown that inhibition of influenza virus replication is due at least in part to impaired recognition of undermethylated viral mRNA cap structures by the influenza virus polymerase complex. These results are consistent with the findings in viral nucleocapsids that mRNAs possessing the cap 1 ($m^7GpppNm$) structure are 14-fold more active in priming than those with a cap O (m^7GpppN) structure (5). These data suggest that the susceptibility of a virus to an AdoHcy hydrolase inhibitor would depend ultimately on the susceptibility of the viral (or cellular) enzyme(s) which methylates the viral mRNA to inhibition by AdoHcy.

A potential disadvantage of selecting AdoHcy hydrolase as a target for the design of antiviral agents is the fact that AdoHcy hydrolase is a cellular enzyme. Thus, inhibition of this cellular enzyme perturbs the cellular metabolism of AdoHcy and homocysteine (32). Since cellular AdoMet-dependent methyltransferases like the viral enzymes are susceptible to inhibition by AdoHcy and cellular AdoMet-dependent methyltransferases are critical to cellular viability (31), why is it possible to selectively inhibit virus replication and not perturb host cell viability? Until recently, it has been difficult to address this question because many AdoHcy hydrolase inhibitors are multifunctional drugs having effects on a variety of molecular targets. For example, neplanocin A is a potent inhibitor of AdoHcy hydrolase (3, 23), but it is also a substrate for adenosine kinase, resulting in the formation of nucleotide metabolites that can be converted to *S*-neplanocylmethionine (9, 10, 14, 15, 20, 21). In addition, neplanocin A also causes reduction in both the purine and pyrimidine nucleotide pools (Table 3). The transformation of neplanocin A to the corresponding carbocyclic nucleotide and *S*-neplanocylmethionine contributes significantly to the cytotoxic properties of this molecule (10, 14, 15, 21), whereas the inhibitory effects of the drug on AdoHcy hydrolase appear to mediate its antiviral effects (3, 6, 7, 17, 19).

Recently, specific inhibitors of AdoHcy hydrolase which are devoid of substrate activity for adenosine kinase and adenosine deaminase have been synthesized. These AdoHcy inhibitors include the carbocyclic analogs of 3-deazaadenosine (24), the 3-deaza derivative of neplanocin A (9, 11; B. Keller, K. Hillgren, C. Tseng, V. Marquez, and R. Borchardt, FASEB [Fed. Am. Soc. Exp. Biol.] J. 2:A1348, 1988) and *trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl derivatives 1 and 2 (Fig. 1) of neplanocin A (4, 12, 13; Keller et al., FASEB J.). With these specific AdoHcy hydrolase inhibitors, it is possible to elucidate the molecular mecha-

TABLE 3. rNTPs in murine L-929 cells treated with compound 1 or 2 or neplanocin A

Treatment[a]	Avg ± SD rNTP content (pmol/10^6 cells)[b]				
	CTP	UTP	ATP	NpcTP[c]	GTP
None (control)	216 ± 31	464 ± 44	1,885 ± 128		365 ± 42
Compound 1	214 ± 8	437 ± 19	1,809 ± 96		389 ± 7
Compound 2	194 ± 8	389 ± 28	1,631 ± 92		334 ± 51
Neplanocin A	102 ± 6	223 ± 9	1,186 ± 16	573 ± 13	297 ± 17

[a] Each compound was used at 10 μM.
[b] The values are averages of triplicate assays.
[c] On the basis of the elution pattern reported by Saunders et al. (29), the peak eluting immediately after the ATP peak in neplanocin-treated cells was assumed to be neplanocin A triphosphate (NpcTP).

nisms which allow for selective inhibition of virus replication and not perturbation of host cell viability.

As shown in this study, the anti-vaccinia virus activities of analogs 1 and 2 in L-929 cells can be closely correlated with the accumulation of intracellular AdoHcy, the increase in the AdoHcy/AdoMet ratios, and the inhibition of methylation of cytoplasmic poly(A)$^+$ mRNA. L-929 cells, which normally maintain low intracellular levels of AdoHcy (approximately 50 pmol/mg of protein) and low ratios of AdoHcy/AdoMet (approximately 0.05 to 0.1), are a conducive environment for vaccinia virus replication. However, slight elevations in the intracellular level of AdoHcy (to approximately 100 to 125 pmol/mg of protein) and elevations in the ratio of AdoHcy/AdoMet (to approximately 0.15 to 0.19) caused by inhibition of cellular AdoHcy hydrolase create an unfavorable environment which suppresses vaccinia virus replication. At concentrations of compounds 1 and 2 which produce these slight elevations in intracellular AdoHcy and the AdoHcy/AdoMet ratio, inhibition of methylation of poly(A)$^+$ RNA also occurs. These results strongly suggest that inhibition of virus replication produced by low concentrations (0.03 to 3.0 μM) of compounds 1 and 2 results from inhibition of highly susceptible AdoMet-dependent methyltransferases that are critical for virus replication. Our working hypothesis is that the highly susceptible AdoMet-dependent enzyme(s) is the viral mRNA methyltransferase responsible for formation of the capped structure (19).

In contrast to the extreme susceptibility of virus replication to slight elevations in the intracellular level of AdoHcy and the AdoHcy/AdoMet ratio, cellular viability is quite tolerant to higher levels of this same cellular metabolite. For example, cellular toxicity was observed at intracellular AdoHcy levels of approximately 825 to 950 pmol/mg of protein and AdoHcy/AdoMet ratios of approximately 1.3. The exact mechanism responsible for the cellular toxicity at high concentrations (10 to 100 μM) of compounds 1 and 2 has yet to be determined. This toxicity could result from inhibition of critical cellular AdoMet-dependent methyltransferases or general disruption of cellular AdoHcy-homocysteine homeostasis (32). Both mechanisms are being examined in our laboratory.

Previously, our laboratory (12) reported that the levels of antiviral effectiveness (ID_{50}/IC_{50} ratios) of analogs 1 and 2 were 61 and 59, respectively. The data reported in this study make it possible to define a therapeutic index for these compounds based on a comparison of the AdoHcy cellular levels at the ID_{50}s and IC_{50}s. These data show that with a specific inhibitor of AdoHcy hydrolase, cellular toxicity occurs only when intracellular concentrations of AdoHcy are elevated seven to eight times above those needed to inhibit virus replication.

Since cells are able to regulate intracellular levels of AdoHcy in part by releasing the metabolite into the extracellular medium (32), we were not surprised to see the differences between the therapeutic index calculated on the basis of the ID_{50}/IC_{50} ratio (approximately 60) and the therapeutic index calculated by measuring the intracellular levels of AdoHcy at the IC_{50}s and ID_{50}s (approximately 7 to 8).

In summary, the data reported in this paper show that virus replication can be inhibited with only slightly elevated levels of intracellular AdoHcy, whereas host cell toxicity is produced at intracellular concentrations of AdoHcy which are seven to eight times higher.

ACKNOWLEDGMENTS

This work was supported by a Public Health Service grant from the National Institutes of Health (GM-29332) and a grant from Glaxo, Inc.

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