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2-METHYLPROPYL ESTER OF 3-(ADENIN-9-YL)-2-HYDROXYPROPANOIC ACID

MECHANISM OF ANTIVIRAL ACTION IN VACCINIA VIRUS-INFECTED L929 CELLS

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Abstract—Alkyl esters of (*RS*)-3-(adenin-9-yl)-2-hydroxypropanoic acid (AHPA) were shown recently to be broad spectrum antiviral agents (De Clercq E and Holy A, *J Med Chem* **28**: 282–287, 1985). It was postulated that these alkyl esters function as prodrugs by undergoing hydrolysis catalyzed by cellular esters to AHPA, a known inhibitor of *S*-adenosyl-L-homocysteine (AdoHcy) hydrolase. In this study, we describe the metabolic fate of the 2-methylpropyl ester of AHPA (AHPA-iBu) in murine L929 cells. When AHPA-iBu was included in the culture medium, it was taken up rapidly by murine L929 cells. The uptake was time- and concentration-dependent, resulting in the intracellular accumulation of the free acid, AHPA. Treatment with AHPA-iBu caused inhibition of cellular AdoHcy hydrolase in both a time- and a dose-dependent manner. Complete inhibition of the enzyme was achieved after a 1-hr incubation in culture medium containing 50 μM AHPA-iBu. The inhibition of the enzyme caused cellular accumulation of AdoHcy and a significant increase in the ratio of AdoHcy/*S*-adenosyl-L-methionine (AdoMet). Partial recovery of the AdoHcy hydrolase activity in L929 cells treated with 50 μM AHPA-iBu was observed after 24 hr. This recovery of enzyme activity was paralleled by a significant decrease in the cellular levels of AdoHcy and the ratio of AdoHcy/AdoMet. AHPA-iBu also exerted an inhibition (IC_{50} = 0.17 μM) of vaccinia virus plaque formation in monolayers of L929 cells. A 1 μM concentration of AHPA-iBu, which caused 80% inhibition of plaque formation, produced a 17-fold increase in AdoHcy content in drug-treated, virus-infected cells versus non-drug-treated, virus-infected cells and a 15% undermethylation of the poly(A)$^+$ RNA. These data show that AHPA-iBu is a prodrug for AHPA which inhibits cellular AdoHcy hydrolase. The inhibition of this enzyme elevates cellular levels of AdoHcy, creating an unfavorable environment which suppresses replication of vaccinia virus.

S-Adenosyl-L-homocysteine (AdoHcy§) hydrolase (EC 3.3.1.1) has become an attractive target for the design of antiviral agents [1, 2]. 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 [3]. Inhibition of cellular AdoHcy hydrolase, however, results in an intracellular accumulation of AdoHcy, causing a significant increase in the intracellular AdoHcy/AdoMet ratio and subsequent inhibition of viral replication [2, 4–6]. The inhibition of viral AdoMet-dependent mRNA methylations caused by the elevated cellular levels of AdoHcy has been proposed to be the molecular mechanism by which AdoHcy hydrolase inhibitors such as neplanocin A, adenosine dialdehyde, and 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-adenine (DHCA) inhibit vaccinia virus replication in murine L929 cells [4–6]. Ransohoff *et al.* [7] 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 structure by the influenza polymerase complex.

Recently, a number of open-chain adenosine analogs have been shown to exhibit reversible [e.g. (*S*)-9-(2,3-dihydroxypropyl)-adenine ((*S*)-DHPA)] [8, 9] and irreversible [e.g. (*RS*)-3-adenine-9-yl)-2-hydroxypropanoic acid (AHPA), eritadenines] [10] inhibition of AdoHcy hydrolase. These acyclic AdoHcy hydrolase inhibitors all display broad-spectrum activity with preference toward (−)RNA viruses (rhabdo-, para-myxoviruses), (±)RNA viruses (reo) and poxviruses [9–12]. The antiviral effects of hydroxylated adenin-9-yl-alkanoic acids (e.g. AHPA, eritadenines) were observed only at relatively high concentrations in comparison to the neutral open-chain analogs (e.g. DHPA) [9–11], and the antiviral effects were strictly dependent upon host cell systems employed in the experiments [11].

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§ Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoHcy hydrolase, *S*-adenosylhomocysteine hydrolase; AdoMet, *S*-adenosyl-L-methionine; AHPA-iBu, 2-methylpropyl ester of (*RS*)-3-(adenin-9-yl)-2-hydroxypropanoic acid; AHPA, (*RS*)-3-(adenin-9-yl)-2-hydroxypropanoic acid; DHCA, 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-adenine; (*S*)-DHPA, (*S*)-9-(2,3-dihydroxypropyl)-adenine; Ado, adenosine; DTT, dithiothreitol; PFU, plaque-forming units; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; M.O.I., multiplicity of infection; and MTA, methylthioadenosine.

The need for higher concentrations of these compounds to produce antiviral effects probably results from their anionic character at physiological pH, which reduces their cell permeability [12]. Esters of adenin-9-yl-hydroxypropanoic acids (e.g. AHPA) exhibit little, if any, inhibitory activity toward purified AdoHcy hydrolase. However, they are much more potent antiviral agents than the parent compounds and, therefore, it was proposed that the esters act as prodrugs [12].

The present study was undertaken to elucidate the mechanism of the antiviral action of the 2-methylpropyl ester of AHPA (AHPA-iBu) in mouse L929 cells infected with vaccinia virus. We report that during uptake into the cell, AHPA-iBu was rapidly converted to AHPA, which inhibited cellular AdoHcy hydrolase and resulted in increases in the cellular level of AdoHcy and the ratio of AdoHcy/AdoMet. This perturbation in cellular metabolism caused undermethylation of poly(A)$^+$ RNA in drug-treated, virus-infected cells and, thus, inhibition of viral replication.

MATERIALS AND METHODS

Materials. Radiochemicals were obtained from the following sources: [*methyl*-^{3}H]methionine, 80 Ci/mmol (Amersham Corp., Arlington Heights, IL); [U-^{14}C]uridine, 517.5 mCi/mmol (New England Nuclear, Boston, MA); and [^{3}H]thymidine, 62 Ci/mmol (ICN Radiochemicals, Irvine, CA). [2,8-^{3}H]AdoHcy (7.72 mCi/mmol) was prepared enzymatically from [2,8-^{3}H]adenosine and D,L-homocysteine using partially purified bovine liver AdoHcy hydrolase according to the method of Chabannes *et al.* [13].

Standard chemicals and supplies were purchased from the following commercial suppliers: calf intestinal adenosine deaminase (Sigma Chemical Co., St Louis, MO); 3a70 scintillation fluid (Research Products International, Mt Prospect, IL); DE-81 disks (Whatman, Clinton, NJ); Waymouth's 752/1 (low calcium) and Waymouth's MB 701/1 medium (Hazleton Research Products, Denver, PA); Zorbax C8 reverse-phase HPLC column (Dupont, Wilmington, DE); oligo(dT)-cellulose Type 7 (Pharmacia Biotechnology, Piscataway, NJ); and cellulose TLC plates (Eastman Kodak, Rochester, NY). AHPA and AHPA-iBu were synthesized as previously described [12, 14].

Cell culture. Stock cultures of clone 929 mouse cells, Strain L (Earle), were grown in suspension at 37° in Waymouth's 752/1 medium with 4% bovine calf serum and 70 μg/mL gentamycin as described previously [15]. Experimental cultures of L929 cells plated in tissue culture dishes were grown in Waymouth's MB 701/1 medium containing 2% calf serum.

Vaccinia virus plaque assay. Murine L929 cells for the anti-vaccinia virus assay were plated in 6-well culture plates (Costar, 30 mm diameter) and grown in Waymouth's MB 701/1 medium containing 2% calf serum. Nearly confluent cell monolayers were infected with vaccinia virus at about 300 plaque-forming units (PFU) per well. After a 60-min virus adsorption period, the viral inoculum was diluted by the addition of the culture medium and then removed completely by aspiration. Immediately after infection, each well containing a cell monolayer was overlaid with 2.6 mL of medium containing 0.1% methylcellulose and the test compound. After incubation for 48 hr at 37°, cultures were washed with phosphate-buffered saline (PBS) and stained with 0.1% crystal violet, and the plaques were then counted. The experiments were conducted in duplicate and the data (see Fig. 6) were expressed as an IC_{50} value (concentration of drug required to reduce viral plaque formation by 50%).

AdoHcy hydrolase assay. Murine L929 cells (3×10^6 cells/60 mm diameter dish) were removed by trypsinization, washed in PBS (containing 1 mM DTT) and lysed in 50 μL of cold hypotonic buffer (10 mM Sorensen phosphate buffer, 1 mM DTT, pH 7.4) by rapid freezing on dry ice. The cell debris was removed by centrifugation, and the AdoHcy hydrolase activity was determined in a reaction mixture which contained 40 mM Sorensen phosphate buffer (pH 7.4), 170 μM [2,8-^{3}H]AdoHcy, 1 mM EDTA, 4 units of intestinal adenosine deaminase and 70 μL of the cell lysate. The incubation was carried out for 40 min at 37°. Aliquots (5 μL) were then withdrawn from the reaction mixture and directly applied to TLC cellulose plates (the reaction was terminated by hot air) previously spotted with cold standards of AdoHcy, inosine and hypoxanthine. After the chromatography in 2-propanol: conc. ammonia: water (7:1:2), the TLC sheets were dried and corresponding spots were visualized using a UV 254 lamp and removed for radioactivity determination in 3a70 scintillation fluid. The radioactivity was determined in both AdoHcy and inosine-hypoxanthine (double spot) areas.

Determination of intracellular levels of AHPA, AHPA-iBu, AdoHcy and AdoMet. In experiments designed to determine the effects of AHPA-iBu on the intracellular levels of AHPA, AdoHcy and AdoMet (Figs 1–3, 5 and 7), murine L929 cells (3×10^6) in 60 mm culture dishes were incubated with Waymouth's MB 701/1 medium containing 2% calf serum with or without the prodrug. Cells were removed from the culture dishes by trypsinization and lysed in 135 μL of 0.25 M perchloric acid by rapid freezing on dry ice. The samples were stored at −70° prior to HPLC analysis. To determine the effects of AHPA-iBu on the intracellular levels of AdoHcy and AdoMet in virus-infected cells, murine L929 cells (3×10^6) in 60 mm culture dishes were infected with vaccinia virus (200 μL, M.O.I > 1) or mock-infected. After a 1-hr infection period, the inoculum was diluted by addition of cold culture medium, and then removed completely by aspiration. Cultures were refed with Waymouth's MB701/1 medium containing 2% calf serum with or without 1 μM AHPA-iBu and incubated for various periods of time. At the indicated times cells were collected into microcentrifuge tubes and prepared for HPLC analysis of the metabolites as described above. In preparation for HPLC analysis, the samples were thawed rapidly and the cell debris was removed by centrifugation. The supernatant fraction (100 μL) was injected into a Perkin–Elmer Series 3 HPLC system equipped with a 15 cm × 4 mm Zorbax C8 reverse-phase column.

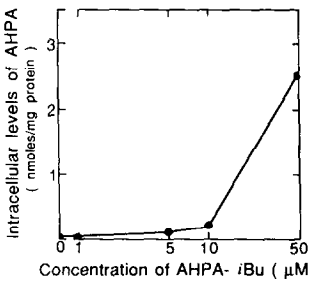

Fig. 1. Effect of AHPA-iBu concentration on intracellular accumulation of AHPA. The L929 cells grown in the presence of AHPA-iBu at the indicated concentrations were harvested by trypsinization after a 1-hr incubation period, and 0.25 M $HClO_4$ extracts were analyzed by reverse-phase HPLC as described in Materials and Methods. Data are averages of duplicate experiments.

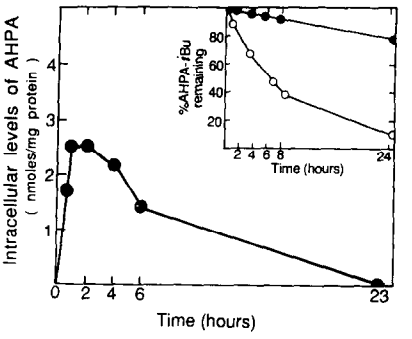

Fig. 2. Time-dependent accumulation of AHPA in L929 cells after treatment with AHPA-iBu. Monolayer cultures of L929 cells were treated with 50 μM AHPA-iBu. At the indicated times, cultures were harvested by trypsinization. The cell pellets were extracted in 0.25 M $HClO_4$ and analyzed by HPLC. Inset: aliquots (65 μL) from the culture medium in the presence (○) or absence (●) of the L cells were analyzed by the same procedure. For details see Materials and Methods. Data are averages of duplicate experiments.

AHPA-iBu, AHPA, AdoHcy and AdoMet were separated by a two-step gradient program at a flow rate of 1 mL/min (see Fig. 3). Solvent A: acetonitrile; solvent B: 50 mM sodium phosphate (pH 3.2), 10 mM heptanesulfonic acid. Program: 5–20% A, 15 min; 20–25% A, 10 min; and quantitated by absorption at 254 nm. Cell pellets were dissolved into 0.1 N NaOH containing 2% $CaCO_3$, and the protein content was determined by the method of Bradford [16].

Cytoplasmic poly(A)$^+$ RNA methylation in vaccinia virus-infected L929 cells. Murine L929 cells were cultured for 12 hr in spinner culture in Waymouth's MB 701/1 medium lacking methionine and Ca^{2+} and containing dialyzed 2% calf serum. The cells were concentrated to a density of 30×10^5 cells/mL in 100-mL bottles and infected with vaccinia virus (M.O.I. = 10). In addition to the virus inoculum, cycloheximide (100 μg/mL) and AHPA-iBu (1 and 50 μM) were added to the cell cultures. After a 1-hr incubation period, adenosine (20 μM) and guanosine (10 μM) were added simultaneously, and 1 hr later the cells were pulse-labeled with [^{3}H-*methyl*]methionine (10 μCi/mL) and [U-^{14}C]uridine (0.5 μCi/mL). After a 3-hr labeling time (total 5-hr incubation), the infected cells were harvested by centrifugation and washed with PBS; total cytoplasmic RNA was then prepared using a modification of the guanidine thiocyanate method (at pH 9) [17, 18]. Total cytoplasmic RNA was fractionated to poly(A)$^-$ RNA and poly(A)$^+$ RNA by oligo(dT)-cellulose affinity chromatography [19] and quantified by binding to DE-81 paper disks and liquid scintillation counting.

RESULTS

Uptake of AHPA-iBu into L929 cells. The accumulation of AHPA in monolayers of L929 cells was shown to be dependent on both the concentration of AHPA-iBu (Fig. 1) and the incubation time (Fig. 2) The penetration of AHPA-iBu was apparently accompanied by its hydrolysis, since intracellular accumulation of the free acid AHPA (Figs 1, 2, and 3C), not the prodrug AHPA-iBu, was observed. After treating the cell monolayer with 50 μM AHPA-iBu, the maximal cellular content of AHPA (approx. 2.5 nmol/mg protein) was achieved in 1–2 hr of incubation (Fig. 2). After 2 hr of incubation, cellular levels of AHPA began to decrease, reaching undetectable levels (sensitivity of the assay method is 50 pmol/mg protein) within 24 hr. When the concentration of the prodrug AHPA-iBu (inset, Fig. 2) in the culture medium was measured simultaneously with the cellular accumulation of AHPA, the data show that the concentration of AHPA-iBu decreased with time and after 24 hr only 10% of the prodrug was detectable in the medium (Fig. 2, inset). AHPA-iBu disappeared much more rapidly from the culture medium in the presence of L929 cells than in culture medium alone (Fig. 2, inset). It should be noted that at concentrations of AHPA-iBu up to 150 μM no significant toxicity to L929 cells was observed.

When L929 cells were treated with AHPA-iBu, trace amounts (e.g. 13% of the initial dose of AHPA-iBu) of the free acid AHPA could be detected in the medium within 1 hr of incubation (Fig. 3B). The concentration of AHPA in the medium increased slightly with time (e.g. 22% of the initial dose of AHPA-iBu, 9 hr), but never exceeded 30% of the initial dose of the prodrug.

Cellular content of AdoHcy, AdoMet and AdoHcy hydrolase activity. AHPA has been shown to be a more potent inhibitor of purified AdoHcy hydrolase than AHPA-iBu [10, 12]. However, when L929 cells were treated with 50 μM AHPA, only partial inhibition (approx. 51%) of cellular AdoHcy hydrolase was observed after 2 hr of incubation (data not shown). In contrast, treatment of L929 cells with 50 μM AHPA-iBu caused complete inhibition of AdoHcy hydrolase within a 1-hr incubation period (Fig. 4). Lower concentrations (1 and 10 μM) of AHPA-iBu resulted in only partial inhibition of the AdoHcy hydrolase (20 and 68%, respectively, at 1 hr) (Fig. 4). Cells treated with 1, 10 or 50 μM AHPA-iBu showed substantial recovery of AdoHcy

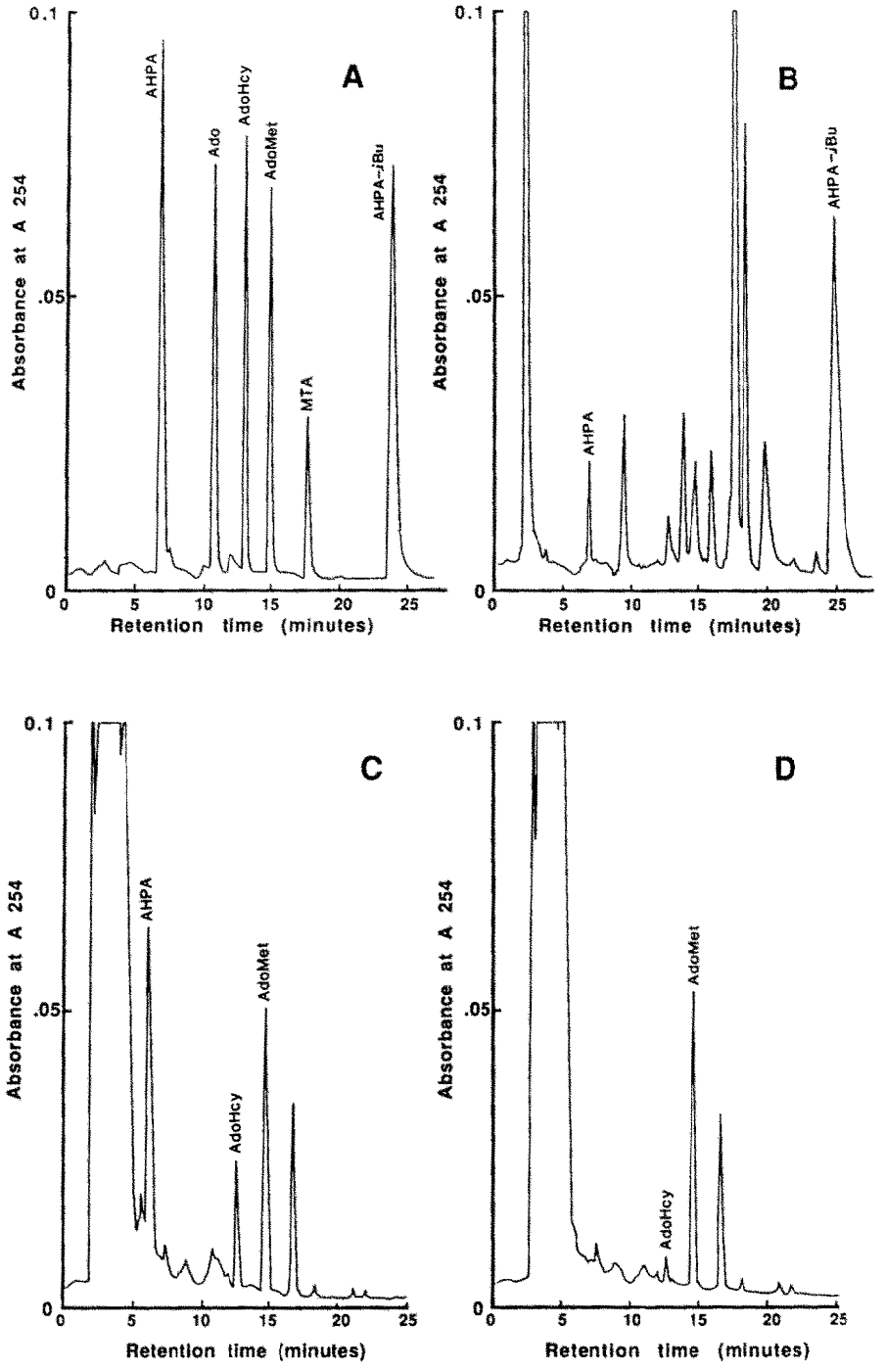

Fig. 3. HPLC chromatograms of cell extracts and culture medium from drug-treated (50 μM AHPA-iBu) and non-drug-treated (control) L929 cells. (A) Standard samples of AHPA, Ado, AdoHcy, AdoMet, MTA and AHPA-iBu. (B) Aliquot of the culture medium from cells treated for 1 hr with AHPA-iBu. (C) Cell extract prepared from cells treated for 1 hr with AHPA-iBu. (D) Cell extract prepared from non-drug-treated cells. Cell extract preparation and HPLC were conducted as described in Materials and Methods.

hydrolase activity (Fig. 4) after 24 hr of incubation. This recovery of enzyme activity was quite consistent with the disappearance of AHPA from the cells (Fig. 2).

The inhibition of AdoHcy hydrolase in the L929 cells with 50 μM AHPA-iBu was accompanied by a rapid accumulation of AdoHcy, causing an elevation in the AdoHcy/AdoMet ratio which reached maximum levels 4–6 hr after prodrug administration (Fig. 5). During the first 4 hr after prodrug administration,

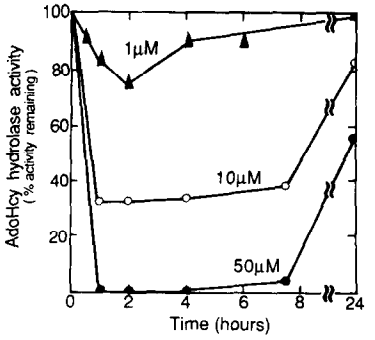

Fig. 4. Time- and concentration-dependence of inhibition of AdoHcy hydrolase activity in cell lysates prepared from AHPA-iBu-treated L929 cells. The monolayers of L cells were trypsinized at the indicated times, and the AdoHcy hydrolase activity in the cell lysates was determined as described in Materials and Methods. Data are averages of duplicate experiments.

a significant increase in AdoMet content was also observed (Fig. 5). Within 23 hr the intracellular AdoHcy content and the AdoHcy/AdoMet ratio decreased substantially from the peak values reached after 4–6 hr of incubation. These results are consistent with the partial recovery of AdoHcy hydrolase activity after 24 hr of incubation (Fig. 4).

When L929 cells were treated with a 50 μM concentration of the free acid AHPA, slight increases in the intracellular level of AdoHcy (0 hr, 47 pmol/mg protein; 1 hr, 87 pmol/mg protein; 2 hr, 107 pmol/mg protein; 3 hr, 118 pmol/mg protein; 6 hr, 131 pmol/mg protein) and the ratio of AdoHcy/AdoMet (0 hr, 0.06; 1 hr, 0.10; 2 hr, 0.11; 3 hr, 0.12; 6 hr, 0.14) were observed. These results are consistent with the fact that AHPA caused only partial inhibition of cellular AdoHcy hydrolase.

Inhibition of vaccinia virus plaque formation. The antiviral activity of AHPA-iBu was determined using confluent monolayers of L929 cells. The prodrug at the indicated concentration was added to the culture medium following the virus adsorption period (60 min) (Fig. 6). From the concentration-dependent inhibition of plaque formation shown in Fig. 6, the IC_{50} value for AHPA-iBu was determined to be 0.17 μM (0.05 μg/mL). This IC_{50} value for inhibition of vaccinia virus replication was approximately 60 times lower than the IC_{50} value reported earlier [12] using primary rabbit kidney cells. This difference in antivaccinia effects in L929 cells versus primary rabbit kidney cells is not unexpected, since similar differences in the antiviral activity of AHPA-iBu have been reported by De Clercq and Holý [12] using vesicular stomatitis virus in different cells. With 1 μM AHPA-iBu, 80% inhibition of vaccinia virus plaque formation was observed. Complete inhibition was observed when concentrations in the range of 5–10 μM AHPA-iBu were employed.

AdoHcy and AdoMet content in vaccinia virus-infected L929 cells. Considering our working hypothesis that the antiviral effects of AdoHcy hydrolase inhibitors are caused by inhibition of maturation of early viral mRNA, we measured the changes in the

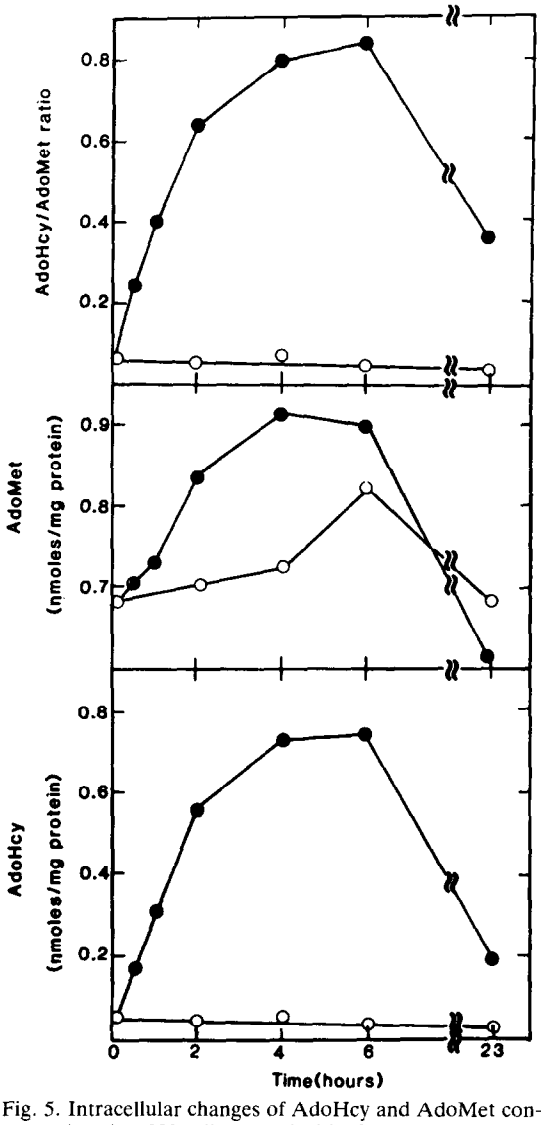

Fig. 5. Intracellular changes of AdoHcy and AdoMet concentrations in L929 cells treated with 50 μM AHPA-iBu. L cells [(○) control cells; (●) AHPA-iBu-treated cells] were treated for the indicated times, and AdoHcy and AdoMet levels were determined as described in Materials and Methods. Data are averages of duplicate experiments.

intracellular levels of AdoHcy and AdoMet in the vaccinia virus-infected cells (at M.O.I. > 1) and mock-infected cells treated with 1 μM AHPA-iBu (this concentration causes 80% reduction in plaque formation) (Fig. 6). As expected, both the AHPA-iBu-treated, virus-infected cells (Fig. 7B) and AHPA-iBu-treated, mock-infected cells (Fig. 7A) showed a time-dependent increase in intracellular levels of AdoHcy which reached maximum levels about 4 hr after drug treatment. The relative maximal increases in AdoHcy content at 4 hr in drug-treated, virus-infected cells versus non-drug-treated, virus-infected cells were 17-fold. In contrast, the relative maximal increases in AdoHcy content at 4 hr in the drug-treated, mock-infected cells versus non-drug-treated, mock-infected cells were only 5-fold.

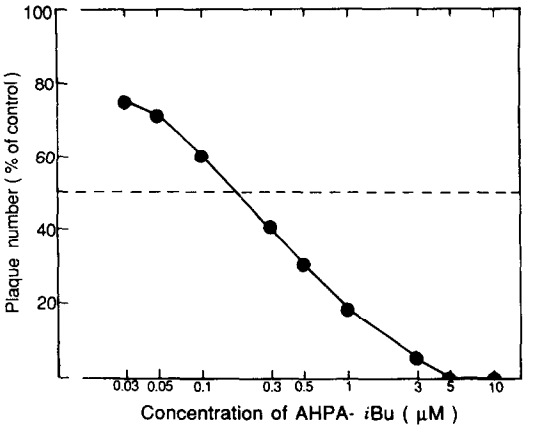

Fig. 6. Effect of AHPA-iBu on vaccinia virus plaque formation in L929 cells. For details, see Materials and Methods. Data are averages of duplicate experiments. Control, non-drug-treated samples had approximately 300 plaques per well.

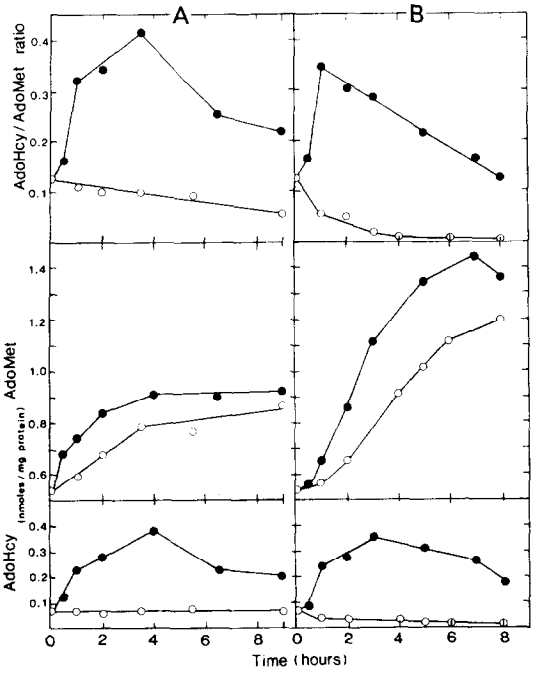

Fig. 7. Comparison of AdoHcy and AdoMet levels in the mock-infected (A) and vaccinia virus-infected (B) cells treated with 1 μM AHPA-iBu. The prodrug [(○) control, non-drug treated; (●) drug-treated] was applied after the virus adsorption period (M.O.I. > 1.0). The experiments were carried out in 60 mm dishes (3×10^6 cells/dish) using the conditions described in Materials and Methods. Data are averages of duplicate experiments.

This difference occurs because of the decrease in AdoHcy levels in non-drug-treated, virus-infected cells. The mechanism responsible for this virus-induced decrease in AdoHcy content is currently under investigation in our laboratories. The absolute cellular levels of AdoHcy observed in drug-treated, virus-infected cells and drug-treated, mock-infected cells were similar. The data also show that in both non-drug-treated and drug-treated, virus-infected and mock-infected cells, time-dependent elevations in AdoMet levels were observed. The elevation in AdoMet was 50% higher in the virus-infected cells (Fig. 7B) than in the mock-infected cells (Fig. 7A). The result of these changes in both virus- and mock-infected cells is that treatment with AHPA-iBu causes a time-dependent increase in the ratio of AdoHcy/AdoMet which reaches a maximum of approximately 0.4 after 2–4 hr of drug treatment.

Methylation of the total cytoplasmic poly(A)$^+$ RNA isolated from L929 cells infected with vaccinia virus. In an attempt to determine if the antiviral activity of AHPA-iBu was caused by undermethylation of the viral mRNA, we purified the total cytoplasmic poly(A)$^+$ RNA from the L929 cells infected with vaccinia virus. AHPA-iBu (1 or 50 μM) was applied to the cells in spinner culture together with the virus (at M.O.I. = 10) and the infected cells were incubated for a total of 5 hr. The non-drug-treated, virus-infected cells were cultured under the same conditions. Two hours into the incubation, both the drug-treated and non-drug-treated cells were pulse-labeled with [^{3}H-*methyl*]methionine and [U-^{14}C]uridine. Three hours later the cells were collected, and total cytoplasmic RNA was isolated using oligo(dT)-cellulose affinity chromatography. The extent of the methylations in both the poly(A)$^-$ and poly(A)$^+$ RNA was then calculated as the ^{3}H/^{14}C ratio. The data presented in Table 1 show that in AHPA-iBu-treated, vaccinia virus-infected cells the ^{3}H/^{14}C ratio in poly(A)$^+$ RNA fractions was markedly lower than in the corresponding non-drug-treated, virus-infected cells. The 5-hr treatment of the infected cells with 1 or 50 μM AHPA-iBu resulted in 15 and 19% undermethylation respectively. Since the degree of incorporation of [^{3}H-*methyl*]methionine and [U-^{14}C]uridine into cytoplasmic RNA and the yield of the cytoplasmic RNA varied from experiment to experiment, the results shown in Table 1 were confirmed in replicate experiments (data not shown).

DISCUSSION

During the past decade, various laboratories have shown that inhibition of AdoHcy hydrolase, which is accompanied by alterations in the intracellular AdoHcy/AdoMet ratio, produces a variety of pharmacological effects [3]. Perhaps the most interesting pharmacological effect, from a therapeutic viewpoint, is the antiviral effect of AdoHcy hydrolase inhibitors [1, 2]. Carbocyclic (e.g. neplanocin A [4]) and acyclic (e.g. AHPA [10, 12]) analogs of adenosine have been shown to exhibit antiviral effects which could be correlated with their ability to inhibit AdoHcy hydrolase. A major limitation of the utility of acyclic alkanoic acids (e.g. AHPA) is their poor cell permeability [10, 12]. However, esterification of the free carboxyl group of AHPA was shown to be an effective means of enhancing its antiviral activity, apparently by enhancing its cellular permeability. The objective of this study was to determine if esters of AHPA (e.g. AHPA-iBu) are,

Table 1. Effect of AHPA-iBu on RNA methylation in vaccinia virus-infected L929 cells

AHPA-iBu (μM)	Sample	[^{3}H-*methyl*]-Methionine (dpm)	[U-^{14}C]-Uridine (dpm)	^{3}H/^{14}C
0	poly(A)$^-$ RNA	2981	1353	2.20
	poly(A)$^+$ RNA	1804	1399	1.29
1	poly(A)$^-$ RNA	2386	1236	1.93
	poly(A)$^+$ RNA	1558	1408	1.10
50	poly(A)$^-$ RNA	4026	1985	2.02
	poly(A)$^+$ RNA	2051	1953	1.05

The spinner cultures of L cells were infected with vaccinia virus (M.O.I. = 10). The prodrug (AHPA-iBu) was added to the culture together with the virus and the cells were incubated for 5 hr. During the last 3 hr, the cells were labeled with [^{3}H-*methyl*]methionine and [U-^{14}C]uridine. The total cytoplasmic RNA was extracted from the cells, and the poly(A)$^+$ RNA was isolated using oligo(dT)-cellulose affinity chromatography. The radioactivity of both poly(A)$^-$ RNA and poly(A)$^+$ RNA was determined in aliquots using the DE-81 paper disk method as described in Materials and Methods.

in fact, prodrugs of AHPA, as suggested earlier by De Clercq and Holý [12].

The results of these studies have shown that the cellular accumulation of AHPA is dependent upon the concentration of AHPA-iBu in the culture medium. In addition to accumulation of AHPA upon treatment of cells with 50 μM AHPA-iBu, cellular AdoHcy hydrolase was inhibited completely with 1 hr of drug treatment, and the cellular levels of AdoHcy and the ratio of AdoHcy/AdoMet increased, reaching maximal levels 4–6 hr after drug treatment. It is important to note that all of the biochemical changes produced by AHPA-iBu treatment (e.g. inhibition of AdoHcy hydrolase, increases in AdoHcy and the ratio of AdoHcy/AdoMet) were reversible with time (Figs 2, 4 and 5). Furthermore, our data show that the reversibility of these changes can be correlated with the decrease in intracellular concentration of AHPA. AHPA apparently is either undergoing metabolism within the cell to a metabolite that does not inhibit AdoHcy hydrolase or, most probably, is effluxed from the cell pool to the culture medium. The high relative content of AHPA in the medium (Fig. 2, inset) was apparently due to the hydrolysis of AHPA-iBu during the permeation. In contrast to the effects seen with AHPA-iBu, exposure of L929 cells to 50 μM AHPA resulted in only partial inhibition of AdoHcy hydrolase and slight increases in intracellular levels of AdoHcy and the ratio of AdoHcy/AdoMet. The data illustrate the poor cellular permeability of AHPA and the advantage of a prodrug form such as AHPA-iBu.

The observation in this study that the inhibition of AdoHcy hydrolase in AHPA-iBu-treated L929 cells is reversible was unexpected. Holý *et al.* [10] have reported that AHPA causes irreversible inactivation of the purified enzyme *in vitro* and Schanche *et al.* [20] have reported the time-dependent inactivation of the enzyme in intact rat hepatocytes treated with eritadenines and AHPA. In contrast to the irreversible inactivation of AdoHcy hydrolase produced by hydroxylated adenin-9-yl-alkanoic acids (e.g. AHPA, eritadenines), the corresponding 9-(hydroxyalkyl)-adenines (e.g. (*S*)-DHPA) have been reported to be reversible inhibitors [9, 21–22].

To confirm the apparent reversibility of the inhibitory effect of AHPA on L929 cell AdoHcy hydrolase observed in this study (Fig. 4), we treated purified bovine liver AdoHcy hydrolase with AHPA. Treatment of this purified AdoHcy hydrolase with 250 μM AHPA resulted in nearly complete inhibition (>97%) of the enzyme. However, dialysis of the AHPA-treated enzyme resulted in partial recovery (approx. 45%) of enzyme activity (Lee Y, Hasobe M and Borchardt RT, unpublished data). The origin of the difference between the data published earlier [10, 20] and the results reported here is unclear and will be the subject of future studies.

With several AdoHcy hydrolase inhibitors (e.g. neplanocin A), it has been shown that the undermethylation of the viral mRNA causes the inhibition of viral replication [2, 4–6]. In the present study, we have shown that a concentration (1 μM) of AHPA which elevated the AdoHcy/AdoMet ratio from 0.05 (control) to 0.35 and increased the AdoHcy level from 23 (control) to 350 pmol/mg protein in virus-infected cells was sufficient to produce a significant (approximately 80%) inhibition of viral plaque formation. Under these conditions, total cytoplasmic poly(A)$^+$ RNA became undermethylated by 15% in comparison with mock-infected cells. The data reported above for AHPA-iBu are in good agreement with the data reported for other AdoHcy hydrolase inhibitors. For example, Hasobe *et al.* [23] reported that the antiviral effects of DHCA could be related to the intracellular concentration of AdoHcy and the ratio of AdoHcy/AdoMet. At the concentration of DHCA that produced 50% inhibition of vaccinia virus replication (IC_{50}) in murine L929 cells, the intracellular levels of AdoHcy increased from approximately 50 pmol/mg protein (controls) to approximately 200 pmol/mg protein and the ratio of AdoHcy/AdoMet increased from approximately 0.05 (controls) to 0.20. Under these conditions, DHCA caused approximately 25% undermethylation of poly(A)$^+$ RNA.

In summary, the data presented in this manuscript show that AHPA-iBu is a prodrug for AHPA, which inhibits cellular AdoHcy hydrolase. The inhibition of this enzyme elevates cellular levels of AHPA,

creating an unfavorable environment which suppresses replication of vaccinia virus.

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REFERENCES

1. De Clercq E, *S*-Adenosylhomocysteine hydrolase inhibitors as broad-spectrum antiviral agents. *Biochem Pharmacol* **36**: 2567–2575, 1987.
2. Keller BT and Borchardt RT, Inhibition of *S*-adenosylmethionine-dependent transmethylation as an approach to the development of antiviral agents. In: *Antiviral Drug Development, A Multidisciplinary Approach* (Eds. De Clercq E and Walker RT), pp. 123–138. Plenum, New York, 1988.
3. Ueland PM, Pharmacological and biochemical aspects of *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-homocysteine hydrolase. *Pharmacol Rev* **34**: 223–253, 1982.
4. Borchardt RT, Keller BT and Patel-Thombre U, Neplanocin A. A potent inhibitor of *S*-adenosylhomocysteine hydrolase and of vaccinia virus multiplication in mouse L929 cells. *J Biol Chem* **259**: 4353–4358, 1984.
5. Keller BT and Borchardt RT, Adenosine dialdehyde: A potent inhibitor of vaccinia virus multiplication in mouse L929 cells. *Mol Pharmacol* **31**: 485–492, 1987.
6. Hasobe M, McKee JG, Borcherding DR, Keller BT and Borchardt RT, Effects of 9-(*trans*-2′-*trans*-3′-dihydroxycyclopent-4′-enyl)-adenine and 3-deaza-adenine on the metabolism of *S*-adenosylhomocysteine in mouse L929 cells. *Mol Pharmacol* **33**: 713–720, 1988.
7. Ransohoff RM, Narayan P, Ayers DF, Rottman FM and Nilsen TW, Priming of influenza mRNA transcription is inhibited in CHO cells treated with the methylation inhibitor, neplanocin A. *Antiviral Res* **7**: 317–327, 1987.
8. Votruba I and Holý A, Inhibition of *S*-adenosyl-L-homocysteine hydrolase by the aliphatic nucleoside analog 9-(*S*)-(2,3-dihydroxypropyl)-adenine. *Collect Czech Chem Commun* **45**: 3039–3044, 1980.
9. Holý A, Votruba I and De Clercq E, Structure–activity studies on open-chain analogs of nucleosides: inhibition of *S*-adenosyl-L-homocysteine hydrolase and antiviral activity. 1. Neutral open-chain analogs. *Collect Czech Chem Commun* **50**: 245–261, 1985.
10. Holý A, Votruba I and De Clercq E, Structure–activity studies on open-chain analogs of nucleosides: inhibition of *S*-adenosyl-L-homocysteine hydrolase and antiviral activity. 2. Acid open-chain analogs. *Collect Czech Chem Commun* **50**: 262–279, 1985.
11. Holý A, Votruba I and De Clercq E, Synthesis and antiviral activity of stereoisomeric eritadenines. *Collect Czech Chem Commun* **47**: 1392–1407, 1982.
12. De Clercq E and Holý A, Alkyl esters of 3-(adenin-9-yl)-2-hydroxypropanoic acid: A new class of broad-spectrum antiviral agents. *J Med Chem* **28**: 282–287, 1985.
13. Chabannes B, Gharib A, Cronenberger L and Pacheco H, *S*-Adenosyl-L-homocysteine: simplified enzymatical preparation with high yield. *Prep Biochem* **12**: 395–415, 1983.
14. Holý A, Preparation and synthetic utilization of 3-(adenine-9-yl)-2-hydroxyalkanoic acids and their derivatives. *Collect Czech Chem Commun* **49**: 2148–2166, 1984.
15. Keller BT, Clark RS, Pegg AE and Borchardt RT, Purification and characterization of some metabolic effects of *S*-neplanocylmethionine. *Mol Pharmacol* **28**: 364–370, 1985.
16. Bradford MM, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
17. Berger SL and Birkenmeier CS, Inhibition of intractable nucleases with ribonucleoside–vanadyl complex: isolation of messenger ribonucleic acid from resting lymphocytes. *Biochemistry* **18**: 5143–5149, 1979.
18. Pacha RF and Condit RC, Characterization of a temperature-sensitive mutant of vaccinia virus reveals a novel function that prevents virus-induced breakdown of RNA. *J Virol* **56**: 395–403, 1985.
19. Maniatis T, Fritch EF and Sambrook J, *Molecular Cloning, A Laboratory Manual*, pp. 197–198. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
20. Schanche J-S, Schanche T, Ueland PM, Holý A and Votruba I, The effect of aliphatic adenine analogs on *S*-adenosylhomocysteine hydrolase in intact rat hepatocytes. *Mol Pharmacol* **26**: 553–558, 1984.
21. Votruba I, Holý A and De Clercq E, Metabolism of the broad-spectrum antiviral agent, 9-(*S*)-(2,3-dihydroxypropyl)adenine in different cell lines. *Acta Virol* **27**: 273–276, 1983.
22. Holý A and Cihak A, Metabolism of 3-(*S*)-(2,3-dihydroxypropyl)adenine, an antiviral agent, in mice. *Biochem Pharmacol* **30**: 2359–2361, 1981.
23. Hasobe M, McKee JG and Borchardt RT, The relationship between the intracellular concentration of *S*-adenosylhomocysteine and inhibition of vaccinia viral replication versus inhibition of murine L929 cell growth. *Antimicrob Agents Chemother* **33**: 828–834, 1989.