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INFLUENCE OF *S*-ADENOSYLHOMOCYSTEINE HYDROLASE INHIBITORS ON *S*-ADENOSYLHOMOCYSTEINE AND *S*-ADENOSYLMETHIONINE POOL LEVELS IN L929 CELLS

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Abstract—*S*-Adenosylhomocysteine hydrolase has been recognized as the target enzyme for the antiviral activity of several carbocyclic and acyclic adenosine analogues. In a previous study [Cools M and De Clercq E, *Biochem Pharmacol* **38**: 1061–1067, 1989], we found a close correlation between the antiviral activity of six adenosine analogues {(*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA], (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid [(*RS*)-AHPA] (isobutyl ester), 3-deazaneplanocin A, carbocyclic 3-deazaadenosine (C-c^{3}Ado), adenosine dialdehyde and neplanocin A} against vaccinia virus and vesicular stomatitis virus and the inhibitory effect of these compounds on purified AdoHcy hydrolase isolated from murine L929 cells. We have now examined the effects of the different adenosine analogues on the intracellular pool levels of *S*-adenosylhomocysteine (AdoHcy) and *S*-adenosylmethionine (AdoMet). Treatment of vaccinia virus-infected L929 cells for 24 hr with the adenosine analogues at a dose that reduced vaccinia virus growth by 90% (ID_{90}) increased the average AdoHcy pool levels from 0.027 nmol/mg protein to approximately 0.3 nmol/mg protein and the AdoHcy/AdoMet ratio from 0.038 to approximately 0.3. Moreover, the AdoHcy/AdoMet ratio correlated closely with the vaccinia virus yield reduction, both determined over the 24-hr post infection period (correlation coefficient of 0.972). These findings indicate that the activity of the AdoHcy hydrolase inhibitors against vaccinia virus may be related to the raise in intracellular AdoHcy pool levels and AdoHcy/AdoMet ratio.

S-Adenosylhomocysteine (AdoHcy) hydrolase is considered as the target enzyme for the antiviral action of several carbocyclic and acyclic adenosine analogues {neplanocin A, adenosine (Ado) dialdehyde, 3-deazaneplanocin A, carbocyclic 3-deazaadenosine (C-c^{3}Ado), (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid [(*RS*)-AHPA] (isobutyl ester) and (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA]} [1, 2]. These adenosine analogues exhibit a characteristic broad-spectrum antiviral activity: they are particularly active against negative-stranded RNA viruses (Rhabdoviridae and Paramyxoviridae), double-stranded RNA viruses (Reoviridae) and Poxviridae [1].

AdoHcy hydrolase catalyses the reversible hydrolysis of AdoHcy to adenosine and homocysteine [3]. In the cell, AdoHcy hydrolase removes the AdoHcy formed by *S*-adenosylmethionine (AdoMet)-dependent methylation reactions. AdoHcy is a product-inhibitor of these reactions. AdoMet-dependent methylations play a crucial role in the cap formation of mRNA. 5′-Capping of mRNA is essential for initiation of protein synthesis [4]. Viruses belonging to the Rhabdoviridae, Reoviridae and Poxviridae are known to contain viral methyltransferases [5–8]. It is assumed that the antiviral activity of AdoHcy hydrolase inhibitors is related to the inhibition of viral methyltransferases. Inhibition of AdoHcy hydrolase would lead to an intracellular accumulation of AdoHcy, which, in turn, would result in the inhibition of viral methyltransferases, and thus, suppression of viral growth. Evidence for this hypothesis has been presented by Hasobe *et al.* [9]. These authors found that the anti-vaccinia virus activity of 9-(*trans*-2′, *trans*-3′-dihydroxycyclopent-4′-enyl)-adenine (DHCA) and -3-deazaadenine (DHCDA), two specific inhibitors of AdoHcy hydrolase, was related to the intracellular concentrations of AdoHcy and AdoHcy/AdoMet ratio.

In a previous study, we established that there was a close correlation between the activity of six adenosine analogues against vaccinia virus (VV) and vesicular stomatitis virus (VSV) and their inhibitory effect on purified AdoHcy hydrolase [2]. These findings pointed to AdoHcy hydrolase as the target for the antiviral activity of these compounds. Here, we examined the effects of different AdoHcy hydrolase inhibitors on the intracellular AdoHcy and AdoMet levels of VV-infected L929 cells. In these experiments, the compounds were used at a dose that reduced vaccinia virus yield by 90%. If the antiviral activity of the compounds is due to their effect on AdoHcy hydrolase, drug doses that achieve similar antiviral activity should elevate AdoHcy pool levels and AdoHcy/AdoMet ratios to a comparable extent.

MATERIALS AND METHODS

Compounds. (*S*)-9-(2,3-Dihydroxypropyl)adenine [(*S*)-DHPA] and (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid [(*RS*)-AHPA] isobutyl ester were provided by A. Holý (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of

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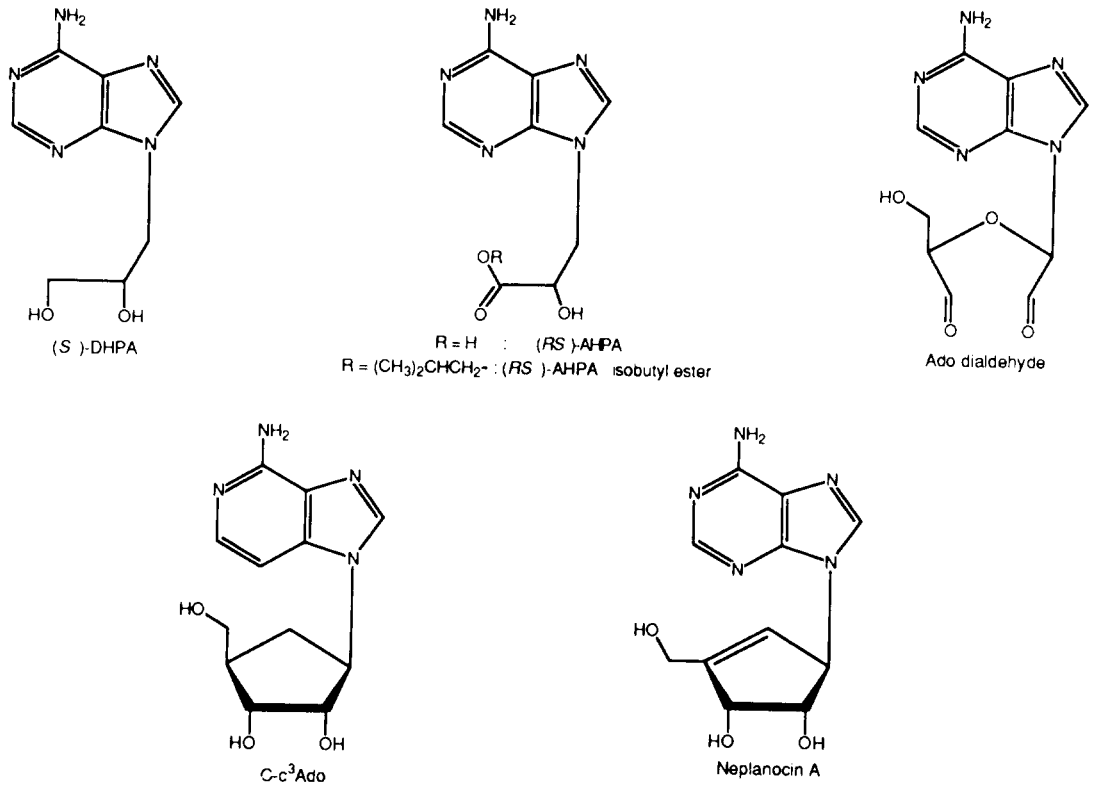

Fig. 1. Structural formulae of (*S*)-DHPA, (*RS*)-AHPA isobutyl ester, C-c^{3}Ado, Ado dialdehyde and neplanocin A.

Sciences, Prague, Czechoslovakia). Carbocyclic 3-deazaadenosine (C-c^{3}Ado) was provided by J. Montgomery (Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL, U.S.A.). Adenosine dialdehyde was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Neplanocin A was obtained from Toyo Jozo Co. (Ohito, Shizuoka-Ken, Japan), through the courtesy of J. Murase. The structural formulae of the compounds are depicted in Fig. 1.

Preparation of cell extracts for HPLC analysis. L929 cells were seeded at 3×10^6 cells / 60-mm dish. When confluent, usually at 24 hr, cell cultures were inoculated with VV at a multiplicity of infection (m.o.i.) of 0.1 plaque forming units (PFU) per cell. After a 1-hr adsorption period, the residual virus was removed and the cells were further incubated in fresh medium (3% fetal calf serum) with or without compound at a dose that reduced VV growth by 90% (ID_{90}). After 4, 8, 12 and 24 hr, cell cultures were washed with phosphate-buffered saline (PBS) and trypsinized. The cells were collected in Eppendorf tubes, centrifuged and the cell pellets were lysed in 200 μL of 10% trichloroacetic acid (TCA) (10 min, 4°). After centrifugation, the supernatant was frozen and stored at −70° (for less than 2 weeks) until HPLC analysis. The acid-insoluble pellets were dissolved overnight in 0.1 N NaOH and the protein content was determined.

HPLC analysis of intracellular AdoHcy and AdoMet pool levels. HPLC analyses were performed with a Perkin-Elmer series 400 liquid chromatograph equipped with a ISS-101 sampling system and a LCI-100 computing integrator (Perkin-Elmer, Uberlingen, F.R.G.). Analysis of AdoHcy and AdoMet was accomplished on a Superspher RP-8 column (0.46 mm × 12.5 cm) (Merck, Darmstadt, F.R.G.). For the elution, acetonitrile (ACN) and a buffer containing 50 mM NaH_2PO_4 and 5 mM heptane sulfonate pH 3.2 was used. At a flow rate of 1 mL / min, nucleosides were eluted with a 15 min linear gradient from 3–20% ACN, followed by a 5 min linear gradient from 20–25% ACN. AdoHcy and AdoMet peaks were monitored at 254 nm. The retention times were 10.0 and 11.1 min, respectively.

Protein assay. Protein content of the TCA-pellet (redissolved in NaOH) was determined using the

Table 1. Antiviral potency of AdoHcy hydrolase inhibitors against vaccinia virus in L929 cells

Compound	ID_{90} (μM)*
(*S*)-DHPA	200
(*RS*)-AHPA isobutyl ester	10
C-c^{3}Ado	3
Ado dialdehyde	0.2
Neplanocin A	0.1

* Data taken from Cools and De Clercq [2].

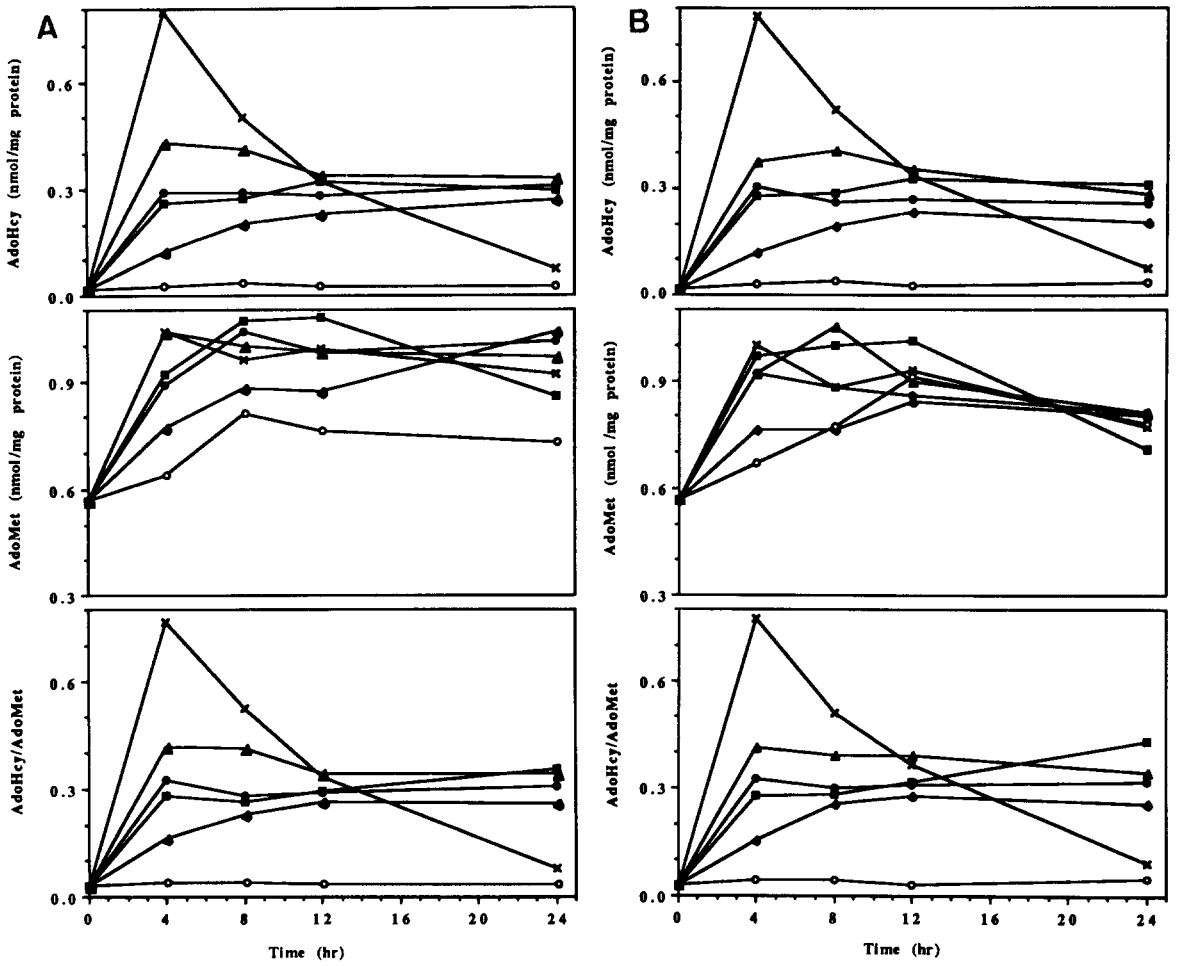

Fig. 2. (A) AdoHcy and AdoMet levels and AdoHcy / AdoMet ratio in L929 cells infected with vaccinia virus and either treated with (*S*)-DHPA (●), (*RS*)-AHPA isobutyl ester (×), C-c^{3}Ado (■), Ado dialdehyde (▲), neplanocin A (◆) at their ID_{90} for vaccinia virus growth or not treated (○). All data represent mean values for at least three separate experiments. (B) AdoHcy and AdoMet levels and AdoHcy / AdoMet ratio in uninfected L929 cells, either treated with (*S*)-DHPA (●), (*RS*)-AHPA isobutyl ester (×), C-c^{3}Ado (■), Ado dialdehyde (▲), neplanocin A (◆) at their ID_{90} for vaccinia virus growth or not treated (○). All data represent mean values for at least three separate experiments.

Bio-Rad protein assay (Bio-Rad, München, F.R.G.) and bovine serum albumin as standard.

Vaccinia virus yield reduction. The procedure for measuring VV production has been described previously [2]. Briefly, 3×10^6 L929 cells per 60-mm dishes were infected with 0.1 PFU of VV per cell during 1 hr. Unadsorbed virus was removed and fresh medium containing the test compound at the ID_{90} (see below) was added. At different time intervals, culture dishes were frozen, and, after freeze–thawing, the cell homogenate was cleared by centrifugation and the virus content of the supernatant was determined by a plaque assay on L929 cells.

RESULTS

AdoHcy and AdoMet levels in VV-infected and mock-infected L929 *cells treated with AdoHcy hydrolase inhibitors*

To compare the effects of the different AdoHcy hydrolase inhibitors on the intracellular AdoHcy and AdoMet pool levels, VV-infected and mock-infected L929 cells were treated with the compounds at a dose that reduced VV growth by 90% (ID_{90}). The ID_{90} values of (*S*)-DHPA, (*RS*)-AHPA isobutyl ester, C-c^{3}Ado, Ado dialdehyde and neplanocin A for VV growth are indicated in Table 1. Since the ID_{90} for VV replication was determined at 24 hr after virus infection, cell extracts for HPLC were prepared within this time period (at 4, 8, 12 and 24 hr post infection). From this study, 3-deazaneplanocin A treatment had to be excluded, because, with the HPLC assay system used, 3-deazaneplanocin A and AdoHcy had the same retention time and, thus, the effect of 3-deazaneplanocin A on the AdoHcy pool levels could not be evaluated. The effects of the other compounds on AdoHcy and AdoMet pool levels and the AdoHcy / AdoMet ratio in VV-infected L929 cells and mock-infected L929 cells are shown in Fig. 2A and B, respectively.

Table 2. Average AdoHcy levels and AdoHcy/AdoMet ratio determined over a 12-hr or 24-hr treatment period of VV-infected L929 cells with AdoHcy hydrolase inhibitors

Compound	Treatment period (12 hr) AdoHcy (nmol/mg)	AdoHcy/AdoMet	Treatment period (24 hr) AdoHcy (nmol/mg)	AdoHcy/AdoMet
(*S*)-DHPA	0.243	0.255	0.269	0.277
(*RS*)-AHPA	0.487	0.491	0.343	0.350
C-c^{3}Ado	0.233	0.235	0.271	0.280
Ado dialdehyde	0.339	0.340	0.337	0.340
Neplanocin A	0.149	0.180	0.199	0.220
Control	0.027	0.038	0.028	0.038

Treatment of the L929 cells with the AdoHcy hydrolase inhibitors at the ID_{90} for vaccinia virus increased the AdoHcy pool levels from 0.02 nmol/mg protein in the untreated cells to approximately 0.3 nmol/mg protein in the drug-treated cells. No differences were observed between the AdoHcy pool levels in VV-infected cells (Fig. 2A) and mock-infected cells (Fig. 2B). Addition of (*RS*)-AHPA isobutyl ester more rapidly raised the AdoHcy pool levels than did addition of the other compounds. Although the AdoHcy pool levels achieved following 8 hr treatment with (*RS*)-AHPA isobutyl ester were higher than with the other compounds, at 24 hr AdoHcy pool levels generated by (*RS*)-AHPA were lower than those generated by the other AdoHcy hydrolase inhibitors.

The AdoMet pool levels of L929 cells did not change upon VV infection or treatment with the AdoHcy hydrolase inhibitors. Treatment of VV-infected or mock-infected L929 cells with the AdoHcy hydrolase inhibitors at their ID_{90} for vaccinia virus growth thus increased the AdoHcy/AdoMet ratio from 0.03 (untreated cells) to approximately 0.3 (drug-treated cells).

Since the AdoHcy pool levels and the AdoHcy/AdoMet ratio may differ as a function of time [particularly striking for (*RS*)-AHPA isobutyl ester], the average AdoHcy pool levels and the average AdoHcy/AdoMet ratio over a 12-hr and 24-hr period were calculated. At 12 hr and 24 hr post-infection, the average AdoHcy levels and AdoHcy/AdoMet ratio in VV-infected cells treated with (*S*)-DHPA, (*RS*)-AHPA isobutyl ester, C-c^{3}Ado or Ado dialdehyde were comparable (Table 2). Treatment of L929 cells with 0.1 μM neplanocin A resulted in slightly lower AdoHcy pool levels and a lower AdoHcy/AdoMet ratio than treatment of the cells with the other AdoHcy hydrolase inhibitors at their ID_{90}.

VV yield after treatment of VV-infected L929 cells with AdoHcy hydrolase inhibitors at their ID_{90} for vaccinia virus

To elucidate whether the differences in average AdoHcy pool levels and AdoHcy/AdoMet ratio corresponded to differences in the antiviral potency of the compounds, L929 cells were infected with vaccinia virus at the same multiplicity of infection as in the previous experiment, and the virus yield was determined at the same time points as the AdoHcy and AdoMet pool levels (Fig. 3). Treatment of VV-infected L929 cells with the AdoHcy hydrolase inhibitors at their ID_{90} for vaccinia virus reduced the virus titer at 12 hr by 90–90%, and at 24 hr by 79–83%. It was immediately clear that neplanocin A, that increased AdoHcy pool levels and the AdoHcy/AdoMet ratio to a lesser extent than the other compounds, also inhibited virus growth to a lesser extent. We then compared the average AdoHcy levels and the AdoHcy/AdoMet ratios with the decrease in virus titer over the same time period (12 or 24 hr) (Fig. 4). For the 24-hr treatment period, a close correlation was found between the AdoHcy pool levels or the AdoHcy/AdoMet ratio and the reduction in virus titer during this period (correlation coefficient: 0.962 and 0.972, respectively). For the 12-hr treatment period, the correlation between the average AdoHcy pool levels or the AdoHcy/AdoMet ratio and the reduction in virus titer gave coefficients of 0.852 and 0.867, respectively.

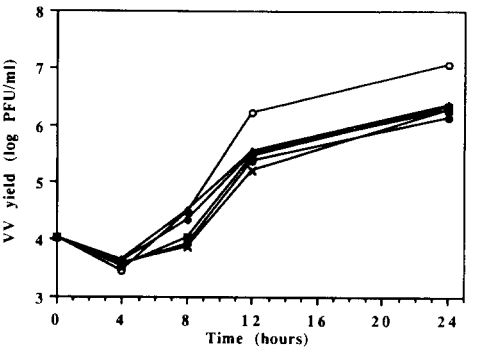

Fig. 3. Vaccinia virus titers after treatment of VV-infected cells (m.o.i.: 0.1) with different AdoHcy hydrolase inhibitors at their ID_{90}: (*S*)-DHPA (●); (*RS*)-AHPA isobutyl ester (×); C-c^{3}Ado (■); Ado dialdehyde (▲); neplanocin A (◆); control (○). All data represent mean values for at least three separate experiments.

DISCUSSION

AdoHcy hydrolase has been proposed as the target enzyme for the antiviral activity of several adenosine analogues [1]. Although it has been clearly shown that different adenosine analogues inhibit AdoHcy hydrolase, both *in vitro* and *in vivo* [10–16], there is

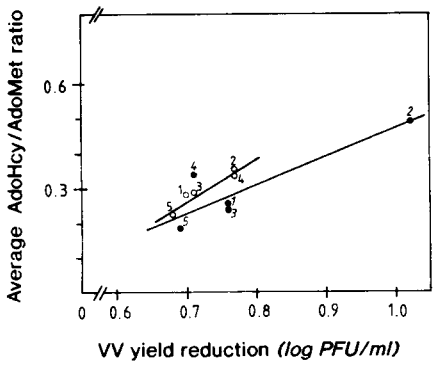

Fig. 4. Correlation between the average AdoHcy / AdoMet ratio of VV-infected L929 cells and the decrease in vaccinia virus titer at 12 hr (●) or 24 hr (○) post infection. 1, (*S*)-DHPA; 2, (*RS*)-AHPA; 3, C-c^{3}Ado; 4, Ado dialdehyde; 5, neplanocin A. Correlation coefficients: 0.867 (12 hr) and 0.972 (24 hr).

little, if any, experimental evidence for a direct causal relationship between the antiviral activity of the AdoHcy hydrolase inhibitors and their inhibitory effect on AdoHcy hydrolase. In a previous study, we found a close correlation between the antiviral activity of several acyclic and carbocyclic adenosine analogues (against vaccinia virus and vesicular stomatitis virus) in murine L929 cells and their inhibitory effect on L929 cell AdoHcy hydrolase [2]. Houston *et al.* [17] also demonstrated a correlation between the activity of a number of 2′,3′-dialdehyde derivatives of adenosine against vaccinia virus and their inhibiting effect on beef liver AdoHcy hydrolase, again pointing to AdoHcy hydrolase as the target enzyme for the antiviral activity of these compounds. Moreover, Hasobe *et al.* [9] showed that the antiviral and cytostatic effects of two AdoHcy hydrolase inhibitors, DHCA and DHCDA was related to the intracellular concentrations of AdoHcy. At concentrations of the compounds that afforded 50% inhibition of vaccinia virus replication, the intracellular AdoHcy levels rose from 0.05 nmol/mg protein in untreated cells to 0.1–0.125 nmol/mg protein in drug-treated cells and the AdoHcy/AdoMet ratio rose from 0.05–0.1 in untreated cells to 0.15–0.19 in drug-treated cells.

The present study provides further evidence that the antiviral activity of the AdoHcy hydrolase inhibitors (*S*)-DHPA, (*RS*)-AHPA isobutyl ester, C-c^{3}Ado, Ado dialdehyde and neplanocin A is targeted at AdoHcy hydrolase. Among the AdoHcy hydrolase inhibitors studied, both reversible and pseudo-irreversible inhibitors were found, which resulted in different patterns of accumulation of AdoHcy; i.e. treatment with (*RS*)-AHPA isobutyl ester led to a rapid increase of the AdoHcy pool levels, rapidly followed by a decrease of the AdoHcy pool levels. Treatment with neplanocin A slowly, but steadily increased the AdoHcy pool levels. When the average AdoHcy pool levels and the AdoHcy / AdoMet ratio over a 24-hr period were calculated, the different AdoHcy hydrolase inhibitors at their ID_{90} for vaccinia virus yield reduction increased the AdoHcy / AdoMet ratio from 0.04 to approximately 0.3 (range from 0.22 to 0.35) over a 24-hr period. The average AdoHcy pool levels and the AdoHcy / AdoMet ratio over the 24-hr period were directly correlated with the extent of vaccinia virus growth reduction.

In summary, the data presented here clearly indicate that the antiviral activity of AdoHcy hydrolase inhibitors against vaccinia virus is related to the effect of the compounds on the intracellular AdoHcy pool levels and the AdoHcy / AdoMet ratio.

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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. Cools M and De Clercq E, Correlation between the antiviral activity of acyclic and carbocyclic adenosine analogues in murine L929 cells and their inhibitory effect on L929 cell *S*-adenosylhomocysteine hydrolase. *Biochem Pharmacol* **38**: 1061–1067, 1989.
3. de la Haba G and Cantoni GL, The enzymatic synthesis of *S*-adenosyl-L-homocysteine from adenosine and homocysteine. *J Biol Chem* **234**: 603–608, 1959.
4. Shatkin AJ, Capping of eucaryotic mRNAs. *Cell* **9**: 645–653, 1975.
5. Furuichi Y, Muthukrishnan S, Tomasz J and Shatkin AJ, Mechanism of formation of reovirus mRNA 5′-terminal blocked and methylated sequence, m^{7}GpppG^{m}pC. *J Biol Chem* **251**: 5043–5053, 1976.
6. Morgan JR, Cohen LK and Roberts BE, Identification of the DNA sequences encoding the large subunit of the mRNA-capping enzyme of vaccinia virus. *J Virol* **52**: 206–214, 1984.
7. Moss B, Replication of poxviruses. In: *Virology* (Ed. Fields BN), pp. 685–703. Raven Press, New York, 1985.
8. Banerjee AK, Transcription and replication of rhabdoviruses. *Microbiol Rev* **51**: 66–87, 1987.
9. Hasobe M, McKee JG and Borchardt RT, Relationship between intracellular concentration of *S*-adenosylhomocysteine and inhibition of vaccinia virus replication and inhibition of murine L-929 cell growth. *Antimicrob Agents Chemother* **33**: 828–834, 1989.
10. Hoffman JL, The rate of transmethylation in mouse liver as measured by trapping *S*-adenosylhomocysteine. *Arch Biochem Biophys* **205**: 132–135, 1980.
11. Bartel RL and Borchardt RT, Effects of adenosine dialdehyde on *S*-adenosylhomocysteine hydrolase and *S*-adenosylmethionine-dependent transmethylations in mouse L929 cells. *Mol Pharmacol* **25**: 418–424, 1984.
12. 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.
13. Schanche J-S, Schanche T, Ueland PM and Montgomery JA, Inactivation and reactivation of intracellular *S*-adenosylhomocysteinase in the presence of nucleoside analogues in rat hepatocytes. *Cancer Res* **44**: 4297–4302, 1984.
14. Aarbakke J, Miura GA, Prytz PS, Bessesen A, Slordal L, Gordon RK and Chiang PK, Induction of HL-60

cell differentiation by 3-deaza-(±)-aristeromycin, an inhibitor of *S*-adenosylhomocysteine hydrolase. *Cancer Res* **46**: 5469–5472, 1986.

15. Glazer RI, Hartman KD, Knode MC, Richard MM, Chiang PK, Tseng CKH and Marquez VE, 3-Deazaneplanocin: a new and potent inhibitor of *S*-adenosylhomocysteine hydrolase and its effect on human promyelitic leukemia cell line HL-60. *Biochem Biophys Res Commun* **135**: 688–694, 1986.
16. Ramakrishnan V and Borchardt RT, Adenosine dialdehyde and neplanocin A: potent inhibitors of *S*-adenosylhomocysteine hydrolase in neuroblastoma N2a cells. *Neurochem Int* **10**: 423–431, 1987.
17. Houston DM, Dolence EK, Keller BT, Patel-Thombre U and Borchardt RT, Potential inhibitors of *S*-adenosylmethionine-dependent methyltransferases. 9. 2′,3′-Dialdehyde derivatives of carbocyclic purine nucleosides as inhibitors of *S*-adenosylhomocysteine hydrolase. *J Med Chem* **28**: 471–477, 1985.