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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

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Abstract—For a series of acyclic and carbocyclic adenosine analogues, a close correlation was found between their inhibitory effect on murine L929 cell *S*-adenosylhomocysteine (AdoHcy) hydrolase and their inhibitory effects on the replication of vaccinia virus and vesicular stomatitis virus (*r*: 0.993 and 0.988, respectively). In terms of their increasing inhibitory action against both virus replication and AdoHcy hydrolase activity the compounds ranked as follows: (*S*)-9-(2,3-dihydroxypropyl)adenine < (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid (isobutyl ester) < 3-deazaneplanocin A ~ carbocyclic 3-deazaadenosine < adenosine dialdehyde < neplanocin A. These findings point to AdoHcy hydrolase as the target for the antiviral action of these adenosine analogues.

S-Adenosylhomocysteine (AdoHcy) hydrolase catalyses the reversible hydrolysis of AdoHcy to adenosine (Ado) and L-homocysteine (Hcy) [1]. The reaction equilibrium favors the synthesis, but in intact cells the reaction proceeds in the hydrolytic direction because Ado and Hcy are further metabolized. In mammals there is no other enzyme known that degrades AdoHcy under physiological conditions.

Various acyclic and carbocyclic analogues of adenosine have been described as inhibitors or inactivators of AdoHcy hydrolase. The best known are: (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA], (D)-eritadenine, (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid [(*RS*)-AHPA], 3-deazaadenosine and carbocyclic 3-deazaadenosine (C-c^{3}Ado), aristeromycin, neplanocin A, 3-deazaneplanocin A and adenosine dialdehyde [2–10].

These adenosine analogues exhibit a marked antiviral activity against negative-stranded (−) RNA viruses [paramyxoviridae (parainfluenza virus, and measles virus), rhabdoviridae (rabies virus and vesicular stomatitis virus, VSV)], double-stranded (±) RNA viruses [reoviridae (reo- and rotavirus)] and poxviridae (vaccinia virus) (for review, see Ref. 11). These viruses are known to depend on a viral methyltransferase for 5′-capping of their mRNA [12–17]. This methylation is essential for maturation of the viral mRNAs and its consequent translation to proteins (for review, see Ref. 18). The virus-specified methyltransferases are more sensitive to product inhibition by AdoHcy than their cellular counterparts [16, 19, 20]. Inhibition of AdoHcy hydrolase by adenosine analogues leads to an accumulation of AdoHcy [5, 10, 21–24], and, thus, indirectly to an inhibition of viral mRNA methylation and maturation.

AdoHcy hydrolase may, therefore, be considered as a putative target enzyme for the antiviral action of adenosine analogues. As these analogues could theoretically be expected to interact with various metabolic steps, it appeared of fundamental interest to sort out whether an inhibitory action at the AdoHcy hydrolase level may indeed account for their antiviral activity. For a set of 4 adenosine analogues, (*S*)-DHPA, (*RS*)-AHPA (isobutyl ester), C-c^{3}Ado and neplanocin A, we found a close correlation between their inhibitory effects on bovine liver AdoHcy hydrolase and their activity against VSV replication [25]. To further delineate the role of AdoHcy hydrolase as target for the antiviral activity of acyclic and carbocyclic adenosine analogues, we included several new compounds in our investigations and extended the measurements of AdoHcy hydrolase activity to AdoHcy hydrolase extracted from the same (murine L929) cells as those used for the antiviral activity determinations. To this end, AdoHcy hydrolase was isolated from murine L929 cells and purified.

A close correlation was established between the inhibitory effects of six adenosine analogues on AdoHcy hydrolase from murine L929 cells and their antiviral potencies against both vesicular stomatitis virus and vaccinia virus.

MATERIALS AND METHODS

Compounds. (*S*)-9-(2,3-Dihydroxypropyl)adenine [(*S*)-DHPA], (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid [(*RS*)-AHPA] and (*RS*)-AHPA isobutyl ester were provided by A. Holy (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). Carbocyclic 3-deazaadenosine (C-c^{3}Ado) was provided by J. Montgomery (Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL). Neplanocin A was obtained from Toyo Jozo Co. (Ohito, Shizuoka, Japan) through the courtesy of J. Murase. 3-Deazaneplanocin A was provided by V. Marquez and J. S. Driscoll (Laboratory of

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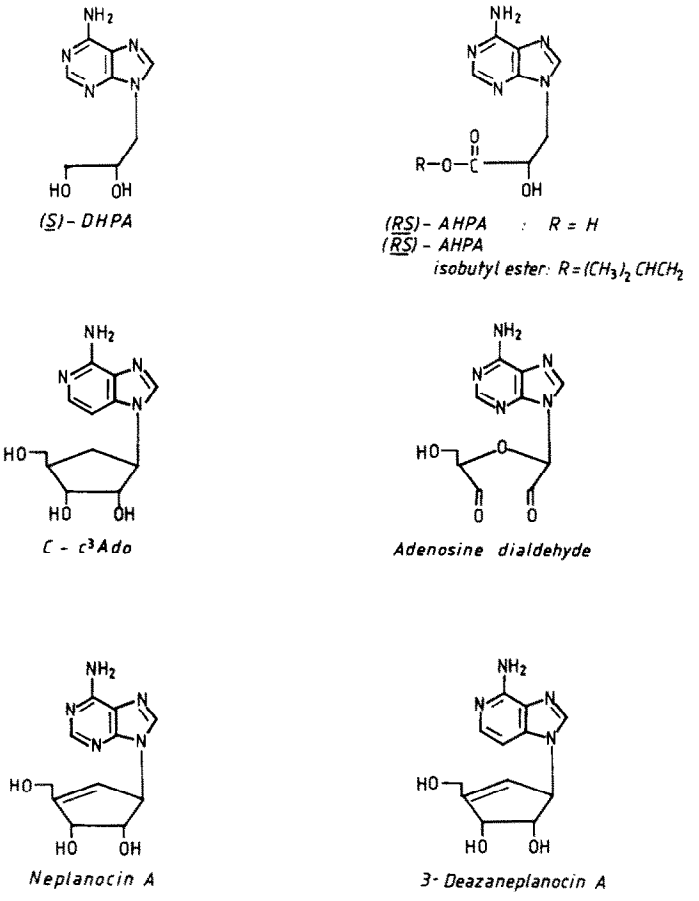

Fig. 1. Structural formulae of (*S*)-DHPA, (*RS*)-AHPA, (*RS*)-AHPA isobutyl ester, adenosine dialdehyde, C-c^{3}Ado, neplanocin A and 3-deazaneplanocin A.

Pharmacology and Experimental Therapeutics, National Cancer Institute, Bethesda, MD) and adenosine dialdehyde was purchased from Sigma Chemical Co. (St. Louis, MO). The structural formulae of the compounds are shown in Fig. 1.

AdoHcy hydrolase assay. AdoHcy hydrolase was purified from murine L929 cells to apparent homogeneity by using affinity chromatography [26], and AdoHcy hydrolase activity was measured in the direction of AdoHcy synthesis. The assay mixture (0.5 ml) contained 12.5 ng purified enzyme, varying amounts of [8-^{14}C]-adenosine (Amersham, Bucks, U.K.), 2 mM D,L-homocysteine, 2 mM dithiothreitol, 1 mM K–EDTA and appropriate concentrations of the test compounds in 150 mM K–phosphate buffer pH 7.6. After 4 min incubation at 37°, the reaction was stopped with 4 N perchloric acid; then 4 N KOH was added to pH 7.0, and unreacted adenosine was converted to inosine with one unit adenosine deaminase (Sigma Chemical Co.). AdoHcy and inosine were separated on a SP-Sephadex C-25 column (Pharmacia Fine Chemicals, Uppsala, Sweden). Inosine was eluted with 0.1 N formic acid and then AdoHcy was eluted with 0.5 N NaOH. The amount AdoHcy formed could be calculated from the ratio of the radioactivity counts found in the two eluates. From this ratio was subtracted the blank (without AdoHcy hydrolase, but with Ado deaminase and test compound at the highest concentration).

Virus yield reduction assay. To determine the inhibitory effects of the compounds on the replication of VSV and vaccinia virus (American Type Culture Collection, Rockville, MD), confluent L929 cells (3×10^6 cells/60 mm dish) were inoculated with either 0.001 or 0.1 plaque-forming units (PFU) per cell (for VSV and vaccinia virus, respectively). After a 1-hr virus adsorption period at 37°, residual non-adsorbed virus was discarded, and the cells were further incubated in fresh medium containing various concentrations of the test compounds. After different time intervals, when virus yields had reached their peak value (data not shown), cell cultures were frozen. For VSV, cell cultures were frozen after 12 and 24 hr, and for vaccinia virus the cells were frozen after 24 and 48 hr. Subsequently, the cells were thawed, and cell debris was removed by centrifugation. The virus content of the supernatant fluid was determined as follows: tenfold dilutions of the supernatant were added to confluent L929 cell cultures, and after a 1-hr incubation period, the medium was replaced by 4 ml agar overlay (VSV) or 4 ml medium (vaccinia virus). After another 2- or 3-day incubation period, plaques were stained with

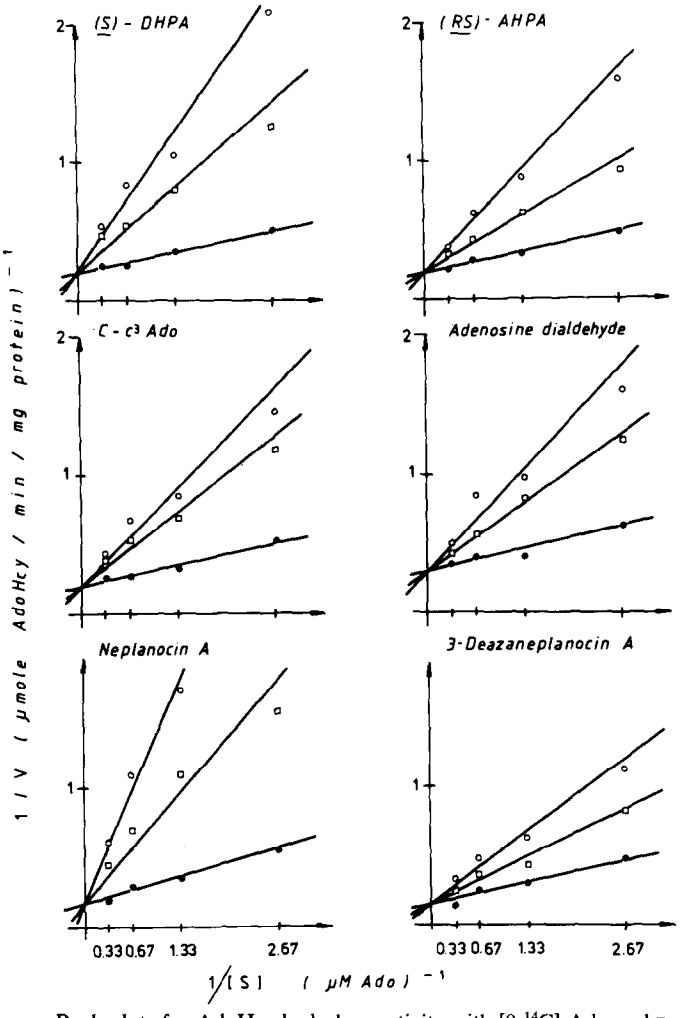

Fig. 2. Lineweaver–Burk plots for AdoHcy hydrolase activity with [8-^{14}C]-Ado and D,L-homocysteine as substrates in the presence of: (A) (*S*)-DHPA at 0 µm (●), 1 µM (□) and 2 µM (○); (B) (*RS*)-AHPA at 0 µM (●), 0.1 µM (□) and 0.25 µM (○); (C) C-c^{3}Ado at 0 µM (●), 0.05 µM (□) and 0.1 µM (○); (D) Adenosine dialdehyde at 0 µM (●), 0.01 µM (□) and 0.025 µM (○); (E) Neplanocin A at 0 µM (●), 0.01 µM (□) and 0.025 µM (○); (F) 3-Deazaneplanocin A at 0 µM (●), 0.025 µM (□) and 0.05 µM (○).

Neutral Red (VSV) or Crystal Violet (vaccinia virus). The concentration of test compound that reduced virus yield by 90% was determined as the 90%-inhibitory dose (ID_{90}).

RESULTS

The inhibitory effects of the test compounds on murine L929 cell AdoHcy hydrolase were monitored by measuring the enzyme activity in the direction of AdoHcy synthesis. The radiolabeled substrate [8-^{14}C]-adenosine was used at concentrations of 3, 1.5, 0.75 and 0.375 µM. The K_m of the purified L929 cell AdoHcy hydrolase for [8-^{14}C]-adenosine was 0.51 µM [26]. The adenosine analogues (*S*)-DHPA, (*RS*)-AHPA, C-c^{3}Ado, 3-deazaneplanocin A, adenosine dialdehyde and neplanocin A exerted an inhibitory effect on the AdoHcy hydrolase, which was competitive with respect to the natural substrate, adenosine. K_i values (Table 1) were calculated from the Lineweaver–Burk plots (Fig. 2). The order of increasing inhibitory potency was: (*S*)-DHPA < (*RS*)-AHPA < 3-deazaneplanocin A < C-c^{3}Ado < adenosine dialdehyde < neplanocin A.

Next, the compounds were evaluated for their antiviral activity against vaccinia virus and VSV. Both viruses encode for a specific methyltransferase and are thus, as explained in the Introduction, susceptible to inhibition by AdoHcy hydrolase inhibitors. To detect distinct differences in antiviral

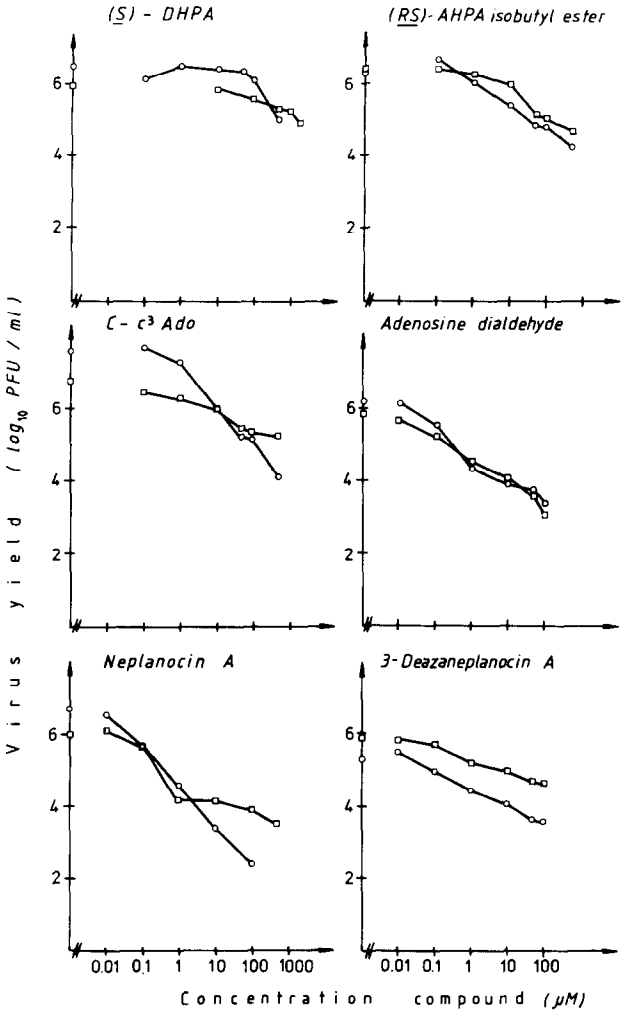

Fig. 3. VSV virus yield (□) and vaccinia virus yield (○), 12 hr (VSV) or 24 hr (vaccinia virus) after the virus-infected cells had been exposed to different concentrations of (*S*)-DHPA, (*RS*)-AHPA isobutyl ester, C-c^{3}Ado, adenosine dialdehyde, neplanocin A or 3-deazaneplanocin A.

properties, virus yield reduction assays were carried out. Figure 3 shows the VSV and vaccinia virus yields obtained 12 hr (VSV) or 24 hr (vaccinia virus) after incubation of the virus-infected cells in the presence of various concentrations of the test compounds. Similar curves were obtained after 24 hr (VSV) or 48 hr (vaccinia virus) (data not shown). From these curves, the dose of compound that reduced virus yield by 90% (ID_{90}) was calculated (Table 1). The order of increasing antiviral potency was: (*S*)-DHPA < (*RS*)-AHPA isobutyl ester < C-c^{3}Ado ~ 3-deazaneplanocin A < adenosine dialdehyde < neplanocin A. Although the order of increasing antiviral potency was identical for vaccinia virus and VSV, higher doses of compound were needed to achieve a similar reduction in the yield of VSV than of vaccinia virus.

Whereas in the AdoHcy hydrolase inhibition assays (*RS*)-AHPA was used in its free acid form, it was used as its isobutyl ester in the antiviral activity studies. (*RS*)-AHPA itself is poorly taken up by the cells because of its highly polar character [27]. In contrast, (*RS*)-AHPA isobutyl ester is rapidly taken up by the cells, and once it has penetrated in the cell it releases the free acid (*RS*)-AHPA [28].

When the K_i values of the compounds for L929 cell AdoHcy hydrolase were plotted as functions of their ID_{90} values for VSV and vaccinia virus replication (Fig. 4), linear regression showed a close correlation between the inhibitory effects on AdoHcy hydrolase and virus replication ($r = 0.993$ and 0.988 for vaccinia virus and VSV, respectively).

DISCUSSION

That adenosine analogues are inhibitory to AdoHcy hydrolase has been reported in several studies. However, as most of these studies were done with AdoHcy hydrolases purified from different sources, it is difficult to compare the relative potency of the adenosine analogues as inhibitors of AdoHcy hydrolase. We have previously shown that neplanocin A is a more potent inhibitor of bovine liver AdoHcy hydrolase (K_i: 2 nM) than C-c^{3}Ado, (*RS*)-AHPA or (*S*)-DHPA (K_i: 0.013, 0.073 and 1.4 μM, respectively) [25].

Borchardt *et al.* [5] determined an inactivation constant (K_I) of 8.39 nM for neplanocin A. For C-c^{3}Ado quite varying data have been reported: thus, Montgomery *et al.* [4] found a K_i value of 3 μM with the bovine liver enzyme and a K_i value of 1 nM with the hamster liver enzyme. Kim *et al.* [29] confirmed the low K_i value of C-c^{3}Ado for hamster liver AdoHcy hydrolase (K_i: 2 nM), whereas Houston *et al.* [7] also found a low K_i value (4 nM) for the bovine liver enzyme. With the (*R*)- and (*S*)-enantiomers of AHPA, Merta *et al.* [30] found for AdoHcy hydrolase extracted from L1210 leukemic cells IC_{50} values of 0.04 and 0.12 μM, respectively. They also mentioned an IC_{50} value of 0.9 μM for (*S*)-DHPA, comparable to a K_i of 3.5 μM for rat liver AdoHcy hydrolase [3]. Patel-Thombre and Borchardt [9] found adenosine dialdehyde to be a more potent inactivator of bovine liver enzyme than neplanocin A (K_I: 2.39 and 8.39 nM, respectively). Recently, Glazer *et al.* [10] reported that 3-deazaneplanocin A was the most potent AdoHcy hydrolase inhibitor ever found. For the hamster liver enzyme, they estimated a K_i value of 0.05 nM. However, from the data published by Glazer *et al.* [10], we calculated a K_i value of 5 nM, thus 100-fold that reported, and comparable to those found with neplanocin A and adenosine dialdehyde.

According to our own experience with AdoHcy hydrolase from murine L929 cells, the most potent AdoHcy hydrolase inhibitor is neplanocin A, followed by adenosine dialdehyde. 3-Deazaneplanocin is less potent an inhibitor of AdoHcy hydrolase; its potency is comparable to that of C-c^{3}Ado. Finally, (*RS*)-AHPA and (*S*)-DHPA are the least potent inhibitors of AdoHcy hydrolase.

In addition to establishing the comparative inhibitory effect of the adenosine analogues on AdoHcy hydrolase, we have also demonstrated a close correlation between their inhibitory effect on the enzyme and their antiviral potencies against vaccinia virus and vesicular stomatitis virus. In keeping with our previous findings [25], the present investigations evidently point to AdoHcy hydrolase as the target enzyme for the antiviral activity of the acyclic and carbocyclic adenosine analogues. *Stricto sensu*, our data implicate AdoHcy hydrolase as a target in the activity of the adenosine analogues against vaccinia and VSV, but it is likely that the correlation as found here between the inhibitory effect on AdoHcy hydrolase and the activity against vaccinia and VSV, also extends to other viruses depending for their replication on virus-specific methyltransferases. Furthermore, the correlation between antiviral activity and AdoHcy hydrolase inhibition, may extend to various adenosine analogues other than those that were the subject of the present study. In fact, with a number of 2′,3′-dialdehyde derivatives of adenosine, Houston *et al.* [8] also found a correlation between their activity against vaccinia virus and their inhibitory effect on AdoHcy hydrolase..

Although some of the test compounds are also known to irreversibly inactivate AdoHcy hydrolase, only the competitive inhibitory constants were taken up in the correlation determinations. Neplanocin A is supposed to be a mixed-type inhibitor of AdoHcy hydrolase: it can competitively inhibit the enzyme but also inactivate it irreversibly [5, 25, 31]. However, when the K_i for inhibition and the K_I for inactivation were compared, these values were almost identical, as shown by Chiang and Miura [31]. Chiang and Miura [31] postulated that mixed-type inhibitors may well act as pseudo-irreversible inac-

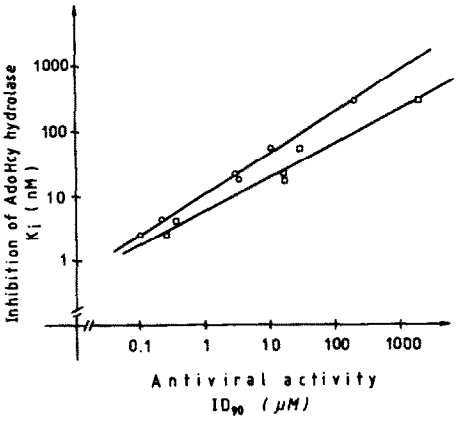

Fig. 4. Linear regression for K_i of (*S*)-DHPA, (*RS*)-AHPA (isobutyl ester), C.c^{3}Ado, 3-deazaneplanocin A, adenosine dialdehyde and neplanocin A for L929 cell AdoHcy hydrolase as a function of their antiviral ID_{90} values for the replication of vaccinia virus (○) or VSV (□).

Table 1. K_i values of acyclic and carbocyclic adenosine analogues for murine L929 cell AdoHcy hydrolase and antiviral activity of these compounds against vaccinia virus and VSV in L929 cells

Compound	Inhibition of AdoHcy hydrolase K_i (nM)	Antiviral potency ID_{90} (μM) against vaccinia virus	against VSV
(*S*)-DHPA	280	200	1900
(*RS*)-AHPA (isobutyl ester)	52	10	28.5
C-c^{3}Ado	18	3	16
3-Deazaneplanocin A	22	2.7	16
Adenosine dialdehyde	4.3	0.2	0.34
Neplanocin A	2.6	0.1	0.25

* ID_{90} = dose required to reduce virus yield by 90%.

tivators, which means that under the right conditions the enzyme activity may be recovered. This hypothesis is supported by the findings of Matuszewska and Borchardt [32], who were able to recover enzyme activity from AdoHcy hydrolase inactivated by neplanocin A upon incubation with NAD^+. Adenosine dialdehyde is the only other adenosine analogue subjected to the presented study that has been described as a pseudo-irreversible inactivator of AdoHcy hydrolase [9].

In summary, we have shown that the inhibitory effects of neplanocin A, adenosine dialdehyde, 3-deazaneplanocin A, C-c^3Ado, (*RS*)-AHPA isobutyl ester and (*S*)-DHPA on murine L929 cell AdoHcy hydrolase is closely correlated with their antiviral activity against vaccinia virus and VSV in these cells, thus corroborating the concept that AdoHcy hydrolase serves as target enzyme for the antiviral action of these compounds.

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