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MECHANISM OF THE SYNERGISTIC ANTIVIRAL AND CYTOSTATIC ACTIVITY OF (*RS*)-3-(ADENIN-9-YL)-2-HYDROXYPROPANOIC ACID ISOBUTYL ESTER AND D,L-HOMOCYSTEINE*

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Abstract—In a previous report (De Clercq E, Cools M and Balzarini J, *Biochem Pharmacol* **38**: 1771–1778, 1989) we showed that homocysteine (Hcy) enhanced the antiviral and cytostatic activity of *S*-adenosylhomocysteine (AdoHcy) hydrolase inhibitors. The mechanism of synergistic action between Hcy and the isobutyl ester of (*RS*)-3-(adenin-9-yl)-2-hydroxypropanoic acid [(*RS*)-AHPA] has been the subject of the present study. The selectivity index of (*RS*)-AHPA against vaccinia virus in murine L929 cells was significantly increased if the drug was combined with 1 or 3 mM Hcy. Even if Hcy was added as late as 12 hr after (*RS*)-AHPA, a synergistic antiviral activity was noted. Treatment of the L929 cells with (*RS*)-AHPA caused a significant increase in AdoHcy levels, and these levels were further increased if, in addition to (*RS*)-AHPA, Hcy (1 mM) was added to the cell cultures. Double-pulse label experiments showed that the additional AdoHcy built up after the combined treatment of (*RS*)-AHPA with Hcy did not originate from *S*-adenosylmethionine (via transmethylation reactions), but resulted from residual AdoHcy hydrolase activity (in the synthetic direction). To maintain sufficient levels of AdoHcy, AdoHcy hydrolase activity must be inhibited in the hydrolytic direction.

In recent years, some interest has been focused on inhibitors of *S*-adenosylhomocysteine (AdoHcy) hydrolase as broad-spectrum antiviral agents [1–10]. The spectrum of the viruses which are susceptible to the AdoHcy hydrolase inhibitors mainly consists of (−) and (±)stranded RNA viruses and the Poxviridae (DNA viruses) [1]. Representative examples are the Rhabdoviridae (rabies and vesicular stomatitis virus), the Paramyxoviridae (measles and parainfluenza virus) and the Reoviridae (reo- and rotavirus). We have observed a close correlation between the inhibitory effect of six (acyclic or carbocyclic) adenosine analogues on AdoHcy hydrolase activity and their inhibitory effect on vaccinia virus and vesicular stomatitis virus [2]. AdoHcy hydrolase can thus be considered as the target enzyme for the antiviral action of these adenosine analogues.

However, many of the potent AdoHcy hydrolase inhibitors, i.e. neplanocin A, may not solely be directed at the AdoHcy hydrolase enzyme, but can be recognized by other purine metabolizing enzymes such as adenosine deaminase and adenosine kinase. The cytotoxicity of neplanocin A may be related to its intracellular phosphorylation to the triphosphate [11, 12]. For this reason, some new analogues of neplanocin A were developed which cannot be phosphorylated [13, 14]. As expected, these compounds were less cytotoxic than the parental compound and had a higher selectivity index [9, 13]. However, even those compounds that are assumed to interact only with AdoHcy hydrolase are capable of inhibiting cell growth. Kim *et al.* [15] demonstrated that addition of homocysteine (Hcy) partially reversed the cytostatic effect of carbocyclic 3-deazaadenosine on macrophages. From our experiments, however, Hcy appeared to enhance the cytostatic and antiviral effects of AdoHcy hydrolase inhibitors [16]. Here we demonstrate that the isobutyl ester of (*RS*)-3-(adenin-9-yl)-2-hydroxypropanoic acid [(*RS*)-AHPA] gained increased antiviral, and to a lesser extent, cytostatic activity upon combination with Hcy. This synergistic action could be attributed to an increase in AdoHcy levels resulting from residual AdoHcy hydrolase activity in the synthetic direction.

MATERIALS AND METHODS

Compounds. (*RS*)-AHPA isobutyl ester‖ was kindly provided by Dr A. Holy (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). D,L-Homocysteine (Hcy) was purchased from the Sigma Chemical Co. (St Louis, MO). [2,8-^{3}H]Adenosine (Ado) (37 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA). [2,8-^{3}H]AdoHcy was prepared enzymatically from [2,8-^{3}H]Ado and D,L-homocysteine using purified bovine liver AdoHcy hydrolase [17]. L-[^{35}S]Methionine was purchased from Du Pont (Boston, MA).

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‖ Throughout all experiments (*RS*)-AHPA was used in its isobutyl ester form: for convenience it is referred to as (*RS*)-AHPA in the text.

Murine L929 cell culture. Murine L929 cells were grown as suspension cells in Waymouth's 752/1 medium (Hazelton Research products, Denver, PA) containing 4% calf serum at 37° in a humidified, CO_2-controlled atmosphere. For the experiments, L929 cells were grown as monolayers in Waymouth's MB 752/1 medium (Hazelton) containing 2% calf serum.

Vaccinia virus plaque reduction. L929 cells (25×10^4 cells/well in 24-well dishes) were incubated for 8 hr, until the cell monolayers were confluent. Cells were infected with 100 plaque forming units (PFU) of vaccinia virus per well (50 μL virus inoculum/well) during 1 hr (37°). The inoculum was diluted 1:10 with cold culture medium and removed by aspiration. Culture medium (500 μL) containing 0.1% methylcellulose and varying concentrations of the test compounds was then added. The dishes were incubated for 48 hr in a humidified, CO_2-controlled atmosphere. The plaques were stained with crystal violet, counted under the microscope, and ID_{50} values (50% reduction in number of plaques, as compared to the control) were calculated. Based on the ID_{50}, fractional inhibitory concentrations (FIC) were calculated (FIC_{AHPA} = ID_{50} AHPA in combination/ID_{50} AHPA alone; FIC_{Hcy} = ID_{50} Hcy in combination/ID_{50} Hcy alone; FIC = $FIC_{AHPA} + FIC_{Hcy}$).

Vaccinia virus yield reduction. This method has been described previously [2]. Briefly, 3×10^6 L929 cells per 60-mm dishes were infected with 0.1 PFU of vaccinia virus per cell during 1 hr. Unadsorbed virus was removed and fresh medium containing the test compound was added. After 24 hr, the culture dishes were frozen, and after freeze-thawing the cell homogenate was cleared by centrifugation and the virus content of the supernatant was determined.

Cell growth inhibition. Murine L929 were seeded in 96-well microtiter plates (Falcon, Becton Dickinson, Cowley, Oxford, U.K.) at a density of 10,000 cells/well in Waymouth's MB-752/1 medium containing 4% calf serum. After 4 hr, varying concentrations of the test compounds were added. The cells were then allowed to proliferate during 72 hr at 37° in a humidified, CO_2-controlled atmosphere. The growth of the cells was linear during this period. At the end of the incubation period, cells were trypsinized and enumerated in a Coulter counter (Coulter Electronics Ltd, Harpenden, U.K.). Cell growth inhibiting activity was expressed as ID_{50}, that is the concentration required to reduce cell number increment by 50%. Anticellular FIC values were determined in the same way as the antiviral FIC values.

AdoHcy levels, AdoMet levels and AdoHcy/AdoMet ratio. L929 cell cultures (3×10^6 cells/60-mm dishes) were seeded 24 hr prior to analysis. At different time intervals, cell cultures were washed with cold phosphate-buffered saline (PBS) and trypsinized. The cells were collected in Eppendorf tubes, centrifuged and the cell pellets were lysed in 125 μl 0.25 N perchloric acid. After centrifugation, 100 μL of the supernatant was injected into a Perkin–Elmer Series 3 HPLC system equipped with a Zorbax C-8 reverse phase column, 25 cm × 4.6 mm (Du Pont, Boston, MA). For the elution, acetonitrile (ACN) and a buffer containing 50 mM NaH_2PO_4, and 10 mM heptane sulfonic acid pH 3.2 were used. At a flow rate of 1 mL/min, nucleosides were eluted with a 15 min linear gradient from 4% ACN to 20% ACN followed by a 10 min linear gradient from 20% ACN to 25% ACN. Subsequently, the column was purged with 60% ACN, followed by a 15 min equilibration run with 4% ACN. The peaks of AdoHcy and AdoMet were quantitated at 254 nm. The retention times were 14.7 and 16.8 min, respectively. The acid-insoluble pellets were dissolved in 0.1 N NaOH containing 0.2% Na_2CO_3 and their protein contents were measured.

Pulse-labeling of AdoHcy and AdoMet. L929 cells (3×10^6 cells/60-mm dish) were seeded 24 hr prior to the experiment. One hour after addition of the test compounds, AdoHcy and AdoMet pools were pulse-labeled with [2,8-^{3}H]adenosine (0.25 μM, 37 Ci/mmol) and L-[^{35}S]methionine (6 nM, 1.12 μCi/mmol). After 1 hr pulse-labeling, cell extracts were made as described above. Prior to HPLC analysis, AdoHcy pools were spiked by addition of 2 nmoles AdoHcy. After injection, AdoHcy and AdoMet peaks were collected and [^{3}H] and [^{35}S] radioactivity was measured using a liquid scintillation counter.

Cellular AdoHcy hydrolase activity. L929 cells (10×10^6) in 10-cm dishes were seeded 24 hr prior to analysis. Cells were washed with cold PBS and trypsinized. After centrifugation, the cell pellets were lysed in 400 μL hypotonic buffer (10 mM Na_2HPO_4, 10 mM NaCl, 1.5 mM $MgAc_2$ pH 7.6) by rapid freezing in dry-ice acetone and thawing. After centrifugation in an Eppendorf centrifuge, 320 μL of the supernatant was mixed with 180 μL reaction mixture, containing 150 mM phosphate buffer pH 7.6, 1 mM EDTA, 40 μM [2,8-^{3}H]AdoHcy (7.1 mCi/mmol) and 4 units of adenosine deaminase (Sigma Chemical Co.). Samples were incubated for 30 min at 37° and the reaction was stopped by the addition of 100 μL 5 N formic acid. The reaction samples were then applied onto SP-Sephadex G25 columns, which were equilibrated with 0.1 N formic acid. After washing the test tubes with 1 mL formic acid, [2,8-^{3}H]inosine was eluted with 12 mL of 0.1 N formic acid. The radioactivity in 1 mL-eluates was determined in a liquid scintillation counter.

RESULTS

When the antiviral activity of the combination of (*RS*)-AHPA with Hcy was evaluated by a vaccinia virus plaque reduction assay, Hcy appeared to potentiate the inhibitory effect of (*RS*)-AHPA on vaccinia virus replication (Table 1). Evaluation of the combined antiviral effects of the two compounds by the isobologram procedure (Fig. 1A) indicated that (*RS*)-AHPA acted synergistically with D,L-Hcy. The lowest FIC indices (0.49 and 0.5) were obtained in the presence of 0.8 and 1 mM Hcy, respectively.

In parallel with the evaluation of the antiviral activity, the cell growth-inhibitory effect of both compounds alone or combined were also determined. Addition of Hcy increased the cell growth inhibitory effect of (*RS*)-AHPA (Table 1). The isobologram method revealed that (*RS*)-AHPA and D,L-Hcy acted slightly synergistically in the inhibition

Table 1. Antiviral and cell growth inhibiting effect of (*RS*)-AHPA combined with homocysteine

(*RS*)-AHPA	Activity against vaccinia virus ID_{50} (μM)*	Inhibitory effect on L929 cell growth IC_{50} (μM)*	Antiviral selectivity index
+ 0 mM Hcy	0.92	65.9	72
+ 0.3 mM Hcy	0.71	59.4	84
+ 1 mM Hcy	0.26	38.7	150
+ 3 mM Hcy	0.045	28.2	627

* Average values for three to five separate experiments.

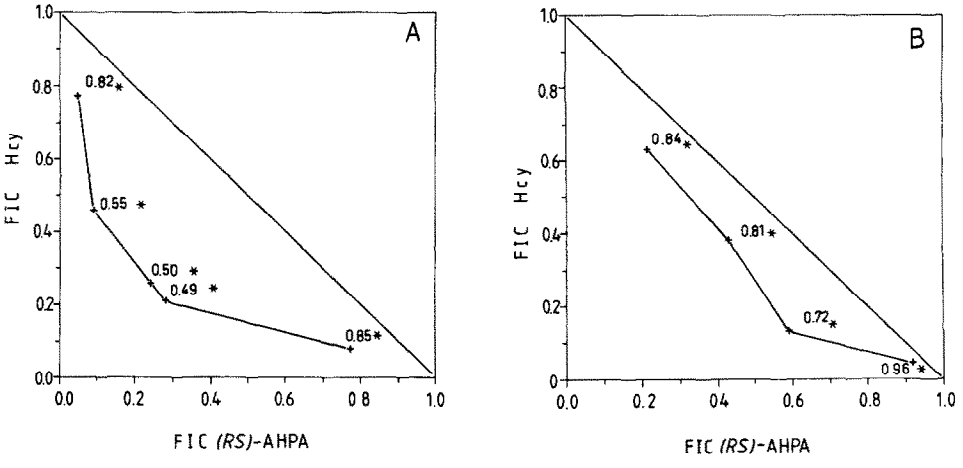

Fig. 1. Isobologram of the anti-vaccinia virus activity (A) and the cytostatic effect (B) of (*RS*)-AHPA combined with Hcy. The values indicated by an asterisk refer to the fractional inhibitory concentrations or FIC ($FIC = FIC_{AHPA} + FIC_{Hcy}$; $FIC_{compound} = ID_{50}$ or IC_{50} of compound in combination/ID_{50} or IC_{50} of compound alone). FIC < 0.5: significant synergism; FIC 0.5–0.9: slight synergism; FIC 1: additive effects; FIC 1.1–1.9: indifference or partial antagonism.

of cell growth (Fig. 1B): the lowest FIC indices were 0.72 and 0.81.

Addition of Hcy enhanced the antiviral activity to a greater extent than the cell growth inhibitory activity of (*RS*)-AHPA, so that the antiviral selectivity index of (*RS*)-AHPA gradually increased with increasing concentrations of Hcy (Table 1). The selectivity index of (*RS*)-AHPA combined with 3 mM Hcy was about 10-fold higher than the selectivity index of (*RS*)-AHPA alone. The selectivity index of Hcy against vaccinia virus was 2 (data not shown).

For the determination of the intracellular AdoHcy and AdoMet levels, uninfected cell cultures were used throughout. In additional experiments it was ascertained that vaccinia virus infection at a multiplicity of infection of 0.1 did not influence AdoHcy or AdoMet pools of L929 cells whether or not these cells were treated with (*RS*)-AHPA (unpublished data). Treatment of L929 cells with (*RS*)-AHPA caused a dose-dependent and a time-dependent increase in AdoHcy pools (Fig. 2A). Since the intracellular AdoMet pools (approximately 1.0–1.4 nmol/mg protein) were not altered by treatment with (*RS*)-AHPA (data not shown), the AdoHcy/AdoMet ratio showed the same pattern as the AdoHcy pools. Upon addition of Hcy, AdoHcy pools further increased as compared to those obtained in the absence of Hcy (Fig. 2B: compare with Fig. 2A). Combined (*RS*)-AHPA and Hcy treatment did not affect intracellular AdoMet pools. Again, AdoHcy/AdoMet ratios showed the same pattern as the AdoHcy pools.

Treatment of L929 cells with (*RS*)-AHPA led to a dose-dependent decrease in AdoHcy hydrolase activity (Table 2). If (*RS*)-AHPA was combined with Hcy (1 mM), the values noted for AdoHcy hydrolase activity were slightly decreased as compared to the values obtained in the absence of Hcy, irrespective of the doses used for (*RS*)-AHPA (Table 2).

To determine whether the net increase of AdoHcy levels obtained in the presence of Hcy (Fig. 2B) resulted from transmethylation reactions (AdoMet → AdoHcy) or the active synthesis of AdoHcy by AdoHcy hydrolase (Ado + Hcy → AdoHcy), we conducted double-labeling experiments, using [2,8-^{3}H]adenosine and [^{35}S]methionine. If the increase in AdoHcy results from transmethylation reactions, the [^{3}H]/[^{35}S] ratio should be similar for AdoMet and AdoHcy. If, however, the increased AdoHcy levels result from the condensation of [^{3}H]adenosine with

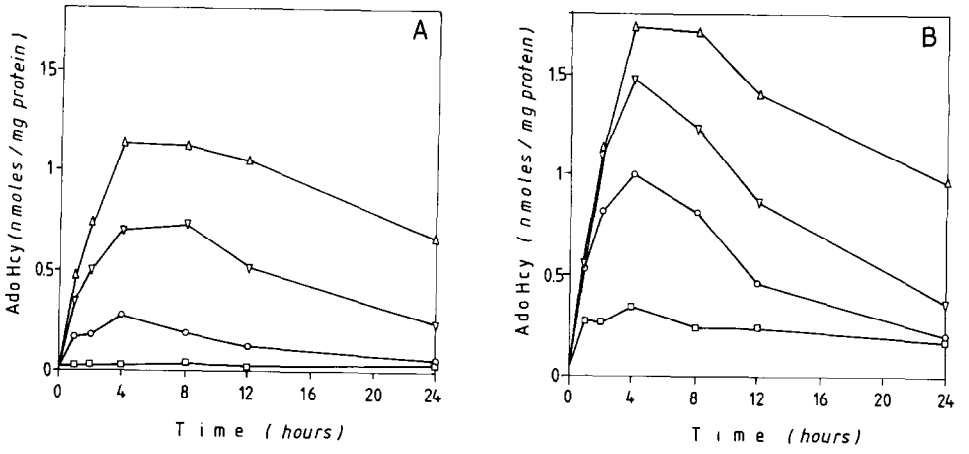

Fig. 2. Intracellular AdoHcy pool levels following treatment of L929 cells with varying concentrations of (*RS*)-AHPA alone (panel A) or in combination with Hcy (1 mM) (panel B). After different time intervals, cells were harvested and AdoHcy content was determined by HPLC analysis as described in Materials and Methods. Concentration of (*RS*)-AHPA: (□) 0 μM; (○) 0.3 μM; (▽) 3.2 μM (△) 32 μM.

Table 2. AdoHcy hydrolase activity in L929 cells treated with (*RS*)-AHPA with or without 1 mM Hcy

	AdoHcy hydrolase activity (%)*			
	After 1 hr treatment		After 2 hr treatment	
(*RS*)-AHPA (μM)	Without Hcy	With Hcy	Without Hcy	With Hcy
0	100	82	100	95
0.3	59	43	67	51
3.2	25	16	31	23
32	4	2	4	2

* Average values for two separate determinations.

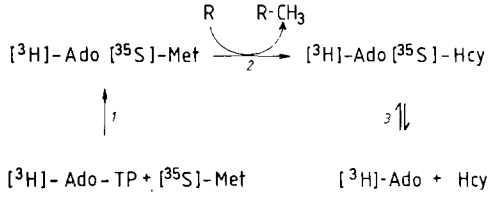

Fig. 3. Pathways for the incorporation of [^{3}H] and [^{35}S] radioactivity in AdoHcy and AdoMet pools after pulse-labeling with [^{3}H]Ado and [^{35}S]methionine. (1) AdoMet synthetase; (2) methyltransferase; (3) AdoHcy hydrolase.

Hcy, the [^{3}H]/[^{35}S] ratio should be higher for AdoHcy than AdoMet (Fig. 3). L929 cells incubated in the presence of increasing concentrations of (*RS*)-AHPA showed an increased incorporation of [^{3}H] and [^{35}S] radioactivity in AdoHcy, but the ratio of [^{3}H] to [^{35}S] radioactivity remained constant (Table 3). The incorporation of [^{3}H] and [^{35}S] radioactivity in AdoMet was not influenced by (*RS*)-AHPA. Upon addition of Hcy, [^{3}H] incorporation into AdoHcy was markedly increased, whereas the [^{35}S] incorporation remained essentially unaltered. The result was an increased [^{3}H]/[^{35}S] ratio in the presence of Hcy. This ratio decreased with increasing concentrations of (*RS*)-AHPA. For the incorporation of [^{3}H] and [^{35}S] radioactivity in AdoMet and the [^{3}H]/[^{35}S] ratio of AdoMet no changes were noted upon addition of Hcy.

These data clearly indicate that the accumulation of AdoHcy after treatment with (*RS*)-AHPA in the absence of Hcy resulted from transmethylation reactions. However, the accumulation of AdoHcy following treatment with both (*RS*)-AHPA and Hcy must have resulted partly from transmethylation reactions ([^{35}S] incorporation) and partly from the condensation of adenosine and homocysteine by AdoHcy hydrolase. The additional build-up of AdoHcy following treatment with (*RS*)-AHPA and Hcy could thus be explained by two factors: (i) increased formation of AdoHcy via residual AdoHcy hydrolase activity in the synthetic direction and (ii) inhibition of AdoHcy hydrolase activity in the hydrolytic direction.

Hcy caused a rapid increase in intracellular AdoHcy pools, irrespective of the time when it was added to the cell cultures, i.e. 0, 1, 2 or 4 hr after (*RS*)-AHPA (Fig. 4). Thus, even delayed addition of Hcy stimulated the synthesis of AdoHcy, and this stimulatory effect of Hcy on AdoHcy synthesis was also reflected by the inhibitory effect that the Hcy addition achieved on virus multiplication: even added 12 hr after (*RS*)-AHPA, Hcy still effected a synergistic reduction in vaccinia virus yield (Fig. 5).

DISCUSSION

(*RS*)-AHPA isobutyl ester could be considered as a prodrug of the free acid (*RS*)-AHPA, which is a

Table 3. Amount of [^{3}H] and [^{35}S] radioactivity incorporated into AdoHcy and AdoMet pools after pulse-labeling of L929 cells with [^{3}H]adenosine and [^{35}S]methionine

(*RS*)-AHPA (μM)	Addition of 1 mM Hcy	AdoHcy [^{3}H] CPM	AdoHcy [^{35}S] CPM	AdoHcy Ratio [^{3}H]/[^{35}S]	AdoMet [^{3}H] CPM	AdoMet [^{35}S] CPM	AdoMet Ratio [^{3}H]/[^{35}S]
0	−	4261	146	29	93285	3080	30
0.3	−	9040	416	22	100376	3884	26
3.2	−	14956	743	20	95660	4402	22
32	−	20134	933	22	92992	3610	26
0	+	39243	374	105	93988	3951	24
0.3	+	42113	484	87	100638	4076	25
3.2	+	34315	616	56	99085	4119	24
32	+	28443	917	31	99680	4138	24

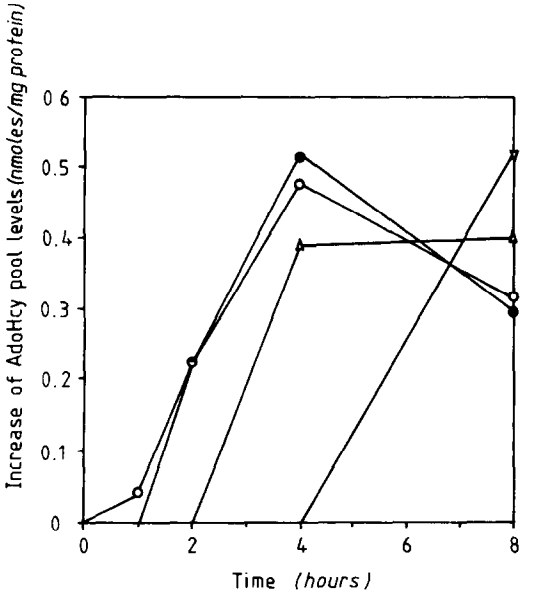

Fig. 4. Increase in intracellular AdoHcy levels following addition of Hcy (1 mM) at different times to L929 cells which were treated with (*RS*)-AHPA (3.2 μM) from time zero: (○) time 0; at 1 hr (●); at 2 hr (△); at 4 hr (▽). After the varying time intervals, cells were harvested and AdoHcy concentrations were determined by HPLC analysis as described in Materials and Methods. The curves presented in the graph represent the increments in AdoHcy pool levels following addition of Hcy over the AdoHcy pool levels in the presence of (*RS*)-AHPA.

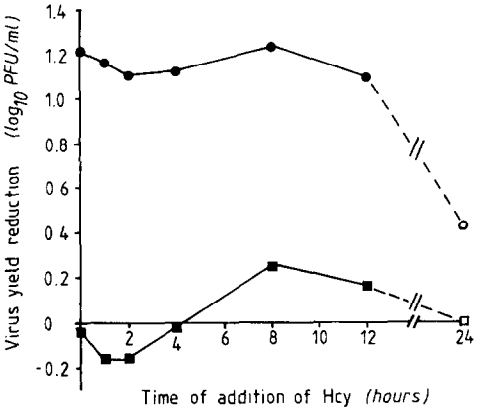

Fig. 5. Synergistic effect of Hcy and (*RS*)-AHPA on vaccinia virus multiplication in L929 cells; Hcy administered at different times after (*RS*)-AHPA treatment. After a 1-hr virus adsorption period, cells were replenished with fresh medium containing 0 μM (■) or 3.2 μM (*RS*)-AHPA (●). Hcy was added at 0, 1, 2, 4, 8 or 12 hr after removal of the virus. Twenty-four hours after virus adsorption, cell cultures were frozen and further processed as described in Materials and Methods.

moderate inhibitor of AdoHcy hydrolase (K_i = 52 nM for AdoHcy hydrolase purified from L929 cells [2]). When exposed to the cells, (*RS*)-AHPA isobutyl ester leads to the intracellular appearance of (*RS*)-AHPA, which causes a time- and dose-dependent inhibition of AdoHcy hydrolase [18]. The antiviral activity of (*RS*)-AHPA can be attributed to the accumulation of AdoHcy.

AdoHcy is the end product of transmethylation reactions starting from *S*-adenosylmethionine (AdoMet) as the methyl donor. AdoHcy is also a potent feedback inhibitor of these transmethylation reactions. Transmethylations play an important role in the maturation of mRNA (i.e. 5′-cap formation) [19]. Inhibition of AdoHcy hydrolase leads to the intracellular accumulation of AdoHcy, which in turn results in the inhibition of viral mRNA methyltransferases responsible for the 5′-capping of viral mRNA. As a rule, viral methyltransferases are more sensitive to inhibition of AdoHcy than cellular methyltransferases [20–22]. Ransohoff *et al.* [23] demonstrated that treatment of the cells with neplanocin A, a potent inhibitor of AdoHcy hydrolase [8], brought about an undermethylation of mRNA cap structures, concomitantly with a reduction in influenza virus replication. Also, neplanocin A was shown to inhibit vaccinia virus replication, most likely due to a reduction in the methylation of the viral mRNA [24]. Furthermore, Hasobe *et al.* [25] ascertained that the anti-vaccinia virus effects of the neplanocin A analogues 9-(*trans*-2′, *trans*-3′-dihydroxycyclopent-4-enyl)-adenine (DHCA) and -3-deazaadenine

(DHCDA) were closely correlated with the accumulation of intracellular AdoHcy, the increase in AdoHcy/AdoMet ratio and the reduction in methylation of poly(A)$^+$ mRNA.

We have previously established that the antiviral activity of AdoHcy hydrolase inhibitors, i.e. (*RS*)-AHPA, can be enhanced by the exogenous addition of homocysteine [16]. As further shown by the present results, addition of homocysteine simultaneously with or after treatment with (*RS*)-AHPA caused a synergistic increase in the antiviral activity of this nucleoside analogue. This synergism can be explained by an additional build-up of AdoHcy. Addition of exogenous Hcy decreases the minimum concentration of (*RS*)-AHPA required to raise the cellular levels of AdoHcy and the AdoHcy/AdoMet ratio that are inhibitory to viral replication. As mentioned above, Hasobe *et al.* [25] demonstrated that the anti-vaccinia activity of AdoHcy hydrolase inhibitors can be explained by the accumulation of AdoHcy. In fact, a two- to four-fold increase in AdoHcy levels and AdoHcy/AdoMet ratio suffices to reduce vaccinia virus growth by 50%. When added at a dose that reduces vaccinia virus growth by 50%, (*RS*)-AHPA, combined with or without Hcy, increases the AdoHcy levels and the AdoHcy/AdoMet ratio by two- to four-fold.

If the cells are treated with (*RS*)-AHPA alone, AdoHcy is formed as the end product of transmethylation reactions and accumulates because the enzyme responsible for the hydrolysis of AdoHcy is inhibited by (*RS*)-AHPA. When Hcy and (*RS*)-AHPA are combined, part of AdoHcy is formed by the transmethylation reactions and part is synthesized from adenosine and Hcy by AdoHcy hydrolase. Compelling evidence for this hypothesis stemmed from double-labeling experiments, where, after labeling with [^{35}S]methionine and [^{3}H]adenosine, the [^{3}H]/[^{35}S] ratio in the final product (AdoHcy) was significantly increased if Hcy had been combined with (*RS*)-AHPA (Table 3). The additional build-up of AdoHcy must be ascribed to the condensation of [^{3}H]adenosine with Hcy by AdoHcy hydrolase.

Backlund *et al.* [26] reported that with 3-deazaadenosine (c^{3}Ado), which is not only an inhibitor but also a substrate of AdoHcy hydrolase, the ratio of c^{3}AdoHcy to AdoHcy decreases with increasing concentrations of c^{3}Ado. The higher the concentrations of c^{3}Ado, the more AdoHcy is formed, whereas the c^{3}AdoHcy pool remains constant. However, the c^{3}AdoHcy pool is increasing following exogenous addition of Hcy. Here again, the synthesis of c^{3}AdoHcy is stimulated by addition of Hcy. Although AdoHcy hydrolase is inhibited in the presence of AdoHcy hydrolase inhibitors such as c^{3}Ado and (*RS*)-AHPA, significant synthesis of AdoHcy or c^{3}AdoHcy still occurs, while enzymatic activity in the hydrolytic direction is almost totally suppressed. Several investigators have shown that even at high concentrations of an AdoHcy hydrolase inhibitor, there is residual AdoHcy hydrolase activity if measured in the hydrolytic direction [8, 27–29]. Hasobe *et al.* [29] postulated that this residual activity stems from an inhibitor-insensitive form of AdoHcy hydrolase. Approximately 10% of the cellular AdoHcy hydrolase would be insensitive to the AdoHcy hydrolase inhibitor DHCA. The exact nature of this inhibitor-insensitive form of AdoHcy hydrolase remains to be elucidated. It would synthesize AdoHcy when the substrates adenosine and Hcy are present. Since the velocity of the AdoHcy hydrolase reaction is higher in the synthetic than hydrolytic direction [30, 31], the AdoHcy would accumulate under these conditions. Further evidence that the synergistic antiviral action of Hcy and (*RS*)-AHPA is due to the increased AdoHcy pools stems from the fact that those adenosine analogues that are not targeted at AdoHcy hydrolase do not show increased antiviral activity in the presence of Hcy [16].

The combined effects of Hcy and c^{3}Ado on the cells have been well studied [26, 32, 33]. Exogenous Hcy stimulates c^{3}AdoHcy build-up and increases the biological activity of the compound (only when the activity is a consequence of inhibition of AdoHcy hydrolase). For the carbocyclic analogue of 3-deazaadenosine (C-c^{3}Ado), the situation is less clear. Kim *et al.* [15] reported that micromolar concentrations of Hcy partially reversed the cytostatic effect of 100 μM C-c^{3}Ado on macrophages. Backlund *et al.* [26] did not observe an alteration in AdoHcy pools upon addition of Hcy (50 μM) to the C-c^{3}Ado-treated macrophages. On the other hand, we have clearly demonstrated that both the antiviral and cytostatic effects of C-c^{3}Ado in different cells are enhanced by addition of millimolar concentrations of Hcy [16]. Addition of lower concentrations of Hcy had no effect. The effect of C-c^{3}Ado on intracellular Hcy levels may differ from one cell line (i.e. macrophages) to another (i.e. fibroblasts). It is known that Hcy is exported from cells into the extracellular medium and only small amounts are retained within the cells [34]. In hepatocytes, inhibition of AdoHcy hydrolase is not associated with a depletion of intracellular Hcy, because the egress of Hcy from cells is strongly suppressed. However, Svardal *et al.* [35] ascertained that the effect of C-c^{3}Ado treatment on intracellular Hcy levels differed from hepatocytes to non-transformed fibroblasts. In fibroblasts, a depletion of intracellular Hcy occurred whereas in hepatocytes no such depletion was observed. Further studies are required to elucidate the effect of AdoHcy hydrolase inhibitors and exogenous Hcy on intracellular Hcy pool levels and Hcy egress from cells.

Hasobe *et al.* [29, 36] also found that the antiviral activity of DHCA can be potentiated by the exogenous addition of Hcy. Addition of Hcy lowered the minimum concentration of DHCA that was required to bring AdoHcy (and AdoHcy/AdoMet) up to the levels that are inhibitory to vaccinia virus replication. The increased AdoHcy levels were shown to arise from the condensation of adenosine and Hcy catalysed by the DHCA-insensitive form of AdoHcy hydrolase.

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