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Elucidation of the Mechanism by which Homocysteine Potentiates the Anti-Vaccinia Virus Effects of the *S*-Adenosylhomocysteine Hydrolase Inhibitor 9-(*trans*-2′,*trans*-3′-Dihydroxycyclopent-4′-enyl)-adenine

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SUMMARY

9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-adenine (DHCA), a specific inhibitor of *S*-adenosyl-*L*-homocysteine (AdoHcy) hydrolase, has been used in this study to elucidate the mechanism by which DL-homocysteine (Hcy) potentiates the antiviral effects of AdoHcy hydrolase inhibitors as reported by De Clercq [*Biochem. Pharmacol.* **36**:2567–2575 (1987)]. The potentiating effects of Hcy on the antiviral effects of DHCA were determined using murine L929 cells infected with vaccinia virus. When virus-infected cells were incubated with DHCA alone or in combination with various concentrations of Hcy, the following IC_{50} values (concentrations of the drug required to reduce by 50% viral plaque formation) were observed: 0.30 μM (0 mM Hcy), 0.15 μM (0.3 mM Hcy), 0.09 μM (1.0 mM Hcy), and 0.04 μM (3.0 mM Hcy). In the drug combination studies, increased cellular toxicity, compared with DHCA alone, was observed only at the highest concentration of Hcy (3.0 mM); thus, at lower concentrations Hcy increased the antiviral effectiveness [ID_{50} (concentration of the drug required to reduce the increase in cell number by 50%)/IC_{50}] of DHCA. For example the following ID_{50}/IC_{50} values were observed for DHCA alone or in combination with Hcy: 64 (0 mM Hcy), 113 (0.3 mM Hcy), 151 (1.0 mM Hcy), and 88 (3.0 mM Hcy). In these studies, Hcy was also observed to potentiate the increase in cellular levels of AdoHcy and the ratio of AdoHcy/*S*-adenosyl-L-methionine (AdoMet) in DHCA-treated cells. In earlier studies, our laboratory has shown that antiviral effects of DHCA are caused by only slight elevations in intracellular levels of AdoHcy [from 50 pmol/mg of protein (controls) to 100–200 pmol/mg of protein (drug-treated)] and slight elevations in the ratios of AdoHcy/AdoMet [from 0.05–0.1 (control) to 0.15–0.20 (drug-treated)]. Thus, in the presence of Hcy, lower concentrations of DHCA are needed to increase the intracellular concentration of AdoHcy and the AdoHcy/AdoMet ratio to levels that suppress replication of vaccinia virus. Murine L929 cells were shown to contain DHCA-sensitive and DHCA-insensitive forms of AdoHcy hydrolase. Based on the results of labeling experiments using [2,8-^{3}H]adenosine and [^{35}S]methionine, the elevated levels of AdoHcy were shown to arise from the reaction of [2,8-^{3}H]adenosine and Hcy, catalyzed by the DHCA-insensitive form of AdoHcy hydrolase.

AdoHcy hydrolase is a ubiquitous eukaryotic enzyme that in a purified form can catalyze the degradation or the synthesis of AdoHcy (1, 2). The presumed physiological function of this enzyme is to catalyze the degradation of AdoHcy to Ado and Hcy, thus maintaining low cellular concentrations of this product inhibitor of AdoMet-dependent methylations (2). Inhibition of cellular AdoHcy hydrolase results in an intracellular accumulation of AdoHcy, causing a significant increase in the intracellular AdoHcy/AdoMet ratio and the subsequent inhibition of AdoMet-dependent methylations (3–8).

Recently, AdoHcy hydrolase has become an attractive target for the design of antiviral agents (8, 9). The interest in AdoHcy hydrolase as a target for the design of antiviral agents has arisen because (a) most plant and animal viruses have a "capped, methylated structure" at the 5′-terminus, which is required for viral mRNA transcription and thus viral replication (10–11); (b) a close correlation exists between the antiviral potency of adenosine analogs and their selective inhibitory effects on AdoHcy hydrolase (12–13); and (c) AdoHcy hydrolase inhibitors have been shown to be broad-spectrum antiviral agents possessing activity against poxviruses (e.g., vaccinia), negative-stranded RNA viruses (e.g., paramyxoviruses parainfluenza and measles; rhabdoviruses rabies and vesicular sto-

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ABBREVIATIONS: AdoHcy hydrolase, *S*-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1); Ado, Adenosine; AdoHcy,*S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; AHPA, 3-(adenin-9-yl)-2-hydroxypropanic acid; DHCA, 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-adenine; HPLC, high performance liquid chromatography; Hcy,DL-homocysteine.

matitis), and double-stranded RNA viruses (reovirus and rotavirus) (9). Herpes viruses and positive-stranded RNA viruses (picornaviruses enterovirus and rhinovirus; togavirus) are virtually resistant to AdoHcy hydrolase inhibitors.

In 1987, De Clercq (9) reported the surprising observation that Hcy potentiated the antiviral activity of AdoHcy hydrolase inhibitors (e.g., neplanocin A). This potentiation by Hcy occurred only with viruses (e.g., vaccinia) that are intrinsically susceptible to the antiviral effects of AdoHcy hydrolase inhibitors. With those viruses (e.g., herpes) not belonging to the activity spectrum of the AdoHcy hydrolase inhibitors, no potentiation was observed upon addition of Hcy.

In this study, we have elucidated the molecular mechanism responsible for the potentiating effects of Hcy on the antiviral activity of DHCA, which is a synthetic analog of neplanocin A (14). DHCA is a "pure" inhibitor of AdoHcy hydrolase, which is devoid of substrate activity for adenosine kinase and adenosine deaminase (8, 15, 16) and which has been shown to be a broad-spectrum antiviral agent with reduced cytotoxicity compared with neplanocin A (17).[1]

Experimental Procedures

Materials. Standard chemicals and supplies were purchased from the following commercial suppliers: calf intestinal adenosine deaminase and SP-Sephadex C-25 (Sigma Chemical Co., St. Louis, MO); DE-81 disks (Whatman, Clinton, NJ); 3a70 scintillation cocktail (Research Products International, Mount Prospect, IL); Waymouth's 752/1 (low calcium) medium for spinner culture, Waymouth's MB 752/1 medium for dish culture, and calf bovine serum (Hazleton Research Products, Denver, PA); 24-well (13-mm diameter) multiculture dishes (Costar, Cambridge, MA); cell culture dishes (100-mm diameter) (Corning, Corning, NY); 96-well microtest plates (Falcon, Bectron-Dickinson, Cowley, Oxford, England); sodium phosphate monobasic (HPLC grade) and 1-heptanesulfonic acid, sodium salt (HPLC grade) (Fisher Scientific, Springfield, NJ); Zorbax C8 reverse phase HPLC column (DuPont, Wilmington, DE); and [2,8-^{3}H]Ado (36 Ci/mmol), [^{35}S]Met (1134 Ci/mmol), [^{14}C]uridine (517.5 mCi/mmole), and [*methyl*-^{3}H]thymidine (84.1 Ci/mmol) (New England Nuclear, Boston, MA).

[2,8-^{3}H]AdoHcy (7.1 mCi/mmol) was synthesized enzymatically from [^{3}H]Ado and DL-Hcy, using purified bovine liver AdoHcy hydrolase (18), according to the method of Chabannes *et al.* (19). Protein determinations were done according to the method of Bradford (20). DHCA was synthesized according to procedures described earlier by our laboratory (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 supplemented with 4% bovine calf serum, as described previously (4). Experimental cultures of L929 cells were grown as monolayers in Waymouth's MB 752/1 medium containing 2% bovine calf serum, in tissue culture dishes (60-mm or 100-mm diameter), 24-well (13-mm diameter) multiculture dishes, or 96-well microtest plates.

AdoHcy hydrolase assay. Cultures of mouse L-929 cells (approximately 6×10^6 cells/100-mm diameter dish), which were treated with various concentrations of DHCA, were harvested with trypsin treatment and pelleted in Eppendorf tubes (1.5 ml). The cells were lysed in 400 μl of cold hypotonic buffer (10 mM Na_2HPO_4, 10 mM NaCl, 1.5 mM magnesium acetate, pH 7.6) by rapid freezing and thawing. The cell debris was removed by centrifugation in an Eppendorf microcentrifuge (12,400 rpm, 1 min) and the AdoHcy hydrolase activity in the supernatant was determined using [2,8-^{3}H]AdoHcy as the substrate (16). Adenosine deaminase was included in the assay mixture to convert the [2,8-^{3}H]Ado, which was formed from [2,8-^{3}H]AdoHcy, to [2,8-^{3}H]inosine. [2,8-^{3}H]Inosine was eluted on a SP-Sephadex C-25 column, as previously described by our laboratory (16). Experimental results represent averages of five determinations.

Determination of intracellular levels of AdoHcy and AdoMet. Cultures of mouse L929 cells (approximately 2×10^6 cells/60-mm diameter dish) were incubated for various times at 37°, with Waymouth's MB 752/1 medium containing 2% bovine calf serum, with various concentrations of DHCA alone or in combination with various concentrations of Hcy. Cells were removed from the culture dishes at various times by trypsin treatment and lysed in 100 μl of 0.25 N perchloric acid by vigorously vortexing and rapid freezing on dry ice. The samples were stored at −70° before analysis. In preparation for HPLC analysis, the samples were rapidly thawed and the cell debris was removed by centrifugation in an Eppendorf microcentrifuge (12,400 rpm, 1 min). The supernatant (100 μl) was injected into a Perkin-Elmer Series 3 HPLC equipped with a Zorbax C8 reverse phase column (25 cm × 4.6 mm). AdoHcy and AdoMet were separated by a two-step gradient program, at a flow rate of 1.0 ml/min, as follows: solvent A, acetonitrile; solvent B, 50 mM sodium phosphate (pH 3.2), 10 mM heptane sulfonic acid; program, 5–20% A in 15 min then 20–25% A in 10 min; quantitated by absorption at 254 nm; retention times: Ado, 12 min; AdoHcy, 15 min; AdoMet 17 min.

Labeling intracellular pools of AdoMet and AdoHcy with [2,8-^{3}H]Ado and [^{35}S]Met. L929 cell cultures in 6-cm dishes (3×10^6 cells/well) were treated with DHCA and Hcy, alone or in combination, for 1 hr and then the cultures were labeled with [2,8-^{3}H]Ado (7 μCi/ml) and [^{35}S]Met (12 μCi/ml) for 1 additional hr. Cells were harvested and prepared for HPLC analysis as described above, except the acid-soluble supernatants (100 μl) were mixed with 50 μl of 0.25 N perchloric acid containing 150 μM Ado, AdoHcy, and AdoMet. Samples were then injected onto a C8 reverse phase HPLC column, the peak fractions of AdoHcy and AdoMet were collected, and the radioactivity was measured by scintillation counting.

Anti-vaccinia virus assay. Murine L929 cells were seeded (approximately 1.7×10^5 cells/well) in 24-well multiculture plates and grown in Waymouth's MB-752/1 medium containing 2% calf serum. Nearly confluent cell monolayers were infected with vaccinia virus at about 140 plaque-forming units/well. After a virus adsorption period (60 min), the viral inoculum was diluted by the addition of cold culture medium and then completely removed by aspiration. Immediately after infection, each well containing a cell monolayer was overlaid with medium (400 μl) containing 0.1% methylcellulose and 2% calf serum, to which was added medium (100 μl) containing various concentrations of DHCA alone or in combination with Hcy. After incubation for 48 hr at 37°, cultures were washed with phosphate-buffered saline and stained with 0.1% crystal violet. The plaque number was then counted under a microscope. The data are expressed as IC_{50} values (concentrations of the drug required to reduce by 50% viral plaque formation) and are averages of three or four experiments.

Cell growth inhibition assays. Murine L929 cells were seeded in 96-well microtest plates, at a density of 1×10^4 cells/well, in Waymouth's MB-752/1 medium containing 4% bovine calf serum. Various concentrations of DHCA and Hcy alone or in combination were added 4 hr later. The cells were then allowed to proliferate for 72 hr at 37°. The growth of the cells was linear during this period. After the incubation period, cells were removed by trypsin treatment and enumerated in a Coulter counter (Coulter Electronics, Ltd., Harpenden Herts, England). Cell growth-inhibiting activities were expressed as ID_{50} values (concentration of the drug required to reduce the increase in cell number by 50%) and are the results of four to six experiments.

DNA and RNA synthesis assay. Murine L929 cells were seeded (approximately 20×10^4 cells/well) in 24-well multiculture plates and grown in Waymouth's MB 752/1 medium containing 2% calf serum. To nearly confluent cell monolayers were added various concentrations of DHCA and Hcy, alone or in combination. The cells were incubated at 37° for 12 hr and then pulse-labeled with [^{3}H]thymidine (final concentration, 2 μCi/ml) or [^{14}C]uridine (final concentration, 0.2 μCi/ml) for 1 hr. The radioactive culture medium was removed by aspiration

[1] E. De Clercq, M. Cools, J. Balsarini, V. E. Marquez, D. R. Borcherding, R. T. Borchardt, J. C. Drach, S. Kitaoka, and T. Konno. Broad-spectrum antiviral activity of new neplanocin A analogs. Submitted for publication.

and the monolayers were washed with cold phosphate-buffered saline and then lysed with a solution containing 1% sodium dodecyl sulfate, 5 mM EDTA, 30 mM Tris hydrochloride (pH 9.0), 0.1 M NaCl, and 10 mM vanadyl ribonucleoside complex (21). The lysate was transferred onto a DE-81 disk. After being washed five times with 5% Na_2HPO_4 (5 ml/disk) and two times with distilled water, each disk was placed in a scintillation vial, to which was added 10 ml of 3a70 scintillation cocktail, and the radioactivity remaining on the disk was measured by liquid scintillation counting.

Results

Effects of Hcy on the anti-vaccinia virus activity and murine L929 cytotoxicity of DHCA. The anti-vaccinia virus activities of DHCA alone and in combination with Hcy were evaluated by measuring the inhibition of virus plaque formation during a 48-hr exposure to the drug and/or Hcy. The IC_{50} values observed for DHCA alone and in combination with 0.3, 1.0, and 3.0 mM Hcy are presented in Table 1. These data clearly show that Hcy potentiates the anti-vaccinia virus activity of this AdoHcy hydrolase inhibitor.

The effects of DHCA alone and in combination with Hcy on murine L929 cytotoxicity were evaluated after exposure to the drug and/or Hcy for 72 hr. Only slight increases in the cytotoxicity (ID_{50}) of DHCA were observed in cultures treated with the drug and 0.3 or 1.0 mM Hcy, when compared with cells treated with the drug alone. A significant increase in toxicity was observed only in cells treated with DHCA and 3.0 mM Hcy. Because Hcy at low concentrations (0.3 and 1.0 mM) potentiates the antiviral activity of DHCA with minimal increases in cellular toxicity, the net effect is an increase in the antiviral effectiveness (ID_{50}/IC_{50}) of DHCA. For example, at 0, 0.3, and 1.0 mM Hcy the antiviral indices (ID_{50}/IC_{50}) of DHCA were calculated to be 64, 113, and 151, respectively.

The cytotoxic effects of DHCA and Hcy alone or in combination were also determined by measuring the effect on DNA and RNA synthesis in murine L929 cells. As shown in Table 2, DHCA alone at 0.1 and 1.0 μM concentrations had no effect on either DNA or RNA synthesis. Incubation of cells with Hcy (0.3 to 3.0 mM) alone caused a dose-dependent increase in the inhibition of DNA synthesis without affecting RNA synthesis. Treatment of L929 cells with Hcy (0.3 to 3.0 μM) in combination with DHCA (0.1 or 1.0 μM) also caused inhibition of DNA synthesis. The extent of inhibition of DNA synthesis was similar to that seen with Hcy alone. Only at 1.0 μM DHCA in the presence of high concentrations of Hcy (1.0 or 3.0 mM) were inhibitory effects (approximately 21%) on RNA synthesis observed.

TABLE 1

Effects of Hcy on the antiviral activity and cytotoxicity of DHCA

For the antiviral studies, nearly confluent L929 cell monolayers in 24-well multiculture plates were infected with vaccinia virus at 140 plaque-forming units/well. After virus adsorption period (60 min), the viral inoculum was diluted and removed by aspiration. Various concentrations of DHCA alone or in combination with Hcy were added to the monolayer that had been overlaid with medium containing 0.1% methylcellulose and 2% calf serum. After incubation for 48 hr at 37°, cultures were washed and stained with 0.1% crystal violet and plaques were counted as described in Experimental Procedures. Anti-vaccinia viral activities are expressed as IC_{50} values, which are averages of three or four experiments. For the cell growth inhibition assays, L929 cells were seeded in 96-well microtest plates at a density of 1×10^4 cells/well in Waymouth's MB-752/1 medium containing 4% calf serum. After 4 hr, various concentrations of DHCA alone or in combination with various concentrations of Hcy were added and the cells were allowed to proliferate for 72 hr at 37°. After removal by trypsin treatment, cells were counted using a Coulter counter and the cell growth inhibitory activities were expressed as ID_{50} values, which are averages of four to six experiments. Values are means ± standard deviations.

Hcy	Vaccinia virus IC_{50}	Murine L929 ID_{50}	Antiviral effectiveness, ID_{50}/IC_{50}
mM	μM		
0	0.30 ± 0.10	19.1 ± 0.9	64
0.3	0.15 ± 0.03	16.9 ± 1.9	113
1.0	0.09 ± 0.06	13.6 ± 2.9	151
3.0	0.04 ± 0.02	3.5 ± 0.8	88

TABLE 2

Effects of DHCA, alone or in combination with Hcy, on L929 cell DNA and RNA synthesis

Confluent L929 cells monolayers were cultured at 37° in multiwell dishes in Waymouth's MB 752/1 medium containing 2% calf serum, without or with various concentrations of DHCA, alone or in combination with various concentrations of Hcy. After 12 hr of incubation at 37°, the cultures were pulse-labeled for 2 hr with [^{14}C]uridine or [^{3}H]thymidine. The monolayers were lysed and applied to DE-81 paper disks. After the disks were washed as described in Experimental Procedures, radioactivity remaining on the disks was measured by liquid scintillation counting. Assays were done in duplicate.

DHCA	Hcy	Radioactivity remaining on DE-81 paper			
		[^{3}H]Uridine		[^{3}H]Thymidine	
μM	mM	cpm/paper	% of control	cpm/paper	% of control
0	0	19872	100	3232	100
0	0.3	19348	97	3220	100
0	1.0	19401	98	2657	82
0	3.0	19533	98	2115	65
0.1	0	20941	105	3145	97
0.1	0.3	19455	98	3162	98
0.1	1.0	18676	94	2935	91
0.1	3.0	19099	96	2004	62
1.0	0	20063	101	3251	101
1.0	0.3	18431	92	3259	101
1.0	1	17351	87	2812	88
1.0	3	15720	79	2027	64

Effect of DHCA on AdoHcy hydrolase in murine L929 cells. In an earlier study (16), 1.0 μM DHCA was shown to produce rapid inactivation (within 30 min the enzyme was 95% inhibited) of AdoHcy hydrolase activity in murine L929 cells. This level of inhibition (approximately 95%) persisted for at least 72 hr. To establish the dose dependency of this enzyme inactivation in more detail, we studied the effect of the concentration of DHCA on L929 cellular AdoHcy hydrolase activity. As shown in Fig. 1, when murine L929 cells are treated with DHCA for 24 hr we observed that approximately 90% of the AdoHcy hydrolase is very sensitive to low concentrations (3.2 to 100 nM) of this inhibitor. However, approximately 10% of the cellular AdoHcy hydrolase is insensitive to DHCA at concentrations up to 10μM.

Effects of DHCA and Hcy, alone or in combination, on the intracellular levels of AdoHcy and AdoMet. To determine the effects of Hcy on the ability of DHCA to elevate intracellular levels of AdoHcy and the ratio of [AdoHcy]/[AdoMet], time-course experiments were conducted using 0.3 μM DHCA and 2 mM Hcy. This concentration of DHCA was selected because it is the concentration (IC_{50} value) that produces 50% reduction in vaccinia virus replication (see Table 1). This concentration of Hcy was selected because it produced a significant potentiation of the anti-vaccinia virus effects of DHCA. As shown in Fig. 2A, treatment of L929 cells with 0.3 μM DHCA for 5 hr causes increases in the intracellular level of AdoHcy (from 56 to 155 pmol/mg of protein) and the ratio of [AdoHcy]/[AdoMet] (from 0.051 to 0.16). After 24 hr the intracellular levels of AdoHcy (213 compared with 44 pmol/mg

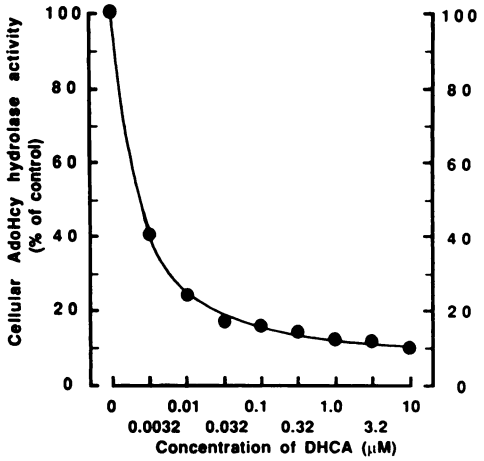

Fig. 1. AdoHcy hydrolase activity in L929 cells treated with various concentrations of DHCA. Cultures of mouse L929 cells (approximately 6 × 10^6 cells/100-mm diameter dish) were incubated with various concentrations of DHCA for 24 hr at 37°. The cells were harvested and lysed and the AdoHcy hydrolase activity was determined as described in Experimental Procedures. The specific activity of AdoHcy in control samples (non-drug-treated) was 463 pmol/min/mg of protein. Experimental results represent averages of five determinations.

of protein) and the ratios of [AdoHcy]/[AdoMet] (0.19 compared with 0.049) are still elevated, compared with non-drug-treated controls. Interestingly, treatment of L929 cells with a 2 mM concentration of Hcy alone also causes transient increases in the intracellular levels of AdoHcy and the ratios of [AdoHcy]/[AdoMet]. Maximal increases in intracellular AdoHcy levels (455 pmol/mg of protein) and the ratio of [AdoHcy]/[AdoMet] (0.316) were observed after 5 hr of incubation with Hcy (2 mM). Both the intracellular concentration of AdoHcy and the ratio of [AdoHcy]/[AdoMet] returned to near control values after 24 hr (AdoHcy = 64 pmol/mg of protein; [AdoHcy]/[AdoMet] = 0.071). In cells treated with both DHCA (0.3 μM) and Hcy (2 mM), substantial increases in AdoHcy cellular levels and [AdoHcy]/[AdoMet] ratios were observed at both 5 hr ([AdoHcy] = 1230 pmol/mg of protein; [AdoHcy]/[AdoMet] = 0.771) and 24 hr ([AdoHcy] = 363 pmol/mg of protein; [AdoHcy]/[AdoMet] = 0.339) of drug treatment.

The dose dependency of these synergistic increases in the intracellular levels of AdoHcy and the ratio of [AdoHcy]/[AdoMet] were examined in more detail by treating L929 cells with various concentrations of DHCA (0.1–1 μM) and Hcy (0.3–3 mM), alone or in combination, and measuring 24 hr after drug treatment the intracellular levels of AdoHcy (Fig. 3A) and the ratio of [AdoHcy]/[AdoMet] (Fig. 3B). From the data presented in Fig. 3, it is obvious that both the intracellular levels of AdoHcy and the ratios of [AdoHcy]/[AdoMet] are increased in cells treated with combinations of Hcy and DHCA, as compared with cells treated with either agent alone. At all concentrations of DHCA tested, Hcy causes a dose-dependent increase in both the cellular levels of AdoHcy and the ratio of [AdoHcy]/[AdoMet].

Relationship between the intracellular concentration of AdoHcy and the anti-vaccinia viral effects of combinations of DHCA and Hcy. The concentration of DHCA needed to produce 50% reduction in virus replication (IC_{50} = 0.30 μM) causes only slight increases in the intracellular levels of AdoHcy (to approximately 200 pmol/mg of protein) and the ratio of [AdoHcy]/[AdoMet] (to 0.2). Recently Hasobe *et al.*

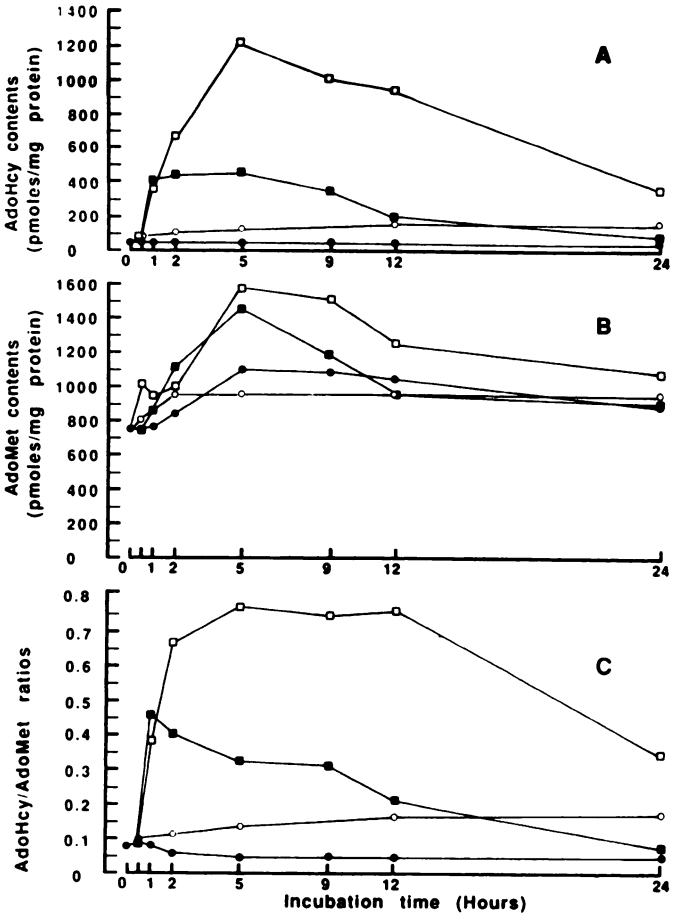

Fig. 2. Time course of the changes in the intracellular contents of AdoHcy and AdoMet and the AdoHcy/AdoMet ratios in L929 cells treated with DHCA alone or in combination with Hcy. Four sets of monolayer cultures of mouse L929 cells (approximately 2 × 10^6 cells/60 cm dish) were treated with or without 0.3 μM DHCA and cultured at 37°. After 30 min of incubation, Hcy (2 mM) was added to two sets of cultures (with or without DHCA). At the indicated times, cultures of control (●), 0.3 μM DHCA (○), 2 mM Hcy (■), and the combination of 0.3 μM DHCA and 2 μM Hcy (□) were harvested and the AdoHcy contents (A) and the AdoMet contents (B) were determined as described in Experimental Procedures. The AdoHcy/AdoMet ratios (C) were calculated from the data shown in A and B. Experiments were carried out in duplicate.

(22) showed that a relationship exists between this slight elevation in the intracellular level of AdoHcy and the antiviral activity of AdoHcy hydrolase inhibitors. As shown in Table 3, the net effect of adding Hcy to the culture medium is to decrease the concentration of DHCA needed to increase the intracellular concentration of AdoHcy and the ratio of [AdoHcy]/[AdoMet] to levels critical for inhibiting viral replication. In the presence of Hcy, lower concentrations of DHCA are needed to achieve this critical intracellular level of AdoHcy; consequently, lower concentrations of DHCA are needed to inhibit viral replication.

Biosynthetic source of AdoHcy in cells treated with Hcy alone or combinations of Hcy and DHCA. In order to determine the biosynthetic source of AdoHcy in cells treated with DHCA and Hcy alone or in combination, we conducted double-labeling experiments using [2,8-^{3}H]Ado and [^{35}S]Met. These double-labeling experiments should allow for determination of the biosynthetic pathway responsible for the formation of AdoHcy, because conversion of [^{35}S]Met and [^{3}H]Ado, the latter via ATP, to AdoHcy via AdoMet should produce [^{3}H]/[^{35}S]AdoHcy having a ^{3}H/^{35}S ratio equal to that observed in

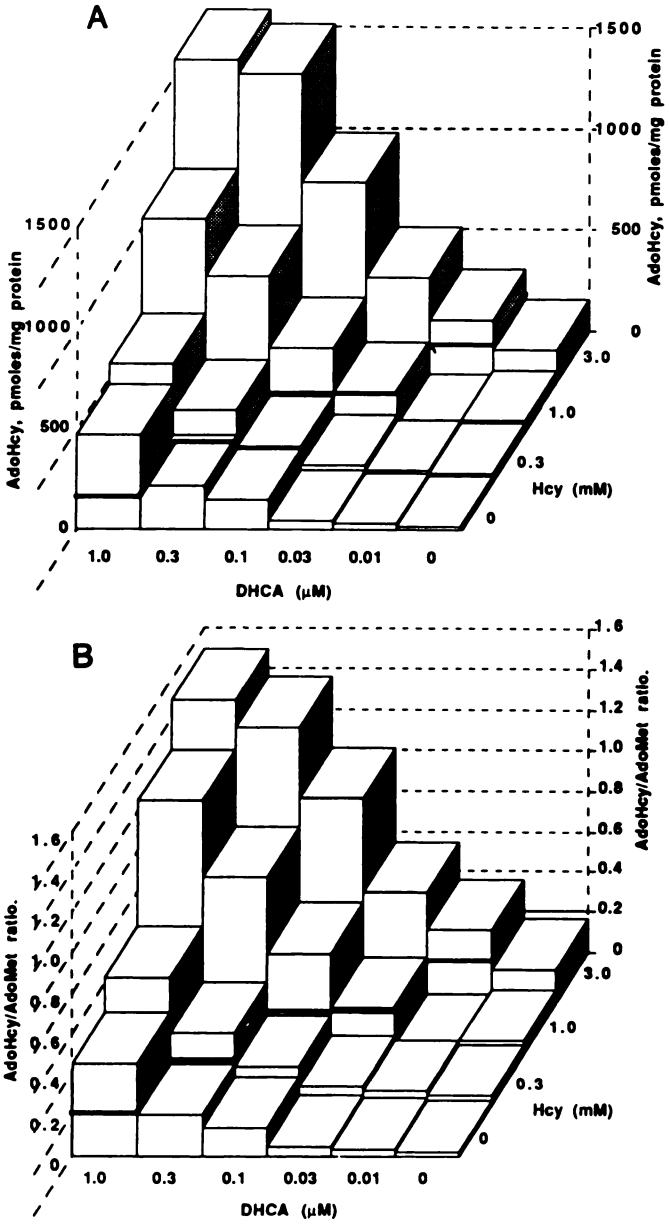

Fig. 3. Intracellular AdoHcy contents and AdoHcy/AdoMet ratios in L929 cells treated with various concentrations of DHCA alone or in combination with various concentrations of Hcy. Cultures of mouse L929 cells (approximately 2 × 10^6 cells/60-mm diameter dish) were incubated at 37° without DHCA and Hcy or with various concentrations of DHCA (0.01–1.0 μM) and Hcy (0.3–3.0 mM), alone or in combination with various concentrations of DHCA and Hcy. Treatment was started by the simultaneous addition of compounds. After 24 hr, the cells were harvested and the AdoHcy contents (A) and AdoMet contents (data not shown) were determined as described in Experimental Procedures. The AdoHcy/AdoMet ratios (B) were calculated from the endogenous AdoHcy and AdoMet contents. The *bold lines* in A and B represent the concentration of AdoHcy and the AdoHcy/AdoMet ratio, respectively, which is needed to inhibit vaccinia viral replication in murine L929 cells (22).

[^{3}H]/[^{35}S]AdoMet. If AdoHcy is being produced by the AdoHcy hydrolase-catalyzed conversion of [^{3}H]Ado and Hcy, the ^{3}H/^{35}S ratio in AdoHcy should be greater than the ratio in AdoMet.

Table 4 shows the results of experiments in which L929 cells were treated with DHCA (0.1 μM) and Hcy (1 mM), alone or in combination, for 1 hr and then pulse-labeled with [^{3}H]Ado and [^{35}S]Met for another hour. In cells receiving no drug treatment,

TABLE 3

Effect of Hcy on the antiviral activity of DHCA and the ability of DHCA to induce changes in the intracellular content of AdoHcy and the ratio of AdoHcy/AdoMet

The IC_{50} values for DHCA were taken from Table 1. AdoHcy content and AdoHcy/AdoMet ratios were determined using the concentrations of DHCA at the IC_{50} values observed for the various concentrations of Hcy. See Experimental Procedures for details. Values are averages ± standard deviations of triplicate assays.

Hcy	Inhibition of vaccinia virus replication IC_{50}	At IC_{50} value	
		AdoHcy content	AdoHcy/AdoMet ratios
mM	*μM*	*pmol/mg of protein*	
0	0.30 ± 0.10	267 ± 19	0.27 ± 0.02
0.3	0.15 ± 0.03	221 ± 7	0.23 ± 0.01
1.0	0.09 ± 0.06	259 ± 25	0.27 ± 0.01
3.0	0.04 ± 0.02	551 ± 55	0.60 ± 0.06

the ^{3}H/^{35}S ratio in AdoHcy and AdoMet were 12 and 6.6, respectively. These results suggest that, under these experimental conditions, a proportion of AdoHcy in murine L929 cells arises from the direct conversion of Ado and Hcy to AdoHcy, thus not involving AdoMet and transmethylation. Similar labeling patterns of AdoHcy and AdoMet were observed in cells treated with 0.1 μM (Table 4) and 1.0 μM (data not shown) DHCA. When cells are treated with Hcy (1 mM) alone or Hcy (1 mM) and DHCA (0.1 μM), a significant change in the labeling pattern of AdoHcy, but not the labeling pattern of AdoMet, was observed. In cells treated with Hcy alone and Hcy plus DHCA, the ^{3}H/^{35}S ratios were 187 and 155, respectively. In contrast, the ^{3}H/^{35}S ratios in AdoMet remained at approximately 6. These data suggest that, in the presence of Hcy in the culture medium, the major source of AdoHcy is via AdoHcy hydrolase-catalyzed condensation of Ado and Hcy and not via AdoMet and transmethylations.

Discussion

In 1987, De Clercq (9) reported that Hcy potentiated the antiviral activity of AdoHcy hydrolase inhibitors (e.g., carbocyclic 3-deazaadenosine, neplanocin A, and AHPA) but not the antiviral activity of those compounds that are not supposed to act via AdoHcy hydrolase (e.g., tuberculin, acyclovir, and ribavirin). In addition, Hcy was observed to potentiate the activity of AdoHcy hydrolase inhibitors only against those viruses that are intrinsically susceptible to their antiviral action. These results with Hcy were surprising because the original rationale for conducting the experiments was that an exogenous supply of Hcy might elevate intracellular levels of Met, hence AdoMet, which might overcome the inhibitory effects of AdoHcy on AdoMet-dependent methyltransferases and thus reverse the antiviral activity of AdoHcy hydrolase inhibitors.

Several key observations have aided us in elucidating the mechanism underlying the Hcy effect on AdoHcy hydrolase inhibitors. The first key observation was made by de la Haba and Cantoni (1), who reported that AdoHcy hydrolase *in vitro* catalyzed the degradation of AdoHcy to Ado and Hcy as well as the biosynthesis of AdoHcy from Ado and Hcy. Another important observation was that addition of exogenous Hcy could stimulate the accumulation of AdoHcy in Ado-treated cells (23) and 3-deazaadenosyl-L-homocysteine in 3-deazaadenosine-treated cells (24–26).

Another key observation was made by Hasobe *et al.* (22), who reported that the antiviral effects and cytotoxic effects of

TABLE 4

Effect of exogenous DHCA and Hcy, alone or in combination, on the cellular synthesis of AdoHcy and AdoMet

L929 cell cultures in 6-cm dishes (3×10^6 cells/well) were treated with DHCA and Hcy alone or in combination for 1 hr and then the cultures were labeled with [2,8-^{3}H] Ado (7 μCi/ml) and [^{35}S]Met (12 μCi/ml) for 1 additional hr. Cells were harvested and prepared for HPLC analysis as described in "Determination of intracellular levels of AdoHcy and AdoMet." The fractions corresponding to the peaks observed for standard samples of AdoHcy and AdoMet were collected and the radioactivity was measured by scintillation counting. The results are expressed as averages of duplicates.

DHCA	Hcy	AdoHcy			AdoMet		
		^{3}H	^{35}S	^{3}H/^{35}S	^{3}H	^{35}S	^{3}H/^{35}S
μM	mM	cpm			cpm		
0	0	7,864	660	12	149,290	22,578	6.6
0	1	51,675	267	187	153,036	25,341	6.0
0.1	0	7,679	698	11	164,225	25,402	6.5
0.1	1	50,072	323	155	145,132	23,837	6.1

DHCA could be related to the intracellular concentrations of AdoHcy and the ratios of [AdoHcy]/[AdoMet]. Earlier De Clercq and Cools (12, 13) had established a correlation between the antiviral activity of acyclic and carbocyclic adenosine analogs and their inhibitory effects on L929 cell AdoHcy hydrolase. The more recent studies by Hasobe *et al.* (22) have extended the observations made by De Clercq and Cools (12, 13), to show that the concentration of DHCA that produces 50% inhibition of vaccinia virus replication (IC_{50}) causes an elevation in intracellular level of AdoHcy [from approximately 50 pmol/mg of protein (controls) to approximately 200 pmol/mg of protein] and an elevation in the ratio of [AdoHcy]/[AdoMet] [from approximately 0.05 (controls) to approximately 0.20]. In contrast to the extreme sensitivity of viral replication to a slight elevation in intracellular AdoHcy, the cell viability is quite tolerant to higher levels of this metabolite. For example, the concentration of DHCA that produces 50% inhibition of L929 cell replication (ID_{50}) causes a significant increase in the intracellular level of AdoHcy (to approximately 825–950 pmol/mg of protein) and a striking elevation in the ratio of [AdoHcy]/[AdoMet] (to approximately 1.3) (22).

Critical to elucidating the mechanism of this Hcy effect on AdoHcy hydrolase inhibitors was the finding that two forms of AdoHcy hydrolase exist in murine L929 cells, one form representing 90–95% of the total enzyme activity, which is very sensitive to inactivation by DHCA, and a second form representing 5–10% of the total enzyme activity, which is very insensitive to inactivation by DHCA (3) (Fig. 1). A similar phenomenon has been observed by Schanche *et al.* (27) in rat hepatocytes, using other AdoHcy hydrolase inhibitors. The exact nature and function of these two forms of AdoHcy hydrolase have yet to be determined. Additional key observations made in this study were (a) that incubation of murine L929 cells with DHCA in the presence of Hcy in the culture medium caused a synergistic increase in the intracellular levels of AdoHcy and the ratio of [AdoHcy]/[AdoMet] (Figs. 2 and 3) and (b) that in Hcy- or Hcy plus DHCA-treated murine L929 cells the elevated intracellular levels of AdoHcy were formed from the AdoHcy hydrolase-catalyzed reaction of Ado and Hcy (Table 4).

Taken together these observations suggest that the pivotal component of the mechanism responsible for the effects of Hcy on the antiviral effects of AdoHcy hydrolase inhibitors is an inhibitor-insensitive form of AdoHcy hydrolase and its ability to catalyze the biosynthesis of AdoHcy from endogenous Ado and the artificially elevated levels of Hcy. The addition of Hcy to DHCA-treated cells causes a synergistic increase in the intracellular levels of AdoHcy. Because the intracellular level of AdoHcy is directly related to the inhibition of viral replication (22), one observes the potentiating effects of Hcy on the antiviral activity of DHCA. As reported earlier by De Clercq (9), Hcy potentiates the antiviral activity of a series of AdoHcy hydrolase inhibitors, including AHPA. Recently, we have shown that Hcy also potentiates the antiviral activity of the 2-methylpropyl ester of AHPA and this effect is mediated by the same mechanism described above for DHCA.[2]

The exact nature and function of the inhibitor-insensitive form of AdoHcy hydrolase in murine L929 cells is unclear. However, it would appear that this form of AdoHcy hydrolase may play a role in regulating the intracellular level of Hcy. This conclusion is based on the observation[3] that, in murine L929 cells treated with high concentrations (e.g., 10 μM) of DHCA, the resulting increase in the intracellular levels of AdoHcy is accompanied by a decrease in the intracellular levels of Hcy. By conducting pulse-labeling experiments with [^{3}H] Ado and [^{35}S]Met, evidence was obtained to support the hypothesis that a significant source of AdoHcy in DHCA-treated cells was from Ado and Hcy and not from AdoMet via transmethylation. The observations described above suggest that AdoHcy hydrolase may play a role in the cell beyond simply regulating cellular levels of AdoHcy. An additional function for AdoHcy hydrolase may be in regulating cellular levels of Hcy.

In summary the results presented in this paper explain at a molecular level the mechanism responsible for potentiating effects of Hcy on the antiviral activity of AdoHcy hydrolase inhibitors. This mechanism, which involves an inhibitor-insensitive form of AdoHcy hydrolase catalyzing the conversion of Ado and Hcy to AdoHcy, has suggested an important new role for AdoHcy hydrolase involving regulation of Hcy metabolism in cells.

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