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HOMOCYSTEINE POTENTIATES THE ANTIVIRAL AND CYTOSTATIC ACTIVITY OF THOSE NUCLEOSIDE ANALOGUES THAT ARE TARGETED AT *S*-ADENOSYLHOMOCYSTEINE HYDROLASE*

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Abstract—Various adenosine analogues, i.e. (*S*)-9-(2,3-dihydroxypropyl)adenine, (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid, carbocyclic 3-deazaadenosine and neplanocin A, which have been previously recognized as specific inhibitors of *S*-adenosyl-L-homocysteine (SAH) hydrolase, gained a marked increase in their cytostatic activity (against tumor cells) and antiviral activity (against vaccinia and vesicular stomatitis virus) in the presence of L-homocysteine (10^{-3} M). Homocysteine did not increase the cytostatic or antiviral activity of those compounds (i.e. tubercidin, ribavirin, acyclovir or vidarabine) that do not achieve their biological activity via SAH hydrolase inhibition. The increased antiviral activity following addition of homocysteine was observed only with those viruses (i.e. vaccinia and vesicular stomatitis virus) that belong to the activity spectrum of SAH hydrolase inhibitors [*Biochem Pharmacol* **36**: 2567–2575, 1987], and only in those cells in which the SAH hydrolase inhibitors are normally active. The enhancing effect of homocysteine on the cytostatic and antiviral activity of the SAH hydrolase inhibitors could not be attributed to a non-specific increase in the cytotoxicity of the compounds, as their effects on host cell macromolecule (DNA, RNA, protein) synthesis was not markedly altered in the presence of homocysteine. Most likely, homocysteine exerted its potentiating effect on the activity of the SAH hydrolase inhibitors through an increase in the intracellular levels of SAH, which is known to act as a product inhibitor of *S*-adenosyl-L-methionine (SAM)-dependent transmethylation reactions.

Adenosine analogues such as (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA] [1], carbocyclic 3-deazaadenosine (C-c^3 Ado) [2], (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid [(*RS*)-AHPA] alkyl esters [3] and neplanocin A [4, 5], have a unique spectrum of antiviral activity, encompassing, in particular, poxviruses (i.e. vaccinia), iridoviruses (i.e. African swine fever), paramyxoviruses (i.e. parainfluenza), rhabdoviruses (i.e. vesicular stomatitis) and reoviruses (i.e. rota) [6]. Also, C-c^3 Ado and neplanocin A show a marked cytostatic action against some tumor cell lines [7–9].

(*S*)-DHPA, C-c^{3}Ado, (*RS*)-AHPA and neplanocin A are assumed to exert their broad-spectrum antiviral activity as well as their specific cytostatic effect on tumor cell lines through the inhibition of transmethylation reactions requiring *S*-adenosyl-L-methionine (SAM) as methyl donor. As the direct target for the action of these adenosine analogues would serve *S*-adenosyl-L-homocysteine (SAH) hydrolase, which is responsible for the (reversible) hydrolysis of SAH to adenosine and L-homocysteine. If SAH hydrolase is inhibited, SAH would accumulate, and, since the latter is a product inhibitor of SAM-dependent transmethylations, accumulation of SAH would result in a decreased rate of methylations, including those that are required for virus replication and tumor cell growth.

If the antiviral and cytostatic effects of adenosine analogues are due to their inhibitory action on SAH hydrolase, the resulting accumulation of SAH should be accompanied by a depletion in the intracellular pool sizes of homocysteine and adenosine (Fig. 1). Since homocysteine is required as substrate for methionine synthesis (catalyzed by 5-methyltetrahydrofolate: L-homocysteine methyltransferase) and homocysteine is supplied exclusively through hydrolysis of SAH, the depletion of homocysteine not only leads to a depletion of methionine and SAM but also affects the synthesis of nucleic acid precursors. In the absence of methionine synthesis, methyltetrahydrofolate is not regenerated to tetrahydrofolate and, thus, not available for nucleic acid precursor biosynthesis [10]. Exogenous addition of homocysteine might be expected to generate sufficient methionine to overcome the consequences of a depletion in the intracellular homocysteine pools. If this reasoning would be correct, the addition of homocysteine should be able to reverse the cytostatic effects of adenosine analogues (at least of those adenosine analogues that are targeted at SAH hydrolase), and, indeed, from a previous report [7] it appears that C-c^{3}Ado loses much of its cytostatic activity upon addition of homocysteine to the cell culture medium.

MATERIALS AND METHODS

Test compounds. (*S*)-DHPA [1] and (*RS*)-AHPA

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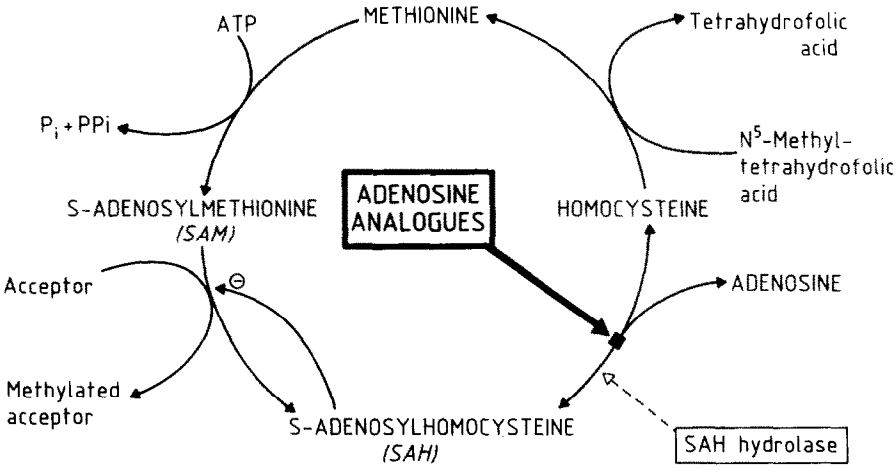

Fig. 1. SAH hydrolase as target for the antiviral and cytostatic action of adenosine analogues [(*S*)-DHPA, (*RS*)-AHPA, C-c^{3}Ado and neplanocin A] and role of SAH hydrolase in transmethylation reactions requiring SAM as the methyl donor.

isobutyl ester [3] were obtained from Dr A. Holy (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). C-c^{3}Ado [2] was obtained from Dr J. A. Montgomery (Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL) and neplanocin A was provided by Dr J. Murase (Toyo Jozo Company, Mifuku Ohito-cho, Tagata-gun, Shizuoka-ken, Japan). Tubercidin was purchased from the Upjohn Company, Fine Chemicals Division (Kalamazoo, MI), ribavirin was obtained from ICN Nutritional Biochemicals (Cleveland, OH); acyclovir from the Wellcome Research Laboratories, Burroughs Wellcome Company (Research Triangle Park, NC); and vidarabine from Parke Davis and Company (Ann Arbor, MI). L-Homocysteine thiolactone was obtained from Calbiochem (San Diego, CA).

Radiolabeled precursors. The radiolabeled precursors [*methyl*-^{3}H]-2′-deoxythymidine (dThd), [5-^{3}H]-uridine (Urd) and [4,5-^{3}H]-leucine (Leu) used to monitor the synthesis of cellular DNA, RNA and protein were obtained from Amersham (Buckinghamshire, U.K.). Their radioactivity was 40, 30 and 52 Ci/mmole, respectively.

Viruses and cells. The origin of the viruses [herpes simplex virus type 1 (HSV-1, strain KOS), herpes simplex virus type 2 (HSV-2, strain G), vaccinia virus (VV) and vesicular stomatitis virus (VSV)] has been documented previously [4].

The cell lines used for the antiviral activity assays were PRK (primary rabbit kidney), HeLa, Vero (African green monkey kidney), E_6SM (human embryonic skin-muscle), BS-C-1A (monkey kidney) and RK13 (rabbit kidney). For further details on the origin and cultivation of these cells, see Ref. 11.

Tumor cells. The tumor cell lines used for the cytostatic activity determinations were murine leukemia (L1210), murine mammary carcinoma (FM3A), human B-lymphoblast (Raji, Namalva) and rat (Novikoff) hepatoma. The methodology for culturing these tumor cell lines has been documented previously [12].

Antiviral activity. Antiviral activity measurements were based on the inhibition of virus-induced cytopathogenicity following well-established procedures [4, 13].

Cytostatic activity. Cytostatic activity determinations were based on the inhibition of cell proliferation during their exponential growth phase [12, 14], i.e. at 2 days for L1210 and FM3A cells, 3 days for Raji, Namalva and hepatoma cells, and 4 days for PRK cells.

Antimetabolic activity. For the evaluation of antimetabolic activity, inhibition of host cell macromolecule (DNA, RNA and protein) synthesis was monitored by the incorporation of [*methyl*-^{3}H]dThd, [5-^{3}H]Urd and [4,5-^{3}H]Leu, respectively (following a 16-hr incubation period [4]).

RESULTS

When (*S*)-DHPA, C-c^{3}Ado and neplanocin A, three adenosine analogues which have been previously recognized as specific inhibitors of SAH hydrolase [6, 15] were evaluated for their cytostatic potential in the absence and presence of homocysteine (Table 1), it appeared that homocysteine markedly potentiated the cytostatic activity of these compounds against all tumor cell lines that were examined. Homocysteine also increased the cytostatic activity of (*S*)-DHPA, C-c^{3}Ado and neplanocin A against PRK cells. The ID_{50} of (*S*)-DHPA and C-c^{3}Ado for cell growth was decreased by approximately 10- to 30-fold if homocysteine had been added to the cell culture medium; and the ID_{50} of C-c^{3}Ado for FM3A cells was decreased by almost 100-fold after addition of homocysteine. For neplanocin A that has potent cytostatic activity in the absence of homocysteine, the increase in cytostatic potency (decrease in ID_{50}) was less pronounced (at an average 3-fold) following addition of homocysteine than for the other SAH hydrolase inhibitors.

In addition to (*S*)-DHPA, C-c^{3}Ado and neplan-

Table 1. Effect of L-homocysteine on the cytostatic activity of various compounds against L1210, FM3A, Raji, Namalva, hepatoma and PRK cells

Compound	Homocysteine (10^{-3} M)	ID_{50} (μg/ml)*					
		L1210	FM3A	Raji	Namalva	Hepatoma	PRK
(*S*)-DHPA	–	163 ± 72	62 ± 21	313 ± 122	220 ± 29	113 ± 48	69 ± 31
	+	18 ± 6	1.7 ± 0.4	12 ± 1	16 ± 5.7	3.6 ± 1.7	3.7 ± 1.4
C-c^{3}Ado	–	1.7 ± 0.2	0.97 ± 0.3	8 ± 1.7	4.7 ± 0.9	1.5 ± 0.3	0.46 ± 0.27
	+	0.24 ± 0.03	0.01 ± 0.001	0.28 ± 0.07	0.3 ± 0.09	0.25 ± 0.2	0.042 ± 0.034
Neplanocin A	–	0.06 ± 0.02	0.029 ± 0.001	0.058 ± 0.0003	0.12 ± 0.05	0.045 ± 0.01	0.052 ± 0.034
	+	0.02 ± 0.001	0.008 ± 0.0003	0.034 ± 0.0006	0.027 ± 0.04	0.002 ± 0.001	0.036 ± 0.030
Tubercidin	–	0.075 ± 0.02	0.18 ± 0.01	0.12 ± 0.002	0.062 ± 0.01	0.046 ± 0.003	0.013 ± 0.006
	+	0.056 ± 0.01	0.19 ± 0.006	0.06 ± 0.004	0.042 ± 0.006	0.023 ± 0.002	0.010 ± 0.006
Ribavirin	–	9.3 ± 3.5	3.3 ± 0.5	33 ± 2	30 ± 2	14 ± 5	7.3 ± 2.7
	+	6.9 ± 2.3	3.8 ± 0.4	48 ± 15	43 ± 4	6.7 ± 2.8	7.7 ± 1.7
Acyclovir	–	38 ± 13	42 ± 13	>100	>100	33 ± 8	23 ± 11
	+	22 ± 5	63 ± 7	>100	>100	40 ± 20	7.5 ± 3.2
Vidarabine	–	14 ± 4	28 ± 2	7.8 ± 0.6	8.3 ± 3.5	6.3 ± 1.7	23 ± 10
	+	8 ± 1.5	20 ± 3	6.4 ± 1.2	6.2 ± 3	5.8 ± 0.8	20 ± 13

* A 50% inhibitory dose, achieving a reduction in the number of cells by 50%. Average values (± standard deviation) for 3 to 5 separate experiments. At 10^{-3} M, homocysteine itself effected a slight reduction in the number of tumor cells: at an average 10.5% for L1210 cells, 25% for FM3A cells, 20% for Raji cells, 30% for Namalva cells, 17% for hepatoma cells and 6.3% for PRK cells.

Table 2. Effect of L-homocysteine on the antiviral activity of various compounds against herpes simplex virus type 1 and type 2, vaccinia virus and vesicular stomatitis virus in primary rabbit kidney cell cultures

Compound	Homocysteine (10^{-3} M)	MCC* (μg/ml)	MIC (μg/ml)†			
			HSV-1 (KOS)	HSV-2 (G)	VV	VSV
(*S*)-DHPA	−	>400	>400	>400	150	70
	+	>400	>400	>400	10	0.7
(*RS*)-AHPA	−	>200	>200	>200	2	0.2
	+	>200	>200	>200	0.07	0.02
C-c^{3}Ado	−	>400	>400	>400	2	0.7
	+	>400	>400	>400	0.2	0.02
Neplanocin A	−	≥10	≥4	≥4	0.07	0.07
	+	≥10	≥4	≥4	0.002	0.007
Tubercidin	−	≥0.4	>0.1	>0.1	0.2	0.04
	+	≥0.4	>0.1	>0.1	0.1	0.04
Ribavirin	−	>400	>400	>400	20	>400
	+	>400	>400	>400	40	>400
Acyclovir	−	>400	0.2	0.1	150	>400
	+	>400	0.2	0.2	300	>400
Vidarabine	−	≥100	20	7	7	70
	+	≥100	20	7	2	7

* Minimum cytotoxic concentration, required to cause a microscopically detectable alteration of normal cell morphology.

† Minimum inhibitory concentration, required to reduce virus-induced cytopathogenicity by 50%.

At 10^{-3} M, homocysteine itself had no effect on normal cell morphology, nor did it affect viral cytopathogenicity at that concentration.

ocin A, four other nucleoside analogues (tubercidin, ribavirin, acyclovir and vidarabine), which are assumed to exert their biological (antiviral and/or antimetabolic) effects through interactions other than SAH hydrolase inhibition, were also examined for their cytostatic activity in the absence and presence of homocysteine (Table 1). These four compounds demonstrated a widely varying cytostatic potency, tubercidin being the most, and acyclovir the least potent, cytostatic agent. None of the four gained a marked increase in cytostatic potency (decrease in ID_{50}) following addition of homocysteine to the cell culture medium.

The antiviral spectrum of those adenosine analogues that are targeted at SAH hydrolase is well characterized [6]. These SAH hydrolase inhibitors are specifically active against such DNA and (−)RNA viruses as VV and VSV, while they are virtually inactive against herpes viruses (HSV-1, HSV-2). This is also evident from the results presented in Table 2. If homocysteine was added to the cell culture medium, the inhibitory activity of (*S*)-DHPA, (*RS*)-AHPA, C-c^{3}Ado and neplanocin A against VV and VSV was markedly enhanced: their MIC for VV and VSV decreased by a factor of 10- to 100 (Table 2). While becoming more potent as inhibitors of VV and VSV, the SAH hydrolase inhibitors did not acquire an increased activity against HSV-1 and HSV-2 following the addition of homocysteine; neither did they become more toxic for the host cells in the presence of homocysteine: their MCC for PRK cells remained unchanged (Table 2).

Tubercidin, ribavirin, acyclovir and vidarabine showed the usual pattern of antiviral and cytotoxic activity (Table 2), conform with data obtained previously [13, 16]. Addition of homocysteine to the cell culture medium had no effect on the MIC or MCC values of these compounds, except for vidarabine which gained a 3- or 10-fold increase in its anti-VV and anti-VSV potency, respectively.

In the above experiments (Table 2), antiviral activity measurements were based upon a reduction of virus-induced cytopathogenicity. With C-c^{3}Ado additional experiments were carried out whereby the compound, both in the absence and presence of homocysteine, was evaluated for its inhibitory effect on the multiplication of VV in PRK cells (Fig. 2). Virus yield was measured at different times (up to 72 hr) after infection, and, as clearly shown in Fig. 2, the inhibitory effect of C-c^{3}Ado on VV progeny formation was markedly increased in the presence of homocysteine. For example, the combination of C-c^{3}Ado (1 μg/ml) and homocysteine (10^{-3} M) achieved a 2 $\log_{10}$ reduction in the 48-hr virus yield, whereas neither compound used individually had any effect on the virus yield (Fig. 2, panel A). At 10 μg/ml, C-c^{3}Ado (in the absence of homocysteine) effected a 1.5 $\log_{10}$ reduction in virus yield, and this virus yield reduction augmented by one more $\log_{10}$ in the presence of homocysteine (Fig. 2, panel B). Thus, at a concentration of 1 μg/ml in the presence of homocysteine, C-c^{3}Ado was more effective an inhibitor of VV multiplication than at 10 μg/ml in the absence of homocysteine.

The dose–response relationship of the potentiating effect of homocysteine on the antiviral activity of C-c^{3}Ado was assessed in different cell lines infected with VSV (Table 3). The optimal dose of homocysteine appeared to be 10^{-3} M. At a concentration of 3×10^{-4} M, homocysteine caused only a slight (and insignificant) decrease in the MIC of C-c^{3}Ado for VSV; and at doses lower than 3×10^{-4} M, it

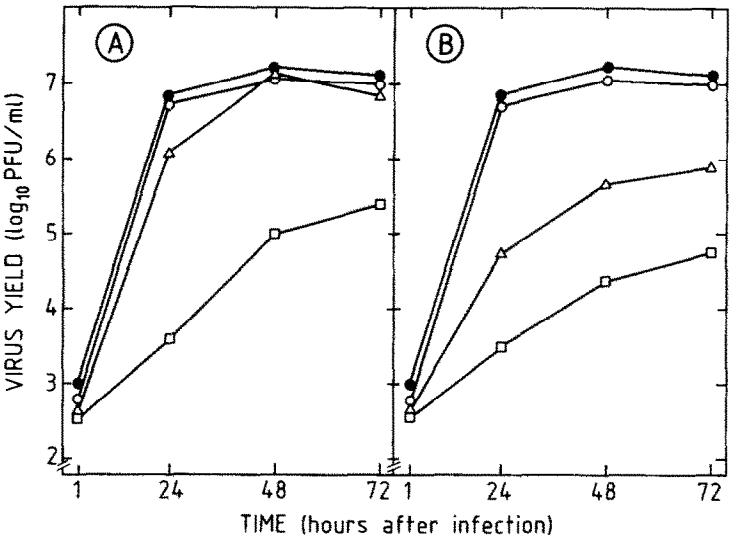

Fig. 2. Inhibitory effect of C-c^{3}Ado, in the absence and presence of L-homocysteine on the multiplication of vaccinia virus in PRK cell cultures. The cells were infected with vaccinia virus (4.5 log_{10} PFU per 0.5 ml per Petri dish) and, after one hour virus adsorption, incubated in the presence of the test compounds. Virus yield was measured at 1, 24, 48 and 72 hr after infection. Therefore, the cells were frozen at −70°, thawed and the cell homogenates were then assayed for virus content by plaque formation in Vero cells. Virus yields are expressed in plaque forming units (PFU). Panel A: ●, control; ○, homocysteine (10^{-3} M); △, C-c^{3}Ado at 1 μg/ml; □, homocysteine (10^{-3} M) + C-c^{3}Ado at 1 μg/ml. Panel B: ●, control; ○, homocysteine (10^{-3} M); △, C-c^{3}Ado at 10 μg/ml; □, homocysteine (10^{-3} M) + C-c^{3}Ado at 10 μg/ml.

Table 3. Effect of different concentrations of L-homocysteine on activity of C-c^{3}Ado against VSV in different cell lines

Homocysteine (M)	MIC (μg/ml)*					
	PRK	HeLa	Vero	E_6SM	BS-C-1A	RK13
3×10^{-3}	toxic	0.07	>400	toxic	toxic	toxic
1×10^{-3}	0.02	0.2	>400	0.2	>400	0.07
3×10^{-4}	0.7	1	>400	0.7	>400	0.7
1×10^{-4}	1	2	>400	2	>400	0.7
3×10^{-5}	1.5	4	>400	2	>400	1
1×10^{-5}	1.5	2	>400	2	>400	2
None	1	4	>400	2	>400	2

* Minimum inhibitory concentration, required to reduce virus-induced cytopathogenicity by 50%.

was essentially ineffective. At a dose of 3×10^{-3} M, homocysteine proved cytotoxic for most of the cell lines used.

While C-c^{3}Ado proved effective in protecting PRK, HeLa, E_6SM, RK13 (Table 3) and murine L-929 cells [2] against the cytopathogenicity of VSV, in other cells (i.e. Vero, BS-C-1A) it failed to do so. Addition of homocysteine potentiated the antiviral activity of C-c^{3}Ado only in those cells where C-c^{3}Ado was normally active. Homocysteine could not make C-c^{3}Ado active in those cells where it was inactive (Table 3).

In addition to homocysteine, adenosine was also investigated for its potentiating effect on the antiviral activity of C-c^{3}Ado. The exogenous supply of adenosine to the cell culture medium at concentrations up to 300 μg/ml (10^{-3} M) did not affect the activity of (*S*)-DHPA, (*RS*)-AHPA, C-c^{3}Ado or neplanocin A against either VV or VSV in primary rabbit kidney cells (data not shown).

To explore the possibility that the enhancing effect of homocysteine on the cytostatic activity of the SAH hydrolase inhibitors might have resulted from an aspecific inhibition of the cell's metabolism, host cell macromolecule (DNA, RNA and protein) synthesis was measured in L1210 cells (as representative of the tumor cell lines), which had been exposed to the SAH hydrolase inhibitors in the presence or absence of homocysteine (Table 4). The ID_{50} values of (*S*)-DHPA, (*RS*)-AHPA, C-c^{3}Ado and neplanocin A for L1210 cell DNA, RNA and protein synthesis (as monitored by the incorporation of [*methyl*-^{3}H]dThd,

Table 4. Effect of L-homocysteine on L1210 cell DNA, RNA and protein synthesis, as monitored by the incorporation of [*methyl*-^{3}H]dThd, [5-^{3}H]Urd and [4,5-^{3}H]Leu, respectively

Compound	Homocysteine (10^{-3} M)	ID_{50} (μg/ml)*		
		[*Methyl*-^{3}H]dThd incorporation	[5-^{3}H]Urd incorporation	[4,5-^{3}H]Leu incorporation
(*S*)-DHPA	−	> 1000	> 1000	793 ± 247
	+	> 1000	> 1000	332 ± 209
(*RS*)-AHPA	−	> 100	> 100	> 100
	+	> 100	> 100	> 100
C-c^{3}Ado	−	> 1000	> 1000	399 ± 152
	+	≥ 1000	> 1000	247 ± 99
Neplanocin A	−	407 ± 80	24 ± 12	1.5 ± 0.5
	+	358 ± 194	54 ± 15	1.3 ± 0.2
Tubercidin	−	1.7 ± 0.8	0.34 ± 0.034	0.13 ± 0.007
	+	2.5 ± 1.2	0.34 ± 0.05	0.12 ± 0.06
Ribavirin	−	4.8 ± 0.8	8.6 ± 2	36 ± 25
	+	6.4 ± 1	23 ± 15	24 ± 14
Acyclovir	−	810 ± 167	> 1000	≥ 1000
	+	> 1000	> 1000	≥ 1000
Vidarabine	−	56 ± 25	251 ± 32	61 ± 32
	+	49 ± 8	243 ± 94	76 ± 57

* A 50% inhibitory dose, achieving a reduction in the incorporation of the radiolabeled precursor by 50%. Average values (± standard deviation) for 3 to 5 separate experiments. At 10^{-3} M, homocysteine itself did not cause any inhibition in the incorporation of [5-^{3}H]Urd or [4,5-^{3}H]Leu, and only 8% inhibition in the incorporation of [*methyl*-^{3}H]dThd.

[5-^{3}H]Urd and [4,5-^{3}H]Leu, respectively) remained essentially unaltered following the addition of homocysteine. Similarly, homocysteine did not influence the inhibitory effects of tubercidin, ribavirin, acyclovir or vidarabine on L1210 cell macromolecule synthesis (Table 4).

Cellular DNA, RNA and protein synthesis was also examined in PRK cells exposed to the test compounds in the presence or absence of homocysteine (Table 5). Here, some striking (10- to 40-fold) reductions were noted in the ID_{50} values of (*RS*)-AHPA and C-c^{3}Ado for DNA synthesis (monitored by [*methyl*-^{3}H]dThd incorporation) following the addition of homocysteine. A less striking (2- to 3-fold) reduction was noted for the corresponding ID_{50} values for RNA synthesis (monitored by [5-^{3}H]Urd incorporation). Small fluctuations in ID_{50} values following addition of homocysteine were also observed in some other instances, i.e. with tubercidin (Table 5). The meaning of these small (2- to 3-fold) changes in ID_{50} remains to be assessed. Of possible interest is the finding that in the presence of homocysteine the ID_{50} of ribavirin for RNA synthesis increased by a factor of 2–3, both in PRK cells (Table 5) and L1210 cells (Table 4). Again, the significance of apparent decrease in the inhibitory effect of ribavirin on RNA synthesis remains to be determined.

DISCUSSION

Based on previous observations of Kim *et al.* [7] that homocysteine reversed the cytostatic activity of C-c^{3}Ado (for the RAW264 mouse macrophage cell line), we expected homocysteine to reverse the cytostatic effects of C-c^{3}Ado on other cell lines as well. However, it did not. On the contrary, homocysteine potentiated the cytostatic activity of C-c^{3}Ado for various tumor cell lines, and this potentiating effect was not only observed with C-c^{3}Ado but also with other inhibitors of SAH hydrolase [6]. In contrast, compounds such as tubercidin, ribavirin, acyclovir, and vidarabine which are not supposed to act *via* inhibition of SAH hydrolase did not acquire increased cytostatic potency following the addition of homocysteine (Table 1). Tubercidin interferes with a number of cellular processes (other than SAH hydrolase) [16]; ribavirin primarily acts at the mRNA 5′-capping level [17]; acyclovir terminates DNA chain elongation after it has been phosphorylated to its triphosphate (in HSV-infected cells) [18, 19]; and vidarabine may owe its biological (cytostatic and antiviral) activity mainly to its inhibitory effect on DNA polymerase and/or incorporation into DNA [20] [although some of the antiviral effects of vidarabine, (i.e. against VSV) may be due to inhibition of SAH hydrolase].

Homocysteine potentiated the antiviral activity of the SAH hydrolase inhibitors (Table 2). This potentiating effect was observed only with those compounds that are assumed to act via SAH hydrolase inhibition, namely (*S*)-DHPA, (*RS*)-AHPA, C-c^{3}Ado, neplanocin A [6]; only for those viruses that are specifically included in the activity spectrum of the SAH hydrolase inhibitors, namely vaccinia virus, vesicular stomatitis virus [6]; and only in those cells that are normally permissive to the antiviral effects of the SAH hydrolase inhibitors, namely PRK, HeLa, E_6SM, RK13. Thus, the enhanced antiviral activity generated by homocysteine strictly depended on the choice of the compounds, viruses and cells. It was not observed with compounds, such as tubercidin, ribavirin and acyclovir, which act by mechanisms

Table 5. Effect of L-homocysteine on PRK cell DNA, RNA and protein synthesis, as monitored by the incorporation of [*methyl*-^{3}H]dThd, [5-^{3}H]Urd and [4,5-^{3}H]Leu, respectively

Compound	Homocysteine (10^{-3} M)	ID_{50} (μg/ml)*		
		[*Methyl*-^{3}H]dThd incorporation	[5-^{3}H]Urd incorporation	[4,5-^{3}H]Leu incorporation
(*S*)-DHPA	−	350 ± 40	>400	>400
	+	240 ± 84	>400	>400
(*RS*)-AHPA	−	65 ± 27	131 ± 55	>400
	+	6.7 ± 3	71 ± 63	>400
C-c^{3}Ado	−	59 ± 51	10 ± 2	>400
	+	1.3 ± 1	3.2 ± 2	>400
Neplanocin A	−	4 ± 2	8.4 ± 5	>400
	+	2 ± 1.3	4.4 ± 3	>400
Tubercidin	−	0.018 ± 0.001	0.03 ± 0.007	0.52 ± 0.3
	+	0.024 ± 0.01	0.08 ± 0.07	0.17 ± 0.03
Ribavirin	−	1.7 ± 0.4	4.9 ± 4	>100
	+	3.2 ± 1.8	13 ± 11	>100
Acyclovir	−	6.8 ± 2.7	>100	>200
	+	11.3 ± 3	>100	>200
Vidarabine	−	7.6 ± 3.4	>100	>400
	+	12 ± 13	>100	>400

* A 50% inhibitory dose, achieving a reduction in the incorporation of the radiolabeled precursor by 50%. Average values (± standard deviation) for 3–4 separate experiments.

At 10^{-3} M, homocysteine itself caused 11%, 6% and 13% inhibition of the incorporation of [*methyl*-^{3}H]dThd, [5-^{3}H]Urd and [4,5-^{3}H]Leu, respectively.

other than SAH hydrolase inhibition. Neither was it demonstrated with viruses, such as HSV-1 or HSV-2, which are not sensitive to SAH hydrolase inhibitors or cells, such as Vero or BS-C-1A, which are not permissive to the antiviral action of SAH hydrolase inhibitors.

The enhancing effects of homocysteine on both the cytostatic and antiviral activity of SAH hydrolase inhibitors should be regarded as a rather specific phenomenon, *not* merely resulting from cytotoxicity for the host cells. Under conditions where homocysteine enhanced the antiviral activity of the SAH hydrolase inhibitors, there was no concomitant increase in cytotoxicity (Table 2), and under conditions where the cytostatic activity of the SAH hydrolase inhibitors was enhanced, their effects on host cell DNA, RNA and protein synthesis remained unaffected (Table 4). Only for DNA synthesis in PRK cells was a significant shift in ID_{50} of (*RS*)-AHPA and C-c^{3}Ado noted following addition of homocysteine (Table 5). However, the latter effect may result from an interference with the formation of methyltetrahydrofolate and, consequently, dTTP pools rather than inhibition of DNA synthesis *per se*.

As a rule, the inhibitory effects of the compounds on cell growth were achieved at much lower concentrations than those required to inhibit host cell DNA, RNA or protein synthesis (Tables 1, 4 and 5). These differences may be related to the methodology used, since inhibition of cell growth was measured after an incubation period of 2–4 days, whereas inhibition of macromolecular syntheses was evaluated after a 16-hr incubation period. The longer incubation times in the cell growth experiments may have contributed to an amplification of the cell growth-inhibitory effects of the compounds relative to their inhibitory effects on macromolecular syntheses.

How could the stimulatory effect of exogenous homocysteine on the biological activity of SAH hydrolase inhibitors be explained? According to Fig. 1, homocysteine may be converted to SAH either directly following condensation with adenosine, or indirectly via the intermediary formation of methionine and SAM. Should the latter pathway occur, however, one might expect a decreased, rather than increased, biological activity of the compounds. Such a decreased activity was found by Kim *et al.* [7] following addition of homocysteine, but not methionine. These authors have suggested that depletion of homocysteine pools leads *via* insufficient regeneration of tetrahydrofolate to a reduction in the biosynthesis of purine and pyrimidines. However, pulse-label experiments with [^{35}S]methionine and [2,8-^{3}H]adenosine have indicated that the elevated SAH levels resulting from exogenous addition of homocysteine must be formed *via* the SAH hydrolase reaction [21]. Apparently, the residual SAH hydrolase activity in cells treated with the SAH hydrolase inhibitors would be sufficient to catalyze the conversion of homocysteine and adenosine to SAH (Fig. 1).

It is thus conceivable that the exogenous homocysteine exerts its enhancing effect on the antiviral and cytostatic activity of the adenosine analogues through increasing the intracellular SAH levels via SAH hydrolase. If homocysteine increases the biological activity of SAH hydrolase inhibitors, one may expect adenosine to behave similarly. Yet, exogenous addition of adenosine failed to enhance the biological activity of the SAH hydrolase inhibitors.

Probably, the intracellular pool levels of adenosine are sufficient for maximal formation of SAH, while homocysteine is present at suboptimal concentrations. This may explain why the exogenous supply of homocysteine, but not adenosine, is able to further boost the formation of SAH, and, hence, augment the inhibitory effect of the latter on the SAM-dependent methylations.

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REFERENCES

1. De Clercq E, Descamps J, De Somer P and Holy A, (*S*)-9-(2,3-Dihydroxypropyl)adenine: an aliphatic nucleoside analog with broad-spectrum antiviral activity. *Science* **200**: 563–565, 1978.
2. De Clercq E and Montgomery JA, Broad-spectrum antiviral activity of the carbocyclic analog of 3-deazaadenosine. *Antiviral Res* **3**: 17–24, 1983.
3. De Clercq E and Holy A, Alkyl esters of 3-adenin-9-yl-2-hydroxypropanoic acid: a new class of broad-spectrum antiviral agents. *J Med Chem* **28**: 282–287, 1985.
4. De Clercq E, Antiviral and antimetabolic activities of neplanocins. *Antimicrob Agents Chemother* **28**: 84–89, 1985.
5. 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.
6. De Clercq E, S-Adenosylhomocysteine hydrolase inhibitors as broad-spectrum antiviral agents. *Biochem Pharmacol* **36**: 2567–2575, 1987.
7. Kim I-K, Aksamit RR and Cantoni GL, Mechanism of the cytostatic activity of 3-deazaaristeromycin, an inhibitor of adenosylhomocysteine hydrolase. *J Biol Chem* **257**: 14726–14729, 1982.
8. Glazer RI and Knode MC, Neplanocin A: a cyclopentenyl analog of adenosine with specificity for inhibiting RNA methylation. *J Biol Chem* **259**: 12964–12969, 1984.
9. Saunders PP, Tan M-T and Robins RK, Metabolism and action of neplanocin A in chinese hamster ovary cells. *Biochem Pharmacol* **34**: 2749–2754, 1985.
10. Cantoni GL, Aksamit RR and Kim I-K, Methionine biosynthesis and vidarabine therapy. *New Engl J Med* **307**: 1079, 1982.
11. De Clercq E, Comparative efficacy of antiherpes drugs in different cell lines. *Antimicrob Agents Chemother* **21**: 661–663, 1982.
12. Balzarini J, De Clercq E, Torrence PF, Mertes MP, Park JS, Schmidt CL, Shugar D, Barr PJ, Jones AS, Verhelst G and Walker RT, Role of thymidine kinase in the inhibitory activity of 5-substituted-2′-deoxyuridines on the growth of human and murine tumor cell lines. *Biochem Pharmacol* **31**: 1089–1095, 1982.
13. De Clercq E, Descamps J, Verhelst G, Walker RT, Jones AS, Torrence PF and Shugar D, Comparative efficacy of antiherpes drugs against different strains of herpes simplex virus. *J Infect Dis* **141**: 563–574, 1980.
14. De Clercq E, Balzarini J, Torrence PF, Mertes MP, Schmidt CL, Shugar D, Barr PJ, Jones AS, Verhelst G and Walker RT, Thymidylate synthetase as target enzyme for the inhibitory activity of 5-substituted 2′-deoxyuridines on mouse leukemia L1210 cell growth. *Molec Pharmacol* **19**: 321–330, 1981.
15. De Clercq E and Cools M, Antiviral potency of adenosine analogues: correlation with inhibition of S-adenosylhomocysteine hydrolase. *Biochem Biophys Res Commun* **129**: 306–311, 1985.
16. Bergstrom DE, Brattesani AJ, Ogawa MK, Reddy PA, Schweickert MJ, Balzarini J and De Clercq E, Antiviral activity of C-5 substituted tubercidin analogs. *J Med Chem* **27**: 285–292, 1984.
17. Smith RA, Mechanisms of action of ribavirin. In: *Ribavirin: A Broad Spectrum Antiviral Agent* (Eds. Smith RA and Kirkpatrick W), pp. 99–118. Academic Press, New York, 1980.
18. Fyfe JA, Keller PM, Furman PA, Miller RL and Elion GB, Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. *J Biol Chem* **253**: 8721–8727, 1978.
19. Furman PA, McGuirt PV, Keller PM, Fyfe JA and Elion GB, Inhibition by acyclovir of cell growth and DNA synthesis of cells biochemically transformed with herpesvirus genetic information. *Virology* **102**: 420–430, 1980.
20. Pelling JC, Drach JC and Shipman C Jr, Internucleotide incorporation of arabinosyladenine into herpes simplex virus and mammalian cell DNA. *Virology* **109**: 323–335, 1981.
21. Hasobe M, Ishii H, McKee JG and Borchardt RT, The effects of 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-adenine (1) in combination with DL-homocysteine (Hcy) on the metabolism of S-adenosylhomocysteine (AdoHcy) and on vaccinia virus replication in murine L929 cells. *FASEB J* **2**: A1752, 1988.