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Intracellular Metabolism of the N7-Substituted Acyclic Nucleoside Analog 2-Amino-7-(1,3-dihydroxy-2-propoxymethyl)purine, a Potent Inhibitor of Herpesvirus Replication

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ABSTRACT

We investigated the intracellular metabolism of S2242 (2-amino-7-(1,3-dihydroxy-2-propoxymethyl)purine), the only known antivirally active acyclic nucleoside analogue with the side chain substituted at the N7 position of the purine ring. Uptake of S2242 by CEM cells increased linearly with increasing extracellular concentrations of the compound and was blocked by inhibitors of nucleoside transport. S2242 was phosphorylated in a time- and concentration-dependent manner to its monophosphates, diphosphates, and triphosphates. Intracellular half-life of the diphosphates and triphosphates in CEM cells was ~3–6 hr. A strong correlation was found between the cytostatic action of the compound and its phosphorylation in different cell lines. In accord with the findings that (1) the cytostatic potential of S2242 is reversed by deoxycytidine (dCyd) and (2) the growth of deoxycytidine kinase-deficient (dCK^-) cells is refractory to the inhibitory effect of S2242, the amount of metabolites formed from S2242 in the dCK^- cell line was approximately one hundredth of that in the wild-type cells. The observation that purified dCK phosphorylates S2242 to its monophosphate further corroborates these results. The activity of S2242 against herpes simplex virus, varicella-zoster virus, and human herpesvirus type 6 was reversed by 50–100-fold on the addition of exogenous dCyd. Compound S2242 was not preferentially phosphorylated in herpes simplex virus 1-, varicella-zoster virus-, or human herpesvirus type 6-infected cells (Vero, human embryonic lung, and HSB-2 cells, respectively), and exogenously added dCyd reduced substantially the formation of S2242 metabolites in these cells. In human cytomegalovirus (HCMV)-infected human embryonic lung cells, a 5–25-fold increase in S2242 metabolite formation was observed compared with the noninfected cells, suggesting that an HCMV-encoded or -induced enzyme causes the specific phosphorylation of S2242. Exogenously added dCyd had little effect on the activity of S2242 against HCMV and on the phosphorylation of the compound in HCMV-infected cells. S2242 was not specifically phosphorylated by the HCMV-encoded UL-97 kinase in cells infected with a vaccinia/UL-97 recombinant. S2242 was found to be a substrate (K_m = 90 μM) for purified human deoxyguanosine kinase; the latter enzyme was stimulated 3–4-fold in HCMV-infected cells.

Recently, we reported on the potent and selective antiherpesvirus activity of S2242 (2-amino-7-[(1,3-dihydroxy-2-propoxymethyl)]purine), the only known antivirally active nucleoside analogue with the side chain substituted at the purine N7 position. Of special interest is the potent activity of the compound against HCMV and TK^- strains of HSV and VZV. In addition, compound S2242 proved to be a highly potent inhibitor of HHV-6 and HHV-8. The activity of the compound against TK^- strains of HSV and VZV is corroborated by the observation that the HSV-1-encoded TK is not responsible for the phosphorylation of S2242 (Jähne *et al.*, 1994; Neyts *et al.*, 1994; Neyts and De Clercq, 1997). The compound has also proved to be effective in several experimental herpesvirus infections in mice. In fact, S2242 seemed to be more efficacious than ACV in the systemic treatment of

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ABBREVIATIONS: HCMV, human cytomegalovirus; HSV, herpes simplex virus; VZV, varicella zoster virus; HHV, human herpes virus; ACV, acyclovir; DHPG, ganciclovir; dCK, deoxycytidine kinase; ara-C, 1-β-D-arabinofuranosylcytosine; MEM, minimum essential medium; HEL, human embryonic lung; FCS, fetal calf serum; TK^-, thymidine kinase-deficient; CC_{50}, 50% cytostatic concentration; HPLC, high performance liquid chromatography; CPE, cytopathic effect; DTT, dithiothreitol; dGK, deoxyguanosine kinase; ara-G, 9-β-D-[^{3}H]arabinofuranosylguanine; dCyd, deoxycytidine; EC_{50}, 50% effective concentration.

HSV-1 infections and topical treatment of intracutaneous HSV-2 infections. It proved to be active against infections with TK$^-$ HSV-1 and double-resistant (foscarnet- and ACV-resistant) strains of HSV-1 and demonstrated superior activity to ganciclovir in the treatment of murine cytomegalovirus infections in either immunocompetent or immunodeficient mice (Neyts *et al.*, 1995b). In view of the unique structure of S2242 and its potency against herpesvirus infections, our aim in the current study was to gain insight into the intracellular metabolism of the compound.

Materials and Methods

Compound. S2242 (Fig. 1) was synthesized according to the method of Jähne *et al.* (1994). ^{14}C-Labeled S2242 was kindly provided by Dr. I. Winkler and Dr. H. Lötzsch (Hoechst, Frankfurt am Main, Germany) (specific radioactivity: batch 1, 3.21 mCi/mmol; batch 2, 44 mCi/mmol; chemical purity, 99.0%). Radiolabeled [^{3}H]ganciclovir (specific activity, 18.6 Ci/mmol) was kinda provided by Dr. H. Maag (Syntex, Palo Alto, CA). Ara-G (6.5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). [^{32}P]ATP (10 mCi/ml) was from DuPont (Bad Homburg, Germany). ACV (Zoviraxâ) was obtained from Glaxo-Wellcome (Aalst, Belgium). DHPG (Cymeveneâ) was obtained from Serva-Syntex (Heidelberg, Germany). The natural nucleosides were purchased from Sigma (Bornem, Belgium).

Cells. CEM/0 cells (a continuous human T cell line) and HSB-2 cells (a continuous human B cell line) were grown in RPMI medium supplemented with 10% calf serum. CEM cells deficient in dCK (CEM dCK) were kindly provided by Dr. J. M. Leeds (Duke University Medical Center, Durham, NC). Murine leukemia L1210/0 cells were grown in MEM supplemented with 10% calf serum. The L1210/ara-C subline has been selected from the parental L1210 cells for its ability to grow in the presence of ara-C (1 μg/ml). This mutant cell line is deficient in dCK (Balzarini and De Clercq, 1983). HEL, VERO, and C127I cells were grown in MEM supplemented with 10% FCS. FM3A cells (subclone F287) were originally established from a spontaneous mammary carcinoma in a C3H/He mouse and were designated FM3A/0.

Viruses. HSV-1 (strain KOS) and HSV-2 (strain G) have been described previously (De Clercq *et al.*, 1980). HCMV (strain Davis) was obtained from American Type Culture Collection (Rockville, MD). HCMV strains 5 and 6 are clinical isolates (kindly provided by Dr. A. Erice, University of Minnesota, Minneapolis, MN). Strain 5 is a wild-type with normal DHPG-phosphorylating capacity, whereas strain 6 results in impaired DHPG phosphorylation in the infected cells. VZV (strains OKA and YS) was obtained from American Type Culture Collection. HHV-6 (strain GS) was kindly provided by Dr. D. Ablashi (National Institutes of Health, Bethesda, MD). The antiviral drug testing assays have been described in detail previously (Neyts *et al.*, 1995a). Recombinant vaccinia virus containing the HCMV UL-97 ORF from a wild-type DHPG-sensitive HCMV strain was used to infect a TK$^-$ human osteosarcoma cell line (143B) as described previously (Metzger *et al.*, 1994). Plaque reduction assays to assess the drug sensitivity of this recombinant vaccinia were carried out as described perviously (Metzger *et al.*, 1994).

Cytostatic assay. HEL cells were seeded at a ratio of 4.5×10^3 cells/well on 96-well microtiter plates in Eagle's MEM containing 20% FCS. Appropriate concentrations of the test compounds were added in medium supplemented with 2% FCS, and the cells were allowed to proliferate for 4 days. Then, the cells were detached by trypsinization and counted with a Coulter counter (Coulter Electronics, Luton, UK). The cytostatic action for Vero cell growth was assessed similarly. The CC_{50} value was estimated from graphic plots. Human (CEM/0 and HSB-2) and murine (L1210) cell lines were seeded in microtiter plates at 5×10^4 cells/well in the absence or presence of compounds. The cells were counted with a Coulter counter after which they were allowed to proliferate for 48 hr.

Uptake studies. CEM/0 cells (5×10^5/ml) were incubated with different concentrations of radiolabeled S2242 for 8 min at 37°. Parallel cultures were incubated with 10 μM dipyridamole from 10 min before the addition of S2242 until the end of the experiment. After the 8-min incubation period, the cultures were washed three times rapidly with cold MEM containing 100 μg/ml unlabeled S2242, after which cell-associated radioactivity was determined.

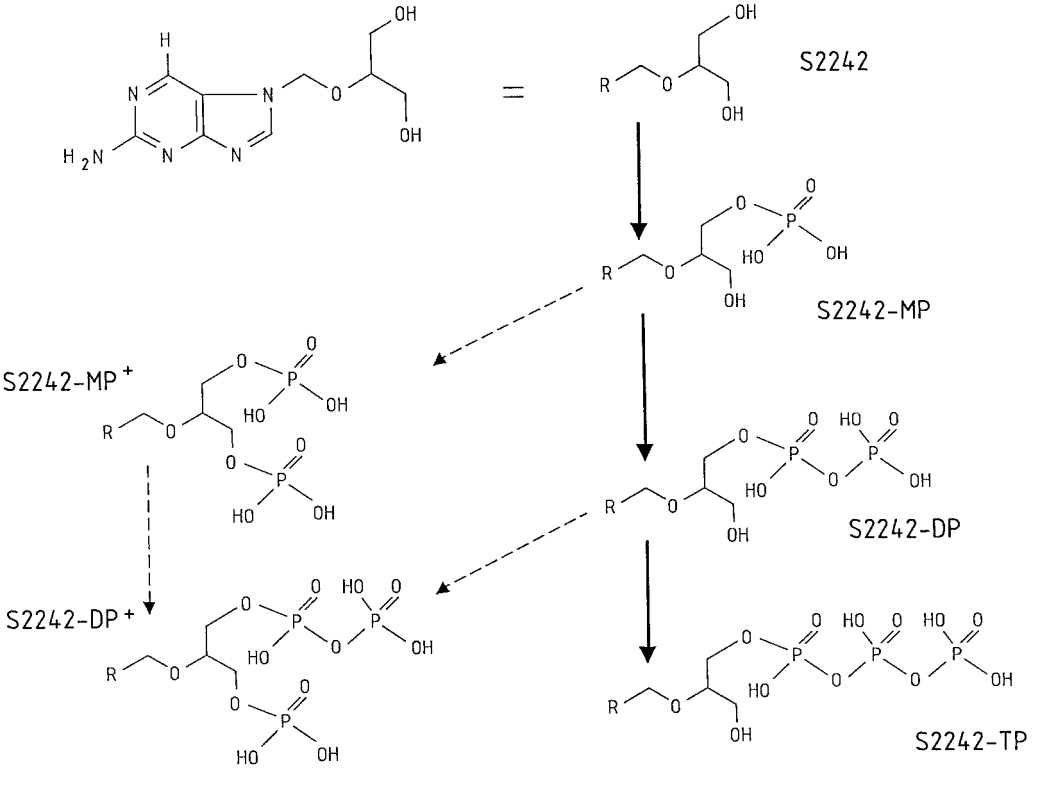

Fig. 1. Formulas of S2242 and presumed metabolites.

Cell metabolism studies. Cultures of CEM/0 or L1210 cells (either wild-type or mutants) at a density of 5×10^5 cells/ml were incubated with the indicated concentration of radiolabeled S2242 for the indicated period of time, after which the cultures were analyzed for metabolite formation. Extracts were prepared at various times after the cultures had been incubated with 50 μM radiolabeled S2242 for 24 hr followed by three washings in drug-free medium (see Table 3).

Monolayers of Vero or HEL cells or cultures of HSB-2 cells were either infected or not infected with HSV-1 (Vero and HEL cells), HCMV (HEL cells), or HHV-6 (HSB-2), after which the cultures were incubated with MEM (2% FCS) containing radiolabeled S2242 (usually 100 μM for 24 hr). In some experiments, unlabeled nucleosides or nucleoside analogues were added at the time of the addition of labeled S2242. Depending on the differential progression of the CPE for the different viruses, the labeled compound was added at the time that the CPE reached ~40% (HSV-1), ~60% (HHV-6), or ~80% (HCMV). At the end of the incubation period, intracellular levels of S2242 and its metabolites were determined by HPLC analysis.

HPLC analysis. Cultures were collected and washed three times with cold MEM containing 100 μg/ml unlabeled S2242. Monolayer cultures were washed rapidly with cold MEM before trypsinization. After centrifugation, the cell pellets were extracted with 70% ice-cold methanol and left on ice for 10 min. After centrifugation at 10,000 rpm, the supernatants were filtered, and quantification of S2242 or DHPG metabolites was accomplished by HPLC analysis using a Partisil-sphere radial compression column (Pharmacia, St. Albans, Herts, UK), as described previously (Balzarini and De Clercq, 1990). Chemically prepared S2242 monophosphate was used as an internal spike. To confirm the identity of the metabolites, "peak shift analysis" experiments were performed. Extracts from CEM/0 cells that had been incubated with 100 μM radiolabeled S2242 were run on the anion-exchange column. The length of the run was extended for these experiments from 50 to 93 min. The different fractions were collected and desalted. Desalting was done by the addition of 0.1 volume of NH_4OH and 3 volumes of methanol. After centrifugation, the resulting supernatant was evaporated to dryness. Two additional rounds of desalting were performed so that the final salt concentration was ≤50 mM (as judged by means of a refractometer). The metabolites were then redissolved in 50 mM Tris buffer, pH 9.8, and incubated overnight with alkaline phosphatase. Samples were then analyzed on a reverse-phase column using the following gradient: 2% acetonitrile (A) in 98% 50 mM NaH_2PO_4/5 mM heptansulfonic acid, pH 3.2 (B), increasing linearly to 50% A and 50% B over a 20-min time interval. Fractions were collected every minute, and radioactivity was determined. Unlabeled and radiolabeled S2242 was used as spike.

dCK assay. Human cytosolic dCK was purified as described previously (Karlsson *et al.*, 1994). The assay mixture contained 50 mM Tris·HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM DTT, 1.0 mg/ml bovine serum albumin, 2.5 mM ATP, 10 mM NaF, different concentrations of [^{14}C]S2242, and cytosolic dCK. After incubation at 37°, the reaction mixture was spotted onto DE81 filter papers (Whatman, Maidstone, UK) and washed twice with 1 mM NH_4COOH, once with water, and twice with ethanol. Filters were air dried, and radioactivity was determined by liquid scintillation.

dGK assay. The phosphorylation of S2242 and DHPG by dGK was measured by the phosphoryl transfer assay as described previously (Eriksson *et al.*, 1991). Recombinant human dGK was expressed and purified as described previously (Johansson and Karlsson, 1996). Different concentrations of S2242 were incubated with dGK in 50 mM Tris, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM ATP, and 0.25 μCi of [^{32}P]ATP. The reaction mixtures were separated on polyethyleneimine cellulose sheets, autoradiographed for 12 hr, and quantified with Image Master system (Pharmacia). The kinetic data were obtained using a hyperbolic regression program. The dGK activity in crude cell extracts was determined using ara-G as substrate based on the radiochemical method described previously (Arner *et al.*, 1992). Extracts from mock- or HCMV-infected cells (80–100% CPE) were prepared as follows. Cell pellets were suspended in 500 μl of extraction buffer (50 mM Tris·HCl, pH 7.6, 2 mM DTT, 5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol, 0.5% Nonidet P40). After freeze-thawing three times, the cell extracts were centrifuged for 20 min at 1300 rpm at 4°. The supernatants were used for the enzyme assay. Protein concentration was determined according to the method of Bradford. dGK activity in crude cell extracts was determined as follows: 20 μg of protein was added to a final reaction mixture of 8 mM unlabeled ara-G, 2 mM ara-G (specific activity, 6.5 Ci/mmol), 50 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, 15 mM NaF, 100 mM KCl, and 0.5 mg/ml bovine serum albumin. The reactions were incubated at 37° and were linear up to 30 min; the reaction mixtures were spotted onto Whatman DE-81 filters. The filters were washed, and radioactivity was determined.

Alkaline phosphatase and phosphodiesterase treatment of cell extracts. CEM/0 cells were incubated with radiolabeled S2242 (100 μM) for 24 hr. Cell extracts were prepared in 70% methanol/H_2O; after centrifugation, the supernatant was evaporated, and the residue was resuspended in 50 mM Tris, pH 9.4, to which ≥1 IU of alkaline phosphatase was added, or in 50 mM Tris·HCl, pH 7.5, and 8 mM $MgCl_2$, to which 0.1 IU snake venom phosphodiesterase was added. After 1 hr at 37°, reactions were stopped by the addition of 300 μl of ice-cold 70% methanol, and metabolites were quantified by HPLC as described above. Alternatively, samples were incubated overnight at 37° with the enzyme.

Phosphorylation of S2242 and DHPG in recombinant vaccinia/UL-97-infected osteosarcoma cells. We used 143B TK$^-$ cells for infection with rVV. Construction of rVV T1 containing the HCMV UL-97 ORF has been described previously (Metzger *et al.*, 1994). Infected or noninfected cells were exposed to radiolabeled DHPG or S2242 for 6 hr, after which metabolites in the cell extracts were analyzed by HPLC.

Results

Uptake of [^{14}C]S2242. The uptake of S2242 in CEM/0 cells proved to be proportional with the extracellular concentration of the compound and was blocked completely by di-

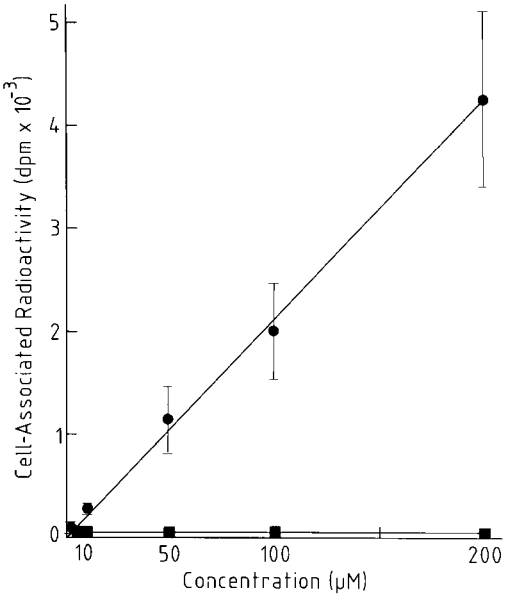

Fig. 2. Uptake of S2242 in CEM cells in the presence (■) or absence (●) of 10 μM dipyridamole. Data are mean values for two separate experiments.

TABLE 1

Concentration- and time-dependent metabolism of S2242 in CEM/0 cells

Cells were incubated for 24 hr at 37° with the indicated concentrations of radiolabeled compound or were incubated for the indicated times with 25 μM radiolabeled S2242 (specific activity, 3.1 mCi/mmol). Data are expressed as pmol/10^6 cells and are the mean for two separate experiments.

	S2242	S2242-MP	SS2242-MP$^+$	S2242-DP	S2242-DP$^+$	S2242-TP
Concentration						
5 μM	10 ± 7	25 ± 1		18 ± 0.7		17 ± 0.1
20 μM	24 ± 8	72 ± 6		52 ± 3	6.0 ± 4.0	50 ± 3
50 μM	46 ± 7	114 ± 27	7.6 ± 1.2	91 ± 20	7.6 ± 0.2	76 ± 27
100 μM	92 ± 14	194 ± 31	1.2 ± 3.3	150 ± 28	12 ± 12	160 ± 18
Time of incubation						
3 hr	57 ± 24	19 ± 19		19 ± 2		9.6 ± 1.7
6 hr	20 ± 4	50 ± 12		22 ± 3		17 ± 0.7
12 hr	55 ± 20	80 ± 0.8		61 ± 4		35 ± 3
24 hr	78 ± 27	130 ± 2	8.0 ± 0.0	95 ± 2		68 ± 0.5

pyridamole (an inhibitor of nucleoside transport) at 10 μM (Fig. 2).

Phosphorylation of [^{14}C]S2242 in CEM/0 cells. Formation in CEM/0 cells of the monophosphate (S2242-MP) and the presumed diphosphate (S2242-DP) and triphosphate (S2242-TP) derivatives of S2242 increased with higher input concentrations (Table 1, Fig. 1). The metabolite eluting at 10 min was identified as S2242-MP because it coeluted with the chemically prepared S2242-MP. All three major metabolites (eluting at 10, 17, and 25 min, respectively) were formed at comparable levels. After a 24-hr incubation period with a 100 μM concentration of compound, the intracellular concentration of the metabolites in CEM cells was 150–190 pmol/10^6 cells. In addition to the major metabolites, two additional peaks were detected eluting shortly after the monophosphate (12 min) and shortly after the presumed diphosphate (19 min) derivative (Table 1). These additional metabolites were designated S2242-MP$^+$ and S2242-DP$^+$, respectively. Further efforts were undertaken to characterize the metabolites formed. The peak eluting at 10 min was identified as the S2242 monophosphate because it coeluted with the chemically synthesized S2242-MP. However, synthesis of the diphosphate and triphosphate metabolites was unsuccessful after repeated trials. In peak shift analysis experiments, all the metabolites that we detected were found to yield the parent compound S2242, indicating that these metabolites consist of phosphorylated forms of S2242. Finally, we also attempted to characterize by mass spectrometry the metabolites for which spiking was not possible. Extracts were prepared of 10^9 CEM cells, which had been incubated with 100 μM of unlabeled S2242 for 24 hr. After repeated extractions, the extracts were run on the anion-exchange HPLC column. Fractions containing the different metabolites were collected, desalted, and analyzed by mass spectrometry. However, sufficient high levels of metabolites could not be collected to allow identification of the metabolites formed against the background of natural nucleotides.

A time-dependent increase in metabolite formation was observed; the formation of metabolites increased up to 24 hr after the addition of the compounds (Table 1). When the cytostatic action of S2242 in different cell lines was compared with the formation of metabolites, a strong correlation was found between the phosphorylation of the compound and its cytostatic potential (r = 0.94, 0.95, and 0.98 for the inverse correlation between the CC_{50} values and the monophosphate, diphosphate, and triphosphate levels in the different cell lines) (Table 2).

Decay over time of S2242 metabolites in CEM/0 cells. Because we found previously that S2242 is endowed with a long-lasting antiviral response, we studied the decay of the S2242 metabolites in CEM cells after the extracellular compound had been removed following a 24-hr incubation period. An initial half-life of 3.6 hr could be calculated for the presumed triphosphate metabolite, 2.8 hr for the diphosphate derivative, and 2.4 hr for the monophosphate derivative. Levels of the diphosphate and triphosphate metabolites remained as high as 9.4% and 7.7%, respectively, of the initial levels 24 hr after removal of the extracellular compound (Table 3).

Alkaline phosphatase and phosphodiesterase treatment. When extracts of CEM cells that had been incubated with S2242 for a 24-hr period were treated for 1 hr with alkaline phosphatase, virtually complete conversion of the metabolites to the parent compound S2242 was observed, except for the presumed S2242-DP derivative that was catabolyzed for ~91% (Table 4). However, after prolonged incubation with alkaline phosphatase (i.e., overnight), all S2242-DP was degraded and converted to the parent com-

TABLE 2

Relation between the cytostatic action of S2242 and metabolite formation in several cell lines

The inverse correlation between the CC_{50} values and S2242-MP, S2242-DP, and S2242-TP levels was .94, .95, and .98, respectively (for this calculation, the CC_{50} value for FM3A/0 cells was considered to be 100 μg/ml).

Cell	CC_{50}	S2242-MP[a]	S2242-DP[a]	S2242-TP[a]
	μg/ml		*pmol/10^6 cells*	
CEM/0	1.0 ± 0.3	194 ± 31	150 ± 28	160 ± 18
Vero	12 ± 3.3	18 ± 8.0	29 ± 5.8	19 ± 3.8
HEL	32 ± 2	9.5 ± 4.2	13 ± 5.7	7.9 ± 3.7
L1210/0	42 ± 9	8.9 ± 3.6	13 ± 9.1	7.9 ± 1.0
FM3A/0	>100	0.95 ± 0.05	1.2 ± 0.1	1.9 ± 0.5

[a] Cells were incubated with 100 μM [^{14}C]S2242 (44 Ci/mmol) for 24 hr at 37°.

TABLE 3

Decay over time of S2242 metabolites in CEM/0 cells after an initial 24-hr incubation period with the compound

Cells were incubated for 24 hr at 37° with 50 μM [^{14}C]S2242 (specific activity, 3.1 mCi/mmol). Data are expressed as pmol/10^6 cells and are mean values for two separate experiments. N.D., not detectable.

Hours after washing	S2242	S2242-MP	S2242-DP	S2242-TP
0	102 ± 40	201 ± 5	195 ± 17	90 ± 4
3	13 ± 3	77 ± 5	91 ± 2	60 ± 10
12	N.D.	19 ± 0.4	46 ± 3	26 ± 7
24	N.D.	N.D.	18 ± 0.2	7 ± 1

TABLE 4

Effect of treatment with phosphodiesterase or alkaline phosphatase of extracts of S2242-incubated CEM cells

Cells were incubated at 37° for a period of 24 hr with 50 μM [^{14}C]S2242 (specific activity, 44 mCi/mmol). Cell extracts were either left untreated or were treated with alkaline phosphatase or phosphodiesterase. Data are expressed as pmol/10^6 cells and are the mean for two to four separate experiments.

	S2242	S2242-MP	S2242-MP$^+$	S2242-DP	S2242-DP$^+$	S2242-TP
Control	98 ± 9.9	134 ± 5.2	12.3 ± 1.5	104 ± 14	7.9 ± 3.3	31.4 ± 14.4
Alkaline phosphatase	351 ± 3.5	0.5 ± 0.7	4.3	11.6 ± 4.7	0.08 ± 0.12	0.03 ± 0.05
Phosphodiesterase	101 ± 5.6	233 ± 79	12.5 ± 2.1	63.7 ± 17.4	7.9 ± 2.9	17.8 ± 10.1

TABLE 5

Effect of natural nucleosides on the antiviral effects of S2242, ACV, and DHPG

All nucleosides were added at a final concentration of 100 μg/ml, except for guanosine, which was added at 25 μg/ml in the HCMV and HSV assays. Data are mean values for at least two or three separate experiments.

Nucleoside added	EC_{50}								
	HCMV (Davis)		HSV-1 (KOS)			VZV[a]			HHV-6 (GS)
	DHPG	S2242	DHPG	ACV	S2242	DHPG	ACV	S2242	S2242
	$\mu g/ml$								
None	0.7	**0.02**	0.008	0.5	**0.2**	0.5	0.1	**0.03**	**0.005**[d]
2'-Deoxythymidine	2.5	0.04	12	>200	0.9	17.5	7.6	0.8	
Cytidine			0.008	0.2	0.9	2.2	1.0	0.6	
2'-Deoxycytidine	1.5	**0.1**	0.24	4.5	**18**	12	11	**1.8**	**0.3**[d]
Adenosine	3.5	0.03	0.008	2.5	0.2	0.94	1.9	0.04	
2'-Deoxyadenosine	0.5	0.04	0.008	0.2	0.6	3.0	1.1	0.09	
Guanosine	3.0	0.03	0.02	0.9	0.2	0.82	0.25	0.02	
2'-Deoxyguanosine	5.0	0.01	0.11	15	0.55	11.9	1.2	0.04	

[a] Mean values for strains Oka and YS.

[b] Similar values were obtained when the cultures were analyzed for HHV-6 antigen expression by FACS analysis (i.e., EC_{50} value for the control, 0.003 μg/ml, compared to 0.25 μg/ml in the presence of dCyd).

pound (as revealed by peak shift analysis). Treatment of the cell extracts with snake venom phosphodiesterase had a much less pronounced effect on the conversion of the different metabolites to S2242-MP. Under the conditions used for the S2242 experiments, phosphodiesterase was found to work appropriately because the natural nucleotides ATP and ADP were completely converted to AMP by the enzyme (data not shown).

Effect of natural nucleosides on the cytostatic and antiviral activities of compound S2242. To obtain initial information on the enzyme responsible for the phosphorylation of S2242 to its monophosphate form, the antiviral activity of S2242 was studied in combination with several natural nucleosides (Table 5). The anti-HSV-1, anti-VZV, and anti-HHV-6 activities of the compound was reversed 20- to 100-fold by exogenously added dCyd. In contrast to its effect on ACV and DHPG, dThd had little effect on the antiviral activity of S2242. None of the other nucleosides tested had any important effect on the antiviral activity of S2242. In contrast to the anti-HSV-1, anti-VZV, and anti-HHV-6 activities, the anti-HCMV activity of S2242 was only reversed 5-fold by exogenously added dCyd (100 μg/ml). None of the other nucleosides listed in Table 5 nor uridine, inosine, xanthosine, or hypoxanthine (data not shown) had any effect on the anti-HCMV activity of S2242. Even when fresh dCyd (100 μg/ml) was added daily to the S2242-treated HCMV-infected cultures, the EC_{50} value of S2242 for inhibition of the HCMV-induced CPE was not increased by >5-fold (data not shown).

We next studied whether the cytostatic effect of S2242 on CEM/0, L1210/0, and HSB-2 cells could be reversed by the addition of exogenous dCyd (Table 6). In accord with the antiviral data, dCyd (at 200 μg/ml) significantly reversed the cytostatic effect of the compound in all three cell lines. In addition, cytidine, although less efficacious, resulted in a 15–19-fold increase in CC_{50} values. None of the other nucleosides tested (including dUrd; see also Table 5) reversed markedly the cytostatic action of S2242 (data not shown). These data were further corroborated by the observation that the cytostatic effect of S2242 on the growth of CEM and L1210 cells deficient in dCK activity was much less pronounced than that for the wild-type cells. CEM cells deficient in thymidine kinase activity, which were included as a negative control, proved equally susceptible to S2242 as the wild-type cells (Table 6).

TABLE 6

Cytostatic action of S2242 in CEM, L1210, and HSB-2 cells in combination with natural nucleosides and in dCyd kinase-deficient CEM and L1210 cell lines

Data are mean for two to six separate determinations.

	CC_{50}			
	No addition	+200 μg/ml dCyd	+200 μg/ml Cyd	+200 μg/ml dUrd
	$\mu g/ml$			
CEM/0	1.0 ± 0.3	43 ± 17	19 ± 5	2.0 ± 0.05
CEM dCK$^-$	52 ± 3			
CEM dTK$^-$	2.3 ± 1.5			
L1210/0	42 ± 9.2	>100	79 ± 16	
L1210 dCK$^-$	>100			
HSB-2	0.59 ± 0.03	16 ± 4	9.3 ± 1.7	1.2 ± 0.7

Phosphorylation of S2242 by dCK. The phosphorylation of S2242 was studied in CEM cells that were incubated with 100 μg/ml exogenously added dCyd (Table 7). In accord with the observation that dCyd reduces the cytostatic action of S2242, almost no S2242 metabolites were detected under these experimental conditions. In addition, in the CEM cell line deficient for dCK activity, the phosphorylation of S2242 was ~1% that of the control wild-type CEM cultures. S2242

TABLE 7

Metabolism of S2242 in wild-type and dCyd kinase-deficient CEM cells

Data are expressed as pmol/10^6 cells and are mean values for two or three separate experiments (one experiment for the CEM/0 + dCyd). Cells were incubated for 24 hr at 37° with 100 μM [^{14}C]S2242 (specific activity, 44 mCi/mmol) at 100 μM. When combined with dCyd, the latter was used at a final concentration of 100 μg/ml. N.D., not detectable.

	S2242	S2242-MP	S2242-MP$^+$	S2242-DP	S2242-DP$^+$	S2242-TP
CEM/0	250 ± 44	280 ± 16	11 ± 0.8	242 ± 14	15 ± 0.8	203 ± 27
CEM/0 + dCyd	414	1.8	N.D.	1.0	N.D.	3.4
CEM/dCK$^-$	204 ± 12	2.4 ± 2.0	0.3 ± 0.4	2.4 ± 1.6	N.D.	3.4 ± 2.0

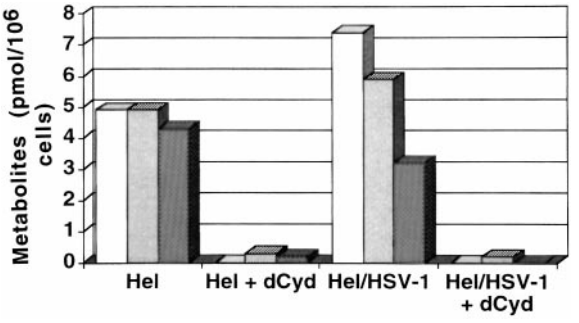

Fig. 3. Phosphorylation of S2242 in HSV-1-infected HEL cells (*Hel*) in the absence or presence of dCyd. HSV-1 (strain KOS) or uninfected HEL cells were incubated with radiolabeled S2242 (100 μM) when 40–50% CPE was noted in the infected cultures. To the medium, no or 100 μg/ml dCyd was added. Metabolites were analyzed 24 hr later. Data are from a representative experiment. □, Monophosphate; ▨, diphosphate; ▩, triphosphate.

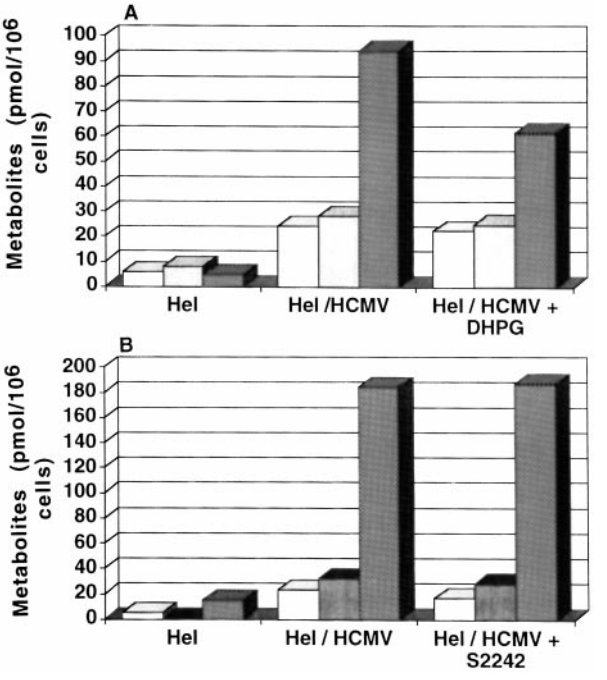

Fig. 4. Effect of excess DHPG (800 μM) on the phosphorylation of S2242 in HCMV-infected HEL cell cultures treated with 50 μM concentration of radiolabeled compound (A) and effect of excess S2242 (800 μM) on the generation of DHPG metabolites (B) in HCMV-infected HEL cells in cultures incubated with 4 μM DHPG. □, Monophosphate; ▨, diphosphate; ▩, triphosphate.

was phosphorylated by purified cytosolic dCK according to Michaelis-Menten kinetics. A K_m value of 4.25 ± 2.72 mM was calculated (compared with 10–20 μM for ara-C), indicating that S2242 is a weak substrate for dCK (data not shown).

Metabolism of S2242 in HSV-1- and HHV-6-infected cells. HEL cells were either mock-infected or infected with HSV-1. When CPE reached ~50%, the cultures were incubated for an additional 24 hr with 100 μM radiolabeled S2242 in the presence or absence of 100 μg/ml dCyd (Fig. 3). Compound S2242 was phosphorylated to a comparable extent in HSV-1-infected and noninfected HEL cells. When the cultures were incubated in the presence of exogenous dCyd, almost no metabolites were detected in either infected or noninfected cultures. The fact that dCyd suppresses S2242 phosphorylation in HSV-1-infected cells corroborates the results presented in Table 5, in which dCyd was found to reverse the anti-HSV-1 activity of S2242. Also in HSV-1-infected VERO cell cultures, S2242 was not preferentially phosphorylated compared with the control cells, and dCyd afforded a drastic reduction in the formation of S2242 metabolites (data not shown for VERO cells). Akin to the situation in HSV-1-infected cells, S2242 was not preferentially phosphorylated in HHV-6-infected cells. In fact, even lower levels of metabolites were detected in the infected cells compared with the noninfected cells. In addition, exogenously added dCyd significantly reduced the formation of S2242 metabolites in both infected and noninfected cells (10, 8, and 12 pmol/10^6 cells for S2242-MP, S2242-DP, and S2242-TP, and 0, 0, and 2 pmol/10^6 cells for S2242-MP, S2242-DP, and S2242-TP in HHV-6-infected HSB-2 cells that had been incubated or not incubated, respectively, with 100 μg/ml dCyd) (data not shown).

Metabolism of S2242 in HCMV-infected HEL cells. In HEL cells that had been infected with a laboratory strain of HCMV (Davis), 5–20-fold increased levels of the presumed S2242-TP metabolites were detected (Table 8, Fig. 4). In

TABLE 8

Metabolism of S2242 in HCMV (strain Davis)-infected and noninfected HEL cells in the absence or presence of dCyd

Data are expressed as pmol/10^6 cells. Values are means for two or three separate experiments. Infection with HCMV (strain Davis) was performed to obtain ~80–90% CPE at day 4 after infection. Infected and noninfected cells were then further incubated for 24 hr at 37° with 100 μM [^{14}C]S2242 (specific activity, 44 mCi/mmol) in the absence or presence of 100 μg/ml dCyd.

	S2242	S2242-MP	S2242-MP$^+$	S2242-DP	S2242-DP$^+$	S2242-TP
HEL	142 ± 7.7	9.5 ± 4.2	3.2 ± 2.1	13 ± 5.7	1.3 ± 0.7	7.9 ± 3.7
HEL + dCyd	110 ± 7.8	N.D.	0.19 ± 0.19	0.38 ± 0.38	0.4 ± 0.4	1.0 ± 0.1
HEL/HCMV	152 ± 43	14 ± 3.0	5.3 ± 0.9	17 ± 3.5	1.1 ± 0.1	43 ± 4.9
HEL/HCMV + dCyd	182 ± 0.7	3.5 ± 0.1	1.1 ± 0.2	4.0 ± 0.03	N.D.	34 ± 4.9

N.D., not detectable.

contrast to the situation in HSV-1- and HHV-6-infected cells, exogenously added dCyd had little effect on the formation of the presumed triphosphate metabolite (although a 3–5-fold reduction in S2242-MP and S2242-DP formation was noted), which is in agreement with the fact that exogenously added dCyd has only a marginal effect on the anti-HCMV activity of S2242 (Table 5). Exogenously added DHPG (800 μM) only slightly reduced the formation of S2242-TP in HCMV-infected cultures that had been incubated with 50 μM radiolabeled S2242 for 24 hr (Fig. 4A). Vice versa, an excess of S2242 (800 μM) had no influence on the specific phosphorylation of DHPG (4 μM for 24 hr) in HCMV-infected cells (Fig. 4B).

The phosphorylation of S2242 and DHPG was evaluated in TK^- osteosarcoma cell lines infected with a recombinant vaccinia virus carrying (and expressing) the UL-97 gene. DHPG (at 20 μM) was phosphorylated 13-fold more efficiently in cells infected with the recombinant virus than in cells infected with wild-type virus (5.7 $\pm$ 0.5 versus 75.7 $\pm$ 4.7 pmol/10^6 cells for the DHPG metabolites in noninfected and infected cells, respectively), whereas the phosphorylation of S2242 was not stimulated (S2242 metabolites: 9.0 $\pm$ 0.7 versus 9.5 $\pm$ 1.5 pmol/10^6 cells in noninfected and infected cells, respectively). In addition, unlabeled S2242 at 200 μM had no effect on the increased phosphorylation of DHPG in osteosarcoma cells infected with the UL-97 recombinant virus (data not shown).

We next evaluated the phosphorylation of S2242 in HEL cells infected with a clinical isolate of HCMV. Strain 6 proved to be markedly deficient in DHPG phosphorylation (we found 20-fold lower levels DHPG-TP in cells infected with the mutant virus than in cells infected with the wild-type virus). Levels of S2242-TP were 4.5- and 13-fold higher in HEL cells infected with the mutant and wild-type virus, respectively, indicating that HCMV strains deficient in DHPG phosphorylation are still able to stimulate the phosphorylation of S2242 in HEL cells. The 3-fold difference in phosphorylation efficiency between cells infected with strains 5 and 6 may result from the slower progression of CPE as observed in cells infected with strain 6.

Although exogenously added guanosine or deoxyguanosine did not reverse the anti-HCMV activity of S2242, this does not exclude a possible role of dGK in the phosphorylation of S2242. Indeed, purine nucleoside phosphorylase rapidly catalyzes the conversion of these nucleosides to the base and sugar-1-phosphate followed by conversion of the base by hypoxanthine guanine phosphoribosyl transferase to GMP. Surprisingly, we found that S2242, but not DHPG, is a substrate for purified recombinant human dGK. The K_m value for S2242 phosphorylation by dGK was 90 μM (the V_{max} value could not be determined by the method used). We therefore compared the levels of dGK activity in HCMV-infected cells with those of mock-infected cells. A 3.6-fold increase in dGK activity was observed in the HCMV-infected cells; ara-G was converted to its monophosphate form at 0.46 and 1.68 pmol/10^6 cells/min in mock- and HCMV-infected HEL cells, respectively.

Discussion

We studied the cellular uptake and metabolism of the only known antivirally active nucleoside with the side chain substituted at the purine N7 position. The cellular uptake of S2242 was proportional to the concentration of the drug in the medium and was blocked by dipyridamole, indicating that the interiorization of the compound occurs via the purine nucleoside carrier. This observation points to the fact that this aberrant molecule may act as a classic nucleoside analogue. The uptake of some other antivirally active nucleoside analogues (e.g., AZT) has been reported not to be influenced by inhibitors of nucleoside transport (Zimmerman *et al.*, 1987) or, as in the case of acyclic nucleoside phosphonates such as PMEA, may occur via endocytosis (Palú *et al.*, 1991).

S2242 shows a straightforward phosphorylation pattern in different cell lines. Phosphorylation increases with time (up to 24 hr) and drug concentration in the external medium. Evidence that the phosphorylated metabolites of S2242 are responsible for the biological activity stems from the observation that there exists a strong correlation between the formation of metabolites and the cytostatic potential in the different cell lines. Significant levels of the presumed diphosphate and triphosphate metabolites were detected up to 24 hr after removal of extracellular compound. This observation may explain the sustained *in vitro* antiviral effect of S2242 after removal of compound (Neyts *et al.*, 1994).

Three major S2242 metabolites were formed. The first peak was designated S2242-MP and coeluted with chemically synthesized S2242-MP. Two minor peaks were detected, one eluting shortly after the monophosphate and one after the presumed diphosphate metabolite. To identify further the nature of the metabolites, a peak shift analysis experiment was performed. All metabolites yielded the parent compound S2242, indicating that the metabolites represent phosphorylated form(s) of S2242. Because S2242 contains two free hydroxyl groups on its open "sugar" chain, phosphorylation may possibly occur at both positions. The major diphosphate and triphosphate metabolite derivatives may be formed on further phosphorylation of one of the two possible monophosphate forms (Fig. 1). An explanation for the formation of the minor metabolites may be that the peak eluting between the monophosphate and diphosphate represents a diphosphate derivative of S2242 consisting of S2242 with a phosphate at each free hydroxyl position. The peak eluting between the presumed S2242 diphosphate and triphosphate may represent a triphosphate of S2242, in which a diphosphate is located at one hydroxyl and a phosphate is located at the other hydroxyl position. The absence of an effect of snake venom phosphodiesterase on the S2242-MP^+ peak is in agreement with the hypothesis that this metabolite would represent a diphosphate, with one phosphate at each of the hydroxyl positions of the open "sugar" part of S2242. The influence of phosphodiesterase on the S2242-DP^+ metabolite also may be expected to be inefficient because only one phosphate will be removed from such type of molecule.

The observations that (1) dCyd reverses the anti-HSV-1, anti-VZV, and anti-HHV-6 activities of S2242, (2) dCyd reverses the cytostatic activity of S2242, (3) S2242 is much less cytostatic to CEM cells deficient in dCK activity than to the wild-type cells, and (4) the formation of S2242 metabolites in CEM cells deficient in dCK activity is ~1% of that in the wild-type cells indicate that dCK is responsible for the phosphorylation of S2242. These findings were corroborated by the observation that highly purified cytosolic dCK phosphorylates S2242, although the compound seems to be a weak substrate for the enzyme [K_m = 4.2 $\pm$ 2.7 mM, which is

~10–20-fold higher than the value reported for the dideoxynucleoside analogues di-dCyd and 2′,3′-dideoxyadenosine (Johnson *et al.*, 1988)]. Further evidence that dCK is the principal S2242 phosphorylating enzyme stems from the observation that S2242 has relatively high cytocidal potential against human lymphoid cells [e.g., CEM, MT-4, and HSB-2 cells (Neyts *et al.*, 1994)], which may be related to the fact that lymphoid cells contain relatively high levels of dCK activity as compared with other tissues. In fact, in normal human tissues the highest dCK activity is present in lymphoid organs such as spleen and bone marrow (Ho, 1973).

dCK (NTP: deoxycytidine-5′-phosphotransferase; EC 2:7.1.74) is a pyrimidine salvage enzyme that catalyzes the phosphorylation of 2′-dCyd to 2′-dCyd monophosphate. The enzyme is known as a multisubstrate enzyme, with dCyd as the preferred substrate; it also phosphorylates deoxyadenosine and deoxyguanosine. It has been suggested that dCK may exist in two different conformational states: one form responsible for the phosphorylation of cytosine nucleosides and another form able to phosphorylate purine nucleosides (Kierdaszuk *et al.*, 1992; Kierdaszuk and Eriksson, 1990). dCK can also use various (deoxy)nucleotides as phosphate donor, with ATP and UTP being the preferred ones (Shewach *et al.*, 1992). dCK can activate, in addition to natural nucleosides, several antiviral and cytotoxic nucleoside analogues. Antitumoral agents phosphorylated by dCK include ara-C (cytarabine), gemcitabine (2′, 2′-difluorodeoxycytidine), cladibine (2-chloro-2′-deoxyadenosine), and fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine). Antivirals activated by dCK include zalcitabine (2′,3′-dideoxycytidine), 3TC [(−)-2′,3′-dideoxy-3′-thiacytidine], 2′,3′-dideoxy-5-fluoro-3′-thiacytidine, and 2′,3′-dideoxyadenosine (Balzarini, 1994; Balzarini *et al.*, 1987; Balzarini and De Clercq, 1994; Ruiz van Haperen and Peters, 1994; Starnes and Cheng, 1987).

In cultures infected with either HSV-1 or HHV-6, no virus-specific phosphorylation of S2242 was observed compared with noninfected cultures. Also, in HSV-1-, VZV-, and HHV-6-infected cell cultures, dCK seems to be responsible for the initial phosphorylation of S2242. This can be concluded from the fact that exogenously added dCyd (1) reverses the anti-HSV-1, anti-VZV, and anti-HHV-6 activities of the compound and (2) abolishes the phosphorylation of S2242 in the infected cells.

In contrast to the situation of HSV-1-, VZV-, and HHV-6-infected cells, S2242 is specifically phosphorylated in HCMV-infected cells. We demonstrated that this also is the case for clinical strains of HCMV, in which, in fact, an even higher intracellular metabolism was observed than with the laboratory strains (Neyts *et al.*, 1995a). Interestingly, this stimulated phosphorylation was not reversed on the addition of exogenously added dCyd. This also was the case in cells infected with murine cytomegalovirus and rat cytomegalovirus (data not shown). In accord with these findings, exogenously added dCyd did not reverse the anti-HCMV activity of the compound. Although it has been reported that dCK activity is stimulated in HCMV-infected cells (Biron *et al.*, 1986), it is unlikely that stimulation of the activity of this enzyme in the HCMV-infected cells is responsible for the increased phosphorylation. If this were true, excess exogenously added dCyd would still reduce the phosphorylation of S2242.

Thus, in the HCMV-infected cell, a virus-stimulated host cell enzyme other than dCK or a virus-encoded enzyme must be responsible for the specific phosphorylation of S2242. It has been well documented that the HCMV UL-97-encoded phosphotransferase is responsible for the specific phosphorylation of DHPG in HCMV-infected cells (Littler *et al.*, 1992; Sullivan *et al.*, 1992). However, we did not observe an inhibitory effect of excess DHPG on the phosphorylation of S2242. Vice versa, excess S2242 had no effect on the phosphorylation of DHPG in HCMV-infected cells. Furthermore, S2242 is as active against HCMV resistant to DHPG (caused by mutations in UL-97) as against wild-type virus (Andrei G, Snoeck R, and DeClercq E, unpublished observations), which again argues against a role of the HCMV UL-97-encoded gene product in the S2242 phosphorylation. Additional evidence that UL-97 is not involved in the phosphorylation of S2242 stems from the observation that the compound is not specifically phosphorylated in cells infected with a recombinant vaccinia virus carrying the UL-97 ORF and expressing high levels of the HCMV-encoded phosphotransferase.

We then found that S2242, but not DHPG, is a relatively good substrate for recombinant human dGK (K_m = 90 μM). Moreover, in crude extracts of HCMV-infected cells, a 3.6-fold increase in dGK activity was observed that may, at least in part, explain the increased intracellular metabolism of S2242 in HCMV-infected cells. Although exogenously added (deoxy)guanosine did not reverse the anti-HCMV activity of S2242, the competition of deoxyguanosine or guanosine in the possible phosphorylation of S2242 by dGK (and thus also the effect of these nucleosides on the anti-HCMV activity of S2242) may be expected to be minimal. Indeed, (deoxy)guanosine is rapidly catabolyzed to the base and sugar-1-phosphate by purine nucleoside phosphorylase, after which guanine can be converted directly by hypoxanthine guanine phosphoribosyl transferase to GMP.

To unravel whether enzymes other than dGK may be able to specifically phosphorylate S2242 in HCMV-infected cells, we are attempting to grow S2242-resistant HCMV. The characterization of S2242-phosphorylating enzyme or enzymes in the HCMV-infected cell is important for our understanding of the intracellular activation of antivirally active nucleosides.

In conclusion, S2242 is transported into the cells by a purine nucleoside carrier and then phosphorylated further by cytoplasmic dCK and mitochondrial dGK. The compound is not preferentially phosphorylated in HSV-1-, VZV-, and HHV-6-infected cells but is specifically metabolized in HCMV-infected cells. Neither dCK nor the HCMV-encoded UL-97 kinase is responsible for this phosphorylation. Purified dGK is able to phosphorylate S2242. The activity of this enzyme is increased by 3–4-fold in HCMV-infected cells. This may, at least in part, explain the specific phosphorylation of S2242 in the HCMV-infected cells. Further studies are required to determine whether enzymes other than dGK also may play a role in the specific activation of this compound in HCMV-infected cells.

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References

Arner ESJ, Spasokoukotskaja T, and Eriksson S (1992) Selective assays for thymidine kinase 1 and 2 and deoxycytidine kinase and their activities in extracts from human cells and tissues. *Biochem Biophys Res Commun* **188:**712–718.

Balzarini J (1994) Metabolism and mechanism of antiretroviral action of purine and pyrimidine derivatives. *Pharm World Sci* **16:**113–126.

Balzarini J, Cooney DA, Dalal M, Kang G-J, Cupp JE, De Clercq E, Broder S, and Johns DG (1987) 2′,3′-Dideoxycytidine: regulation of its metabolism and antiretroviral potency by natural pyrimidine nucleosides and by inhibitors of pyrimidine nucleotide synthesis. *Mol Pharmacol* **32:**798–806.

Balzarini J and De Clercq E (1983) Role of deoxycytidine kinase in the inhibitory activity of 5-substituted 2′-deoxycytidines and cytosine arabinosides on tumor cell growth. *Mol Pharmacol* **23:**175–181.

Balzarini J and De Clercq E (1990) 9-β-Arabinofuranosyladenine 5′-monophosphate (araAMP) is converted directly to its antivirally active 5′-triphosphate form by 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase. *Biochem Biophys Res Commun* **173:**781–787.

Balzarini J and De Clercq E (1994) Biochemical pharmacology of nucleoside analogues active against HIV, in *Textbook of AIDS Medicine* (Broder S, Merigan TC, and Bolognesi DP, eds) pp 751–772, Williams & Wilkins, Baltimore.

Biron KK, Fyfe JA, Stanat SC, Leslie LK, Sorrell JB, Lambe CU, and Coen DM (1986) A human cytomegalovirus mutant resistant to the nucleoside analog 9-([2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine (BW B759U) induces reduced levels of BW B759U triphosphate. *Proc Natl Acad Sci USA* **83:**8769–8773.

DeClercq E, Descamps J, Verhelst G, Walker RT, Jones AS, Torrence PF, Shugar D (1980) Comparative efficacy of antiherpes drugs against different strains of herpes simplex virus. *J Infect Dis* **141:**563–574.

Eriksson S, Kierdaszuk B, Munch-Petersen B, Öberg B, and Johansson N (1991) Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. *Biochem Biophys Res Commun* **176:**586–592.

Ho DH (1973) Distribution of kinase and deaminase of 1-β-D-arabinofuranosylcytosine in tissue of man and mouse. *Cancer Res* **33:**2816–2820.

Jähne S, Kroha H, Müller A, Helsberg M, Winkler I, Gross G, and Scholl T (1994) Regioselective synthesis and antiviral activity of purine nucleoside analogues with acyclic substituents at N7. *Angew Chem* **106:**603–605 (international edition in English **33:**562–563).

Johansson M and Karlsson A (1996) Cloning and expression of human deoxyguanosine kinase cDNA. *Proc Natl Acad Sci USA* **93:**7258–7262.

Johnson MA, Ahluwalia G, Connelly MC, Cooney DA, Broder S, Johns DG, and Fridland A (1988) Metabolic pathways for the activation of the antiretroviral agent 2′,3′-dideoxyadenosine in human lymphoid cells. *J Biol Chem* **263:**15354–15357.

Karlsson A, Johansson M, and Eriksson S (1994) Cloning and expression of mouse deoxycytidine kinase. *J Biol Chem* **269:**24374–24378.

Kierdaszuk B, Bohman C, Ullman B, and Eriksson S (1992) Substrate specificity of human deoxycytidine kinase toward antiviral 2′,3′-dideoxynucleoside analogs. *Biochem Pharmacol* **43:**197–206.

Kierdaszuk B and Eriksson S (1990) Selective inactivation of the deoxyadenosine phosphorylating activity of pure human deoxycytidine kinase: stabilisation of different forms of the enzyme by substrates and biological detergents. *Biochemistry* **29:**4109–4114.

Littler E, Stuart AD, and Chee MS (1992) Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. *Nature (Lond)* **358:**160–162.

Metzger C, Michel D, Schneider K, Luske A, Schlicht HJ, and Mertens T (1994) Human cytomegalovirus UL97 kinase confers susceptibility to recombinant vaccinia virus. *J Virol* **68:**8423–8427.

Neyts J, Andrei G, Snoeck R, Jähne G, Winkler I, Helsberg M, Balzarini J, and De Clercq E (1994) The N-7-substituted acyclic nucleoside analog 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine is a potent and selective inhibitor of herpesvirus replication. *Antimicrob Agents Chemother* **38:**2710–2716.

Neyts J, Andrei G, Balzarini J, Snoeck R, and De Clercq E (1995a) Specific phosphorylation of 2-amino-7-[(1,3-dihydroxy-2-propoxymethyl)]purine (S2242) in human cytomegalovirus-infected human embryonic lung fibroblasts. *Scand J Infect Dis (Suppl)* **99:**115–116.

Neyts J and De Clercq E (1997) Antiviral drug susceptibility of human herpes virus type 8. *Antimicrob Agents Chemother* **41:**2754–2756.

Neyts J, Jähne G, Andrei A, Snoeck R, Winkler I, and De Clercq E (1995b) In vivo antiherpesvirus activity of N-7-substituted acyclic nucleoside analog 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine. *Antimicrob Agents Chemother* **39:**56–60.

Palú G, Stefanelli S, Rassu M, Parolin C, Balzarini J, and De Clercq E (1991) Cellular uptake of phosphonylmethoxyalkylpurine derivatives. *Antiviral Res* **16:**115–119.

Ruiz van Haperen VWT and Peters GJ (1994) New targets for pyrimidine antimetabolites for the treatment of solid tumors: deoxycytidine kinase. *Pharm World Sci* **16:**104–112.

Shewach DS, Reynolds KK, and Hertel L (1992) Nucleotide specificity of human deoxycytidine kinase. *Mol Pharmacol* **42:**518–524.

Starnes MC and Cheng Y-C (1987) Cellular metabolism of 2′,3′-dideoxycytidine, a compound active against human immunodeficiency virus in vitro. *J Biol Chem* **262:**988–991.

Sullivan V, Talarico CL, Stanat SC, Davis M, Coen DM, and Biron KK (1992) A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. *Nature (Lond)* **358:**162–164 [published errata appear in *Nature (Lond)* **359:**85 (1992) and **366**:756 (1993)].

Zimmerman TP, Mahony WB, and Prus KL (1987) 3′-Azido-3′-deoxythymidine and acyclovir: antiviral nucleoside analogues with unusual cell membrane permeation properties. *J Biol Chem* **262:**5748–5754.

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