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EFFECTS OF ACYCLOVIR AND ITS METABOLITES ON HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE

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Abstract—Acyclovir [9-(2-hydroxyethoxymethyl)guanine], a clinically useful anti-herpesvirus agent, was a weak inhibitor (K_i = 190 μM) of hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) from human erythrocytes. Nevertheless, this acyclic nucleoside analog was a more effective inhibitor than were its natural counterparts, guanosine (K_i = 1400 μM) and deoxyguanosine (K_i = 570 μM). The two oxidized metabolites of acyclovir, 9-carboxymethoxymethylguanine (K_i = 720 μM) and 8-hydroxy-9-(2-hydroxyethoxymethyl)guanine (K_i > 2000 μM), were less inhibitory than was the parent drug. None of the phosphorylated metabolites of acyclovir was as potent an inhibitor of HGPRTase as was GMP (K_i = 4 μM). However, the K_i value for acyclovir monophosphate was similar to that of dGMP (12 μM). The K_i values for acyclovir diphosphate (8.3 μM) and triphosphate (30 μM) were less than those for dGDP (110 μM) and dGTP (140 μM). The levels of these phosphate esters of acyclovir in cultured monkey kidney (Vero) and human embryo fibroblast (WI38) cells exposed to therapeutic levels of the drug were well below the observed K_i values. However, in herpesvirus-infected WI38 cells the levels of the phosphate esters of acyclovir were high enough potentially to inhibit the enzyme. Although inhibition of this enzyme by the phosphorylated metabolites of acyclovir may occur in these infected cells, concentrations of the drug very much higher than the EC_{50} concentration were required to achieve inhibitory levels. It is, therefore, unlikely that this inhibition contributes significantly to the antiviral activity.

Acyclovir [9-(2-hydroxyethoxymethyl)guanine] has been introduced recently to medical practice as an anti-herpetic agent. Metabolism studies have shown that a small percentage of the drug is oxidized to 8-hydroxy-9-(2-hydroxyethoxymethyl)guanine (8-hydroxyacyclovir†) and to 9-carboxymethoxymethylguanine in experimental animals [1, 2] and in human subjects [3]. In herpesvirus-infected cells, acyclovir is readily converted to its mono-, di-, and triphosphate derivatives [4, 5]. In non-infected cells, little or no phosphorylation occurs [4, 5]. The metabolism of acyclovir is summarized in Fig. 1.

It has been reported that the purine salvage enzyme, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8, HGPRTase), is inhibited and/or inactivated by periodate-oxidized ribonucleosides, ribonucleotides, and their derivatives [10–12]. Since these "acyclic" compounds have some structural similarity to acyclovir, their inhibitory properties suggested that acyclovir and/or its metabolites might inhibit HGPRTase. This together with the widespread clinical use of acyclovir prompted an investigation of the effects of this drug and its metabolites on this enzyme. A preliminary report of this work has been presented [13].

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† Abbreviations: 8-hydroxyacyclovir, 8-hydroxy-9-(2-hydroxyethoxymethyl)guanine; PRPP, 5-phosphoribosyl-1-pyrophosphate; HGPRTase, hypoxanthine-guanine phosphoribosyltransferase; and HPLC, high performance liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials. Guanine, hypoxanthine, guanosine, guanosine-2′-phosphate (sodium salt), guanosine-3′-phosphate (sodium salt), 2′-deoxyguanosine-3′-phosphate (ammonium salt), guanosine-5′-diphosphate (sodium salt), and 2′-deoxyguanosine-5′-diphosphate (sodium salt) were purchased from P.L. Biochemicals, Milwaukee, WI. Deoxyguanosine, guanosine-5′-phosphate (sodium salt), 2′-deoxyguanosine-5′-phosphate (sodium salt), 5-phosphoribosyl-1-pyrophosphate (sodium salt), orotate phosphoribosyltransferase/OMP decarboxylase, orotic acid, agarose-GMP, and bovine serum albumin (fraction V) were purchased from the Sigma Chemical Co., St. Lious, MO. Guanosine-5′-triphosphate (sodium salt) and 2′-deoxyguanosine-5′-triphosphate (sodium salt) were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. Biogel P-2, a polyacrylamide gel for gel filtration chromatography, was purchased from Bio-Rad Laboratories, Richmond, CA. Lichroprep RP-18, an octadecyl derivatized silica gel for reversed phase chromatography, was purchased from EM Laboratories, Elmsford, NY. Acyclovir [14], acyclovir monophosphate [15], acyclovir triphosphate [15], 8-hydroxyguanine [16], and 4-hydroxy-6-aminopyrazolo[3,4-*d*]pyrimidine [17] were synthesized in these laboratories according to published procedures. Acyclovir diphosphate occurred as a by-product in the acyclovir triphosphate synthesis reaction mixture, previously

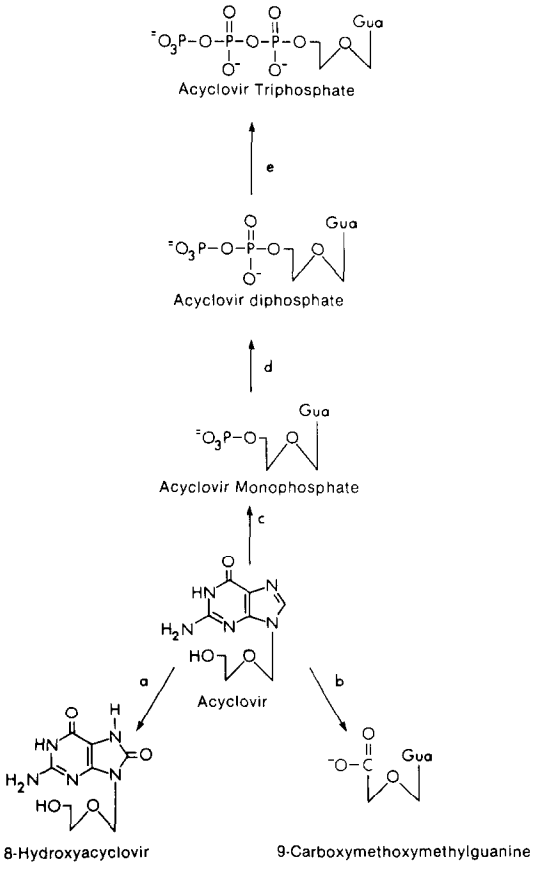

Fig. 1. Structural formulas for acyclovir and its metabolites. The enzymes which catalyze the above reactions have been elucidated by other studies and are as follows: (a) Aldehyde oxidase (W. W. Hall and T. A. Krenitsky, unpublished observations). (b) Alcohol dehydrogenase [6]. (c) Thymidine kinase in herpes-infected cells [7]. In uninfected cells the enzyme has not been identified. (d) GMP kinase [8]. (e) Phosphoglycerate kinase and a variety of other kinases [9]. Gua = guanyl moiety.

described [15]. It was purified as described elsewhere [9]. 9-Carboxymethoxymethylguanine and 8-hydroxyacyclovir were synthesized by James L. Kelley and Lilia M. Beauchamp of these laboratories using procedures which will be published elsewhere.

Compound purification. To avoid artifacts resulting from contamination of guanine nucleosides, nucleotides, or their acyclic analogues by guanine and/or guanine nucleosides, further purification of the compounds from the sources stated above was performed. Traces of guanine were removed from acyclovir by gel filtration using a 5 × 90 cm Biogel P-2 column equilibrated with H_2O at 25°. After elution with H_2O, the acyclovir-containing fractions were combined and stored at −20°. This same procedure was also used to remove traces of 8-hydroxyguanine from 8-hydroxyacyclovir. GMP, dGMP, acyclovir monophosphate, 9-carboxymethoxymethylguanine, guanosine-2′-phosphate, guanosine-3′-phosphate, and 2′-deoxyguanosine-3′-phosphate were purified further by preparative HPLC using a 1.5 × 50 cm Lichroprep RP-18 column equilibrated with H_2O at 25°. After elution with H_2O, the pH of the combined fractions containing the desired compound was adjusted to 7.0 with 0.1 N NaOH and stored at −20°. Deoxyguanosine was purified further by HPLC as described above, except that the column was equilibrated and eluted with 2% (v/v) ethanol/water. The combined fractions containing deoxyguanosine were evaporated to dryness at 40° under reduced pressure to remove residual ethanol. The compound was then dissolved in H_2O and stored at −20°. Acyclovir di- and triphosphates, GDP, and dGDP were purified further by ion exchange chromatography using a 0.8 × 12 cm DEAE-Sephadex A-25 column equilibrated with 50 mM ammonium bicarbonate at 25°. Compounds were eluted with a 200 ml linear ammonium bicarbonate gradient (0.05 to 1.0 M). The fractions containing the desired compound were combined and then evaporated to dryness at 40° under reduced pressure. The residue was dissolved in H_2O and evaporated to dryness again in order to remove residual ammonium bicarbonate. This procedure was repeated several times after which the compound was dissolved in H_2O and stored at −20°. GTP and dGTP were purified by isocratic elution from a 1 × 5 cm PEI-cellulose column with 0.1 M triethylamine-bicarbonate buffer, pH 7.5. After removal of triethylamine-bicarbonate, as described above for ammonium bicarbonate, the compounds were dissolved in H_2O and stored at −20°.

Determination of compound purity. Compound purity was determined by reversed phase HPLC. For the analysis of nucleosides, nucleoside monophosphates, and their acyclic analogues, a Waters μBondapak C_{18} column (0.39 × 30 cm) was used. The column was eluted (80 ml/hr) at 25° with 2% (v/v) ethanol/water. Retention times (sec) were as follows: GMP, 90; acyclovir monophosphate, 90; 9-carboxymethoxymethylguanine, 100; dGMP, 110; guanosine-3′-phosphate, 110; 2′-deoxyguanosine-3′-phosphate, 110; guanosine-2′-phosphate, 140; guanine, 320; 8-hydroxyguanine, 370; acyclovir, 480; guanosine, 540; 8-hydroxyacyclovir, 560; and deoxyguanosine, 740. Ultraviolet absorption was monitored at 246 nm. The relative concentration of each compound was calculated from its peak area and extinction coefficient at 246 nm. Extinction coefficients ($mM^{-1} \cdot cm^{-1}$) used were 10.7 for guanine, 12.9 for guanine nucleosides, nucleotides, and their acyclic analogues, 11.3 for 8-hydroxyguanosine, and 12.4 for 8-hydroxyacyclovir. Nucleoside di- and triphosphates and their analogues were analyzed by anion-exchange HPLC as previously described [4].

The relative amount of contaminant(s) listed below is expressed on a molar basis. The values given are for the compounds after purification as described above, except for guanosine which was not purified further. Guanosine, deoxyguanosine and acyclovir contained <0.01% guanine. GMP, dGMP, and acyclovir monophosphate contained <0.01% guanine and <0.2% of their respective nucleosides. 8-Hydroxyacyclovir contained <0.02% 8-hydroxyguanine, <0.2% guanine, and <0.02% acyclovir. 9-Carboxymethoxymethylguanine contained <0.01% guanine and <0.2% acyclovir. Guanosine-2′-phosphate, guanosine-3′-phosphate, and 2′-deoxyguanosine-3′-phosphate contained <0.1% guanine and <0.1% of their respective

nucleosides. GDP, dGDP, and acyclovir diphosphate contained <0.02% guanine and <0.2% of their respective nucleosides. GDP contained 0.7% GMP, dGDP contained 0.7% dGMP, and acyclovir diphosphate contained 0.3% acyclovir monophosphate. GTP, dGTP, and acyclovir triphosphate contained <0.1% guanine and <0.1% of their respective nucleosides. GTP contained 0.2% GMP and 5% GDP. dGTP contained <0.05% dGMP and 1.5% dGDP. Acyclovir triphosphate contained <0.5% acyclovir monophosphate and 0.5% acyclovir diphosphate.

Enzyme assays. Reaction rates were monitored spectrophotometrically at 25°. The rate of phosphoribosyl transfer to guanine ($\Delta\varepsilon = 4.2\ mM^{-1} \cdot cm^{-1}$) or to 4-hydroxy-6-aminopyrazolo[3,4-*d*]pyrimidine ($\Delta\varepsilon = 7.5\ mM^{-1} \cdot cm^{-1}$) catalyzed by HGPRTase was monitored at 255 nm in 100 mM Tris·HCl buffer (pH 7.4), 5 mM $MgSO_4$ as previously described [18]. The levels of interfering enzyme activities in the HGPRTase preparation were also determined. Purine nucleoside phosphorylase (EC 2.4.2.1) was assayed using 0.1 mM guanosine as substrate as described elsewhere [19]. Guanase (EC 3.5.4.3) and nucleotidase were assayed as previously described except that the concentration of IMP was increased to 1.0 mM in the nucleotidase assay [18].

Kinetic constants were determined with the computer programs described by Cleland [20]. Inhibition constants were determined in the presence and absence of a single concentration of inhibitor. One unit of HGPRTase is defined as the amount of enzyme that catalyzes the conversion of 1 μmole of guanine to GMP per min at 25° under the specified conditions.

Other determinations. The concentration of PRPP was determined using a published procedure [21]. Protein was quantitated using the Coomassie Blue method with bovine serum albumin as the standard [22].

Enzyme purification. HGPRTase was purified from human erythrocytes using the procedure of Holden and Kelley [23]. The specific activity of the resulting preparation was 104 units/mg protein with 0.04 mM guanine as the substrate. This preparation contained no detectable guanase (<0.02% of the rate of phosphoribosyl transfer to guanine). It did contain a trace of nucleotidase (0.06%) and purine nucleoside phosphorylase (0.10%). However, under the conditions of the HGPRTase assay (i.e. no added phosphate), the rate of guanosine phosphorolysis in the presence of this enzyme was <0.002% (at 0.1 mM guanosine) of the rate of phosphoribosyl transfer to guanine. This preparation was stable for at least 1 year when stored at −70° in 10 mM Tris·HCl buffer (pH 7.4) which also contained 5 mM $MgSO_4$, 1 mM Na_4PRPP, and 1 mg bovine serum albumin/ml.

Table 1. Inhibition constants for hypoxanthine–guanine phosphoribosyltransferase from human erythrocytes

Inhibitor	Variable substrate*	K_i† (μM)
Acyclovir	GPP‡ (Gua)§	190 ± 23
9-Carboxymethoxymethylguanine	GPP	720 ± 280
8-Hydroxyacyclovir	GPP	>2000
Deoxyguanosine	GPP	570 ± 120
Guanosine	GPP	1400 ± 460
Acyclovir monophosphate	PRPP‖	11 ± 0.59
GMP	PRPP	4.0 ± 0.33
dGMP	PRPP	12 ± 0.78
2′-Deoxyguanosine-3′-phosphate	PRPP	100 ± 7.5
Guanosine-3′-phosphate	PRPP	200 ± 18
Guanosine-2′-phosphate	PRPP	1100 ± 390
Acyclovir diphosphate	PRPP	8.3 ± 0.43
GDP	PRPP	91 ± 6.8
dGDP	PRPP	110 ± 10
Acyclovir triphosphate	PRPP	30 ± 2.0
GTP	PRPP	130 ± 12
dGTP	PRPP	140 ± 13

* The reaction mixture concentration of $MgSO_4$ was kept constant at 5.0 mM.

† Inhibition was competitive with respect to the variable substrate. Values are means ± standard error.

‡ GPP, 4-hydroxy-6-aminopyrazole[3,4-*d*]pyrimidine. The sensitivity of the assays was increased as a result of the larger $\Delta\varepsilon$ value for the phosphoribosylation of this compound as compared with that of guanine. Also, as a result of its relatively large K'_m value (260 vs 3 μM for guanine), enzyme inhibition could be detected at much lower inhibitor concentrations. A minimum of six concentrations of this substrate, ranging from 130 to 10 μM, was used. The reaction concentration of 5-phosphoribosyl-1-pyrophosphate was kept constant at 1.0 mM.

§ When guanine (K'_m =3 μM) was varied from 30 to 2 μM, replacing GPP as the variable substrate, identical K_i values were obtained for acyclovir.

‖ PRPP, 5-phosphoribosyl-1-pyrophosphate. A minimum of six concentrations of this substrate (K'_m =66 μM), ranging from 400 to 20 μM, was used. The reaction concentration of guanine was kept constant at 30 μM.

RESULTS

Inhibition constants for HGPRTase are listed in Table 1. All the nonphosphorylated inhibitors exhibited competitive inhibition with the heterocyclic substrate, whereas all the phosphorylated inhibitors exhibited competitive inhibition with PRPP. When the phosphorylated inhibitors were tested using the heterocyclic substrate as the variable substrate, the inhibition was weak and appeared to be noncompetitive. A similar result was obtained when nonphosphorylated inhibitors were tested using PRPP as the variable substrate. The inhibitors listed in Table 1 were competitive with either PRPP or heterocyclic base. These substrates have been shown to bind to this enzyme in an ordered sequence with PRPP first [24, 25]. Therefore, the K_i values of inhibitors that compete with PRPP should be equal to dissociation constants of the inhibitors from the enzyme–inhibitor complex at any fixed concentration of the second substrate [26]. Furthermore, when the second substrate (heterocyclic base) was varied, the first substrate (PRPP) was fixed at a saturating concentration (fifteen times its K_m' value). Therefore, the K_i values for the inhibitors that compete with the heterocyclic base should approximate dissociation constants of the inhibitors from the enzyme–PRPP–inhibitor complex [26].

The K_m' values obtained with guanine (3 μM) and PRPP (66 μM) were similar to those previously reported (4 and 66 μM respectively) [24, 25]. The K_i value for GMP (4 μM) was within the range of values (1.3 to 14 μM) previously reported [24, 27], and the K_i values for GDP (91 μM) and GTP (130 μM) were similar to the values (90 and 150 μM respectively) reported for the enzyme from rat brain [28]. The relatively large K_i values for guanosine (K_i = 1400 μM) and deoxyguanosine (K_i = 570 μM) are consistent with the observations that this enzyme was inhibited only slightly or not at all by these compounds [10, 29].

Of the nucleosides and their acyclic analogs tested, acyclovir had the lowest K_i value (190 μM). The two oxidized metabolites of acyclovir, 9-carboxymethoxymethylguanine (K_i = 720 μM) and 8-hydroxyacyclovir (K_i > 2000), were weaker inhibitors than acyclovir.

Of the nucleotides and their acyclic analogs tested, GMP had the lowest K_i value (4 μM). The K_i values for acyclovir monophosphate (11 μM) and dGMP (12 μM) were similar. In contrast, the K_i values for acyclovir diphosphate (8.3 μM) and triphosphate (30 μM) were less than those for the di- and triphosphate esters of guanosine and deoxyguanosine.

DISCUSSION

A basic question is whether HGPRTase would be inhibited significantly by acyclovir or its metabolites under therapeutic conditions. A corollary to this question is whether or not inhibition of this enzyme in virus-infected cells contributes to the antiviral effects of acyclovir. These questions have been approached by comparing the concentration of these compounds found in biological systems with their K_i values for this enzyme. Such a comparison can only indicate whether these compounds are present at a concentration sufficient to theoretically inhibit the enzyme in the absence of any competing substrates. The magnitude of any inhibition would, of course, depend on the levels of endogenous substrates and/or inhibitors, their K_s or K_i values, and the extent that substrates accumulate in the presence of the exogenous inhibitor.

In human patients infused intravenously with 5 mg/kg of acyclovir, the steady-state plasma acyclovir label observed was 43.2 μM [30]. The intra-erythrocyte concentration was found to be the same as the plasma concentration [3]. This concentration is one-third the K_i value for HGPRTase (Table 1). In patients treated with 10 mg/kg acyclovir, the steady-state plasma level was 88.9 μM [30], a concentration which was still well below the K_i value for acyclovir with HGPRTase. It, therefore, appears unlikely that acyclovir itself significantly inhibits HGPRTase in erythrocytes under therapeutic conditions.

The tissue distribution of acyclovir has been studied in the mouse and rat [1]. In these species the level of acyclovir found in various tissues was not markedly higher than that found in plasma, except in kidney, where the higher level can be attributed to contamination of the tissue samples with acyclovir in urine. Preliminary results indicate that the distribution of acyclovir in human tissues is similar to that found in the above species (P. de Miranda, personal communication). As noted above, the steady-state plasma concentration of acyclovir is less than its K_i value for HGPRTase under therapeutic conditions. These results indicate that this enzyme would not be appreciably inhibited by the intracellular concentrations of acyclovir found in human tissues.

The intracellular or plasma concentration of the two oxidized metabolites of acyclovir has not been determined in humans. However, since the concentration of these metabolites found in the urine of patients treated with acyclovir was much less than the concentration of acyclovir [3], the plasma concentrations of these metabolites also can be inferred to be much less than the acyclovir concentration. Furthermore, the high K_i values for these oxidized metabolites render them less likely than the parent drug to inhibit this enzyme *in vivo*.

The levels of the phosphorylated metabolites of acyclovir in tissue extracts of herpesvirus-infected and uninfected mice that were treated with acyclovir (50 mg/kg, s.c.) have been studied by Biron *et al.* [31]. In both infected and uninfected animals, the levels of the phosphorylated metabolites were below their corresponding K_i values for HGPRTase.

Since the percentage of infected cells in whole tissues *in vivo* is small, the levels of acyclovir phosphates in herpesvirus-infected cells in culture would perhaps provide data more relevant to the possible role that inhibition of HGPRTase might play in the virus-infected cell. Such levels* have been published by Furman *et al.* [5]. After monkey kidney (Vero)

* These levels, which were expressed as pmoles/10^6 cells, were converted to μmolar values for comparison with K_i values. The basis for this conversion is that 10^6 Vero or WI38 cells have a packed volume of 5 μl.

cells which were infected with a variety of strains of herpes simplex virus types I and II were incubated with 100 μM acyclovir, the monophosphate levels ranged from 0.4 to 10 μM, diphosphate from 0.4 to 6 μM, and triphosphate from 3 to 75 μM. With the H29 strain of herpes simplex virus (type I) grown in human embryonic fibroblasts (WI38), these values were significantly higher: 29, 54, and 417 μM respectively.

A comparision of these values with the K_i values for these phosphorylated metabolites of acyclovir with HGPRTase (Table 1) suggests that in the virus-infected cell in the presence of high drug concentrations some inhibition of HGPRTase might occur. However, the relatively high level of acyclovir (100 μM) required to achieve concentrations of its phosphorylated metabolites that are above their K_i values for HGPRTase is 100 to 1000 times greater than the EC_{50} values (0.1 to 1.4 μM) for a variety of herpes simplex virus strains in Vero cells [5]. With lower levels of acyclovir that were still well above the EC_{50} values, the levels of phosphorylated metabolites were well below their K_i values for HGPRTase. For example, at an acyclovir concentration of 10 μM, the acyclovir phosphates in Vero cells infected with herpes simplex virus (type I, strain H29) were 0.14, 0.024, and 1.5 μM for the mono-, di-, and triphosphate respectively (P. A. Furman and M. H. St. Clair, personal communication). This acyclovir concentration was 100 times its EC_{50} value (0.1 μM) for the virus.

The data presented here on the inhibition of HGPRTase by acyclovir and its metabolites, together with the published data on the distribution and metabolism of the drug, suggest that, although some inhibition of HGPRTase might be encountered at high levels of the drug, especially in virus-infected cells, it appears unlikely that this inhibition contributes significantly to the antiviral activity.

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