Effects of Acyclovir and Its Metabolites on Purine Nucleoside Phosphorylase*

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Acyclovir (9-(2-hydroxyethoxy)methylguanine), the clinically useful antiviral agent, is an "acyclic" analogue of 2′-deoxyguanosine. Purine nucleoside phosphorylase partially purified from human erythrocytes did not catalyze detectable phosphorylation of this drug or any of its metabolites (<0.07% of the rate with Guo). However, these compounds were competitive inhibitors of this enzyme with Ino as the variable substrate. Acyclovir per se was a relatively weak inhibitor. Its Ki value (91 μM) was much greater than that for its 8-hydroxy metabolite (Ki = 4.7 μM) but less than that for its carboxylic acid metabolite (9-carboxyhydroxymethylguanine) (Ki = 960 μM). The phosphorylated metabolites of acyclovir were more potent inhibitors than were their guanine nucleotide counterparts. At a phosphate concentration of 50 mM, the apparent Ki values for the mono- (120 μM), di- (0.51 μM), and tri (43 μM)-phosphate esters of acyclovir were 1/4, 1/200, and 1/6 those for dGMP, dGDP, and dGTP, respectively. The concentration of phosphate did not markedly affect the Ki value of acyclovir but dramatically affected those of its phosphorylated metabolites and their nucleotide counterparts. Decreasing phosphate to a physiological concentration (1 mM) decreased the apparent Ki values for the mono-, di-, and triphosphate esters of acyclovir to 6.6, 0.0087, and 0.31 μM, respectively. Inhibition of the enzyme by acyclovir diphosphate was also influenced by pH. This metabolite of acyclovir is the most potent inhibitor of purine nucleoside phosphorylase reported to date. It has some features of a "multisubstrate" analogue inhibitor.

In this study, acyclovir and its metabolites were tested as substrates and inhibitors of purine nucleoside phosphorylase (EC 2.4.2.1) from human erythrocytes. This endeavor was prompted by the structural similarities between acyclovir and natural substrates of this enzyme. Kinetic constants were determined for acyclic compounds and compared with those for their naturally occurring counterparts. Some of the metabolites of acyclovir were found to be much more potent inhibitors than were their natural counterparts.

EXPERIMENTAL PROCEDURES

RESULTS

The partially purified purine nucleoside phosphorylase from human erythrocytes did not catalyze detectable phosphorylation of acyclovir, or any of its metabolites, even at concentrations as high as 1 mM (Table I). As is characteristic of this enzyme (16), double reciprocal plots of initial velocity versus concentrations of Ino, Guo, or dGuo were concave downward at substrate concentrations above 0.1 mM. For this reason, the kinetic constants listed in Table I were determined from the linear portion of the double reciprocal plots, i.e., at substrate concentrations below 0.1 mM. The Ki values for Ino, Guo, and dGuo and the Ki values for Guo and GMP (Table I) were similar to reported values (16–18).

Inhibition constants for acyclovir and its metabolites and for some of their natural counterparts were determined at 50 mM phosphate (Table I). The Ki value for acyclovir (91 μM) was more similar to the Ki values for dGuo (65 μM) and Guo (46 μM) than to the Ki value for Guo (4.6 μM). 9-Carboxymethoxymethylguanosine (Ki = 960 μM) was a less potent inhibitor of this enzyme than was acyclovir, whereas 8-hydroxyacyclovir (Ki = 4.7 μM) was much more potent. The phosphorylated metabolites of acyclovir were more potent inhibitors than were their nucleotide counterparts. Surprisingly, acyclovir diphosphate was the most potent inhibitor tested. The apparent Ki value at 50 mM phosphate for this inhibitor (0.51 μM) was 1/200 that for dGDP. Acyclovir triphosphate (Ki = 43 μM) was a more potent inhibitor than was acyclovir monophosphate (Ki = 120 μM). Of the naturally occurring guanine nucleotides examined, 2′-deoxyguanosine-3′-phosphate was the most potent inhibitor (Ki = 390 μM) and guanosine-2′-phosphate was the least potent inhibitor (Ki = 3200 μM). The 2′-deoxyribonucleotides were more potent.

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† The trivial name used is: acyclovir, 9-(2-hydroxyethoxy)methylguanine.
The concentration of phosphate, pH 7.5, in the assays was kept constant at the concentration indicated. Unless noted otherwise, the concentration of other assay components was as described under “Experimental Procedures.”

**Kinetic constants for human purine nucleoside phosphorylase**

| Substrate             | 50 mM phosphate | K_i at 1.0 mM phosphate* |
|-----------------------|-----------------|--------------------------|
|                       | μM              | μM                       |
| Inosine               | 40 ± 4.7        | 110                      |
| Guanosine             | 46 ± 2.3        | 100                      |
| 2'-Deoxyguanosine     | 65 ± 3.5        | 91                       |
| Guanine               |                 | 4.6 ± 0.37               |
| 8-Hydroxyguanine      |                 | 19 ± 1.4                 |
| Acyclovir             |                 | <0.02 (<0.002)           |
| 8-Hydroxyacyclovir    |                 | 91 ± 9.7                 |
| 9-Carboxymethoxyethylguanine | <0.07 (<0.002) | 4.7 ± 0.28               |
| Acyclovir monophosphate | <0.07            | 960 ± 87                 |
| 2'-Deoxyguanosine-3'-phosphate | <0.03 | 120 ± 6.4               |
| dGMP                  |                 | 390 ± 38                 |
| Guanosine-3'-phosphate|                 | 1400 ± 220               |
| dGMP                  |                 | 2000 ± 140               |
| Guanosine-2'-phosphate|                 | 3200 ± 770               |
| Acyclovir diphosphate |                 | 0.51 ± 0.072             |
| dGDP                  |                 | 590 ± 79                 |
| GDP                   |                 | 1500 ± 330               |
| Acyclovir triphosphate|                 | 43' ± 3.0                |
| dGTP                  |                 | 1100 ± 110               |
| GTP                   |                 | 2400 ± 440               |

*In addition to desalted purine nucleoside phosphorylase, these assay mixtures also contained 100 mM Tris-HCl, pH 7.5, and 0.04 IU/ml desalted xanthine oxidase.

The above kinetic constants were determined at 50 mM phosphate. This is a customary concentration for studies with this enzyme since phosphate is used both as substrate and by acyclovir. In contrast, the degree of inhibition by the potent inhibitor, acyclovir diphosphate, was phosphate rather than potassium ion or ionic strength which was responsible for this effect.

**DISCUSSION**

Neither acyclovir nor its metabolites were phosphorolyzed by purine nucleoside phosphorylase from human erythrocytes (Table I). This is consistent with the finding that there is no detectable cleavage of acyclovir in vivo (1, 4, 5).
Acyclovir per se was a weak inhibitor of purine nucleoside phosphorylase. The marked increase in affinity for the enzyme resulting from the substitution of an amino group at the 8-position of Gua and Guo (20). On the other hand, substitution of a hydroxy group at the 8-position of Gua diminished the affinity for the enzyme (Table I).

The finding that the apparent $K_i$ value for the phosphorylated inhibitors was a function of the concentration of phosphate (Fig. 4) has important implications when nucleotides or their analogues are examined as inhibitors of this enzyme. In a recent study, purine nucleotides were found to be weak inhibitors of the enzyme from human granulocytes (21). However, the weak inhibition reported for these nucleotides may reflect the high concentration of phosphate (100 mM) used in the assays. For example, the apparent $K_i$ values for dGMP, dGDP, and dGTP at 50 mM phosphate were 5, 15, and 34 times those at 1 mM phosphate (Table I).

From the effects of phosphate on the inhibition by guanine nucleotides revealed in this study, it would appear that these compounds and their acyclic analogues possess binding determinants for both the guanine/guanosine and phosphate/ribose-1-phosphate-binding regions of the enzyme. This suggests that these phosphate esters can be considered as "multisubstrate" analogue inhibitors (22). The markedly increased affinity of the enzyme for the phosphorylated metabolites of acyclovir, as compared with their nucleotide counterparts, may be due, in part, to the flexibility of the acyclic side chain. This might allow the phosphate moiety to be positioned in closer proximity to the phosphate-binding site of the enzyme. Such flexibility is readily apparent from space-filling models.

The diphosphate ester of acyclovir was a more potent inhibitor than its mono- and triphosphate esters (Table I). An optimum distance between guanine and the terminal phosphate moiety in acyclovir diphosphate might account for its greater inhibitory properties. It is noteworthy that acyclovir diphosphate is the most potent inhibitor of purine nucleoside phosphorylase of all the compounds and their acyclic analogues possess binding determinants for both the guanine/guanosine and phosphate/ribose-1-phosphate-binding regions of the enzyme. This suggests that these phosphate esters can be considered as "multisubstrate" analogue inhibitors (22). The markedly increased affinity of the enzyme for the phosphorylated metabolites of acyclovir, as compared with their nucleotide counterparts, may be due, in part, to the flexibility of the acyclic side chain. This might allow the phosphate moiety to be positioned in closer proximity to the phosphate-binding site of the enzyme. Such flexibility is readily apparent from space-filling models.

Because of its clinical use, the question arises whether or not acyclovir and its metabolites might significantly inhibit purine nucleoside phosphorylase in vivo. This question can be approached by comparing the published intracellular concentrations of these compounds with the $K_i$ values presented here. 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 actual magnitude of inhibition would, of course, depend on the levels of endogenous competing substrates and/or inhibitors and their $K_m$ or $K_i$ values.

In patients infused intravenously with the recommended dose of 5 mg/kg of acyclovir, the mean steady state peak plasma acyclovir level observed was 44 µM (23). The erythrocyte concentration was found to be nearly the same as the plasma concentration (4). This concentration is one-half the $K_i$ value for purine nucleoside phosphorylase. It is therefore unlikely that this enzyme would be significantly inhibited at this concentration of acyclovir. However, in patients treated with the higher dose of 10 mg/kg acyclovir (intravenously), the steady state plasma level was 92 µM (23), a concentration which might theoretically result in some inhibition.

The intracellular or plasma concentrations of the two oxidized metabolites of acyclovir have not been determined in humans. However, the concentration of these metabolites found in the urine of patients treated with acyclovir was much lower than the concentration of acyclovir (4). Therefore, it can be inferred that their plasma concentrations are much lower than that of acyclovir. Little inhibition of purine nucleoside phosphorylase by the carboxylic acid metabolite of acyclovir would be expected since its $K_i$ value is greater than that of acyclovir. Even though the $K_i$ value for 8-hydroxyacyclo- vir was 1/5 that for acyclovir, it is unlikely that it would inhibit significantly since only trace amounts of this metabolite are found in human urine (4).

The levels of the phosphorylated metabolites of acyclovir in animal tissues have been determined in vivo (24). At a relatively high dose (50 mg/kg, subcutaneously), the peak levels of acyclovir diphosphate found in the three tissues examined (brain, 0.06 µM; liver, 0.14 µM; and kidney, 0.24 µM) were greater than its apparent $K_i$ value at 1 mM phosphate (Table I). This suggests that some inhibition of the enzyme in vivo might occur. However, the dose of acyclovir used was 10-fold higher than the recommended intravenous dose for human use.

One of the prime motivating factors in the search for inhibitors of purine nucleoside phosphorylase has been the discovery that patients who are genetically deficient in this enzyme have impaired cellular, but not humoral, immunity (25). It is possible that an inhibitor of this enzyme that is effective in vivo might be a useful selective immunosuppressive agent. In view of the inhibition of purine nucleoside phosphorylase by acyclovir and its metabolites reported here, it is relevant to note that acyclovir has been shown to have little or no effect on a variety of immune-related functions in vivo at doses 10 to 40 times that recommended for intravenous use (26).

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SUPPLEMENTARY MATERIAL TO
EFFECTS OF ACYCLOVIR AND ITS METABOLITES ON PURINE NUCLEOSIDE PHOSPHORYLASE
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EXPERIMENTAL PROCEDURES

Materials - Inosine was purchased from P.L. Biochemicals, Milwaukee, WI. Borate-free saline solution (BSS) was prepared from Ringers Mammalian Bicarbonate, Indianapolis, IN. The source or synthesis of [14C]-GDP, [14C]-GTP, and [14C]-adenosine 3', 5'-cyclic phosphate have been previously described (18). The sources for other nucleotides and compounds and their purification methods were as previously described (18). In all cases, the presence of ADP was used.

Enzyme Assay - Enzymes were assayed spectrophotometrically at 25°C. Rates of phosphorylation of guanine nucleotides, precursors, and their adenosine analogues were monitored at 560 nm (A = 6.3 X 10⁻³ M⁻¹ cm⁻¹) in 50 mM phosphate buffer, pH 7.5, unless otherwise specified. 1-Phospho-adenosine 5'-phosphate was similarly monitored for phosphorylase activity except that the wavelength was 370 nm (A = 3.7 X 10⁻³ M⁻¹ cm⁻¹). Phosphorylation of GDP was similarly monitored at 340 nm (A = 13.3 X 10⁻³ M⁻¹ cm⁻¹) in the presence of saline solution (50 mM NaCl, 0.5% albumin or saline solution) and EDTA (0.1 mM).

In all cases, the saline solution or saline solution was used to determine the assay activity. The assay mixture contained the same as the assay mixture except that the concentration of Mg²⁺ was increased to 15.0 mM in the saline solution assay. Phosphate concentrations were determined using the phosphate assay procedure described by Cleland (28). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the conversion of 1 micromole of substrate per minute at 37°C under the assay conditions.

Preparation of inosine deaminase - Preparations were made by previous methods (18). In all cases, the enzyme was purified to homogeneity by a combination of CM-cellulose and DEAE-cellulose chromatography. The specific activity of the enzyme was consistently determined by the procedure described above.

Preparation of guanines - Guanines were prepared by the method of Biron et al. (21). It had a specific activity of 1.2 units per gram with 0.1 M NaOH at the standard PH.

Results - The inhibition of Purine Nucleoside phosphorylase was partially purified from human erythrocytes using the procedure of Biron et al. (21). It had a specific activity of 1.2 units per gram with 0.1 M NaOH at the standard PH.

A similar experiment was performed with LDL as the variable substrate (Figure 3). In this case, the double reciprocal plot was linear at substrate concentrations of 8.0 nm. The linear intercepts in the presence and absence of acyclovir diaphosphate were similar (9.4-97) indicating competitive inhibition. The results of another experiment designed to reveal whether or not this competitive inhibition was linear is shown in Figure 3. When acyclovir diaphosphate was varied between 0 and 8.4 g M⁻¹ at 1 mM phosphate and 0.68 mM LDL, a linear Dixon plot was obtained.

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Inhibition of Purine Nucleoside Phosphorylase

Figure 1: Inhibition of purine nucleoside phosphorylase by acyclovir diphosphate with phosphate as the variable substrate. The concentration of lipoic acid was kept constant at 5 mM. In addition to deoxynucleoside phosphorylase, the assay also contained 180 mM Tris-Cl, pH 7.4, and 6.64 mM of deoxynucleoside diphosphate. When present, the concentration of acyclovir diphosphate was 5 mM.

Figure 2: Inhibition of purine nucleoside phosphorylase by acyclovir diphosphate with lipoic acid as the variable substrate. The concentration of phosphate was kept constant at 1 mM. When present, the concentration of acyclovir diphosphate was 3 mM. Other assay conditions were as described for Figure 1. Under these conditions, the apparent K_i value was calculated to be 2.7 ± 0.4 mM.

Figure 3: Double plot of the inhibition of purine nucleoside phosphorylase by acyclovir diphosphate. The concentrations of the substrates lipoic acid and phosphate were 0.65 mM and 1 mM, respectively. Other assay conditions were as described for Figure 1.

Because the kinetic with lipoic acid had a clearly linear range, the effects of phosphate were further studied by determining the K_i values of acyclovir diphosphate at various fixed concentrations of phosphate with lipoic acid as the variable substrate. A plot of the K_i values vs. phosphate concentration was not linear (Figure 4). The slope changed most markedly near 5 mM phosphate, which corresponds to the region of greatest non-linearity in Figure 1. From the ordinate intercept (see inset of Figure 4), a K_i value of 3.5 × 10^{-10} M was determined for acyclovir diphosphate in the absence of lipoic acid.1

Figure 4: Effects of the phosphate concentration on the apparent K_i value for acyclovir diphosphate with purine nucleoside phosphorylase. A minimum of six concentrations of phosphate ranging from 10 to 100 mM were used to determine each K_i value. The concentration of phosphate was kept constant at the value indicated on the abscissa. Other assay conditions were as described for Figure 1. The y-intercept was not constant with lipoic acid at all phosphate concentrations. The vertical bars indicate the standard error of each apparent K_i value. The limit is an extension of the 95% confidence interval of the plot. The K_i value of acyclovir diphosphate extrapolated to zero phosphate was 0.28 mM.

Effects of pH on inhibition: Since the phosphorylated metabolites of acyclovir are in the physiological pH range, the effects of pH on the inhibition by these compounds are of some importance. Figure 5 shows the effect of pH on inhibition by acyclovir diphosphate. The inhibition decreased dramatically from pH 6 to pH 8. The K_i for lipoic acid has been found to increase with increasing pH over this pH range (51). Therefore, the decrease in inhibition shown in Figure 5 cannot be due to a corresponding decrease in the K_i for lipoic acid. The effect of pH on the inhibition by acyclovir diphosphate and DMP-ribose (data not shown) was similar to that shown for acyclovir diphosphate (Fig. 5).

Figure 5: Effect of pH on the inhibition of purine nucleoside phosphorylase by acyclovir diphosphate. Phosphate was kept constant at 1 mM. When present, the concentration of acyclovir diphosphate was 180 mM. In addition to deoxynucleoside phosphorylase, the reaction mixture also contained 0.8 mM Tris-Cl (pH 8.0) or Tris-Cl (pH 6.0) buffer and 6.64 mM of deoxynucleoside diphosphate.

1Purification of deoxynucleoside for 10 min at 10°C with 25 mM acyclovir diphosphate did not increase the percent inhibition. This suggests that the inhibition by this compound is not progressive.
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