Biochemical Pharmacology 81 (2011) 43–49

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Phosphorylation of antiviral and endogenous nucleotides to di- and triphosphates by guanosine monophosphate kinase

Brian G. Gentry [a,1], Sara N. Gentry [b], Trachette L. Jackson [b], Jiri Zemlicka [c], John C. Drach [a,*]

[a] Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, MI 48109-1078, United States
[b] Department of Mathematics, College of Literature, Science, and the Arts, University of Michigan, Ann Arbor, MI 48109-1043, United States
[c] Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201-1397, United States

ARTICLE INFO

Article history:
Received 1 July 2010
Accepted 7 September 2010

Keywords:
Cytomegalovirus
Guanosine monophosphate kinase
Cyclopropavir
GMP
Antivirals

ABSTRACT

Many fraudulent nucleosides including the antivirals acyclovir (ACV) and ganciclovir (GCV) must be metabolized to triphosphates to be active. Cyclopropavir (CPV) is a newer, related guanosine nucleoside analog that is active against human cytomegalovirus (HCMV) *in vitro* and *in vivo*. We have previously demonstrated that CPV is phosphorylated to its monophosphate (CPV-MP) by the HCMV pUL97 kinase. Consequently, like other nucleoside analogs phosphorylated by viral kinases, CPV most likely must be converted to a triphosphate (CPV-TP) in order to elicit antiviral activity. Once formed by pUL97, we hypothesized that guanosine monophosphate kinase (GMPK) is the enzyme responsible for the conversion of CPV-MP to CPV-DP. Incubation of CPV-MP with GMPK resulted in the formation of CPV-DP and, surprisingly, CPV-TP. When CPV-DP was incubated with GMPK, a time-dependent increase in CPV-TP occurred corresponding to a decrease in CPV-DP thereby demonstrating that CPV-DP is a substrate for GMPK. Substrate specificity experiments revealed that GMP, dGMP, GDP, and dGDP are substrates for GMPK. In contrast, GMPK recognized only acyclovir and ganciclovir monophosphates as substrates, not their diphosphates. Kinetic studies demonstrated that CPV-DP has a K_M value of 45 ± 15 μM. We were, however, unable to determine the K_M value for CPV-MP directly, but a mathematical model of experimental data gave a theoretical K_M value for CPV-MP of 332 ± 60 μM. We conclude that unlike many other antivirals, cyclopropavir can be converted to its active triphosphate by a single cellular enzyme once the monophosphate is formed by a virally encoded kinase.

1. Introduction

Human cytomegalovirus (HCMV) is a beta herpesvirus with wide distribution in the human population. It is a pathogen that can result in severe disease such as interstitial pneumonia, mental retardation, hearing loss, and a mononucleosis-like syndrome in immunocompromised and immunologically immature individuals [1,2]. Currently, drugs such as ganciclovir (GCV), foscarnet, cidofovir, and fomivirsen are used for the treatment or prophylaxis of HCMV disease, but the development of drug resistance and severe side effects can occur due to the long-term therapy that is generally required [2–7].

Cyclopropavir (CPV) is a guanosine nucleoside analog in which the ribose has been replaced with a bis-hydroxymethyl cyclopropylidene moiety (Fig. 1). It is active against HCMV *in vitro* at 10-fold lower concentrations compared to GCV (e.g. $EC_{50's}$ = 0.46 and 4.1 μM, respectively) [8–10]. *In vivo*, CPV was highly effective in preventing mortality when administered orally to murine cytomegalovirus-infected normal mice. It also reduced viral replication in human fetal tissue implanted in severe combined immunodeficient mice infected with HCMV [11]. Its increased antiviral activity without increased toxicity compared to GCV prompted our studies into the metabolism and mechanism of action of CPV.

Guanosine monophosphate (guanylate) kinase (GMPK) was first characterized by Miech and Parks [12]. It is a highly specific enzyme with regard to both phosphate acceptor (guanosine monophosphate) and donor (ATP). Later it was determined that formation of guanosine triphosphate (GTP) from guanosine monophosphate (GMP) required two different enzymes indicating that GMPK was responsible solely for the phosphorylation of monophosphate to diphosphate while another enzyme performed the catalytic conversion of diphosphate to triphosphate [13,14]. With the advent of nucleoside analogs as antiviral drugs that must

* Corresponding author at: Department of Biologic and Materials Sciences, University of Michigan, 1210 Eisenhower Place, Ann Arbor, MI 48108-3218, United States. Tel.: +1 734 975 9402; fax: +1 734 975 9329.
E-mail addresses: brian.gentry@drake.edu (B.G. Gentry), jcdrach@umich.edu (J.C. Drach).
[1] Present address: Drake University College of Pharmacy, 2507 University Ave, Des Moines, IA 50311-4505, United States. Tel.: +1 515 271 2980; fax: +1 515 271 1867.

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.09.005

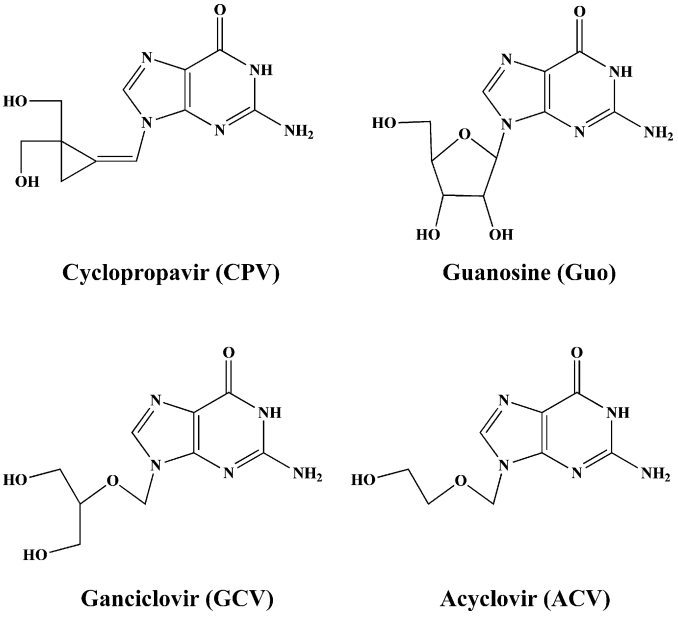

Fig. 1. Structures of cyclopropavir, guanosine, ganciclovir, and acyclovir.

be phosphorylated to a triphosphate to be active, examination of GMPK as one of the enzymes responsible for the conversion of guanosine nucleoside analogs to active compounds was crucial for the understanding of the mechanism of action of these drugs [3,7,15–18]. These studies demonstrated that the phosphorylation of nucleoside analogs, such as acyclovir (ACV) and GCV (Fig. 1), from a monophosphate to diphosphate was catalyzed by GMPK indicating an increased range of substrates that can be utilized by this enzyme [16,19,20].

Like GCV [5,21–24], the initial phosphorylation of CPV to a monophosphate (CPV-MP) is performed by the viral protein kinase pUL97 [25]. Also like GCV, incubation of CPV in HCMV infected cells results in inhibition of viral DNA synthesis and viral replication [10]. Therefore, we hypothesize that CPV must be phosphorylated to a triphosphate (CPV-TP) in order to elicit antiviral activity and that endogenous cellular kinases are responsible for the conversion of CPV-MP to CPV-TP. We have investigated the second part of this hypothesis and herein describe the role of GMPK in the phosphorylation of CPV-MP.

2. Materials and methods

2.1. Nucleoside analogs

Cyclopropavir [(Z)-9-{[2,2-bis-(hydroxymethyl)cyclopropylidene]methyl}guanine, CPV]; the (+)- and (−)-enantiomers of cyclopropavir monophosphate (CPV-MP); and racemic mixtures of CPVdi- and triphosphates [CPV-DP, CPV-TP] were provided by Dr. Zemlicka [9,26]. GMP, dGMP, GDP, dGDP, GTP and dGTP were purchased from Sigma Aldrich (St. Louis, MO). Ganciclovir (GCV) was obtained from Syntex (Roche, Basel, Switzerland). Acyclovir monophosphate (ACV-MP) and [8-^{3}H]ganciclovir were purchased from Moravek Biochemicals and Radiochemicals (Brea, CA). The radiochemical purity of [8-^{3}H]ganciclovir was 99.3% and the specific activity was 3.4 Ci/mmol.

2.2. Guanosine monophosphate kinase

Porcine guanosine monophosphate kinase (GMPK) was purchased from Sigma Aldrich (St. Louis, MO) at a concentration of 3.1 units/mL with <0.3% myokinase. (A unit is defined as the amount of enzyme necessary to convert 1.0 μmol of GMP to GDP in 1.0 min at pH 7.5 and 30 °C.)

2.3. HCMV pUL97 protein kinase

GST-UL97 (pUL97) was prepared and purified as previously described [25] and provided through the courtesy of Dr. Donald M. Coen (Harvard Medical School, Boston, MA).

2.4. Enzyme assay

The enzyme assay was adopted from Marshalko et al. [27]. Briefly, kinase buffer (0.05 M Tris, pH 7.6; 0.05 M KCl; 5.0 mM $MgCl_2$), ATP (2.0 mM), BSA (0.1 mg/mL) (all final concentrations), and substrate (GMP, dGMP, GDP, dGDP, CPV-MP, CPV-DP, ACV-MP, GCV; 10.0–500 μM) were incubated at 37 °C for 20 min prior to introduction of enzyme. In experiments in which GCV was the substrate, 4.0 μCi of [^{3}H]GCV per 100 μL of reaction mixture was added. At time zero, porcine GMPK was added to the solution to give a final concentration ranging from 0.01 to 0.2 units/mL. (In experiments with GCV, pUL97 was also added at time zero at a final concentration of 5.0 ng/μL.) At the designated times, aliquots were removed, placed on ice, and proteins precipitated with 0.04 volumes of 10N perchloric acid. The samples were centrifuged, supernatants neutralized with KOH, and stored at −20 °C until analyzed by HPLC.

2.5. Reverse-phase high performance liquid chromatography (HPLC)

CPV and its phosphorylated derivatives (CPV-MP, CPV-DP, CPV-TP) were separated by reverse-phase HPLC [Beckman Coulter (Fullerton, CA) System Gold Programmable Solvent Module 125 and System Gold Programmable Detector Module 166 controlled by 32 Karat Software (version 7.0)]. Reverse-phase HPLC was employed because ADP and ATP were present in the reaction mixtures and have retention times similar to CPV-DP and CPV-TP, respectively, on strong anion exchange HPLC. Before injection, each sample was centrifuged at 15,000 × *g* for 5 min to remove any remaining particulate matter. Supernatants were loaded onto a 10 μm Alphabond C18 300 mm × 3.9 mm reverse phase column (Alltech, Deerfield, IL) at a flow rate of 2.0 mL/min. CPV and its phosphorylated derivatives were eluted using 300 mM ammonium phosphate (pH 3.0) and 100% methanol (linear gradient of 5% methanol over 30 min). Each compound was quantified by comparing their peak area with that of a known amount of the appropriate standard at a wavelength of 254 nm. Retention times for CPV, CPV-MP, CPV-DP, and CPV-TP were 30.3, 18.6, 13.0, and 10.0 min, respectively.

2.6. Strong anion-exchange HPLC

Nucleotides (GMP, dGMP, GDP, dGDP, GTP, dGTP, ADP, ATP) and antiviral nucleotide analogs (ACV-MP, GCV-MP, ACV-DP, GCV-DP) were separated by strong anion-exchange HPLC [Beckman Coulter (Fullerton, CA) Proteome Lab PF 2D Protein Fractionation System controlled by 32 Karat Software (version 7.0)]. Before injection, each sample was centrifuged at 15,000 × *g* for 5 min to remove any remaining particulate matter. Supernatants were loaded onto a Partisil 5 SAX analytical 4.6 mm × 250 mm column (Whatman, Clifton, NJ) at a flow rate of 1.0 mL/min and nucleotides were eluted using a 60 min linear gradient of 10 mM ammonium phosphate (pH 3.0) to 500 mM ammonium phosphate (pH 3.0) followed by 30 min of 500 mM ammonium phosphate (pH 3.0). Nucleotides were quantified by comparing their peak area with that of a known amount of the appropriate standard at a wavelength of 254 nm. For assays with [^{3}H]GCV, 1-min fractions were collected, analyzed, and quantified by liquid scintillation

spectrometry using a Tri-Carb liquid scintillation analyzer (Canberra, Meriden, CT).

2.7. Model development and numerical methods

For both substrates (GMP, CPV-MP), the variables were defined as follows: A is the monophosphate, A^* is the GMPK-bound monophosphate, B is the diphosphate, B^* is the GMPK-bound diphosphate, C is the triphosphate, and E is GMPK. A general schematic of the enzymatic reaction for both substrates is:

$$\mathbf{A} + \mathbf{E} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathbf{A}^* \xrightarrow{k_2} \mathbf{B} + \mathbf{E} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathbf{B}^* \xrightarrow{k_4} \mathbf{C} + \mathbf{E}$$

where all $k_{\pm n}$ are rates of enzyme binding or dissociation. Preliminary experiments determined that the rates of de-phosphorylation are insignificant in comparison with the rates of phosphorylation, and, therefore, not included in this model. The values of $k_{\pm n}$ are unknown. However, the K_M values for the formation of triphosphates from diphosphates ($KM_{BC} = (k_{-3} + k_4)/k_3$) were determined experimentally. The K_M for the formation of GDP from GMP ($KM_{AB} = (k_{-1} + k_2)/k_1$) was also determined experimentally. These K_M values are reported in Section 3.4. Since the K_M for the formation of CPV-DP from CPV-MP could not be determined experimentally, mathematical modeling was employed to estimate this value based on the experimental data presented herein.

The mathematical model consists of six ordinary differential equations with six unknown parameters. We extend the traditional framework commonly used for enzyme kinetics modeling of two substances to account for three forms of substance [28]. This model assumes that the total amount of compound (the sum of the mono-, di-, and triphosphate forms) and enzyme is conserved. Furthermore, it assumes that once phosphorylated, a substance does not dephosphorylate to degrade to a simpler form. Under such assumptions, we arrive at the following system of equations:

$$\frac{dA}{dt} = -k_1AE + k_{-1}A^*$$

$$\frac{dA^*}{dt} = k_1AE - k_{-1}A^* - k_2A^*$$

$$\frac{dB}{dt} = k_2A^* - k_3BE + k_{-3}B^*$$

$$\frac{dB^*}{dt} = k_3BE - k_{-3}B^* - k_4B^*$$

$$\frac{dC}{dt} = k_4B^*$$

$$\frac{dE}{dt} = -k_1AE + k_{-1}A^* + k_2A^* - k_3BE + k_{-3}B^*$$

To solve the system of equations, we used the MATLAB (MathWorks, Natick, MA) solver *ode15s*. Data points for mono-, di-, and triphosphate were randomly selected within the experimentally recorded ranges shown in Figs. 2a and 3a. To reduce the number of parameters, we note that $k_{-3} = (KM_{BC})(k_3) - k_4$, since KM_{BC} is a known value for both substrates. The MATLAB command *lsqcurvefit* was used to determine the optimal values of the remaining unknown parameters according to least-squares curve fitting. The K_M value for the conversion of A to B was then calculated according to $KM_{AB} = (k_{-1} + k_2)/k_1$. The process was repeated for a total of 500 random samples each resulting in a model-derived KM_{AB} value. From this large sample of KM_{AB} values, the mean and median values were calculated to predict the actual value of KM_{AB} based on the mathematical model and existing experimental data.

3. Results

3.1. Phosphorylation of CPV-MP by GMPK

We have previously reported the enantioselective phosphorylation of (+)-CPV-MP by bovine GMPK and that (+)-CPV-MP has K_M and V_{Max} values similar to those of GCV-MP [19,26]. We have now examined more extensively the role of porcine GMPK in the phosphorylation of CPV-MP. A time-course study revealed that this enzyme is responsible not only for the formation of CPV-DP, but CPV-TP as well (Fig. 2a). In fact, after 4 h of incubation, the formation of CPV-TP was approximately 2.5 times greater than that of CPV-DP.

To determine directly if CPV-DP is a substrate for GMPK or if GMPK mediates the transfer of a pyrophosphate to CPV-MP resulting in the one-step formation of CPV-TP, a racemic mixture of CPV-DP was incubated with enzyme (it was necessary to use the racemic mixture because the separated enantiomers of CPV-DP were not available). The formation of CPV-TP occurred in a time-dependent manner corresponding to the loss of the (+)-CPV-DP (Fig. 2b). In addition, no AMP formation was detected in the HPLC effluent at any time, which would occur if a pyrophosphate from ATP was transferred to CPV-MP. Therefore, we conclude that both CPV-MP and CPV-DP are substrates for GMPK and that this enzyme is responsible for mediating the formation of CPV-TP from CPV-DP.

3.2. GMPK phosphorylation of naturally occurring guanosine nucleotides

Based upon these results, we also studied naturally occurring guanosine nucleotides as substrates for porcine GMPK. Incubation of GMP with enzyme resulted in the formation of both GDP and GTP (Fig. 3a). Unlike CPV-MP, incubation of this natural substrate with GMPK resulted in approximately two-times greater formation of diphosphate than triphosphate. To confirm the phosphorylation of GDP to GTP with GMPK, we incubated GDP with enzyme

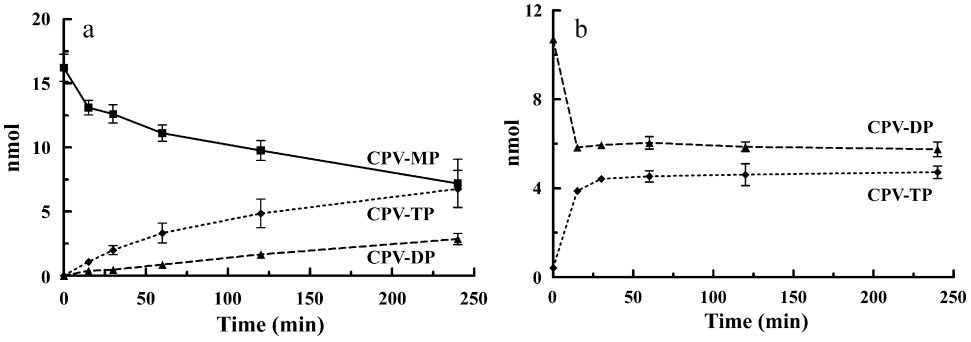

Fig. 2. GMPK catalyzed phosphorylation of CPV-MP and CPV-DP. GMPK was incubated with 500 μM CPV-MP (a) or CPV-DP (b) and the levels of mono- (■), di- (▲), and triphosphates (◆) were determined by UV absorption of HPLC effluent. The values represent the mean ± standard deviation from at least two experiments.

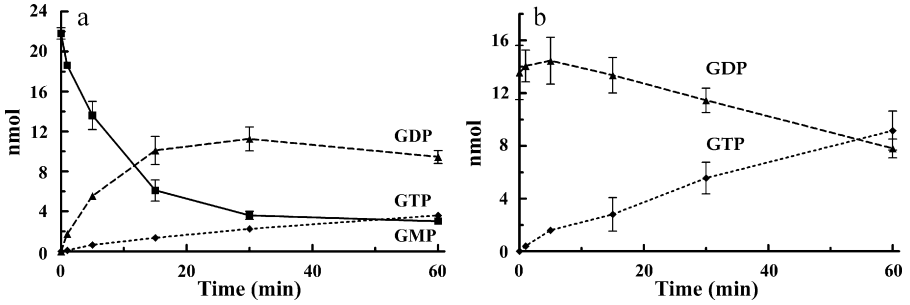

Fig. 3. GMPK catalyzed phosphorylation of GMP and GDP. GMPK was incubated with 500 μM of either GMP (a) or GDP (b) and the levels of mono- (■), di- (▲), and triphosphates (◆) were determined by UV absorption of HPLC effluent. The values represent the mean ± standard deviation from at least two experiments.

and observed that GMPK did phosphorylate GDP to GTP (Fig. 3b). Similar time course experiments with dGMP and dGDP as substrates gave dGDP and dGTP as products with time-course results nearly identical to those seen in Fig. 3 (data not presented). Therefore, we conclude that like GMP and GDP, their 5′-deoxy homologs are substrates for porcine GMPK as well.

3.3. GMPK phosphorylation of antiviral guanosine nucleotide analogs

We also examined the phosphorylation of the monophosphates of the structurally related antiviral drugs acyclovir and ganciclovir (Fig. 1). As previously reported by Miller and Miller [16], acyclovir monophosphate (ACV-MP) is a substrate for GMPK. Incubation of ACV-MP with GMPK resulted in the formation of ACV-DP with a corresponding decline of ACV-MP (Fig. 4). However, unlike CPV-MP, GMP, and dGMP, no triphosphate (ACV-TP) was formed.

Similar results were obtained with the monophosphate of ganciclovir (GCV-MP) as a substrate. Even though this compound itself was not available, we formed it *in situ* and then studied its phosphorylation by incubating GCV with both pUL97 and GMPK [19,25]. GCV-MP formed by pUL97 was phosphorylated to GCV-DP by GMPK but no GCV-TP was detected (Fig. 5). Together, these results demonstrate a previously unreported substrate specificity for GMPK.

3.4. Determination of kinetic constants

With the discovery that GMPK recognized both mono- and diphosphates as substrates, we determined the kinetic constants for both types of substrates. Preliminary experiments established that all conditions for Michaelis–Menten kinetic analysis including steady-state, saturation of substrate, and multiple turnovers, were met; kinetic constants were determined by Lineweaver–Burk methodology (Table 1). The K_M concentrations obtained for GMP and dGMP (69 ± 22 and 80 ± 25 μM, respectively) are similar to those previously reported [16,19]. The K_M for ACV-MP (410 ± 40 μM) was approximately six-times greater than GMP, which is also similar to what has been previously reported [16]. The theoretical K_M value for CPV-MP (as determined by the math model described below) was somewhat lower than that for ACV-MP but higher than the values for the natural substrates GMP and dGMP.

Interestingly, the K_M value for CPV-DP (45 ± 15 μM) was significantly lower than the value for CPV-MP (332 ± 60 μM). This is consistent with the greater accumulation of CPV-TP noted in Fig. 2a. The K_M value for CPV-DP is similar to those for GMP and dGMP (Table 1), as well as that reported for GCV-MP [19] suggesting that the diphosphate form of CPV has at least as good an affinity for the substrate binding pocket of GMPK as that of the monophosphate forms of the natural substrates and the antiviral used as the standard of treatment for HCMV, GCV.

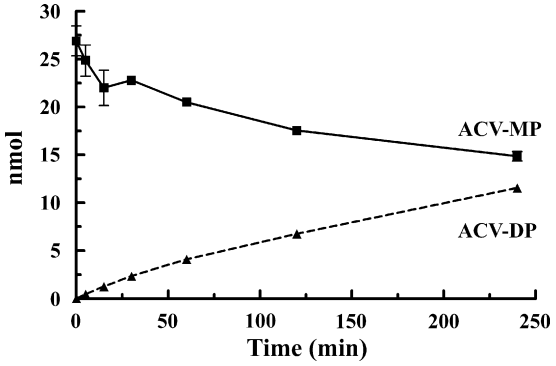

Fig. 4. Phosphorylation of ACV-MP by GMPK. The time-dependent formation of ACV-DP (▲) was determined by incubating 500 μM ACV-MP (■) with GMPK, separating products by strong anion exchange HPLC and monitoring UV absorption of effluents. The values represent the mean ± standard deviation from at least two experiments.

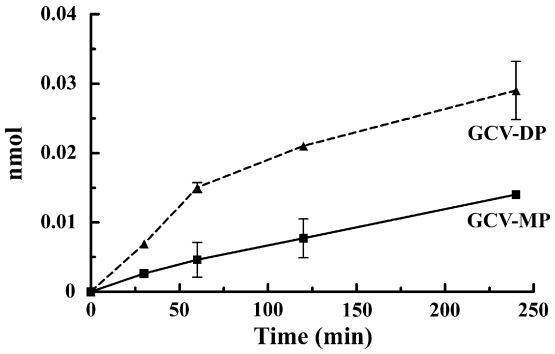

Fig. 5. Phosphorylation of GCV to GCV-DP by pUL97 and GMPK. The time-dependent formation of GCV-MP (■) and GCV-DP (▲) was determined by incubating 500 μM [^{3}H]GCV with both enzymes, separating products by strong anion exchange HPLC, collecting 1 min fractions of effluent, and analysis by liquid scintillation spectrometry. The values represent the mean ± standard deviation from two experiments.

Table 1
Kinetic constants for substrates of porcine GMPK.

Substrate	K_M (μM)	V_{Max} (nmol/min)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1} M^{-1}$)
Monophosphates				
GMP[a]	69 ± 22	0.31 ± 0.05	0.06	870
dGMP[a]	80 ± 25	0.18 ± 0.09	0.03	375
ACV-MP[a]	410 ± 40	0.09 ± 0.01	0.004	10
CPV-MP[b]	332 ± 60	N.D.	N.D.	N.D.
Diphosphates				
GDP[a]	520 ± 50	0.41 ± 0.14	0.08	150
dGDP[a]	220 ± 110	0.33 ± 0.17	0.06	270
CPV-DP[a]	45 ± 15	0.17 ± 0.06	0.008	180

N.D.: value not determined.
[a] Values determined by Lineweaver–Burk analysis of experimental data.
[b] Value determined by mathematical model based on data presented in Figs. 2a and 3a.

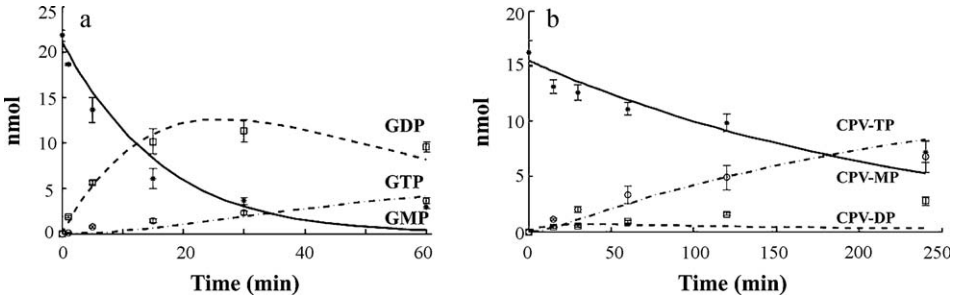

Fig. 6. Mathematical model simulations. For both GMP (a) and CPV-MP (b), the mathematically obtained median KM_{AB} values and experimentally reported KM_{BC} values (Table 1) were used. The smooth curves are the solution curves for the mono- (*), di- (□), and triphosphates (○) based on the mathematical model equations. The error bars denote the experimentally recorded data.

3.5. Mathematical model to determine K_M value for CPV-MP

We were unable to experimentally determine the K_M value for CPV-MP with GMPK because CPV-TP formed more rapidly and to a greater extent than the product to be measured, CPV-DP (Fig. 2a). Therefore, a mathematical model using the existing data from Figs. 2a and 3a was used to determine a theoretical K_M value for CPV-MP. To validate the mathematical model, simulations were first run for GMP to compare the experimentally obtained K_M value with the mathematical prediction (KM_{AB} value in math model). For this endogenous substrate, the experimental K_M was 69 ± 22 μM; likewise, the experimental K_M for GDP (KM_{BC} in math model) was 520 ± 50 μM (Table 1). Using a random sample of 500 trials in which KM_{BC} was fixed at 520 μM, the mathematical model predicted a range of KM_{AB} values lying between 64 and 654 μM, with a mean value of 125 ± 100 μM, a median value of 78 μM, and the interquartile range lying between 72 and 129 μM. A simulation of the rate of phosphorylation of GMP to GDP and GTP using the mathematically obtained median KM_{AB} value and experimentally reported K_M value for GDP is depicted in Fig. 6a. It is worth noting that outliers in the model calculations of KM_{AB} skew the derivation of the mean value, and, therefore, the median value is a more accurate prediction. For example, elimination of values beyond two standard deviations of the mean gives the mean value of the remaining data of 107 μM, while the median still remains 78 μM. Thus the close correspondence between the median value of 78 μM and the experimentally determined value of 69 ± 22 μM (Table 1) validates the model for the natural substrate.

Next, the mathematical model was employed to make predictions for the unknown KM_{AB} value for CPV-MP. Experimental data demonstrated that the K_M for CPV-DP was 45 ± 15 μM (Table 1). Using a random sample of 500 trials in which KM_{BC} was fixed at 45 μM, the mathematical model predicted a range of KM_{AB} values lying between 211 and 532 μM, with a mean KM_{AB} value of 332 ± 60 μM (Table 1), a median value of 327 μM, with the interquartile range lying between 284 and 371 μM. A simulation of the rate of phosphorylation of CPV-MP to CPV-DP and CPV-TP using the mathematically obtained median KM_{AB} value and experimentally reported K_M value for CPV-DP is depicted in Fig. 6b. It is important to note that there are many less outliers in the model calculations of KM_{AB} for CPV-MP compared to GMP, and, therefore, the mean was not skewed. This is apparent in that the mean and median are very close in value.

4. Discussion

We have previously demonstrated that CPV is phosphorylated to its monophosphate by the HCMV pUL97 phosphotransferase [25]. It also has been demonstrated that CPV inhibits HCMV DNA synthesis resulting in inhibition of viral replication [10]. Therefore, we hypothesize that CPV elicits its antiviral effect in a manner similar to that of GCV; namely, phosphorylation of parent compound to a triphosphate resulting in inhibition of the viral DNA polymerase [3,7]. We have now demonstrated that like GCV-MP, CPV-MP is phosphorylated to its diphosphate by GMPK [19]. However, unlike GCV-MP, we observed that CPV-MP is not only phosphorylated to a diphosphate by GMPK, but to its triphosphate as well. Substrate specificity studies with GMPK revealed that this enzyme also phosphorylates the natural substrates GMP and dGMP to triphosphates but not the antiviral drugs acyclovir and ganciclovir.

Similar to the current results, we previously reported that bovine GMPK phosphorylates CPV-MP to a diphosphate with the (+)-enantiomer as the preferred substrate [26]. In those experiments with the bovine enzyme, triphosphate was produced but only at levels that were 16 times less than that of diphosphate (data not shown). In fact, when CPV-DP was incubated with bovine GMPK for 4 h, little to no triphosphate formation was observed (data not shown). In the same manner, when GMP was incubated with bovine GMPK, little to no GTP formed (data not shown). These results are in contrast to those for porcine GMPK reported herein. There is a 90% amino acid sequence homology amongst human, bovine, and porcine GMPK; in those regions identified as integral for the function of the enzyme (substrate binding pocket, ATP binding site), there is no amino acid difference between the enzymes [29]. We, therefore, hypothesize that the amino acid differences found outside of the substrate binding pocket result in small, but significant structural changes which allow one enzyme (porcine) to accept a diphosphate substrate whereas the other (bovine) prefers monophosphates. Differences in substrate specificity between the porcine and bovine enzymes should not be surprising. Despite extensive sequence similarity to its eukaryotic counterparts, *E. coli* GMPK is structurally and enzymatically different [30].

To our knowledge, this is the first report of nucleoside diphosphates as substrates for GMPK when the natural phosphate donor, ATP, was used. Previous reports demonstrated the formation of GTP from GDP by GMPK, but GDP was both the phosphate donor and acceptor in those experiments and no kinetic values for GDP were provided [31]. Other reports measuring GMPK activity may have failed to detect triphosphate formation because products were not measured directly [12,16,19,32]. Instead, the indirect classical assay developed by Agarwal et al. [33] was used. This coupled GMPK/pyruvate kinase–lactate dehydrogenase assay does not detect nucleotide products directly, it only measures NADH absorbance changes at 340 nm. Thus our use of direct HPLC assay establishes that GMPK is capable of accommodating more than just monophosphate substrates.

Even though CPV-DP is phosphorylated by GMPK, it is possible that other cellular enzymes also recognize it as a substrate. Similar

to the phosphorylation of ACV-DP to its triphosphate by numerous cellular enzymes, CPV-DP could also be phosphorylated to its triphosphate by some or all of these enzymes including phosphoglycerate kinase, pyruvate kinase, and nucleoside diphosphate kinase [17]. If true, multiple cellular pathways that facilitate the conversion of CPV to a triphosphate would aid in its effectiveness as an antiviral agent.

Because other enzymes phosphorylate GDP and dGDP and these could phosphorylate CPV-DP as well, we have considered the possibility that triphosphate formation in our experiments was not due to GMPK, but rather was caused by a contaminating enzyme. We view this to be highly unlikely, however, because the only detected contaminating enzymatic activity was $\leq$0.3% myokinase (AMP kinase) for both the bovine and porcine GMPK preparations (see Section 2.2). This enzyme phosphorylates monophosphates, not diphosphates, and it does not recognize GMP as a substrate [34], making it even less likely that a myokinase contamination could be responsible for the triphosphate formation we observed. Our observation that the levels of CPV-TP and GTP formed by the bovine preparation were only 10–20% of those observed for the porcine preparation supports this conclusion because the reported levels of myokinase were similar in each preparation. It is also highly unlikely that enzymes known to phosphorylate antiviral nucleoside diphosphate analogs – such as phosphoglycerate kinase, pyruvate kinase, and nucleoside diphosphokinase – were present in the GMPK preparations. Our results demonstrating the formation of ACV-DP but not ACV-TP (Fig. 4) argue that such contaminants were not present because ACV-DP is a known substrate of these enzymes [17].

Due to its methylenecyclopropane ring, the chemical properties of CPV are significantly different from those of GMP. Although both CPV-MP and GMP are capable of being converted to their respective triphosphates by GMPK, it is clear that the diphosphate of CPV is a better substrate for GMPK than the monophosphate. This is in stark contrast to the natural substrate in which GMP is clearly the better substrate compared to GDP. Thus we hypothesize that the highly hydrophobic methylenecyclopropane ring of CPV binds to GMPK differently than that of the ribose sugar ring of GMP. We further hypothesize that the substrate binding pocket of GMPK is more suited to accommodate CPV-DP than CPV-MP.

Because the K_M for CPV-DP formation from CPV-MP could not be determined experimentally (due to the rapid formation of CPV-TP), mathematical modeling was utilized to predict this value. In order to test the model's effectiveness, simulations were first run with GMP to mathematically derive a K_M (KM_{AB} in the math model) value that could be compared to the experimentally derived K_M. For calculations using data for this endogenous substrate (Fig. 3a), we found that outlier data significantly skewed the calculation of the mean value. This occurred because the model assumes that the total amount of substrate is conserved throughout the course of the experiment whereas experimental data exhibited a slight loss of substrate compared to the initial amount of monophosphate with the total of mono-, di-, and triphosphates at the end of the time course (Fig. 3a). This lack of conservation caused deviations in parameter estimation, which in turn contributed to the production of outliers in calculations of the KM_{AB} value. In contrast, the median value of KM_{AB} = 78 μM was more robust and in line with the experimentally determined value of 69 ± 22 μM (Table 1).

When the model was used to predict the unknown KM_{AB} value for CPV-MP, outlier data were not as significant and the mean and median values, 332 and 327, respectively, were very close. We note that the experimentally recorded observations for the mono-, di-, and triphosphate forms are better conserved throughout the course for CPV-MP (Fig. 2a) than for GMP (Fig. 3a), which explains why outlier values are less significant for the CPV data. The experimentally determined K_M for CPV-DP (KM_{BC} value in math model) of 45 ± 15 μM is significantly lower than all mathematically predicted values for KM_{AB}, which ranged from 211 to 532 μM. Therefore, the model predicts that $KM_{AB} > KM_{BC}$ for CPV-MP.

For each substrate, KM_{BC} values were calculated using early time points with respect to the entire duration of the experiment. As a result, the solution curves produced by the mathematical model are more accurate for the earlier stages of the experiment compared to the later time points. Steady-state analysis of the model equations predicted mono- and diphosphate substrates will eventually approach zero, while the triphosphate products will approach the initial monophosphate value. If experimental evidence to the contrary were found in which mono- and diphosphates approach a non-zero steady state, then the mathematical equations would be more accurate for modeling earlier stages of the experimental data and less accurate for determining the long-run equilibrium amongst the substrate. However, since the known K_M values are calculated from data in the initial stages of each experiment, the mathematical equations employed here are sufficient to predict the unknown K_M value for CPV-MP.

When examining the binding of each substrate to GMPK and its subsequent enzyme catalyzed phosphorylation, it appears that a rigid structure attached to the guanine base – such as those in CPV-MP, GMP and dGMP – is necessary for further phosphorylation to a triphosphate. Without a rigid structure, such as the aliphatic chains of ACV-MP and GCV-MP, the enzyme appears to be incapable of binding the diphosphate and, as a result, cannot produce a triphosphate. Therefore, we hypothesize that a rigid structure, such as a methylenecyclopropane ring or ribose sugar, is necessary for diphosphate binding and enzyme-mediated phosphorylation to a triphosphate.

The data presented herein demonstrate different phosphorylation patterns of GMPK substrates that have different chemical and structural properties. CPV-MP combines the properties of the natural substrate GMP – which allows phosphorylation to a triphosphate by GMPK – with the antiviral action of ACV and GCV [3,5,7] – which are not converted to triphosphates by GMPK. Compared to GCV, the current standard of treatment for HCMV diseases, CPV is a better substrate for pUL97, the enzyme responsible for the initial phosphorylation of both drugs [25]. When combined with the facts that CPV has increased antiviral activity compared to GCV [9] and it does not need to be made into a prodrug to give good oral bioavailability [35,36], further examination into the mechanism of action and pre-clinical development of this compound appears to be warranted.

Conflicts of interest

None.

Acknowledgments

We thank Yasuo Yamakoshi and James Simmer for assistance with the use of Dr. Simmer's HPLC system. This work was supported by grants from NIH (CA32779) and Microbiotix, Inc., plus funds from the University of Michigan.

References

[1] Ho M. The history of cytomegalovirus and its diseases. Med Microbiol Immunol 2008;197:65–73.
[2] Whitley RJ, Jacobson MA, Friedberg DN, Holland GN, Jabs DA, Dieterich DT, et al. Guidelines for the treatment of cytomegalovirus diseases in patients with AIDS in the era of potent antiretroviral therapy. Arch Intern Med 1998;158:957–69.
[3] Faulds D, Heel RC. Ganciclovir: A review of its antiviral activity pharmacokinetic properties and therapeutic efficacy in cytomegalovirus infections. Drugs 1990;39:597–638.

[4] Alrabiah FA, Sacks SL. New antiherpesvirus agents: their targets and therapeutic potential. Drugs 1996;52:17–32.
[5] Noble S, Faulds D. Ganciclovir: An update of its use in the prevention of cytomegalovirus infection and disease in transplant recipients. Drugs 1998; 56:115–46.
[6] Emery VC, Hassan-Walker AF. Focus on new drugs in development against human cytomegalovirus. Drugs 2002;62:1853–8.
[7] Biron KK. Antiviral drugs for cytomegalovirus diseases. Antiviral Res 2006; 71:154–63.
[8] Zemlicka J, Chen X. Methylenecyclopropane analogs of nucleosides as antiviral agents. In: Schinazi RF, Liotta DC, editors. Frontiers in nucleosides and nucleic acids. College Park, Georgia: IHL Press; 2004. p. 267–307.
[9] Zhou S, Breitenbach JM, Borysko KZ, Drach JC, Kern ER, Gullen E, et al. Synthesis and antiviral activity of (*Z*)- and (*E*)-2,2-[bis(hydroxymethyl)cyclopropylidene]methylpurines and –pyrimidines: second-generation methylenecyclopropane analogues of nucleosides. J Med Chem 2004;47:566–75.
[10] Kern ER, Kushner NL, Hartline CB, Williams-Aziz SL, Harden EA, Zhou S, et al. In vitro activity and mechanism of action of methylenecyclopropane analogs of nucleosides against herpesvirus replication. Antimicrob Agents Chemother 2005;49:1039–45.
[11] Kern ER, Bidanset DJ, Harline CB, Yan Z, Zamlicka J, Quenelle DC. Oral activity of a methylenecyclopropane analog, cyclopropavir, in animal models for cytomegalovirus infections. Antimicrob Agents Chemother 2004;48:4745–53.
[12] Miech RP, Parks Jr RE. Adenosine triphosphate: guanosine monophosphate phosphotransferase. J Biol Chem 1965;240:351–7.
[13] Griffith TJ, Helleiner CW. The partial purification of deoxynucleoside monophosphate kinases from l cells. Biochem Biophys Acta 1965;108:114–24.
[14] Kimura N, Shimada N, Fukuda M, Ishijima Y, Miyazaki H, Ishii A, et al. Regulation of cellular functions by nucleoside diphosphate kinases in mammals. J Bioenerg Biomembr 2000;32:309–15.
[15] Elion GB, Furman PA, Fyfe JA, De Miranda P, Beauchamp L, Schaeffer HJ. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)-guanine. Proc Natl Acad Sci USA 1977;74:5716–20.
[16] Miller WH, Miller RL. Phosphorylation of acyclovir (acycloguanosine) monophosphate by gmp kinase. J Biol Chem 1980;255:7204–7.
[17] Miller WH, Miller RL. Phosphorylation of acyclovir diphosphate by cellular enzymes. Biochem Pharmacol 1982;31:3879–84.
[18] Biron KK, Stanat SC, Sorrell JB, Fyfe JA, Keller PM, Lambe CU, et al. Metabolic activation of the nucleoside analog 9-{[2-hydroxy-1-(hydroxymethyl)-ethoxy]methyl}guanine in human diploid fibroblasts infected with human cytomegalovirus. Proc Natl Acad Sci USA 1985;82:2473–7.
[19] Boehme RE. Phosphorylation of the antiviral precursor 9-(1,3-dihydroxy-2-propoxymethyl)guanine monophosphate by guanylate kinase isozymes. J Biol Chem 1984;259:12346–9.
[20] Smee DF, Boehme R, Chernow M, Binko BP, Matthews TR. Intracellular metabolism and enzymatic phosphorylation of 9-(1,3-dihydroxy-2-propoxymethyl)guanine and acyclovir in herpes simplex virus-infected and uninfected cells. Biochem Pharmacol 1985;34:1049–56.
[21] Chou S, Waldemer RH, Senters AE, Michels KS, Kemble GW, Miner RC, et al. Cytomegalovirus UL97 phosphotransferase mutations that affect susceptibility to ganciclovir. J Infect Dis 2002;185:162–9.
[22] Gilbert C, Boivin G. Human cytomegalovirus resistance to antiviral drugs. Antimicrob Agents Chemother 2005;49:873–83.
[23] Gohring K, Mikeler E, Jahn G, Hamprecht. Rapid simultaneous detection by real-time PCR of cytomegalovirus ul97 mutations in codons 460 and 520 conferring ganciclovir resistance. J Clin Microbio 2006;44:4541–4.
[24] Schregel V, Auerochs S, Jochmann R, Maurer K, Stamminger T, Marschall M. Mapping of a self-interaction domain of the cytomegalovirus protein kinase pUL97. J Gen Virol 2007;88:395–404.
[25] Gentry BG, Kamil JP, Coen DM, Zemlicka J, Drach JC. Stereoselective phosphorylation of cyclopropavir by pUL97 and competitive inhibition by maribavir. Antimicrob Agents Chemother 2010;54:3093–8.
[26] Li C, Gentry BG, Drach JC, Zemlicka J. Synthesis and enantioselectivity of cyclopropavir phosphates for cellular GMP kinase. Nucleos Nucleot Nucleic Acids 2009;28:795–808.
[27] Marshalko SJ, Schweitzer BI, Beardsley GP. Chiral chemical synthesis of DNA containing (S)-9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) and effects on thermal stability, duplex structure, and thermodynamics of duplex formation. Biochemistry 1995;34:9235–48.
[28] Edelstein-Keshet L. Models for molecular events. In: Mathematical models in biology. Boston, MA: McGraw Hill; 1988. pp. 310.
[29] Fitzgibbon J, Katsanis N, Wells D, Delhanty J, Vallins W, Hunt DM. Human guanylate kinase (GUK1): cDNA sequence, expression, and chromosomal localization. FEBS Lett 1996;385:185–8.
[30] Gentry D, Bengraj C, Ikeharall K, Cashel M. Guanylate kinase of *Escherichia coli* K-12. J Biol Chem 1993;268:14316–21.
[31] Prinz H, Lavie A, Scheidig AJ, Spangenberg O, Konrad M. Binding of nucleotides to guanylate kinase, $p21^{ras}$, and nucleoside-disphosphate kinase studied by nano-electrospray mass spectrometry. J Biol Chem 1999;274:35337–42.
[32] Auvynet C, Topalis D, Caillat C, Munier-Lehmann H, Seclaman E, Balzarini J, et al. Phosphorylation of dGMP analogs by vaccinia virus TMP kinase and human GMP kinase. Biochem Biophys Res Commun 2009;388:6–11.
[33] Agarwal KC, Miech RP, Parks Jr RE. Guanylate kinases from human erythrocytes, hog brain, and rat liver. In: Hoffee PA, Jones ME, editors. Methods in enzymology, vol. LI. New York: Academic Press Inc.; 1978. p. 483–90.
[34] Tomasselli AG, Noda LH. Mitochondria1 ATP: AMP phosphotransferase from beef heart: purification and properties. Eur J Biochem 1980;103:481–91.
[35] Curran M, Noble S. Valganciclovir. Drugs 2001;61:1145–50.
[36] Bowlin TL, Brooks JL, Zemlicka J. Preclinical pharmacokinetic, toxicokinetic and toxicology results for cyclopropavir, a promising new agent for the treatment of beta- and gamma-herpesviruses. Antiviral Res 2009;82:A46–7.