Published in final edited form as:

Antiviral Res. 2015 July ; 119: 23–27. doi:10.1016/j.antiviral.2015.04.001.

TAOK3 Phosphorylates the Methylenecyclopropane Nucleoside MBX 2168 to its Monophosphate

Gloria Komazin-Meredith^{1,*}, Steven C. Cardinale¹, Katelyn Comeau¹, Kevin J. Magalhaes¹, Carol B. Hartline², John D. Williams¹, Timothy J. Opperman¹, Mark N. Prichard², and Terry L. Bowlin¹

¹Microbiotix, Inc., Worcester MA, USA

²University of Alabama at Birmingham, Birmingham, AL, USA

Abstract

Monohydroxymethyl methylenecyclopropane nucleosides (MCPNs) with ether or thioether substituents at the 6-position show promise as broad-spectrum herpes virus inhibitors. Their proposed mechanism of action involves sequential phosphorylation to a triphosphate, which can then inhibit viral DNA polymerase. The inhibition of herpes simplex virus (HSV) by these compounds is not dependent on the viral thymidine kinase (TK), which is known to phosphorylate acyclovir (ACV), a standard treatment for HSV infections. Previous studies on the mechanism of action of these compounds against human cytomegalovirus (HCMV) implicated a host kinase in addition to HCMV UL97 kinase in performing the initial phosphorylation. After first eliminating other candidate HSV-1 encoded kinases (UL13 and US3) as well as potential host nucleoside kinases, using activity-based fractionation, we have now identified the host serine-threonine protein kinase TAOK3 as the kinase responsible for transforming the representative monohydroxymethyl MCPN analog MBX 2168 to its monophosphate.

Keywords

Mechanism of Action; Monohydroxymethyl methylenecyclopropane nucleosides; Herpes simplex virus; TAOK3

1. Introduction

Herpes simplex viruses (HSV) cause a wide spectrum of diseases in humans, which may be life-threatening in immunodeficient patients and neonates (Ann Arvin, 2007). Following primary infection, HSV establishes a long-term latency in the ganglia of the sensory neurons, and can reactivate episodically. The antiviral drug acyclovir (ACV), a guanosine analog, is the therapy of choice for HSV infections (Ann Arvin, 2007; Chou et al., 2014;

*Corresponding author. Current address: Pennsylvania State University, Department of Biochemistry and Molecular Biology, South Frear 207, University Park, PA 16802. Phone: (814) 867-6165. gmk21@psu.edu.

Conflicts of interest statement

Gloria Komazin-Meredith, Steven Cardinale, John Williams, Timothy Opperman, and Terry Bowlin are former or current employees of Microbiotix Inc.

Rozenberg et al., 2011; Whitley, 2012). Upon host cell entry, ACV is sequentially phosphorylated to an active ACV-triphosphate metabolite before it can inhibit the viral DNA polymerase (Derse et al., 1981; Furman et al., 1984; St Clair et al., 1984). The first phosphate is added by a viral thymidine kinase (TK) and mutations in HSV that impart resistance to ACV typically map to this kinase although mutations can occur in the DNA polymerase (Piret and Boivin, 2011). While ACV is an effective treatment, the emergence of HSV strains resistant to ACV is a significant problem (Piret and Boivin, 2011).

A number of monohydroxymethyl methylenecyclopropane nucleosides (MCPNs) with ether or thioether substituents at the 6-position show promise as broad spectrum anti-herpes agents as they inhibit HSV-1 and 2, varicella-zoster virus (VZV), human cytomegalovirus (HCMV), human herpes virus type 6 (HHV-6), human herpes virus type 8 (HHV-8) and Epstein-Barr virus (EBV) (Prichard et al., 2013). They are analogs of dihydroxymethyl MCPN cyclopropavir (CPV) (Figure 1), which is currently in a Phase I clinical trial for the treatment of HCMV infections. CPV also inhibits EBV, HHV-6 and HHV-8 but does not effectively inhibit HSV (Kern et al., 2005). The proposed mechanism of action of these compounds involves their sequential phosphorylation to a triphosphate, which then inhibits viral DNA polymerase. We previously showed that the HCMV protein kinase UL97 and the HHV-6 protein kinase U69 are involved in phosphorylation of MCPNs to their monophosphates (Komazin-Meredith et al., 2013; Komazin-Meredith et al., 2014). The antiviral activity of these compounds was, however, less dependent on the activity of the HCMV UL97 kinase than was CPV, suggesting the involvement of a host enzyme in their phosphorylation (Komazin-Meredith et al., 2014). Unlike ACV, monohydroxymethyl MCPNs also do not require HSV TK for activity, which could be promising for treatment of ACV-resistant infections with resistance mapping to the viral TK (Prichard et al., 2013). In this study, we investigated the mechanism of activation of a representative monohydroxymethyl MCPN analog with a 6-ether moiety, MBX 2168 (Figure 1), against HSV-1 by identifying a host kinase that phosphorylates MBX2168 to form the monophosphate.

2. Materials and Methods

2.1 Antiviral Compounds

The MCPN analogs MBX 2168, CPV and synguanol were synthesized at Microbiotix Inc. (Worcester, MA). Ganciclovir (GCV), acyclovir (ACV), and cidofovir (CDV) were obtained from Sigma-Aldrich (St. Louis, MO).

2.2 Cells culture and virus strains

Vero and Human Foreskin Fibroblast (HFF) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The HSV-1 strain F was obtained from the ATCC. Recombinant viruses R4970 (UL13⁻, TK⁻, HCMV UL97⁺), R7041 (UL13⁻), and R7356 (US3⁻) were a generous gift from Dr. Bernard Roizman (The University of Chicago, Chicago IL). The E-377 and DM2.1 strains of HSV-1 were characterized as reported previously (Prichard et al., 2009).

2.3 Plaque reduction assay

Vero cells were seeded in 6-well plates at 300,000 cells per well. The next day, they were infected with 100 plaque forming units (PFU) per well in DMEM media plus 5 % FBS. One to two hours post-infection, media containing selected drug dilutions and final FBS concentration of 5% and 0.5% methylcellulose were added. All drug dilutions, ranging in concentration from 0.01 μ M to 100 μ M, were tested in duplicate. After incubation at 37 °C for 2 to 3 days, cell monolayers were stained with crystal violet, and plaques were enumerated under light microscopy. The concentration of compound that reduced plaque formation by 50% (EC_{50}) was calculated using GraphPad Prism 5 software.

2.4 DNA synthesis assay

HFF cells were seeded in 96-well plates at a concentration of 2.5×10^4 cells/well in tissue culture media with 10% FBS and were incubated 24 h at 37 °C. Tissue culture media was removed from wells and two-fold serial dilutions of test compounds were prepared directly in the plates in media with 2% FBS with the highest compound concentration at 50 μ M. Cell monolayers were then infected at an MOI of 0.05 PFU/cell and incubated for 24 h. Total DNA was extracted using Wizard SV 96 Genomic DNA Purification System (Promega, Madison, WI). The total genome copy number was determined by real-time PCR using an ABI 7300 real-time PCR system as described previously (Prichard et al., 2011). Genome copy number was quantified using plasmid pMP031 to provide absolute quantification. The primers used were 5'-ACC GCC GAA CTG AGC-3' and 5'-TGA GCT TGT AAT ACA C-3', with the probe 5'-6FAM-CGC GTA CAC CAA CAA GCG CC-BHQ. They detect sequences within the DNA polymerase open reading frame that correspond to coordinates 65824 to 65899. (GenBank accession number GU734771.1) The total genome copy number for each compound concentration was determined from triplicate wells and compared to the virus control wells that did not contain compound.

2.5 Identification of host kinase that phosphorylates MBX 2168

To identify host kinase responsible for phosphorylating MBX 2168, HFF cells were grown in ten T150 flasks until they were confluent. HFF extracts were loaded onto an anion exchange HiTrap Q HP column (GE Healthcare) and eluted using a 100 mM to 1 M NaCl linear gradient in a buffer containing 50 mM Tris-HCl, pH 8.0, 2 mM DTT, and 20% glycerol. The flow-through as well as eluting fractions were assayed for MBX 2168 kinase activity. The active protein was in the flow-through, which was then loaded onto a cation exchange HiTrap S HP column (GE Healthcare) and eluted using a 100 mM to 1 M NaCl linear gradient. The active fractions from the SP column were then loaded onto a gel filtration column, Superdex200 (GE Healthcare), which was pre-equilibrated in buffer containing 5% glycerol, 20 mM Tris, pH 8.0, 1 mM DTT and 150 mM NaCl. The active fraction from the gel filtration column was run on an unresolved short SDS polyacrylamide gel and sent for Electrospray Ionization (LC-MS/MS) analysis at the Proteomics and Mass Spectrometry Facility at the University of Massachusetts Medical School, Shrewsbury, MA. Recombinant human adenylate kinase 5 was purchased from ProSpec-Tany TEchnoGene Ltd. (Ness Ziona, Israel) and recombinant human PKN1 and PKN2 serine/threonine protein kinases were purchased from Invitrogen.

2.6 Protein expression and purification

Human deoxyguanosine kinase (dGK) in a pUC57 vector was purchased from Genscript. It was cloned into a pET19b vector (Novagen), transformed into BL21 DE3 pLysS cells (Novagen) and grown until O.D. reached 0.6, before inducing protein expression with IPTG. After IPTG induction, cells were grown for 20 hours at 16 °C. Cells were lysed using a French Press and sonication, and the extracted protein was purified with a nickel column (Qiagen) followed by anion exchange column HiTrap Q HP (GE Healthcare). Protein expression was confirmed using SDS-PAGE gel.

Invitrogen Bac-to-Bac® system was used to construct baculovirus expressing GST-TAOK3. SF21 cells infected with GST-TAOK3 baculovirus were harvested 65 h post infection and lysed using a french press in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 1 M NaCl, 2 mM DTT, 10 mM EDTA, 10% glycerol, and SIGMAFAST™ protease inhibitor tablets (Sigma). After clarification by centrifugation, the lysate was loaded on a 1 ml GStrap column (GE Healthcare) using BioLogic DuoFlow 10 System (BioRad). The column was washed with lysis buffer and eluted in buffer containing 10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM DTT, and 20% glycerol. This protein solution was loaded on a cation exchange HiTrap S HP column (GE Healthcare) and eluted using a 50 mM to 1M NaCl linear gradient in a buffer containing 50 mM Tris-HCl, pH 8.0, 2 mM DTT, and 20% glycerol. Fractions containing purified protein were pooled, concentrated, and frozen at –80 °C.

2.7 TAOK3 kinase assay

TAOK3 was either purified at Microbiotix or purchased from Sigma (catalog # SRP5239) or Invitrogen (catalog # PV3652). The TAOK3 kinase assay was performed in an assay mix containing 50 mM Tris, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, and 3 mM ATP. MBX 2168 (0.2 – 4 mM) or 1 mM CPV and synguanol were incubated in the kinase assay mix for 20 min at 37 °C before the TAOK3 enzyme (10 ng/μl) was added. At designated times, aliquots were removed and the reactions were stopped by addition of 2% trifluoroacetic acid (TFA) (0.2% [Final]). For kinetic analysis, enzyme velocity was measured during the linear phase of drug monophosphate formation (360 minutes). Samples were analyzed using reverse phase HPLC (Gilson 322 HPLC/Gilson 215 liquid handler, Gilson Inc., Middleton, WI). Solvent A was 0.1% TFA (Sigma Chemical, St. Louis, MO) in H₂O (EMD Chemicals Inc., Gibbstown, NJ) and Solvent B was 0.1% TFA in 100% Acetonitrile (EMD Chemicals Inc., Gibbstown, NJ). The samples (100 μL injections) were resolved using a Grace Alltima (Grace Davison Discovery Sciences, Deerfield, IL) 150 mm×4.6 mm 5 μm C18 Column with a gradient of 5 % Solvent B to 40 % Solvent B over 25 minutes. The effluent was monitored at 280 nm. Individual HPLC peaks were collected and analyzed utilizing direct infusion ESI/MS to confirm the identity of the drug monophosphate formed in the reaction.

2.8 Deoxyguanosine kinase assay

A pyruvate kinase lactate dehydrogenase-coupled assay was done in a reaction buffer containing 50 mM Tris pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 5 mM ATP, 1 mM PEP, 0.1 mM NADH, 4.5–7 U/ml of Pyruvate Kinase Lactate Dehydrogenase enzyme

mixture (Sigma) and various concentrations of dG, MBX 2168 or GCV. After 15 minutes at 37 °C, dGK was added to the wells of a 384 well plate containing reaction mixture in a 20 µl total volume. Fluorescence at 460 nm was measured on a SpectraMax plate reader (Molecular Devices).

3. Results

3.1 Antiviral activity of MBX 2168 against TK deficient HSV-1

We previously showed that a thioether MCPN analog did not require active viral TK for antiviral activity against HSV-1 (Prichard et al., 2013). We performed a detailed study to investigate whether the antiviral activity of another member of the MCPN series, MBX 2168, is independent of viral TK. The efficacy of MBX 2168 was evaluated against a TK-positive (E-377) and a TK-negative (DM2.1) HSV-1 isolate, and ACV and CDV were used as a positive and negative control, respectively (Table 1, Figure 1). CDV is already a monophosphate and therefore does not need viral TK for activity. As expected, the TK-negative mutant was completely resistant to ACV. By contrast, MBX 2168 remained effective against the resistant isolate with EC₅₀ value elevated 3.2 fold and comparable to that observed for the CDV negative control (2.8-fold). Similarly, the absence of active viral TK did not affect the inhibition of viral DNA synthesis by MBX 2168 (fold resistance of 1.4). These data suggest that viral TK is not required the antiviral activity of MBX 2168.

3.2 Antiviral activity of MBX 2168 against recombinant HSV-1 strains lacking viral protein kinases

MBX 2168 is phosphorylated by UL97, a serine-threonine protein kinase encoded by HCMV and resistance to MBX 2168 maps to this viral kinase (Komazin-Meredith et al., 2014). MBX 2168 is also a substrate of the HHV-6 U69 protein kinase, which is a homologue of UL97 (Komazin-Meredith et al., 2013). The genome of HSV-1 encodes two protein kinases, US3 and UL13. Even though they are protein kinases with no reported ability to phosphorylate nucleosides or nucleoside analogs, since HCMV UL97 and HHV-6 U69 can phosphorylate both proteins and nucleoside analogs including MBX 2168 we wanted to investigate whether US3 and UL13 can phosphorylate MBX 2168. To determine if they are involved in phosphorylating MBX 2168 we tested efficacy of this compound against recombinant HSV-1 strains lacking either US3 or UL13. There was no difference between efficacy of MBX 2168 against wild-type virus in comparison with strains lacking either UL13 or US3 (Table 2). The same result was observed with the control compound ACV. Improved activity of MBX 2168 was seen with the recombinant HSV-1 virus lacking TK and UL13 but harboring HCMV UL97 kinase further confirming previously shown involvement of this viral kinase in activity of MBX 2168 against HCMV. This virus was also more sensitive to CPV, which shows very little activity against wt-HSV-1. These data indicate that HSV-1 protein kinases UL13 and US3 are not required for MBX 2168 activity.

3.3 Phosphorylation of MBX 2168 by host kinases

Since kinases encoded by HSV-1 were not required for MBX 2168 phosphorylation we investigated the possibility that this process is carried out by a host kinase. Because MBX 2168 is a guanosine analog so we first tested whether human deoxyguanosine kinase (dGK)

can phosphorylate MBX 2168. As expected, purified dGK efficiently phosphorylated its natural substrate dG ($V_{\max}$ 1.1 $\mu\text{M}/\text{min}$, K_m 16 μM). In addition, dGK was able to phosphorylate the guanosine analog GCV ($V_{\max}$ 0.8 $\mu\text{M}/\text{min}$, K_m 740 μM). However, no detectable phosphorylation of MBX 2168 was observed (Figure 2). The human deoxycytosine kinase also failed to phosphorylate MBX 2168 (data not shown).

To identify the host kinase that phosphorylates MBX 2168 to its monophosphate form we used extracts of human foreskin fibroblasts (HFF), which had MBX 2168 kinase activity. We purified the active fraction by passing HFF extracts sequentially over three different protein purification columns and following activity by kinase assays. The final active fraction was sent for tandem mass spectrometry electrospray ionization (LC-ESI-MS/MS) analysis, which identified 152 different proteins (Supplemental Table 1) of which seven were known kinases (Table 3). The following purified kinases were purchased and tested for MBX 2168 kinase activity: adenylate kinase isoenzyme 5, serine-threonine-protein kinase TAOK3, and serine-threonine-protein kinases PKN1 and PKN2. Only the serine-threonine protein kinase thousand and one amino acid protein 3 (TAOK3) was able to phosphorylate MBX 1268 *in vitro*, and was thus investigated further.

3.4 Phosphorylation of MBX 2168 by TAOK3

Purified TAOK3 was purchased from two different suppliers as either a truncated 463 amino acid protein (amino acids 1-463), or as full-length GST-tagged recombinant protein. We tested both of these enzymes in an *in vitro* biochemical assay to determine whether they could phosphorylate MBX 2168. Both of these proteins were able to phosphorylate MBX 2168 (data not shown). In addition, we purified the full length TAOK3 with a GST affinity tag from recombinant baculovirus infected insect cells, which was used for kinetic studies. Time-dependent phosphorylation of MBX 2168 was observed in the presence of purified GST-TAOK3 (Figure 3A), confirming that this compound is a substrate of this host kinase. An analysis of the kinetics of MBX 2168 phosphorylation by TAOK3 was used to determine an apparent Michaelis-Menten constant (K_m) of 4.1 mM, a maximum enzyme velocity ($V_{\max}$) of 2.4 pmol/min, an enzyme turnover number (k_{cat}) of 0.005 s^{-1} , with a catalytic efficiency value (k_{cat}/K_m) of 1.2 $\text{s}^{-1}\text{M}^{-1}$ (Figure 3B). No detectable TAOK3 phosphorylation of CPV or synguanol, a monohydroxymethyl MCPN without any substituents at the 6-position was observed (data not shown).

4. Discussion

In vitro selection of HCMV resistance to monohydroxymethyl MCPNs with ether or thioether substituents at the 6-position resulted in UL97-defective mutants, although the compounds still retained measurable activity (Komazin-Meredith et al., 2014). This, together with their lack of dependence on HSV thymidine kinase, suggested the involvement of a host enzyme in their phosphorylation. In this study we showed that monohydroxymethyl MCPN analog MBX 2168 also does not require HSV-1 UL13 or US3 protein kinase for activity, indicating the activity of the MCPN compound class may be independent of viral kinase activity. After first looking at host nucleoside kinases without success we identified TAOK3, a serine-threonine protein kinase, as the active kinase by

activity based fractionation of HFF cell extracts. TAOK3 has not been shown previously to phosphorylate nucleosides or nucleoside analogs. It is member of the STE20 kinase family and is involved in MAPK signaling through the p38 and JNK pathways, and is an essential gene for cell viability (Kapfhamer et al., 2012; Raman et al., 2007; Tassi et al., 1999). HCMV UL97 and HHV- 6 U69 are also serine-threonine protein kinases with the ability to phosphorylate certain nucleoside analogs (De Bolle et al., 2002; Gentry et al., 2010; Komazin-Meredith et al., 2013; Komazin-Meredith et al., 2014; Littler et al., 1992; Sullivan et al., 1992; Talarico et al., 1999). The kinetics of MBX 2168 phosphorylation by TAOK3 are similar to those previously reported for UL97 and U69 (Komazin-Meredith et al., 2013; Komazin-Meredith et al., 2014), having comparable binding affinity (apparent K_m of 5 mM) but ~50-fold lower V_{max} and k_{cat} values.

TAOK3 did not phosphorylate CPV or synguanol, which explains in part the lack of activity of these compounds against HSV, and could explain the increased broad-spectrum anti-herpes activity of monohydroxymethyl MCPNs with ether or thioether substituents at the 6-position. It would be interesting to further examine the structure activity relationship of TAOK3 and other MCPN analogs as well as to investigate whether TAOK3 can phosphorylate other types of nucleoside analogs. The lack of absolute viral TK dependence for MBX 2168 activity is promising for the treatment of ACV-resistant infections since the majority of them are associated with mutations in the viral TK (Piret and Boivin, 2011). Preliminary studies also showed that the expression of TAOK3 mRNA was not significantly affected by infection with HSV-1 or HSV-2 or by the addition of MBX 2168. Thus this kinase may prove to provide a predictable means of activation for this class of compounds. By utilizing both viral and essential host kinases for phosphorylation, MCPN analogs hold promise for a more favorable single step kinase related resistance profile.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are thankful to Karin M. Green for help with LC-MS/MS analysis. These studies were funded with Federal funds from the National Institutes of Allergy and Infectious Diseases under R44 AI 082799.

References

- Arvin, Ann; C-F, G.; Mocarski, Edward; Moore, Patrick S.; Roizman, Bernard; Whitley, Richard; Yamanishi, Koichi, editors. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge: Cambridge University Press; 2007.
- Chou S, Komazin-Meredith G, Williams JD, Bowlin TL. Cytomegalovirus mutants resistant to ganciclovir and cidofovir differ in susceptibilities to synguanol and its 6-ether and 6-thioether derivatives. *Antimicrobial agents and chemotherapy*. 2014; 58:1809–1812. [PubMed: 24379208]
- De Bolle L, Michel D, Mertens T, Manichanh C, Agut H, De Clercq E, Naesens L. Role of the human herpesvirus 6 u69-encoded kinase in the phosphorylation of ganciclovir. *Mol Pharmacol*. 2002; 62:714–721. [PubMed: 12181449]
- Derse D, Cheng YC, Furman PA, St Clair MH, Elion GB. Inhibition of purified human and herpes simplex virus-induced DNA polymerases by 9-(2-hydroxyethoxymethyl)guanine triphosphate.

Effects on primer-template function. *The Journal of biological chemistry*. 1981; 256:11447–11451. [PubMed: 6271750]

Furman PA, St Clair MH, Spector T. Acyclovir triphosphate is a suicide inactivator of the herpes simplex virus DNA polymerase. *The Journal of biological chemistry*. 1984; 259:9575–9579. [PubMed: 6086640]

Gentry BG, Kamil JP, Coen DM, Zemlicka J, Drach JC. Stereoselective phosphorylation of cyclopropavir by pUL97 and competitive inhibition by maribavir. *Antimicrobial agents and chemotherapy*. 2010; 54:3093–3098. [PubMed: 20547817]

Kapfhamer D, King I, Zou ME, Lim JP, Heberlein U, Wolf FW. JNK pathway activation is controlled by Tao/TAOK3 to modulate ethanol sensitivity. *PloS one*. 2012; 7:e50594. [PubMed: 23227189]

Kern ER, Kushner NL, Hartline CB, Williams-Aziz SL, Harden EA, Zhou S, Zemlicka J, Prichard MN. In vitro activity and mechanism of action of methylenecyclopropane analogs of nucleosides against herpesvirus replication. *Antimicrobial agents and chemotherapy*. 2005; 49:1039–1045. [PubMed: 15728900]

Komazin-Meredith G, Cardinale SC, Williams JD, Peet NP, Prichard MN, Bowlin TL. Human herpesvirus 6 U69 kinase phosphorylates the methylenecyclopropane nucleosides cyclopropavir, MBX 2168, and MBX 1616 to their monophosphates. *Antimicrobial agents and chemotherapy*. 2013; 57:5760–5762. [PubMed: 23979753]

Komazin-Meredith G, Chou S, Prichard MN, Hartline CB, Cardinale SC, Comeau K, Williams JD, Khan AR, Peet NP, Bowlin TL. Human cytomegalovirus UL97 kinase is involved in the mechanism of action of methylenecyclopropane analogs with 6-ether and -thioether substitutions. *Antimicrobial agents and chemotherapy*. 2014; 58:274–278. [PubMed: 24145545]

Littler E, Stuart AD, Chee MS. Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. *Nature*. 1992; 358:160–162. [PubMed: 1319559]

Piret J, Boivin G. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. *Antimicrobial agents and chemotherapy*. 2011; 55:459–472. [PubMed: 21078929]

Prichard MN, Kern ER, Hartline CB, Lanier ER, Quenelle DC. CMX001 potentiates the efficacy of acyclovir in herpes simplex virus infections. *Antimicrobial agents and chemotherapy*. 2011; 55:4728–4734. [PubMed: 21788472]

Prichard MN, Quenelle DC, Hartline CB, Harden EA, Jefferson G, Frederick SL, Daily SL, Whitley RJ, Tiwari KN, Maddry JA, Secrist JA 3rd, Kern ER. Inhibition of herpesvirus replication by 5-substituted 4'-thiopyrimidine nucleosides. *Antimicrobial agents and chemotherapy*. 2009; 53:5251–5258. [PubMed: 19770274]

Prichard MN, Williams JD, Komazin-Meredith G, Khan AR, Price NB, Jefferson GM, Harden EA, Hartline CB, Peet NP, Bowlin TL. Synthesis and antiviral activities of methylenecyclopropane analogs with 6-alkoxy and 6-alkylthio substitutions that exhibit broad-spectrum antiviral activity against human herpesviruses. *Antimicrobial agents and chemotherapy*. 2013; 57:3518–3527. [PubMed: 23669381]

Raman M, Earnest S, Zhang K, Zhao Y, Cobb MH. TAO kinases mediate activation of p38 in response to DNA damage. *The EMBO journal*. 2007; 26:2005–2014. [PubMed: 17396146]

Rozenberg F, Deback C, Agut H. Herpes simplex encephalitis : from virus to therapy. *Infectious disorders drug targets*. 2011; 11:235–250. [PubMed: 21488834]

St Clair MH, Miller WH, Miller RL, Lambe CU, Furman PA. Inhibition of cellular alpha DNA polymerase and herpes simplex virus-induced DNA polymerases by the triphosphate of BW759U. *Antimicrobial agents and chemotherapy*. 1984; 25:191–194. [PubMed: 6324666]

Sullivan V, Talarico CL, Stanat SC, Davis M, Coen DM, Biron KK. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. *Nature*. 1992; 359:85. [PubMed: 1326083]

Talarico CL, Burnette TC, Miller WH, Smith SL, Davis MG, Stanat SC, Ng TI, He Z, Coen DM, Roizman B, Biron KK. Acyclovir is phosphorylated by the human cytomegalovirus UL97 protein. *Antimicrobial agents and chemotherapy*. 1999; 43:1941–1946. [PubMed: 10428917]

- Tassi E, Biesova Z, Di Fiore PP, Gutkind JS, Wong WT. Human JIK, a novel member of the STE20 kinase family that inhibits JNK and is negatively regulated by epidermal growth factor. *The Journal of biological chemistry*. 1999; 274:33287–33295. [PubMed: 10559204]
- Whitley RJ. The use of antiviral drugs during the neonatal period. *Clin Perinatol*. 2012; 39:69–81. [PubMed: 22341538]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.
Chemical structures of compounds used in the study.

Figure 2.

Compound phosphorylation by human deoxyguanosine kinase (dGK). Formation of drug monophosphate formed was measured indirectly by a pyruvate kinase lactate dehydrogenase-coupled assay. Deoxyguanosine, red circles; ganciclovir, green squares; MBX 2168, purple triangles. Data are representative of two experiments done in duplicate. Data were analyzed using GraphPad Prism 5 software.

Figure 3.

Time course (A) and kinetics (B) of MBX 2168 phosphorylation by TAOK3. Formation of drug monophosphate formed was measured by UV-absorbance on HPLC. The concentration of phosphorylated species was determined using standard curves of the corresponding unphosphorylated parent (molar absorptivity assumed to be equal). Enzyme velocity was measured during the linear phase of drug monophosphate formation (360 minutes). Data are representative of two experiments done in duplicates. The error bars show standard error of the means. Data were analyzed using GraphPad Prism 5 software.

Table 1
Antiviral activity of 6-substituted methylenecyclopropane analog MBX 2168 against ACV-resistant HSV

Compound	Efficacy against HSV-1		DNA Synthesis		
	EC ₅₀ (μM) ^a :		EC ₉₀ (μM) ^b :		Fold resistance
	E-377	DM2.1	E-377	DM2.1	
2168	6.7 ± 2.1	22	1.4	2.4	1.4
ACV	1.0 ± 0.2	>100 ± 0	0.15	>50	>333
CDV	0.8 ± 0.7	2.3 ± 0.1	0.97	0.58	0.59

^aValues are from single assays or the means with standard deviations from two separate plaque reduction assays.

^bValues shown reflect the concentration of the compounds required to reduce the absolute DNA copy number by 90% as determined by real time PCR.

Table 2

Antiviral activity of MBX 2168 against recombinant HSV viruses

Compound	EC ₅₀ (μM) for HSV-1 ^a :			
	Wild-Type	R4970 (UL13 ,TK , HCMV UL97+)	R7041 (UL13)	R7356 (US3)
2168	9.8 ± 3.0	1.5 ± 0.2	9.1 ± 3.6	9 ± 4.5
CPV	420 ± 33	48 ± 35	NT	NT
ACV	0.34 ± 0.12	NT	0.19 ± 0.04	0.33 ± 0.14

^aData are shown as means from two to six separate plaque reduction assays with standard deviations.^bNT, not tested

Table 3

Kinases present in protein fraction with MBX 2168 phosphorylation activity

Kinase	MBX 2168-MP activity
Adenylate kinase isoenzyme 5	No
Serine/threonine-protein kinase TAOK3	Yes
Serine/threonine-protein kinase PKN2	No
Serine/threonine-protein kinase PKN1	No
Integrin linked protein kinase	Not tested
Myosin light chain kinase	Not tested
Phosphatidylinositol 5-phosphate 4-kinase type-2	Not tested