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Distinct thymidine kinases encoded by cowpox virus and herpes simplex virus contribute significantly to the differential antiviral activity of nucleoside analogs

Mark N. Prichard*, Angela D. Williams, Kathy A. Keith, Emma A. Harden, Earl R. Kern

University of Alabama School of Medicine, Department of Pediatrics, 128 Childrens Harbor Building, 1600 6th Avenue South, Birmingham, AL 35233, USA

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Abstract

Orthopoxviruses and herpesviruses are both large enveloped DNA viruses, yet these virus families exhibit very different susceptibilities to antiviral drugs. We investigated the activation of nucleoside analogs by the types I and II thymidine kinase (TK) homologs expressed by herpes simplex virus type 1 (HSV-1) and cowpox virus (CV). Antiviral activity against TK^- and TK^+ strains of HSV-1 and CV was determined, and the ratio of the EC_{50} values was used as a measurement of TK dependence. As to HSV-1, most of the selected compounds were markedly less effective against the TK^- strains, suggesting that this enzyme was required for the activation of these nucleoside analogs. This differs from the results for CV where only idoxuridine and bromodeoxyuridine appeared to be activated, putatively by the type II TK expressed by this virus. These data confirm that the type II TK encoded by CV exhibits a more limited substrate specificity than the type I TK encoded by HSV-1. These data suggest that the inefficient activation of nucleoside analogs by the orthopoxvirus TK significantly limits their activity. Additional screening against orthopoxviruses will be required to identify nucleoside analogs that are efficiently activated by their type II TK.

Keywords: Orthopoxviruses; Herpesviruses; Thymidine

1. Introduction

Herpesviruses and orthopoxviruses are large double-stranded DNA viruses and encode DNA polymerases that share significant homology in the conserved catalytic domains (Boehmer and Lehman, 1997; Hwang et al., 2004; Traktman, 1990). Given the relative similarity of these polymerases, it is not unexpected that nucleoside analogs might inhibit both enzymes. Cidofovir (CDV) and related nucleotide analogs exhibit good antiviral activity against both virus families (De Clercq and Neyts, 2004), but precious few nucleosides are effective against both herpesviruses and orthopoxviruses. The relative substrate specificities of the DNA polymerases encoded by these different viruses certainly contribute to the differential activity of nucleoside inhibitors; however, the activation of these molecules by the nucleoside kinases encoded by these viruses will also significantly impact their activity. Indeed, a previous study demonstrated that vaccinia virus (VV) became fully sensitive to acyclovir (ACV) when the herpes simplex virus (HSV) thymidine kinase (TK) was supplied in *trans* to activate the drug (Darby et al., 1980). Nucleosides used to treat herpesvirus infections require a virus-induced thymidine kinase for the first steps of the phosphorylation cascade that leads to the active triphosphate metabolite (Elion, 1983). The selective phosphorylation of nucleoside analogs by these viral enzymes has proven to be a tremendously effective strategy to confer specificity to these compounds to be highly effective in treating herpesvirus infections (Whitley and Roizman, 2001).

Both HSV and VV express TK activity and each of these enzymes can phosphorylate thymidine (Kit et al., 1967, 1963). These enzymes differ in that the herpesvirus TK is a type I enzyme, whereas the VV TK is a type II enzyme (Black and Hruby, 1992a,b). The HSV type I TK is the product of the of the *UL23* gene (McGeoch et al., 1988), and like all type I enzymes, is active as a homodimer and lacks allosteric control (Jamieson and Subak-Sharpe, 1974). This enzyme also exhibits a rather broad substrate specificity, including thymidine, 2′-deoxycytidine and synthetic nucleoside analogs (Coen

* Corresponding author.
E-mail address: mprichard@peds.uab.edu (M.N. Prichard).

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and Schaffer, 1980; Jamieson and Subak-Sharpe, 1974). VV TK is the prototypic type II enzyme and is encoded by the J2R gene (Hruby et al., 1983). Like other members of this class, it is active as a homotetramer (Hruby and Ball, 1982) and is allosterically inhibited by both dTTP and dTDP (Black and Hruby, 1992a). The human TK is also a type II enzyme and like its homolog in VV, exhibits a restricted substrate specificity limited to thymidine and closely related analogs.

We hypothesized that the observed differences in susceptibility to a number of antiviral drugs might be related to the distinct classes of TK enzymes expressed in these virus families. Thus, the poor activity of many nucleoside analogs against cowpox virus (CV) may reflect their inability to be activated by the type II TK expressed by this virus. To test this hypothesis, we examined the antiviral activity of selected compounds against TK^+ and TK^- strains of both HSV-1 and CV. Most of the compounds examined in this study did not exhibit HSV-1 TK dependence. This is consistent with the notion that the poor phosphorylation of nucleoside analogs contributes significantly to the lack of activity for many compounds against orthopoxviruses. The approach described here to investigate TK dependence in CV could also be used to identify novel compounds that are preferentially activated by the orthopoxvirus TK.

2. Materials and methods

2.1. Cells and viruses

Primary human foreskin fibroblast (HFF) cells were prepared and passaged by methods described previously (Rybak et al., 2000). Vero cells were obtained from the American Type Culture Collection (Manassas, VA). The wild type (wt) HSV-1 strain F and TK^- strain DM2.1 were described and propagated as reported previously (Hartline et al., 2005). CV strains delta crmA (TK^+) and TK:GFP lacZ (TK^-) were obtained from Pete Turner (University of Florida, Gainesville, FL) and were described previously (Ali et al., 1994). Cidofovir, idoxuridine (IDU), 5-bromodeoxyuridine (BrdU), vidarabine (AraA), 5-fluorodeoxyuridine (FdU), trifluridine (TFT), fialuridine (FIAU), fiacitabine (FIAC), sorivudine (BVAU), ACV and brivudin (BVDU) were either purchased (Sigma–Aldrich, St. Louis, MO) or were obtained through the NIAID, NIH, Bethesda, MD.

2.2. CV β-galactosidase assay

Monolayers of Vero cells in 96-well plates were incubated at 37 °C for 24 h in a humidified incubator. Drugs were then diluted in the plates and either TK^+ or TK^- strains of CV were added at a multiplicity of infection of 0.05 PFU/cell. At 72 h post infection, the medium was removed and the β-galactosidase substrate, chlorophenol red-β-galactopyranoside, was added at a final concentration of 50 μg/ml in a phosphate-buffered saline solution. The conversion of the colorimetric substrate was determined by measuring the absorbance at 570 nm, and 50% effective concentration (EC_{50}) values were calculated. The TK^-/TK^+ EC_{50} ratio was calculated in each experiment and an average ratio and standard deviation were calculated for all experiments.

2.3. HSV-1 plaque reduction assay

HFF cells were seeded into six-well plates and incubated at 37 °C. Two days later, drug was serially diluted 1:5 in MEM with 2% FBS using six concentrations of drug with a starting concentration of 100 μg/ml. Viruses were diluted in MEM containing 10% FBS to a concentration that yielded 20–30 plaques per well. The media were then aspirated from the wells and 0.2 ml of virus was added to each of triplicate wells with 0.2 ml of medium being added to control wells. The plates were then incubated for 1 h with shaking every 15 min and drug was added to appropriate wells. After an incubation period of 3 days, the cells were stained with 0.1% crystal violet in 20% MeOH. The stain was aspirated, the wells washed with PBS and the plaques enumerated using a stereomicroscope. EC_{50} values were calculated in a standard manner.

2.4. Cytotoxicity assay

To determine the toxicity of drugs, HFF cells were seeded into 96-well plates at a concentration of 2.5×10^4 cells/well in growth media. After 24 h, the media were aspirated and 100 μg/ml of drug was added to the first row of the plate and five-fold serial dilutions were performed. Following a 7-day incubation, the media were aspirated and 660 μg/ml of neutral red stain was added and incubated for 1 h. The monolayers were then washed and the dye was dissolved in a solution containing 50% ethanol and 1% glacial acetic acid. The plates were mixed for 15 min on a rotating shaker and the optical densities were determined at 550 nm. CC_{50} values were interpolated from the data.

2.5. Genetic resistance assays

The principle behind both the CV and HSV-1 genetic resistance assays is the same. The efficacy of antiviral drugs was determined in TK^+ and TK^- strains of the same virus and the ratio of the EC_{50} against the TK^- virus to the EC_{50} against the TK^+ virus was used as a measure of TK dependence. The nucleotide analog CDV was used as a negative control for both these viruses. As positive controls, ACV and IDU were used in HSV-1 and CV assays, respectively, since these drugs require phosphorylation for their antiviral activity.

2.6. Amino acid alignment and phylogeny

Amino acid sequences for TK homologs were downloaded from GenBank and subjected to a clustal W alignment and unrooted phylogenetic trees were constructed using Vector NTI (Invitrogen, San Diego, CA). Gene ID numbers in the analysis and abbreviations used are VV, 29692200; HSV-1, 9629403; HSV-2, 9629292; *Homo sapiens* (human), 4507519; goatpox virus, 55274605; VZV, 118822; EBV, 23893647; variola virus,

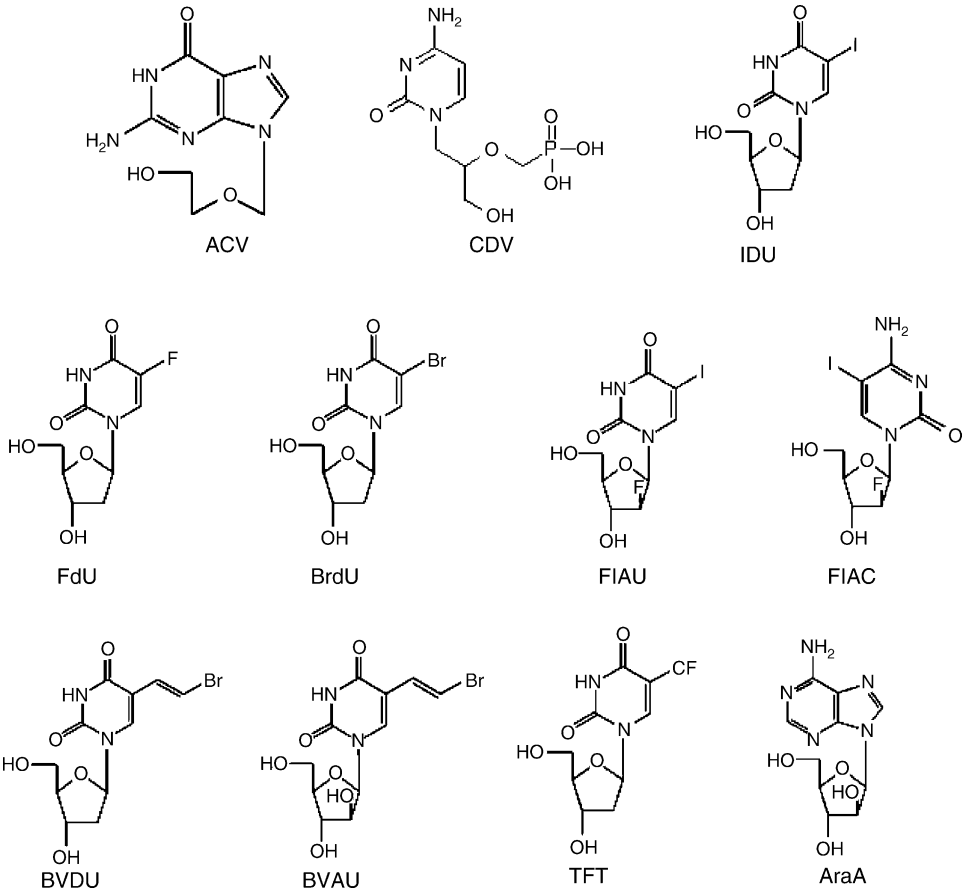

Fig. 1. Structures of nucleotide analogs tested.

66679; fowlpox virus, 221413; monkeypox virus, 22096356; CV, 20178469.

3. Results

A set of antiviral drugs was selected based on previously described activity against HSV-1 and orthopoxviruses. Since IDU has been used in the laboratory to select for TK-deficient orthopoxviruses (Byrd and Hruby, 2004) and a number of analogs have good activity against VV (De Clercq, 1980), we studied additional analogs to determine if this gene was involved in the activation of other compounds in this series (Fig. 1). A total of 11 compounds including control drugs were tested in the genetic resistance assays and the EC_{50} ratios were calculated (Table 1). For HSV-1, the wt strain was very sensitive to ACV, while the TK^- strain was highly resistant to the drug and yielded an EC_{50} ratio of 286. In contrast, EC_{50} values for the CDV negative control were indistinguishable between these two strains yielding an EC_{50} ratio of 1, confirming that the assay could identify compounds that require TK in their mechanism of action. TFT, FdU and AraA did not appear to be selectively phosphorylated to a significant extent by the HSV-1 TK since the EC_{50} ratios were very close to 1, but each of the remaining compounds was significantly less effective against the TK^- virus suggesting not only that this enzyme was required for their activation. Among these, ACV, BVAU, BVDU, FIAC and FIAU appeared to be the most dependent on this enzyme suggesting that they are not phosphorylated to a significant extent by host kinases. IDU and BrDU were dependent on TK, but retained some activity in the TK^- virus suggesting that they might be activated to some degree by enzymes other than the HSV-1 TK (type 1).

This same set of compounds was also tested against CV, and EC_{50} ratios were calculated to assess the ability of the type II TK from this virus to activate these same compounds (Table 1). Both strains of CV were fully sensitive to the CDV control and yielded an EC_{50} ratio of 0.54. The TK^+ strain of CV was sensitive to IDU as reported previously for CV (Kern, 2003; Smee and Sidwell, 2004) and vaccinia virus (Kern, 2003; Neyts et al., 2002), while the TK^- CV strain was significantly less sensitive to the drug and yielded an EC_{50} ratio of 7.3. These results indicated that the assay was capable of identifying compounds that require TK for their activity. The closely related analog, BrdU, was also much less effective against the TK^- virus and yielded a TK^-/TK^+ EC_{50} ratio of >50. Both TFT and FdU were equally effective in inhibiting both strains of CV and HSV-1, as was expected since both these molecules are inhibitors of thymidylate synthetase (Emura et al., 2004). The antiviral activity of AraA was also independent of TK and is consistent with results obtained with HSV-1. ACV, BVDU and BVAU did not significantly inhibit replication of either CV strain, suggesting that either they are not activated by the CV-encoded TK or they are not substrates for the

Table 1
Antiviral activity of compounds against TK^+ and TK^- strains of HSV-1 and CV

	HSV-1			CV			
	F[a] EC_{50} (TK^+)	DM2.1[a] EC_{50} (TK^-)	EC_{50} ratio[b]	delta crmA[c] EC_{50} (TK^+)	TK:GFP[c] lacZ EC_{50} (TK^-)	EC_{50} ratio[b]	Toxicity[d] CC_{50}
ACV	0.35 ± 0.2	>100 ± 0	>286	>30	>30	1	>100 ± 0
CDV	1.5 ± 1.1	1.5 ± 1.4	1	6.1 ± 0.30	3.3 ± 2.3	0.54	>100 ± 0
IDU	2.1 ± 0.1	54 ± 8.1	26	2.2 ± 0.87	16 ± 9.3	7.3	>100 ± 0
AraA	6.4 ± 3.4	4.9 ± 0	0.78	0.91 ± .005	0.78 ± 0.2	0.86	>100 ± 0
BrdU	1.6 ± 0.2	39 ± 6.3	24	0.37[a] ± 0	19[a] ± 15	51	>100 ± 0
FdU	2.5 ± 1.7	4.5 ± 3.3	1.8	0.37[a] ± 0	0.37[a] ± 0	1	60 ± 0.7
TFT	1.0 ± 0.2	1.1 ± 0.3	1.1	0.42 ± 0.33	0.72 ± 0.28	1.7	>100 ± 0
FIAU	0.05 ± 0.03	3.9 ± 3.3	78	14 ± 7.0	19 ± 6.7	1.4	>100 ± 0
FIAC	0.06 ± 0.02	8.0 ± 1.3	133	18 ± 0.25	9.2 ± 3.7	0.51	>100 ± 0
BVDU	0.09 ± 0	100 ± 0	1111	>30	>30	1	>100 ± 0
BVAU	0.05 ± 0.02	>100 ± 0	>2000	>30	>30	1	>100 ± 0

[a] Average of two experiments ± standard deviation (μg/ml).
[b] Ratio of EC_{50} values for TK^- and TK^+ strains of the virus.
[c] Average of five experiments ± standard deviation.
[d] Determined by duplicate neutral red uptake assays.

viral DNA polymerase. Both FIAC and FIAU exhibited modest activity against TK^- and TK^+ strains of the virus suggesting that the compounds can inhibit the CV DNA polymerase, but also that the TK expressed by CV does not appear to activate the compounds to a measurable extent. This contrasts with the high EC_{50} ratios for FIAC and FIAU in HSV-1 of 78 and 133, respectively.

4. Discussion

We investigated the effect of HSV-1 TK and CV TK on the antiviral activity of several related nucleoside analogs. In these assays, a positive result is significant in that it suggests that TK is involved in the mechanism of action of the drug, and also implies selectivity since viral TK gene must be dominant over cellular kinases. It does not demonstrate the phosphorylation of the drugs directly, but suggests that it may be involved given the kinase activity of the enzymes. There are many potential explanations for negative results including the possibility that both cellular and viral enzymes could be activating the drug.

Closely related nucleoside analogs were selected because the orthopoxviruses express type II TK enzymes and were expected to have a narrow substrate specificity. Results presented were consistent with this prediction since very few analogs of IDU appear to be activated by CV TK. The narrow substrate specificity of this enzyme was apparent even with the small set of compounds presented here. As expected, IDU was dependent on the CV TK and the substitution of bromine for iodine was well tolerated by the enzyme as evidenced by the high TK^-/TK^+ EC_{50} ratio of 51. However, the substitution of a larger bromovinyl group at this same position eliminated its activity against both strains of CV and this is consistent with a lack of activation by CV TK, but it is also possible that the triphosphate metabolite of this compound did not inhibit the CV DNA polymerase. The introduction of either fluorine or a trifluoromethyl group at the C-5 position makes FdU and TFT inhibitors of thymidylate synthetase and predictably, both these molecules inhibit TK^+ and TK^- strains of both HSV-1 and CV. Although TFT does not exhibit TK dependence in HSV, the viral enzyme can phosphorylate the drug to a limited extent and the monophosphorylated drug can be detected in TK^- cells (Field et al., 1981). Substitutions on the sugar moiety also appear to be poorly tolerated since the addition of a fluorine at the 2′ position in FIAU rendered it equally effective against both strains of CV. Results from FIAC and FIAU were particularly informative since both these drugs exhibit modest antiviral activity against CV, and this is consistent with the compounds inhibiting the DNA polymerase. However, neither of these compounds appeared to be activated by the type II TK encoded by this virus and contrasts with results in HSV-1, where both these compounds were efficiently phosphorylated by the type I TK encoded by this herpesvirus. The fact that the relative potency of these compounds against CV is similar to that against the TK^- strain of HSV-1 is also consistent with this interpretation of the data.

Results presented here confirm that the CV TK has a rather limited substrate specificity compared to the TK encoded by HSV-1. This outcome was expected since this virus encodes a type II TK homolog that is more closely related to the human enzyme than the enzyme from HSV-1 (Fig. 2). TK homologs encoded by VV, CV, variola virus and monkeypox virus are all quite similar compared to the high degree of sequence divergence in the type I enzymes encoded by the herpesviruses (Fig. 3). The high degree of similarity among the human orthopoxviruses TK homologs is important and suggests that the assay reported will be predictive for variola virus and monkeypox virus. The high degree of amino acid identity among the orthopoxvirus enzymes and the human homolog as well as the narrow substrate specificity that is characteristic of type II enzymes will likely make it difficult to identify nucleoside analogs that are only activated by these enzymes. Nevertheless, it should be possible to identify nucleosides that are selectively phosphorylated by the orthopoxvirus TK homolog (Fig. 3). Once activated in cells infected with CV, cellular enzymes could phosphorylate the compounds to the level of the triphosphate to make them inhibitors of the viral DNA polymerase. Further studies will be required to identify inhibitors with this mechanism of

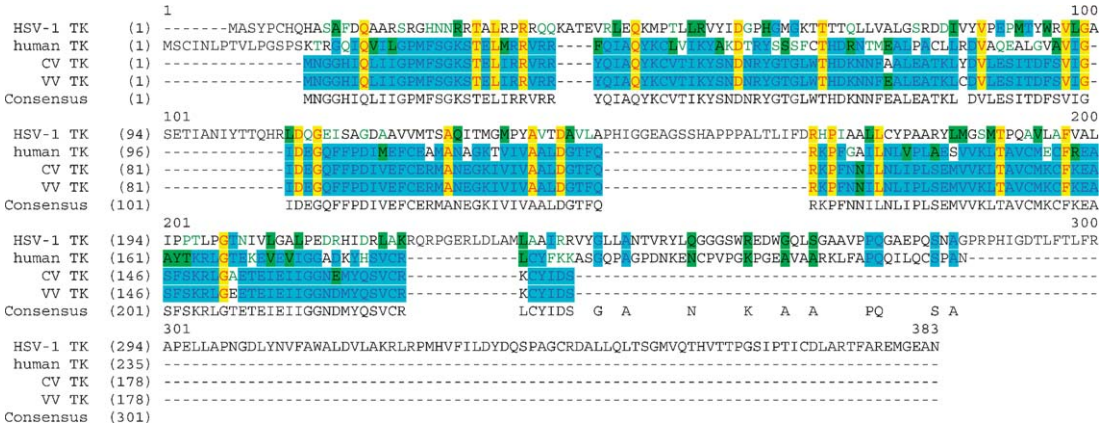

Fig. 2. Multiple amino acid alignments of thymidine kinase homologs expressed in HSV-1, CV, VV and humans, including a consensus line. Identity and similarity are depicted as light gray and dark gray boxes, respectively.

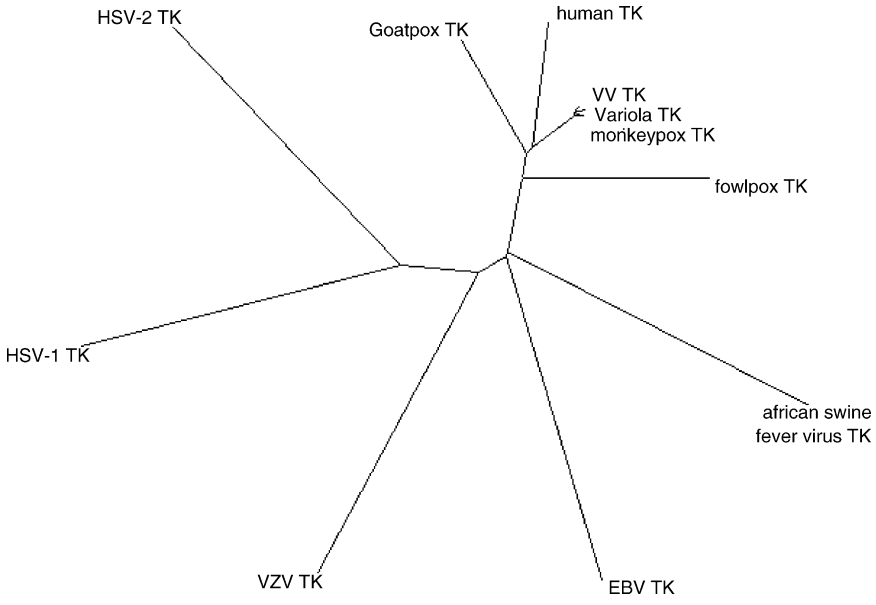

Fig. 3. Unrooted phylogenetic tree based on the amino acid sequence of the TK homologs is shown. Type II orthopoxvirus TK homologs are closely related to the human enzyme that is a member of this same class.

action. The CV genetic resistance assay described here will be an important first step in this process since it is capable of identifying inhibitors that depend on the viral TK for their activity.

The development of DNA polymerase inhibitors that are specifically activated by the type I TK molecules encoded by the herpesviruses was a comparatively simple task given the broad substrate specificity of this class of enzymes. However, it should be considered possible to develop antiviral drugs with this same mechanism of actions against orthopoxviruses. Lessons learned from the development of inhibitors of herpesviruses such as ACV and ganciclovir demonstrate that specificity derived through selective phosphorylation is a highly effective strategy and one that should be actively pursued for the treatment of orthopoxvirus infections. The approach described here will help identify compounds that act by such a mechanism by identifying drugs that require the viral TK for their activity. Resulting lead compounds should have a relatively good toxicity profile since, if they remain unphosphorylated, they are unlikely to inhibit cellular polymerases. This strategy should hold promise in the identification of new therapies for the treatment of orthopoxvirus infections.

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