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Selective Phosphorylation of Antiviral Drugs by Vaccinia Virus Thymidine Kinase▽

Mark N. Prichard,[1]* Kathy A. Keith,[1] Mary P. Johnson,[1] Emma A. Harden,[1] Alexis McBrayer,[1] Ming Luo,[2] Shihong Qiu,[2] Debasish Chattopadhyay,[2] Xuesen Fan,[3] Paul F. Torrence,[3] and Earl R. Kern[1]

Departments of Pediatrics[1] and Microbiology,[2] University of Alabama School of Medicine, Birmingham, Alabama 35233, and Department of Chemistry and Biochemistry, Northern Arizona University, Flagstaff, Arizona 86011[3]

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The antiviral activity of a new series of thymidine analogs was determined against vaccinia virus (VV), cowpox virus (CV), herpes simplex virus, and varicella-zoster virus. Several compounds were identified that had good activity against each of the viruses, including a set of novel 5-substituted deoxyuridine analogs. To investigate the possibility that these drugs might be phosphorylated preferentially by the viral thymidine kinase (TK) homologs, the antiviral activities of these compounds were also assessed using TK-deficient strains of some of these viruses. Some of these compounds were shown to be much less effective in the absence of a functional TK gene in CV, which was unexpected given the high degree of amino acid identity between this enzyme and its cellular homolog. This unanticipated result suggested that the CV TK was important in the mechanism of action of these compounds and also that it might phosphorylate a wider variety of substrates than other type II enzymes. To confirm these data, we expressed the VV TK and human TK1 in bacteria and isolated the purified enzymes. Enzymatic assays demonstrated that the viral TK could efficiently phosphorylate many of these compounds, whereas most of the compounds were very poor substrates for the cellular kinase, TK1. Thus, the specific phosphorylation of these compounds by the viral kinase may be sufficient to explain the TK dependence. This unexpected result suggests that selective phosphorylation by the viral kinase may be a promising new approach in the discovery of highly selective inhibitors of orthopoxvirus replication.

Effective therapies for orthopoxvirus infections are required to combat potential infections of variola virus or monkeypox virus and also to treat adverse events associated with vaccination with vaccinia virus (VV) (7, 8, 26). Cidofovir (CDV) exhibits good antiviral activity against a wide spectrum of orthopoxviruses, including VV, cowpox virus (CV), variola virus, ectromelia virus, and monkeypox virus (3, 18, 35, 43, 51). There is also a small body of clinical experience using CDV to treat molluscum contagiosum and orf virus infections (15, 27). Thus, CDV is a potentially useful drug for the treatment of orthopoxvirus infections and there is an Investigational New Drug Approval for the emergency treatment of smallpox and complications from vaccination. Unfortunately, the utility of this compound is limited by the lack of oral bioavailability and inherent toxicity reduces its usefulness in the clinic (13).

Recent advances in the development of therapeutics for these infections have identified a number of highly active compounds (52). Among these, inhibitors of the VV I7L proteinase have been identified that block virion maturation (9). Inhibitors of the p37 major envelope protein (F13L) are also good inhibitors of viral replication both in vitro and in vivo (54). Ether lipid analogs of CDV have also been shown to be orally bioavailable and highly effective inhibitors of orthopoxvirus infection both in vitro and in vivo (35, 36, 38). The thymidine analog, (*N*)-methanocarbathymidine, is also active against orthopoxviruses both in vitro and in vivo, and its activity appears to be dependent on a functional orthopoxvirus TK gene (45). This mechanism of action has historically proven to be extraordinarily effective in the therapy of herpesvirus infections, so we sought to identify other potential nucleoside analogs that could be selectively phosphorylated by this viral enzyme.

While herpesviruses and orthopoxviruses both express proteins with TK activity (39, 40), the enzymes are distinct in several fundamental respects, including molecular weight, quaternary structure, and substrate specificity. Herpesvirus enzymes belong to the type I family of TKs, whereas the VV TK is a type II enzyme (5). The type I enzyme encoded by the herpes simplex virus (HSV) *UL23* gene (42) is active as a homodimer and is unaffected by allosteric effectors (34). This enzyme, like other members of this family, can phosphorylate a broad range of substrates, including thymidine, 2′-deoxycytidine, and many synthetic nucleoside analogs (19, 25, 34). The prototypic type II TK is encoded by the J2R gene in VV and is closely related to the human cytosolic TK1, which is also a member of this family (32). This group of enzymes is active as homotetramers (31) and is allosterically controlled by both dTTP and dTDP (6, 30). Members of this family are also characterized by a very narrow substrate specificity limited to thymidine and a few closely related analogs.

Early studies by Prusoff and coworkers identified a number of 5-substituted 2′-deoxyuridine analogs, such as idoxuridine (IDU) and trifluoridine (TFT), which exhibited antiviral activity (28). Although some of these compounds were associated with significant toxicity, they could selectively inhibit the replication of both HSV (1, 11, 12) and VV (33, 44). Early studies

* Corresponding author. Mailing address: 128 Children's Harbor Building, 1600 6th Ave. South, Birmingham, AL 35233. Phone: (205) 975-1995. Fax: (205) 975-1992. E-mail: mprichard@peds.uab.edu.

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with VV demonstrated that IDU competed with thymidine as a substrate for the DNA polymerase and was incorporated in viral DNA (48). Interestingly, a functional TK was apparently involved in the mechanism of action of the drug, since recombinant viruses that did not express this enzyme were comparatively resistant to its activity (10). HSV was also sensitive to this compound and similarly required a functional virus TK for activity (25). Subsequent studies identified related compounds, such as brivudine, that were remarkably active against HSV yet did not exhibit the toxicity of earlier compounds (16, 17). Like IDU, these compounds derive their remarkable specificity through selective phosphorylation by herpesvirus TK homologs and remained unactivated in uninfected cells, since they are not substrates for cellular nucleoside kinases (55). However, these compounds were inactive against the orthopoxviruses, since they were not phosphorylated by the viral type II TK homologs and were not converted to active metabolite.

Recently, a new series of deoxyuridine analogs with large substituents at the 5 position were described that retained activity against both VV and CV (21–24). Here, we report that the compounds exhibit an unexpected TK dependence in orthopoxviruses. Enzymatic assays demonstrated that these novel compounds were good substrates for the VV TK, whereas they were poor substrates for the human homolog, TK1. These results suggest that although these enzymes are closely related, selective activation of antiviral drugs by the VV TK is a viable approach in the discovery of highly specific drugs to treat orthopoxvirus infections. Studies presented here were designed to describe the unique substrate specificity of the VV TK in an effort to develop better antiviral drugs for the treatment of orthopoxvirus infections.

MATERIALS AND METHODS

Cells, viruses, and drugs. Methods for obtaining and passaging human foreskin fibroblast (HFF) cells were described previously (49). Culture medium for all cell lines was minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) and standard concentrations of L-glutamine, penicillin, and gentamicin. VV strains WR, Copenhagen, and IHD were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Working stocks of these viruses were propagated in Vero cells obtained from the ATCC. CV strain Brighton was kindly provided by John W. Huggins (Department of Viral Therapeutics, Virology Division, U.S. Army Medical Research Institute of Infectious Disease, Frederick, MD). CV strains delta crmA (TK^+) and TK:GFP lacZ (TK^-) were obtained from Pete Turner (University of Florida, Gainesville, FL) and were described previously (2). The wild-type (WT) HSV-1 strain F and the TK-deficient DM2.1, as well as the WT HSV-2 strain MS and the TK-altered strain AG-3, were described and propagated as reported previously (29). CDV (Vistide) was a gift from Mick Hitchcock (Gilead Sciences Inc., Foster City, CA), and other compounds were either purchased (Sigma Chemical company, St. Louis, MO) or provided through the NIAID, NIH, Bethesda, MD. Substituted 2′-deoxyuridine derivatives were synthesized in the lab of Paul Torrence and are as follows: (1) 5-(2,2-dicyanovinyl)-2′-deoxyuridine; (2) 5-(2-carboxyethyl-2-cyanovinyl)-2′-deoxyuridine; (3) 5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4H-chromen-4-yl)-1-(2-deoxypento-furanosyl)-pyrimidine-2,4(1H,3H)-dione; (4) 1-(2-deoxypentofuranosyl)-5-(3-methyl-5-oxo-1-phenyl-4,5-dihydro-4H-pyrazol-4-ylidene)pyrimidine-2,4(1H,3H)-dione; (5) 5-bis(3-methyl-5-oxo-1-phenyl-4,5-dihydro-4H-pyrazol-4-yl)methyl-1-(2-deoxypentofuranosyl)pyrimidine-2,4(1H,3H)-dione; (6) 5-(2-amino-3-cyano-5-oxo-6,6-dimethyl-5,6,7,8-tetrahydro-4H-chromen-4-yl)-1-(2-deoxypento-furanosyl)-pyrimidine-2,4(1H,3H)-dione.

VV, CV, and HSV plaque reduction assays. For assays against VV and CV, HFF cells were added to six-well plates and incubated for two days at 37°C with 5% CO_2 and 90% humidity. On the day of the assay, the drug at two times the final desired concentration was diluted serially 1:5 in 2× MEM with 10% FBS to provide six concentrations. Aspiration of culture medium from triplicate wells for each drug concentration was followed by the addition of 0.2 ml per well of diluted virus which would give 20 to 30 plaques per well in MEM containing 10% FBS or 0.2 ml medium for drug toxicity wells. The plates were incubated for 1 h with shaking every 15 min. An equal amount of 1% agarose was added to an equal volume of each drug dilution; this mixture was added to each well in 2-ml volumes, and the plates were incubated for three days. The cells were stained with a solution of 0.01% neutral red in phosphate-buffered saline (PBS) and incubated for 5 to 6 h. The stain was aspirated, and plaques were counted using a stereomicroscope at ×10 magnification and 50% effective concentration (EC_{50}) values were calculated by standard methods. The HSV plaque reduction assays were essentially the same as for VV and CV with the following changes. The drug solutions were prepared at the desired concentration in MEM with 2% FBS, and a liquid overlay with pooled human serum containing antibodies to HSV instead of agarose was used. At 72 h following infection, the media containing the drug was aspirated and the monolayers were stained with 1 ml of a solution of 0.01% crystal violet in 60% methanol for 10 min. Residual stain was then washed from the wells with 1 ml PBS, and plaques were counted. Varicella-zoster virus (VZV) assays were performed in the same manner, except the plaques were stained at 10 days following infection.

CV β-galactosidase assay. Monolayers of HFF 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 48 h postinfection, the medium was removed and the β-galactosidase substrate, chlorophenol red-β-galactopyranoside, was added at a final concentration of 50 μg/ml in PBS. The conversion of the colorimetric substrate was determined by measuring the absorbance at 570 nm, and EC_{50} values were calculated by standard methods (46). The EC_{50} ratio for TK^- and TK^+ viruses, respectively, was calculated and used as a measure of TK dependence.

Cytotoxicity determination. HFF cells were added to 96-well, black-walled plates at a concentration of 2.5×10^4 cells per well. After 24 h, the media was aspirated and 125 μl of each drug concentration in MEM with 2% FBS was added to the first row of wells in triplicate. Serial 1:5 dilutions were performed using a Beckman BioMek liquid handling system. After compound addition, the plates were incubated for 7 days in a 5% CO_2 incubator at 37°C. To each well 35 μl of CellTiter-Glo (Promega, Madison, WI) reagent was added, and luminescence was measured with a luminometer. Standard methods were used to determine the drug concentration which inhibited cell proliferation by 50% (IC_{50}).

Enzyme preparation. The full-length open reading frame for the TK encoded by the WR strain of VV and a cDNA encoding human TK1 were amplified and cloned into pET15b (Novagen, Madison, WI) and pET151 d vector (Invitrogen, Carlsbad, CA), respectively, to amino-terminal His tags. Primers for amplifying J2R were 5′-CAC CAT GAA CGG CGG ACA TAT TC-3′ and 5′-TGA GTC GAT GTA ACA CTT TCT TAA-3′, and primers for amplifying TK1 were 5′-CAC CAT GAG CTG CAT TAA CCT GCC CAC T-3′ and 5′-CTA GTT GGC AGG GCT GCA TT-3′. These plasmids were transformed into *Escherichia coli* strain BL21(DE3) (Invitrogen), grown to exponential phase (optical density at 600 nm of 0.4 to 0.6), and induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma) at 37°C for 4 h. Cells were collected by centrifugation, and pellets were stored at −80°C. Pellets were thawed on ice and resuspended in enzyme lysis buffer consisting of 50 mM Tris, pH 8.0, 500 mM NaCl, 2 mM $MgCl_2$, 2 mM imidazole, 0.05% Tween 20, 10% sucrose, 1 mg/ml lysozyme, and protease inhibitor cocktail (Sigma Chemical Company, St. Louis, MO). Samples were incubated on ice for 30 min and sonicated (10 six-second bursts), and 0.5 mg/ml DNase I from bovine pancreas (Sigma Chemical Company) was added to the lysate, which was incubated an additional 15 min on ice. The lysate was clarified by centrifugation for 30 min at 25,000 rpm. Ni-nitrilotriacetic agarose beads (QIAGEN, Germantown, MD) were equilibrated in lysis buffer and added to the supernatant. The agarose bead suspension was agitated for 2 h at 4°C and then loaded onto a column for subsequent purification steps. The column was washed with 10 bed volumes of wash buffer (20 mM Tris, 500 mM NaCl, 2 mM $MgCl_2$, 20 mM imidazole, 0.5% Tween 20). Proteins were eluted with 5 bed volumes of elution buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 2 mM $MgCl_2$, 250 mM imidazole, 0.05% Tween 20), aliquoted, and stored at −80°C. The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to assess purity. Higher-molecular-weight species were observed in the human TK1 preparation, so mass spectrometry was used to identify these proteins. This analysis identified these products as human TK1, consistent with multimeric forms of the enzyme. This analysis also identified one contaminating bacterial protein (b2255, gi16130190) that is of unknown function but is not homologous to any known kinases. It also does not exhibit detectable ATPase activity, since the concentrated enzyme did not significantly degrade ATP in 1 h of incubation.

Enzyme activity assays. Kinetic parameters for substrates of TK1 and VV TK were determined by measuring ATP utilization with a luciferase-based assay.

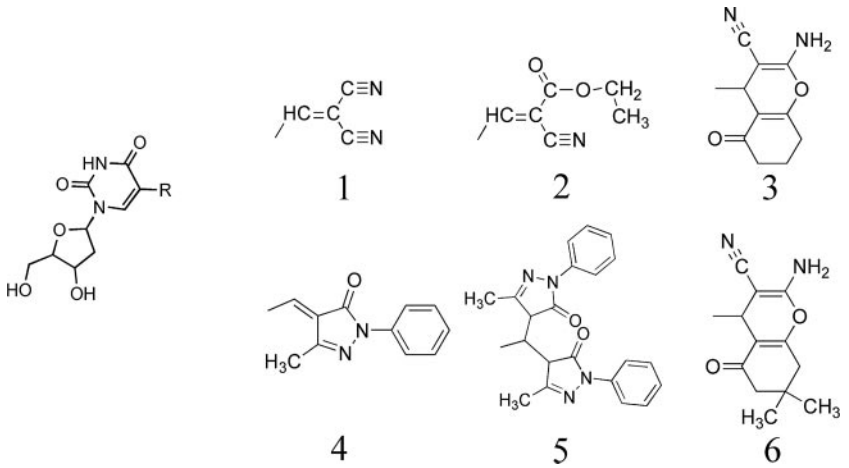

FIG. 1. Structures of 5-substituted deoxyuridine analogs. Compounds 1 to 6 are analogs of deoxyuridine and contain the substitutions shown.

Briefly, substrates were diluted in black clear-bottom, 96-well plates in buffer containing 20 mM HEPES, pH 7.4, 250 mM NaCl, 2 mM dithiothreitol, 1 mM $MgCl_2$, and 50 μM ATP. Reactions were initiated by the addition of the purified enzyme, and the mixtures were incubated at room temperature for 1 h, after which the Kinase-Glo luciferase reagent (Promega) was added. The resulting luminescence was used to measure the quantity of residual ATP in the reaction. Reactions were linear over 90 min and were dependent on the addition of dThd or other suitable substrates.

RESULTS

Antiviral activity and TK dependence in orthopoxviruses. Recently described 5-substituted deoxyuridine analogs that were shown to possess antiviral activity are related in that they have rather bulky substituents in the 5 position of the pyrimidine ring (Fig. 1). These compounds proved to be very active against VV and CV in plaque assays and were relatively nontoxic (Table 1). We hypothesized that these compounds might be selective inhibitors of orthopoxvirus replication because they were preferentially phosphorylated by the viral TK homologs. A recently reported TK dependence assay was used to see if the presence of the CV TK homolog affected the activity of the compounds (47). In this assay, the CDV negative control was equally effective against the TK^+ and TK^- strains of the virus, since it is a monophosphate analog and does not require the first phosphorylation step (Table 1). By contrast, the IDU positive control requires an initial phosphorylation by the viral kinase and consequently was much less active in a TK^- strain, as reported previously (10). In this series of experiments, each of the analogs was more than 20-fold less active in the TK^- strain, which suggested that they required the viral TK for optimal activity. This unexpected result was intriguing, since it suggested that the orthopoxvirus TK activity expressed in infected cells might be able to confer sensitivity to antiviral drugs in the same manner as the herpesvirus TK homologs impart sensitivity to acyclovir.

Antiviral activity against herpesviruses. The TK dependence observed in the first set of experiments prompted an examination of the efficacy of these compounds against herpesviruses, which also express enzymes with TK activity. This series of drugs was evaluated against HSV-1, HSV-2, and VZV using standard plaque assays. Each of these compounds had good antiviral activity against WT strains of HSV-1, HSV-2, and VZV (Table 2). These compounds were also tested in TK-deficient strains of HSV-1 and HSV-2 to see if the drugs also exhibited TK dependence in these viruses. Only compound 5 was substantially less effective in the TK-negative strains of these viruses. This contrasts with the results obtained

TABLE 1. Antiviral activity against orthopoxviruses

Compound	EC_{50} (μM)[a]		TK dependence[b] (μM)			Toxicity[c] (μM)
	VV	CV	TK^-	TK^+	TK^-/TK^+ ratio	
1	17 ± 6.4	21 ± 12	46 ± 15	1.9 ± 0.6	23 ± 1.2	180 ± 73
2	18 ± 14	11 ± 9.2	71 ± 25	1.9 ± 0.5	35 ± 9.3	>300 ± 0
3	4.6 ± 2.0	2.0 ± 0.3	67 ± 6	1.9 ± 0.2	36 ± 4.6	200 ± 11
4	6.9 ± 0.9	5.6 ± 5.2	73 ± 1.2	0.9 ± 0.2	84 ± 14	159 ± 13
5	11 ± 1.0	9.0 ± 7.0	51 ± 0	2.7 ± 1.7	23 ± 12	>234 ± 58
6	7.9 ± 2.5	8.4 ± 3.6	67 ± 0	3.1 ± 2.4	38 ± 32	>233 ± 96
IDU	5.6 ± 0.2	1.8 ± 0.2	76 ± 8.4	1.1 ± 0.3	75 ± 15	>293 ± 0
CDV	11 ± 3.8	32 ± 7.5	16 ± 12	10 ± 3.5	1.9 ± 1.9	>317 ± 0

[a] Effective concentrations that reduced plaque formation by 50% (EC_{50}). Some values were reported previously (22).
[b] EC_{50} values were determined in β-galactosidase assays in TK^- and TK+ strains of CV as described previously (47).
[c] Cytotoxicity was determined by CellTiter-Glo assays (Promega) with standard deviation values shown.

TABLE 2. Antiviral activity against HSV and VZV

Compound	EC_{50} (μM)[a]				
	HSV-1 TK^+ (E-377)	HSV-1 TK^- (DM2.1)	HSV-2 TK^+ (MS)	HSV-2 TK^- (AG-3)	VZV (Ellen)
1	11 ± 0.3	14 ± 4.6	11 ± 2.6	12 ± 4.6	15 ± 1.5
2	7.6 ± 0.8	16 ± 5.7	8.3 ± 2.6	11 ± 1.1	48 ± 11
3	8.6 ± 4.8	13 ± 1.3	12 ± 3.5	12 ± 4.4	16 ± 11
4	7.9 ± 5.5	9.6 ± 2.9	4.2 ± 2.6	7.6 ± 0.9	15 ± 4.5
5	7.9 ± 0.3	25 ± 6.0	10 ± 0.7	39 ± 12	10 ± 1.8
6	7.3 ± 1.9	17 ± 0.6	13 ± 5.3	14 ± 2.9	9.2 ± 3.8
ACV[b]	1.3 ± 0	>444 ± 0	3.1 ± 0	>444 ± 0	8.5 ± 6.3

[a] EC_{50}, effective concentration that reduced plaque formation by 50%. Virus strains used were described previously (47).
[b] ACV, acyclovir.

with CV and suggests that other viral targets may also be involved in the mechanism of action of these compounds in herpesviruses.

Purification of VV TK and characterization of its enzymatic activity. The TK dependence observed in CV suggested that this enzyme was important in the mechanism of action of these new drugs. We hypothesized that the viral TK might be activating the drugs directly. To test this hypothesis, we wanted to determine the relative phosphorylation efficiency of these substrates by human TK1 and J2 in VV. The VV enzyme was selected rather than the CV kinase, since it is more closely related to the enzyme expressed by variola virus. And since the CV and VV kinases are 98% identical at the amino level, they might be expected to exhibit similar activities. Both VV TK and human TK1 were expressed in bacteria, and the histidine-tagged enzymes were purified to see if the viral enzyme selectively phosphorylated these compounds. Enzymatic activity was determined in an ATP utilization assay using luciferase as a reporter. For VV TK, we demonstrated that the utilization of ATP was dependent on thymidine or IDU as phosphate acceptors, and reactions were linear over the 60-min assay period (Fig. 2A). Assays were also optimized with respect to pH (Fig. 2B), salt concentration, and Mg^{2+} requirements (data not shown). The enzyme required Mg^{2+}, and concentrations of salt over 100 mM reduced its activity, while the pH optimum was approximately 9. A previous report identified dTTP and dTDP as allosteric effectors of the enzyme (30). We confirmed the allosteric inhibition with dTTP with the new assay and also observed >50% inhibition of enzymatic activity for dUTP concentrations greater than 60 μM (Fig. 2C). The assay was reproducible, and Lineweaver-Burk plots for thymidine also yielded K_m values between 13 and 49 μM (Fig. 2D; Tables 3 and 4). K_m values were confirmed in a standard spectrophotometric assay (50) and an isothermal calorimetry assay that directly measures the heat of binding of the substrate to the enzyme and generated values of 27 and 24 μM, respectively (P. F. Torrence and R. F. Smith, unpublished data). These values agree well with the reported value of 15 μM for the human TK1 homolog purified from *E. coli*, which occurs predominantly as a dimer (4). These data suggested that the assay conditions were suitable for assessing the kinetic parameters of other potential substrates. Human TK1 was also purified by similar methods (Fig. 2E). Higher-molecular-weight species were observed and were confirmed by mass spectrometry to be TK1, consistent with multimers of this enzyme. Kinase assays with this enzyme yielded results similar to those described for the viral enzyme, including thymidine-dependent ATP utilization, reactions that were linear over the assay period, and K_m values that were similar to those reported in the literature (Fig. 2F) (4).

A series of known pyrimidine analogs was assayed with purified VV TK and human TK1 to further characterize this system. The viral enzyme appeared to phosphorylate dThd, IDU, and bromodeoxyuridine (BrdU) with similar efficiencies, while the phosphorylation of fluorodeoxyuridine (FDU) appeared to be somewhat less efficient (Table 3). TFT and fialuridine (FIAU) appeared to be superior substrates for the VV TK, whereas the cytidine analog, fiacitabine (FIAC), was not a substrate for the viral enzyme. Human TK1 also appeared to phosphorylate most of the substrates with comparable efficiencies, with the single exception of FIAU. This drug had a K_m with the viral TK that was significantly lower than with TK1, suggesting that it was a better substrate for the viral enzyme (Table 1). The efficiency with which the viral enzyme phosphorylates this compound was also more than sixfold higher than that observed with TK1. This selective phosphorylation by the viral enzyme is also consistent with the previously reported activity of this compound against VV and CV (37). The potential phosphorylation of several other compounds was also evaluated in this system, but they did not appear to be suitable substrates, including CDV, acyclovir, penciclovir, ganciclovir, brivudine, and sorivudine (data not shown). These results demonstrate that, in many respects, the substrate specificities of these enzymes are related but also that there are significant differences that might be exploited in the discovery of new antiviral agents.

5-Substituted deoxyuridine derivatives as substrates for VV TK and TK1. The TK dependence exhibited by the compounds in Table 1 suggested that the CV TK was important for their antiviral activity. To test the hypothesis that this might be mediated through selective phosphorylation by the viral TK, we determined their kinetic parameters using purified VV TK and human TK1 (Table 4). This analysis confirmed that all six analogs were substrates for the viral TK, and many of them had K_m values that were similar to thymidine, the natural substrate for this enzyme. Compounds 1, 2, and 4 were phosphorylated with the highest efficiency. These results contrast with those obtained with the cellular enzyme, where only compounds 1 and 2 were substrates, and these had K_m values that exceeded 100 μM and were not

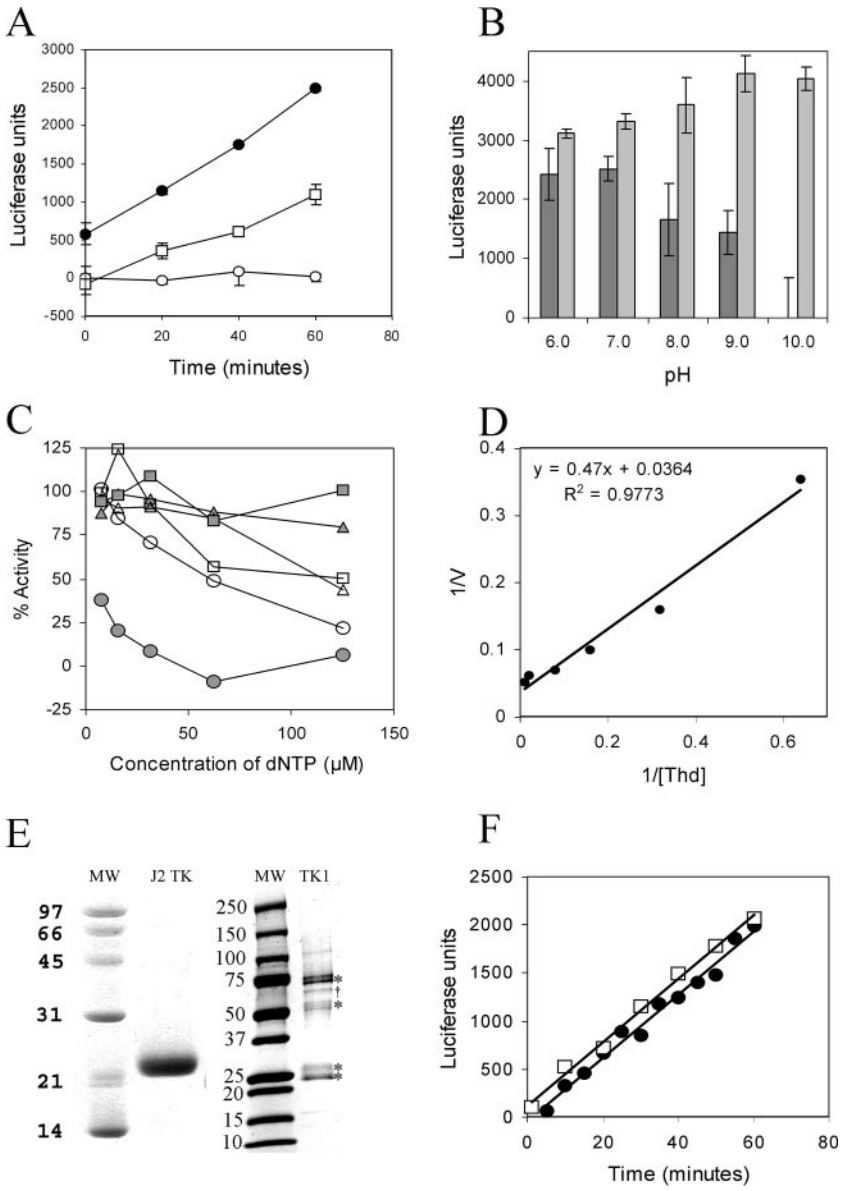

FIG. 2. Characteristics of the enzymatic activity of J2 TK and human TK1. (A) ATP utilization by VV TK was dependent on either 100 μM thymidine (filled circles) or 100 μM IDU (open squares), while no ATP was consumed in the absence of a nucleoside substrate (open circles). (B) Thymidine-dependent utilization of ATP by VV TK was determined at pH 6, 7, 8, 9, and 10. Luciferase activity was measured following a kinase reaction in the presence (dark gray bars) and absence (light gray bars) of 50 μM thymidine. (C) VV TK enzymatic activity was determined in the presence of the potential allosteric effector molecules shown. Shaded circles and open circles represent dTTP and dUTP, respectively, while shaded squares represent the control without added inhibitors. Open triangles, shaded triangles, and open squares represent dGTP, dCTP, and dATP, respectively. (D) A Lineweaver-Burk plot for thymidine as a substrate of VV TK. (E) Coomassie-stained gels of purified J2 TK and human TK1. Higher-molecular-weight species indicated by asterisks in the human TK1 lane were identified as TK1 by mass spectrometry and are consistent with multimers. One contaminating bacterial protein was also identified as indicated by the dagger (†) symbol and is a bacterial protein of unknown function (b2255, gi16130190). (F) Enzymatic assays for both J2 (filled circles) and TK1 (open squares) are linear with time, and both require thymidine or a related substrate for ATP utilization.

phosphorylated efficiently. Of interest, four analogs were phosphorylated by the viral TK with efficiencies that were 10-fold greater than those measured for TK1. Compound 4 appeared to be one of the best substrates for the viral kinase, and its phosphorylation efficiency was 34-fold higher for the viral enzyme. This result is consistent with the TK dependence data, where this compound also exhibited the greatest TK dependence (Table 1). Since the viral enzyme preferentially phosphorylates this series of deoxyuridine analogs as well as FIAU, we conclude that the substrate specificity of the viral enzyme is broader than had been suspected and is distinct from that of TK1. These data, taken together with the TK dependence data, suggest that selective phosphorylation is important in the mechanism of action of these compounds and that orthopoxvirus TK homologs can confer specificity to this series of compounds.

TABLE 3. Kinetic parameters for substrates of VV TK and human TK1 using known antiviral agents

Substrate	VV TK			Human TK1		
	K_m (μM)[a]	V_{max} (μM min^{-1} mg^{-1})	V_{max}/K_m[b]	K_m (μM)[a]	V_{max} (μM min^{-1} mg^{-1})	V_{max}/K_m[b]
dThd	49 ± 7.6	289 ± 137	5.9	60 ± 13	619 ± 70	10
BrdU	30 ± 6.2	281 ± 59	9.4	46 ± 0	573 ± 62	12
IDU	31 ± 4.7	222 ± 58	7.2	38 ± 11	577 ± 26	15
FDU	113 ± 85	108 ± 63	0.96	96 ± 11	472 ± 22	5
TFT	14 ± 4.3	333 ± 44	24	47 ± 2.8	545 ± 68	12
FIAU	4.3 ± 2.5	99 ± 41	22.7	66 ± 3.5	233 ± 6	3.5
FIAC	>135	<0.14	<0.001	>135	<25	<0.19
CDV	>158	<0.025	<0.0002	>159	<25	<0.16

[a] Average of three or more determinations with standard deviations shown.
[b] Calculated efficiency using 0.5 μg of purified VV TK and TK1 in each assay.

Predicted structure of J2. The distinct substrate specificity of the J2R kinase was interesting, since this enzyme is so closely related to the human homolog, TK1. Although the conserved domains in these proteins share 70% identity at the amino acid level, there are regions where they diverge (Fig. 3). To see if these regions might be near the substrate recognition site, we modeled the structure of this enzyme based on an amino acid alignment with four crystal structures of other TKs, including human (Protein Database [PDB] IDs 1XBT and 1W4R), *Clostridium acetobutylicum* (PDB ID 1XX6), and *Ureaplasma parvum* (PDB ID 2B8T). Each of these structures is a type II enzyme and would be predicted to be structurally related. The identity was 70, 37, and 35% between the conserved domains of the viral enzyme and the human, *C. acetobutylicum*, and *U. parvum* TKs, respectively. The coordinates of human TK 1XBT were used as the template to model VV TK, using the program Modeler (41). Since the identity between the conserved domains of the two proteins is 70% (Fig. 3), the reliability of the model for VV TK is high. The active site residues are rather well conserved, suggesting the enzyme specificity of VV TK is similar to that of human TK. This is consistent with results obtained for most compounds in this report; however, significant differences were also observed and may be related to the subtle differences between the two enzymes. One potentially important difference is a substitution of a slightly smaller serine residue for the threonine 163 in TK1 that is in close contact with the 5 methyl group in thymidine. Another difference is the conformation of the side chain of residue Arg45 (Fig. 4). In the crystal structure of 1XBT, the human TK is in complex with the allosteric inhibitor dTTP (53), where the side chain of its active site arginine (Arg60) coordinates with the triphosphate of the inhibitor. The loop next to this arginine (residues 61 to 74) is disordered in this structure. The homologous Arg45 in VV TK was modeled without the bound inhibitor, and this amino acid is located within the space to be occupied by dTTP if present. The conformation of the loop next to Arg45 (residues 46 to 59) was modeled in VV TK (Fig. 4). This loop contains seven residues with significant differences from those in the human TK (Fig. 3). The difference in this loop may offer an opportunity for exploitation of VV TK-specific substrates, especially the five residues (47 to 51) next to Arg45. Moieties representing modifications of the triphosphate of dTTP may result in selective binding to VV TK. Residues within 12 Å of the active site (Fig. 3) may also contribute to the selectivity of a potential inhibitor of VV TK. In the human TK1 structure, the binding pocket is rather open around the 3′ and 4′ carbons of the deoxyribose portion of the molecule (53). Thus by extension, it may be possible to modify this region of the molecule to increase its selectivity for the viral enzyme, which appears to be borne out by the selective phosphorylation of FIAU, which has a fluorine in the 2′ position of an arabinose sugar. After the manuscript was submitted, we determined the crystal structure of the VV TK, and it was also reported by another laboratory (20). The model presented here is very close to the actual structure for the enzyme, and continued efforts with compounds crystallized in the active site will help define the molecular basis of the observed differences in substrate specificity.

TABLE 4. Kinetic parameters for substrates of VV TK and human TK1 using new experimental compounds

Substrate	VV TK			Human TK1		
	K_m (μM)[a]	V_{max} (μM min^{-1} mg^{-1})[a]	V_{max}/K_m[b]	K_m (μM)[a]	V_{max} (μM min^{-1} mg^{-1})[a]	V_{max}/K_m[b]
1	9.6 ± 5.5	120 ± 46	12	113 ± 20	130 ± 65	1.2
2	13 ± 5.2	119 ± 6	9	118 ± 14	98 ± 21	0.8
3	103 ± 2.9	91 ± 33.4	0.9	>200	<55	<0.28
4	11 ± 7.9	106 ± 7	9.6	>200	<55	<0.28
5	21 ± 1.1	91 ± 3	4.3	>200	<55	<0.28
6	97 ± 26	78 ± 7	0.8	>200	<55	<0.28
dThd	21 ± 12	302 ± 7	15	23 ± 13	334 ± 192	14.5
CDV	>200	<55	<0.28	>200	<55	<0.28

[a] Average of three or more determinations with standard deviations shown.
[b] Calculated efficiency using 0.5 μg of purified VV TK and TK1 in each assay.

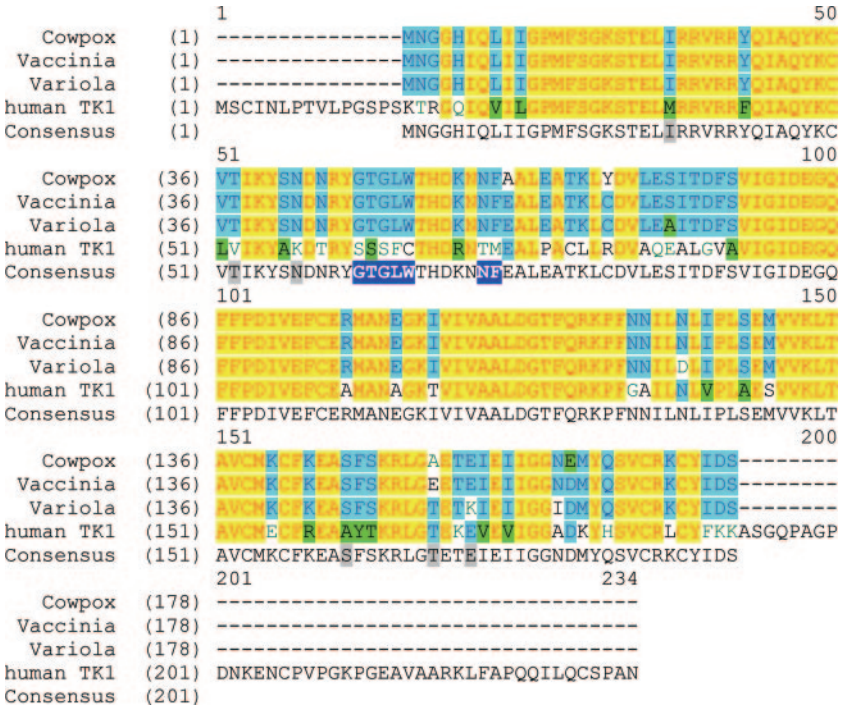

FIG. 3. Alignment of amino acid sequences for selected TK homologs. Amino acid sequences of TK homologs are shown for cowpox virus (NP_619890), vaccinia virus (YP_232976), variola virus (NP_042123), and human TK1 (AAH07986) with the consensus sequence shown below. In the consensus line, text in dark blue boxes represents the significant differences in the loop next to the active site residue Arg45. Text highlighted in gray denotes amino acid differences within 12 Å of the bound inhibitor dTTP.

DISCUSSION

Experiments summarized here described the antiviral activities of a new series of 5-substituted pyrimidine analogs against selected orthopoxviruses and herpesviruses. These data led us to conduct additional experiments examining the mechanisms of action of these compounds. Results from these studies suggested that (i) VV TK can selectively phosphorylate deoxyuridine nucleosides with rather large substituents at the 5 position, (ii) the phosphorylation of these drugs by orthopoxvirus TK homologs is important in the mechanisms of action of these compounds, and (iii) the strategy of selective phosphorylation can be used in the discovery of new inhibitors against orthopoxviruses. These results were unexpected, inasmuch as TK1 shares a high degree of amino acid identity with the VV homolog and was thought to share a similarly narrow substrate specificity (53). They are also important because selective phosphorylation has proven to be one of the most successful strategies for the treatment of herpesvirus infections. Indeed, the treatments of choice for HSV, VZV, Epstein-Barr virus, and cytomegalovirus infections each rely on a viral-encoded enzyme to selectively phosphorylate the drugs, and this activation is required for the compounds to be metabolized to the active form that inhibits the viral DNA polymerase. Unfortunately, these compounds are not active in orthopoxvirus infections, primarily because the initial phosphorylation step is not catalyzed by the TK homologs in these viruses (47). This view is supported by a previous report in which VV became fully sensitive to acyclovir when the HSV TK homolog was supplied in *trans* to activate the drug (14). Thus, a strategy of improving the selective activation of nucleosides could be used to improve the efficacy of drug candidates. Results presented here also support this idea and demonstrate that selective phosphorylation is a feasible strategy for orthopoxviruses.

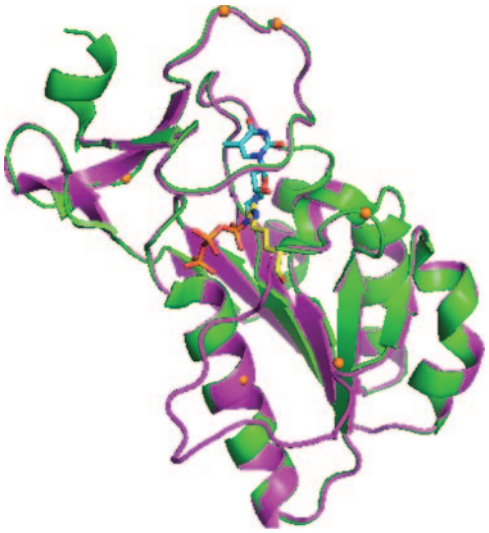

FIG. 4. Model of VV TK structure derived from the crystal structure of 1XBT by blastp (www.ncbi.nlm.nih.gov). Modeled J2R TK structure is shown in magenta overlaid on the reported structure of human TK1 1XBT (green). The modeled side chain of Arg45 (yellow) was shown to overlap with the bound dTTP in 1XBT. Orange dots represent the amino acid differences near the active site. The drawing was created with the program PyMol (Delano Scientific, San Carlos, CA).

The activation of the compounds by the VV TK suggests

that the substrate specificities of the VV TK and TK1 are sufficiently different for such a strategy to be effective. The modeled structure of this enzyme suggests that there are a few amino acid differences near the active sites of these two enzymes that may contribute to the broader substrate specificity observed with the viral enzyme. In this regard, solving the three-dimensional structure of this enzyme should facilitate the design of new inhibitors that are better substrates for the viral kinase, and this project is well under way. This information will represent an important step in the development of even more selective and effective pyrimidine analogs for use in treating orthopoxviruses that rely on the selective activation by the viral kinase, resulting in a highly effective and nontoxic drug. In this endeavor it will also be important to consider differences in the J2R homologs in other orthopoxviruses, particularly variola and monkeypox, which each have 5 amino acid substitutions compared to VV (http://www.poxvirus.org/data.asp). Of particular interest are the E153T and E156K polymorphisms in variola, compared to VV, since each of these amino acids is predicted to lie within 12 Å of the active site of the bound dTTP inhibitor. Also of interest will be the coordinates of substrates cocrystallized in the active sites of the enzymes. These and other similar experiments remain on the critical path of development of these and related compounds.

The TK dependence observed with this series of compounds in CV taken together with the preferential phosphorylation by the VV TK are consistent and suggest a mechanism of action for this series of compounds. We propose that the viral TK activates these compounds by catalyzing the addition of the alpha phosphate on these compounds. Subsequent phosphorylation events by viral or cellular enzymes further phosphorylate the compound to the level of the triphosphate, which in turn inhibits the viral DNA polymerase. Studies presented here do not directly demonstrate the addition of a phosphate to the 5′ position of these compounds, and it remains possible that some other metabolite is the active form and that viral DNA synthesis is inhibited indirectly through the inhibition of thymidylate synthetase or other cellular targets. It is also possible that these compounds are also substrates for other kinases, including the mitochondrial thymidine kinase, TK2, or even the thymidylate kinase encoded by VV, and that other phosphorylation steps are also important in the mechanism of action of these compounds. Nevertheless, the TK dependence data in CV suggests that the viral TK exerts a major influence on the activities of the compounds regardless of the final targets of the inhibitors. Data in favor of our proposed mechanism of action including direct phosphorylation of the drugs are (i) the compounds are analogs of known substrates that are phosphorylated by the VV TK, (ii) the luminescent TK assays presented require a phosphate acceptor for the hydrolysis of ATP and each of the compounds exhibit this property, (iii) the isothermal calorimetry study demonstrated direct binding of the compounds to the enzyme, (iv) the compounds require the viral TK to be active in vitro, and (v) the potent inhibition of DNA synthesis by each of the analogs (EC_{50} values of less than 2 μM) is consistent with direct inhibition of the DNA polymerase by triphosphate metabolites. Further experiments are required to identify the active metabolites and the final targets of these compounds.

Perhaps the most significant aspect of these studies is how they affect the perception of the potential of the orthopoxvirus TK homologs to play a role in the therapy of these infections. The data presented here suggest that the orthopoxvirus and herpesviruses TK homologs can each be used in a common approach towards the development of specific antiviral therapies. While neither enzyme is required for viral replication, both can be exploited by drugs used to inhibit the replication of these viruses. To be sure, the range of substrates that the VV TK will phosphorylate is more restricted than those phosphorylated by the herpesvirus TK homologs, and the TK dependence in herpesviruses is more robust than that seen in the orthopoxviruses, but opportunities remain. Additional experiments may identify better substrates for the kinase that have the potential to be highly selective therapies for the treatment of orthopoxvirus infections.

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