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Inhibition of Herpesvirus Replication and Herpesvirus-Induced Deoxyribonucleic Acid Polymerase by Phosphonoformate†

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Phosphonoformate was found to be an inhibitor of the deoxyribonucleic acid polymerase induced by the herpesvirus of turkeys. The apparent inhibition constants were 1 to 3 μM. Phosphonoformate was also able to block the replication in cell culture of Marek's disease herpesvirus, the herpesvirus of turkeys, and herpes simplex virus. It was as effective as phosphonoacetate. Phosphonoformate was not an effective inhibitor of a phosphonoacetate-resistant mutant of the herpesvirus of turkeys nor of its induced deoxyribonucleic acid polymerase.

Phosphonoacetate is an effective inhibitor of the replication of herpesviruses (11, 13, 23, 25). The inhibition of herpesvirus replication is through an effect on the viral-induced deoxyribonucleic acid (DNA) polymerase (9, 14-16, 18). In animal model studies, the efficacy of phosphonoacetate as an antiherpesvirus drug has been clearly demonstrated (7, 8, 12). Its clinical use, however, may be limited because it is somewhat toxic to test animals and because it is accumulated in bone (4).

Other phosphonate compounds are of interest as inhibitors of herpesvirus replication because they might exhibit an improved therapeutic ratio over phosphonoacetate either by being more effective inhibitors of virus replication or by being less toxic to animals. These compounds are also of interest at the enzymological level for the information that they might provide about the binding site on the herpesvirus-induced DNA polymerase. Consequently, using as an assay procedure the ability to inhibit the herpesvirus-induced DNA polymerase or the ability to block herpesvirus replication in cell culture or in animals, many other phosphonates have been looked at (10, 13, 14, 23). Only the low-molecular-weight carboxyl esters of phosphoacetate have proven to be effective inhibitors.

In this report, we demonstrate that phosphonoformate, a compound first synthesized in 1924 (17), is an effective inhibitor of the DNA polymerase induced by the herpesvirus of turkeys (HVT) and blocks the replication of Marek's disease herpesvirus, HVT, and herpes simplex virus (HSV). Further, we compare the effectiveness of phosphonoformate with phosphonoacetate. We also report the effect that phosphonoformate has on a phosphonoacetate-resistant mutant of HVT and point out that phosphonoformate inhibits herpesvirus replication through an effect on the DNA polymerase in an analogous manner to phosphonoacetate.

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EXPERIMENTAL PROCEDURE

Reagents. Phosphonoacetate, disodium salt, was a gift from Abbott Laboratories. Triethyl phosphite and ethyl chloroformate were purchased from Aldrich Chemical Co. Triethyl phosphite was redistilled before use. Other reagents were from sources previously described (3) or were from the usual commercial sources.

Synthesis of triethyl phosphonoformate and trisodium phosphonoformate. Both triethyl phosphonoformate and trisodium phosphonoformate were prepared following the procedure of Nylen (17) as described by Warren and Williams (26). In brief, triethyl phosphonoformate was prepared by an Arbuzov reaction with ethyl chloroformate and triethyl phosphite and was purified by vacuum distillation. The infrared spectrum and proton nuclear magnetic resonance spectrum agreed with published spectra (21, 22). The ^{13}C-nuclear magnetic resonance proton-decoupled spectrum gave resonances at 14.6 ppm ($CO_2CH_2\mathit{C}H_3$), 16.8 ppm (doublet, J_{P-C} = 5.9 Hz, $POCH_2\mathit{C}H_3$), 62.6 ppm (doublet, J_{P-C} = 4.4 Hz, $CO_2\mathit{C}H_2CH_3$), 64.9 ppm (doublet, J_{P-C} = 6.6 Hz, $PO\mathit{C}H_2CH_3$), and 168.2 ppm (doublet, J_{P-C} = 265.4 Hz, C = O). The spectrum was obtained using a Bruker WP-60 spectrometer, and all chemical shifts are relative to tetramethylsilane. The triethyl ester was saponified

with NaOH, and the product was recrystallized several times from water to give trisodium phosphonoformate hexahydrate. Phosphorus analysis (1) for $CNa_3O_5P \cdot 6H_2O$ gave a molecular weight of 299 (in theory, 300). The ^{13}C-nuclear magnetic resonance proton-decoupled spectrum gave a resonance at 180 ppm (doublet, $J_{P\text{-}C}$ = 229.3 Hz). Trisodium phosphonoformate was further characterized by descending paper chromatography with the following solvent systems: (i) isopropyl alcohol-water-concentrated ammonia (7:2:1), R_f = 0.03; (ii) methanol-water-concentrated ammonia (6:1:3), R_f = 0.63; (iii) ethanol-1 M ammonium acetate, pH 7.5 (5:2), R_f = 0.06. The chromatograms were sprayed as described by Bandurski and Axelrod (2), and only a single blue spot was detected with each solvent system.

Virus strains. Marek's disease herpesvirus strain GA (MDHV) (19) was propagated in primary duck embryo fibroblasts as previously described (24). HVT strain FC-126 (HVT_{wt}) (28) and a phosphonoacetate resistant mutant of this strain (HVT_{pa}) (L. F. Lee, K. Nazerian, R. Witter, S. Leinbach, and J. Boezi, manuscript in preparation) were also propagated in primary duck embryo fibroblasts as previously described. The HSV type 1 used was the MP strain (20) adapted to duck embryo fibroblasts.

Assessment of phosphonate effect on herpesvirus replication. Triplicate duck embryo fibroblasts culture samples were inoculated with MDHV and incubated with various concentrations of phosphonoformate or phosphonoacetate according to the following regimen: with 0.035 mM phosphonate, cultures were inoculated with 50, 100, 500, and 1,000 plaque-forming units (PFU); with 0.07 mM phosphonate, cultures were inoculated with 100, 500, 1,000, and 5,000 PFU; at 0.14 mM phosphonate, with 5,000 and 10,000 PFU; and at 0.28 mM, with 50,000 and 100,000, and 500,000 PFU. Cultures were incubated, and plaques were counted 6 or 7 days postinfection. Relative numbers of plaques were determined by dividing the observed number of plaques formed by the input PFU.

Identical culture conditions were used for HVT_{wt} and HVT_{pa} except that triplicate cultures were all inoculated with 100 PFU in various concentrations of either phosphonate, and plaques were counted 5 days postinfection. The percentage of plaques surviving was calculated from cultures containing no phosphonate.

Identical culture conditions were also used for HSV except that it was inoculated at about 1,000 PFU/plate into cultures containing various concentrations of either phosphonate.

Preparation of HVT-induced DNA polymerase. Both HVT_{wt} and HVT_{pa} were treated in an identical manner. The preparation and growth of duck embryo fibroblasts and infection with virus was as previously described (3). The partial purification of the HVT-induced DNA polymerase was from the nuclear fraction of infected cells by phosphocellulose chromatography as described by Leinbach et al. (14). The specific enzymatic activity of the preparation used in the kinetic studies reported here was about 1,000 nmol of deoxynucleoside monophosphate incorporated into DNA/30 min per mg of protein. This enzyme fraction, when tested using the standard assay conditions for DNA polymerization, contained no detectable deoxyribonuclease activity, deoxyribonucleoside triphosphatase activity, or inorganic pyrophosphatase activity. The kinetic experiment with HVT_{wt}-induced DNA polymerase was also performed with a more highly purified preparation which had been purified by phosphocellulose and hydroxylapatite chromatography. No differences in the results were seen.

Inhibition patterns. Inhibition patterns and kinetic constants were defined according to the nomenclature of Cleland (5, 6). The data for the double reciprocal plots were evaluated using a computer program based on the method of Wilkinson (27). For evaluation of the apparent inhibition constants, replots of the intercepts and slopes of the double reciprocal plots were analyzed by the method of least squares.

RESULTS

Phosphonoformate inhibition of the DNA polymerization reaction catalyzed by HVT_{wt}-induced DNA polymerase. In the course of a study examining phosphonate compounds for their ability to inhibit the herpesvirus-induced DNA polymerase, it was discovered that phosphonoformate was an effective inhibitor of the HVT_{wt}-induced DNA polymerase. The addition of 2 to 3 μM phosphonoformate to the standard assay mixture resulted in a decrease in the rate of the DNA polymerization reaction by about 50%. The inhibition patterns produced by phosphonoformate were examined, and as shown in Fig. 1, phosphonoformate gave linear noncompetitive inhibition with the four deoxynucleoside triphosphates (dNTP's) as variable substrate and activated DNA at a saturating concentration of 200 μg/ml. The apparent inhibition constant determined from the replot of the vertical intercepts against phosphonoformate concentration (K_{ii}) was 1.1 μM. The apparent inhibition constant determined from the replot of the slopes against phosphonoformate concentration (K_{is}) was 0.9 μM. With activated DNA as the variable substrate and the four dNTP's at their apparent Michaelis constant

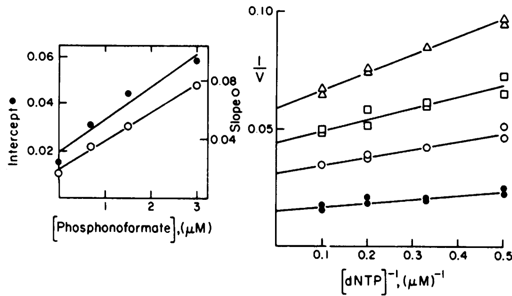

FIG. 1. *Double reciprocal plots of the HVT_{wt}-induced DNA polymerase-catalyzed reaction with the four dNTP's as the variable substrate and phosphonoformate as inhibitor. Activated DNA was at a concentration of 200 μg/ml. The initial velocities were expressed as picomoles of [^{3}H]thymidine 5'-monophosphate incorporated into DNA/30 min. Phosphonoformate concentrations were 0 (●), 0.7 μM (○), 1.5 μM (□), and 3.0 μM (△). Equimolar concentrations of each of the four dNTP's were present in the different reaction mixtures. The replots of the slopes (○) and intercepts (●) as a function of phosphonoformate concentration are shown in the left panel.*

concentration of 2.5 μM each, phosphonoformate gave linear noncompetitive inhibition (data not shown). A replot of the vertical intercepts yielded a K_{ii} of 2.6 μM, and a replot of the slopes yielded a K_{is} of 2.3 μM. The inhibition patterns and the apparent inhibition constants are similar to those obtained with phosphonoacetate (14).

Effect of phosphonoformate on the replication of herpesviruses in cell culture. After this discovery, phosphonoformate was tested for its ability to block the replication of HVT_{wt}, MDHV, and HSV in cell culture. Again, phosphonoformate was an effective inhibitor. With HVT_{wt}, phosphonoformate was as effective an inhibitor as phosphonoacetate (Fig. 2). The addition of 0.06 to 0.07 mM of either phosphonate to the culture medium brought about a 50% reduction in the number of plaques observed. Essentially no plaques were observed at concentrations above 0.3 mM.

Phosphonoformate and phosphonoacetate also exhibited a parallel ability to block the replication of MDHV. The addition of either phosphonate to a final concentration of about 0.02 mM brought about a 50% reduction in the number of plaques produced. Either phosphonate at 0.14 mM reduced the number of plaques by more than three orders of magnitude (Fig. 3). At 0.28 mM, no plaques at all were observed (data not shown).

Phosphonoformate also inhibited the replication of HSV type 1 in cell culture (data not shown). Again, phosphonoformate was about as effective an inhibitor as phosphonoacetate.

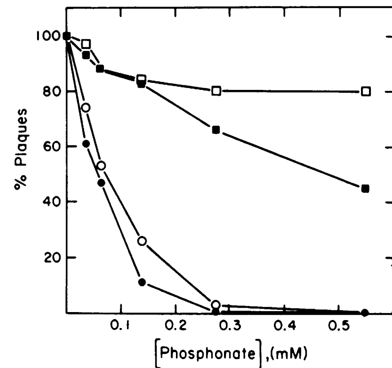

FIG. 2. *Effect of phosphonoformate and phosphonoacetate on the replication of HVT_{wt} and HVT_{pa}. A known titer of each virus preparation was used to infect secondary duck embryo fibroblast cultures in growth media without and with different concentrations of phosphonate. Plaques were enumerated 5 days postinfection. Each point represents the average of three experiments, and the number of plaques enumerated in cultures without phosphonate was considered as 100%. Viruses and inhibitors were HVT_{wt} with phosphonoformate (●), HVT_{wt} with phosphonoacetate (○), HVT_{pa} with phosphonoformate (■), and HVT_{pa} with phosphonoacetate (□).*

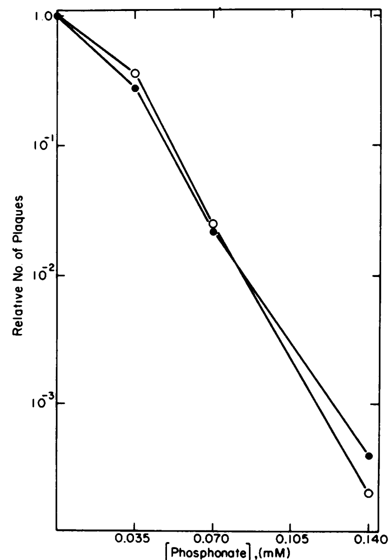

FIG. 3. *Semilog plot of the effect of phosphonoformate (●) and phosphonoacetate (○) on the replication of MDHV. A known titer of virus was used to infect secondary duck embryo fibroblast cultures in growth media without and with different concentrations of phosphonate. Plaques were enumerated 6 to 7 days postinfection. Each point represents the average of three experiments. The number of plaques observed in each experiment was normalized to the input number of PFU.*

At concentrations up to 0.55 mM, phosphonoformate had no obvious cytotoxic effect on the growth of normal duck embryo fibroblasts. The treated cells formed a confluent monolayer in the usual time, and maintenance of the monolayer was normal.

In the above studies, phosphonoformate was an effective inhibitor of the replication of MDHV, HVT_{wt}, and HSV in cell culture. Phosphonoformate, however, was not an effective inhibitor of the replication of HVT_{pa} in cell culture. As seen in Fig. 2, the replication of HVT_{pa} is less sensitive to phosphonoformate than is the replication of HVT_{wt}. This indicates that the mutation to phosphonoacetate resistance also leads to phosphonoformate resistance and suggests that the DNA polymerase of HVT_{pa} is altered so as to be less sensitive to phosphonoformate as well as phosphonoacetate.

Phosphonoformate inhibition of the DNA polymerization reaction catalyzed by the HVT_{pa}-induced DNA polymerase. Indeed, phosphonoformate, like phosphonoacetate, was not an effective inhibitor of the DNA polymerase of this phosphonoacetate-resistant mutant. With the four dNTP's as the variable substrate, phosphonoformate again gave linear noncompetitive inhibition (Fig. 4). The K_{ii} was 8 μM and K_{is} was 18 μM. This represents an increase of 10 to 20 times over the values obtained for the HVT_{wt}-induced DNA polymerase and is similar to the increase seen with phosphonoacetate (L. F. Lee et al., manuscript in preparation). With activated DNA as the variable substrate and the four dNTP's at their apparent Michaelis concentration of 5 μM, phosphonoformate gave linear noncompetitive inhibition, a K_{ii} of 13 μM, and a K_{is} of 43 μM. Again, this represents an increase of about 10 to 20 times over the same values obtained for the HVT_{wt}-induced DNA polymerase, as was also seen with phosphonoacetate.

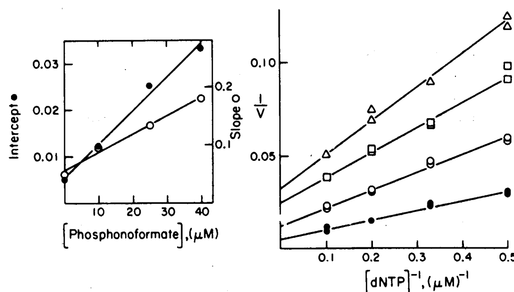

FIG. 4. *Double reciprocal plots of the HVT_{pa}-induced DNA polymerase-catalyzed reaction with the four dNTP's as the variable substrate and phosphonoformate as inhibitor. Activated DNA was at a concentration of 200 μg/ml. The initial velocities were expressed as picomoles of [^{3}H]thymidine 5'-monophosphate incorporated into DNA/30 min. Phosphonoformate concentrations were 0 (●), 10 μM (○), 25 μM (□), and 40 μM (△). Equimolar concentrations of each of the four dNTP's were present in the reaction mixtures. The replots of the slopes (○) and intercepts (●) as a function of phosphonoformate concentration are shown in the left panel.*

DISCUSSION

The replication of MDHV, HVT_{wt}, and HSV in cell culture was effectively inhibited by phosphonoformate. This inhibition was as effective as the inhibition by phosphonoacetate. The inhibition patterns seen in the steady-state enzyme kinetic analysis of the HVT_{wt}-induced DNA polymerase with phosphonoformate as inhibitor were identical to those reported for phosphonoacetate (14). Both phosphonates showed noncompetitive inhibition with the four dNTP's as variable substrate. With activated DNA as the variable substrate and the four dNTP's at their Michaelis concentration, noncompetitive inhibition was also observed. The apparent inhibition constant values were similar. These observations, taken together with the resistance of HVT_{pa} replication to the effect of phosphonoformate and the much higher inhibition constant values obtained for the HVT_{pa}-induced DNA polymerase, indicate that the inhibition of herpesvirus replication by phosphonoformate is through an effect on the herpesvirus-induced DNA polymerase in a manner analogous to the inhibition by phosphonoacetate (9, 14–16).

Although phosphonoformate is a potent inhibitor of the herpesvirus-induced DNA polymerase, it is not entirely specific. Recent experiments in this laboratory have shown that the α-polymerase of Hela, KB, and Wi-38 cells was inhibited by phosphonoformate (C. L. K. Sabourin, J. Reno, and J. Boezi, manuscript in preparation). Phosphonoacetate also inhibits these enzymes. The apparent inhibition constant values for either phosphonate were about 30 μM. The β and γ polymerases are not effectively inhibited by phosphonoformate or phosphonoacetate.

The results of previous studies on analogs of phosphonoacetate demonstrated that the structural requirements for inhibition were rather narrowly defined (10, 13, 14, 23). For example, analogs containing a mono- or diester on the phosphono group or containing a carboxyl or sulfo substitution for the phosphono group were not inhibitors. Analogs that contained a methylamino- or phenyl-substituted methylene carbon also were not inhibitors. Apparently some modification at the carboxyl end of phosphonoacetate is permissible. Low-molecular-weight carboxyl esters are reported to be effective inhibi-

tors (10). Aldehyde, amide, and acetonyl substitutions for the carboxyl group of phosphonoacetate, however, did not yield effective inhibitors (J. A. Boezi, unpublished results). Phosphonates having longer carbon chain length than phosphonoacetate (for example, phosphonopropionate or phosphonobutyrate) were not inhibitors. This report demonstrates that the shorter chain length of phosphonoformate yielded an effective inhibitor.

Now that phosphonoformate has been shown to be an effective inhibitor of herpesvirus replication, its efficacy as an antiherpesvirus drug in animals must be determined. Recent results have shown that phosphonoformate is as effective as phosphonoacetate against HSV types 1 and 2 in mice and guinea pigs (E. R. Kern, J. Overall, L. Glasgow, J. Reno, and J. Boezi, manuscript in preparation). Additional animal model systems will be tested, and toxicity studies will follow. Phosphonoformate may be of sufficiently different chemistry that it would be less toxic, would not be accumulated in bone, and might become a useful drug in the treatment of herpesvirus infections.

ACKNOWLEDGMENTS

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