Activity of the Cytomegalovirus Genome in the Presence of PP₁ Analogs

B. WAHREN,¹ U. RUDÉN,¹ H. GADLER,¹ B. ÖBERG,² AND B. ERIKSSON²

Department of Virology, National Bacteriological Laboratory, S-105 21 Stockholm, Sweden,¹ and Department of Antiviral Chemotherapy, Research and Development Laboratories, S-151 85 Södertälje, Sweden²

Received 2 July 1985/Accepted 9 August 1985

PP₁ analogs and esters of these were studied for their effect on cytomegalovirus (CMV) multiplication. Five aromatic monoesters of phosphonofomate esterified either in the phosphono or the carboxylic group and two diesters were demonstrated to inhibit CMV DNA synthesis and late viral protein synthesis. In a direct assay, the monoesters but not the diesters inhibited CMV DNA polymerase activity. The production of early CMV antigens was not inhibited by any of the compounds. After incubation with either drug for periods up to 7 days, renewed viral production occurred on withdrawal of the compound. All inhibitory esters as well as PP₁ analogs showed a CMV multiplicity dependence. This was demonstrated both for CMV strain Ad.169 and for all tested CMV isolates. Evidence was found that the esters are hydrolyzed to phosphonofomate and, therefore, may be of importance as useful produgs in the specific therapy of CMV infections. The general phenomenon of reversibility to the productive state and the multiplicity dependence of CMV are important factors in any treatment schedule.

In recent years it has become possible to study the molecular events of compounds that inhibit viral replication. The interaction between virus-specified enzymes and analogs to substrates or produgs participating in the enzyme reactions are especially important (10, 14). Cytomegalovirus (CMV) encodes a DNA-dependent DNA polymerase which is inhibited by analogs of PP₁, such as phosphonofomate (PFA) and phosphonoacetate (PAA), at concentrations which do not inhibit cellular polymerases (5). PAA inhibits the activity of herpes simplex virus (HSV) DNA polymerase by interacting with the enzyme rather than the template (12). By binding to the enzyme, it has been suggested that PAA interacts at the site at which PP₁ is split off during the elongation process of growing viral DNA (11, 15). The same mechanism seems to hold true for the effect of PFA and most other PP₁ analogs on CMV DNA polymerase activity (4). The inhibition of herpesvirus DNA polymerases by PFA and PAA is noncompetitive with respect to deoxynucleotide triphosphates and uncompetitive with respect to templates (3, 5, 11, 12).

Certain newly synthesized derivatives of PP₁ analogs were found to possess CMV-inhibiting activity in vitro. Esterification of different negatively charged groups of PAA (1) or PFA (13) previously was shown not to inhibit the HSV DNA polymerase activity. We found five monoesters of PFA that directly inhibited the activity of CMV DNA polymerase and two diesters that inhibited CMV multiplication in tissue culture. The active derivatives were monoesters with an aromatic substituent in either the phosphono or the carboxylic group.

MATERIALS AND METHODS

Cells and virus. Human embryonic lung fibroblasts were cultured in Eagle minimal essential medium supplemented with 2% calf serum and antibiotics. The cellular toxicity (Table 1) was evaluated with a microscope and consisted of poorly growing, nonconfluent, slender, or fragmented cells, as compared with control cells without any added compound.

CMV Ad.169 was plaque purified twice, and either of the first two passages at low multiplicities of infection was used in the experiments. Primary CMV was isolated in 1984 from urine or blood of adults who had received bone marrow transplants or renal transplants. Virus from the lowest possible passage on human embryonic lung fibroblast cells (passages two through six) was used.

Antiviral compounds. All compounds were synthesized at or received from the Department of Antiviral Chemotherapy, Astra Läkemedel AB, Sweden. The basic structure of the PFA esters is given in Fig. 1. The detailed syntheses of the PP₁ analogs and derivatives have been described previously (3, 13). The esters were synthesized from PFA. Analyses of the PFA content of all active drugs were made by high-pressure liquid chromatography with a reversed-phase ion-pair technique on a C₁₈ column and an electrochemical detector ESA 5100 A (Coalochem).

Viral sensitivity assay. The viral sensitivity assay (VSA) was performed as described previously (20). Briefly, about 3 × 10⁵ human embryonic lung fibroblast cells growing in 24-well plastic plates (Linbro; Flow Laboratories, Hamden, Conn.) were incubated in duplicate for 1 h with different dilutions of CMV. Subsequently, the excess inoculate was removed, and dilutions of antiviral compounds were added and allowed to remain for the entire period of cultivation, usually 4 to 6 days. The CMV antigen content of the cell lysate was determined as enzyme-linked immunosorbent assay (ELISA;17, 20). Monkey anti-CMV immunoglobulin G (IgG) was used to coat 96-well microplates (M129AR; Dynatech, Zug, Switzerland) and incubated with 100 µl of viral antigen dilutions, each in duplicate. After washing, rabbit anti-CMV was added, followed by swine anti-rabbit IgG labeled with alkaline phosphatase, and after further washing, p-nitrophenyl phosphate was added as substrate. A₄₁₀ was measured in a Dynatech MR600 spectrometer (Arlington, Va.) Data were analyzed by a specially designed computer program (23). The concentration of antiviral compounds needed to reduce the amount of viral antigens to 50% (50% inhibiting concentration [IC₅₀]) was related to the amount of viral antigen after growth in culture without the

* Corresponding author.
The presence of 100% partially purified polymerase from each of enzyme 200 mM triphosphates was determined in precipitation activity of CMV-infected DNA. Incubation incorporation of DNA was calculated for hybridization. Whatman filter paper was dried and baked at 80°C for 2 h, and hybridizations were carried out with a denatured mixture of 3P-labeled, nick-translated CMV DNA. After autoradiography, the spots were also cut out and counted in a scintillation counter for accurate measurement of the IC50 of the various compounds.

**Immunofluorescence.** Staining for CMV early antigens (EA) was performed by anticomplement immunofluorescence (19, 21) 1 day after infection. The serum was a rabbit anti-CMV DNA polymerase with an anticomplement immunofluorescent titer of 80. CMV late antigens (LA) as well as CMV titers (fluorescence focus units (21)) were determined on day 6 by indirect immunofluorescence with a human anti-CMV nucleoprotein IgG with an indirect immunofluorescent titer of 160. The IC50 of antiviral drug for reduction of EA or LA were determined.

**RESULTS**

**Production of CMV proteins in the presence of PP, analogs and esters.** Analogs of PP and esters of PFA were assayed in tissue culture for their inhibitory activity to CMV Ad.169 and a primary isolate as determined by the VSA method (Table 1). Seven esters of PFA were found to possess an inhibitory potential. Of these, three were monosubstituted in the phosphono moiety (B041, B059, B442) and two in the carboxyl moiety (B130 and B239). Two derivatives were diesters (B043 and B065). Four of the most active PP, analogs known are also listed: PFA, PAA, carbonyl diphosphonate (CDP), and α-hydroxy phosphonoacetate (HPA).

Derivatives that were nontoxic and inactive as inhibitors at the concentrations tested (25 to 800 μM) were five monooesters of PFA (B440, B452, B238, B242, and B420), one diester (B848), and two triesters (B056, B057). Inactive and nontoxic PP, analogs were phosphonoacetamide (B755), hypophosphate (B785), and α-substituted phosphonoacetates (B608, B623, B985). One monooester of PFA (B135) and a PAA analog with a long chain in the α-position (B624) were toxic to cells.

The hydrolysis of the diester B043 with regard to PFA formation was studied. A solution of 400 μM B043 contained <3 μM PFA. After addition to CMV-infected cells 10 μM PFA was formed after incubation for 5 days. In uninfected cells 6 μM PFA was formed after incubation for 5 days. The amount of PFA in the stock solution of 400 μM B043 did not change after 10 days of storage at room temperature. Thus both uninfected and CMV-infected cultures converted this PFA ester to PFA.

**Multiplicity dependence.** Different multiplicities of virus inoculates were assayed with various concentrations of the inhibitory compounds. An analysis with PFA, HPA, and the seven active esters demonstrated a multiplicity dependence for all compounds (Table 2). PFA was the compound studied most thoroughly for multiplicity dependence. Of 40 primary isolates from different patients, multiplicity dependence was clearly seen in 36 cases. In the remaining four cases similar IC50 values of PFA were obtained at the two virus dilutions. With a fourfold difference in viral input, the IC50 difference among the 36 CMV isolates was 127 ± 98 μM for PFA (mean ± standard deviation). Three PFA esters were assayed with five primary CMV isolates. For B041 the mean IC50 difference between fourfold virus dilutions was 100 μM, for B065 it was 40 μM, for B239 it was 50 μM.

**EA persistence and reversibility of CMV inhibition.** The activity of the compounds on formation of CMV EA and CMV LA was assayed by studying the appearance of CMV.
antigens in individual cells by immunofluorescence. Neither the seven esters nor the two other active PP₁ analogs inhibited EA formation. However, all effectively inhibited the formation of LA and cytopathic effect (CPE). Table 3 shows the inhibition of LA but not EA by three PFA esters and two other PP₁ analogs with CMV Ad.169 and a primary CMV isolate.

It previously has been shown that the inhibitory effect of PFA on CMV multiplication is reversible (22). CMV-infected cells therefore were incubated with 125 to 625 μM PFA, HPA, and three active esters (B041, B239, and B065) to completely inhibit virus replication. After 7 days, the compounds were withdrawn, and the possible subsequent appearance of CPE and CMV LA were studied. The two tested CMV strains both resumed replication and late protein synthesis, as determined by the occurrence of LA and CPE. The time required for measurable amounts of LA to appear was 3 to 9 days after withdrawal of the compounds (data not shown). The observed variation in time may be an effect of the initial viral dose or an effect of the highest (625 μM) drug concentrations on cellular metabolism or both.

CMV Ad.169 and four additional primary CMV isolates were assayed for reversion after incubation for 12 days with 300 μM PFA. After they were suppressed, as judged by the lack of CPE and LA, virus reappeared in all cases with a yield of 0.1 × 10⁵ to 2.5 × 10⁵ fluorescence focus units per ml.

**TABLE 1. Structures of PP₁ analogs and esters**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitory PFA esters</td>
<td>C₆H₅</td>
<td>4-CH₃C₆H₅</td>
<td>5-Indanyl</td>
</tr>
<tr>
<td>B041</td>
<td>I-P</td>
<td>C₆H₅</td>
<td>4-CH₃C₆H₅</td>
</tr>
<tr>
<td>B059</td>
<td>I-P</td>
<td>C₂H₅</td>
<td>4-CH₃C₆H₅</td>
</tr>
<tr>
<td>B442</td>
<td>I-P</td>
<td>C₂H₅</td>
<td>4-CH₃C₆H₅</td>
</tr>
<tr>
<td>B130</td>
<td>I-C</td>
<td>C₂H₅</td>
<td>4-CH₃C₆H₅</td>
</tr>
<tr>
<td>B239</td>
<td>I-C</td>
<td>C₂H₅</td>
<td>4-CH₃C₆H₅</td>
</tr>
<tr>
<td>B043</td>
<td>II</td>
<td>C₂H₅</td>
<td>4-CH₃C₆H₅</td>
</tr>
<tr>
<td>B065</td>
<td>II</td>
<td>C₂H₅</td>
<td>4-CH₃C₆H₅</td>
</tr>
<tr>
<td>Nonactive and nontoxic PFA esters</td>
<td>4-Cl C₆H₄</td>
<td>4-CH₃C₆H₄</td>
<td>4-CH₃C₆H₄</td>
</tr>
<tr>
<td>B440</td>
<td>I-P</td>
<td>2,6-(CH₃)₂C₄H₃</td>
<td>4-Cl C₆H₄</td>
</tr>
<tr>
<td>B452</td>
<td>I-P</td>
<td>C₂H₅</td>
<td>4-Cl C₆H₄</td>
</tr>
<tr>
<td>B238</td>
<td>I-C</td>
<td>C₂H₅</td>
<td>4-Cl C₆H₄</td>
</tr>
<tr>
<td>B242</td>
<td>I-C</td>
<td>C₂H₅</td>
<td>4-Cl C₆H₄</td>
</tr>
<tr>
<td>B420</td>
<td>I-C</td>
<td>CH₃</td>
<td>iso-C₆H₄</td>
</tr>
<tr>
<td>B848</td>
<td>II</td>
<td>CH₃</td>
<td>iso-C₆H₄</td>
</tr>
<tr>
<td>B056</td>
<td>III</td>
<td>4-CH₃C₆H₄</td>
<td>iso-C₆H₄</td>
</tr>
<tr>
<td>B057</td>
<td>III</td>
<td>4-CH₃C₆H₄</td>
<td>iso-C₆H₄</td>
</tr>
</tbody>
</table>

* Effect on CMV Ad. 169 and a CMV isolate (V2428) multiplication in tissue culture VSA.

**TABLE 2. Effect of PP₁ analogs and derivatives on CMV by VSA**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM ± SD) for viral antigen production at the following multiplicities of infection:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>B041</td>
<td>25 ± 9</td>
</tr>
<tr>
<td>B059</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>B442</td>
<td>31 ± 15</td>
</tr>
<tr>
<td>B130</td>
<td>75 ± 9</td>
</tr>
<tr>
<td>B239</td>
<td>60 ± 24</td>
</tr>
<tr>
<td>B043</td>
<td>48 ± 12</td>
</tr>
<tr>
<td>B065</td>
<td>50 ± 14</td>
</tr>
<tr>
<td>PFA</td>
<td>37 ± 35</td>
</tr>
<tr>
<td>HPA</td>
<td>58 ± 31</td>
</tr>
</tbody>
</table>

**TABLE 3. Inhibition of late but not of early CMV antigens by PFA, esters, and HPA**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Multiplicity of infection</th>
<th>CMV antigen</th>
<th>IC₅₀ (μM) for CMV antigens in ACIF or IF:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B041</td>
</tr>
<tr>
<td>CMV AD.169</td>
<td>1.0</td>
<td>EA</td>
<td>&gt;400</td>
</tr>
<tr>
<td>V2828</td>
<td>1.0</td>
<td>EA</td>
<td>&gt;400</td>
</tr>
</tbody>
</table>

* ACIF, Anticomplement immunofluorescence; IF, indirect immunofluorescence.
merase activity were PAA, CDP, and HPA (Table 5). Among the compounds active in VSA, PFA had the highest ratio of 133 between its activity on cellular polymerase α and CMV DNA polymerase. B239 was the most effective ester in this respect with a ratio of 89.

The content of PFA was analyzed in the solutions of all esters active on CMV DNA polymerase, to exclude the possibility that contaminated PFA mediated the inhibitory activities. The amount of PFA in the solutions varied from 0.005 to 1.3%. The PFA concentration in the dilutions of monoesters giving 50% CMV DNA polymerase inhibition (Fig. 2) was 0.03 μM for B041, 0.05 μM for B239, 0.13 μM for B130, 0.03 μM for B442, and 0.17 μM for B059, and for the diester B043 it was 0.28 μM. Only for the diester B043 would the PFA content explain the observed inhibition.

Among the esters which were not inhibitory to CMV in tissue culture, two (B238 and B242) inhibited the CMV DNA polymerase activity at low concentrations. Among the compounds toxic to cells, two (B135 and B624) were directly inhibitory to the CMV DNA polymerase activity. B242 and B624 had activity ratios of 142 and 250, respectively. It was not meaningful to calculate ratios for additional compounds since their effects on CMV DNA polymerase was low or the compounds were inactive in tissue culture.

**DISCUSSION**

It was shown that seven esters of PFA inhibited replication in vitro of both CMV Ad.169 and patient isolates of CMV. CMV strains passaged in human fibroblasts are often used for in vitro studies of CMV properties. It was shown, however, that CMV Ad.169 is more susceptible to inhibition by PFA than are the majority of CMV isolates passaged a few times (23). Also, infection of monocytes by CMV is feasible by early nonfibroblast-adapted CMV isolates but not by CMV Ad.169 (2, 16). Thus, it appears to be important to include primary patient isolates in studies of CMV biology.

Aromatic mono- and diesters of PFA inhibited HSV type 1 (HSV-1) plaque formation (13). Some monoesters and diesters were also active against cutaneous HSV-1 infection in guinea pigs. However, none of the esters inhibited the activity of isolated HSV-1 polymerases (13). We found, however, that seven aromatic mono- and diesters inhibit CMV DNA synthesis and late CMV protein synthesis. Five monoesters, among which the monoesters B041 and B239 appeared to be most potent, inhibited the CMV DNA polymerase but not the cellular DNA polymerase α. This indicates that the CMV polymerase PP binding site is available for larger molecules than both cellular and herpes simplex virus DNA polymerases. The correlation between Pd values of three PP analogs and their inhibition of CMV DNA polymerase at different pH values indicates that the most active compounds have three negatively charged groups (23a). PFA, which has three negatively charged groups, remains the most active and selective compound in the virus polymerase assay.

Of the two diesters that were inhibitory to CMV in tissue culture VSA, one had no effect on the CMV DNA polymerase activity, and the activity of the other could be ascribed to contaminating PFA. The latter diester (B043) was shown to be hydrolyzed in cells and thereafter to contain an increased amount of 6 to 10 μM PFA. Since 10 μM PFA is not sufficient to inhibit CMV in tissue culture, the diesters may be inhibitory to CMV in cells as monoesters after partial hydrolysis, or the intracellular concentration of PFA may be higher than the extracellular concentration. These two diesters were not active in plaque reduction of HSV-1 in Vero cells (13). The reason for this may be differences in cellular esterase activities, which is also indicated by the fact that the
Thus, inhibitory mono- and diesters are presumably hydro-
two derivatives were effective inhibitors in the cutaneous
HSV-1 infection of guinea pigs (13).

In the VSA tissue culture assay, the IC50 values of active
monoesters and diesters were comparable to those of PFA.
Thus, inhibitory mono- and diesters are presumably hy-
drolyzed to a large extent to PFA. Both the free PFA and
several of the monoesters would then inhibit the viral
polymerase. Some of these esters, further activated after cell
metabolism, may be considered pro-drugs to PFA.

It is not clear why some relatively simple monoesters and
and a diester were nonactive. Perhaps these compounds do
not enter the infected cells or, more likely, are not easily
metabolized. PAA analogs with long α-substituents were
included in the assays because phosphonoacetates carrying
large or bulky α chains or both were strong inhibitors of the
CMV DNA polymerase activity (5). Here, one was toxic and
two were inactive to CMV in cell culture.

With PFA, ester derivatives, and a PAA analog, the
multiplicity dependence of inhibition was demonstrated for
CMV Ad.169 and several isolates. Multiplicity dependence
is a phenomenon in which higher multiplicities of virus
require higher concentrations of inhibitors to give a certain
predetermined inhibition value, e.g., a 50% reduction of viral
DNA production, yield, plaque formation, or protein syn-
thesis (7a, 7b, 8, 18, 20). The CMV isolates were plated at
the concentrations judged from our experience to give
replication of isolates and before the true multiplicity was
known. The concentrations of 40 to 239 μM to obtain IC50
with fourfold differences in multiplicities thus only serve
to indicate the level of increased amounts of drug needed to
inhibit a larger dose of CMV. With very careful titrations of
the isolates, it is possible that lower inhibiting concentra-
tions would have been observed. For that, however, several
tissue culture passages would be required, which would
probably change the properties of the primary isolate. An
increase in the levels of viral polymerase in the cell in vivo
may decrease the relative availability, for PFA or analogs, to
the PP, binding site. The multiplicity dependence phenome-
on has also been observed with nucleoside analogs acti-
vated by viral thymidine kinases (6, 7a, 7b). The levels of
inhibition by different PP, analogs can be distinguished in our
experiments. Viral uptake and early antigen synthesis were
seen in the presence of all analogs and derivatives and with
all CMV isolates. The activity of CMV DNA polymerase in
vitro was inhibited by PFA and five monoesters. Two
diesters had to be metabolized to PFA in the cell, after which
they, as well as the previously mentioned compounds,
inhibited CMV DNA synthesis and CMV late protein syn-
thesis.

It is interesting that at higher doses, PFA as well as the
esters could completely inhibit CMV LA synthesis as judged
by the absence of LA synthesis and CPE. However, when
the drugs were withdrawn, CMV replication resumed, and
infectious virus was recovered in all cases. This phenome-
non has been demonstrated previously (22) and is now
extended to four compounds and several primary virus
isolates. Huang (9) has shown that after removal of PAA,
CMV DNA synthesis resumes at a reduced rate after pro-
longed incubation. It is thus a general phenomenon that the
CMV genome persists in a latent form in vitro during
treatment with PP, analogs. Varicella zoster virus was shown
to persist in the presence in vitro for many weeks (24) and
resumed viral replication after removal of PAA. Inhibition of this reversibility would be an
important acquisition, particularly for CMV treatment.

ACKNOWLEDGMENTS

We thank Sir Nordlund, Ellen Sølver, and Annelie Åkesson for excellent technical assistance and Nils G. Johansson, Susanna
Kovacs, Björn Lindborg, Alfons Misjorn, Jan O. Norén, and Göran
Stening for providing PP, analogs.

LITERATURE CITED

1. Boezi, J. A. 1973. The antiherpes virus action of phospho-
naacetate. Pharmacol. Ther. 4:231–143.


analogues as inhibitors of DNA polymerases of cyto-


9. Huang, E.-S. 1975. Human cytomegalovirus. IV. Specific inhibi-

mechanisms and consequences. Antiviral Res. 4:1–42.


herpes simplex virus DNA polymerase by phosphonoacetate.
Biochemistry 14:5475-5479.