**Human Cytomegalovirus Infections in vitro after Treatment with Arildone**

By A. S. TYMS,* R. J. STEVENS, MARGARET A. MOBBERLEY, 1 T. A. RYDER 1 AND D. J. JEFFRIES

Department of Medical Microbiology, St Mary's Hospital Medical School, Paddington, London W2 1PG and 1Electron Microscope Unit, Queen Charlotte's Hospital for Women, Goldhawk Road, London W6, U.K.

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**SUMMARY**

Arildone (WIN 38020), a broad spectrum antiviral, aryl-β-diketone (4-[6-(2-chloro-4-methoxy)phenoxy]hexyl-3,5-heptanedione), blocks the replication of human cytomegalovirus at a stage prior to the synthesis of virus-specific DNA. Inhibitory action was demonstrated against a number of virus isolates from neonates and immune-compromised patients. Intranuclear sites of virus replication, highlighted by DNA-staining methods or immunofluorescence, were absent after Arildone treatment and corresponded with the lack of ultrastructural changes associated with productive infection. The abundance of early antigens in cells treated with Arildone was evidence for expression of the viral genome and this was confirmed by detection of immediate-early viral proteins in the presence of the drug. The results suggest that Arildone prevents the replication of human cytomegalovirus at a stage after virion uncoating but prior to viral DNA synthesis.

**INTRODUCTION**

Human cytomegalovirus (HCMV) is an important pathogen in the newborn and the immune-compromised host (Stern, 1979; Betts, 1982). The increasing practice of organ transplantation and the advent of the Acquired Immunodeficiency Syndrome (AIDS) has led to a significant increase in the numbers of susceptible individuals. Immune compromise, either naturally occurring or iatrogenic, may result in disseminated infections with HCMV, particularly pneumonia, and this is a significant cause of morbidity and mortality (Marker et al., 1981; Meyers et al., 1982; Macher et al., 1983). Detailed prospective studies have shown that congenital abnormalities and progressive deafness are major problems after HCMV infection (Peckham et al., 1983) and these conditions are probably influenced by the long-term persistence of virus in children (Plotkin et al., 1982). Equally important are post-natal infections which may result in severe and often fatal disease in susceptible neonates (Yeager et al., 1981). There is an urgent need for effective antiviral chemotherapy for such infections.

Several compounds with established modes of action have proven activity in vitro against HCMV infections. Phosphonoacetic and phosphonoformic acids are structural analogues of pyrophosphoric acid with activity against HCMV-encoded DNA polymerase (Huang, 1975; Eriksson et al., 1982) but their therapeutic value is hampered by potential deposition in bone (Kung et al., 1978; Helgstrand et al., 1979). The antiviral activity of Acyclovir (Zovirax®) against HCMV in vitro is strain-variable (Tyms et al., 1981; Plotkin et al., 1982) and at least 100-fold lower than against herpes simplex virus (HSV). The apparent benefit of Acyclovir treatment in some patients suffering from CMV infections (Balfour et al., 1982; Gluckman et al., 1983) has not been shown in others (Wade et al., 1982).

A recently described compound, known as BWB759U, DHPG or 2'NDG, has similarities to Acyclovir in both structure and mechanism of action (Ashton et al., 1982; Cheng et al., 1983) and has potent activity in vitro against HCMV infections (Smee et al., 1983; Mar et al., 1983; A. S. Tyms et al., unpublished observations). Nucleoside analogues in the series of 2'-fluoro-5-
substituted arabinofuranosylcytosines and -uracils (FMAU, FIAU, FMAC and FIAIC) were also highly effective against HCMV in cell culture (Mar et al., 1984).

These anti-herpes agents offer promise for treatment of HCMV disease but their common target of viral DNA synthesis is accompanied by potential problems of co-resistant mutants and the recognized lack of effect against latent infections. New compounds are required with novel mechanisms of action preferably targeted at or near to events in the initiation of virus replication. Such a mode of action has been demonstrated for Arildone (WIN 38020), an aryl-β-diketone (4-6-[2-chloro-4-methoxy]phenoxyl)hexyl-3,5-heptanedione), against poliovirus replication in cell culture (McSharry et al., 1979) and the drug protects mice from lethal infection with this virus (McKinlay et al., 1982). Arildone is well tolerated in cellular and animal experimental models and its broad-spectrum activity in vitro includes HSV types 1 and 2 (Kuhrt et al., 1979; Kim et al., 1980) with activity against herpetic infections in guinea-pigs (McSharry & Pancic, 1982). We reported recently that Arildone inhibited the replication of HCMV (Jeffries & Tyms, 1983) and the present report is directed towards elucidating the mode of action of the drug against this virus.

**METHODS**

Cells. Human embryo fibroblast cells (HEF) used during the study were either MRC-5 cells or the WF strain of skin/muscle embryo fibroblasts derived in this laboratory. Growth medium consisted of Eagle's MEM supplemented with 10% foetal calf serum and confluent cell cultures were maintained in the same defined medium containing 2% foetal calf serum. Cells were propagated as previously described (Tyms & Williamson, 1980) using 13 mm glass coverslips, which supported about 10⁵ HEF cells, for cytological studies.

Viruses. The nine human cytomegaloviruses used included the prototype strains AD169 and Kett obtained from Professor H. Stern, St George's Hospital, London, U.K., two clinical isolates (Ai and Wi) supplied by Dr C. Peckham, Charing Cross Hospital, London and five clinical isolates from immune-compromised patients in this hospital (Br, Ha, La, Re, West). All viruses were characterized by restriction enzyme analysis of viral DNA (Tyms, 1983), and virus suspensions were prepared and titrated as previously described (Tyms & Williamson, 1980).

Procedure for infection. Confluent cell cultures were infected by exposure for 2 h at 20°C to a virus inoculum calculated to give a high m.o.i. (5 to 10 p.f.u./cell) except in some immunofluorescence experiments with AD169 virus and in the screening of clinical isolates when a low multiplicity of infection (0.05 to 0.2 p.f.u./cell) was used.

Chemicals and restriction enzymes. Arildone was dissolved in DMSO (BDH) as a 1 mg/ml working solution. Arildone-free controls consisted of cell cultures treated with DMSO alone or left untreated. Cycloheximide and actinomycin D were purchased from Sigma, and [32P]orthophosphate (carrier-free), L-[35S]methionine (1170 Ci/mmol) and restriction enzymes from Amersham.

Cytological procedures. The presence of intranuclear, DNA-containing inclusions in HEF cells infected with the different HCMVs at a high or low m.o.i. was visualized by an acidine staining method (Williamson, 1976).

Immunofluorescence was performed by the indirect method using human convalescent serum with high complement fixation test antibody titre to HCMV (≥ 1:2048) and rabbit anti-human globulin labelled with fluorescein isothiocyanate. In certain experiments cells infected at low multiplicity were preferred, to provide un-dyed short wave-length illuminator. The volume of each DNA preparation was adjusted for restriction enzyme cleavage. The method was similar to those previously described (Lonsdale, 1979; Tyms, 1983).

**Restriction enzyme analysis.** DNA preparations were cleaved with restriction endonuclease (EcoRI) under the manufacturer's recommended conditions using approximately 2 μg total DNA in a final volume of 30 μl with sufficient enzyme for complete digestion in 2 h at 37°C. The reaction was stopped with 15 μl 0.1 M-Tris–HCl pH 8.0, 0.05 M-EDTA, 5% Ficoll, 0.125% bromophenol blue and DNA fragments were separated by overnight electrophoresis in 0.6% agarose gels (2 V/cm) in running buffer comprising 0.04 M-Tris–HCl pH 7.5, 0.02 M-sodium acetate and 0.003 M-EDTA. DNA bands were located by u.v. fluorescence after ethidium bromide staining (0.5...
Arildone treatment of CMV infection

\( \mu g/ml \) and by autoradiography (Fuji RX film) after drying. DNA from mock-infected cells cleaved with \( \text{EcoRI} \) was resolved into discrete bands in only two regions of the gel (data not shown).

**Polypeptide analysis.** HEF cells were infected in the presence of cycloheximide (50 \( \mu g/ml \)) and incubated for 16 h. Actinomycin D (5 \( \mu g/ml \)) was then added to the medium for 15 min before rinsing repeatedly in phosphate-buffered saline (PBS) containing this inhibitor. Cells were incubated for a further 2 h with \( ^{35} \text{S} \) methionine (5 \( \mu Ci/ml \)) in methionine-free medium containing actinomycin D (5 \( \mu g/ml \)). Immediately after the pulse-labelling, cells were lysed in situ in 0.08 M-Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1 M-dithiothreitol and 0.01% bromophenol blue by heating to 90 °C for 5 min. Samples were subjected to discontinuous SDS-PAGE with molecular weight protein standards with a 5% stacking gel and 9 or 12.5% running gel. Coomassie Brilliant Blue-stained gels were dried and autoradiographed (Fuji RX film). Details of the electrophoresis were similar to those published elsewhere (Laemmli, 1970).

**Ultrastructural studies.** Representative cell cultures (about 1.5 \( \times \) 10^6 cells) were either gently removed from the glass surface using a rubber policeman, or released by 0.01% Pronase in PBS. The cells were washed in PBS, pelleted by centrifugation, then fixed for 2 h in 3% glutaraldehyde in HEPES buffer pH 7.4 (Williamson & Cox, 1968). The samples were washed for a minimum of 12 h in fresh HEPES buffer before post-fixing for 2 h in 1% osmium tetroxide, which was freshly prepared in HEPES buffer. The cells were washed in 50% alcohol, pelleted and encased in agar using the method of Ryder & MacKenzie (1981). The resulting 'blocks' of cells were dehydrated through a graded series of alcohols and embedded in Araldite using propylene oxide as the transitional fluid. Ultrathin sections were cut on glass knives using a Reichert OMU 3 ultramicrotome and treated with saturated alcoholic uranyl acetate and Reynolds' lead citrate prior to examination with an Hitachi HU 12A electron microscope.

**RESULTS**

**Cytology**

The phases of virus replication can be conveniently divided into early and late by the synthesis of genomic nucleic acid. In the case of HCMV, the development of intranuclear inclusions, as determined by acridine orange staining (McAllister et al., 1963), provides an accurate and convenient assessment of virus-specific DNA synthesis (Goodheart et al., 1964; Huang et al., 1973). In the present study HEF cells infected with AD169 virus and treated with Arildone (1 to 5 \( \mu g/ml \)) for 144 h post-infection were examined for viral inclusions. Infected control cells, with or without the respective concentration of DMSO, showed cytomegaly and the characteristic DNA-containing inclusions were present in the nucleus. Infected cells treated with Arildone at a concentration of 3 \( \mu g/ml \) or more remained in the early rounded form and were devoid of inclusions (Fig. 1). Lower concentrations of Arildone had correspondingly less effect on inclusion development. This procedure was used to monitor the activity of Arildone against a number of HCMV isolates obtained from clinical material. All virus isolates tested were unable to replicate in the presence of Arildone (3 \( \mu g/ml \)) when assessed for the development of intranuclear inclusions after 144 h incubation. If Arildone was removed from infected cultures at this time, intranuclear inclusions developed within 3 days of reincubation, demonstrating the Arildone block to be reversible.

**Viral DNA synthesis**

HCMV-specific DNA can be readily detected by restriction enzyme analysis in total DNA extracts prepared from infected cultures (Tyms, 1983) and this procedure was used in the present study to ascertain the effect of Arildone on the synthesis of viral DNA.

HEF cells infected with AD169 virus were treated with various concentrations of Arildone or DMSO and labelled with \( ^{32} \text{P} \) orthophosphate. Equal amounts of the total DNA recovered from each sample were digested to completion with \( \text{EcoRI} \) and the DNA fragments were separated by gel electrophoresis into the characteristic profile for AD169 (Spector et al., 1982). The radio-labelling was equally intense in viral DNA from DMSO-treated or untreated controls but significantly reduced if infected cultures were exposed to 1.5 \( \mu g/ml \) Arildone and very weak or absent at higher drug concentrations. Similar results were obtained when DNA fragments were resolved by ethidium bromide staining and this procedure gave an indication of the relative amounts of viral and cellular DNA in each digest (Fig. 2).
Fig. 1. HEF cells were infected with Br, a low passage HCMV isolate, and stained with acridine orange at 144 h post-infection. Uninfected cells (a); infected cells treated with 3 μg/ml Arildone (b) remained in an early rounded form and no intranuclear inclusions were seen; infected cells treated with 0.3% DMSO (c) showed cytomegaly and prominent DNA-containing intranuclear inclusions (IN). Infected cells in maintenance medium (results not shown) were indistinguishable from those treated with 0.3% DMSO.

Fig. 2. Total DNA from HEF cells infected with AD169 radiolabelled with [32P]orthophosphate was extracted at 120 h post-infection and digested to completion with EcoRI. The viral DNA content was resolved into discrete bands by gel electrophoresis in 0.6% agarose and (a) stained by 0.5 μg/ml ethidium bromide then (b) dried and autoradiographed at −60 °C using intensifying screens. Cells were treated as follows: lane 1, 0.3% DMSO; lane 2, 0.6% DMSO; lane 3, untreated; lane 4, 1.5 μg/ml Arildone; lane 5, 3 μg/ml Arildone; lane 6, 6 μg/ml Arildone. Only the high molecular weight portion of the gel is shown.

Virus-specific antigens

Inhibition of HCMV DNA synthesis per se, or of events leading to its synthesis, would preclude the production of late infected cell-specific proteins (ICSPs) but may allow the synthesis of early ICSPs. As an indication of the synthesis of early ICSPs, Arildone-treated cultures were investigated for the presence of virus-specific antigens by immunofluorescence (IF) (Fig. 3). By 24 h post-infection, infected cells exposed to Arildone developed the characteristic rounded form seen in control cultures. A diffuse IF reaction was present in the nucleus and cytoplasm of these cells, with localized staining at the nuclear membrane, nucleolus and intracytoplasmic
Arildone treatment of CMV infection

Fig. 3. Effect of Arildone on the synthesis of virus-specific antigens in HEF cells infected with AD169 virus. (a, b) Cells infected at 0.2 p.f.u./cell, treated with 3 μg/ml Arildone and examined at 24 h post-infection. (a) Antigens detected by immunofluorescence observed in the nucleus and cytoplasm of the rounded infected cells. (b) The same cells shown by phase-contrast microscopy. Infected cells untreated or treated with 0.3% DMSO (not shown) were indistinguishable from those in (a) and (b). (c, d) Cells infected at 5 p.f.u./cell and examined by immunofluorescence at 120 h post-infection. (c) Cells treated with 0.3% DMSO showing cytomegaly and large aggregates of late antigen (L) in the nucleus. (d) Cells treated with 3 μg/ml Arildone showing the early rounded form with diffuse nuclear and cytoplasmic fluorescence similar to that observed early in virus infection.

Polypeptide analysis

The presence of virus-specific antigens in Arildone-treated cells is evidence for the synthesis of virus-coded proteins. Blanton & Trevethia (1981) identified at least 20 ICSPs by SDS-PAGE after immunoprecipitation of infected cell extracts. Four proteins were identified as immediate-
Fig. 4. HEF cells were infected with AD169 virus (V) or Kerr virus (V/Ke) and IE proteins were analysed by SDS–PAGE. Infected or mock-infected (M) cells were incubated for 16 h with or without cycloheximide (CH), then exposed to [35S]methionine in the presence of actinomycin D. (a) Cell cultures treated with 3 µg/ml Arildone during transcription (lanes 1, 2 and 5) and translation (lanes 1, 2 and 6) or left untreated and polypeptides separated in 9% polyacrylamide gels. In control experiments 0.3% DMSO was substituted for Arildone and showed similar results to above. (b) Infected or mock-infected cells treated continuously during transcription and translation with Arildone at 12 µg/ml (lanes 1 and 7), 6 µg/ml (lane 2), 3 µg/ml (lane 3) or 1.2% DMSO (lanes 5 and 8) or left untreated (lanes 4 and 6). Polypeptides were resolved in 12.5% polyacrylamide gels. Gels were vacuum-dried and exposed to X-ray film at −60 °C for autoradiography. Apparent molecular weights (× 10⁻³) of infected cell-specific polypeptides are shown.

ey early (IE) by their synthesis in the presence of actinomycin D, after release from a cycloheximide block. Using this protocol, up to 11 IE proteins have been identified in HCMV-infected cells including the predominant IE protein, which is readily resolved after cycloheximide enhancement (Stinski, 1978; Cameron & Preston, 1981; Gibson, 1981). In the present study, expression of IE proteins of HCMV and the predominant IE protein in particular was used as a measure of early transcription and translation after virus infection.

The effect of Arildone on accumulation of IE transcripts during cycloheximide enhancement and the subsequent translation in the presence of [35S]methionine and actinomycin D was investigated. The apparent molecular weight of the predominant IE protein of AD169 virus determined by SDS–PAGE was 74 000 and 70 000 for the less abundant satellite protein. Strain differences in the electrophoretic mobility of the predominant IE protein (Cameron & Preston, 1981; Gibson, 1981, 1983; Stevens et al., 1984) are supportive evidence of the viral nature of this protein and this point is illustrated in Fig. 4(a) by comparison of AD169 virus and prototype virus Kerr. Expression of the predominant IE complex was observed when Arildone was present during the transcription period or during translation. The omission of the cycloheximide block resulted in failure to resolve the IE proteins, whether Arildone was present or not, and was evidence that the compound did not substitute directly for the inhibitor of protein synthesis (Fig. 4a). IE proteins continued to be expressed at concentrations of Arildone two- or fourfold higher than that required to prevent virus growth in conditions where the drug was maintained throughout transcription and translation (Fig. 4b). The highest concentration of Arildone or DMSO used had no demonstrable effect on the radiolabelling of proteins after mock-infection.
Arildone treatment of CMV infection

Ultrastructural studies

The results so far described have shown that early expression of the HCMV genome occurs during Arildone treatment but the synthesis of viral DNA and subsequent production of late viral ICSPs is prevented. The progression of HCMV replication can be readily determined by electron microscopy (Iwasaki et al., 1973) and this procedure was used to study the effect of Arildone treatment on the fine structure of infected cells at early and late times after infection. HEF cells infected with AD169 virus were treated with Arildone (3 μg/ml) or DMSO (0.3%) for 16 or 120 h post-infection and processed as described earlier. At the late time in virus replication, the Arildone-free controls showed an abundance of nucleocapsids associated with the skein-like...
Table 1. Frequency of nucleocapsids in nuclei of HEF cells infected with AD169 virus and treated with Arildone or DMSO at 120 h post-infection

<table>
<thead>
<tr>
<th>Nucleocapsids</th>
<th>Arildone (3 μg/ml)</th>
<th>DMSO (0.3%)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>97.8 ± 0.8*</td>
<td>11.2 ± 0.8*</td>
</tr>
<tr>
<td>1-10</td>
<td>1.4 ± 0.9</td>
<td>6.2 ± 5.2</td>
</tr>
<tr>
<td>10-50</td>
<td>0.6</td>
<td>10.2 ± 3.7</td>
</tr>
<tr>
<td>51 or more</td>
<td>0.2</td>
<td>72.4 ± 4.3</td>
</tr>
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* Standard deviation was determined from 5 × 100 cells.

intranuclear inclusion; enveloped virions and dense bodies were present in the cytoplasmic region adjacent to the nucleus (Fig. 5). These features of productive infection were recorded in very few cells treated with Arildone when examined at 120 h post-infection. The fine structure of the treated cells was indistinguishable at early or late times after infection and was similar to the DMSO-treated cells at 16 h post-infection (results not shown). The effect of Arildone treatment was quantified by scoring 500 cell nuclei for the presence of nucleocapsids at late times and the results, along with the respective control counts, are presented in Table 1. This clearly demonstrates the suppression of progeny virus production in cells treated with Arildone.

**DISCUSSION**

It is essential to investigate the mechanism of action of potential antiviral agents to define the rationale for their specificity and to characterize alternative targets for chemotherapy. Arildone has been reported to act on picornavirus replication by stabilizing the nucleocapsid structure with a consequent failure of virion uncoating (McSharry et al., 1979; Caliguiri et al., 1980). This may be a common target for lipophilic compounds (Tisdale & Selway, 1984). It was considered feasible that similar mechanisms may be operative for other viruses sensitive to Arildone and might explain the apparent broad-spectrum activity of this compound (Kim et al., 1980). In the case of HCMV, exponential growth was blocked by Arildone (Jeffries & Tyms, 1983) and results of the present study have shown that the inhibitory step is prior to the synthesis of virus-specific DNA. The DNA-containing inclusions are a useful marker of drug inhibition (McAllister et al., 1967; Tyms & Williamson, 1982) and this test showed broad-spectrum sensitivity of clinical isolates to Arildone. The reversible effect of the drug seen in these studies demonstrated the continued viability of infected cells during the Arildone block. Quantification of viral DNA by restriction enzyme analysis provided a sensitive and unequivocal assessment of viral DNA synthesis but failed to measure virus-induced cellular DNA synthesis (Furukawa et al., 1976; St Jeor & Hutt, 1977), a feature of the overall stimulation of host cell functions after HCMV infection in vitro (Isom, 1979; see Introduction). It is assumed that Arildone had no serious effects on host-macromolecular synthesis at drug concentrations that allowed cell proliferation but prevented HCMV growth (Jeffries & Tyms, 1983) but this may not be the case if cellular activity is under virus control. Preliminary results show, for example, that polyamine biosynthesis, which is essential for cell proliferation (Pegg & McCann, 1982) and HCMV replication (Tyms et al., 1979; Tyms & Williamson, 1982) appears to be affected by Arildone treatment only when this pathway is virus-induced (A. S. Tyms, unpublished data).

The lack of progression in virus growth seen in the presence of Arildone was also evident in electron micrographs. In particular, the absence of nucleocapsids at 120 h post-infection correlated with a failure to demonstrate aggregates of late antigens at the site of the intranuclear inclusion. A block in the synthesis of late antigens would not be unexpected due to the observed inhibition by Arildone of viral DNA synthesis. This has recently been confirmed by the inability to detect late ICSPs in Arildone-treated cells by SDS–PAGE after pulse-labelling with [35S]-methionine at 120 h post-infection (A. S. Tyms, unpublished observations). The early IF reaction observed in the cytoplasm of HCMV-infected cells was blocked by cycloheximide or actinomycin D (Reynolds, 1978) indicating a requirement for de novo protein synthesis. In con-
Arildone treatment of CMV infection

contrast, an IF reaction observed in the nucleus within 60 min of infection only responded to cycloheximide if treatment commenced 18 to 24 h pre-infection (Michelson-Fiske et al., 1977; Reynolds, 1978) and may represent virus-induced modification of pre-existing host cell proteins. The early nuclear antigen, as well as cytoplasmic antigens, detected after Arildone treatment were probably a consequence of de novo protein synthesis because novel IE proteins are known to contribute to the early IF reaction associated with the nucleus after HCMV infection (Michelson et al., 1979). The nature of the antigens detected after Arildone treatment is presently under investigation using monospecific antibodies.

Selective enhancement of IE proteins is possible through accumulation of IE transcripts by cycloheximide treatment, although it is more difficult to detect the subsequent production of early proteins in HCMV-infected cells (Wathen & Stinski, 1982). Transcription of the IE genes is restricted to a region between 0.66 and 0.77 map units in the long unique section of the genome of prototype CMV strains (Wathen et al., 1981; De Marchi, 1981; Wathen & Stinski, 1982). In the present study, the 74000 mol. wt. predominant IE protein and its associated 70000 IE protein were synthesized at concentrations of Arildone that inhibited the replication of AD169 virus. These IE proteins correspond to the 72000 and 68000 mol. wt. predominant IE proteins of Towne virus (Stinski, 1978) which appear to be derived from a 75000 IE precursor molecule by modification in vivo (Stinski et al., 1983). Expression of the IE genes is obligatory for sequential transcription of the viral genome (Stinski, 1978) and the predominant IE protein appears to have a key regulatory function in virus replication (Stinski et al., 1982; Stenberg et al., 1984).

The synthesis of viral DNA and the major ICSPs was inhibited by Arildone in cells infected with HSV type 2 but the effect of the drug on the synthesis of early ICSPs was not resolved (Kuhrt et al., 1979). It seems unlikely that the drug prevented virion uncoating, however, because HSV growth remained susceptible to Arildone treatment for up to 4 h post-infection and did not require an eclipse phase on release from an Arildone block at 24 h post-infection. These observations, coupled with the results of the present study, suggest that Arildone does not inhibit the replication of certain herpesviruses prior to release and early expression of the viral genome.

In conclusion, Arildone is an effective inhibitor of HCMV and HSV replication in vitro at concentrations that have little or no effect on propagation of host cells. The drug had efficacy in treating infections in guinea-pigs and protected mice from otherwise fatal intracerebral infection with poliovirus. This aryl-β-diketone or analogues with similar antiviral activity are therefore of potential therapeutic value in treating infections with HCMV.

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2138 A. S. TYMS AND OTHERS


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