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Preliminary Studies of the Mode of Action of Arildone, a Novel Antiviral Agent

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Arildone (also known as Win 38020), a novel aryl diketone, inhibited replication of herpes simplex virus type 2 in tissue culture by interfering with an event that occurs prior to 6 h postinfection. The inhibition could be partially reversed by washing. Although the exact mechanism of action is unknown, neither viral deoxyribonucleic acid nor viral proteins were synthesized in the presence of arildone.

Several aryl diketone compounds have been shown to have antiviral activity. A member of this series, 4-[6-(2-chloro-4-methoxy) phenoxyl] hexyl -3,5-heptanedione, designated arildone, was evaluated in cell culture and animals and was active against ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) viruses (3; L. Caliguiri, J. McSharry, and H. Eggers, submitted for publication). The properties of this drug make it an excellent candidate for clinical evaluation. This report describes studies of the effect of arildone on the replication of herpes simplex virus type 2 (HSV-2) in tissue culture and on macromolecular synthesis in infected and uninfected host cells.

MATERIALS AND METHODS

Virus and cell lines. The Curtis strain of HSV-2 was used throughout. Monkey kidney cells (BSC_1) were used except where noted and were grown in test tubes (16 by 150 mm), 25-cm^2 plastic flasks (Falcon, Los Angeles, Calif.), 32-oz. (ca. 0.95-liter) glass bottles, and Pavitzki bottles (560 cm^2).

Plaque assay. HSV-2 was quantitated by plaque assay. Serial 10-fold dilutions were made in Leibovitz medium (L-15), and 2-ml portions of the dilutions were pipetted onto 3- or 4-day-old monolayers of BSC_1 cells grown in 25-cm^2 plastic flasks. After 1 h at 33°C, the inoculum was removed and replaced with 5 ml of L-15 supplemented with 5% calf serum (Reheis, Chicago, Ill.) and 0.65% Ionagar (Colab, Chicago Heights, Ill.). Infected cultures were incubated for 3 days at 33°C and flooded with a solution of crystal violet stain (0.25%), prepared with 10% Formalin containing 2% sodium acetate. Plaques were counted after the agar was removed from the flasks and expressed as plaque-forming units per flask.

Cell viability. The effect of arildone on the viability of host cells was evaluated by culturing BSC_1 cells for 7 days in 32-oz. (ca. 0.95-liter) glass bottles seeded at a density of about 7×10^6 cells per bottle. The monolayers were incubated at 33°C with Eagle medium containing 10% calf serum and arildone at a concentration of 3 μg/ml. Cells incubated with Eagle medium containing 10% calf serum served as a control. Culture fluid containing arildone was renewed after 5 days of incubation. At the end of the 5-day incubation period, cells were removed from the bottles by treatment with 0.1% ethylenediaminetetraacetate (EDTA), stained with trypan blue, and counted with a hemacytometer chamber.

Time of addition and reversal studies. Arildone (insoluble in aqueous systems) was dissolved in 100% dimethylsulfoxide (DMSO) at a concentration of 1 mg/ml and diluted to the desired final concentration with L-15 containing 5% serum. Preliminary studies showed that DMSO had no effect on HSV-2 replication at the concentrations employed. Three- or 4-day-old monolayers of BSC_1 cells in test tubes or Falcon flasks were used to determine the effect of arildone when added at various times after infection. HSV-2 Curtis strain, containing from 5×10^5 to 3×10^6 plaque-forming units per ml, was used to inoculate the cells, usually at a multiplicity of infection of about 1. Virus dilutions were prepared in L-15, and inoculum volumes were 0.2 ml and 2 ml for tubes and flasks, respectively. Cultures were incubated at 33°C for 1 h, the inoculum was removed by aspiration, and the monolayers were washed twice with 2-ml or 5-ml volumes of L-15. Fresh L-15 containing 5% calf serum and various concentrations of arildone was added, and incubation was continued at 33°C. In all experiments, 0 h postinfection (PI) refers to the time of addition of the fresh medium. For time of addition experiments, medium without arildone was added at 0 h PI and replaced with medium containing 3 μg of arildone per ml at the indicated time. Reversal of drug action was initiated by removing the culture fluid containing arildone, washing the monolayers twice with 1 or 2 ml of L-15, and finally continuing the incubation with fresh medium devoid of arildone. Virus yield was determined by discarding the culture fluid from monolayers, replacing it with 0.1% EDTA, and then assaying the contents after two cycles of freezing and thawing. Pilot studies had shown that prior to lysis the culture fluid contained less than 2% of the total virus.

Preliminary single-cycle growth curve experiments with BSC_1 cells infected with HSV-2 indicated that virus recovery was lowest at 6 h PI and increased to a maximum at 16 to 24 h PI. These events were determined by separately assaying total virus yield hourly from 0 to 24 h PI.

Electron microscopy. For these studies BSC_1 cells grown in 25-cm^2 Falcon flasks were infected with HSV-2 at a multiplicity of infection of 10. At the desired times, cultures were cooled to 0°C, rinsed twice with 0.1 M cacodylate buffer (pH 7.3), and fixed in situ (15 min) with 3% glutaraldehyde in 0.1 M cacodylate buffer at 0°C. Cells were gently scraped from the flask surface into fresh fixing solution, fixed for an additional 20 min, pelleted by low-speed centrifugation, washed in the buffer, and postfixed in 1.33% osmium tetroxide in 0.067 M *s*-collidine buffer (pH 7.3) for 45 min at 0°C. The cells were pelleted, rinsed in double-distilled water, and infiltrated in 2% molten agar. The cell pellet was cut into cubes, which were dehydrated in a graded series of ethanols and propylene oxide and embedded in an Epon 812-Araldite 502 mixture. Ultrathin sections were examined and photographed with an RCA electron microscope. Virus particles were counted using enlarged photographic prints of micrographs of infected or infected and treated cells. At least 25 micrographs of both infected or infected and treated cells were examined.

Isotopic labeling. In vitro isotopic labeling of host cell macromolecules was accomplished with [^{14}C]uridine (0.1 μCi/ml), [^{14}C]thymidine (0.5 μCi/ml), or DL-[^{14}C]valine (0.5 μCi/ml), diluted with Eagle medium containing 10% calf serum. For experiments involving polyacrylamide gel electrophoresis, proteins were labeled with a mixture of uniformly labeled ^{14}C-amino acids (New England Nuclear Corp., Boston, Mass.; NEC 445) diluted to a concentration of 2.5 μCi/ml with L-15 containing 1% calf serum.

In vitro assay of precursor uptake and macromolecular synthesis by host cells. When arildone was used in this system, it was dissolved in 100% DMSO at a concentration of 1 mg/ml and diluted to 3 μg/ml with Eagle medium containing 5% calf serum. Confluent monolayers of BSC_1 cells in Falcon flasks were cultured with Eagle medium containing 10% calf serum and either 3 μg of arildone per ml or 0.30% DMSO as solvent control for 3, 6, 9, 12, or 15 h at 37°C. Culture fluid (control or containing arildone or DMSO) was renewed every 3 h to maintain constant levels of nutrients and pH. At sampling times, cells were pulse-labeled with the isotopic mixture for 30 min, fluid was removed from the flasks, and the cells were washed twice and then flooded with 0.1% EDTA. After 20 min the cells were freed from the plastic plates, and triplicate samples were pooled and mixed gently. Total uptake and specific incorporation of isotopes into DNA, RNA, and proteins were then determined using the method of Roodyn and Mandel (11).

DNA isolation. Confluent BSC_1 cells in 560-cm^2 bottles were infected with HSV-2 at a multiplicity of infection of about 1 and incubated for 24 or 42 h at 33°C. Maintenance medium during incubation consisted of 400 ml of L-15 containing 5% serum. Arildone was added from a stock solution dissolved in 100% DMSO. The cells were periodically examined microscopically for evidence of cytopathic effect. One bottle of cells was assayed for total infectious virus. At harvest (24 or 42 h PI), maintenance medium in the remaining bottles was replaced with 50 ml of saline–EDTA (7), and after 20 min cells could be removed from the glass with gentle shaking. The cells were pelleted by centrifugation (900 × *g*) and suspended in about 10 ml of saline–EDTA. DNA was extracted by a modification of the method of Russell and Crawford (12). Sodium dodecyl sulfate was added to the cell suspension to a final concentration of 1% by volume. The suspension was shaken gently for 3 min, and sodium perchlorate was added to a final concentration of 1 M. An equal volume of chloroform–isoamyl alcohol (24:1) was added, and the mixture was shaken gently for 2 min and then centrifuged for 20 min (5,000 × *g*) to separate the phases. The aqueous phase (upper layer) was removed with a wide-bore pipette. Further purification was achieved by adding ethanol and "spooling" precipitated DNA from the liquid interface. The spooled DNA was squeezed to remove traces of solvent and then slowly dissolved in dilute saline citrate. Samples were preserved with 1 drop of chloroform.

Analytical CsCl equilibrium density gradient centrifugation. A solution (ρ = 1.700) was prepared by adding a combination of saturated CsCl and buffer (dilute saline citrate) to a sample of extracted DNA and adjusting the ratio of components until the correct density was obtained as determined by refractive index measurements. The sample and solvent blank were placed in a 2° double-sectored charcoal-filled Epon centerpiece. The sample centerpiece was centrifuged for 24 h at 34,000 rpm and 20°C in a Beckman model E analytical ultracentrifuge equipped with an AN-D or AN-F rotor. The centrifuge was equipped with high-intensity ultraviolet optics and a multiplexer scanner attachment. All density gradient profiles were scanned at 265 nm. The image speed was 3 cm/min, and the chart speed was 1 mm/s. Purified DNA (ρ = 1.7107) from *Escherichia coli* was centrifuged with each sample as an internal reference.

Polyacrylamide gel electrophoresis. Preparation of samples and gels for electrophoresis followed the methods of Kuhrt and Pate (6) with minor modifications. Cell monolayers were carefully scraped from the bottle into 0.1 M sodium phosphate (pH 7.2). Cells were washed twice with the same buffer and suspended in a solvent containing 3% sodium dodecyl sulfate, 0.15% 2-mercaptoethanol, and 7.5 mM EDTA, all dissolved in 0.1 M sodium phosphate buffer (pH 7.2). After overnight dialysis against buffer containing 8 M urea, the samples were boiled for 2 min and mixed 1:1 with 40% sucrose containing a trace of bromophenol blue. Samples of 50 μl were loaded atop polyacrylamide gels (6 by 150 mm) and run for about 20 h at a constant current of 5 mA per gel toward the anode. The bath buffer was 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% sodium dodecyl sulfate. A standard reference mixture which contained human gamma-globulin, bovine serum albumin, ovalbumin, and trypsin, each at a concentration of 1.25 mg/ml, was run along with the samples.

After electrophoresis, gels were removed from the gel tubes and either stained with 0.25% Coomassie

blue (reference gels) or frozen (samples gels with ^{14}C-labeled proteins). Sample gels were later thawed and sliced into 1.25-mm segments with a model no. 83 gel slicer (Earl Sandbeck Medical Instruments, Baltimore, Md.). The slices were placed in 0.5-ml portions of tissue solubilizer (Soluene-100, Packard, Downers Grove, Ill.) and heated at 50°C for 2 h. Scintillation fluid was then added to each sample, and they were analyzed for ^{14}C content on a Packard Tricarb scintillation counter.

RESULTS

Activity of arildone against HSV-2. Figure 1 illustrates that 3 μg of arildone per ml, added at 0 h PI, suppressed production of infectious virus by 3 logs in BSC_1 cells. A concentration of 2 μg/ml reduced virus replication by over 2 logs, and as little as 1 μg/ml also significantly reduced virus replication. Preliminary studies indicated that arildone at 10 μg/ml or less had no detectable virucidal acitvity.

Time of addition studies. Figure 2 illustrates an experiment in which arildone was added at 0, 4, and 6 h PI. It can be seen that synthesis of infectious virus was inhibited by 3 μg of arildone per ml added to BSC_1 cells as late as 4 h after infection with HSV-2. Addition of arildone at 6 h PI did not significantly affect synthesis of infectious virus. Addition of arildone at 8 and 10 h PI gave similar results (data not shown). From these data, it appears that arildone interferes with some step of HSV-2 replication that occurs before 6 h PI.

Reversibility of antiviral activity. Cells

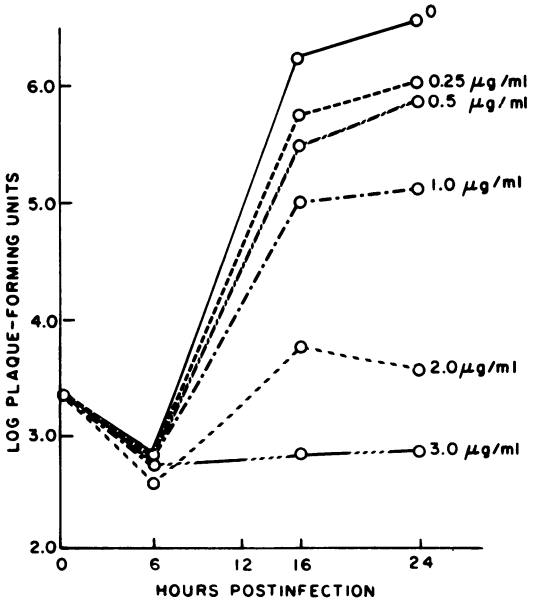

FIG. 1. *Effect of arildone on a single cycle of HSV-2 replication in BSC_1 monolayers.*

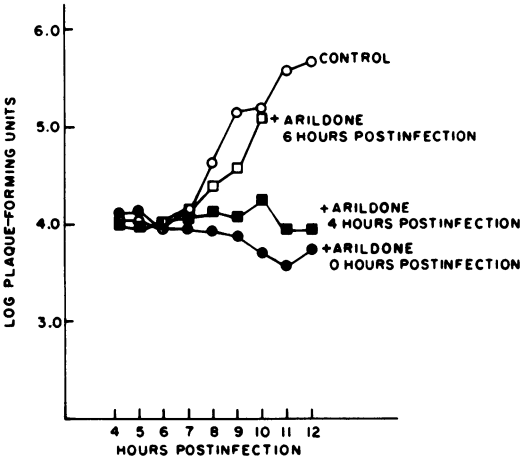

FIG. 2. *Effect of adding arildone (3 μg/ml) at various times PI to HSV-2-infected BSC_1 monolayers.*

treated immediately after infection were washed at different times PI to determine if the compound could be removed. The results of an experiment in which cells were washed 3 h after addition of arildone can be seen in Fig 3. Infectious virus was produced at essentially the same rate as in untreated cells (including a lag period which lasted until 7 to 8 h PI). Similar results were obtained when washing was delayed until 5 h PI. Figure 4 shows results with cells washed with a delay as long as 24 h after addition of arildone. These cells also produced virus, and synthesis of virus was observed within 1 h after washing. The extent of reversal is considerable but may not be complete.

Electron microscopy. Untreated and arildone-treated (3 μg/ml) BSC_1 cells infected with HSV-2 (multiplicity of infection ≥ 10) were examined by electron microscopy. At 18 h PI, treated cells contained fewer virus particles within their nuclei than untreated cells, and fewer enveloped virus particles could be found attached to the cell membrane of treated cells (Table 1). Often large virus crystals were present in untreated cells. These crystals were seen in only two of several hundred treated cells examined. The number of virus particles visible in sections of treated and untreated cells correlated well with titers of virus present in both samples determined by plaque assay. Margination of chromatin was obvious in both treated and untreated infected cells. Since the input titer was high, and the actual particle count could be one or two orders of magnitude greater than the plaque-forming unit determination, the margination and some of the virus particles seen in treated cells could be due to input virus rather than de novo synthesis.

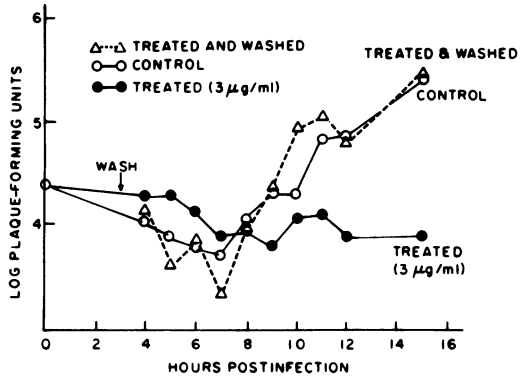

FIG. 3. *Reversal of antiviral activity of arildone. BSC_1 cells infected with HSV-2 were treated with arildone (3 μg/ml) at 0 h PI and washed at 3 h PI.*

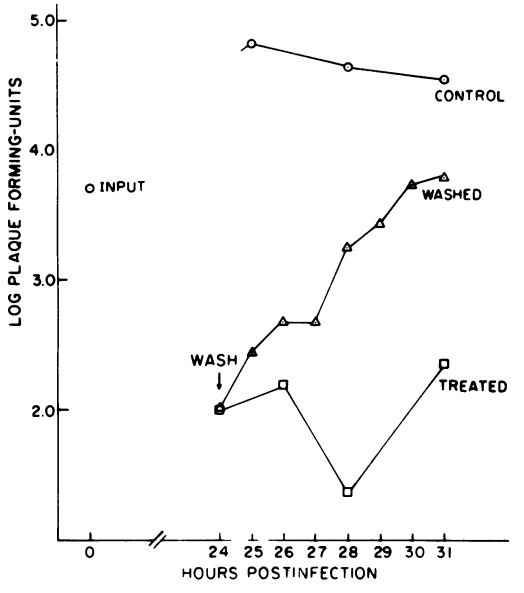

FIG. 4. *Reversal of antiviral activity of arildone. BSC_1 cells infected with HSV-2 were treated with arildone (3 μg/ml) at 0 h PI and washed at 24 h PI.*

Effect on precursor uptake and RNA, DNA and protein synthesis in uninfected cells. In vitro synthesis of macromolecules by uninfected BSC_1 cells was monitored by measuring incorporation of ^{14}C-labeled precursors into acid-precipitable material, and uptake of precursors was determined by measuring the total of radiolabeled precursors in cells and subtracting acid-precipitable label. Uptake of precursors for macromolecular synthesis was inhibited by about 30% by 3 μg of arildone per ml (Fig. 5). Incorporation of radiolabeled precursors into RNA and DNA was also inhibited by about 30% by 3 μg of arildone per ml. Incorporation of precursors into protein was inhibited by about 12%. This apparent inhibition of synthesis may only reflect inhibited transport (uptake) of the necessary precursors. Similar results (data not shown) were obtained with several other cell lines, both in this laboratory and by J. McSharry and L. Caliguiri (personal communication). The concentration of DMSO present with 3 μg of arildone per ml (0.3%) did not inhibit uptake or incorporation of radiolabeled precursors over the 15-h test period.

BSC_1 cells were cultured for 7 days in the presence of 3 μg of arildone per ml. No differences in viability or cell morphology were detectable by microscopic examination of trypan blue-stained cells.

Density gradient analysis of viral DNA. Replication of HSV-2 DNA was monitored by density gradient analysis of total DNA extracted from infected cells. Purified DNA was subjected to analytical ultracentrifugation, and the amount of DNA banding at a density range between 1.727 and 1.729 in the CsCl equilibrium density gradients was taken as a direct measure

TABLE 1. *HSV-2 particles detected in BSC_1 cells by electron microscopy*

Sample	Avg. no. of virus particles[a]	
	Within nucleus	Within cytoplasm
Infected cells	103.0	41.7
Infected and treated cells[b]	26.5	21.4
% Decrease	74%	48%

[a] At least 25 separate cells of each type were examined. Cells were chosen at random from those with the greatest exposed cross-sectional area.

[b] Treated with 3 μg of arildone per ml and observed at 18 h PI.

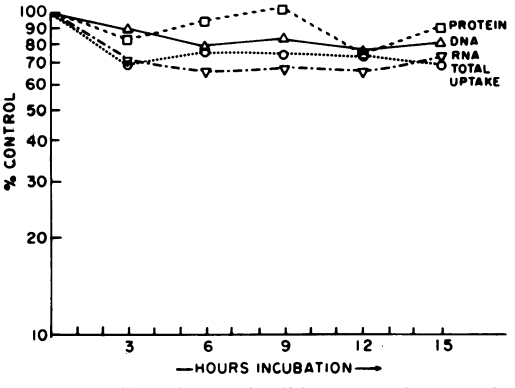

FIG. 5. *Effect of 3 μg of arildone per ml on uptake of precursors and incorporation of precursors into macromolecules by BSC_1 monolayers.*

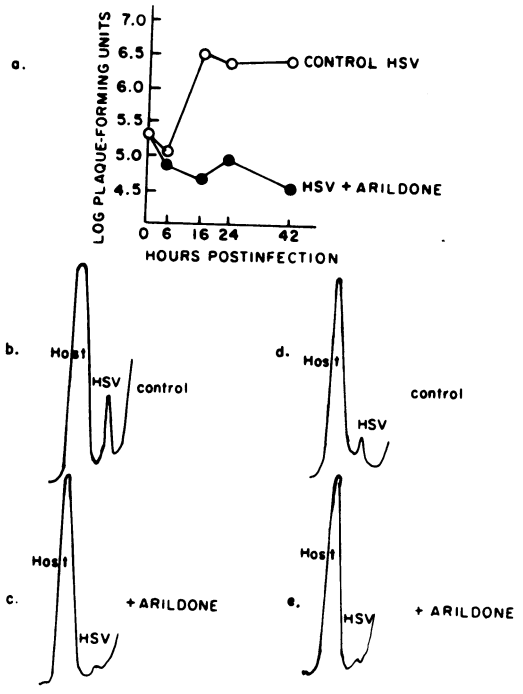

FIG. 6. *Effect of 3 μg of arildone per ml on replication of HSV-2 DNA by BSC$_1$ monolayers. (a) Titer of infectious particles; (b) host and viral DNA, no drug, 24 h PI; (c) host and viral DNA, 3 μg of arildone per ml, 24 h PI; (d) host and viral DNA, no drug, 42 h PI; (e) host and viral DNA, 3 μg of arildone per ml, 42 h PI. (b-e) Density gradient tracings.*

of HSV-2 DNA. Maximum infectious titer was attained after 16 to 24 h (Fig. 6a). At this time, extracts from the infected cells contained a significant amount of DNA, which banded at a density of 1.727 in equilibrium CsCl density gradients (HSV-2 DNA, Fig. 6b). Extracts from cells infected with HSV-2 but treated with 3 μg of arildone per ml contained only barely detectable amounts of HSV-2 DNA (Fig. 6c) at 24 h. After 42 h of incubation, the amount of HSV-2 DNA present in cells infected with HSV-2 and treated with 3 μg of arildone per ml was still barely discernible (Fig. 6e).

Viral protein synthesis. Normal proteins of BSC$_1$ cells were uniformly labeled by growing cells in the presence of ^{14}C-labeled amino acids for 24 h. Proteins extracted from the cells, dissolved with sodium dodecyl sulfate, and separated into a distinct pattern by polyacrylamide gel electrophoresis are shown in Fig. 7. In cells infected with HSV-2 before labeling, synthesis of normal cell proteins was halted and virus-specific proteins were produced. About nine distinct proteins were detected in the high-molecular-weight range (>40,000) of the gels. Five of these, numbered 1, 2, 5, 6, and 8, were clearly unique to HSV infection and could be used as an indicator of infection. These proteins correlated closely with those reported by other investigators to be found in HSV infections (4). When arildone was present (3 μg/ml) after infection, synthesis of virus-specific proteins was greatly inhibited. The major capsid protein of HSV-2 is represented by protein 2 in our scheme (molecular weight = 159,000). Synthesis of this protein was at least 90% inhibited by 3 μg of arildone per ml. These experiments do not differentiate between early and late protein synthesis but rather represent total protein synthesis over a 24-h period after infection. Some early viral protein synthesis may be unaffected by the drug and the activity of these proteins could account for the continued shutdown of host protein synthesis during treatment. Input toxicity due to the HSV-2 particles themselves may also account for this shutdown. Treatment of uninfected cells with arildone (3 μg/ml) did not significantly alter the electrophoretic pattern of their cell proteins (data not shown), although overall synthesis was somewhat less.

DISCUSSION

The antiviral activity of arildone can be halted by washing, and time-of-addition studies demonstrate that the drug inhibits a step in virus replication prior to 6 h PI. Although the evi-

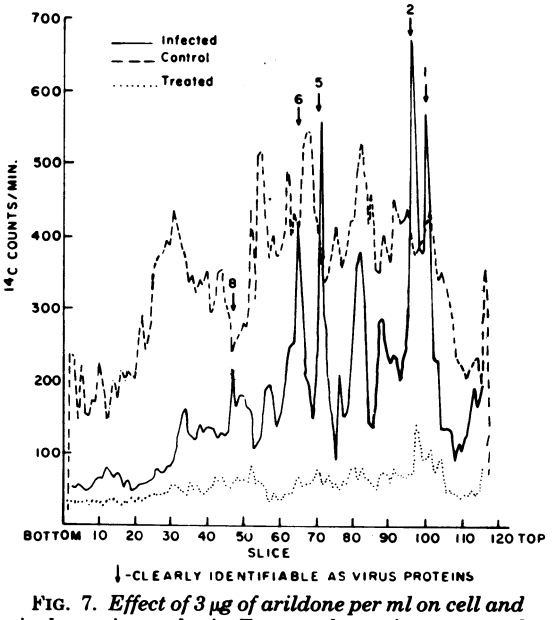

FIG. 7. *Effect of 3 μg of arildone per ml on cell and viral protein synthesis. Extracted proteins separated by electrophoresis. Numbered arrows refer to identifiable viral proteins.*

dence is indirect, the finding that exponential virus replication takes place within 60 to 90 min after arildone is washed from infected cells (at times later than 6 h PI) strongly suggests that the drug does not exert its antiviral effect via inhibition of attachment, penetration, or uncoating. One would expect a longer lag period between washing and virus production if a very early replication step were inhibited, i.e., the eclipse period typically seen in this system was 5 to 7 h. Ultrastructural studies, while not entirely quantitative, strengthen the interpretation that HSV-2 is uncoated and that other very early events (0 to 4 h PI) associated with herpesvirus infection, such as margination of chromatin, proceed in the presence of the drug. When arildone is added after 6 h PI, it has no apparent effect on the cycle of replication underway. It is known (1, 2, 8, 9, 13) that, by 6 to 8 h PI, HSV-2 is assembled and enveloped in a number of cell culture systems.

Centrifugal analysis of DNA from cells infected with HSV-2 and treated with arildone showed that little or no DNA with a buoyant density different from host cell DNA was synthesized. Synthesis of viral capsid proteins is also inhibited by arildone. Ultrastructural examination revealed no unusual accumulation of incomplete or morphologically altered forms of virus in cells treated with arildone. This evidence indicates that actual synthesis of virus parts does not take place in treated cells.

Arildone does not interfere directly with macromolecular synthesis in host cells, but does inhibit transport of precursors. However, this inhibition is not extensive enough to cause any detectable morphological changes in cells even after 5 days of exposure.

Margination of chromatin can be interpreted as a sign of early steps of virus replication. In these experiments, this interpretation is complicated by the high virus input titer used for electron microscopy. The presence of large numbers of virus particles, even some that may be noninfective, can cause some toxic effects including margination. It is possible that some of these noninfective particles may also be seen at the outer boundary of the cell membrane. Whether some of the particles within the cytoplasm and nucleus are due to input or de novo synthesis could only be determined by further experiments with more sophisticated techniques such as autoradiography.

Arildone has shown activity against a wide spectrum of viruses, and in this respect it mimics an interferon inducer. However, interferon could not be detected in several cell culture systems or in mice treated with arildone (unpublished data). Also, the antiviral activity seen in arildone-treated monolayers could readily be reversed by washing, and it is known that washing does not reverse the action of interferon. Because arildone does not structurally resemble a purine or pyrimidine it is not likely to be directly incorporated into DNA, but binding to DNA has not been ruled out. Centrifugal and ultrastructural evidence indicates that little or no viral DNA synthesis takes place in infected and treated cells, and in this respect arildone behaves similarly to phosphonoacetic acid (10). However, unlike phosphonoacetic acid, arildone also inhibits viral protein synthesis. Cells treated with iododeoxyuridine synthesize viral DNA, but it is denser than normal due to the incorporation of the base analog (5).

In summary, arildone inhibits replication of HSV-2 by interfering with a sensitive event prior to 6 h PI and possibly after 4 h PI. Neither viral DNA nor viral proteins are synthesized in the presence of arildone when the drug is added prior to 6 h PI, but the inhibition is at least partially reversible. The precise step of early viral replication that is blocked by arildone is as yet unknown.

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