Selective Anabolism of 6-Methoxypurine Arabinoside in Varicella-Zoster Virus-Infected Cells

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Received 11 February 1991/Accepted 3 June 1991

6-Methoxypurine arabinoside (ara-M) is a highly selective inhibitor of varicella-zoster virus (VZV). It belongs to a class of purine arabinosides whose anti-VZV activity in vitro correlates with substrate utilization by the VZV-encoded thymidine kinase (TK) (D. R. Avrett, G. W. Koszalka, J. A. Fyfe, G. B. Roberts, D. J. M. Purifoy, and T. A. Krenitsky, Antimicrob Agents Chemother. 35:851–857, 1991). In this study, the mechanism of action of ara-M was explored. VZV-infected human fibroblasts selectively accumulated ara-M and its phosphorylated metabolites, whereas in uninfected fibroblasts or in those infected with a TK-deficient strain of VZV, there was virtually no cellular uptake of ara-M. The major intracellular metabolite of ara-M in VZV-infected cells was identified as the triphosphate of adenine arabinoside (ara-ATP). Applicable levels of ara-ADP, ara-AMP, and ara-MMP were also detected. However, di- or triphosphorylated forms of ara-M were not detected. Moreover, in VZV-infected cells, the concentrations of ara-ATP which accumulated in the presence of ara-M were up to eightfold higher than those generated with ara-A itself. In contrast, in uninfected cells, the levels of ara-ATP which accumulated in the presence of ara-M were barely detectable. Clearly, ara-M activation was dependent on the activity of the virus-encoded TK, while ara-M anabolism resulted primarily from the activity of host cell enzymes. Therefore, ara-M selectively generates the DNA polymerase inhibitor ara-ATP in the VZV-infected cell.

The chemotherapy of herpes virus infections has evolved with the introduction of antiviral nucleoside analogs with increasing selectivity for virus-encoded target enzymes. Vidarabine (adenine arabinoside [ara-A]) was initially approved for the treatment of certain herpes virus infections. It reduced the spread of both herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections and decreased new-lesion formation in the immunocompromised-patient population (22–24). Ara-A has also shown efficacy in the treatment of HSV- and VZV-induced encephalitis (20, 24) and is equivalent to acyclovir [ACV; 9-(2-hydroxyethoxymethyl) guanine, Zovirax] in the management of neonatal herpes virus infections (21). However, its therapeutic use is complicated by problems of solubility and toxicity (6, 24). ACV is a more potent nucleoside analog approved for the treatment of infections caused by HSV and VZV. ACV has been reported effective in the treatment of VZV infections in both the immunocompetent (9, 13) and the immunocompromised (18) host, although VZV is less susceptible than HSV to ACV inhibition in vitro (2, 4). A comparison of the efficacy of ara-A and ACV in VZV infections in immunocompromised patients showed ACV to be superior by several parameters (1a, 18). In view of the comparable potency of ara-A and ACV against VZV replication in vitro, the superi-er efficacy and safety of ACV in the clinic is due in part to the selective activation of this nucleoside analog in virus-infected cells by the herpesvirus-encoded deoxythymidine kinase.

A series of purine arabinosides has been described in a preceding paper (1). The most active member of this series, 6-methoxypurine arabinoside (ara-M), has potent and selective anti-VZV activity but no appreciable activity against the other human herpesviruses (HSV types 1 and 2), human cytomegalovirus or Epstein-Barr virus. In the studies described here, the intracellular metabolism of ara-M was investigated in uninfected and VZV-infected human fibroblasts and compared with that of ara-A.

MATERIALS AND METHODS

Chemicals. Ara-M was synthesized (1) and provided to Moravek Biochemicals, Brea, Calif., for titration. The radiochemical purity of [2,8-3H]ara-M ([3H]ara-M) as determined by high-pressure liquid chromatography (HPLC) was >99% at a specific activity of 16 Ci/mmols. [2,8-3H]ara-A ([3H]ara-A) with a specific activity of 40 Ci/mmols was obtained from Moravek, and [2,8-3H]hypoxanthine arabinoside ([3H]ara-H) was obtained by the treatment of [1H]ara-A with adenosine deaminase. The monophosphate and triphosphate of ara-M (ara-MMP and ara-MTP, respectively) were synthesized by procedures previously described (8, 25). All other purines, nucleosides, and nucleotides used as HPLC standards and the solvents used in HPLC buffers were of the highest grade commercially available.

Virus and cells. Monolayer cultures of human foreskin fibroblasts (HFF), derived in these laboratories, were main-tained in Eagle minimal essential medium supplemented with glutamine and fetal bovine serum (HyClone; Sterile Sys-tems, Logan, Utah). VZV Oka and Ellen (American Type Culture Collection, Rockville, Md.) and the thymidine kinase (TK)-deficient strain were maintained as cell-associated virus by passage in human fibroblasts as previously de-scribed (2). Cell lines and virus strains were negative for mycoplasmas by Hoescht staining methods or by GenProbe assay (GenProbe Inc., San Diego, Calif.). The TK-deficient variant of strain Ellen was selected under ACV pressure; the resulting virus drug phenotype (3) and sequence alteration (15) have been defined elsewhere.

Cellular metabolism studies. Subconfluent monolayers of HFF cells in 150-cm² flasks were mixed with VZV-infected cells at a ratio of 1:5 infected to uninfected cells. Infections
were allowed to establish in each experiment before the appropriate radiolabeled compounds were added. All experiments were carried out in duplicate, and the duplicate samples were pooled at the time of harvest in order to provide sufficient radioactivity for quantitation. Cells were harvested at times indicated in the figure legends for nucleotide analysis by trypsin release from the monolayer. Cell numbers were then determined, and the cells were washed, pelleted, and extracted with 60% methanol. The intracellular accumulation of radioactivity was determined by direct liquid scintillation counting of an aliquot of the methanolic extracts. The methanolic extracts were evaporated to dryness, reconstituted with deionized water, and applied to a Partisil SAX analytical anion-exchange column (particle size of column material, 10 μm; inner diameter, 25 mm by 4.6 mm; Alltech Associates/Applied Science, Avondale, Pa.). The column was eluted with 10 mM and 1 M ammonium phosphate buffers, pH 3.5 (mobile phases A and B, respectively), both containing 6% ethanol, as follows: (i) a 17-min isocratic elution with 100% buffer A, (ii) a 67-min linear gradient from 100% buffer A to 50% each buffers A and B, and (iii) a 17-min linear gradient from 50% each buffers A and B to 100% buffer B. After each sample was taken, the column was purged with 100% buffer B for 5 min and then reequilibrated in 100% buffer A for 15 min. The UV A254 and A280 of the column effluent were monitored. The radioactivity was monitored with a Flo-One/Beta Radioactive Flow Detector (model IC; Radiomatic Instrument and Chemical Co., Inc., Tampa, Fla.).

The identification of metabolites was based on a comparison of the retention times of the peaks of radioactivity with those of radiolabeled standards (i.e., [3H]ara-M, [3H]ara-A, and [3H]ara-H) and/or unlabeled standards. In addition, spectral analysis of the HPLC effluent by diode array detection was used for identification of the ara-M anabolites. The UV spectral characteristics of the radiolabeled nucleotides were compared with those of authentic standards analyzed under identical conditions. Phosphorylated anabolites were further characterized by treatment of the cell extracts with alkaline phosphatase for conversion of the nucleotides to nucleosides. The digestion was performed by incubating an aliquot of the concentrated cell extract with alkaline phosphatase (from calf intestine, 78 U/ml; Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) in pH 9 buffer containing 50 mM glyciglycerine, 2.5 mM magnesium acetate, and 2.5 mM zinc acetate for 1 h at room temperature. Protein was removed by ultrafiltration prior to analysis by reversed-phase HPLC (3a).

In order to measure the intracellular half-life of ara-ATP, VZV-infected cultures were incubated with [3H]ara-M (50 μM) for 12 h. Samples were then harvested for determination of initial ara-ATP levels. Drug-containing medium was decanted from the remaining samples, and cell monolayers were rinsed twice with phosphate-buffered saline, replenished with medium lacking drug, and further incubated at 37°C until harvest.

RESULTS

HFF mock infected or infected with VZV Oka were treated with radiolabeled ara-M for intervals of up to 24 h. Parallel incubations were also carried out with radiolabeled ara-A and ara-H. The difference in uptake of radioactivity in uninfected and VZV-infected cultures treated with ara-M was striking. Virus-infected cells accumulated up to 76-fold-higher levels of radioactivity than did uninfected fibroblasts.

![Figure 1](http://aac.asm.org/DownloadedFrom/aac.asm.org.png)

**FIG. 1.** Effect of VZV infection on cell uptake of radioactivity (A) and triphosphate formation (B) of radiolabeled ara-H, ara-A, and ara-M. Nine hours after infection with VZV Oka, the radiolabeled compounds were added to HFF cultures at final concentrations of 100 μM. Incubation was continued for 24 h, and samples were processed for analysis by liquid scintillation counting and anion-exchange HPLC.

(Fig. 1A). Intracellular accumulation of radioactivity with ara-A or ara-H treatment was relatively inefficient in either virus-infected or uninfected cells. Moreover, the difference in uptake of radioactivity between uninfected and infected cells was less marked with ara-H and ara-A.

Extracts of VZV-infected or uninfected cells treated with radiolabeled ara-M were analyzed by anion-exchange HPLC. Elution profiles of the infected-cell extracts indicated extensive anabolism of ara-M to mono-, di-, and triphosphorylated nucleosides (Fig. 2B). The only phosphorylated form

![Figure 2](http://aac.asm.org/DownloadedFrom/aac.asm.org.png)

**FIG. 2.** Relative anabolism of ara-A and ara-M in VZV-infected HFF. Experimental infections and incubations were as described in the legend to Fig. 1. Methanolic extracts (100 μl) of cells treated with [3H]ara-A (A) or [3H]ara-M (B) were analyzed by anion-exchange HPLC as described in Materials and Methods. BT, breakthrough region.
formed methanol-soluble lites of Radioactive ara-IMP, triphosphates. The alkaline ara-M in -ATP confirmed nucleotides eluted and chromatogram radiolabeled onstrated some shift of anabolism. from ara-A in cells. for ara-ATP than in infected, in infected cultures. Therefore, in the infected cell, ara-M was a much better precursor for ara-ATP than was ara-A (Fig. 1B and Fig. 2A and B). Moreover, a significant amount of ara-A radioactivity was incorporated into adenine nucleotides, particularly in infected cells (Fig. 2B), via ara-A's extensive deamination to ara-hypoxanthine and subsequent cleavage to labeled hypoxanthine.

The obligatory role of virus-encoded TK in the metabolic activation of ara-M was suggested when a TK-deficient mutant of strain Ellen was used (Table 1). Clearly, in the absence of a functional virus TK, cells treated with ara-M failed to accumulate ara-ATP. This failure is consistent with the lack of antiviral activity by ara-M against TK-deficient strains of VZV (1). However, in cells treated with ara-A, the differences in ara-ATP formation in the presence or absence of a functional viral TK were much less dramatic.

The relationship of drug concentration in the medium to both the uptake and phosphorylation of ara-M was investigated (Fig. 4). HPLC analyses revealed that intracellular accumulation of total radioactivity and intracellular levels of both ara-MMP and ara-ATP increased as a function of concentration of drug in the medium. The levels of the active antiviral ara-ATP generated with 10 μM ara-M (the in vitro 50% effective dose of ara-M for VZV is 1 μM) were detectable (80 to 100 pmol/10^6 cells) by these experimental methods. Most experiments utilized 50 or 100 μM ara-M in order to generate quantitative data in the variable VZV infection system. The cytotoxic endpoint of ara-M for MRC-5 or HFF was not established; no growth inhibition was observed at 100 μM (data not shown).

The intracellular half-life of ara-ATP formed in VZV-infected cells by incubation with 50 μM ara-M was determined (Fig. 5). The levels which had accumulated after 12 h of drug exposure declined 90% by 12 h after drug removal.

![Graph of methanol-soluble lites of Radioactive ara-IMP, triphosphates](image)

**Fig. 3.** Effect of alkaline phosphatase treatment on intracellular anabolites formed in [3H]ara-M-treated VZV-infected cells. Samples of methanol-soluble extracts were analyzed by reverse-phase HPLC before (A) and after (B) alkaline phosphatase treatment, as described in Materials and Methods. *"BT,"* breakthrough region of the chromatogram, in which the nucleoside mono-, di-, and triphosphates were eluted.

![Graph of intracellular anabolites](image)

**Table 1.** Concentrations of radiolabeled nucleotides in cells infected with TK⁺ or TK⁻ strain of VZV and incubated with 100 μM ara-M or ara-A

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Concn (pmol/10^6 cells) in cells treated with*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ara-A</td>
</tr>
<tr>
<td>TK⁺</td>
<td>TK⁻</td>
</tr>
<tr>
<td>AMP or ara-AMP</td>
<td>170</td>
</tr>
<tr>
<td>Ara-MMP</td>
<td>NA</td>
</tr>
<tr>
<td>Ara-IMP</td>
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<tr>
<td>Ara-ADP</td>
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</tr>
<tr>
<td>ATP</td>
<td>1,840</td>
</tr>
<tr>
<td>Ara-ATP</td>
<td>450</td>
</tr>
</tbody>
</table>

* Virus TK phenotype is indicated by + (competent strain Ellen) or − (negative ACV-resistant mutant of strain Ellen ACV² Cl 3-5-1 [3, 13]). NA, not applicable.

* Total radioactivity.

![Graph of ara-M concentration on uptake of radioactivity and intracellular anabolism in VZV Oka-infected HFF](image)

**Fig. 4.** Effect of ara-M concentration on uptake of radioactivity and intracellular anabolism in VZV Oka-infected HFF. Cells were treated with various concentrations of [3H]ara-M for 18 h, beginning 12 h postinfection. Methanolic extracts were analyzed directly for total uptake of radioactivity and for ara-MMP and ara-ATP levels.
The half-life for ara-ATP persistence in the ara-M treated cultures was calculated to be approximately 2.6 h.

**DISCUSSION**

Ara-M exerts potent and selective antiviral activity against VZV (1). The activity of this antiviral agent was highly dependent on a functional virus-encoded TK. In addition, ara-M was an excellent substrate for the virus-encoded TK, with a relative phosphorylation rate 3-fold higher than that of the natural substrate, deoxycytidine, and roughly 20-fold higher than that measured for ACV (1). In this study, we show that ara-M is a more effective precursor to ara-ATP than is ara-A itself. The pathway for the subsequent anabolism of ara-MMP to ara-ATP in VZV-infected cells is the subject of a forthcoming paper (4a).

The uptake of ara-M into VZV-infected cells exceeded that into uninfected cells. ACV, which is a less-efficient substrate for VZV TK than is ara-M, was not appreciably taken up or anabolized in the VZV-infected cells compared with uptake and anabolism of ara-M (data not shown). These observations are consistent with data derived from comparative substrate studies with VZV TK (1) and previous studies of ACV anabolism in VZV-infected cells (2). However, since ACV is an effective inhibitor of VZV replication in vitro, ACV triphosphate is likely a potent inhibitor of viral DNA polymerase.

Ara-M anabolism to ara-MMP occurred selectively in the TK-competent VZV-infected cell. In uninfected cells treated with ara-M, the levels of ara-ATP formed were at the lower limits of detection when the experimental methods described here were used, regardless of cell growth rate. In contrast, the growth states of the cell cultures played a major role in the ultimate levels of ara-ATP formed in either uninfected or VZV-infected cells.

Once formed, ara-ATP ultimately inhibits HSV replication at the level of viral DNA synthesis (11, 17, 19). Ara-ATP serves as a competitive inhibitor of the HSV DNA polymerase-catalyzed incorporation of dATP into viral DNA (12). The HSV type 1-specified DNA polymerase can internalize ara-AMP into the growing DNA chain (14). However, the similarity in \( K_s \) for polymerase inhibition and \( K_{i\text{m}} \) for the normal substrate dATP implies that ara-ATP is not a highly selective substrate for this viral enzyme (12). Inhibition of the VZV-encoded DNA polymerase by ara-ATP has not been studied. Moreover, ara-ATP is a relatively efficient inhibitor of mammalian DNA polymerases alpha and beta (7), on which it may act as a pseudodeterminator, with little incorporation into elongating DNA molecules (14).

In this study, the ara-ATP formed in VZV-infected cells after treatment with [\(^3\text{H}\)ara-M persisted for approximately 2.5 h (two independent experiments). Similar half-life results were obtained with ara-A (data not shown). Moreover, the stability of ara-ATP formed in uninfected HFF after incubation with [\(^3\text{H}\)ara-A was similar to that measured for ara-ATP in VZV-infected cells (data not shown). This half-life is similar to the half-life of ara-ATP in uninfected Vero cells previously reported (16). However, those same investigators measured a threefold increase in ara-ATP half-life in Vero cells infected with HSV (16). This contrasts with the results described here, in which ara-ATP half-lives in uninfected and VZV-infected HFF were comparable. The reasons for these differences are not apparent, but they may be related to cell type or to differences inherent in the intracellular environment of cells infected with HSV as opposed to VZV.

The marginal selectivity of ara-ATP at the level of viral DNA polymerase compared with the host mammalian cell DNA polymerase underscores the importance of selectivity in the generation of ara-ATP pools in the virus-infected cell. This study and another (16) indicate that there is little specificity of ara-A itself for the herpes virus-infected cell at the level of drug uptake and phosphorylation. However, ara-M offers an effective means of selectively targeting VZV-infected cells with the active anabolite ara-ATP.

**ACKNOWLEDGMENTS**

We thank Richard L. Miller and Donald J. Nelson for critical review of the manuscript, Beverly Nobles for artwork, and Janice Wilson for skilful manuscript preparation.

**REFERENCES**


