Metabolism of Ribavirin in Respiratory Syncytial Virus-Infected and Uninfected Cells

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The metabolism of ribavirin to its mono-, di-, and triphosphate derivatives was examined in uninfected and respiratory syncytial virus-infected cells. The degree of phosphorylation was dose dependent upon extracellular ribavirin concentration. The major species formed was the triphosphate, with mono- and diphosphates being approximately 12 and 4% of the triphosphate, respectively. Amounts of triphosphate formed in infected cells were up to 2.6-fold greater than those in uninfected cells. Upon drug removal, ribavirin triphosphate degradation was very rapid, with decay half-lives of 70 to 100 min. Actinomycin D inhibited triphosphate production and also neutralized the antiviral effect of ribavirin.

The broad-spectrum antiviral substance ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (19) has been shown to inhibit human respiratory syncytial virus (RSV) in vitro (11), in experimental animals (12), and in human subjects (9, 27). The mode of action of the compound involves phosphorylation to ribavirin monophosphate (RMP) by cellular adenosine kinase (28), followed by metabolism to di- and triphosphates (RDP and RTP) (33) by unidentified cellular enzymes. The phosphate derivatives of ribavirin inhibit other enzymes such as cellular IMP dehydrogenase (25), influenza virus RNA polymerase (4), and vaccinia virus and Venezuelan equine encephalitis virus RNA-capping enzymes (2, 8). The inhibition of these processes leads to virus inhibition. The specific mode of action of ribavirin against RSV may involve similar activities, although little if any research has been done in this area.

The pharmacokinetics of ribavirin in animals and humans has been investigated (3, 15). The compound has poor efficacy when administered systemically against naturally occurring influenza infections in humans (24), probably because it concentrates in erythrocytes and is rapidly metabolized in the liver. These problems have largely been bypassed by aerosol delivery of the agent, and successful treatment of respiratory infections has been achieved (9, 27). Because viral replication and drug inhibition of viral replication are both intracellular processes, it is important to understand the intracellular pharmacology of ribavirin, not just its pharmacokinetic behavior. For this reason, the present study was initiated to evaluate those parameters in RSV-infected and uninfected cells.

MATERIALS AND METHODS

Cells and virus. African green monkey kidney (MA-104) and human epidermoid larynx carcinoma (HEp-2) cells were purchased from MA Bioproducts, Walkersville, Md. They were propagated in Eagle minimal essential medium supplemented with 10% fetal bovine serum and 0.12% NaHCO3. RSV strain Long was obtained from the American Type Culture Collection, Rockville, Md. Initially, the virus pools were prepared in HEp-2 cells in T-150 flasks. High-titer virus (107 to 109 PFU/ml) was produced in T-850 roller bottles of MA-104 cells. Methods used to obtain high-titer RSV and to retain its viability upon freezing have been described before (10, 13, 16).

Compounds and enzymes. Ribavirin was synthesized by Ernie Prisbe at Syntex USA, Palo Alto, Calif., by a published procedure (29). [14C]ribavirin (56.3 mCi/mmol) and [3H]adenosine (10 Ci/mmol) were purchased from ICN Radiochemicals, Inc., Irvine, Calif. Adenosine, guanosine, and actinomycin D were purchased from Sigma Chemical Co., St. Louis, Mo. Erythro-9-(2-hydroxy-3-onyl)adenine, an adenosine deaminase inhibitor, was obtained from the National Cancer Institute, Bethesda, Md.

Antiviral and cytopathicity experiments. The activity of ribavirin against RSV was evaluated by cytopathic effect inhibition (22), plaque reduction (21), and virus yield reduction assays in MA-104 cells. For virus yield reduction, RSV at 5 PFU per cell was incubated for 24 h in the presence of various ribavirin concentrations in 24-well plates. Sucrose in medium at a 20% final concentration was added to wells (10), followed by sonication for 30 s and freezing at −80°C. Later, 50% endpoint dilution titrations (17) of the virus samples were conducted in 96-well plates. Fourfold dilutions were made by using a Pro/Pette apparatus (Cetus Instrument Systems, Emeryville, Calif.), and the amount of virus in each well was determined at 4 to 7 days. The cytoxicity of ribavirin was evaluated by cell proliferation assay in six-well plates. Nonconfluent monolayers were allowed to replicate for 4 days in the presence of drug or control. Cells were collected by trypsinization, dispersed by pipetting, stained with trypan blue, and counted with a hemacytometer.

Preparation of cell extracts and nucleotide analysis. Experiments were conducted in T-25 flasks of MA-104 cells. RSV at 5 PFU per cell was used except where noted. Uninfected, drug-treated cells were run in parallel. After a 1.25-h virus adsorption period, various concentrations of [14C]ribavirin from 10 to 100 μM (56.3 mCi/mmol) and 300 μM (28.15 mCi/mmol) were applied to cells in medium devoid of serum. Drug-free cultures were also included for determining the GTP intracellular pool size in infected and uninfected controls. After appropriate incubations, medium was aspirated from each container, and 330 μl of 3.5% perchloric acid was added to cell monolayers. After 15 min at 4°C, the perchloric acid was neutralized with 120 μl of 1 M KOH–0.4 M imidazole. Extracts were frozen at −80°C until analyzed by ion-exchange high-pressure liquid chromatography as de-
controls had to phosphate, ammonium ribavirin. The ATP) on the activity determined in reaction tubes of modifications and formate to N.J.) (100 reduce was at a 50% -80°C. The rate degree abnormalities a Determined conncn of RMP RSV infected c Percentage of the uninfected control or the RSV-infected control. The two controls had approximately the same GTP pool size. c Uninfected cells. d RSV infected (5 PFU per cell).

Adenosine kinase assays. RSV-infected and uninfected cells from T-25 flasks were trypsinized at various times after infection, pelleted at 600 × g for 5 min, and stored dry at -80°C. The cells were suspended in hypotonic buffer (10 mM Tris hydrochloride [pH 7.5], 10 mM KCl, 0.5 mM MgCl₂, 1 mM dithiothreitol) and freeze-thawed through three cycles. The supernatant was clarified by centrifugation at 50,000 × g for 10 min. Adenosine kinase activity was determined in reaction tubes (0.1 ml) at 37°C by using modifications of a published procedure (32). The assay mixture contained 64 mM Tris hydrochloride (pH 7.5), 40 mM KCl, 1 mM ATP, 0.5 mM MgCl₂, 100 mM erythro-9-(2-hydroxy-3-nonyl)adenine, 1 µM [³H]adenosine, and crude enzyme extract. The amount of enzyme used was that predetermined to convert about 10% of the substrate to product. At 60 min, DE-81 disks (Whatman, Inc., Clifton, N.J.) were spotted, washed three times in 2 mM ammonium formate and once in ethanol, and counted for radioactivity.

RESULTS

Antiviral activity of ribavirin against RSV. In plaque reduction assays, ribavirin inhibited RSV plaque numbers by 50% at a concentration of 35 µM. This concentration of the drug was found to reduce RSV cytopathic effect by 50% and to reduce virus yield by 90%. The antiviral activity of ribavirin was unaffected by the presence of adenosine or guanosine (100 to 1,000 µM) in the culture medium. Actinomycin D at 0.3 µM (higher concentrations were toxic) completely eliminated the anti-RSV activity of ribavirin.

Cytotoxicity determination. Ribavirin inhibited the proliferation of uninfected MA-104 cells by 50% at 75 µM. Since the virus was inhibited at a concentration of 35 µM, a slight degree of selectivity was present favoring viral over cell growth inhibition. Confluent, nonreplicating cells were more resistant to ribavirin, showing no microscopically discernable abnormalities at ≤320 µM.

Ribavirin anabolism in uninfected and RSV-infected cells. The rate of formation of ribavirin nucleotides in RSV-infected cells was similar but not identical to that observed in uninfected MA-104 cells. In the first analysis, different extracellular concentrations of ribavirin were used, and nucleotides were evaluated at a single time point (8 h) (Table 1). There was up to 140% more ribavirin nucleotides present in RSV-infected cells (depending upon the extracellular drug concentration) than in uninfected cells, indicating that the virus induced enzymes leading to ribavirin nucleotide formation. In both infected and uninfected cells, the predominant nucleotide present was RTP. At 300 µM in the medium, there was an enormous amount of intracellular RTP. Estimating the cell volume at 1 × 10⁻⁶ to 1.5 × 10⁻⁶ liters/10⁶ cells (26), 7,300 pmol/10⁶ cells would correspond to intracellular RTP concentrations of 5 to 7 mM.

One of the effects of ribavirin is to reduce the GTP pool size, as RMP inhibits IMP dehydrogenase (25) (a process which can inhibit the replication of some viruses by starving the virus-induced RNA polymerase of substrate). The relative sizes of the GTP pools at each ribavirin concentration are shown in Table 1. Slightly greater degrees of inhibition of the GTP pool size were noted in RSV-infected cells compared with uninfected cells at 10 to 300 µM, which may reflect greater amounts of RMP present in the infected cells.

The anabolism of ribavirin was evaluated over 24 h with a single drug concentration of 30 µM (Fig. 1). The amount of RTP formed in infected cells was greater than that in uninfected cells (Table 1). RTP in uninfected cells reached its maximum at 8 h, was static through 12 h, and then slowly declined at 18 to 24 h. RTP anabolism in RSV-infected cells continued through 18 h and then declined by 24 h. Amounts of RMP and RDP in the cells at the various time points were an average of 12 and 4% of RTP, respectively. In a study conducted in parallel to that shown in Fig. 1, ribavirin was removed from the culture medium at 8 h postinfection (Fig. 2). The catabolism of RTP was monitored for 16 h (or 24 h postinfection). In both the infected and uninfected cells, RTP degradation was very rapid. The half-lives of RTP catabolism were 70 and 100 min in the uninfected and infected cells, respectively. Ribavirin was rapidly excreted from cells into the culture medium after drug removal (Fig. 2).

![FIG. 1. Formation of RTP in RSV-infected (○) and uninfected (□) cells. Infecting virus was at 5 PFU per cell. Extracellular ribavirin concentration was 30 µM.](http://aac.asm.org/)

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**TABLE 1. Ribavirin nucleotide formation in RSV-infected and uninfected cells, and effects on GTP pool sizes**

<table>
<thead>
<tr>
<th>Extracellular concn of ribavirin (µM)</th>
<th>pmol/10⁶ cells</th>
<th>GTP pool size (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RMP</td>
<td>RDP</td>
</tr>
<tr>
<td>10</td>
<td>20 26</td>
<td>7 12</td>
</tr>
<tr>
<td>30</td>
<td>64 77</td>
<td>27 39</td>
</tr>
<tr>
<td>100</td>
<td>199 236</td>
<td>124 171</td>
</tr>
<tr>
<td>300</td>
<td>475 518</td>
<td>338 384</td>
</tr>
</tbody>
</table>

* Determined after an 8-h drug incubation.
* Percentage of the uninfected control or the RSV-infected control. The two controls had approximately the same GTP pool size.
* Uninfected cells.
* RSV infected (5 PFU per cell).
Because RSV appeared to stimulate the production of ribavirin nucleotides, most likely by cellular enzyme induction, the effects of varying the virus input were studied (Table 2). Virus multiplicities ranging from 0 to 20 PFU per cell were used, ribavirin was applied, and nucleotides were analyzed at 8 h. The maximum stimulation of ribavirin nucleotide formation was observed with virus multiplicities of infection (MOIs) of 2.5 and 5 PFU per cell. At 10 to 20 PFU per cell, the stimulation was slight or negligible. These effects were observed at extracellular ribavirin concentrations of 30 and 100 μM.

The anti-RSV activity of ribavirin was shown to be antagonized by actinomycin D, but not by adenosine or guanosine (see above). To explain these results, we investigated the effects of these substances on ribavirin nucleotide formation (Table 3). Actinomycin D treatment decreased amounts of RTP by 40% in uninfected cells and by 63% in RSV-infected cells. In fact, infected and uninfected cells treated with actinomycin D had nearly identical amounts of ribavirin nucleotides. Adenosine caused 15 to 30% reductions of RTP in cells, whereas RTP in guanosine-treated cells was 93 to 125% of the ribavirin-treated controls. The amount of RMP was elevated in guanosine-treated, infected cells. Actinomycin D treatment restored the GTP pool size in ribavirin-treated cells, whereas adenosine did not. In guanosine-treated cells, GTP pools were 210 and 270% of drug-free uninfected and infected controls, respectively.

Adenosine kinase activity in cells. Since ribavirin metabolism was stimulated in in RSV-infected cells, it was of interest to know whether adenosine kinase (the enzyme responsible for the first step in ribavirin phosphorylation (28)) activity was also increased. Cell homogenates from RSV-infected (2.5 to 5 PFU per cell) and uninfected cells harvested at 6, 12, and 24 h postinfection were assayed for adenosine kinase activity. The RSV-infected homogenates had no more than a 1.4-fold increase in adenosine kinase activity, compared with uninfected cell homogenates.

### Table 2. Effect of virus input on the phosphorylation of ribavirin

<table>
<thead>
<tr>
<th>Virus input (MOI)</th>
<th>30 μM ribavirin&lt;sup&gt;*&lt;/sup&gt;</th>
<th>100 μM ribavirin&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RDP</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>RTP</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>*</sup> MOI, Ratio of infecting virus titer to cell number.
<sup>∗</sup> Determined after an 8-h drug incubation.
<sup>∗</sup> Extracellular concentration of ribavirin.

### DISCUSSION

Ribavirin was shown to have in vitro anti-RSV activity in three different assays at 35 μM. This inhibitory dose is similar to that reported elsewhere (11). The mode of action of ribavirin against RSV is unknown, although one possible mode, the inhibition of IMP dehydrogenase (25), can be ruled out as having little, if any, bearing on the anti-RSV effect of ribavirin. This conclusion was reached because exogenous guanosine was able to restore the size of the intracellular GTP pool (ribavirin reduces that pool via IMP dehydrogenase inhibition) but yet not affect the virus-inhibitory activity of ribavirin. This implies that ribavirin acts on an RSV-specific event, such as on the RNA polymerase or RNA-capping process. Support for this conclusion comes from the observation that ribavirin inhibits viral but not cellular polypeptide synthesis (the consequence of inhibiting viral mRNA synthesis) in RSV-infected cells (Sme, unpublished data). In a previous report, ribavirin was shown to inhibit influenza virus even with exogenous guanosine present in the culture medium (30). It has been further demonstrated that the major site of action of RTP was on the virus-specified RNA polymerase (31).

The effect of actinomycin D in neutralizing the antiviral activity of ribavirin was reported by others who evaluated Sindbis virus (14) and rotavirus (23). The authors of the article on Sindbis virus felt that actinomycin D acted by maintaining the GTP pool size at normal levels, thus allowing viral RNA polymerase to function adequately (14). Although we observed that the amounts of GTP were normalized by actinomycin D in ribavirin-treated cells, it was shown above that the anti-RSV activity of ribavirin is independent of the GTP pool size. We conclude that actinomycin D is able to alter the anti-RSV activity of ribavirin by inhibiting RTP formation, implying that RTP is the biologically active form of the drug against the virus. Since actinomycin D also acts to stimulate RSV production by as much as 1 log<sub>10</sub> of infectious virus (13), this would also adversely affect the effectiveness of ribavirin against RSV.

Adenosine inhibited RTP production in cells by a moderate degree, probably as a result of competing with ribavirin for phosphorylation by adenosine kinase and myokinase. Since adenosine did not influence the activity of ribavirin against RSV, the slight decrease in intracellular RTP may be inconsequential. Alternatively, with a longer incubation time, the amounts of RTP may eventually reach levels that are achieved in adenosine-free cultures.

Because of the importance of the nucleotides of ribavirin for its antiviral activity, it was important to monitor the intracellular anabolism and catabolism of these substances.
As with other antiviral agents (7, 18, 20), ribavirin metabolism showed a linear relationship of extracellular drug concentration to intracellular nucleotides. The enhancement of ribavirin phosphorylation in RSV-infected cells was most likely due to the slight virus-induced stimulation of adenosine kinase and, possibly, other cell enzymes. This phenomenon has been demonstrated in herpesvirus-infected cells treated with arabinosyladenine (18) and in human cytomegalovirus-infected cells treated with 9-(1,3-dihydroxy-2-propoxyxymethyl)guanine (DHGP or BW759 [1, 6]). In each case, the increase in the active antiviral metabolite in infected cells is favorable because the virus is inhibited at extracellular concentrations lower than those required to inhibit uninfected cells. Although high MOIs of RSV were less stimulatory to ribavirin metabolism, it is doubtful that these high MOIs are achieved in vivo.

In the study in which RTP accumulation over 24 h was reported (Fig. 1), decreases in amounts of RTP were observed at the latter time points. In uninfected cells, this may have been the result of accumulated toxicity or perhaps of an induction of phosphatase activity. In infected cells, the above events could also explain the decline in RTP at 24 h. More likely, though, ribavirin nucleotide decay was the result of cell death and disruption following the completion of the virus life cycle.

The catabolism of ribavirin nucleotides after drug removal was very rapid and may partly explain why the compound is not more active in vivo than one might imagine by in vitro data. It seems that steady-state concentrations of drug would have to be maintained in patients for a therapeutic benefit to be realized. The delivery of ribavirin by a continuous small-particle aerosol achieves this effect, and consequently the drug is clinically active under this regimen against RSV (9, 27). Of course, targeting of the drug to the respiratory tract also plays a role.

The catabolism of ribavirin nucleotides was slightly slower in RSV-infected than in uninfected cells. This might be accounted for by an increase in anabolic enzyme activities (such as adenosine kinase), a decrease in dephosphorylating enzyme activities, or both. The catabolic rates in infected and uninfected cells were probably not sufficiently different to be of any clinical significance.

To emphasize the importance of the intracellular half-life of the active metabolite on the in vivo activity of a compound, the examples of DHGP and acyclovir are presented. Both compounds are equally active against herpesvirus in vitro, yet DHGP is far superior in vivo (5, 21). The pharmacologic effects of both substances in herpesvirus-infected animals are similar. Yet DHGP triphosphate, the active metabolite of DHGP, undergoes an extremely slow intracellular degradation after drug removal from the culture medium, whereas acyclovir triphosphate catabolizes much more rapidly (20). This difference most likely accounts for the greater in vivo potency of DHGP. That ribavirin nucleotides do not show much intracellular stability should illustrate the importance of using steady-state treatment regimens with the drug.

In summary, the metabolism of ribavirin in RSV-infected and uninfected cells was shown to lead primarily to RTP formation. Evidence that RTP is the active form of ribavirin against RSV is presented. Finally, the half-life of RTP in cells was short after drug removal, leading to the suggestion that steady-state conditions must be maintained in vivo for maximum drug efficacy.

LITERATURE CITED


METABOLISM OF RIBAVIRIN