In Vitro Activity of Ketoconazole against Herpes Simplex Virus

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The effects of ketoconazole alone and in combination with acyclovir and adenine arabinoside upon the replication of herpes simplex virus types 1 and 2 (HSV-1 and -2) were investigated by using a yield reduction assay. Ketoconazole demonstrated antiviral activity against HSV-1 and -2 and synergistic antiviral activity when it was combined with acyclovir. Combinations of ketoconazole with adenine arabinoside resulted in either interference or indifference. The effects of ketoconazole upon the protein synthesis of HSV-2-infected cells were also determined in an effort to define the mechanism of action for the antiviral activity of ketoconazole. There was no reduction of HSV proteins when compared with acyclovir. These findings suggest that further investigations of the use of ketoconazole for the treatment of HSV infections are warranted.

Recently, several reports have described a novel approach to antiviral chemotherapy with the antifungal agent amphotericin B. Amphotericin B, a polyene antibiotic, exerts its antifungal effect by binding to sterol components in the fungal cell membrane, which results in increased permeability of the membrane (11). By using the water-soluble ester of amphotericin B, Jordan and Sett demonstrated antiviral effects against enveloped viruses in plaque reduction assays (5). Similarly, Kessler et al. have shown that treatment of hepatitis B virus with increasing doses of amphotericin B caused disruption of the viral particles (6). Extending these observations, Malewicz et al. investigated the combined effects of amphotericin B and acyclovir upon the replication of pseudorabies virus. Low doses of amphotericin B in combination with acyclovir produced a synergistic reduction in viral replication as shown with a plaque reduction assay. The exact mechanism for this synergistic effect was not elucidated (9).

In view of this promising work, we investigated the possibility of antiviral activity for a different antifungal agent, ketoconazole. Ketoconazole is an imidazole compound which exerts its antifungal activity through inhibition of lanosterol demethylation. This blocks the synthesis of ergosterol, the major sterol component of the fungal cell membrane (2). In mammalian cells, ketoconazole also inhibits lanosterol demethylation, with a subsequent decrease in the biosynthesis of cholesterol, the major sterol component of mammalian cell membranes. In addition, ketoconazole interferes with cellular fatty acid and phospholipid biosynthesis (2). In this study, we examined the effects of ketoconazole alone and in combination with acyclovir and adenine arabinoside upon herpes simplex virus types 1 and 2 (HSV-1 and -2) replication with a yield reduction assay.

MATERIALS AND METHODS

Viruses. HSV-1 (F strain) and -2 (G strain) were obtained from the American Type Culture Collection, Rockville, Md. An acyclovir-resistant HSV-1 thymidine kinase-deficient (TK-) mutant (VL 8738) was provided kindly by Sandra Nusinoff-Lehrman (Burroughs Wellcome Co., Research Triangle Park, N.C.). Virus stocks were titered with titers of approximately 107 PFU/ml were grown in monolayers of Vero cells and stored at -70°C.

Cells. Vero and human lung (HL) cells (derived from an HL carcinoma) were used in all assays. Vero cells were grown in RPMI 1640 supplemented with 10% inactivated fetal bovine serum, 1% glutamine, 100 U of penicillin per ml, and 50 μg of streptomycin per ml; and HL cells were grown in Hanks balanced salt solution, basal medium Eagle improved, supplemented with 10% inactivated fetal bovine serum, 1% glutamine, 100 U of penicillin per ml, and 50 μg of streptomycin per ml. Maintenance medium for both cell lines was supplemented with 1% inactivated fetal bovine serum in place of 10% inactivated fetal bovine serum.

Drugs. Ketoconazole was provided kindly by Janssen Pharmaceutical, New Brunswick, N.J. Acyclovir was provided kindly by Burroughs Wellcome Co., Research Triangle Park, N.C., and adenine arabinoside was obtained from Sigma Chemical Co., St. Louis, Mo.

Yield reduction assay. Vero and HL cells were grown to 90% confluency in six-well disposable trays (Costar, Cambridge, Mass.). Growth medium was removed, and the cells were washed three times with phosphate-buffered saline, pH 7.2. Virus in maintenance medium (0.5 ml) was added at a multiplicity of infection of 0.5. After 1 h of adsorption at 37°C, the inoculum was removed, and maintenance medium with various concentrations and combinations of ketoconazole, acyclovir, and adenine arabinoside was added. Control cells were inoculated with virus and treated with maintenance medium without drugs. The plates were incubated at 37°C in 5% CO2 for 24 h, after which they were frozen at -70°C until titration by plaque assay.

Virus plaque assay. HL cells grown to confluence in sterile plastic six-well plates (Costar) were inoculated with log10 dilutions of supernatants from the yield reduction assay in triplicate. The plates were incubated at 37°C in 5% CO2 for 1 h with intermittent rocking at 15-min. intervals. After adsorption, 5 ml of carboxymethyl cellulose overlay was added to each well, and the plates were incubated for an additional 72 h at 37°C in 5% CO2. Monolayers were then fixed for 2 h in a solution of ethanol, glacial acetic acid, and Formalin (6:2:1) and stained with 1% crystal violet. Plaques were quantitated with a dissecting stereomicroscope.

Preincubation of virus in the presence of ketoconazole. HSV-2 (G strain) was incubated with ketoconazole in tripli-
cate under various conditions before plaque assay. The variables included time (0, 20, 40, or 60 min), temperature (4, 25, or 37°C), and ketoconazole concentrations (0, 5, or 10 μg/ml). The reaction mixture consisted of growth media, HSV-2 (5 × 10^5 PFU/ml), and the appropriate concentration of ketoconazole. The final volume of the reaction mixture was 2 ml. After the appropriate incubation period, the viral titer of the reaction mixture was determined by plaque assay and compared with proper control mixtures.

**Protein synthesis studies.** The effect of ketoconazole upon total protein synthesis by HSV-infected cells was determined by incorporation of [35S]methionine into thioracil-acid-precipitable material. Confluent Vero cells were infected with HSV-2 (G strain) at a multiplicity of infection of 5.0. After 1 h of adsorption, fresh medium with and without ketoconazole (10 μg/ml) was added. At various times after infection (3, 5, 9, 12, 15, and 18 h), the monolayers were pulsed with 20 μCi of [35S]methionine for 2 h. The medium was removed, and the monolayers were dissolved in polyacrylamide gel electrophoresis (PAGE) solubilization buffer. Solubilized monolayers were then boiled for 4 min and precipitated with 5% trichloroacetic acid. The precipitates were collected on membrane filters (Millipore Corp.) (0.45-μm pore size), washed, and counted in a liquid scintillation counter. Results were expressed as a percentage of uninfected controls.

**PAGE.** Vero cells were grown in 24-well disposable trays (Costar) until confluent (2.2 × 10^5 cells per tray). The cells were infected with HSV-2 at a multiplicity of infection of 5.0. The monolayers were pulsed with 20 μCi of [35S]methionine per ml for 2 h periods at 3, 6, 9, 12, 15, and 18 h postinfection. After pulse labeling, the medium was removed, and the monolayers were washed and solubilized as described above. Sodium dodecyl sulfate-PAGE was performed by the method of Laemmli (7). The final concentration of acrylamide in the separating gel was 8.5%. The gels were fixed, stained with Coomassie blue, dried, and autoradiographed at ~70°C.

**Definition of drug interactions.** The definitions of drug interactions are as described by Schinazi et al. (14). Briefly, the yield of control for drug A (Y_A) or drug B (Y_B) is the virus titer produced in the presence of drug, divided by the virus titer produced in the absence of drug. The yield of control for the combination of two drugs (Y_A/B) is the virus titer produced in the presence of these two drugs, divided by the virus titer produced in the absence of these two drugs. The calculated or expected yield of control (Y_C) is the product of Y_A and Y_B. If Y_A/B is equal to Y_C, the interaction of the drugs in combination is additive. If Y_A/B is less than Y_C, then the interaction is synergistic. If Y_A/B is greater than the most effective agent alone, then the interaction is antagonism. When the inhibition is not greater than the most effective drug alone, the interaction is defined as indifference (14).

**Drug toxicity assay.** Approximately 5 × 10^5 cells per ml of Vero and HL cells in growth medium were inoculated into 25-cm² tissue culture flasks. The cells were incubated for 24 h at 37°C in 5% CO₂, after which the growth medium was replaced with fresh growth medium with various concentrations of ketoconazole, acyclovir, and adenine arabinoside alone and in combination. Controls were grown in growth medium without drugs. At 24-h intervals for 72 h, the monolayers from three flasks were trypsinized, and viable cells were quantitated by the trypsin blue exclusion method.

## RESULTS

Treatment of HSV-1 and -2 with ketoconazole in HL cells resulted in a dose-dependent reduction in viral titer (Table 1). The greatest effect occurred at a ketoconazole concentration of 10 μg/ml. Of note, the antiviral effect of ketoconazole was similar for both HSV-1 and -2. The HSV-1 TK− strain (VL 8737) was also tested against ketoconazole (Table 1). Although there was a dose-dependent reduction in viral titer, the reduction was of a lesser degree than that of the HSV-1 (F strain). The reasons for the decreased susceptibility of the TK− mutant are not known and will require further evaluation. Similar results were obtained in Vero cells (data not shown).

All combinations and concentrations of ketoconazole and acyclovir demonstrated synergy against both HSV-1 and -2. This was particularly obvious at high concentrations of ketoconazole and acyclovir (Table 2). The antiviral activity of ketoconazole alone and in combination with acyclovir was similarly demonstrated in Vero cells. In contrast, combinations of ketoconazole and adenine arabinoside demonstrated either indifference, indifference, or in one case, antagonism (Table 2). These effects occurred in both cell lines tested.

Pretreatment of the HSV-2 strain with various concentrations of ketoconazole for periods up to 60 min at the various

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**TABLE 1.** Yield reduction assay of HSV with ketoconazole, acyclovir, and adenine arabinoside in HL cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc (μg/ml)</th>
<th>HSV-1 (F strain)</th>
<th>HSV-2 (G strain)</th>
<th>HSV-1 (VL 8737)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Titer × 10^6</td>
<td>% of control</td>
<td>Titer × 10^6</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>4.1 × 10^6</td>
<td>100</td>
<td>1.5 × 10^6</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>1</td>
<td>3.6 × 10^6</td>
<td>87</td>
<td>1.1 × 10^6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.3 × 10^6</td>
<td>33</td>
<td>4.2 × 10^6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.4 × 10^5</td>
<td>5</td>
<td>7.5 × 10^6</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>1</td>
<td>1.4 × 10^6</td>
<td>33</td>
<td>6.4 × 10^5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.0 × 10^5</td>
<td>2.5</td>
<td>3.0 × 10^4</td>
</tr>
<tr>
<td>Adenine arabinoside</td>
<td>1</td>
<td>1.2 × 10^6</td>
<td>29</td>
<td>1.5 × 10^4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.9 × 10^4</td>
<td>4</td>
<td>1.5 × 10^4</td>
</tr>
</tbody>
</table>

* Acyclovir-resistant thymidine kinase-negative mutant.

* Virus titer, PFU/ml.
TABLE 2. Yield reduction assay of HSV with combinations of ketoconazole, acyclovir, and adenine arabinoside in HL cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc (μg/ml)</th>
<th>HSV-1 (F strain)</th>
<th>HSV-2 (G strain)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Titer a</td>
<td>Yield</td>
</tr>
<tr>
<td>Ketoconazole/acyclovir</td>
<td>5/1</td>
<td>3.9 x 10^5</td>
<td>9e</td>
</tr>
<tr>
<td></td>
<td>10/1</td>
<td>4.6 x 10^4</td>
<td>1f</td>
</tr>
<tr>
<td></td>
<td>5/5</td>
<td>1.2 x 10^4</td>
<td>0.2d</td>
</tr>
<tr>
<td></td>
<td>10/5</td>
<td>1.3 x 10^3</td>
<td>0.03d</td>
</tr>
<tr>
<td>Ketoconazole/ad. arabinoside</td>
<td>5/1</td>
<td>8.9 x 10^5</td>
<td>2f</td>
</tr>
<tr>
<td></td>
<td>10/1</td>
<td>6.6 x 10^5</td>
<td>15e</td>
</tr>
<tr>
<td></td>
<td>5/5</td>
<td>6.6 x 10^5</td>
<td>15e</td>
</tr>
<tr>
<td></td>
<td>10/5</td>
<td>4.8 x 10^5</td>
<td>11e</td>
</tr>
</tbody>
</table>

a Viral titer, PFU per milliliter.  
b Yield observed is the titer of virus produced in presence of drug divided by titer of virus produced in absence of drug times 100.  
c Yield calculated for additive interaction is the product of observed yields of the control for each drug in combination.  
d Synergistic interaction.  
e Additive interaction.  
f Indifferent interaction.  
g Interference interaction.  
h Antagonistic interaction.

We have previously shown that ketoconazole inhibits hepatitis B surface antigen production by a transformed human hepatoma cell line which is chronically infected with hepatitis B virus (13).

In the present study we have shown that ketoconazole at a concentration of 10 μg/ml causes an approximate 1-log reduction of HSV titer by an in vitro yield reduction assay. A yield reduction assay was chosen for these experiments to give a better estimate of the antiviral activity of ketoconazole. The yield reduction assay allows for the use of a greater multiplicity of infection of virus and evaluation of higher concentrations of antiviral drugs in combination studies (14). In addition, this assay evaluates the antiviral activity of the various drugs within 24 h of inoculation, which eliminates some of the nonspecific cytotoxicity which can be seen with assays requiring 48 to 72 h of incubation. When tested in a standard plaque reduction assay, ketoconazole at a concentration of 10 μg/ml causes a 3- to 4-log reduction in HSV titer (J. Pottage, H. Kessler, J. Goodrich, T. Hurley, and S. Levin, Clin. Res. 31:736A, 1983). Of potential clinical significance is the finding of a synergistic antiviral effect against HSV-1 and -2 when ketoconazole is combined with acyclovir. Like acyclovir, ketoconazole is an oral agent. The levels of ketoconazole studied in these experiments are obtainable in the serum of humans. Whether the combination of acyclovir and ketoconazole will be clinically effective in HSV infections remains to be seen and will require additional in vivo animal studies.

The mechanism of action of the antiviral activity of ketoconazole is unknown. Our experiments show that there seems to be no direct effect upon the virus particles by ketoconazole. In mammalian cells, ketoconazole interferes with cholesterol biosynthesis by inhibiting lanosterol demethylation (2). It also interferes with phospholipid biosynthesis by the cell (2). There is a paucity of information regarding the role of lipid biosynthesis in HSV replication. A possible site of action for the antiviral activity of ketoconazole is interference with lipid metabolism resulting in the production of a defective lipid envelope for the virus, an action that should decrease virus infectivity. Partial support for this hypothesis comes from the observation that ketoconazole does not demonstrate antiviral activity against the

**DISCUSSION**

Ketoconazole is an imidazole antibiotic which has been used extensively for the treatment of fungal infections. Ketoconazole has also been found to have in vitro inhibitory activity for other microorganisms, including bacteria such as *Staphylococcus aureus* (15), and the protozoans *Plasmodium falciparum* (12), *Trypanosoma cruzi* (10), and *Leishmania tropica* (1). The exact mechanism for the inhibitory activity against these diverse organisms is not known, but is thought to be related to alterations in their lipid metabolism.
novenveloped poliovirus in a similar yield reduction assay (unpublished data). We have also shown that the triazole antifungal agents, itraconazole and R47,829, which are more specific for inhibition of fungal sterol biosynthesis and do not interfere with cholesterol biosynthesis in mammalian cells, have no antiviral activity against HSV-1 and -2 (J. Pottage, H. Kessler, J. Goodrich, K. Kapell, and S. Levin, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 120, 1985). Alternatively, the antiviral effect of ketoconazole could be caused by a specific alteration in DNA or protein synthesis. Ketoconazole has been demonstrated to inhibit DNA synthesis of stimulated lymphocytes (3). Although our studies with [35S]methionine incorporation and PAGE suggest that ketoconazole does not affect the production of the total quantity of HSV proteins in infected cells, studies of the effects of ketoconazole upon individual proteins will need to be performed. The mechanism for the synergistic antiviral effects when ketoconazole was combined with acyclovir and interference when combined with adenine arabinoside is unexplained at this time. Further studies will be required to determine the exact mechanism of action of the anti-HSV activity of ketoconazole.

Whether these in vitro observations regarding the antiviral activity of ketoconazole will correlate with clinical efficacy in humans is unknown. There is a report of a patient with recurrent HSV-2 genital infection who favorably responded to ketoconazole (16). The patient, a female with a 4-year history of recurrent genital HSV infections (recurring every 2 weeks) and concomitant candida vaginitis had no further recurrences of genital HSV over a 5-month follow-up period (16). Although this may be a coincidental effect, this case report and our in vitro data suggest there may be a role for ketoconazole in the treatment of HSV infections.

Although ketoconazole is a relatively safe antifungal agent, it has been associated with significant hepatic toxicity in approximately 1 in 10,000 persons (8). Further evaluation of ketoconazole will be required before it can be investigated for use in the treatment of human HSV infections. Importantly, however, ketoconazole may prove to be a valuable agent for the study of lipid biosynthesis and metabolism in HSV-infected cells. Additionally, these observations may provide the start for a new approach in the development of antiviral agents.

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LITERATURE CITED


