Novel Synergism of Two Antifungal Agents, Copiamycin and Imidazole

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Copiamycin, a macrocyclic lactone antifungal antibiotic, was found to potentiate the antifungal effect of imidazole compounds, ketoconazole in particular. The potentiation of two nominally fungistatic agents in vitro was substantiated by a marked reduction of the minimum inhibitory and minimum fungicidal concentrations when the drugs were used in combination. The effectiveness of this synergistic combination was also demonstrated in experimental murine candidosis. Evidence is presented to suggest that this combined effect is due, at least in part, to the ionophoretic property of copiamycin. Whereas amphotericin B induces a marked increase in cellular permeability, this antibiotic does not possess the ionophoretic action of copiamycin, indicating that the enhancement of copiamycin activity and significant reduction of amphotericin B activity by ketoconazole pretreatment can be ascribed not only to changes in membrane permeability of the test organisms, but also to the different action mechanisms of copiamycin and amphotericin B. It is thus plausible that the strong synergism of copiamycin with imidazole compounds is related to the ionophoretic activity of the antibiotic. Further studies on the biochemical mechanism of this synergistic effect are being conducted together with an assessment of the clinical significance of this drug combination.

Among the various infectious diseases, the chemotherapy for systemic fungal infections in patients, particularly those with impaired host defense mechanisms, has been most discouraging. Until recently, amphotericin B was the only chemotherapeutic agent available for use against these infections. Recently, 5-fluorocytosine was developed and shown to be effective against candidosis and cryptococcosis. However, the major defect of 5-fluorocytosine therapy is the rapid development of organisms resistant to this antymycotic. In addition, the compound is primarily fungistatic. More recently, a number of chlorinated imidazole compounds have been found to have antifungal as well as antibacterial activity. Among them, clotrimazole, miconazole, and econazole have already been used in topical and in some cases systemic therapy. Ketoconazole is a newer dioxolane imidazole which shows promise as a broad-spectrum antifungal agent effective by oral administration. In our studies on the mode of action of ketoconazole (24, 25, 27), we demonstrated that the mechanism of action of this compound is somewhat different from those of the other imidazole antifungal agents. Our results indicated that the primary site of action of ketoconazole is cytochrome c oxidase (25). On the other hand, van den Bossche et al. reported that ketoconazole had an effect on cytochrome P-450 with subsequent inhibition of sterol biosynthesis (28, 29).

Although preliminary clinical studies seem to indicate the potential usefulness of this drug for the treatment of systemic mycoses, its mode of action seems to be more fungistatic rather than fungicidal at clinically achievable concentrations.

On the other hand, potential antifungal chemotherapeutics must be fungicidal or cause a marked reduction in viability to be effective on opportunistic fungal infections, because the patient's defense mechanisms are usually markedly impaired in these cases. Therefore, to provide safer and more effective antifungal treatment, newer protocol concepts must be developed. One method has been to use combination therapy of amphotericin B with other antimicrobial agents or an intermediate of sterol biosynthesis (19). Others have used two different antymycotics (3).

Antifungal activity of preferentially antibacterial antibiotics when combined with amphotericin B was first described by Kwan et al. in 1972 (16). This kind of potentiation of amphotericin B was also observed with tetracycline (14), minocycline (12, 17), rifampin (2, 9), bleomycin, and
fusidic acid (13). Combination therapy of amphotericin B with such antibacterial antibiotics is unquestionably interesting from the viewpoint not only of clinical application but also of the biochemical basis of such potentiation.

Combinations of two kinds of antifungal agents, such as amphotericin B and 5-fluorocytosine, have already been used clinically. More recently, some authors have reported the antifungal action of amphotericin B in combination with other polyenes, 5-fluorocytosine, or imidazoles (4, 21, 22). In contrast, there are also some studies of antagonism in vitro or in vivo between amphotericin B and imidazole antifungal agents (7, 26).

Copiamycin is an antifungal antibiotic isolated by Arai et al. in 1965 (1). This antibiotic is slightly soluble in water and mostly fungistatic even at a high concentration; it was not studied in detail. The skeletal structure of copiamycin was elucidated recently (10), and the antibiotic was found to belong to the macrocyclic lactone antibiotics such as polyene macrolides. However, it does not possess a conjugated double-bond structure, as do polyenes.

The macrocyclic lactone structure of copiamycin suggested that the antibiotic might interfere with the activity of imidazole compounds as amphotericin B does. In striking contrast, the combination proved to be strongly synergistic in vitro as well as in vivo.

MATERIALS AND METHODS

Chemicals. Copiamycin was produced by fermentation of Streptomyces hygroscopicus subsp. crystallogenes as described by Arai et al. (1). Ketoconazole was a gift from Kyowa Hakko & Co., Tokyo, Japan. Clotrimazole, miconazole, and econazole were supplied by Bayer Yakuhin Co., Ltd. (Osaka), Eisai Co., Ltd. (Tokyo), and Otsuka Pharmaceutical Co., Ltd. (Tokyo), respectively.

Organisms. Candida albicans 7N (ATCC 48130, serotype A), an isolate from a clinical case of pulmonary candidosis which is highly virulent to mice, and all the other test fungal strains were obtained from our culture collection (IFM), Medical School of Chiba University and Narashino National Hospital, Japan, and maintained in our laboratory on Sabouraud dextrose (2%) agar slants.

Determination of antifungal activity of copiamycin. Inocula were prepared by homogenizing each strain with glass beads in sterile 0.9% NaCl. The final concentration of organisms was about 10⁸ cells per ml. The minimal inhibitory concentration (MIC) of copiamycin against test fungi was determined by the twofold agar dilution method on Sabouraud dextrose (2%) agar. Solidified plates were inoculated with a loopful of fungal suspension and incubated at 27 or 36°C, depending on the microorganism, until growth appeared on drug-free control plates. The MIC was defined as the lowest concentration of the drug which gave no visible growth.

Combined effect of ketoconazole with copiamycin, clotrimazole, or amphotericin B on agar plates. A paper strip soaked in a solution of ketoconazole (100 μg/ml) was placed across the diameter of a yeast morphology agar plate seeded with C. albicans 7N. Three shorter strips, each soaked in solution containing 100 μg of copiamycin, clotrimazole, or amphotericin B per ml, were placed perpendicular to the ketoconazole strip. The plate was incubated at 37°C for 24 h.

Synergism of copiamycin with representative imidazole drugs. The combined effects of copiamycin, clotrimazole, miconazole, and econazole were compared with those when used alone against 26 isolates of C. albicans. Drugs were tested in the ratios of 4:1, 1:1, and 1:4 by weight. MICs were determined by the twofold agar dilution method on Sabouraud dextrose agar after incubation at 37°C for 24 h.

Cumulative percentage of C. albicans inhibited by combinations of copiamycin and ketoconazole. The susceptibility of 41 strains of C. albicans to copiamycin or ketoconazole alone and in combination was tested by the checkerboard method as follows. The organisms were incubated overnight at 37°C in Sabouraud dextrose agar slants. The organisms were then suspended in sterile 0.9% NaCl to a final concentration of about 10⁶ cells per ml. Serial twofold dilutions of antibiotics (0.006 to 100 μg/ml) were prepared in Sabouraud dextrose agar. The organisms were then plated on solidified plates with a multipoint inoculator (Denly Instruments Co. Ltd., Sussex RH14, England) and were incubated at 37°C until growth was visible on drug-free control plates.

Fungicidal effect of combined copiamycin and ketoconazole. Concentrations of drugs (0.006 to 100 μg/ml) alone or in combination were prepared in Sabouraud broth and then inoculated with C. albicans 7N or Trichophyton mentagrophytes to a final concentration of 6 × 10⁵ by counting with a hemacytometer. The fungicidal effect was evaluated by triplicate plating on Sabouraud dextrose agar and counting the CFU after incubation for 24 h (C. albicans) or 5 days (T. mentagrophytes) at 37°C. The minimal fungicidal concentration was the lowest concentration of the drug which gave no more than 10 colonies per ml.

Synergistic or antagonistic effect of ketoconazole plus copiamycin or amphotericin B on growth rate. The synergism between ketoconazole and copiamycin and the antagonism between ketoconazole and amphotericin B which affected the growth rate of C. albicans were determined by CFU. The starting concentration was 2 × 10⁶ cells per ml in Sabouraud broth, and incubation was for 24 h at 37°C. Drug additions were done at the start or at log phase.

Experimental murine candidosis. Four-week-old male ddY inbred mice were purchased from the Shizuoka Agriculture Cooperative Association for Experimental Animals, Shizuoka, Japan. They were challenged intravenously with 5 × 10⁶ washed cells from an 18-h culture of C. albicans 7N. Each group consisted of 10 animals. Mice were treated intravenously with copiamycin (0.4 mg per mouse) alone, ketoconazole (0.2 mg) alone, or copiamycin (0.2 mg) plus ketoconazole (0.1 mg), starting on day 2 after challenge.

Respiration, swelling, and movements of monovalent cations in rat liver mitochondria. Rat liver mitochondria were isolated by the method of Johnson and Lardy (15). Protein concentrations were determined.
The Pathogenic antibiotic. as concentration were reported compounds (8), ed guanidine lactone droxy compared strains of 30°C with electrodes the data is potassium and of a type G2040C recording the swelling, chondrial (pH mitochondria) um phosphate, 10 mM K+, and 15 mM succinate, and 1.5 mg of rat liver mitochondria (pH 7.4). For the determination of mitochondrial swelling, light scattering was measured by recording the absorbance at 540 nm (Hitachi digital spectrophotometer model 191E). The concentrations of K+ and H+ were determined with a Radiometer model PHM 84 research pH meter in combination with a type F2312 potassium ion selectrode for K+ or with a type G2040C glass electrode for H+. Both reference electrodes were of type K701 with an electrolytic solution of LiCl, COO. After suitable amplification, the data were taken with a Hitachi recorder (model 056). The cuvette (3-ml volume) was maintained at 30°C with a constant-temperature jacket.

RESULTS

The skeletal structure of copiamycin (10) is compared with that of amphotericin B in Fig. 1. Copiamycin consists of a 32-membered polyhydroxy lactone ring and an unsaturated ester group, as well as a side chain with a disubstituted guanidine moiety as its terminal. Table 1 shows the antifungal spectrum of copiamycin. It is interesting to note that Geotrichum candidum, which is reported to be resistant to known imidazole compounds (8), is susceptible to this antibiotic. Pathogenic yeasts such as some strains of C. albicans and Candida stellatoidea were less susceptible, being inhibited at the concentration of 50 µg/ml.

The combination of ketoconazole and amphotericin B seemed to be mostly antagonistic as revealed by a constriction of the inhibition zone (Fig. 2). In contrast, the combination of copiamycin and ketoconazole was evidently synergistic. The combined effect of ketoconazole and clotrimazole was not significant under the present experimental conditions.

Table 2 illustrates the results of the combined activity of copiamycin and such imidazole compounds as clotrimazole, miconazole, and econazole in the ratios of 4:1, 1:1, and 1:4 by weight, in comparison with those of compiamycin or ketoconazole alone against 26 isolates of C. albicans. MICs of ketoconazole were much

![FIG. 1. Structure of copiamycin (a) and amphotericin B (b).](http://aac.asm.org/)

![FIG. 2. Antagonistic (ketoconazole-amphotericin B) or synergistic (ketoconazole-copiamycin) effects on agar plate seeded with C. albicans 7N. Note that clotrimazole is apparently neither antagonistic nor synergistic with ketoconazole.](http://aac.asm.org/)
TABLE 2. Effects of copiamycin and imidazole drug in combination against 26 strains of *C. albicans*.

<table>
<thead>
<tr>
<th>Drug (ratio)</th>
<th>No. of strains inhibited by MIC (μg/ml) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;100</td>
</tr>
<tr>
<td>CPM alone</td>
<td>1</td>
</tr>
<tr>
<td>CPM + CTZ (4:1)</td>
<td>1</td>
</tr>
<tr>
<td>CPM + CTZ (1:1)</td>
<td>8</td>
</tr>
<tr>
<td>CPM + CTZ (1:4)</td>
<td>4</td>
</tr>
<tr>
<td>CTZ alone</td>
<td>1</td>
</tr>
<tr>
<td>CPM + MCZ (4:1)</td>
<td>1</td>
</tr>
<tr>
<td>CPM + MCZ (1:1)</td>
<td>8</td>
</tr>
<tr>
<td>CPM + MCZ (1:4)</td>
<td>5</td>
</tr>
<tr>
<td>MCZ alone</td>
<td>3</td>
</tr>
<tr>
<td>CPM + ECZ (4:1)</td>
<td>3</td>
</tr>
<tr>
<td>CPM + ECZ (1:1)</td>
<td>4</td>
</tr>
<tr>
<td>ECZ alone</td>
<td>3</td>
</tr>
<tr>
<td>CPM + KCZ (4:1)</td>
<td>2</td>
</tr>
<tr>
<td>CPM + KCZ (1:1)</td>
<td>3</td>
</tr>
<tr>
<td>CPM + KCZ (1:4)</td>
<td>1</td>
</tr>
<tr>
<td>KCZ alone</td>
<td>4</td>
</tr>
</tbody>
</table>

a Figures denote number of strains inhibited completely. MICs were determined by the agar dilution method in Sabouraud dextrose agar after incubation for 24 h at 37°C. CPM, Copiamycin; CTZ, clotrimazole; MCZ, miconazole; ECZ, econazole; KCZ, ketoconazole.

higher than those of clotrimazole, miconazole, and econazole. The highest synergism was obtained with the combination of copiamycin and ketoconazole at the ratio of 4:1, followed by 1:1 and 1:4. Synergism was also observed with the combination of copiamycin and other imidazole compounds, although less significantly than with ketoconazole. Again, the highest potentiation was obtained with copiamycin and these imidazole compounds in the combination ratio of 4:1.

Figure 3 illustrates the cumulative percentage of 41 *C. albicans* strains inhibited by combinations of copiamycin and ketoconazole, as examined by checkerboard titration method. Ketoconazole, in a single use, inhibited only 40.6% of test strains even at a concentration as high as 50 ug/ml.

**FIG. 3.** Distribution of MICs of ketoconazole (KCZ) alone with various concentrations of copiamycin (CPM) against 41 strains of *C. albicans*. - - - -; Copiamycin used alone.
TABLE 3. Fungicidal effects of copiamycin and ketoconazole in combination

<table>
<thead>
<tr>
<th>Drug</th>
<th>C. albicans</th>
<th>T. mentagrophytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (µg/ml)</td>
<td>MCC (µg/ml)</td>
</tr>
<tr>
<td>Copiamycin</td>
<td>50.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>50.0</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td>Copiamycin + ketocona-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>zole (1:1)</td>
<td>0.78</td>
<td>3.12</td>
</tr>
</tbody>
</table>

a Conditions: medium, Sabouraud glucose broth; 37°C; 24 h for C. albicans and 5 days for T. mentagrophytes.

µg/ml. However, all the test strains were inhibited when 0.2 µg of copiamycin per ml was added. The case was similar when 6.23 µg of ketoconazole per ml and 0.78 µg of copiamycin per ml were used in combination. Even the combination of ketoconazole and copiamycin at concentrations far below their respective MICs, i.e., 0.78 µg of copiamycin and 1.56 µg of ketoconazole per ml, was found to be highly active, suppressing the growth of all the test strains.

In our preceding experiments with ketoconazole (27), we noted an unusually wide range of MIC and maximum growth allowance concentration. The same feature of antibiotic activity was observed with copiamycin against T. mentagrophytes in particular. These experimental results indicated that both drugs are mostly fungistatic rather than fungicidal. However, the combination of ketoconazole and copiamycin at a 1:1 ratio had fungicidal properties (Table 3). Similarly, the combination of copiamycin with other imidazole antifungal agents not only proved to be synergistic but also resulted in enhanced fungicidal activity.

The effectiveness of the combination was also evident in experimental murine candidosis (Fig. 4). Neither copiamycin nor ketoconazole alone at given doses protected mice against infection, whereas one-half doses of each drug in combination were distinctly effective (60% of mice survived more than 20 days). The effect was further confirmed by the decrease in CFU in the kidneys of surviving mice (data not shown).

The effect of copiamycin on the rate of respiration of mitochondria from rat liver was compared to those of valinomycin, amphotericin B, and antimycin A (Fig. 5). Similar studies were made of the effect of copiamycin on mitochondria light scattering and on K+ and H+ movements (Fig. 6). In a medium containing K+, copiamycin and valinomycin significantly stimulated respiration, whereas amphotericin B exhibited entirely no effect and, by contrast, antimycin A caused immediate cessation of respiration. The stimulation of respiration without ADP presents direct evidence of the uncoupling effect of copiamycin. Rapid release of K+ was observed immediately after the addition of copiamycin (Fig. 6). Mitochondrial swelling, as monitored by light scattering at 540 nm, was also observed in parallel with this K+ exit. On the other hand, the uptake of H+ from the medium was revealed by an increase in pH.

FIG. 4. Synergistic effect of copiamycin and ketoconazole on experimental candidosis in mice. Infection with C. albicans 7N, 5 × 10^5 cells per mouse by intravenous injection. Treatment was once a day, for 5 consecutive days, by intravenous injection. Symbols: (—) Control, (-----) copiamycin (0.4 mg per mouse), (———) ketoconazole (0.2 mg per mouse), (———) copiamycin plus ketoconazole (0.2 + 0.1 mg per mouse, respectively).
In a preliminary attempt to analyze the mechanism involved in the synergistic (ketoconazole-copiamycin) or antagonistic (ketoconazole-amphotericin B) effect, the effectiveness of these antimycotics alone or in combination on the growth rate of *C. albicans* was studied (Fig. 7). When sub-inhibitory doses of ketoconazole were added to cultures growing for 12 h in the presence of a sub-inhibitory concentration of copiamycin, little growth retardation of the test organism was observed. On the other hand, when copiamycin was added to cultures pretreated with ketoconazole, an instantaneous lethal effect took place (Fig. 7A). This effect was comparable to that of both drugs added at the beginning of incubation. The results of similar experiments in which copiamycin was replaced by amphotericin B are illustrated in Fig. 7B. No such potentiation of the effect of amphotericin B, i.e., killing of the fungus at one-half MIC in the presence of ketoconazole, occurred in these experiments. On the contrary, even highly fung-
cidal doses of amphotericin B, added to the culture containing a sub-inhibitory dose of ketoconazole, failed to cause significant growth retardation (Fig. 7C). In this case, antagonism between ketoconazole and amphotericin B was evident.

**DISCUSSION**

In our studies on the mode of action of 5-fluorocytosine (31) and ketoconazole (24, 25, 27), we showed evidence that these two drugs have one character in common, i.e., they are predominantly fungistatic at clinically achievable levels as compared to those more toxic known antifungal agents.

5-Fluorocytosine is a novel antimycotic with minimal toxicity. The sophisticated mechanism of the selective toxicity of 5-fluorocytosine to yeasts is explained by its selective incorporation into yeast cells (31). However, the inhibitory effect of the compound against a variety of fungi is not complete in common complex organic media.

Ketoconazole is unusual in that it is orally active and has much better chemotherapeutic use due to a lower toxicity than already known imidazole derivatives.

These facts seem to suggest that more beneficial antifungal chemotherapeutics can be found among the compounds which have an extraordinarily wide fungistatic concentration range.

However, fungicidal rather than fungistatic chemotherapeutics are required to cope particularly with opportunistic infections in patients with impaired host defense mechanisms. Therefore, the present studies were undertaken in a bid to solve this contradictory problem by combination therapy. Copiamycin, a macrocyclic lactone antifungal antibiotic, in combination with imidazole antifungal agents exhibited marked synergism in vitro and in vivo. The assessment of the combined effect of antifungal agents in vitro and in vivo is sometimes difficult, and conflicting reports have been published, as in the case of the combination of amphotericin B with imidazole antifungal agents. With copiamycin plus imidazole antifungal agents, the potentiation of antifungal activity was 50- to 100-fold with regard to the respective MICs; these results were unequivocal. This augmented potency was further substantiated by the fact that the combination became fungicidal, unlike when each drug was exerted alone.

At first sight of the structure of copiamycin, whose backbone is capable of assuming a conformation that focuses the oxygens about a ring or cavity in space to complex various cations, one may infer an ionophoretic activity of the antibiotic (23). The present experimental results met this expectation quite well. The antibiotic

![Graph](image_url)
induced energy-linked mitochondrial ejection of K+ which was accompanied by a roughly equivalent disappearance of H+ in the medium (i.e., an increase in pH). A decrease in light scattering is indicative of an increased mitochondrial volume. Taken together with the uncoupling effect, it is apparent that copiaymicin is an ionophore antibiotic and that its mode of action is different from those of such known ionophores as valinomycin (20), macrolide actin groups (11), and other ionophore antibiotics (5, 6, 23, 30).

LITERATURE CITED