Effect of Ketoconazole in Combination with Other Inhibitors of Sterol Synthesis on Fungal Growth

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Received 6 May 1985/Accepted 25 July 1985

The effect of combinations of ketoconazole with other sterol synthesis inhibitors on fungal growth was tested against a variety of fungi selected for resistance to ketoconazole. All of the sterol inhibitors, at concentrations lower than their MICs, caused an increase greater than fourfold in the ketoconazole susceptibility of some fungi. Some of the sterol synthesis inhibitors showed this effect with ketoconazole at levels that may be achieved clinically.

Sterols and the sterol biosynthetic pathways are major targets for antifungal drugs. Amphotericin B and other polyenes selectively damage membranes that contain ergosterol, the major fungal sterol (5). The imidazole antifungycotics inhibit fungal growth by preventing 14-demethylation of lanosterol and effectively block synthesis of ergosterol (6, 11), although some of these agents at higher concentrations destroy membranes directly, causing rapid killing (10).

In our search for improved antifungal drugs or drug combinations, we examined the inhibition of fungal growth by pairs of drugs that inhibit sterol synthesis. Each pair contained ketoconazole. After preliminary experiments with 15 known sterol inhibitors, we chose 4 to examine in combination with ketoconazole: naftifine, an allylamine derivative that inhibits squalene epoxidase (7); 15-azasterol, which inhibits the reduction of the 14(15) double bond in the sterol nucleus (12); triarimol, an inhibitor of 14-demethylation (8); and mevinolin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (1).

A variety of fungal pathogens, including yeast and filamentous fungi, were used. The organisms chosen were selected in part for their relative resistance to ketoconazole. Carefully standardized methods of determining MICs were adopted to ensure comparable data in the various experiments.

The aims of the study were to determine whether such combinations have clinical potential and to understand better the biosynthesis and the role of sterols in fungal membranes.

MATERIALS AND METHODS

All fungi tested were originally isolated from clinical sources, with the exception of Rhizopus rhizopodiformis, which was obtained from the American Type Culture Collection (Rockville, Md.). Candida albicans VA was susceptible to ketoconazole. The other organisms selected for study were relatively resistant to ketoconazole. C. albicans 7.22 and Candida tropicalis were obtained from Smith Shadomy (Medical College of Virginia, Richmond, Va.). C. albicans 7.22 was originally isolated by Kirkpatrick et al. (4) and studied by Ryley et al. (9). Torulopsis glabrata was obtained from David Stevens (Santa Clara Valley Medical Center, San Jose, Calif.). Aspergillus fumigatus and Aspergillus niger were obtained from Michael Lew (Childrens' Hospital Medical Center, Boston, Mass.). The organisms were maintained by subculture on Sabouraud dextrose agar.

The sterol inhibitors used in this study were ketoconazole (Janssen R&D Inc., New Brunswick, N.J.), naftifine hydrochloride (Allergan Pharmaceuticals, Irvine, Calif.), mevinolin (Merck Sharp & Dohme, Rahway, N.J.), and triarimol and 15-azasterol (both from Eli Lilly & Co., Indianapolis, Ind.). A stock solution of mevinolin was prepared as described previously (1). Stock solutions of the remaining chemicals were prepared in dimethyl sulfoxide.

All susceptibility tests were performed in the defined synthetic medium of Hoeprich and Finn (3), which is well buffered near the physiological range, pH 7.2 to 7.4. Yeast inocula were prepared from 18- to 24-h cultures in a liquid medium by dilution in sterile physiological saline. These were adjusted to a turbidity reading of 90% transmission at 540 nm. A further one- to fourfold dilution was made in saline, and this suspension was used for inoculation, giving a final inoculum of about 5 x 10^3 CFU/ml. For mycelial fungi, 24- to 48-h cultures in a liquid medium were vigorously vortexed with glass beads (2 mm in diameter), and the suspension was passed through a pad of sterile glass wool. The homogeneously turbid effluent was used for inoculation after adjustment of transmission and further dilution as described above. Tubes were incubated at 30°C for 48 h.

MICs of each drug were first determined by twofold dilutions of the drug. For drug interaction studies, ketoconazole was tested separately with each of the remaining drugs by a checkerboard technique, using twofold dilutions of both drugs. The MIC, determined visually, was the lowest drug concentration that resulted in a sharp decrease in growth. In tubes where fungal growth was diffuse, we compared this method of reading the MICs with that reported by Galgiani and Stevens (2) where the IC_{1/2} (50% inhibition of growth) is determined spectrophotometrically. There was excellent correlation between the two methods. All results herein are reported in terms of visual endpoints.

RESULTS

Table 1 shows the susceptibilities of the tested fungi to the sterol synthesis inhibitors used in this study. C. albicans VA was susceptible to ketoconazole. The other yeasts were from 16- to 64-fold more resistant to the drug. The filamentous fungi were also quite resistant to ketoconazole. As yet there is no standardized method for testing fungal susceptibility to...
ketoconazole and relating it to clinical susceptibility of an infecting organism to ketoconazole treatment.

None of the other sterol synthesis inhibitors showed low MICs for any of the fungi. Azasterol was the most potent of the four agents. Naftifine had a much lower MIC for the Aspergillus species than for any of the other fungi. Triarimol and mevinolin had MICs of 6.25 μg/ml or higher for all the organisms.

The relevant data from the two drug checkerboard experiments are shown in Table 2. We defined a significant result with a drug combination when a concentration of the second inhibitor below its MIC caused at least a fourfold decrease in the MIC of ketoconazole for that fungus.

With mevinolin, significant drug interactions as defined above were seen with C. albicans VA and the four filamentous fungi (Table 2). The naftifine-ketoconazole combination was effective with three filamentous fungi, especially with A. niger, for which a 32-fold decrease in the MIC of ketoconazole was seen. A second A. niger isolate from a different source was examined, and a similar dramatic drug interaction was observed (data not shown). Triarimol decreased the MICs of ketoconazole by 16- to 32-fold in the case of A. fumigatus, A. niger, and the Rhizopus isolate. The azasterol-ketoconazole combination was effective in six of the eight fungi tested, especially with T. glabrata and A. fumigatus 173. In general, yeasts were found to be less susceptible to drug combinations than were filamentous fungi.

### DISCUSSION

There is a pressing need for improvement in therapy for fungal infections. Amphotericin B is often an effective agent but is potentially a very toxic agent. Ketoconazole is often excellent for treatment of infection caused by susceptible fungi, but many pathogens are resistant to the imidazole. In the studies reported here we examined the hypothesis that other sterol synthesis inhibitors may significantly decrease ketoconazole requirement. This hypothesis was based in part on the synergy shown by two inhibitors in the folic acid pathway, sulfamethoxazole and trimethoprim, against many bacteria.

Since we have clinical experience only with ketoconazole among the drugs tested, the data were expressed as the increased susceptibility of the fungi to ketoconazole in the presence of the second agent. A fourfold or greater increase in susceptibility to ketoconazole in the presence of concentrations of the other agents below their MICs was seen with many of the fungi and all of the combinations.

Do any of these combinations have clinical potential? This cannot be answered with certainty since we do not know the blood levels of the other sterol synthesis inhibitors that can be achieved, their effect on fungi in vivo, or their toxicities in humans. If we make the conservative assumption that levels of 1 μg/ml are achievable in serum, then some of the pairs have potential significance against several of the fungi. Mevinolin sensitizes A. fumigatus and A. niger to keto-
azole. Naftifine dramatically sensitizes *A. niger* to ketoconazole. Azasterol shows azole.

Studies in animals and humans must ultimately support suggestions of clinically relevant interactions from in vivo studies.

Would combinations of sterol synthesis inhibitors be too toxic to use in humans? Assuming no action other than the effect on sterol synthesis, it is unlikely that, at least with short-term treatment, humans would suffer toxicities from even profound inhibition of sterol synthesis. Unlike the fungi, humans can satisfy major cholesterol requirements from dietary sources and thus bypass a drug-induced block in cholesterol synthesis.

Based on work from our laboratory, we suggest that ketoconazole resistance depends on how well the organisms adjust to ergosterol depletion and to elevated levels of precursor sterols. With our present understanding there is no way to predict the effect an additional block of sterol synthesis at another site would have on a particular fungus and thus there will likely be no shortcut for testing the different combinations against ketoconazole-resistant organisms. The uniquely effective combinations observed, such as ketoconazole and naftifine versus *A. niger* or ketoconazole and azasterol versus *C. albicans* 7.22, the yeast with exceptionally high ketoconazole resistance, could not have been predicted.

ACKNOWLEDGMENTS

This work was supported by the Veterans Administration and Public Health Service grant AI-19931 from the National Institutes of Health.

LITERATURE CITED