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In Vitro Activity of SCH-56592 and Comparison with Activities of Amphotericin B and Itraconazole against *Aspergillus* spp.

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In this study, we investigated the in vitro activity of SCH-56592 (SCH), a new triazole antifungal agent. We compared the activity of SCH with those of itraconazole (ITZ) and amphotericin B (AB) against 60 clinical isolates of *Aspergillus* spp. by using a microtiter format. Incubation was done at 37°C for 48 h, and MIC endpoints (no growth) were read visually. The medium used for all of the drugs was RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS) and supplemented with 2% glucose. MICs and minimum fungicidal concentrations (MFCs; killing of ≥99.99%) were measured for all isolates. The geometric mean (GM) MICs and ranges (in micrograms per milliliter) were as follows: SCH, 0.09 and ≤0.01 to 1; ITZ, 0.25 and 0.06 to 32; AB, 1.46 and 0.25 to 32. *Aspergillus terreus* ($n = 7$) was markedly more susceptible to SCH (GM, 0.05 μg/ml) and ITZ (GM, 0.07 μg/ml) than to AB (GM, 8.8 μg/ml). For all isolates, the GM MFCs and ranges (in micrograms per milliliter) were as follows: SCH, 3.64 and 0.125 to 16; ITZ, 15.09 and 0.125 to 32; AB, 10.3 and 1 to 32. In the drug concentration range tested, 71, 32, and 64% of the isolates against which SCH, ITZ, and AB, respectively, were tested were killed. A reproducibility study was performed with 20% of the isolates; for 11 of the 12 isolates retested, the MIC was the same or within 1 well of the original MIC of each drug. Therefore, in vitro mould testing of SCH is feasible and reproducible. SCH was found to be very active against all species of *Aspergillus* and at lower concentrations than either ITZ or AB.

In recent years, the incidence of serious fungal infection has been shown to be increasing (9). This is due to a number of factors, including the rising incidence of AIDS (2) and the increase in the types and numbers of organ transplants with consequent use of immunosuppressant chemotherapy. The increase in invasive aspergillosis has led to a greater need for antifungal agents active against *Aspergillus* spp. to combat these infections. Invasive aspergillosis is particularly problematic, as response rates to amphotericin B (AB) are poor (1). The only efficacious systemically available azole for aspergillosis is itraconazole (ITZ), but responses to this drug are variable, especially in AIDS patients, in whom ITZ concentrations in serum can be very low (4).

SCH-56592 (SCH) is a new triazole antifungal agent (Fig. 1) with broad-spectrum activity against the majority of fungal pathogens, including *Candida*, *Aspergillus*, and *Cryptococcus* spp. In this study, we tested the in vitro activity of SCH against a range of *Aspergillus* species and compared it directly with those of AB and ITZ. We used a microtiter method that reliably detects resistance to ITZ (5, 13) and are optimistic that the same method will do so for SCH in the future.

MATERIALS AND METHODS

Strains. The 60 *Aspergillus* isolates tested were from clinical sources and included 39 isolates of *Aspergillus fumigatus*, 7 of *A. terreus*, 7 of *A. flavus*, and 7 of *A. niger*. The organisms were grown on Sabouraud dextrose agar (Lab M, Bury, United Kingdom [UK]) at 30°C for 2 to 3 days or until sufficient conidia had formed. Each isolate was simultaneously tested against all three drugs.

Antifungal agents. SCH (Schering-Plough Research Institute, Bloomfield, N.J.) was supplied as pure drug powder and dissolved in dimethyl sulfoxide (Sigma, Dorset, UK) to produce a stock solution of 1,280 μg/ml. The formula of SCH is $C_{37}H_{42}F_2N_8O_4$; (−)-4-[4-[4-[4-[[(2R-*cis*)-5-(2,4-difluorophenyl)-tetrahydro-5-(1H-1,2,4-triazol-1-ylmethyl)furan-3-yl]methoxy]phenyl]-2,4-dihydro-2-[(S)-1-ethyl-2(S)-hydroxypropyl]-3H-1,2,4-triazol-3-one. Its molecular weight is 700.8, its melting point is 164 to 165°C, and its $[\alpha]_D^{25}$ is −29 ± 3°C (C = 1.00, $CHCl_3$).

ITZ (Janssen Research Foundation, Beerse, Belgium) was dissolved in a 1:1 solution of acetone and 0.2 M HCl. To aid solubilization, the drug was vortexed and heated in a water bath at 60°C for 1 to 2 h or until it had completely dissolved. The drug was stored at a final concentration of 3,200 μg/ml.

AB was supplied as the deoxycholate form of the drug, Fungizone (Squibb, Middlesex, UK). The drug was suspended in sterile water to produce a final concentration of 1,600 μg/ml. All drug stocks were stored in the dark in 0.5-ml aliquots at −20°C until used.

Susceptibility testing. A broth microdilution procedure was used to test all *Aspergillus* isolates. We used RPMI 1640 medium (Sigma) buffered with MOPS (morpholinepropanesulfonic acid; Sigma) and supplemented with 2% glucose for all drugs. To prepare the drug dilution series, start solutions of the drugs were made in RPMI medium and dispensed into the first well of a microtiter tray. Doubling dilutions were prepared in microtiter trays to give the following ranges of drug dilutions: SCH, 0.01 to 16 μg/ml; ITZ, 0.03 to 32 μg/ml; AB, 0.03 to 32 μg/ml. Controls included a solvent control to check the effect of solvent on the growth of the fungus, a positive control to check the viability of the conidia, and a negative control to ensure the sterility of the medium.

The inoculum was prepared by wetting a sterile loop with phosphate-buffered saline (PBS) containing 0.05% Tween 80 (PBS-Tween) and transferring a loopful of *Aspergillus* spores into sterile PBS-Tween. The conidial suspension was vortexed vigorously to prevent clumping of the spores and then counted with a hemocytometer. The conidia were then diluted to a concentration of 10^6 spores/ml by using RPMI medium. A 100-μl volume of this inoculum was added to each well of the drug dilution series, including the positive and solvent controls, so that the final volume in the microtiter plates was 200 μl and the final inoculum was 5×10^5 spores/ml. The plates were incubated in a moist tray (to avoid drying out of the plates) at 37°C for 48 h. The MIC was defined as the lowest concentration of drug to completely inhibit the growth of *Aspergillus* spp.

Reproducibility was assessed by randomly retesting 20% of the isolates.

Minimum fungicidal concentrations (MFCs). A 100-μl sample was removed from each of the wells showing no growth and the last well to show growth and transferred onto horse blood agar. The liquid was allowed to soak into the agar, and when dry, the plate was streaked with a sterile loop to separate any spores present and to remove them from the drug. The plates were then incubated at 37°C for 48 h. The MFC was defined as the lowest drug concentration to allow the growth of five or fewer colonies. This represents killing of ≥99.99% of the original inoculum.

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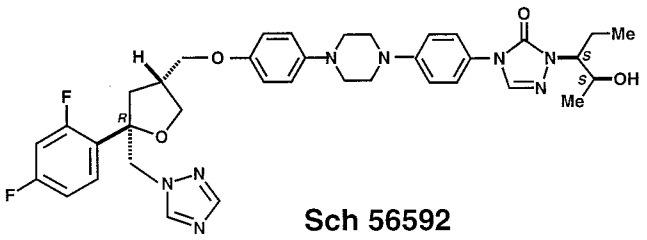

FIG. 1. Structure of SCH-56592.

Analysis of data. A one-way analysis of variance was used to compare the differences in susceptibility of the four *Aspergillus* species to each of the three drugs (SCH, ITZ, and AB), with a Bonferroni correction for multiple-comparison tests (SPSS for Windows).

RESULTS

The MICs of SCH ranged from ≤0.01 to 1 μg/ml, and the geometric mean (GM) MIC was 0.09 μg/ml. Differences in susceptibility between species were seen, with *A. terreus* ($n = 7$) being the most susceptible to SCH, whose GM MIC was 0.05 μg/ml and whose MIC range was 0.03 to 0.125 μg/ml. *A. flavus* was the least susceptible; the GM MIC was 0.22 μg/ml, and the MIC range was 0.125 to 0.5 μg/ml. *A. terreus* was significantly more susceptible than *A. flavus* ($P \leq 0.05$). The MFCs of SCH ranged from 0.125 to 16 μg/ml with a GM of 3.64 μg/ml. SCH was fungicidal for 71% of the isolates in the drug concentration range tested.

The GM MIC of ITZ was 0.25 μg/ml, and the range was 0.06 to 32 μg/ml. For three *A. fumigatus* isolates, the MICs were >16 μg/ml. *A. terreus* also appeared to be the species most susceptible to ITZ, whose GM MIC was 0.07 μg/ml and whose MIC range was 0.06 to 0.125 μg/ml. This species was significantly more susceptible than *A. fumigatus* ($P \leq 0.05$). Against *A. flavus*, the MIC range was 0.125 to 0.5 μg/ml with a GM of 0.18 μg/ml. ITZ was fungicidal for 32% of the isolates in the drug concentration range tested. The ITZ MFCs ranged from 0.125 to 32 μg/ml with a GM of 15.09 μg/ml.

The GM MIC of AB was 1.46 μg/ml, and the MIC range was 0.25 to 32 μg/ml. Differences in susceptibility were seen. For *A. terreus*, which was significantly less susceptible to AB than was any other species ($P \leq 0.05$), the AB MICs ranged from 4 to 32 μg/ml and the GM MIC was 8.8 μg/ml. For the species most susceptible to AB, *A. niger*, the GM MIC was 0.5 μg/ml and the MIC range was 0.25 to 1 μg/ml. This species was significantly more susceptible to AB than any other species ($P \leq 0.05$). Against *A. flavus*, AB MICs ranged from 2 to 4 μg/ml, with a GM MIC of 2.43 μg/ml. AB was fungicidal for 64% of the isolates in the range of drug concentrations tested. The GM MFC of AB was 10.3 μg/ml, and the MFCs ranged from 1 to 32 μg/ml. For all of the *A. terreus* isolates tested against AB, the MFCs were >16 μg/ml.

SCH was found to have the lowest MICs and MFCs (Fig. 2 and 3, respectively). The SCH MICs for the three isolates resistant to ITZ (all MICs were >16 μg/ml) were slightly raised and ranged from 0.5 to 1 μg/ml. The relatively high AB MICs seen with *A. terreus* were not found with SCH or ITZ.

Reproducibility was assessed by retesting a random sample of 12 isolates against all three of the agents tested. The MICs were reproducible; the MICs for 11 of the 12 isolates were the same result or within 1 well of the original MIC.

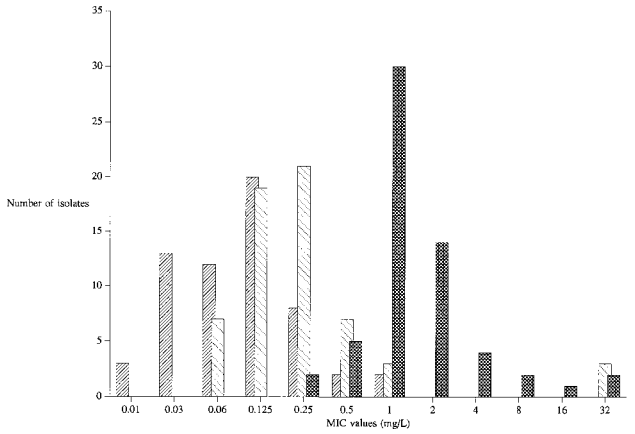

FIG. 2. MICs of SCH (▨), compared with those of AB (▩) and ITZ (▧), for 60 *Aspergillus* isolates.

DISCUSSION

Only two agents are currently available for the treatment of invasive aspergillosis—AB and ITZ. Response rates to either drug are suboptimal, with mortality rates in highly immunocompromised patients, such as bone marrow transplant recipients, of about 90% (1). Immunocompromised patients with cerebral aspergillosis also fare badly (≈99% mortality). Thus, there is a need for new and better anti-*Aspergillus* antifungal agents.

SCH is a new azole antifungal agent with a broad spectrum of action which includes all *Candida* (8, 12) and *Cryptococcus* (8) species and some newer invasive fungal pathogens. Our data is consistent with good anti-*Aspergillus* activity in that GM MICs of SCH were about 3-fold lower than those of ITZ and 20-fold lower than those of AB. In addition, SCH was fungicidal at the exceptionally stringent criterion of 99.99% for 71% of the isolates tested. It was fungicidal substantially more often than ITZ (32%). In vivo work with *Aspergillus* also confirmed its in vitro activity (11).

No standards exist for in vitro testing of *Aspergillus* spp. or any other moulds, and the methodology used in previous work is highly variable (3). Recent attempts to introduce standards for filamentous fungi have used adaptations of the proposed reference method of the National Committee for Clinical Lab-

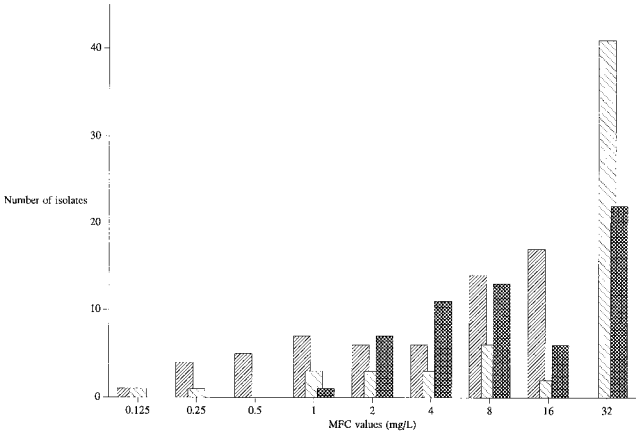

FIG. 3. MFCs of SCH (▨) compared with those of AB (▩) and ITZ (▧), for 60 *Aspergillus* isolates.

oratory Standards for yeast. Progress has been made for AB in yielding reproducible results (7), and in a more recent evaluation an improvement in interlaboratory agreement was seen with both AB and ITZ (6). We have recently validated microtiter and agar dilution testing methods for in vitro susceptibility testing of ITZ by using an animal model and susceptible and resistant isolates of *A. fumigatus* (5, 13). In the present study, we used this microtiter methodology for all three drugs. We included three ITZ-resistant isolates in the study, and it was interesting that SCH was active against these, although less so than against the generally susceptible isolates. Whether SCH is clinically active against these isolates remains to be seen.

It is encouraging that SCH is frequently fungicidal. *A. fumigatus* has a doubling time of 48 min in the presence of hydrocortisone in vitro (10). Primary defenses against hyphal invasion are neutrophils (15) and monocytes (14), which are absent in neutropenic patients and functionally impaired in corticosteroid-treated and AIDS patients. In addition, pharmacological concentrations of hydrocortisone increase the growth rate of *A. fumigatus* (and *A. flavus*) (10). It is therefore hardly surprising that invasive aspergillosis is a rapidly fatal disease in highly immunocompromised patients. It may therefore be valuable clinically to use antifungal agents that are fungicidal in invasive aspergillosis, although this is not proven.

SCH is a valuable new anti-*Aspergillus* agent based on its in vitro activity and efficacy in animal models. Whether it will be useful clinically depends on many factors, including its pharmacokinetic profile and its toxicity potential. Phase II clinical work is certainly indicated on the basis of its in vitro activity.

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