Selection of Orally Active Antifungal Agents from 3,5-Substituted Isoxazolidine Derivatives Based on Acute Efficacy-Safety Profiles

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Routine in vitro screening of a new synthetic series of 3,5-substituted 2-methylisoxazolines revealed that three imidazole analogs (PR 967-248, PR 967-234, and PR 969-566) and, to a lesser extent, a triazole analog (PR 988-399) exerted rather potent antifungal activity against three systemic and four dermatophytic classes of fungi. When tested in vivo for ability to eradicate Candida vaginitis in the rat, the triazole derivative, PR 988-399, was effective after oral administration. In this in vivo test for efficacy, PR 967-234 and PR 969-566 reduced but did not eradicate the infection, while PR 967-248 was inactive. PR 988-399 was, moreover, 4- to 13-fold less potent than the three imidazoles in inhibiting testosterone synthesis in isolated rat Leydig cells. After oral or intravenous administration, PR 988-399 and PR 969-566 elicited the fewest cardiovascular and behavioral side effects in conscious dogs. The rat safety study consisted of oral dosing followed by evaluation of the exploratory motor activity of the naive animals in a novel environment. Motor activity was suppressed least by PR 988-399 and most by PR 969-566. In a battery of mouse behavioral-neuromuscular-drug interaction tests, PR 988-399 and PR 969-566 produced the fewest central-behavioral-neuromuscular signs. These efficacy-safety evaluations were performed with ketoconazole as a positive reference standard. The sequence of drug testing with respect to efficacy-safety considerations appears to be a suitable approach for early detection of orally active antifungal agents such as PR 988-399 for more advanced development.

The incidence of systemic fungal infections, such as those caused by Candida albicans, has been on the rise. The most likely explanation is the increasing number of immunocompromised patients (13, 14, 20, 30) as well as, for example, a recently reported syndrome of C. albicans sepsicemia in heroin addicts (13). Major problems with earlier antifungal therapies were the requirement for parenteral administration and untoward effects. Not until the advent of theazole derivative ketoconazole in the 1970s were orally effective agents available (5, 13, 14, 19). Ketoconazole and other developed azoles were not without their respective liabilities (13, 14, 32, 34, 36). Therefore, a continuing need exists to develop newer agents which possess the following properties: novel structures, fungicidal activity, highly specific or broad-spectrum profiles of antifungal activity, orally active, safer, better tissue distribution (especially penetrability in the cerebrospinal fluid, body fluids, and urinary tract), longer duration of action, less propensity to induce liver enzymes, limited or no action on steroidogenesis and associated endocrine organs, and novel sites of action (14, 30; also see preceding reviews [5, 13, 14, 19, 32, 36] for discussion). New azole derivatives and triazole antifungal agents have been synthesized, resulting in advanced clinical trials for the promising agents itraconazole and fluconazole (13, 32).

We have developed four new synthetic series of 3,5-substituted 2-methylisoxazolines which proved active when screened for antifungal efficacy under in vitro conditions (for syntheses, see references 4, 10, 22, and 23). However, since in vitro activity does not consistently correlate with in vivo efficacy (notably with testing for fluconazole) (32), we developed a rational approach for early identification of promising orally active compounds based on acute in vitro-in vivo efficacy-safety profiles. For in vivo efficacy, the rat model of Candida vaginitis was employed (1). Safety considerations were based on endocrine (inhibition of testosterone synthesis in rat Leydig cells), central nervous system (dog, rat, and mouse models), and cardiovascular (dog) paradigms. In this manner, we report findings for the four most promising compounds from the separate chemical series, namely, PR 988-399, PR 969-566, PR 967-248, and PR 967-234 (see structures in Fig. 1). Ketoconazole was used as a positive reference standard. The testing sequence identified PR 988-399 as the compound with the highest potential for further extensive development.

MATERIALS AND METHODS

Compounds. The chemical names of the antifungal compounds used in the present investigation are listed below. The syntheses have been recently published (4, 10, 22, 23). Refer to Fig. 1 for appropriate structures. The free base derivatives of the Fisons compounds were prepared. The chemical names are as follows: (i) PR 988-399FB (4), cis-1-[3,5-bis(4-chlorophenyl)-2-methyl-3-isoxazolidinyl]methyl]-1H-1,2,4-triazole; (ii) PR 969-566FB (10), cis-3-(4-chlorophenyl)-3-[1H-imidazol-1-yl]-2-methyl-5-[(4-methylphenyl)thio]methyl]isoxazolidine; (iii) PR 967-248FB (23), cis-5-[[4-(chlorophenoxy)methyl]-3-(4-chlorophenyl)-3(1H-imidazol-1-yl)-methyl]-2-methylisoxazolidine; (iv) PR 967-234FB (the imidazole analog of PR 988-399FB) (22), cis-3,5-bis(4-chlorophenyl)-3-[1H-imidazol-1-yl]-2-methylisoxazolidine; (v) ketoconazole, cis-1-acetyl-4-[2-(2,4-dichlorophenyl)-2(1H-imidazol-1-yl)methyl]-1,3-dioxolan-4-yl)methoxy]phenyl]piperazine.

In vitro antifungal activity. The lowest drug concentration
in vitro at which fungal growth was arrested (MIC) was determined by using a solid agar technique as described by McGinnis (18). All the fungi were grown on antibiotic-testing medium no. 3 (Difco Laboratories, Detroit, Mich.). All tests were performed in triplicate, and incubations were conducted at 30°C. The test compounds were initially dissolved in 100% dimethyl sulfoxide, whereas ketoconazole was dissolved in 0.2 N HCl. Portions (3 ml) of various drug concentrations were added to 27 ml of warm liquid medium 3, yielding a final concentration range from 0.2 to 70 μg/ml. Final solvent concentrations did not exceed 0.03%.

The cultured organisms from the American Type Culture Collection (Rockville, Md.) included \textit{C. albicans} ATCC 10259, \textit{Candida stellatoidea} ATCC 36232, \textit{Aspergillus fumigatus} ATCC 28212, \textit{Trichophyton mentagrophytes} ATCC 9533, \textit{Trichophyton rubrum} ATCC 18762, \textit{Microsporum audouinii} ATCC 14057, and \textit{Epidermophyton floccosum} ATCC 18397.

For determination of MICs for the \textit{Candida} species, a vial of the appropriate strain was grown for 48 h on Sabouraud dextrose agar (SDA) (Difco), and then the strain was suspended in sterile distilled water and visually enumerated on a hemacytometer and adjusted to a final concentration of 10,000 cells (CFU) per ml. After solidification, all test plates (including the appropriate solvent controls) were inoculated with 0.05 ml of the \textit{Candida} suspension and left undisturbed until the inoculum had been absorbed into the SDA. The plates were then incubated for 48 h.

For the mold species, the technique was performed essentially as just described. If the mold was not a good spore-former, the mycelia were carefully scraped from the surface of the plate, ground up with a tissue grinder, and adjusted to an optical density of 0.5 to 0.6 at 450 nm. After solidification, all test plates were inoculated with 0.05 ml of the fungal suspension and left undisturbed until the inoculum had been absorbed into the SDA. The plates were then incubated until visible growth appeared in the drug-free control.

The MICs of individual antifungal compounds against specific fungal strains were assigned rank orders of potency according to the following criteria: rank 1 = >70 μg/ml, rank 2 = 20 μg/ml, rank 3 = 7 μg/ml, rank 4 = 2 μg/ml, rank 5 = 0.7 μg/ml, rank 6 = 0.2 μg/ml, and rank 7 = <0.2 μg/ml. These MIC ranks were compared among the different antifungal compounds by Friedman’s test as described in the BMDP statistical manual (2), using the BMDP-3S program (nonparametric statistics).

\textbf{In vivo rat vaginal candidiasis.} The in vivo rat candidiasis studies utilized a modification (1) of the method described by Polak (27). Ovariectomized female Sprague-Dawley rats, 150 to 175 g (Harlan Laboratories, Inc., Indianapolis, Ind.), were maintained on a diet of laboratory rat chow and water ad libitum. A 0.1-ml subcutaneous injection of estradiol valerate (0.5 mg of Delestrogen; E. R. Squibb & Sons, Princeton, N.J.) was given 4 days prior to infection to induce pseudoestrus. Animals were maintained in pseudoestrus by weekly injections of 0.5 mg of estradiol valerate.

The appropriate clinical \textit{C. albicans} isolate was initially cultured on SDA. Isolated colonies were picked from the surface of the plate, placed in 50 ml of Sabouraud dextrose broth (Difco), and allowed to grow for 22 h on a clinical rotator at room temperature. After 22 h of incubation, the broth appeared to be turbid, indicating active microbial growth. Aliquots (1 ml) of this stock broth culture were frozen at -20°C until further use.

To prepare sufficient numbers of organisms to induce vaginal candidiasis, we placed a 1-ml aliquot of the specific clinical isolate of \textit{C. albicans} in a 50-ml flask containing 10 ml of 1% Phytohe-peptone broth (BBL Microbiology Systems, Cockeysville, Md.) and 1% glucose. The flask were incubated on a clinical rotator for 36 h at room temperature. At the end of the incubation period, the contents were centrifuged at room temperature at 500 × g for 10 min. The supernatant was removed, and the pellet was suspended in RPMI 1640 medium containing 100 U of penicillin G per ml and 100 μg of streptomycin per ml, all purchased from Sigma Chemical Co. (St. Louis, Mo.). The suspended yeasts were enumerated by hemacytometer counting and diluted to a concentration of 10^7 organisms per ml.

Four days after the first estradiol injection, the animals were tranquilized by an intraperitoneal injection of 10 mg of ketamine hydrochloride (Vetalar; Parke-Davis, Morris Plains, N.J.). Each animal was suspended head down, and a dry cotton swab was inserted into the vagina to dilute it. Following this dilation, 0.1 ml of the \textit{Candida} inoculum (10^7 CFU) was inserted into the vagina with a 1-ml tuberculin syringe with a 1-cm length of butterfly tubing attached.

To sample repetitively each vagina for recoverable \textit{Candida} organisms, we first anesthetized the animal with ketamine as described above. Then a sterile swab moistened with sterile phosphate-buffered saline was inserted into the vagina and twisted several times before removal. The swab was then swirled in 0.3 ml of sterile phosphate-buffered saline. The resultant suspension of vaginal lavage fluid was then logarithmically diluted, and a 0.1-ml sample was placed on an SDA plate which was prepared 72 h earlier and dried at room temperature. The lavage fluid was spread evenly...
over the SDA plate with an alcohol-flamed bent glass rod until the plate was dry. All plates so processed were then incubated at 24 to 26°C for 48 h. Following this incubation, those plates with 15 to 150 C. albicans colonies were counted.

All test drugs were suspended in 1% Clearjel and orally administered twice daily at 20 mg/kg. Drug dosing regimens were initiated no earlier than 24 h after establishment of vaginal candidiasis infection.

To evaluate fungal virulence, persistence, and reproducibility across different studies, as well as to compare antifungal activity between antifungal compounds, a within-subject, repeated-measures analysis of variance (ANOVA) was performed. When the slope of the time-response curves for two given drug treatments were not different (treatment day interaction was not significant), either Newman-Keuls or pairwise ANOVA tests were used to compare relative effects of specific drug and dose treatments across days. When the slopes of the time-response curves for any two drug treatments were different (significant interactions among drug or doses across days), Newman-Keuls tests were performed for each day individually with no attempt to analyze drug effects over time.

In vitro inhibition of testosterone synthesis in rat Leydig cell suspensions. Leydig cells were isolated and prepared from analyze drug effects over time.

The concentration of antifungal agent required to inhibit in vitro testosterone synthesis by 50% (50% inhibitory concentration) was obtained by combining the data from five separate experiments, each with at least seven concentrations (0.5 M increments from 10⁻¹⁴ to 10⁻⁹ M, obtained by pretesting the drugs at log increments) of drugs, and by using the ALL-FIT analysis, an iterative curve-fitting program for families of dose-response curves based on a four-parameter logistic equation (11). Best fit was achieved when curves were constrained such that all a parameters were shared (i.e., drug responses at zero dose) and all b parameters were equal to 100% (i.e., 100% inhibition at infinite dose).

Mouse screen for acute pharmacological, toxicological, and behavioral signs. Male CF-1 mice (obtained from Harlan Laboratories and weighing 20 to 30 g) were housed for at least 5 days prior to initiation of experimentation under conditions of a 12-h light-dark cycle, at a temperature of 70 to 74°F and at a relative humidity of 30 to 50%. Standard food (Purina Rodent Lab Chow 5001) and water were available ad libitum until the time of drug testing. Prior to initiation of testing, any mice exhibiting abnormal behavior, body weight loss, or signs of ill health were discarded.

Separate groups of mice were examined at various times after oral administration of graded doses of antifungal agents. The screening technique is an examination of toxic, chemical, and behavioral signs and reflects acute drug actions with regard to bioactivity or toxicity (6, 16). In this test, five groups of three mice each were treated with different doses of test compound. A sixth group received the vehicle and served as a control. The groups were evaluated for 27 physical signs including mortality. Observations were made immediately after oral dosing and at 0.5, 3, and 24 h thereafter. For each individual test, the initial dose tested was 400 mg/kg and additional doses were routinely graded downward.

Neural impairment tests. The neural impairment screens were conducted in three separate but neurologically related tests: (i) the inverted screen, a measure of vestibuloneuromuscular coordination (7), (ii) the inclined screen, principally a measure of neuromuscular coordination (9), and (iii) the wire maneuver, which assesses limb reflex involving forelimb and hindlimb grip strength (9). Mice were dosed orally with at least five concentrations of antifungal agents, selected over a wide dosing range (10 mice per dose, initial dose was 400 mg/kg). At 1 h postdose, testing commenced. Because the three tests were neurologically related in terms of overall central effects and it was apparent that the drugs tested did not produce dose-related decrements in performance, the statistical comparisons were made with two doses, namely, 600 and 900 mg/kg. Thus, chi-square comparisons were made between control scores for the five drug treatments and between ketoconazole and the four Fisons compounds combined across three separate tests for neural impairment. In each of the three tests, the frequency of subjects from a total number of each control, drug, and dose group represented a measure of the degree of impairment produced by the five drugs.

LD₉₀. Acute oral doses as high as 1,200 mg/kg were administered to mice (10 per dose) which were subsequently monitored for death over a 24-h period. The intravenous lethal dose (LD₉₀) was obtained by injecting the two water soluble compounds (PR 967-234 and ketoconazole) into the tail vein and monitoring the mice for death over a 24-h period. Ten animals per dose were used in the study, with six to seven doses used per drug.

Supplementary central nervous system tests in mice. In the
initial testing for behavioral signs and neural impairment, the compounds under investigation revealed nonquantifiable central actions. A battery of supplemental tests was used to determine more specifically the nature of the central interactions and to provide clues whether these effects might be harmful or protective. The tests were initially conducted with oral administration of high initial doses (~400 mg/kg) of compounds. If a positive or negative drug action was observed, the test was repeated with at least five doses with 10 mice per dose.

(i) Anticonvulsant. Mice were dosed with antifungal compounds or vehicle, and 30 min later, separate groups (six per group) of animals were evaluated for protection against convulsions elicited by either maximal electroshock or pentyleneetetrazol according to the criteria of Porter et al. (29).

(ii) Phenyl-p-benzoquinone-induced writhing. The phenyl-p-benzoquinone-induced writhing test (25) detects compounds having possible nonopiate analgesic or nonsteroidal anti-inflammatory activity. Separate groups of mice (five per group) were treated with antifungal compounds or appropriate vehicle. At 30 min later, each mouse received 0.25 ml of 0.02% phenyl-p-benzoquinone (dissolved in ethanol) solution intraperitoneally. Mice were then placed individually into small plastic cubes and monitored for inhibition of the writhing response for the ensuing 15 min.

(iii) Oxotremorine-induced tremor and salivation. Oxotremorine is a potent parasympathomimetic agent which when administered to mice produces central symptoms of tremor, muscular rigidity, weakness, analgesia, and hypothermia. In addition, peripheral symptoms of diarrhea, salivation, and lacrimation are prominent. Thus, the test is able to differentiate central anticholinergic (antitremor) from peripheral anticholinergic (inhibition of salivation) actions. The antifungal agents or the vehicle was administered to groups of mice (six per group). At 30 min later, oxotremorine was injected intraperitoneally (1 mg/kg), and 15 min later, the degree of tremor and salivation was scored for each animal according to the criteria established by Lesley and Maxwell (17).

(iv) Tremors induced by LON 954. LON 954 [N-carbamoyl-2(2,6-dichlorophenyl)acetamide hydrochloride] produces transient tremors which are antagonized by dopamine agonists and potentiated by dopamine antagonists (8). Separate groups of mice (five per group) having fasted for 4 h were treated with antifungal compounds or the vehicle. At 30 min later, 50 mg of LON 954 per kg (made up within 1 h of use) was administered orally. The mice were then observed for occurrence and degree of tremors for the ensuing 15 min.

(v) Antihypoxia screen. Separate groups of mice were given oral doses of antifungal agents or the distilled water vehicle. At 15 min later, the mice were placed into a tubular Plexiglas container having five interconnecting compartments, which was in turn placed into a QUEUE environmental chamber maintained at 35°C. The mice were then allowed to remain in this thermally regulated chamber for 15 min prior to exposure to controlled hypoxia (96% N₂ and 4% O₂.). The hypoxic survival time was then monitored and recorded as the time to last gasp (3). Initial screening for antihypoxia actions entailed evaluation of antifungal agents at oral doses of 300 to 400 mg/kg (five per group). Based on positive results from this preliminary study, a more detailed experiment was conducted which compared the effects of orally administered antifungal compounds at two lower doses (50 and 100 mg/kg) with 10 mice per dose.

Acute exploratory motor activity in naive rats. Rats (not fasted, male, Sprague-Dawley rats from Harlan Laborato-

ries, weighing 100 to 150 g) were dosed orally with selected concentrations of either antifungal agents or vehicle. At 1 h after dosing, these naive rats were removed from a lighted laboratory environment and placed into individual activity chambers (Optovarimex, four-chamber, autotrack system; Columbus Instruments) (12). Initial exploratory activity was measured as the distance traveled (centimeters) for a 5-min period. At the end of this 5-min monitoring period, the rats were returned to their home cages and subsequently retested for 5 min at 6 and 24 h after dosing. In each series of experiments, the animals were tested in a randomized fashion and the findings were compared with those of a respective control group which received water only. The resultant data were analyzed by ANOVA and covariance with repeated measures (a BMDP-2V program) (2) which determined treatment effect, time effect, and interaction of treatment with time. This overall analysis was followed, when applicable, by the Newman-Keuls RANGE test to determine levels of significance between individual data points.

Behavioral and cardiovascular effects in conscious dogs. Following anesthesia (sodium pentobarbital, 30 mg/kg, intravenous [i.v.]) of male and female beagle dogs, an arterial catheter was implanted under aseptic conditions for the subsequent measurement of femoral arterial blood pressure. During the experimental procedure, continuous measurements were made of the electrocardiogram, heart rate, systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MAP). Pretreatment values were recorded. Antifungal agents were then administered either orally (at 50 or 100 mg/kg suspended in 4% Clearjel at a volume of 2 ml/kg) by gavage or i.v. (1, 3, 10, and 30 mg/kg). The dogs were then observed for the ensuing 6 h. In the i.v. study, dogs received incremental doses of 1, 3, 10, and 30 mg of compound per kg or vehicle (0.1 N HCl). The drug doses of 1 and 3 mg/kg and the corresponding vehicle treatments were administered as bolus injections, and each dose was followed by a 15-min observation period. The 10- and 30-mg/kg doses and corresponding vehicle treatments were infused at a rate of 2 mg/ml per min. A 30-min observation period followed the 10-mg/kg infusion, and 60 min of observation followed the highest dose.

Statistical evaluation of the results compared responses obtained between treatment groups for each route of administration. Thus, groups treated with PR 988-399FB or its vehicle were compared for differences by an ANOVA with a repeated-measures design (BMDP-2V) (2). If the response patterns were significantly different, the Newman-Keuls procedure (RANGE test) was applied to compare the two groups in terms of change from pretreatment level at each posttreatment time point.

RESULTS

In vitro antifungal activity. Table 1 contains a detailed summary of the range of the MICs (expressed as micrograms per milliliter) at which fungal growth was arrested by the reference agent ketoconazole and the four newly synthesized antifungal agents. Using Friedman's test, we compared the rank order of potencies of the MICs for the five antifungal compounds against three strains of systemic fungi and four strains of dermatophytic fungi. The rank order for inhibition of fungal growth was not significant among the four most potent compounds, namely, ketoconazole, PR 969-566, PR 967-248, and PR 967-234. The triazole derivative, PR 988-399, possessed considerably less in vitro antifungal potency (Friedman's test statistic = 13.71, P =...
0.0083) when compared against the other four compounds (statistical details are presented in Table 1, footnote b).

In the subsequent section, the test compounds were evaluated in vivo against two strains of C. albicans causing vaginal candidiasis, namely, SC9172 and FM391. The compounds were likewise tested for in vitro efficacy against these two strains. The MICs and the rank orders of potency (in parentheses) for the in vitro tests were as follows: (i) strain SC9172, ketoconazole = <0.2 μg/ml (7); PR 988-399 = 0.2 to 0.7 μg/ml (6); PR 969-566 = 0.2 to 2 μg/ml (5); PR 967-248 = 0.7 to 2 μg/ml (5); PR 967-234 = 2 to 7 μg/ml (4); and (ii) strain FM391, ketoconazole = 2 to 7 μg/ml (4); PR 988-399 = 2 to 7 μg/ml (4), PR 969-566 = 0.7 to 2 μg/ml (5), PR 967-248 = 0.7 to 2 μg/ml (5), PR 967-234 = 2 to 7 μg/ml (4). The Friedman's test statistic equaled 2.00 (P = 0.1573), indicating no significant difference in rank order of potency among the compounds for the two strains.

In vivo rat vaginal candidiasis. The in vivo rat model of vaginal candidiasis was used to determine the efficacy of the compounds. Our criterion, prior to more extensive development, for examining and screening promising compounds was a requirement that the test drug be as effective as 20 mg/kg orally as the reference agent, ketoconazole. Ovariectomized rats (eight per group) were injected with 0.5 mg of estradiol 4 days prior to vaginal infection with one of two strains of C. albicans. At 24 h postinfection, animals were administered the appropriate antifungal agent or vehicle (20 mg/kg twice a day orally). The vaginal tracts were sampled daily to determine the number of recoverable viable organisms. The virulence and persistence of strain SC9172 was comparable over days of infection to strain FM391 (a × 6 ANOVA test revealed no significant strain differences across 8 days of infection) (Fig. 2).

In strain SC9172, a (overall) 4 × 8 ANOVA revealed a significant antifungal treatment effect for PR 969-566, PR 988-399, and ketoconazole, plus significant day and treatment-by-days interaction effects. The post hoc Newman-Keuls test indicated that all treatments from days 2 to 8 (except PR 988-399 on day 2) were significantly different from controls. The individual 2 × 2 ANOVAs for comparisons of each drug-treated group with controls or comparisons of the two Fisons drugs with ketoconazole showed that PR 969-566 was not significantly different from controls but that ketoconazole was more potent than controls, PR 969-566, or PR 988-399. PR 988-399 was likewise significantly different from controls. A Newman-Keuls analysis proved that by day 2, both PR 969-566 and ketoconazole had significantly more rapid onset of antifungal activity than PR 988-399. In fact, from days 2 to 4, when ketoconazole eradicated the infection, its activity was significantly more potent than that of PR 988-399. Despite the delay in onset of antifungal activity, PR 988-399 eradicated the infection by day 8, while PR 969-566 treatment was significantly less potent throughout the course of the infection (Fig. 2, upper panel; statistical details in legend).

In testing for antifungal activity against C. albicans FM391, the overall 4 × 8 ANOVA denoted significant treatment, day, and treatment-by-day effects. The post hoc Newman-Keuls test indicated that from treatment days 2 to 8, PR 967-234, PR 988-399, and ketoconazole differed significantly from controls. PR 967-248 was active to a significant degree at day 2 only. The 2 × 2 ANOVA for comparisons of individual drug groups against controls or for comparison of ketoconazole with the Fisons compounds pointed out that, with the exception of PR 967-248, the efficacy of all agents tested against this Candida strain was significant when compared with controls. Moreover, ketoconazole again displayed greater efficacy over the time course of the infection (significant compared with the three Fisons compounds). Ketoconazole had a significantly faster (Newman-Keuls, P < 0.05) onset of antifungal activity from days 2 to 4 than did PR 988-399. However, by day 8, eradication of the disease was evident with both PR 988-399 and ketoconazole (Fig. 2, lower panel; statistical analysis in legend). PR 967-234 did not completely eliminate the infection.

In vitro inhibition of testosterone synthesis in rat Leydig cell suspensions. When a comparison was made among the respective concentrations for 50% inhibition of testosterone synthesis in isolated rat Leydig cells, PR 988-399 was significantly less potent (P < 0.05) than ketoconazole, PR 969-566, PR 967-248, and PR 967-234. These four compounds did not differ significantly in potency among one another (Table 2).

Behavioral signs in mice after oral dosing. Table 3 summarizes and compares behavioral signs exhibited by the four Fisons antifungal agents with those of the reference agent, ketoconazole. Ketoconazole was essentially devoid of behavioral effects until high oral doses of 200 and 400 mg/kg were administered, when symptoms of physical depression and hypothermia occurred. Mice receiving PR 988-399 and especially PR 969-566 were essentially devoid of behavioral signs.

The most prominent and severe symptoms following oral administration were seen in mice receiving doses of 200 and

### Table 1. Statistical comparison of in vitro antifungal MICs against candida, aspergillus, and dermatophyte strains

<table>
<thead>
<tr>
<th>Fungal type</th>
<th>Ketoconazole</th>
<th>PR 988-399</th>
<th>PR 969-566</th>
<th>PR 967-248</th>
<th>PR 967-234</th>
</tr>
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<tr>
<td><strong>Systemic</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Candida albicans</td>
<td>7 (3)</td>
<td>7 (3)</td>
<td>2 (4)</td>
<td>0.7 (5)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Candida stellatoidea</td>
<td>2 (4)</td>
<td>7 (3)</td>
<td>&lt;0.2 (7)</td>
<td>&lt;0.2 (7)</td>
<td>0.7 (5)</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>2 (4)</td>
<td>&gt;70 (1)</td>
<td>2 (4)</td>
<td>20 (2)</td>
<td>2 (4)</td>
</tr>
<tr>
<td><strong>Dermatophytic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trichophyton mentagrophytes</td>
<td>0.2 (6)</td>
<td>7 (3)</td>
<td>0.2 (6)</td>
<td>0.7 (5)</td>
<td>&lt;0.2 (7)</td>
</tr>
<tr>
<td>Trichophyton rubrum</td>
<td>0.2 (6)</td>
<td>0.7 (5)</td>
<td>0.2 (6)</td>
<td>0.6 (2)</td>
<td>0.6 (2)</td>
</tr>
<tr>
<td>Microsporum audouini</td>
<td>20 (2)</td>
<td>2 (4)</td>
<td>0.2 (6)</td>
<td>0.7 (5)</td>
<td></td>
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<tr>
<td>Epidermophyton floccosum</td>
<td>&lt;0.2 (7)</td>
<td>0.7 (5)</td>
<td>&lt;0.2 (7)</td>
<td>&lt;0.2 (7)</td>
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</tr>
</tbody>
</table>

* MICs for antifungal activity of five compounds against seven strains were determined with six dilutions (70, 20, 7, 2, 0.7, and 0.2 μg/ml).

* MIC rank: 1, >70 μg/ml; 2, 20 μg/ml; 3, 7 μg/ml; 4, 2 μg/ml; 5, 0.7 μg/ml; 6, 0.2 μg/ml; 7, <0.2 μg/ml. The MIC rankes were analyzed by Friedman's test as described in the BMDF-2V program. The computer rank sums were as follows: ketoconazole = 21.5, PR 988-399 = 7.5, PR 969-566 = 24.5, PR 967-248 = 25.5, and PR 967-234 = 26.0. Assuming a chi-square distribution with 4 df, the Friedman test statistic = 13.71, P = 0.0003, and the Kendall coefficient of concordance = 0.49. Therefore, PR 988-399 possessed significantly weaker in vitro potency than the other compounds.
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FIG. 2. Efficacy of orally administered ketoconazole (Keto), PR 988-399 (399), PR 969-566 (566), PR 967-248 (248), or PR 967-234 (234) against two strains of C. albicans (SC9172 and FM391) with the rat model of vaginal candidiasis. Drugs were administered at 20 mg/kg twice a day for 7 days beginning at 24 h postinfection. Vaginal tracts were sampled daily to determine the number of recoverable viable organisms (eight animals per experimental condition). The statistical evaluation of the data is as follows. (Upper panel) In the overall 4 × 8 ANOVA: treatment effect, F (3 of 22) = 13.9; P = 0.0001; day effect, F (5 of 110) = 40.3; P = 0.0001; Treatment by days, F (15 of 110) = 11.5; P = 0.001. The post hoc Newman-Keuls test revealed that all drug-treated groups from day 2 to 8 were significantly different (P < 0.05) from controls, except PR 988-399 at day 2. The 2 × 2 ANOVAs for comparisons of drug treatments with controls or for comparisons of Fisons compounds versus ketoconazole are as follows: control versus Keto, F (1 of 8) = 176.9; P < 0.001; control versus 566, F (1 of 8) = 4.5; P = 0.07; control versus 399, F (1 of 8) = 17.0; P = 0.003; Keto versus 566, F (1 of 14) = 20.0; P < 0.001; Keto versus 399: F (1 of 14) = 17.3; P = 0.001. (Lower panel) In the overall 4 × 8 ANOVA: treatment effect, F (4 of 35) = 67.2; P = 0.0001; day effect, F (5 of 175) = 101.0; P = 0.0001; treatment by days, F (20 of 175) = 16.7, P = 0.0001. The post hoc Newman-Keuls test revealed that all drug-treated groups from days 2 to 8 for PR 967-234, PR 988-399, and ketoconazole were significantly different (P < 0.05) from controls. Only day 2 was significantly different from control for PR 967-248. The 2 × 2 ANOVAs for comparisons of drug treatments with controls or for comparisons of Fisons compounds versus ketoconazole are as follows: control versus Keto, F (1 of 14) = 838; P < 0.001; control versus 248, F (1 of 14) = 1.2; P is not significant; control versus 234; F (1 of 14) = 18.7; P < 0.001; control versus 399, F (1 of 14) = 174; P < 0.001; Keto versus 248, F (1 of 14) = 183; P < 0.001; Keto versus 234, F (1 of 14) = 65; P < 0.001; Keto versus 399, F (1 of 14) = 14.4; P = 0.002.

TABLE 2. In vitro inhibition of testosterone synthesis in rat Leydig cell suspensions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antifungal IC₅₀ (µg/ml) (mean ± SE)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole</td>
<td>0.4 ± 0.13</td>
<td>5</td>
</tr>
<tr>
<td>PR 988-399</td>
<td>2.7 ± 0.82</td>
<td>5</td>
</tr>
<tr>
<td>PR 969-566</td>
<td>0.2 ± 0.08</td>
<td>5</td>
</tr>
<tr>
<td>PR 967-248</td>
<td>0.3 ± 0.05</td>
<td>4</td>
</tr>
<tr>
<td>PR 967-234</td>
<td>0.8 ± 0.43</td>
<td>5</td>
</tr>
</tbody>
</table>

* The 50% inhibitory concentration (IC₅₀) derived from ALL-FIT analysis as described in Materials and Methods.
* n, Number of individual determinations.

PR 988-399 is significantly less potent (P < 0.05) than all other compounds (Student's pooled t test).

400 mg of PR 967-248 and PR 967-234 per kg. PR967-248 produced mydriasis, stereotyped locomotor activity, seven signs of physical depression, failure on the wire maneuver, severe hypothermia (−10°C), convulsions, and death in two mice by 96 h. At 200 mg/kg, PR 967-234 produced failure on the wire maneuver, loss of the pinna reflex, and diminished respiration. Additional signs consisting of hypothermia, ataxia, and hypogait were elicited at the higher dose (Table 3).

A perusal of the summary of findings in Table 3 revealed that with respect to oral dosing, the subjective rank order of safety for the five antifungal agents in mice (fewest to most severe symptoms) was PR 969-566 (1), PR 988-399 (2), PR 967-234 (3), ketoconazole (4), and PR 967-248 (5).

Neural impairment. Initial dosing was 400 mg/kg (oral), and doses were scaled upward or downward depending on responses observed for a particular drug under a specific neural impairment-testing situation. It became readily apparent that in the inverted screen test, failures of mice to negotiate the task did not follow dose-response relationships even if acute doses as high as 1,000 to 1,200 mg/kg were attempted. The exception was PR 967-234, for which doses higher than 800 mg/kg resulted in death. The observed lack of dose-response relationships was also evident for the inclined screen and wire maneuver tasks. The exception again was PR 967-234 and also PR 967-248 (wire maneuver only), for which toxic doses that inhibited the response by 50% (TD₅₀) were obtained. Respective TD₅₀s calculated by the AEL-50 program were as follows: (i) PR 967-234, inclined screen, TD₅₀ = 230 mg/kg (321 to 166); slope = 4.7 ± 1.2; (ii) PR 967-234, wire maneuver, TD₅₀ = 247 mg/kg (343 to 179), slope = 4.9 ± 1.2; and (iii) PR 967-248, wire maneuver, TD₅₀ = 627 mg/kg (1,349 to 459), slope = 3.4 ± 1.1. The values in parentheses are 95% confidence limits, and the slope is the mean ± the standard error (SE).

As described in the Materials and Methods, chi-square comparisons were made between compounds and across the three neurologically related neural impairment tests by measuring responses obtained at 600 and 900 mg/kg. The first relationship compared all five compounds with controls, and a significant degree of neural impairment was observed across the three tests at the 900-mg/kg dose (see Table 4 for chi-square analyses). At the 600-mg/kg dose, compounds PR 988-399 and PR 969-566 did not produce significant impairment, whereas PR 967-234, as expected from the TD₅₀ analyses, produced the worst impairment, while ketoconazole and PR 967-248 followed in rank order of significance (Table 4).

The second statistical evaluation for neural impairment compared the four Fisons compounds with the reference.
TABLE 3. Screen for behavioral signs in mice after oral doses of antifungal compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole</td>
<td>None</td>
<td>Mydriasis</td>
<td>Mydriasis</td>
<td>Mydriasis, hypothermia (−2°C), analgesia, ptosis, hypogagit ataxia, loss of pinna reflex, hypomotility</td>
<td>Same as 200 mg/kg plus failed wire maneuver, hypothermia (−6°C), one dead at 48 h</td>
</tr>
<tr>
<td>PR 988-399</td>
<td>Diuresis</td>
<td>Diuresis</td>
<td>Diuresis</td>
<td>Same as 100 mg/kg plus loss of righting reflex</td>
<td>Same as 200 mg/kg, two dead at 72 h</td>
</tr>
<tr>
<td>PR 969-566</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Mydriasis, irritability</td>
</tr>
<tr>
<td>PR 967-248</td>
<td>None</td>
<td>None</td>
<td>Irritability</td>
<td>Same as 100 mg/kg plus mydriasis, failed wire maneuver, stereotypy</td>
<td></td>
</tr>
<tr>
<td>PR 967-234</td>
<td>None</td>
<td>None</td>
<td>Mydriasis, irritability</td>
<td>Same as 100 mg/kg plus decreased respiration, loss of pinna reflex, failed wire maneuver</td>
<td>Same as 200 mg/kg plus ataxia, hypergait, hypothermia (−3°C), one dead at 96 h</td>
</tr>
</tbody>
</table>

a Irwin (16) test; three mice per dose, 27 symptoms evaluated immediately postdose and at 30 min, 3 h, and 24 h. Mice were checked daily for mortality, etc., for 7 days.

agent, ketoconazole. At the 900-mg/kg dose, compounds PR 988-399, PR 969-566, and PR 967-248 produced significantly less neural impairment than ketoconazole, while the comparison for PR 967-234 was not significant. With the 600-mg/kg dose, compounds PR 969-566, PR 988-399, and PR 967-234 produced significantly less impairment than ketoconazole, whereas the impairment for PR 967-248 was not different from that for ketoconazole (Table 4).

LD<sub>50</sub>. Acute administration of large oral doses (900 to 1,200 mg/kg) of the antifungal compounds did not produce a sufficient number of deaths in mice to obtain LD<sub>50</sub>s over a 24-h period. Only two compounds, ketoconazole and PR 967-234, possessed a suitable degree of solubility for i.v. administration to mice. Both ketoconazole and PR 967-234 readily produced immediate (1 to 2 min) death after i.v. injection. Symptoms in survivors usually waned by 10 min after injection. The LD<sub>50</sub>s and 95% confidence limits (in parentheses) for ketoconazole and PR 967-234 were 32 mg/kg (30 to 35) (slope ± SE = 17.5 ± 4) and 20 mg/kg (19 to 21) (slope ± SE = 18 ± 4.2), respectively.

Supplementary central nervous system tests in mice. (i) Anticonvulsant. PR 967-248 was the only antifungal compound to protect mice against seizures elicited by maximal electroshock. A relatively potent oral 50% effective dose of 46 mg/kg was obtained (95% confidence limits = 39 to 55, slope ± SE = 6.6 ± 1.4). No antifungal agents protected mice from pentylenetetrazol-induced convulsions. Alternatively, at 400 mg/kg, PR 967-234 promoted death in all animals injected with pentylenetetrazol.

(ii) Phenyl-p-benzoquinone-induced writhing. At the highest oral doses tested, PR 967-248 (300 mg/kg) protected 20% of mice from phenyl-p-benzoquinone-induced writhing. Similarly, PR 967-234 afforded a 40% protection at 400 mg/kg. No other agents were effective in this test.

(iii) Oxotremorine-induced tremor and salivation. The antifungal compounds when administered at large oral doses were all inactive in the oxotremorine test.

(iv) Tremors induced by LON 954. All antifungal compounds except PR 967-234 were unable to protect mice against tremors elicited by LON 954. The oral 50% effective dose was 154 mg/kg (95% confidence limits, 107 to 234) with a slope ± SE of 2.22 ± 0.5 (data obtained by the AEL-50 program).

(v) Antihypoxia screen. The initial test dose to determine whether the antifungal compounds would extend survival time when mice were exposed to a hypoxic environment was 400 mg/kg orally, except for PR 967-248 (300 mg/kg). The survival time (mean ± SE) of normal mice exposed to the

TABLE 4. Chi-square comparisons of drug-induced neural impairments with controls and with ketoconazole

<table>
<thead>
<tr>
<th>Comparison</th>
<th>600 mg/kg</th>
<th>900 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freq/total</td>
<td>x²</td>
</tr>
<tr>
<td>Control vs PR 988-399</td>
<td>156/166</td>
<td>0.4</td>
</tr>
<tr>
<td>PR 988-399 vs PR 969-566</td>
<td>30/32</td>
<td>3.5</td>
</tr>
<tr>
<td>PR 967-248 vs PR 967-234</td>
<td>22/32</td>
<td>18.8</td>
</tr>
<tr>
<td>PR 967-234 vs Ketoconazole</td>
<td>7/32</td>
<td>95.8</td>
</tr>
<tr>
<td>Ketoconazole vs PR 988-399</td>
<td>20/32</td>
<td>26.9</td>
</tr>
</tbody>
</table>

a Chi-square comparisons of neural impairments were made across three neural impairment tests. Freq/total, Frequency (frequency is the number of animals successfully completing the task)/total animals tested at that dose. Drugs were administered orally.

b NS, not significant.
TABLE 5. Effect of antifungal agents on acute exploratory motor activity in naive rats

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>Mean distance traveled (cm) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Study 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>29</td>
<td>1,131</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>320</td>
<td>30</td>
<td>792b</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>640</td>
<td>30</td>
<td>638b</td>
</tr>
<tr>
<td>PR 988-399</td>
<td>320</td>
<td>30</td>
<td>918b</td>
</tr>
<tr>
<td>PR 988-399</td>
<td>640</td>
<td>30</td>
<td>757b</td>
</tr>
<tr>
<td>Study 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>16</td>
<td>1,020</td>
</tr>
<tr>
<td>PR 969-566</td>
<td>100</td>
<td>16</td>
<td>940</td>
</tr>
<tr>
<td>PR 969-566</td>
<td>200</td>
<td>16</td>
<td>846</td>
</tr>
<tr>
<td>Study 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>10</td>
<td>1,218</td>
</tr>
<tr>
<td>PR 967-248</td>
<td>100</td>
<td>10</td>
<td>1,070b</td>
</tr>
<tr>
<td>PR 967-248</td>
<td>200</td>
<td>10</td>
<td>894b</td>
</tr>
<tr>
<td>PR 967-234</td>
<td>100</td>
<td>10</td>
<td>759b</td>
</tr>
<tr>
<td>PR 967-234</td>
<td>200</td>
<td>10</td>
<td>807</td>
</tr>
</tbody>
</table>

* BMDPV-2V analysis for individual studies was as follows. Study 1, ketoconazole and PR 988-399, five treatments, three times: treatment, F(D of 144) = 16.66, P = 0.0000; interval: F(2 of 288) = 71.3, P = 0.0000; treatment by interval: F(8 of 288) = 2.85, P = 0.0004. Study 2, PR 969-566, three treatments, three times: treatment, F(2 of 45) = 5.06, P = 0.01; interval: F(2 of 90) = 13.28, P = 0.01; treatment by interval: F(4 of 90) = 1.19, P = 0.32. Study 3, PR 967-248 and PR 967-234, five treatments, three times: treatment, F(4 of 45) = 4.91, P = 0.002; interval: F(2 of 90) = 12.47, P = 0.0000; treatment by interval: F(8 of 90) = 3.2, P = 0.003.

* Newman-Keuls analysis significantly different from control (P < 0.05).

The hypoxic environment was 2.3 ± 0.2 min (n = 25). The percent extension of survival times over respective control groups for the initial high oral doses of antifungal agents were (mean percent ± SE): ketoconazole, 71 ± 12 (P = 0.06; Student's pooled t test); PR 988-399, 60 ± 5 (P = 0.001); PR 969-566, 48 ± 4 (P = 0.001); PR 967-248, 57 ± 8 (P = 0.001); and PR 967-234, 20 ± 5 (not significant). The large SE of the mean for ketoconazole with respect to its control group accounts for the lack of significance. The study was then redesigned to obtain direct comparisons of anxyhpic activity with two doses (50 and 100 mg/kg) and 10 mice per dose. The 50-mg/kg dose did not significantly influence survival. However, the 100-mg/kg dose was effective, with ketoconazole extending survival time by 82% (P = 0.001) and PR 967-248 extending survival time by 72% (P = 0.001). The mean survival time for controls in this experiment was 2.2 ± 0.09 min (n = 30).

Acute exploratory motor activity in naive rats. In the three separate studies utilizing the Optovarimex system to monitor acute exploratory motor activity in naive rats, the total distance traveled by controls was consistently greater upon the initial exposure (1-h testing time) of the animals to the apparatus. At the 6- and 24-h testing times, the total motor activity was from 10 to 35% less than that at 1 h. During dose-ranging studies, both ketoconazole and PR 988-399 were found to be ineffective at doses of 7, 20, 40, 80, and 160 mg/kg (orally). At higher oral doses (320 and 640 mg/kg) of ketoconazole and PR 988-399, there were significant decrements in distance traveled over the time course of the experiment. The BMDPV-2V analysis of the ketoconazole and PR 988-399 findings showed significant effects owing to treatment, interval, and treatment-by-interaction.
3-mg/kg i.v. doses were unremarkable \((n = 4)\). However, the 10-mg/kg i.v. doses elicited increased DBP (from 91 ± 5 to 111 ± 4 mm Hg, \(P < 0.001\)) and MABP (from 108 ± 6 to 133 ± 3 mm Hg, \(P < 0.001\)), while ectopic beats were evident on lead II of the electrocardiogram. Two dogs had tonic-clonic seizures, and preictal behavior was present in the remaining two, one of which was overly aggressive.

An empirical judgment of the findings indicates that ketoconazole, PR 988-399, and PR 969-566 were relatively safe in conscious dogs with regard to oral dosing. Ketoconazole was the safest agent after i.v. infusion, followed by PR 988-399 and PR 969-566. Compound PR 967-234 produced the highest degree of untoward actions.

**DISCUSSION**

Since the incidence of *C. albicans* infections is rising owing to better diagnosis, appearance of drug-resistant organisms, and the susceptibility of the increasing numbers of immunocompromised patients \(13, 14, 19, 20, 30, 36\), our therapeutic goal was to develop an effective and safe oral treatment for humans. A candidate compound should be of equal or greater potency and safety than the leading marketed compound, ketoconazole \(5, 14, 26, 35\). Critical factors in the rational development of new orally active antifungal agents are standardized, quantitative, and coordinated approaches involving chemical discovery and acute preclinical efficacy-safety evaluations of promising novel antifungal candidates compared with positive reference compounds. One of the problems faced by us was the paucity of pharmacological data available for such a study. Most reports would describe in vitro antifungal efficacy, but the safety as well as reliable in vivo tests for efficacy were usually lacking. One report \(24\) described ketoconazole, but the array of testing procedures was too extensive for simultaneous evaluation of several active compounds. From the four chemical series described here \(4, 10, 22, 33\), active compounds emerged from in vitro testing for antifungal activity. Thus, the testing sequence next examined all active compounds for acute safety utilizing the rapid tests for behavioral signs \(6, 16\) and neural impairment \(7, 9\) in mice. The four compounds, PR 988-399, PR 969-566, PR 967-248, and PR 967-234, described in this report emerged from their respective synthetic series as the most likely candidates for the ensuing concurrent stages of development, namely, evaluation for in vivo efficacy in the rat model of vaginal candidal infection \(1\), inhibition of testosterone synthesis in incubated rat Leydig cells \(21\), and safety and behavioral (exploratory motor activity) effects in the rat \(12\). Following acquisition of these data, drug and mechanistic interaction studies were conducted in mice to obtain clues about the possible salutary or adverse central sites of actions exerted by the compounds. Last, the compounds were tested for acute behavioral and cardiovascular safety in dogs. The lead candidate selected from these acute studies would then be targeted for extensive development regarding efficacy and chronic toxicity.

When the imidazole antifungal agents \(PR 969-566, PR 967-248, and PR 967-234\) and ketoconazole were tested in vitro for efficacy against three strains of systemic fungi and four strains of dermatophytic fungi, the rank orders of potency for inhibition of fungal growth among these four compounds were not significant. Alternatively, the triazole derivative, PR 988-399, was significantly less potent than the imidazole derivatives and ketoconazole. Work by other investigators \(15, 31, 32\) has also shown weaker in vitro antifungal activity for triazole compounds including new drugs in advanced clinical trials, namely, itraconazole and fluconazole. Notably, the triazole analog of ketoconazole was also less potent. Triazoles usually have limited solubility \(15\). The findings with ketoconazole support earlier work describing its ability to inhibit in vitro fungal growth under a variety of testing conditions \(15, 26, 35\).

The initial safety screens in mice indicated that with regard to production of behavioral signs \(6, 16\), PR 969-566 followed by PR 988-399 were the safest drugs, while PR 967-248 was the least safe. However, high oral doses of 200 to 400 mg/kg were required to produce symptoms with any of the compounds. Both PR 969-566 and PR 988-399 elicited the lowest incidences of neural impairment \(7, 9\). In these tests, PR 967-234 was the most toxic compound. Acute doses as high as 1,000 to 1,200 mg/kg rarely caused mortality, except for PR 967-234. The hypothermia produced by ketoconazole and its relative ineffectiveness on motor performance confirm earlier work \(24\).

Limited oral in vivo efficacy with respect to anticandidal activity was observed for PR 969-566 and PR 967-234, while PR 988-399 and ketoconazole eradicated the infections caused by two equally virulent *Candida* strains, and PR 967-248 was inactive. Moreover, PR 988-399 demonstrated a 2-day lag period for onset of antifungal activity, but eradication by day 8 was similar to that of the reference agent. The data for ketoconazole are in agreement with other investigations which reveal efficacy against a variety of candidal infections involving laboratory animals and humans \(5, 15, 26, 31, 33, 35\).

Active imidazoles exert antifungal activity by increasing membrane permeability by interfering with ergosterol synthesis, the main sterol in fungal membranes. In mammals, acute administration of ketoconazole prevents formation of testosterone and higher sustained doses suppress cortisol as well. The site of action is a selective inhibition of C_{17-20} lyase enzyme involved in the initial steps in the synthesis of the two steroids \(14, 34, 36\). Ketoconazole likewise was a potent inhibitor of testosterone synthesis in isolated rat Leydig cells also see reference \(28\). The Fisons imidazoles did effectively suppress testosterone formation in incubated rat Leydig cells. On the other hand, the triazole, PR 988-399, was significantly less potent \(3\) to 13-fold) in this test situation. Triazoles, however, are generally less soluble than imidazoles, a condition that could bias in vitro investigations \(15\). Nevertheless, with regard to inhibition of testosterone, PR 988-399 emerged as the safest candidate.

Oral ketoconazole at 100 mg/kg was shown by Nakamura et al. \(24\) to reduce spontaneous motor activity in mice, a finding not observed by us in rats with the Optovarimex activity monitor \(12\) until larger doses were used. The Fisons agents with the most potent effects on spontaneous motor activity were the imidazoles, while PR 988-399 was of lesser potency, analogous to ketoconazole.

Test compounds found to modify behavior in mice were evaluated in six drug-neurological interaction procedures designed to determine possible sites of action in the brain, as well as whether the responses were harmful or even beneficial. Antifungal agents like ketoconazole are known to penetrate poorly into the brain, and the resultant concentrations are insufficient to treat fungal meningitis \(32, 36\). In the present study and earlier work, only the highest doses of ketoconazole exerted central analgesic \(24\), anticonvulsant \(24\), or antihypoxic properties. The latter action could be a result of hypothermia; however, an attempt was made to control for this problem \(3\). Of the Fisons compounds, PR
967-248 exhibited cerebral protective actions; it was a potent anticonvulsant and possessed antihypoxic properties. PR 967-234 displayed antiparkinsonian actions but adversely potentiated the central actions of the cholinergic agent oxotremorine. These findings along with the behavioral and neurological effects associated with PR 967-234 and PR 967-248 in mice, rats, and dogs indicate the ability of both compounds to penetrate into the brain, while PR 969-566 and PR 988-399 most likely are not as effective.

Conscious dogs were utilized in the final test of central nervous system and cardiovascular safety. Oral administration revealed the safest compounds to be PR 988-399 followed by ketoconazole and PR 969-566. PR 967-234 was the least safe as evidenced by convulsions and increased MAP at the lower dose of 30 mg/kg. Doses of 10 mg/kg i.v. caused symptoms with all compounds, the most severe occurring with PR 967-234 followed by PR 967-248. At the highest i.v. dose, both PR 988-399 and PR 969-566 produced a transient rise in blood pressure and convulsions while ketoconazole was relatively inactive (emesis and an elevated DBP). Nakakura et al. (24) found no effect of oral ketoconazole on heart rate in rabbits, but large concentrations depressed atrial concentration in guinea pig myocardium in vitro and i.v. administration decreased the heart rate in dogs. In addition, i.v. administration of ketoconazole to anesthetized dogs transiently reversed the increase in blood pressure in response to a norepinephrine challenge. In this study, ketoconazole transiently decreased both SBP and DBP and increased blood flow measured at the femoral artery.

In conclusion, the acute approach described here optimizes the identification of promising antifungal agents based on efficacy-safety considerations. The behavioral tests in mice showed a slightly more favorable degree of safety for PR 969-566 than for PR 988-399. Both compounds exhibited equal degrees of safety in the tests for cardiovascular and behavioral actions in dogs. On the other hand, PR 988-399 demonstrated a better safety profile in tests for neural impairment in mice, exploratory motor activity in rats, and inhibition of testosterone synthesis in vitro. Despite its weaker antifungal properties in vitro, PR 988-399 was as effective, albeit with a delayed onset of action, as ketoconazole in vivo toward eradication of vaginal candidial infections in rats. Therefore, PR 988-399 emerged from the series as the best candidate for further extensive development.

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LITERATURE CITED


dichlorophenyl)]-2-[(1H-imidazol-1-ylmethyl)]-1,3-dioxolan-