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In Vitro and In Vivo Activities of Piritetrate (M-732), a New Antidermatophytic Thiocarbamate

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Piritetrate (M-732), a new topical antifungal agent belonging chemically to the thiocarbamates, was demonstrated to possess a potent selective antidermatophytic activity. In terms of its MICs in susceptibility testing, mainly done by using Sabouraud dextrose agar plates, piritetrate exhibited several- to 10-fold-stronger antidermatophytic activity than tolnaftate, a reference thiocarbamate. Furthermore, piritetrate was found to show a broader antifungal spectrum than tolnaftate; relatively many species and strains of dematiaceous fungi, dimorphic fungi, and some other filamentous fungi as well as a few strains of *Cryptococcus neoformans* were fairly susceptible to piritetrate, while almost all the tested species and strains were resistant to tolnaftate. All the tested species of the genus *Candida* were, however, resistant to both compounds. Variables which can influence antimicrobial activity caused few changes in the MICs of either compound against *Trichophyton mentagrophytes*; however, an increase in the inoculum size resulted in a significant increase in the MICs. The antidermatophytic activities of piritetrate and tolnaftate were fungistatic but not fungicidal. Piritetrate also exhibited a more potent in vitro anti-*T. mentagrophytes* activity than clotrimazole or tolciclate. Piritetrate and tolnaftate had no antibacterial activity. The in vivo activity of topically administered piritetrate against experimental dermal infection of guinea pigs with *T. mentagrophytes* was more effective than that of tolnaftate both mycologically and clinically. Piritetrate manifested no acute toxicity in laboratory animals when administered even in large quantities by the oral, intraperitoneal, and topical routes.

Piritetrate (M-732) is a new thiocarbamate compound [methyl(6-methoxy-2-pyridyl)carbamothioic acid *O*-5,6,7,8-tetrahydro-2-naphthalenyl ester ($C_{18}H_{20}N_2O_2S$)] which was synthesized by the Chemical Research Laboratory, Tosoh Corporation (formerly, Toyo Soda Manufacturing Co., Ltd.), Yamaguchi-ken, Japan. The structure of piritetrate is shown in Fig. 1, together with that of a reference thiocarbamate, tolnaftate [methyl(3-methylphenyl)carbamothioic acid *O*-2-naphthalenyl ester ($C_{19}H_{17}NOS$)] (12). Piritetrate is a white crystalline powder with a melting point of 98.5 to 99.5°C. It is not hygroscopic but is stable in air, insoluble in water, slightly soluble in *n*-hexane, methanol, and ethanol, and readily soluble in benzene, ether, acetone, chloroform, dimethyl sulfoxide, and *N*,*N*-dimethylformamide.

The potent in vitro and in vivo antidermatophytic activities of piritetrate were initially identified in our screening program on a number of members of the thiocarbamate series which had been synthesized as agricultural herbicides by the Chemical Research Laboratory. In this report, we describe the results of comparisons of the in vitro antifungal and antibacterial activities of piritetrate, tolnaftate, tolciclate [a recently developed thiocarbamate compound; *O*-(1,2,3,4-tetrahydro-1,4-methanonaphthalen-6-yl)-*m*-*N*-dimethylthiocarbanilate; 2,3], and clotrimazole [a classic azole compound; 1-[(2-chlorophenyl-)diphenylmethyl]imidazole; 13,16]. The activities of piritetrate and tolnaftate were determined particularly against dermatophytes, including *Trichophyton mentagrophytes*. We also compared the in vivo activities of piritetrate and tolnaftate in dermal model infections of guinea pigs with *T. mentagrophytes*. In addition, the acute toxicity of piritetrate for laboratory animals was investigated.

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MATERIALS AND METHODS

Antifungal agents. Piritetrate, tolnaftate, and tolciclate were provided by Tosoh Corporation. Clotrimazole was a gift from Bayer Yakuhin Co., Ltd., Tokyo, Japan. For in vitro antifungal and antibacterial tests, stock solutions at a concentration of 10 mg/ml were prepared by dissolving the compounds in dimethyl sulfoxide, which proved to be stable for many months when stored at 4°C in the dark. For in vivo antifungal tests, piritetrate and tolnaftate were formulated as 1 and 2% creams suspended in a mixture of polyethylene glycol 400 and 1540 (6:4 [vol/vol]) and were administered topically. Piritetrate was also tested for acute toxicity, the method for which will be described later.

Organisms. For in vitro tests, we used a total of 357 clinical isolates of human pathogenic and opportunistic fungi comprising 302 stock cultures and 55 recent clinical isolates of dermatophytes. The stock cultures included dermatophytes, dematiaceous fungi, dimorphic fungi, other filamentous fungi, and yeasts (see Table 1). The recent isolates of dermatophytes, generously provided by S. Kagawa, Department of Dermatology, Tokyo Medical and Dental University, Tokyo, Japan (see Table 2), were compared with the stock cultures of dermatophytes for susceptibility to piritetrate and the three reference drugs. These fungal strains have been maintained in our laboratory by subculturing on Sabouraud dextrose agar (SDA; 2% dextrose, pH 6.3 to 6.5) slants. The effects of various factors which may affect measured antifungal activities of piritetrate and tolnaftate were examined with two strains of *T. mentagrophytes*, one a

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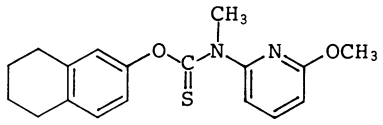
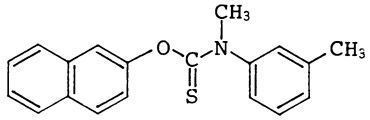


Piritetrate



Tolnaftate

FIG. 1. Structures of piritetrate and tolnaftate. Piritetrate is methyl(6-methoxy-2-pyridyl)carbamothioic acid *O*-5,6,7,8-tetrahydro-2-naphthalenyl ester ($C_{18}H_{20}N_2O_2S$). Tolnaftate is methyl(3-methylphenyl)carbamothioic acid *O*-2-naphthalenyl ester ($C_{19}H_{17}$ NOS).

more susceptible strain (MTU 19021) and the other strain (MTU 19026) a little less susceptible to both compounds. Some hazardous fungi, particularly dimorphic fungi, were handled in a safety cabinet (model SCV-1303ECIIA; Hitachi, Tokyo).

Four strains of each of four species of bacteria, viz., *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, and *Nocardia asteroides*, which were also part of our collection, were studied to determine the antibacterial activities of both compounds.

For in vivo tests, *T. mentagrophytes* MTU 19021 was used. This strain had been isolated by us from a patient with tinea pedis. Its virulence for guinea pigs upon dermal infection was relatively high; it was capable of producing typical severe lesions by inoculation of ca. 10^6 CFU of conidia obtained from a culture grown at 27°C for 4 or 5 weeks. CFU were determined by plate counts.

Media. SDA, used for maintenance of the fungal strains, was also used for susceptibility testing on plates, unless otherwise specified. Sabouraud dextrose broth (SDB; 2% dextrose, pH 6.3 to 6.5) was used for fungicidal activity testing. Brain heart infusion agar (Eiken) was used to test for antibacterial activity, except for *N. asteroides*, for which SDA was used. For in vivo tests, two media were used: ordinary SDA (4% dextrose, pH ~6.5) plates were used to obtain inocula of the test organism for infection, and SDA plates containing 50 μg of chloramphenicol per ml and 200 μg of cycloheximide per ml were used to recover organisms from experimentally infected skin foci. All media other than brain heart infusion agar were prepared in our laboratory.

Animals. Male guinea pigs, each weighing about 400 g, were purchased from Shizuoka Laboratory Animals Corporation, Ltd., Shizuoka-shi, Japan. They were used to examine the efficacy of piritetrate and tolnaftate on experimental dermal infections with *T. mentagrophytes* and to determine the acute toxicity of piritetrate. Male albino mice of the CRJ:ICR strain, each weighing about 20 g, were purchased from the Central Institute for Experimental Animals, Tokyo, Japan. They were also used to test the acute toxicity of piritetrate. These animals were maintained in an air-conditioned room at 23 ± 2°C and were provided with food and water ad lib.

Susceptibility testing. The fungistatic and fungicidal activities of piritetrate and tolnaftate were examined.

(i) Technique for determination of fungistatic activity. Fungistatic activity was determined in terms of the MIC by the agar dilution method using plates containing 20 ml of SDA. Each compound was incorporated in the plates at appropriate concentrations in a twofold dilution series. Inocula were prepared on ordinary SDA slants incubated at 27°C for 4 to 5 weeks for dermatophytes, dematiaceous fungi, and dimorphic fungi; for 2 weeks for other filamentous fungi; and for 48 h for yeast fungi at 30 or 37°C, depending on the growth rate of the test strain. Some fastidious fungal species were grown on ordinary SDA supplemented with 1% yeast extract for preincubation and also for susceptibility testing. The actual inocula were prepared to contain 10^5 CFU of organisms per ml by appropriate dilution, in sterile normal saline, of conidia of filamentous fungi and dimorphic fungi or of yeast cells grown under the same culture conditions as those used for preincubation. By using a calibrated loop (American Opticals Corp., Buffalo, N.Y.), a loopful of each cell suspension (about 10 μl) was inoculated onto each octant of a test compound-containing plate in an ordinary petri dish by making a streak of ca. 3 cm. The final inoculum was therefore 10^3 CFU. The plates were incubated at the same temperature and for the same period of time as those used for preincubation; the lowest concentration of compound at which no growth occurred was taken as the MIC. Dimethyl sulfoxide and saline up to concentrations of 4 and 1%, respectively, were confirmed to exert little or no influence on the growth of the test strains. These test conditions were used for all susceptibility tests, except where noted. In addition, the MIC_{90}s (MICs for 90% of the test strains) were recorded for species for which there were 13 or more strains.

(ii) Technique for determination of fungicidal activity. The rates of killing of fungi by piritetrate and tolnaftate were examined by counting the number of viable cells (conidia) of *T. mentagrophytes* MTU 19021 grown in SDB at 27°C with shaking as a function of the time of exposure to the compound. Samples were withdrawn from the test cultures at intervals, and 10-fold dilutions were made in SDB. Volumes (0.1 ml) of each dilution were spread on SDA plates. After incubation at 27°C for 2 weeks, the CFU per milliliter in each culture was determined.

Testing for effect of incubation time on MICs. The MICs of piritetrate and tolnaftate against two strains of *T. mentagrophytes* (MTU 19021 and MTU 19026) were measured after 2 to 5 weeks of incubation at 27°C.

Testing for effects of inoculum size. The effects of varied inoculum size of conidia (ranging from 10^2 to 10^7 CFU per plate) on the MICs were evaluated. A conidium suspension was prepared by the procedures described above for susceptibility testing.

Testing for the effect of medium pH on MICs. The effects of different pH values, ranging from 4.5 to 8.5 (adjusted with HCl or NaOH), on the MICs were examined in the same manner as described above.

Testing for effect of blood on MICs. To check the effect of addition of human blood on the MICs, blood collected without anticoagulant was incorporated into SDA at a concentration of 10%.

Testing for antibacterial activity. The MICs were likewise determined for bacteria by the agar dilution technique by using brain heart infusion agar and SDA plates. Incubation was performed at 37°C for 24 h with *S. aureus*, *E. coli*, and

TABLE 1. Comparative in vitro activities of piritetrate and tolnaftate against 302 stock cultures of various pathogenic and opportunistic fungi[a] as well as 55 recent isolates of dermatophytes[b]

Organism type and species (no. of strains)	MIC (μg/ml) range[c]	
	Piritetrate	Tolnaftate
Dermatophytes (172)		
Trichophyton mentagrophytes (35)	0.002–0.039 (0.019)	0.078–0.625 (0.156)
T. mentagrophytes (23)[b]	0.004–0.078 (0.039)	0.078–0.625 (0.156)
T. rubrum (22)	0.002–0.039 (0.019)	0.019–0.312 (0.156)
T. tonsurans (5)	0.004–0.019	0.019–0.312
T. schoenleinii (5)	0.002–0.009	0.019–0.625
T. violaceum (5)	0.004–0.019	0.019–0.039
T. verrucosa (5)	0.009–0.039	0.039–0.078
Microsporum audouinii (5)	0.002–0.039	0.039–0.312
M. canis (14)	0.004–0.078 (0.039)	0.012–0.156 (0.156)
M. canis (18)[b]	0.009–0.078 (0.019)	0.156–0.312 (0.156)
M. gypseum (8)	0.009–0.019	0.039–0.312
Epidermophyton floccosum (13)	0.004–0.078 (0.039)	0.039–0.625 (0.312)
E. floccosum (14)[b]	0.009–0.156 (0.039)	0.078–0.625 (0.312)
Dematiaceous fungi (35)		
Fonsecaea pedrosoi (14)	0.312–40 (20)	>80
F. compacta (6)	0.156–0.625	>80
Wangiella dermatitidis (7)	0.312–>80	20–>80
Phialophora verrucosa (80)	0.156–>80	>80
Dimorphic fungi (33)		
Sporothrix schenckii (19)	>80	>80
Histoplasma capsulatum (6)	0.312–0.625	>80
Blastomyces dermatitidis (5)	0.156–0.625	>80
Paracocccidioides brasiliensis (3)	>80	>80
Other filamentous fungi (32)		
Aspergillus fumigatus (21)	0.0312–5 (1.25)	>80
A. nidurans (2)	0.009, 1.25	0.078, >80
A. niger (3)	0.004	0.039–0.078
A. flavus (2)	0.625	0.312, >80
Penicillium notatum (2)	0.004	0.004
P. citereo-viride (2)	0.312, 80	>80
Yeasts (85)		
Candida albicans (20)	>80	>80
C. tropicalis (5)	>80	>80
C. krusei (5)	>80	>80
C. parapsilosis (5)	2.5–>80	>80
C. stellatoidea (5)	>80	>80
C. guilliermondii (5)	>80	>80
C. glabrata (15)	>80	>80
Cryptococcus neoformans (25)	0.156–>80 (>80)	20–>80 (>80)

[a] These were all stock cultures of clinical isolates except for *Aspergillus flavus*, *Penicillium notatum*, and *P. citereo-viride*.
[b] All strains were recent clinical isolates.
[c] Figures in parentheses show the MIC_{90}s that were determined for species represented by 13 or more strains.

S. typhimurium and at 27°C for 1 week with *N. asteroides*. The inoculum size was ca. 10^3 CFU per plate for each bacterium.

Production of *T. mentagrophytes* infection. To prepare the inoculum, conidia from a culture of *T. mentagrophytes* MTU 19021 grown on ordinary SDA plates at 27°C for 5 weeks were suspended in saline containing 0.1% Tween 80 and filtered through sterile folded gauze. The conidium suspension for inoculation was then diluted to 2×10^7 CFU/ml with the same saline. Prior to infection, an area (ca. 8 by 10 cm) of the dorsal trunk of each guinea pig was shaved, and then circular areas ca. 2 cm in diameter at three or four sites at appropriate intervals within the shaved zone were abraded with sandpaper to cause a slight degree of hyperemia. Volumes (0.05 ml) of the conidium suspension (inoculum size, 10^6 CFU) were inoculated with a tuberculin syringe onto each circular area to produce infection and then spread with a Conradi pestle. These procedures had been confirmed in advance to produce a typical infection in 100% of the experimental animals without any recognizable individual variation.

Regimens of treatment and evaluation of results. Guinea pigs were randomly divided into test groups, and 0.2 g of a 1 or 2% cream of a test compound (piritetrate or tolnaftate) was topically applied to each infected area and spread with a Conradi pestle. The drug treatment was started 72 h after infection and continued once daily for 2 weeks. Groups of animals were maintained alongside the treated animal groups to serve as controls, receiving either only the drug vehicle (the polyethylene glycol mixture described above) or no treatment. In an attempt to assess the efficacy of the two compounds, the following two experiments were conducted.

Experiment 1. First, both the clinical and mycological efficacies of treatment were examined. Each of the treated and control groups comprised 20 animals. In the clinical assessment of focal changes of the infected skin areas, scores from 0 (no findings) to 4+ (severe lesions, equivalent to the untreated control) were recorded, according to the criteria of Weinstein et al. (18). Scoring, commencing 3 days after infection, was performed every 3 days, and average scores (total lesion score during the period of observation divided by the total number of animals tested) were also recorded. On the day after the last treatment, all animals were sacrificed to assess the mycological status by recovery of viable cells. Each infected focus was excised and then divided into cubical pieces 2 mm^3 in size with a scalpel, and 10 randomly selected pieces were placed on SDA plates containing chloramphenicol and cycloheximide and incubated at 27°C for 2 weeks to check for fungal growth. Two hundred samples were tested for each group. The comparative mycological efficacies of the 1 and 2% cream preparations of piritetrate and tolnaftate were evaluated in terms of the reduction in the number of positive skin samples in the treated groups, expressed as a percentage.

Experiment 2. To monitor the mycological status during the course of infection and treatment, a similar test was conducted over a period of 3 weeks, using only the 1% cream of each of the two compounds. Two treated and two control (vehicle only and untreated) groups comprised 6 animals each. Three areas on the back of each animal, pretreated as described above, were infected and treated under the same conditions as in experiment 1. Every week, two animals in each group were randomly selected and sacrificed to assess the mycological status by using 10 pieces 2 mm^3 in size from each focus, totaling 60 samples for each group.

Statistical analysis. For comparative estimation of the mycological efficacy, the Mann-Whitney test and the χ^2 test were applied to the results at the end of therapy in experiments 1 and 2, respectively.

Acute toxicity testing. Piritetrate was examined for acute toxicity in view of there being a number of reports on the toxicity of tolnaftate (5, 6, 11). Piritetrate was suspended in 0.3% sodium carboxymethyl cellulose for oral and intraperitoneal administration or in the polyethylene glycol mixture for topical administration. The compound was administered to groups of 20 mice orally (by gavage) by a single dose of 3,000 mg/kg of body weight (BW) and multiple doses of 500 mg/kg of BW once daily for 6 consecutive days or intraperitoneally by a single dose of 500 mg/kg of BW and multiple doses of 100 mg/kg of BW once daily for 6 consecutive days. Dermal toxicity was studied by topical application of 0.2 g of a 2% cream of the compound onto a circular area (ca. 3 cm^2) on the back of each guinea pig, which was pretreated in the same fashion as for production of *T. mentagrophytes* infection as described above. Control suspensions (0.3% sodium methylcellulose and polyethylene glycol mixture) were also tested by these three different routes of administration (see Table 4). The animals were observed for death, development of symptoms including irritative skin responses, and fluctuation of BW for 1 month after the final administration.

RESULTS

Antifungal spectrum and MICs. Table 1 shows the comparative overall activities of piritetrate and tolnaftate against various fungal species. Piritetrate was more active than tolnaftate against all the test species of both stock cultures and recent clinical isolates of dermatophytes, with a relatively narrow range of MICs and MIC_{90}s. The recent isolates of those species appeared to be a little less (but not significantly) susceptible to both piritetrate and tolnaftate than the stock strains. In addition, the antidermatophytic activity of piritetrate was not only confirmed to be superior to that of tolnaftate but was also even more superior to the activities of tolciclate and clotrimazole against the recent isolates of dermatophytes; MICs (MIC_{90}s) of tolciclate and clotrimazole, respectively, were 0.312 to 1.25 μg/ml (0.625 μg/ml) and 0.156 to 0.625 μg/ml (0.312 μg/ml) for *T. mentagrophytes*, 0.156 to 0.625 μg/ml (0.312 μg/ml) and 0.312 to 1.25 μg/ml (0.312 μg/ml) for *Microsporum canis*, and 0.312 to 1.25 μg/ml (0.625 μg/ml) for *Epidermophyton floccosum*, respectively (these data were not included in the table).

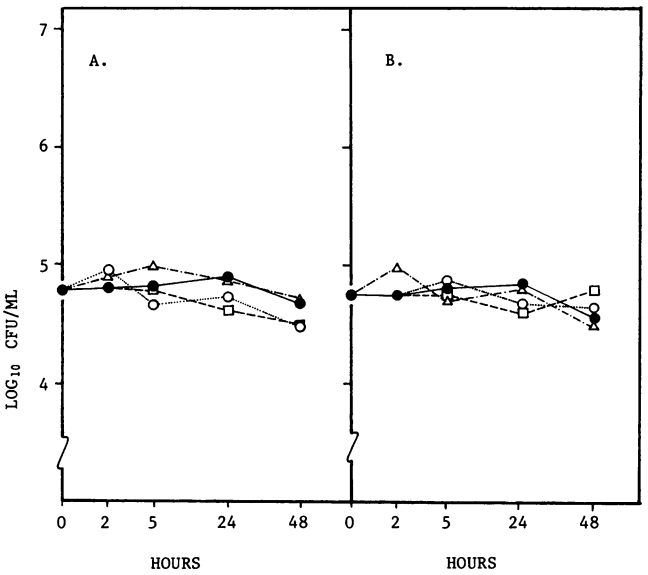


FIG. 2. Fungicidal activities of piritetrate (A) and tolnaftate (B) against *T. mentagrophytes* MTU 19021. The initial inoculum was 7.5 × 10^4 CFU/ml. Symbols: ●, control culture (no added agents); ○, 1 μg/ml; □, 10 μg/ml; △, 80 μg/ml.

Similar results were observed against other species of dermatophytes for which several strains were tested, viz., *T. tonsurans* and others. Dematiaceous fungi appeared to be rather resistant to these thiocarbamates compared with the test strains of dermatophytes. However, almost all the strains of *Fonsecaea pedrosoi* and *F. compacta*, as well as a few strains of *Wangiella dermatitidis* and *Phialophora verrucosa*, manifested quite high susceptibilities to piritetrate, while all these strains were apparently resistant to tolnaftate. Members of the dimorphic fungi showed diverse susceptibilities to piritetrate, whereas all the species and strains of this fungal group were resistant to tolnaftate. Strains of other filamentous fungi, members of the genera *Aspergillus* and *Penicillium*, were highly or quite susceptible to piritetrate, whereas there was great strain difference in susceptibilities to tolnaftate. The most marked difference in susceptibility to the two compounds was seen in *Aspergillus fumigatus*; many strains of this species were quite susceptible to piritetrate, while no strains were susceptible to tolnaftate. None of the yeast fungi were susceptible to either compound; all the species of the genus *Candida* were highly resistant except for a single strain of *Candida parapsilosis*, which was found to be fairly susceptible to piritetrate. It is noteworthy, however, that some strains of *Cryptococcus neoformans* were moderately susceptible to piritetrate.

TABLE 2. Efficacy of piritetrate and tolnaftate against dermal infection in guinea pigs with *T. mentagrophytes*: clinical and mycological studies (experiment 1)

Regimen	No. of days after infection	No. of days after first drug or vehicle administration[a]	No. of infected foci with a lesion score of:					Avg lesion score	No. (%) of skin pieces with positive culture of 200 skin pieces tested from infected foci on the last day of drug administration
			0	1+	2+	3+	4+		
Untreated control	3		0	0	14	6	0	2.30	
	6		0	0	2	18	0	2.90	
	9		0	0	0	14	6	3.30	
	12		0	0	0	2	18	3.90	
	15		0	0	0	3	17	3.85	
	18		0	0	0	5	15	3.75	200 (100)
Vehicle control	3		0	0	15	5	0	2.25	
	6	4	0	0	6	14	0	2.70	
	9	7	0	0	6	12	2	2.80	
	12	10	0	0	2	5	13	3.55	
	15	13	0	1	2	5	12	3.40	
	18	16	0	1	2	4	11	3.05	200 (100)
1% Piritetrate	3		0	0	15	5	0	2.25	
	6	4	5	9	6	0	0	1.05	
	9	7	6	12	2	0	0	0.80	
	12	10	10	9	1	0	0	0.55	
	15	13	16	3	1	0	0	0.25	
	18	16	18	2	0	0	0	0.10	3 (1.5)
1% Tolnaftate	3		0	2	15	3	0	2.05	
	6	4	2	2	14	2	0	1.80	
	9	7	4	7	8	1	0	1.30	
	12	10	5	6	8	1	0	1.25	
	15	13	7	5	7	0	0	0.95	
	18	16	10	9	1	0	0	0.55	17 (8.5)
2% Piritetrate	3		0	0	14	6	0	2.30	
	6	4	2	5	12	1	0	1.60	
	9	7	6	4	10	0	0	1.20	
	12	10	7	10	3	0	0	0.80	
	15	13	13	5	2	0	0	0.45	
	18	16	19	1	0	0	0	0.05	2 (1)
2% Tolnaftate	3		0	1	15	4	0	2.15	
	6	4	1	4	13	2	0	1.80	
	9	7	3	8	7	2	0	1.40	
	12	10	6	7	5	1	0	1.00	
	15	13	8	10	2	0	0	0.70	
	18	16	12	7	1	0	0	0.45	14 (7)

[a] Topical administration of cream and vehicle preparations was performed once daily for 2 consecutive weeks, commencing 3 days after infection.

Fungicidal activity. In time kill studies with *T. mentagrophytes* MTU 19021, neither piritetrate nor tolnaftate decreased the conidial population from 8×10^4 CFU/ml in SDB during a 48-h period even at the highest concentration used (80 μg/ml), but at a concentration as low as 1 μg/ml, both compounds were able to inhibit the growth completely (Fig. 2). These results show that piritetrate and tolnaftate were fungistatic but not fungicidal, at least under the test conditions used for the test strain.

Effects of variables which affect measured antifungal activity. (i) Effect of incubation time. The MICs of piritetrate and tolnaftate for the MTU 19021 and MTU 19026 strains of *T. mentagrophytes* measured after 2 weeks of incubation were the same as those after 3 weeks of incubation, and even after 4 and 5 weeks of incubation, these values increased only two and four times, respectively, over the initial susceptibility, remaining at low levels.

(ii) Effect of inoculum size. The increase in the inoculum size from 10^3 to 10^5 CFU caused no significant increase in the MIC of either compound for the conidia of either of the two strains. When the inoculum size was increased from 10^5 to 10^7 CFU, however, the activities of both compounds were significantly reduced. Conversely, a decrease in the inoculum size from 10^3 to 10^2 CFU caused significant decreases in the MICs of both compounds.

(iii) Effect of pH. Variation in the pH of SDA between 5.5 and 8.5 had little or no effect on the MICs of either compound for both strains, but at pH 4.5 the MICs were twofold lower than at pH 5.5 or higher.

(iv) Effect of blood. The addition of 10% human blood resulted in four- and twofold increases in the MICs of the compounds for both strains. However, the MICs remained low.

Antibacterial effect. Piritetrate and tolnaftate were unable to inhibit the growth of any of the test strains of *S. aureus*, *E. coli*, *S. typhimurium*, and *N. asteroides* even at the highest concentration used (80 μg/ml).

Efficacy against experimental dermal infection of guinea pigs with *T. mentagrophytes*. As shown in Table 2 (experiment 1), all the animals in the two control groups for

TABLE 3. Efficacy of piritetrate and tolnaftate against dermal infection in guinea pigs with *T. mentagrophytes*: mycological studies (experiment 2)

Regimen	No. of wk after infection	No. of days after first drug or vehicle administration[a]	No. of skin pieces with positive culture of 60 skin pieces tested from infected foci (%)	No. of animals with positive culture[b]
Untreated	1		60 (100)	6
	2		60 (100)	6
	3		59 (98.3)	6
Vehicle control	1	3	60 (100)	6
	2	10	60 (100)	6
	3	17	56 (93.3)	6
1% Piritetrate	1	3	24 (40)	6
	2	10	2 (3.3)	1
	3	17	1 (1.5)	1
1% Tolnaftate	1	3	38 (63.3)	6
	2	10	12 (20)	3
	3	17	9 (15)	2

[a] Topical administration of cream and vehicle preparations was performed once daily for consecutive weeks.
[b] Number of animals (of six tested in each case) which had culture-positive skin pieces from the infected foci, even when only one piece was culture positive.

infection alone and for infection plus vehicle application showed increased lesion scores as early as 3 days after the infection. Conversely, for all the animals in the groups treated with piritetrate or tolnaftate, the lesion score began to decrease as early as 3 days after treatment and was reduced remarkably on the day after the last application of either compound. Regarding the comparative activity, piritetrate decreased the lesion score more rapidly than tolnaftate in all four groups treated with the 1 and 2% creams. However, little or no difference in lesion-suppressing effect was observed between the groups treated with the 1 and 2% creams of piritetrate. The same was true for tolnaftate.

These lesion-suppressing effects mirrored the suppression of fungal growth. That is, the piritetrate-treated groups showed fewer positive cultures than the tolnaftate-treated groups in reisolation of inoculated organisms from the foci on the final day of observation. Statistical analysis of these results using the Mann-Whitney test revealed significant superiority of the 1% ($P < 0.01$) and 2% ($P < 0.005$) creams of piritetrate compared with the tolnaftate creams and the two control groups ($P < 0.001$). No significant difference was found between the 1 and 2% piritetrate creams ($P > 0.05$) or between the two tolnaftate creams.

Table 3 shows the results of the follow-up studies on the mycological response to the topical consecutive drug administration as treatment of dermal infections in guinea pigs (experiment 2). Almost all of the foci of the animals in both control groups remained culture positive over the 4-week period of observation, whereas the number of positive cultures after 4 consecutive days of drug application decreased more with piritetrate than with tolnaftate. Prolonged application of piritetrate for 1 or 2 more weeks caused a decisive infection-suppressing effect, and a considerable effect was also seen with tolnaftate. Statistical analysis by the χ^2 test also showed outstanding superiority of the 1% piritetrate cream over the 1% tolnaftate cream ($P < 0.05$) after 10 and 17 days of treatment, and significant efficacy was observed with both piritetrate and tolnaftate compared with the two control groups, especially after 11 days of the drug application ($P < 0.001$).

Acute toxicity of piritetrate. As seen in Table 4, all the animals in the groups which had received single or multiple high doses of piritetrate by various routes survived without any toxic effects and increased in body weight normally for at least 1 month of observation after the last administration. Likewise, all the animals in the three vehicle suspension control groups manifested no toxic responses and increased in body weight normally.

DISCUSSION

Although the excellent antidermatophytic activity of tolnaftate in vitro and in vivo as well as in clinics has already been reported (1, 4, 8, 12, 14, 17), in this study piritetrate was found to be more active than tolnaftate in vitro and in vivo, primarily against dermatophytes, irrespective of the tested genera and strains. Furthermore, piritetrate exhibited considerable in vitro activity against other groups of fungi, such as dematiaceous fungi, dimorphic fungi, and some other filamentous fungi, showing some difference among strains, whereas almost all the test species and strains of these groups were much less susceptible to tolnaftate. The yeast fungi were not susceptible to either compound, with the exception of a few strains of *Cryptococcus neoformans* which were considerably susceptible to piritetrate. Such

TABLE 4. Acute toxicity of piritetrate for laboratory animals

Animal (no. tested)	Daily piritetrate dose	Administration route	Duration of administration (days)[a]	No. of animals		
				Dead	With symptoms	With wt loss
Mouse (3)	None; 0.3% sodium carboxymethyl cellulose[b]	Oral	1	0	0	0
Mouse (20)	3,000 mg/kg of BW	Oral	1	0	0	0
Mouse (20)	500 mg/kg of BW	Oral	6	0	0	0
Mouse (20)	None; 0.3% sodium carboxymethyl cellulose[b]	Intraperitoneal	6	0	0	0
Mouse (20)	500 mg/kg of BW	Intraperitoneal	0	0	0	
Mouse (20)	100 mg/kg of BW	Intraperitoneal	6	0	0	0
Guinea pig (2)	None; polyethylene glycol mixture[b]	Topical	14	0	0	0
Guinea pig (5)	0.5 g of 2% cream[c]	Topical	14	0	0	0
Guinea pig (5)	0.5 g of 1% cream[c]	Topical	14	0	0	0

[a] The agent was administered once a day.
[b] Vehicle suspension control group.
[c] Administered onto pretreated skin on the back of each guinea pig.

outstanding differences between the two thiocarbamates in in vitro activity against some fungal species, including the pathogenic fungi of medical importance, will be further studied with an increased number of strains and from various other viewpoints. The recent clinical isolates of dermatophytes were as highly susceptible as their stock strains to piritetrate, which exhibited more potent activity than tolnaftate, tolciclate, and clotrimazole. Comparative in vitro antidermatophytic activities of these reference compounds and other antifungal agents, such as undecylenic acid, miconazole, etc., have been studied; the results obtained seem to be variable (1, 5, 7, 17, 19).

Variation of incubation time, inoculum size, and medium pH within a limited range did not significantly affect the antidermatophytic activity of piritetrate or tolnaftate. However, a substantial increase in the incubation time or inoculum size caused a considerable decrease in the activities of both compounds. Addition of blood to SDA also exerted very little effect on the antifungal activity of both compounds. Luehrs (9) and Weinstein et al. (18) reported that tolnaftate manifested fungicidal activity against dermatophytes. In contrast to this, our findings obtained with the kill curve technique indicated that the antifungal activities of both piritetrate and tolnaftate are fungistatic, not fungicidal, against growing cells (conidia) of *T. mentagrophytes*. Such differences might be due to differences in the test conditions.

We showed that neither piritetrate nor tolnaftate had antibacterial activity against several gram-positive and gram-negative bacteria. This is considered to be a characteristic feature differentiating the in vitro activity of these thiocarbamates from those of the azoles, which are known to possess fairly potent activities against gram-positive bacteria, although with a certain degree of species and genus difference (13, 15).

It is evident from all of the above-mentioned data that piritetrate manifests significantly more potent in vitro antifungal activity, with a broader spectrum, than tolnaftate. However, the overall activity of piritetrate may be considered to be quite comparable with that of tolnaftate, since both are thiocarbamates. Thus, the difference in their activity is regarded as quantitative, not qualitative, particularly in view of the occurrence of clear shifts in their MICs in response to variation of assay factors, such as the incubation time, etc.

The more potent in vitro antidermatophytic activity of piritetrate compared with tolnaftate was also confirmed in vivo in the guinea pig experimental dermal infection models, using a virulent strain of *T. mentagrophytes* with a higher in vitro susceptibility to piritetrate than to tolnaftate. That is, piritetrate was more effective in suppressing the production of lesions and the multiplication of the infecting organisms.

No significant difference was found in the effectiveness of the 1 and 2% piritetrate creams in the suppression of both pathological and mycological responses. This suggests that a 1% piritetrate cream would be preferable for clinical treatment of dermatophytoses, since it would provide sufficient efficacy and also reduce the risk of undesirable side effects. Nevertheless, no symptoms of side effects, such as local irritation or allergy, developed in any of the present experiments on treatment with the cream preparations or in the test for acute toxicity with a massive dose of piritetrate. Our preliminary study showed that a 0.5% piritetrate cream was considerably less effective in the suppression of infection under the same test conditions than the 1% cream and also that no side effects were seen.

The marked efficacy of piritetrate in the treatment of experimental trichophytoses might also hold true for experimental infections with other species of the genus *Trichophyton* and other genera of dermatophytes, such as *Microsporum* and *Epidermophyton*, in view of its potent in vitro antidermatophytic spectrum.

It seems likely that the superior in vitro and in vivo activities of piritetrate compared with tolnaftate are primarily attributable to its pyridine nucleus with a methoxy group (the counterpart moiety in tolnaftate is a benzene nucleus with a methyl group). However, whether the tetrahydronaphthalene moiety of piritetrate (the counterpart moiety in tolnaftate is naphthalene) plays some role the superior experimental results obtained with piritetrate is not clear. In this connection, studies by Morita et al. (10) on the action mechanism of piritetrate in comparison with tolnaftate are of particular interest. They found that piritetrate was a much more potent inhibitor of fungal squalene epoxidation than tolnaftate; in other words, the greater antifungal efficacy of piritetrate compared with tolnaftate was reflected in its greater inhibitory action on sterol biosynthesis.

As noted above, piritetrate showed no acute toxicity in mice or guinea pigs even in large doses by the oral, intraperitoneal, and topical routes when evaluated in terms of mortality, development of symptoms (including signs in systemic and cutaneous allergic responses), and body weight gain. A subacute toxicity study on piritetrate is under way.

In summary, a new thiocarbamate antifungal agent, piritetrate, was found to be much more potent than an analog, tolnaftate, both in vitro and in vivo and did not manifest acute toxicity in laboratory animals. These results suggest great potential for effective clinical use of piritetrate in the treatment of human dermatophytoses.

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