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Anti-*Trichophyton mentagrophytes* Activity and Percutaneous Permeation of Butenafine in Guinea Pigs

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We examined anti-*Trichophyton mentagrophytes* activity, cutaneous penetration, and skin localization of butenafine, a novel benzylamine antifungal agent. The following results were obtained. (i) In the guinea pig dorsal skin trichophytosis model, butenafine produced complete eradication of fungi from infected sites. Clotrimazole was active when animals were infected with 10^4 or 10^5 cells but was almost inactive when the inoculum size was 10^6 cells. (ii) The MICs of butenafine and clotrimazole against arthrospores of *T. mentagrophytes* KD-04 were 0.025 and 0.39 μg/ml, respectively. (iii) When 0.2 ml of a 1% ^{14}C-butenafine solution was applied for 23 h/day for 7 days, high radioactivity corresponding to 250 to 500 μg of butenafine per g of skin in the epidermis, including the horny layer, was observed. (iv) Butenafine penetrates through transepidermal and transfollicular routes. The excellent therapeutic efficacy of butenafine on experimental dermatophytosis may be attributed to its low MIC and good penetration and distribution in the horny layer and hair follicles, where fungi reside.

Butenafine has a broad spectrum of antifungal activity and is particularly active against dermatophytes (1, 5). In experimental fungal infections and clinical studies on superficial mycosis, butenafine demonstrated excellent therapeutic effects (1, 2, 4).

The efficacy of an antimycotic agent for local application is usually determined (i) by its antifungal activity, that is, its potency and spectrum of activity, (ii) by its local tolerance, and (iii) by its pharmacokinetic properties (8). Therapeutic success of antimycotic agents depends on their high bioavailability in sites where fungi reside.

When butenafine was topically applied on the dorsal skin of guinea pigs 48 h before infection with *Trichophyton mentagrophytes*, it produced prophylaxis against the infection (1). Concentrations of butenafine above its minimal fungicidal concentration were achieved in the infected site and maintained for a sufficiently long period of time (1).

In this study, we examined relationships between in vitro and in vivo anti-*T. mentagrophytes* activities of butenafine and the extent of penetration and depth localization in the skin of guinea pigs.

Male Hartley strain guinea pigs (weight, 500 to 700 g) were divided into groups of three to five animals.

Microconidia were harvested from *T. mentagrophytes* KD-04 cultures grown on Sabouraud dextrose agar slants for 3 weeks at 27°C. The culture was flooded with sterile saline containing 0.1% Tween 80. After shaking, the suspension was filtered through gauze to remove hyphal fragments and agar blocks. The suspension was adjusted to make conidial concentrations of 2×10^7, 2×10^6, and 2×10^5 cells per ml by counting with a hemacytometer.

Arthrospores of *T. mentagrophytes* were prepared by the method of Fujita et al. (3). A microconidial suspension (0.1 ml, 10^6 cells) was applied to a membrane filter (diameter, 47 mm; pore size, 0.45 μm; Millipore Corp.), placed on a brain heart infusion agar plate (Nissui Seiyaku, Tokyo, Japan), and cultured in the presence of 17% CO_2 for 7 days at 30°C. Arthrospores were suspended in saline containing 0.1% Tween 80. The cell count was adjusted to 2×10^6/ml.

Butenafine (lot 2000), ^{14}C-labelled butenafine (lot 900118; radioactivity, 44.2 μCi/mg), and clotrimazole (lot 840614) were synthesized in our laboratories. Each drug was dissolved in dimethyl sulfoxide to a concentration of 10 mg/ml. The solution was serially diluted twofold with dimethyl sulfoxide from 1.25 to 10,000 μg/ml for the in vitro test. For in vivo tests, butenafine was dissolved in polyethylene glycol 400–ethanol–water (30:22:50, wt/wt), and clotrimazole was dissolved in polyethylene glycol 400–ethanol (75:25, vol/vol).

Infection was established by the procedure described previously (1). Briefly, hair was plucked by hand from an area (3 by 3 cm) on the backs of the guinea pigs. The skin was lightly abraded with sandpaper, and 50 μl of the inoculum (10^4, 10^5, or 10^6 cells per lesion) was applied with glass rods (day 0). Each guinea pig was topically treated with 0.2 ml of test compound solution. The treatment was started on day 2 postinfection and continued for 10 days. Two days after the last treatment, all animals were sacrificed under ether anesthesia, and 10 skin sections were obtained from each treated site. Each section was implanted into a Sabouraud dextrose agar plate containing 500 μg of cycloheximide per ml, 50 μg of kanamycin per ml, and 5 μg of gentamicin per ml. All plates were incubated at 27°C for 10 days. The treatment was considered effective if no fungal growth was detected. Statistical analyses were done by the Student *t* test. *P* values of less than 0.05 were considered significant.

MICs were determined by the agar dilution method using brain heart infusion agar. One hundred microliters of each diluted drug solution was mixed with 9.9 ml of brain heart infusion agar. Five microliters of the suspension of arthrospores of *T. mentagrophytes* KD-04, corresponding to about 10^4 CFU/ml, was spotted with an inoculator (Microplanter; Sakuma Seisakusho, Tokyo, Japan). The plates were incubated in the presence of 17% CO_2 for 7 days at 30°C. The MICs were defined as the lowest drug concentrations to inhibit visible growth of the fungi.

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TABLE 1. Therapeutic efficacies of butenafine and clotrimazole[a]

Inoculum size (cells/lesion)	Treatment	No. (%) of skin sections with negative cultures
10^4	None (control)	7 (14)
	Butenafine	50 (100)[b]
	Clotrimazole	49 (98)[b]
10^5	None (control)	4 (8)
	Butenafine	50 (100)[b,c]
	Clotrimazole	23 (46)[b]
10^6	None (control)	0 (0)
	Butenafine	50 (100)[b,c]
	Clotrimazole	10 (20)

[a] Administered by a once-daily application for 10 days starting on day 2 after infection. $n = 50$.

[b] Significantly different from the corresponding value for the control group ($P < 0.05$).

[c] Significantly different from the corresponding value for the clotrimazole group ($P < 0.05$).

The permeation of butenafine in the skin was examined by the procedure of Takahashi et al. (7). One day prior to the test, the hair on the dorsal skin was removed successively by electric clippers, a shaver, and hair remover cream, with special care to prevent skin damage. A 200-μl aliquot of a 1% ^{14}C-labelled butenafine solution was applied by sterilized gauze (2 by 2 cm^2), stuck on the prepared site, and covered with Parafilm and an adhesive elastic tape (Elastopore; Nichiban Co., Tokyo, Japan). At 23 h after the application, the gauze was removed. Unabsorbed drug was wiped off with gauze soaked in hot water. The same procedure was repeated at 7 days. At 24, 48, 72, 120, and 168 h after application, the skin section (3 by 3 cm^2) was removed under anesthesia and frozen. Then, each 25-μm piece of thin film was sliced from the superficial side with a microtome. The sliced film was solubilized with Soluene-350 (Packard), neutralized with acetic acid, and measured for radioactivity by a liquid scintillation counter.

The effects of butenafine and clotrimazole on experimental dermatophytosis produced in the dorsal skin of guinea pigs were examined. The animals were infected with 10^4, 10^5, or 10^6 cells of *T. mentagrophytes* KD-04 per lesion. A single daily topical treatment with a 1% solution of butenafine or the reference drug was started on day 2 postinfection and continued for 10 days. As shown in Table 1, butenafine induced complete eradication of fungi from the infected site. Clotrimazole was not active when the animals were infected with 10^6 cells of *T. mentagrophytes*. However, when the animals were infected with 10^4 or 10^5 cells, the rates of negative culture were 98 and 46%, respectively.

T. mentagrophytes parasitizes keratinized tissues such as the horny layer of the epidermis, hair, and nails. It exists in either a hyphal form or an arthrosporic form. Therefore, the susceptibilities of the arthrospores to the drugs were examined. The MICs of butenafine and clotrimazole against arthrospores of *T. mentagrophytes* KD-04 were 0.025 and 0.39 μg/ml, respectively. The activity of butenafine was 16 times greater than that of the reference drug.

The concentrations of butenafine in different layers of guinea pig skin after topical application of a 1% ^{14}C-labelled butenafine solution were examined. As shown in Fig. 1, the highest radioactivity, estimated at 250 to 500 μg of butenafine per g of skin, was observed in the epidermis including the horny layer (depth, 0 to 50 μm). The concentration in this

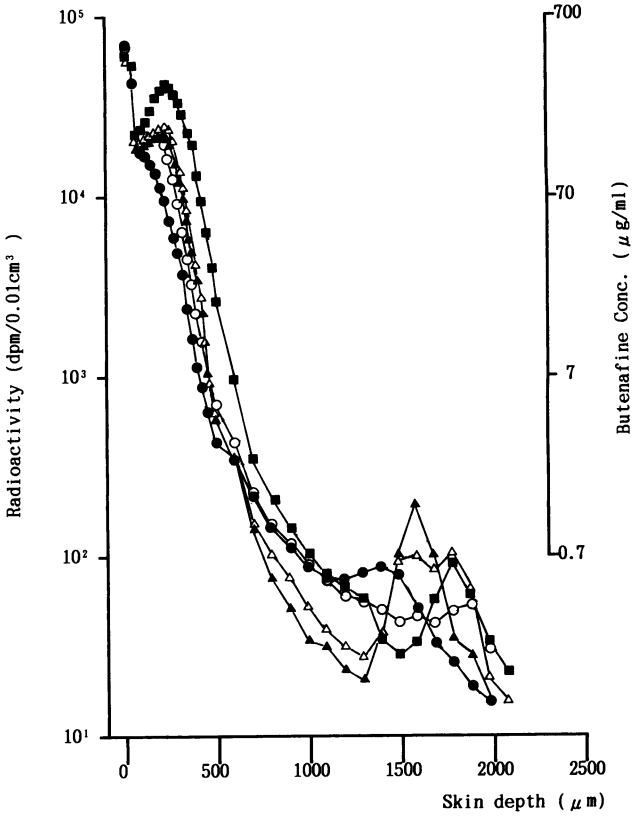

FIG. 1. Distribution of ^{14}C-butenafine in the guinea pig skin after topical application of a 1% solution under occulsion once daily ($n = 3$) on days 1 (●), 2 (○), 3 (▲), 5 (△), and 7 (■).

layer reached a plateau on day 1 or 2 postapplication and remained constant for 7 days. Retention between 100 and 300 μm from the epidermis, where the sebaceous glands are located, was observed. Rapid diffusion and dilution of the drug occurred at deeper levels, and only a low level of radioactivity (less than 1.0 μg per g of butenafine) was found in layers deeper than 1,000 μm. A small peak was detected in the skin as deep as 1,300 to 1,600 μm, indicating that butenafine was absorbed through the hair follicle after accumulation at the sebaceus gland (7). These results demonstrate that butenafine accumulates at a high concentration in the epidermis and penetrates through transepidermal and transfollicular routes.

In the trichophytosis model produced on the backs of guinea pigs, the fungi invade the hair follicle and cause Kerion celsi-like lesions on the dorsal skin. Takahashi reported that this Kerion-producing tendency could be decreased by decreasing the inoculum size (6). The effect of clotrimazole on trichophytosis in guinea pigs was dependent on the inoculum size of *T. mentagrophytes*. However, butenafine had excellent efficacy regardless of inoculum size. The difference between butenafine and clotrimazole in efficacy against *T. mentagrophytes* infection may be associated with their transfollicular penetration as well as their potencies against *T. mentagrophytes*.

These results indicate that butenafine exerts excellent therapeutic efficacy due to potent antifungal activity and good penetration and distribution in the skin.

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