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The anti-*Malassezia furfur* activity *in vitro* and in experimental dermatitis of six imidazole antifungal agents: bifonazole, clotrimazole, flutrimazole, ketoconazole, miconazole and sertaconazole

Die Aktivität von sechs Imidazol-Antimykotika (Bifonazol, Clotrimazol, Flutrimazol, Ketoconazol, Miconazol und Sertaconazol) gegen *Malassezia furfur in vitro* und bei experimenteller Dermatitis

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Key words. *Malassezia furfur*, dandruff, seborrhoeic dermatitis, azole antifungals.
Schlüsselwörter. *Malassezia furfur*, Schuppen, seborrhoische Dermatitis, Azole.

Summary. Bifonazole, clotrimazole, flutrimazole, ketoconazole, miconazole and sertaconazole were tested for their activity against 23 isolates of *Malassezia furfur* by agar dilution *in vitro*. Topical formulations of the same agents were evaluated for efficacy against *M. furfur* skin infections in guinea pigs *in vivo*. The most potent inhibitor *in vitro* was ketoconazole (geometric mean minimum inhibitory concentration 0.51 μg ml^{-1}), followed by bifonazole (8.1 μg ml^{-1}), then miconazole (14 μg ml^{-1}), clotrimazole (15 μg ml^{-1}) and flutrimazole (16 μg ml^{-1}), with sertaconazole the least active (52 μg ml^{-1}). In animal experiments involving three consecutive days of topical treatments, bifonazole 1% cream, clotrimazole 1% cream, flutrimazole 1% and 2% creams, ketoconazole 2% cream and shampoo and miconazole 2% cream all reduced *M. furfur* dermatitis lesion severity below that of untreated control animals; however, sertaconazole 2% gel and cream showed no reduction in lesion severity below control. The results confirm that ketoconazole is a more potent inhibitor of *M. furfur in vitro* than other topical antifungal agents of its class and suggest that sertaconazole is the least effective of such agents among those tested.

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Zusammenfassung. Bifonazol, Clotrimazol, Flutrimazol, Ketoconazol, Miconazol und Sertaconazol wurden *in vitro* mittels Agardilution auf ihre Aktivität gegen 23 Isolate von *Malassezia furfur* getestet. Topische Formulierungen der gleichen Imidazole wurden *in vivo* auf ihre Wirksamkeit gegen *M. furfur*-Hautinfektionen in Meerschweinchen untersucht. *In vitro* war der potenteste Inhibitor Ketoconazol (geometrisches Mittel MIC = 0.51 μg ml^{-1}), gefolgt von Bifonazol (8.1 μg ml^{-1}), Miconazol (14 μg ml^{-1}), Clotrimazol (15 μg ml^{-1}), Flutrimazol (16 μg ml^{-1}) und dem am wenigsten aktiven Sertaconazol (52 μg ml^{-1}). In Tierexperimenten, die drei aufeinanderfolgende Tage topische Behandlung umfaßten, reduzierten Bifonazol 1% Creme, Clotrimazol 1% Creme, Flutrimazol 1% und 2% Cremes, Ketoconazol 2% Creme und Shampoo sowie Miconazol 2% Creme alle den Schweregrad der *M. furfur* Hautläsionen unter den von unbehandelten Kontrolltieren; Sertaconazol 2% Gel und Creme zeigte jedoch keine Verringerung des Schweregrades der Läsionen unter die der Kontrolle. Die Ergebnisse bestätigen, daß Ketoconazol in vitro ein potenterer Inhibitor von *M. furfur* als andere topische antifungale Wirkstoffe seiner Klasse ist und sprechen dafür, daß Sertaconazol unter den getesteten Imidazolen am wenigsten wirksam ist.

Introduction

Ketoconazole 2% cream and 1% and 2% shampoo preparations are effective medications for the

treatment of seborrhoeic dermatitis and dandruff [1–7], multifactorial conditions in which the yeast *Malassezia furfur* plays a dominant contributory role [8]. Other azole antifungal agents, including econazole [9], miconazole [10] and sertaconazole [11], have been evaluated clinically as topical therapies for *M. furfur*-related dermatoses. The present study was undertaken to re-examine the comparative potencies of several azole derivatives against *M. furfur in vitro* and in an animal model of cutaneous *M. furfur* infection that produces erythema, folliculitis and hyperkeratosis characteristic of *M. furfur*-related dermatoses in man.

Materials and methods

Culture medium

For all experiments, Dixon medium was used to cultivate *Malassezia furfur* isolates. The medium comprised (g l^{-1}) malt extract (Difco, Detroit, USA) 40 g, Bacto oxgall (Difco) 20 g, Tween 40 (Merck, Darmstadt, Germany) 10 ml, glycerol (Difco) 2.5 ml and agar, when required, 20 g. The medium was sterilized in an autoclave at 115 °C for 10 min.

Test compounds

Miconazole and ketoconazole pure compounds were synthesized at the Janssen Research Foundation, Beerse, Belgium. Clotrimazole and bifonazole were obtained from Bayer, Wuppertal, Germany. Flutrimazole and sertaconazole were synthesized at the Janssen Research Foundation, Toledo, Spain. Stock solutions of all agents were prepared at concentrations of 5 mg ml^{-1} in dimethylsulphoxide (DMSO), and doubling dilutions of these solutions were also prepared in DMSO. One millilitre volumes of each dilution were then added to 49-ml volumes of Dixon agar held molten at 60 °C in a water bath, thus producing a dilution series with final concentrations from 100 μg ml^{-1} to 0.0063 μg ml^{-1}. For control plates, 1 ml of DMSO was added to 50 ml of molten medium. The mixtures were stirred and distributed in 1-ml volumes in the chambers of Repli-dishes (Sterilin).

For animal experiments, only flutrimazole was unavailable in a commercial formulation for testing, and this compound was prepared as 1% and 2% creams in a carbowax excipient. Bifonazole 1% cream (Mycospor®, Bayer), clotrimazole 1% cream (Canesten®, Bayer), ketoconazole 2% cream and 2% shampoo (Nizoral®, Janssen), miconazole 2% cream (Daktarin®, Janssen) and sertaconazole 2% cream and 2% gel (Ferrer) were purchased for the experiments.

Susceptibility of Malassezia furfur *in vitro*

A total of 23 *M. furfur* isolates were tested for susceptibility to the various azole antifungal agents *in vitro*. The isolates all came originally from clinical samples and had been maintained at −70 °C with glycerol as cryoprotectant. Inocula were prepared as 4-day static cultures in Dixon broth incubated at 37 °C. The suspensions were diluted with sterile water so that a 10-fold dilution gave an OD of 0.1 at 530 nm. A 25-loop multiple inoculating device was used to place volumes of approximately 30 μl on the test media. The cultures were incubated at 37 °C for 4 days and minimum inhibitory concentrations (MICs) were recorded as the lowest concentrations at which no growth of *M. furfur* was visible.

Animal model of Malassezia furfur *infection*

The method used was that devised by Van Cutsem *et al.* [12]. *M. furfur* isolate B39387 was cultured for 7 days in Dixon broth at 37 °C. The cultures were centrifuged and resuspended in one-tenth of the original volume of sterile physiological saline, to produce a suspension containing $1–5 \times 10^7$ CFU ml^{-1} (confirmed by plating on Dixon agar). The suspension was divided into smaller lots and stored at 4 °C.

Eighty-eight albino guinea pigs were used for the experiments. All animals received water and non-medicated food *ad libitum* and were caged individually. Dorsal hair was removed with an electric clipper, then 0.25-ml volumes of inoculum suspension were applied to the central area of intact dorsal skin once each day for seven consecutive days. The infection site was not occluded. The lesions were scored for severity 24 h after the last inoculation. A score of 4 indicated severe erythema, folliculitis and hyperkeratosis. Scores of 3, 2 and 1 indicated lesion severity judged as approximating to 75%, 50% and 25% of maximal severity respectively. A score of 0 indicated no visible lesion. The animals were divided into test groups for treatment on the basis of the lesion scores 24 h after the last infection so as to include animals with an equivalent range of initial lesion severity in each group.

Treatments were begun 24 h after the last infecting dose of *M. furfur* was applied and was repeated on the two following days (i.e. a total of three treatments per animal). For all products, 1 g or 1 ml was applied to the infected site. In the case of ketoconazole shampoo and sertaconazole

gel, the product was left in contact with the infected area for 30 min then the area was thoroughly rinsed off with warm water. The severity of the lesions was rescored 1 week and 2 weeks after the first treatment day.

Results

Activity of azole antifungal agents against Malassezia furfur *in vitro*

The MICs of six azole derivatives for 23 *M. furfur* isolates are summarized in Table 1. Ketoconazole was clearly the most potent inhibitor of *M. furfur* in these agar dilution tests: all of the test isolates were inhibited by ketoconazole at 1.6 $\mu g\ ml^{-1}$ or lower concentrations. Sertaconazole was the least potent antifungal tested, with only 13 of the 23 isolates inhibited at 100 $\mu g\ ml^{-1}$, the highest concentration tested. The other four azole derivatives had anti-*M. furfur* activity *in vitro* intermediate between the activities of ketoconazole and sertaconazole.

Activity of azole antifungal agents against Malassezia furfur *infections in vivo*

In all of the animals treated with three of the test preparations (bifonazole, clotrimazole and miconazole 2% creams) markedly erythematous areas, sometimes associated with hyperkeratosis, were seen 1 week after the first treatment. The area involved was much broader than the original infected areas and the reaction was therefore assumed to be treatment related. No lesion score was recorded for these animals at this time point.

Table 2 summarizes the results of the animal tests. Of the agents tested, only sertaconazole 2% gel and 2% cream appeared to be ineffective in influencing the evolution of the infection: the lesion scores for these preparations were equivalent to those for untreated control guinea pigs both 1

Table 1. Activity of six azole antifungal agents against 23 isolates of *M. furfur* in agar dilution tests *in vitro*

Antifungal agent	Cumulative percentage of 23 *M. furfur* isolates inhibited at ($\mu g\ ml^{-1}$)													MIC statistics ($\mu g\ ml^{-1}$)		
	0.05	0.1	0.2	0.4	0.8	1.6	3.2	6.3	13	25	50	100	>100	MIC_{50}*	MIC_{90}*	Gmean*
Bifonazole	0	0	4	4	4	35	39	52	61	65	100	100	100	6.3	50	8.1
Clotrimazole	0	0	0	0	0	9	30	48	52	61	74	100	100	13	100	15
Flutrimazole	0	0	0	0	0	9	35	52	52	57	61	100	100	6.3	100	16
Ketoconazole	0	13	39	48	65	100	100	100	100	100	100	100	100	0.8	1.6	0.51
Miconazole	0	0	0	0	4	13	43	57	57	61	61	91	100	6.3	100	14
Sertaconazole	0	0	0	0	4	4	4	13	22	43	48	57	100	100	>100	52

* MIC_{50}: MIC value for 50% of isolates tested. MIC_{90}: MIC value for 90% of isolates tested. Gmean: geometric mean MIC.

Table 2. Activity of topical formulations of azole antifungal agents *vs. M. furfur* infections in guinea pigs. Data show the mean ± SD values of lesion scores for each group of animals tested, at weekly intervals. Treatments were applied daily for three consecutive days

Treatment	No. of animals	Mean lesion score ± SD on		
		Day of first treatment	After 1 week	After 2 weeks
None (control)	14	2.7 ± 1.1	2.4 ± 1.2	1.6 ± 1.3
Bifonazole 1% cream	6	2.7 ± 1.0	Not read*	0.6 ± 0.4
Clotrimazole 1% cream	6	2.7 ± 1.0	Not read*	0.5 ± 0.4
Flutrimazole 1%†	6	2.8 ± 1.2	1.1 ± 0.8	0.2 ± 0.3
Flutrimazole 2%†	6	2.7 ± 1.4	1.3 ± 1.1	0.5 ± 0.8
Ketoconazole 2% cream	14	2.6 ± 1.1	1.6 ± 1.2	0.4 ± 0.5
Ketoconazole 2% shampoo	14	2.6 ± 1.0	1.0 ± 1.1	0.3 ± 0.4
Miconazole 2% cream	6	2.5 ± 1.0	Not read*	0.3 ± 0.3
Sertaconazole 2% cream	8	2.8 ± 1.0	3.9 ± 0.4	1.6 ± 0.7
Sertaconazole 2% gel	8	2.8 ± 1.0	2.9 ± 1.4	1.3 ± 0.8

* Area of erythema/hyperkeratosis appeared to be related to zone of treatment, not to *M. furfur* lesions.
† prepared in carbowax excipient.

week and 2 weeks after the first treatment day. All the other preparations tested appeared to be similarly active in reducing the severity of experimental skin lesions resulting from *M. furfur* infection.

Discussion

Relatively few studies have examined the comparative anti *M. furfur* activity of azole derivatives that can be formulated for topical use. Although ketoconazole is probably the most extensively tested of such agents in clinical trials [1–8], other azole compounds are candidates for current or possible future clinical development against dandruff and seborrhoeic dermatitis [9–11]. The efficacy of such agents ultimately requires evaluation in clinical trials, but data from preclinical experiments with *M. furfur* can also provide useful predictive information for selection of other candidates.

Our finding that ketoconazole was the most active of several azole antifungals tested against 23 *M. furfur* isolates *in vitro* matches those obtained by Faergemann [13] with clotrimazole, econazole, itraconazole, ketoconazole and miconazole and by Nenoff & Haustein [14] with bifonazole, clotrimazole, fluconazole, itraconazole, ketoconazole and tioconazole. In both studies the MIC values for ketoconazole were approximately 10 times lower than for the other azole derivatives studied, with the exception of itraconazole, which approached ketoconazole in potency [14]. This is a difference of a magnitude similar to that found in the present study. Drouhet & Dupont [15] compared MICs for miconazole and sertaconazole against three *M. furfur* isolates and found that their activities *in vitro* were of a similar order. If these various published findings and those of the present study are considered together, the conclusion that ketoconazole is a more potent inhibitor of *M. furfur* than virtually all other known topical azole antifungal agents would seem inescapable. The ketoconazole MIC_{90} of 1.6 μg ml^{-1} determined in this study (Table 1) is identical to the results published by Van Cutsem *et al.* [16] for 83 *M. furfur* isolates also tested in Dixon broth but at 25 °C.

Our studies *in vivo* showed treatment efficacy relative to control for bifonazole, clotrimazole, flutrimazole, ketoconazole and miconazole topical formulations against experimental skin infections caused by *M. furfur*. Animals treated with sertaconazole gel and sertaconazole cream, however, showed no difference in lesion severity in comparison with untreated control animals. The treatments were applied daily for only 3 days, and the quantitative results with ketoconazole (Table 2) confirm those previously obtained with this agent [12]. So far, no comparative, double-blind clinical studies have yet been published in which other azole-derivative antifungal agents are directly evaluated against ketoconazole. The results of the present study suggest that ketoconazole possesses a more potent anti- *M. furfur* activity *in vitro* and *in vivo* than other compounds of its chemical type, and that it should retain its present position as gold standard among the topical azoles for the treatment of *M. furfur*-related diseases.

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References

1 Cauwenbergh, G., De Doncker, P., Schrooten, P. & DeGreef, H. (1986) Treatment of dandruff with a 2% ketoconazole scalp gel. *Int. J. Dermatol.* **25**, 541.
2 Faergemann, J. (1990) Treatment of seborrhoeic dermatitis of the scalp with ketoconazole shampoo. *Acta Dermatol. Venereol.* **70**, 171–172.
3 Berger, R., Mills, O. H., Jones, E. L. & Mrusek, S. (1990) Double-blind, placebo-controlled trial of ketoconazole 2% shampoo in the treatment of moderate to severe dandruff. *Adv. Ther.* **7**, 247–255.
4 Ive, F. A. (1991) An overview of experience with ketoconazole shampoo. *Br. J. Clin. Pract.* **45**, 279–284.
5 Arlette, J., Giroux, J. M., Maddin, S. W., Ross, B. & Shear, N. H. (1991) Ketoconazole shampoo in seborrheic dermatitis. *Can. J. Dermatol.* **3**, 175–180.
6 Danby, F. W., Maddin, W. S., Margesson, L. J. & Rosenthal, D. (1993) A randomized, double-blind, placebo-controlled trial of ketoconazole 2% shampoo versus selenium sulfide 2.5% shampoo in the treatment of moderate to severe dandruff. *J. Am. Acad. Dermatol.* **29**, 1008–1012.
7 Go, I. H., Wientjens, D. P. W. M. & Koster, M. (1992) A double-blind trial of 1% ketoconazole shampoo versus placebo in the treatment of dandruff. *mycoses* **35**, 103–105.
8 Degreef, H., Jacobs, P. H., Rosenberg, E. W. & Shuster, S. (eds.) (1989) *Ketoconazole in Seborrhoeic Dermatitis and Dandruff: A Review.* Manchester: ADIS Press, pp. 1–11, 32–48.
9 Aron-Brunetiere, R., Dompmartin-Pernot, D. & Drouhet, E. (1977) Treatment of pityriasis capitis (dandruff) with econazole nitrate. *Acta Dermatovenereol.* **57**, 77–80.
10 Sheth, R. A. (1983) A comparison of miconazole nitrate and selenium disulfide as anti-dandruff agents. *Int. J. Dermatol.* **22**, 123–125.
11 Alsina, M. & Zemba, C. (1994) Sertaconazol en la dermatitis seborreica del cuero cabelludo. *Med. Cut.* **22**, 111–115.
12 Van Cutsem, J., Van Gerven, F., Fransen, J., Schrooten, P. & Janssen, P. A. J. (1990) The *in vitro* antifungal activity of ketoconazole, zinc pyrithione, and selenium sulfide against *Pityrosporum* and their efficacy as a shampoo in the treatment of experimental pityrosporosis in guinea pigs. *J. Am. Acad. Dermatol.* **22**, 993–998.
13 Faergemann, J. (1988) *In vitro/in vivo* sensitivity of *Pityrosporum ovale* to antifungal agents. 2nd International

Skin Therapy Symposium, Antwerp, May 5, 1988. Janssen Pharmaceutica, pp. 24–26.
14 Nenoff, P. & Haustein, U.-F. (1994) Der Effekt antiseborrhoischer Substanzen gegenüber *Pityrosporum ovale in vitro*. *Hautarzt* **45**, 464–467.
15 Drouhet, E. & Dupont, B. (1992) *In vitro* antifungal activity of sertaconazole. *Arzneim. Forsch.* **42**, 705–710.
16 Van Cutsem, J., Van Gerven, F., Van Peer, A., Woestenborghs, R., Fransen, J. & Janssen, P. A. J. (1988) Kétoconazole: activité *in vitro* sur *Pityrosporum*. Efficacité dans la pityrosporose expérimentale du cobaye et dans le pityriasis capitis humain. *Bull. Soc. Fr. Mycol. Méd.* **17**, 283–294.