Original article

Itraconazole corneofungimetry bioassay on *Malassezia* species

Itraconazol-Korneofungimetrie-Bioassay an Malassezia-Arten

G. E. Piérard, J. E. Arrese and C. Piérard-Franchimont

Department of Dermatopathology, University Medical Center of Liège, Liège, Belgium

Summary

Yeasts of the genus *Malassezia* are part of the normal skin biocenosis and are involved in a series of distinct skin disorders and specific dermatomycoses in man and animals. Several species are currently distinguished. Their relative *in vitro* susceptibility to antifungals appears different according to the species and to the nature and route of administration of the drug. Corneofungimetry is an *ex vivo* bioassay allowing to test the fungal response on human stratum corneum following oral intake of a given antifungal by volunteers. Two series of cyanoacrylate skin surface strippings (CSSS) were harvested from the volar forearm of 30 volunteers before and after a 2-week treatment with itraconazole 200 mg daily. They were coated by olive oil and inoculated with suspensions of seven different *Malassezia* spp. After a 1-week culture on CSSS, the amount of viable yeasts was assessed using neutral red staining assisted by computerized image analysis. Growth of the seven species was not similar on the CSSS from untreated stratum corneum. The ranking order from the most proliferative to the least was *M. restricta*, *M. sympodialis*, *M. globosa*, *M. furfur*, *M. obtusa*, *M. slooffiae* and *M. pachydermatis*. Their growth was abated to almost the same level after itraconazole treatment. It is concluded that *in vivo* treatment with itraconazole is highly active against all *Malassezia* spp. colonizing the human stratum corneum.

Zusammenfassung

Hefen der Gattung Malassezia sind Teil der normalen Hautbiozönose und beteiligt an einer Reihe von Hautkrankheiten und spezifischen Dermatomykosen bei Mensch und Tier. Gegenwärtig werden einige Arten unterschieden. Ihre relative In-vitro-Suszeptibilität für Antimykotika ist abhängig von der Erregerart, den antimyzetischen Eigenschaften und der Anwendungsweise. Die Korneofungimetrie ist ein Ex-vivo-Ansatz der es erlaubt, die Pilzreaktion nach oraler Wirkstoff-Aufnahme auf dem Stratum corneum Freiwilliger auszutesten. Zwei Zyanoakrylat-Haut-Strippingserien (CSSS) wurden volarseitig am Unterarm von 30 Freiwilligen entnommen, vor und nach einer 2-Wochen-Itraconazol-Anwendung von 200 mg täglich. Sie wurden mit Olivenöl überschichtet und mit Suspension der sieben Malassezia-Arten beimpft. Nach einwöchiger CSSS-Kultur wurde die Lebendkeimzahl mittels Neutralrot-Färbung im computerisierten Bildzählverfahren bestimmt. Das Hefe-Wachstum in den unbehandelten Kontrollen unterschied sich von Art zu Art: Die Wachstumsdichte nahm in der Reihenfolge *M. restricta*, *M. sympodialis*, *M. globosa*, *M. furfur*, *M. obtusa*, *M. slooffiae* und *M. pachydermatis* ab. Diseses Wachstum wurde durch Itraconazol auf etwa gleich niedrige Raten verringert. Daraus wird geschlossen, dass

Correspondence: Professor Gérald E. Piérard, Department of Dermatopathology, CHU Sart Tilman, B-4000 Liège, Belgium.
Tel.: 32-4366-24-08. Fax: 32-4366-29-76.
E-mail: gerald.pierard@ulg.ac.be

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Itraconazol in vivo hochaktiv gegen alle Malassezia-Arten ist, welche das messnchliche Stratum corneum besiedeln.

Key words: *Malassezia*, corneofungimetry, stripping, bioassay, itraconazole.

Schlüsselwörter: *Malassezia*, Korneofungimetrie, Stripping, Bioassay, Itraconazol.

Introduction

Yeasts of the genus *Malassezia* are components of the normal skin bioecene. They are opportunistic microorganisms involved in or responsible for a series of skin disorders including dandruff, seborrheic dermatitis, pityriasis versicolor, seborrheic blepharitis, *Malassezia* folliculitis, confluent and reticulate papillomatosis and even septicemia in some susceptible individuals. The genus *Malassezia* comprises species distinct by morphological, ultrastructural, physiological and molecular aspects.[1–3] Currently, several distinct species are taxonomically distinguished. They are *M. furfur*, *M. globosa*, *M. obtusa*, *M. pachydermatis*, *M. restricta*, *M. slooffiae*, *M. sympodialis* and a few others.

Variations in the *in vitro* susceptibility to different antifungals were reported for the seven *Malassezia* species.[4] The clinical relevance of the *in vitro* data is indirect because the antifungal activity of metabolites of the antifungals may be involved *in vivo* as well. Many other pharmacokinetic factors also participate in the clinical efficacy, including penetration and accumulation in the stratum corneum, as well as synergism or counteracting effect mediated by molecular components of the stratum corneum. To overcome these drawbacks, the corneofungimetry test was designed in order to complement the classical *in vitro* antifungal testing.[5] This *ex vivo* bioassay is based on the fact that different fungal pathogens can grow on normal stratum corneum harvested from human skin.[6–10] The efficacy of antifungals can be assessed using this method after applying them topically[11–15] or after oral intake.[16–19] Corneofungimetry has several advantages over some other current *in vitro* tests. It is indeed possible to test the final formulations of the antifungals as they are used in clinical practice. In addition, the fungus has to grow on human stratum corneum which is the natural substrate relevant for superficial dermatomycoses. This material contains both growth-promoting and growth-inhibiting factors for fungal pathogens.[18]

This study was performed to assess and compare the efficacy of oral itraconazole on the seven *Malassezia* species using the corneofungimetry bioassay.

Materials and methods

Two series of seven cyanoacrylate skin surface strippings (CSSS) were harvested from the volar aspect of the forearms in 30 healthy volunteers. Briefly, a cyanoacrylate adhesive (Super Glue; Loctite, Brussels, Belgium) was dropped onto a supple 175 μm-thick sheet of terephthalate polyethylene (Melinex O; ICI Plastics Division, Snij-Unie Hifi Zoutketen, Enkhuisen, The Netherlands), which was then deposited onto the skin. After about 30 s, a sheet of stratum corneum was harvested by gently lifting the piece of polyethylene. The first series of CSSS was collected at baseline when the subjects were out of any drug for at least 4 months. The second series of CSSS was collected from the same subjects after a 2-week treatment with itraconazole 200 mg daily. Each CSSS was dipped into olive oil and any excess was absorbed by sterile gauze. These CSSS served as substrate for the *ex vivo* cultures of fungi on the human stratum corneum.

Seven *Malassezia* species were provided by the Fungal Biodiversity Centre, Utrecht (Table 1). They were inoculated on fresh Modified Leeming and Notman Agar medium. After a 2-week primary culture, fungal cells were collected, dispersed in physiological saline and adjusted to a concentration of 10^4–10^5 cells ml^{-1}. A sample of 250 μl of each suspension was deposited onto the centre of the oil-coated CSSS. Indeed, this substrate corresponds to the underface of the intact stratum corneum sample which never harbour fungi of the skin surface resident flora. The material was placed on trays, covered with lids and stored at 27 °C in a moist environment (relative

Table 1 Test microorganisms of the genus *Malassezia.*

Species	Strains
M. furfur	CBS 7019
M. globosa	CBS 7966
M. obtusa	CBS 7876
M. pachydermatis	CBS 1879
M. restricta	CBS 7877
M. slooffiae	CBS 7956
M. sympodialis	CBS 7222

humidity 80 ± 2%) for 1 week. Samples were stained with the vital stain neutral red in order to reveal the living fungal cells.[18, 20] The area covered by the living *Malassezia* cells was expressed in arbitrary units by means of computerized image analysis (Analysis Olympus, Osaka, Japan).

For each fungal species, the mycelial area was expressed as medians and ranges. As inter-sample differences were observed in the growth of the different fungal species on untreated CSSS, growth inhibition was also expressed as a percentage relative to the baseline untreated specimens. Growth of the different *Malassezia* species was compared both before and after itraconazole treatment using the paired non-parametric Friedman test followed by the Dunn test. The statistical comparison between the controls and the itraconazole-treated specimens was made by the two-tailed paired Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Growth of the seven *Malassezia* species was not similar on the untreated human stratum corneum (Table 2). *Malassezia pachydermatis* grew significantly to a lesser extent than *M. slooffiae* ($P < 0.05$), *M. furfur* ($P < 0.001$), *M. globosa* ($P < 0.001$), *M. restricta* ($P < 0.001$) and *M. sympodialis* ($P < 0.001$). *Malassezia obtusa* also grew significantly to a lesser extent than *M. globosa* ($P < 0.01$), *M. restricta* ($P < 0.001$) and *M. sympodialis* ($P < 0.001$). *Malassezia slooffiae* grew significantly to a lesser extent than *M. restricta* ($P < 0.05$) and *M. sympodialis* ($P < 0.001$).

Growth of all *Malassezia* species was significantly abated ($P < 0.001$) after the 2-week itraconazole treatment. In addition, the inter-species differences in growth became much less prominent after treatment (Table 2). As a consequence, the percentages of *Malassezia* growth inhibition by itraconazole relative to the baseline untreated situation were different for the seven species (Table 3). *Malassezia pachydermatis* grew significantly less than *M. globosa* ($P < 0.05$), *M. obtusa* ($P < 0.01$) and *M. sympodialis* ($P < 0.05$).

Table 2 Corneofungimetry data on *Malassezia* species (arbitrary units, median and range).

Species	Untreated CSSS at baseline	2-week itraconazole treatment
M. restricta	7010 (5114–8227)	3600 (2276–4863)
M. sympodialis	7007 (5399–8538)	3779 (2777–4680)
M. globosa	6558 (5062–8933)	3653 (2536–5846)
M. furfur	6445 (3255–8320)	3478 (1985–5647)
M. obtusa	5934 (3996–8041)	3598 (2342–5280)
M. slooffiae	5931 (4111–8004)	3687 (2619–5132)
M. pachydermatis	4107 (2115–6982)	3066 (1899–6859)

Table 3 Percentage of *Malassezia* growth inhibition by itraconazole (%, median and range).

Species	%
M. restricta	49.5 (23.1–64.3)
M. sympodialis	47.4 (28.4–61.8)
M. globosa	44.2 (8.6–63.7)
M. furfur	44.1 (–18.6–71.8)
M. slooffiae	39.6 (–0.4–61.1)
M. obtusa	32.6 (–14.1–61.3)
M. pachydermatis	19.6 (–59.1–63.5)

Discussion

It is acknowledged that the non-lipophilic *M. pachydermatis* is associated with animals. By contrast, the six lipid-dependent species are recovered from normal human skin, dandruff and diseased human skin. Among these fungal species, *M. slooffiae* is also present in animal skin. Previous observations had shown that *Malassezia* spp. were able to grow *in vitro* on human stratum corneum.[7, 8] In these studies, the species had not been identified according to the current taxonomy. The present findings might indicate that *M. pachydermatis* adhere less to and/or proliferate less than the other species on unmedicated human stratum corneum. This situation might play a role for the non-involvement of this yeast in the human skin biocenosis and physiopathology. The bioassay also suggests that *M. globosa*, *M. restricta* and *M. sympodialis* are the lipid-dependent species which are the most prone to colonize the human stratum corneum.

A previous study demonstrated the *in vitro* efficacy of itraconazole against the various *Malassezia* species.[4] The drug appeared almost equally active against them, although the sensitivity may somewhat differ according to strains. The present corneofungimetry bioassay brings new insight. Data are likely close to the clinical situation because the tested antifungal activity is dependent on all the pharmacokinetic variables of the drug and its metabolites. It also integrates any influence of human stratum corneum components on the *Malassezia* biocenosis. Data show that itraconazole abates the density of living yeasts on human stratum corneum to a level almost similar for all *Malassezia* species. Possible differences between strain responses were not tested in this bioassay.

The inter-sample range of variation in the fungal spread was almost similar on untreated and itraconazole-treated specimens. Previous unpublished observations had shown similar variability when a given fungal strain was tested on different CSSS harvested from the same individual. Thus, we interpret the variability as a limitation in precision of the bioassay rather than any interindividual differences in stratum corneum capacity to harbour *Malassezia* yeasts. It is inferred that the present variability cannot be ascribed to therapeutic inconsistency.

In conclusion, corneofungimetry showed that natural growth of seven *Malassezia* species on the human stratum corneum is not equal. Oral itraconazole at usual therapeutic dosage abates the accumulation of yeasts to almost the same level for different *Malassezia* species. This implies that the precise species identification of *Malassezia* is not necessary for the *Malassezia*-driven disorders before initiating oral itraconazole treatment.

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