Article:
Gaitanis, G, Magiatis, P, Mexia, N et al. (4 more authors) (2019) Antifungal activity of selected Malassezia indolic compounds detected in culture. Mycoses, 62 (7). pp. 597-603. ISSN 0933-7407

https://doi.org/10.1111/myc.12893

PROFESSOR GEORGIOS GAITANIS (Orcid ID : 0000-0001-5482-1304)

Article type : Original Article

Antifungal activity of selected Malassezia indolic compounds detected in culture

Georgios Gaitanis,*[1] Prokopios Magiatis,[2] Nikitia Mexia,[2] Eleni Melliou,[2] Maria A. Efstratiou,[3] Ioannis D. Bassukas,[1] Aristea Velegraki[4,5]

[1]Department of Skin and Venereal Diseases, Faculty of Medicine, School of Health Sciences, University of Ioannina, Ioannina, Greece

[2] Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, National and Kapodistrian University of Athens, Panepistimioupolis, Athens, Greece.

[3] Department of Marine Sciences, University of the Aegean, Mytilene, Greece

[4] Mycology Research Laboratory and UOA/HCPF Culture Collection, Department of Microbiology, Medical School, National and Kapodistrian University of Athens

[5]Bioiatriki SA, Athens, Greece

Short Title: Antifungal activity of Malassezia indoles

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/myc.12893

Key words: Malassezia, indoles, tryptophan, indirubin, antifungal

Corresponding Author:

Georgios Gaitanis

"S. Niarchou" Avenue, Department of Skin and Venereal Diseases, Faculty of Medicine, School of Health Sciences, University of Ioannina, Ioannina, Greece

Tel: 00302651007530 Fax: 00302651007031

Email: ggaitan@cc.uoi.gr

Abstract

Background

Malassezia yeasts produce bioactive indolic substances when grown on L-tryptophan agar. A panel of these substances was tested against commensal and opportunistic fungi, the Minimum Inhibitory Concentration (MIC) was determined and the potential for in loco antifungal activity on the skin was assessed.

Materials and Methods

Eight indoles were included (malassezin, pityriacitrin, indirubin, indolo[3,2b]carbazole, formyl-indolo[3,2 b]carbazole, tryptanthrin, 6-hydroxymethyl-indolo[3,2-b]carbazole and 6-methyl-indolo[3,2-b]carbazole) and were tested against 40 fungal strains [yeasts: Malassezia spp.(N=9); Cryptococcus spp.(N=10); Candida spp.(N=7); Yarrowia lipolytica(N=1); Exophialla dermatitidis

(N=2); molds: Aspergillus spp.(N=7); Fusarium spp.(N=2); Rhizopus oryzae(N=2)]. The concentration of 5/8 of the tested indoles on diseased skin was calculated from published data. Kruskal-Wallis and U-Mann-Whitney tests were employed for group susceptibility evaluation in 33 strains.

Results

The MIC range was 0.125-32μg/ml and the median log_2MIC was 4. Indirubin was the most potent antifungal agent and differed significantly from the others. The highest median MIC was found for FICZ.

Malassezia with Candida strains were more susceptible compared to Cryptococcus and Aspergillus and this inhibitory activity was predicted to be valid also on human skin.

Conclusions

Malassezia yeasts produce indolic species that inhibit an array of clinically significant yeasts and molds.

Introduction

The human skin harbors an array of microbial species and microorganisms with Malassezia yeasts being the principal eukaryotic organisms.[1,2] This genus currently includes 17 species [3] and in humans has been associated with the development of pityriasis versicolor (PV), dandruff (DF)/seborrheic dermatits (SD) and certain head and neck manifestations of atopic dermatitis.[4] All these common skin conditions are characterized by a variably compromised skin barrier.[5,6]

Initially it was reported that M. furfur strains have the ability to produce indolic substances when grown in a medium with L-tryptophan as the exclusive nitrogen source.[7] Subsequently, it was found that under the aforementioned culture conditions M. furfur strains isolated from SD and PV lesions have the ability to synthesize in vitro significantly larger quantities of indoles compared to those isolated from healthy skin.[8] Furthermore, it has become clear that this property is not restricted to M. furfur but is wider distributed among more species within the genus, while Malassezia and their respective indoles can be also traced in skin scales from SD and PV patients.[9] Important molecules of this group include malassezin, indirubin, indolo-[3,2b]-carbazole (ICZ), pityriacitrin [10] and formyl-indolo–[3,2b]-carbazole (FICZ). Among potential biological effects of these substances preliminary data indicate that indirubin possesses some antifungal[11] and antiparasitic[12] activity, whilst it could also potentiate the antibacterial effect of ciprofloxacin against resistant S. aureus strains in vitro.[13] Moreover, pityriacitrin has a small, yet measurable, sunlight protective factor (SPF: 1.7) at 5% concentration[14] and FICZ is considered a candidate endogenous aryl-hydrocarbon receptor (AhR) ligand and an ultraviolet damage mediator on human skin.[15] Regarding indirubin it mediates functions as inflammation and tumorigenesis[16] and is currently suggested as a potent topical treatment for psoriasis.[17] The production of these ligands from L-tryptophan is not restricted to yeasts of the Malassezia genus but has also been described in the ascomycetous gastrointestinal truck commensal C. glabrata underscoring the wider biological significance of this pathway.[18]

Aim of this study was to screen indoles detectable in Malassezia cultures for determining antifungal activity against a selection of commensal and opportunistic pathogenic fungi. The determined MIC values in μg/ml were interpreted with regard

to the concentrations of the respective indoles in skin scales in order to predict potential in loco antifungal activity of Malassezia related indoles.

Materials and Methods

Forty fungal strains maintained at the Hellenic Collection of Pathogenic Fungi-UOA/HCPF were tested against 8 Malassezia indoles (Table 1) including the 5 indoles previously isolated from diseased skin (malassezin, pityriacitrin, ICZ, indirubin, FICZ) and 3 additional ones tryptanthrin, 6-hydroxymethyl-indolo [3,2-b] carbazole (H) and 6-methyl-indolo [3,2-b] carbazole (M) (Table 2). The tested strains included 27 yeasts comprising Malassezia spp. (N=9); Cryptococcus spp. (N=10); Candida spp. (N=7); Yarrowia lipolytica (N=1); the black yeast Exophialla dermatitidis (N=2); and 13 molds Aspergillus spp. (N=7); Fusarium spp. (N=2); Rhizopus oryzae (N=2). The used indoles were synthesized as previously[8,9,10] and were maintained in 10^{-2}M dimethyl sulfoxide stock solutions. For the broth microdilution method the CLSI M27-A3 and M38-A2 guidelines for yeasts and molds respectively were followed. Each strain was tested twice and on each testing occasion the quality control and reference strains respectively (Candida parapsilosis ATCC90018 and Candida albicans ATCC90028 for yeasts, and for molds the quality control and reference strains respectively Paecilomyces variotii ATCC MYA-3630 and Aspergillus fumigatus ATCC MYA-3626) were included. If the results for the quality control strains differed >2 dilutions the experiment was discarded. Regarding the results from the tested fungal strains, the higher MIC of the 2 experiments is recorded in Table 1. This was performed in order to compensate for likely dilution issues of the tested indoles to avoid underscoring the MIC value.

The concentration of the applied indoles on human skin was calculated from data published previously.[9] As the skin extracts mostly consist of skin lipids the density was taken at 0.9 gr/ml. For the transformation of the concentration of indoles in the skin extracts to corresponding μg/ml the following formula was employed:

Cx (mol/mg extract) * Molecular weight * 10^9/ 0.9 (g/ml extract)= Cx (μg/ml)

Statistical analysis

In the statistical analysis the results from 33 strains were included as the yeast Y. lipolytica and the molds Rhizopus, Fusarium and Exophiala were excluded due to the small number of strains tested. The group susceptibility of each of the indoles was evaluated with the Kruskal-Wallis and U Mann-Whitney tests employing the SPSS software (v22, Chicago, IL, USA

Results

Table 1 compiles the MIC values for the strains of the fungal species tested. Over all tested yeast strains and indolic substances median $\log_2$MIC was 4 (Fig. 1). Based on the presently employed array of fungal strains, from the indolic substances tested, indirubin (median $\log_2$ MIC=3) was the most potent antifungal agent and the only one that differed significantly in activity from any of the other ones: 6-methyl-indolo [3,2-b] carbazole (p=.015), malassezin (p=.004), pityriacitrin (p=.003), 6-hydroxymethyl-indolo [3,2-b] carbazole (p=.003) and FICZ (p<.001). On the other hand the highest median MIC ($\log_2$MIC=5) was found for FICZ (Fig. 1).

Regarding susceptibility by fungal genera two groups were discerned: Malassezia with Candida and Cryptococcus with Aspergillus. The former group was significantly more sensitive as compared to the latter group over all tested indoles

(Figure 1; Panels A, C). The pattern of susceptibility was equivalent for each genus for pityriacitrin, FICZ and 6-hydroxymethyl-indolo [3,2-b] carbazole (Figure 1).

The concentration of the Malassezia associated indoles isolated from lesional skin scales were transformed to μg/ml (Table 2) in order to compare the above susceptibility data to possible antifungal activity on the corresponding skin surface. From the 9 skin scales extracts 3 (2SD; 1PV) contain indole concentrations that would inhibit all fungal strains, including the Malassezia strains tested in this study. In two cases (SD2 and PV2) the respective inhibitory activity could be attributed to the indirubin concentration and in one to pityriactrin (SD3).

Discussion

Herein we demonstrate that indolic molecular species isolated from Malassezia cultures have antifungal activity in vitro at concentrations detected in scale probes selected form lesional SD and PV skin. From the indolic substances tested, indirubin is significantly the most active one. This molecule is the target of intense investigation as it possesses anti-inflammatory and anticancer properties and it is the active ingredient of indigo naturalis, a popular Chinese traditional medicine.[16] As already mentioned it is evaluated as a topical agent in the treatment of psoriasis[17] and the antifungal properties detected herein could be an additional indication. Furthermore, low water solubility that characterizes indirubin could be an asset on the skin as it would not be easily removed from sweat. Strict, numerical comparison of the MIC with established antifungals[19] of indirubin or the other indoles should not be performed as the antifungal function would be performed in loco on the skin and not after systemic intake in blood or tissue level. Thus, within the context that they are presently assessed, they do achieve on the skin concentrations that could be

active in vivo against commensal (Malassezia and Candida), opportunistic pathogenic yeasts (Cryptococcus) and molds (Aspergillus). Furthermore, the existence of a synergistic action that adds to this effect cannot be excluded.

A notable observation is that they show antifungal activity against their own producers, i.e. Malassezia and Candida species. The indole concentration achieved in vitro by Malassezia species when grown on L-tryptophan agar are 2-3 dilutions lower than the herein recorded MIC.[8] This means that production of indoles in vitro does not inhibit Malassezia growth up to a point, yet the toxicity of these substances might explain the preferentially use of other nitrogen sources as is glycine and resolution to L-tryptophan use when these sources have been exhausted.[20] As for Candida species, only C. glabrata has been found to produce indolic substances and from these, only tryptanthrin (Mexia and Magiatis unpublished data).

Another issue underscored by the findings of this study is the exact role of Malassezia in the accumulation of indolic compounds in association with yeast colonies in vivo and in vitro.[9] Most probably, on the skin the production of indolic compounds represents an adaptive phenomenon of certain Malassezia stains that can exploit their physico-chemical milieu in order to improve their survival competence by optimizing the bioenergetics of the assembly and accumulation of bioproducts in their environment. It has been proposed that the formation of these substances begins with an enzyme-mediated formation of indolepyruvate from L-tryptophan and the subsequent 'avital' transformation of the precursor substance into a whole array of further indolic compounds.[21] Accordingly, malassezin can easily transform to ICZ[22] and additional tryptophan metabolites can be formed on the skin through the action of the omnipresent UV radiation or further oxidative reactions.[23] Taken all these observations together, we would like to suggest that the decisive trait that

controls the variation in the capacity of different Malassezia strains to accumulate indolic compounds in their environment is their ability to uptake and metabolize available L-tryptophan into indolepyruvate and to excrete it into the environment where subsequent formation of further molecular species continues depending on the available milieu conditions. Hence, effective antifungal concentrations of these substances might not be spatially related to the Malassezia microcolonies on the skin, thus not actually inhibiting the producer strains.

In conclusion, Malassezia yeasts produce indolic species that have the ability to inhibit an array of yeast and mold strains at clinically meaningful concentrations. Future research should elaborate on the clinical significance of this observation by expanding our knowledge on the distribution of these bioactive substances on healthy and diseased skin.

Acknowledgements. The Authors wish to express their appreciation to S. Kritikou and A. Milioni for expert technical assistance.

Conflict of Interest

None

References

1. Findley K, Oh J, Yang J, et al. Topographic diversity of fungal and bacterial communities in human skin. Nature 2013;**498**:367-370

2. Huttenhower C, Gevers D, Knight R, et al. Structure, function and diversity of the healthy human microbiome. Nature. 2012;**486**:207-214

3. Theelen B, Cafarchia C, Gaitanis G, Bassukas ID, Boekhout T, Dawson TL. Malassezia ecology, pathophysiology, and treatment. Med Mycol. 2018;**56S**:10-25

4. Gaitanis G, Velegraki A, Mayser P, Bassukas ID. Skin diseases associated with Malassezia yeasts: Facts and controversies. Clin Dermatol. 2013;**31**:455-463.

5. Park HJ, Lee YW, Choe YB, Ahn KJ. Skin Characteristics in Patients with Pityriasis Versicolor Using Non-Invasive Method, MPA5. Ann Dermatol. 2012;**24**:444-452.

6. Lee WJ, Kim JY, Song CH, et al. Disruption of barrier function in dermatophytosis and pityriasis versicolor. J Dermatol. 2011;**38**:1049-1053

7. Mayser P, Wille G, Imkampe A, Thoma W, Arnold N, Monsees T. Synthesis of fluorochromes and pigments in Malassezia furfur by use of tryptophan as the single nitrogen source. Mycoses 1998;**41**:265-71

8. Gaitanis G, Magiatis P, Stathopoulou K, et al. AhR ligands, malassezin, and indolo[3,2-b]carbazole are selectively produced by Malassezia furfur strains isolated from seborrheic dermatitis. J Invest Dermatol 2008;**128**:1620-5

9. Magiatis P, Pappas P, Gaitanis G, et al. Malassezia yeasts produce a collection of exceptionally potent activators of the ah (dioxin) receptor detected in diseased human skin. J Invest Dermatol. 2013;**133**:2023-30

10. Mexia N, Gaitanis G, Velegraki A, Soshilov A, Denison MS, Magiatis P. Pityriazepin and other potent AhR ligands isolated from Malassezia furfur yeast. Arch Biochem Biophys 2015;**571**:16-20.

11. Ponnusamy K, Petchiammal C, Mohankumar R, Hopper W. In vitro antifungal activity of indirubin isolated from a South Indian ethnomedicinal plant Wrightia tinctoria R. Br. J Ethnopharmacol 2010;**132**:349-354

12. Krivogorsky B, Grundt P, Yolken R, Jones-Brando L. Inhibition of Toxoplasma gondii by indirubin and tryptanthrin analogs. Antimicrob Agents Chemother 2008;**52**:4466-4469.

13. Ponnusamy K, Ramasamy M, Savarimuthu I, Paulraj MG. Indirubin potentiates ciprofloxacin activity in the NorA efflux pump of Staphylococcus aureus. Scand J Infect Dis 2010;**42**:500-505

14. Gambichler T, Krämer H-J, Boms S, et al. Quantification of ultraviolet protective effects of pityriacitrin in humans. Arch Dermatol Res 2007;**299**:517-520

15. Park SL, Justiniano R, Williams JD, Cabello CM, Qiao S, Wondrak GT. The Tryptophan-Derived Endogenous Aryl Hydrocarbon Receptor Ligand 6-Formylindolo[3,2-b]Carbazole Is a Nanomolar UVA Photosensitizer in Epidermal Keratinocytes. J Invest Dermatol. 2015;**135**:1649-1658

16. Cheng X, Merz K-H. The Role of Indirubins in Inflammation and Associated Tumorigenesis. In: Advances in Experimental Medicine and Biology. Vol 929. ; 2016:269-290. doi:10.1007/978-3-319-41342-6_12.

17. Lin Y-K, See L-C, Huang Y-H, Chi C-C, Hui RC-Y. Comparison of indirubin concentrations in indigo naturalis ointment for psoriasis treatment: a randomized,

double-blind, dosage-controlled trial. Br J Dermatol 2018;**178**:124-131

18. Mayser P, Wenzel M, Krämer H-J, Kindler BLJ, Spiteller P, Haase G. Production of indole pigments by Candida glabrata. Med Mycol 2007;**45**:519-524

19. Lewis RE. Current Concepts in Antifungal Pharmacology. Mayo Clin Proc 2011;**86**:805-817.

20. Barchmann T, Hort W, Krämer H-J, Mayser P. Glycine as a regulator of tryptophan-dependent pigment synthesis in Malassezia furfur. Mycoses 2011;**54**:17-22

21. Zuther K, Mayser P, Hettwer U, et al. The tryptophan aminotransferase Tam1 catalyses the single biosynthetic step for tryptophan-dependent pigment synthesis in Ustilago maydis. Mol Microbiol 2008;**68**:152-72

22. Wille G, Mayser P, Thoma W, et al. Malassezin--A novel agonist of the arylhydrocarbon receptor from the yeast Malassezia furfur. Bioorg Med Chem 2001;**9**:955-60

23. Rannug A, Rannug U, Rosenkranz HS, et al. Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances. J Biol Chem 1987;**262**:15422-7

Table 1. The fungal species included in the study and the corresponding Minimum Inhibitory Concentrations (MIC) of the 4 Malassezia indoles tested (μg/ml).

		Malassezin	ICZ	Pityriacitrin	Indirubin	FICZ	6-hydroxy-methyl-indolo[3,2-b]carbazole	Tryptanthrin	6-methyl-indolo[3,2-b]carbazole
Yeasts	*Candida albicans* ATCC90028	8	8	8	8	32	16	8	16
	Candida albicans CBS 562	0.125	4	4	0.5	32	0.0125	2	16
	Candida glabrata CBS 7904	8	8	16	4	32	8	8	8
	Candida krusei ATCC6258	8	4	16	4	16	8	4	8
	Candida parapsilosis ATCC90018	8	8	8	8	32	16	8	8
	Candida tropicalis CBS94	8	4	8	8	8	8	8	16
	Candida tropicalis IP 2148-93	0.032	32	32	2	32	0.25	2	8
	Cryptococcus albidus CR 127	32	32	32	16	32	32	32	32
	Cryptococcus gattii CR86	32	32	16	16	16	32	16	32
	Cryptococcus laurentii CR 125	32	32	16	16	32	32	32	32
	Cryptococcus neoformans CR192	32	32	16	16	16	32	4	32
	Cryptococcus neoformans UOA/HCPF 9235	32	32	32	32	32	32	32	32
	Cryptococcus neoformans sero A CR 12	32	32	32	16	32	16	32	8
	Cryptococcus neoformans sero AD CR 48	32	32	32	16	32	32	32	8
	Cryptococcus neoformans sero AD CR 49	32	32	32	16	32	32	32	16
	Cryptococcus neoformans sero C CR 81	32	32	32	16	32	32	32	32
	Cryptococcus neoformans sero D CR 96	32	32	32	16	32	32	32	32
	Malassezia dermatis CBS9145	16	4	32	4	8	16	4	16
	Malassezia furfur CBS 7983	16	8	16	8	16	16	8	16
	Malassezia globosa UOA/HCPF 15443	8	2	16	2	2	8	1	8
	Malassezia nana CBS9559	16	8	16	8	16	8	16	16
	Malassezia pachydermatis CBS1880	16	8	16	8	16	16	8	8
	Malassezia restricta UOA/HCPF15428	16	4	4	2	4	8	16	8
	Malassezia slooffiae CBS7956	4	2	16	4	8	8	1	4
	Malassezia sympodialis CBS7222	16	16	8	4	32	16	8	16
	Malassezia yamatoensis CBS 9725	16	8	16	8	16	16	16	16
	Yarrowia lipolytica CBS6124-1	8	8	16	4	16	8	8	8
Molds	*Aspergillus flavus* UOA/HCPF 12726	32	32	32	32	32	32	32	32
	Aspergillus flavus UOA/HCPF15587	16	8	8	8	8	16	8	16
	Aspergillus fumigatus UOA/HCPF 14662	8	16	32	16	32	4	32	8
	Aspergillus fumigatus UOA/HCPF 7431	32	32	32	32	32	32	32	32
	Aspergillus fumigatus UOA/HCPF15831	16	32	16	8	8	32	8	16
	Aspergillus niger UOA/HCPF 14744	32	32	32	32	32	32	32	32
	Aspergillus niger UOA/HCPF15749	8	4	4	8	8	8	8	4
	Exophiala dermatitidis UOA/HCPF 3801	32	32	32	32	32	32	32	32
	Exophiala dermatitidis UOA/HCPF3801	16	16	8	16	16	16	16	16
	Fusarium solani UOA/HCFP 2213	32	32	16	16	16	32	16	32
	Fusarium oxysporum UOA/HCFP 12739	32	32	32	32	32	32	32	32
	Rhrizopus oryzae UOA/HCPF 3908	32	32	32	32	32	32	32	32
	Rhrizopus oryzae UOA/HCPF15489	8	4	8	4	8	16	8	16

Table 2. Malassezia associated indole concentration (µg/ml) on skin extracts and respective fungal genera that at least one strain tested would be inhibited on diseased human skin. Concentrations are adapted from[9]. PV: Pityriasis versicolor; SD: Seborrheic dermatitis; ICZ: indolo-[3,2b] –carbazole

	Malassezin	ICZ	Pityriacitrin	Indirubin	Species inhibited
SD1				23.57	*Candida,Cryptococcus, Malassezia*
SD2				43.66	*Aspergillus, Cryptococcus, Fusarium, Candida, Malassezia*
SD3	0.06		34.24		*Aspergillus, Cryptococcus, Fusarium, Candida Malassezia*
SD4				1.22	*Candida*
SD6	0.06	2.84	1.87		*Candida*
PV1		5.97	6.84		*Candida, Malassezia*
PV2	0.76			75.68	*Aspergillus, Cryptococcus, Fusarium, Candida, Malassezia*
PV3	0.33			3.78	*Candida, Malassezia*

Figure 1. Susceptibility of the 33 fungal strains included in the study. Panel A. Susceptibility of the fungal genera included in the study against each indole. Panel B. Antifungal activity of the tested indoles against all strains of the genera included in the study i.e. Malassezia, Candida, Cryptococcus and Aspergillus. Indirubin was the most active substance and FICZ the less active one Panel B. Susceptibility of the 4 genera included in the study against the array of indoles employed. FICZ: formyl-indolo – [3,2 b] – carbazole; ICZ: indolo- [3,2b] -carbazole. The asterisk denotes statistical significance.