Malassezia Yeasts Produce a Collection of Exceptionally Potent Activators of the Ah (Dioxin) Receptor Detected in Diseased Human Skin

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Malassezia yeasts are commensal microorganisms, which under insufficiently understood conditions can become pathogenic. We have previously shown that specific strains isolated from diseased human skin can preferentially produce agonists of the aryl hydrocarbon receptor (AhR), whose activation has been linked to certain skin diseases. Investigation of skin scale extracts from patients with *Malassezia*-associated diseases demonstrated 10- to 1,000-fold higher AhR-activating capacity than control skin extracts. Liquid chromatography–tandem mass spectrometry analysis of the patients' extracts revealed the presence of indirubin, 6-formylindolo[3,2-b]carbazole (FICZ), indolo[3,2-b]carbazole (ICZ), malassezin, and pityriacitrin. The same compounds were also identified in 9 out of 12 *Malassezia* species culture extracts tested, connecting their presence in skin scales with this yeast. Studying the activity of the *Malassezia* culture extracts and pure metabolites in HaCaT cells by reverse transcriptase real-time PCR revealed significant alterations in mRNA levels of the endogenous AhR-responsive genes *Cyp1A1*, *Cyp1B1*, and *AhRR*. Indirubin- and FICZ-activated AhR in HaCaT and human HepG2 cells with significantly higher, yet transient, potency as compared with the prototypical AhR ligand, dioxin. *In loco* synthesis of these highly potent AhR inducers by *Malassezia* yeasts could have a significant impact on skin homeostatic mechanisms and disease development.

Journal of Investigative Dermatology (2013) **133,** 2023–2030; doi:10.1038/jid.2013.92; published online 4 April 2013

INTRODUCTION

Healthy human skin harbors a significant amount of commensal yeasts belonging to the genus *Malassezia* that can become pathogenic under currently inadequately understood conditions (Ashbee, 2007; Gaitanis *et al.*, 2012). These yeasts are implicated in the pathogenesis of skin diseases with diverse clinical presentation. *Malassezia* yeasts can cause pityriasis versicolor (PV), a minimally inflammatory condition despite a concomitant heavy fungal load, which, however, modifies the function of melanocytes, as evidenced by the formation of hyper- or hypopigmented plaques (Thoma *et al.*, 2005). On the other hand, *Malassezia* yeasts are also implicated in exacerbations of certain inflammatory dermatoses, such as atopic dermatitis and seborrheic dermatitis (SD; Gupta *et al.*, 2004). All the aforementioned skin conditions are common and may have a significant impact on the quality of life of afflicted individuals (Sugita *et al.*, 2010). Currently, the genus *Malassezia* comprises 14 species (Gaitanis *et al.*, 2012). The most prevalent species in humans are *M. globosa*, *M. restricta*, *M. sympodialis,* and *M. furfur,* with the first two species present on the skin of almost all individuals (Tajima *et al.*, 2008). A major challenge in our effort to comprehend the role of *Malassezia* yeasts in skin diseases is to delineate pathogenic species or strains and to attribute virulence factors. During the last decade, an array of indolic substances synthesized *in vitro* by *M. furfur* has been identified. These substances have been associated with the pathogenic potential of this species (Mayser and Gaitainis, 2010). We have previously observed that *M. furfur* strains isolated from SD lesions (and from PV; unpublished

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Abbreviations: AhR, aryl hydrocarbon receptor; AHRR, AhR repressor; CALUX, Chemical Activated Luciferase gene expression; CDK, cyclin dependent kinase; FICZ, formyl-indolo[3,2-b]carbazole; GSK, glycogen synthase kinase; GST, glutathione S-transferase; ICZ, indolo[3,2-b]carbazole; LC/MS/MS, liquid chromatography–tandem mass spectrometry; MS, mass spectroscopy; PV, pityriasis versicolor; SD, seborrheic dermatitis; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin

Received 2 September 2012; revised 19 January 2013; accepted 5 February 2013; accepted article preview online 28 February 2013; published online 4 April 2013

observation) synthesize substances as are malassezin, indolo[3,2-b]carbazole (ICZ), and pityriacitrin in significantly higher quantities *in vitro* compared with healthy skin isolates (Gaitanis *et al.*, 2008). Malassezin has been shown to induce apoptosis in human melanocytes through aryl hydrocarbon receptor (AhR) activation, whereas ICZ is a known potent ligand of this receptor. Furthermore, the existence of malassezin on human skin could be a chemical marker for the presence of *M. furfur,* as the former has been described to be produced only by this species. To date, the production of these bioactive indoles had been shown only *in vitro* and only for *M. furfur* species, thus causing reservation with respect to their clinical relevance.

AhR is an orphan, ligand-dependent nuclear receptor with multifaceted biological functions. It has been shown to participate in the maintenance of skin homeostasis (Bock and Kohle, 2009), the enhancement of wound healing (Barouti *et al.*, 2009), and the partial mediation of UVR damage through the photochemical intracellular production of the potent AhR ligand, 6-formylindolo[3,2-b]carbazole (FICZ; Fritsche *et al.*, 2007). Regarding skin pathophysiology, it is well known that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes its detrimental effects through persistent activation of this receptor and the downstream signaling pathway. The appearance of chloracne, a characteristic acne-like eruption, is one of the heralding signs of TCDD intoxication in humans (Panteleyev and Bickers, 2006; Sorg *et al.*, 2009). Furthermore, activation of the AhR has an important role in mediating the biological/toxicological effects of a variety of environmental xenobiotics, including the *Malassezia* produced indirubin and ICZ, on the immune system (Esser *et al.*, 2009; Vlachos *et al.*, 2012).

After documenting the preferential production of the AhR ligands ICZ and malassezin in *M. furfur* SD isolates, we decided to validate their *in vivo* existence in *Malassezia*-associated skin diseases (SD and PV) as compared with appropriate control samples. This was carried out by employing the very sensitive chemical activated luceferase gene expression (CALUX) bioassay (Denison *et al.*, 2004) that measures the ability and relative potency of a chemical or extract to activate the AhR and AhR-dependent gene expression. The extracts from diseased skin scales revealed significantly higher AhR activation potential in the CALUX bioassay as compared with controls. In parallel and in order to expand the biological significance of AhR ligands synthesis by *Malassezia* yeasts, we: (1) analyzed *Malassezia* culture and skin scale extracts for the existence of additional indolic AhR activators; (2) screened *Malassezia* species for the production of AhR ligands in order to establish the wider significance of this biochemical trait in this genus; (3) assessed the effect of *Malassezia* culture extracts and indolic ingredients on HaCaT keratinocytes.

RESULTS

AhR activation by human skin scale extracts

Organic solvent skin scale extracts were prepared from 10 patients with SD ($N=6$) or PV ($N=4$) and 6 healthy volunteers and evaluated for their ability to activate AhR-dependent gene expression in recombinant human hepatoma (HG2L7.5c1) cells containing a stably transfected AhR-responsive luciferase reporter gene. The extracts originating from the patients' lesional skin scales were between 10 and 1,000 times more potent in stimulating AhR-dependent luciferase activity than those from control healthy facial skin samples ($N=3$) or skin samples from anatomic areas that are not expected under physiological conditions to be colonized by *Malassezia* yeasts ($N=3$; Table 1).

Identification, isolation, and quantitation of indolic compounds from *M. furfur* culture extracts

Using our previously described methodology (Gaitanis *et al.*, 2008), we re-evaluated the *M. furfur* culture extracts that had been included in the previous study. By chromatographic separations, we isolated and identified by nuclear magnetic resonance and mass spectroscopy three additional indolic compounds known to be potent AhR activators, namely indirubin, FICZ, and tryptanthrin. The quantitation of the more prevalent indolic AhR ligands in culture extracts of *M. furfur* strains was performed by HPLC/UV or liquid chromatography–tandem mass spectrometry (LC/MS/MS) using as analytical standards pure synthetic substances (Supplementary Figure S1 online). In order to rule out the likelihood of the photochemical production of these indoles, all cultures were performed in the dark. As a negative control, extracts from agar slants without *Malassezia* inoculum were included. Comparison of *M. furfur* strains isolated from healthy skin with SD isolates showed that the latter had elevated levels of FICZ ($P=0.001$), indirubin ($P=0.001$), tryptanthrin ($P=0.005$), and pityriacitrin ($P=0.001$; Table 2), in addition to the previously documented elevated levels of ICZ ($P<0.001$) and malassezin ($P=0.001$; Gaitanis *et al.*, 2008).

Identification and quantitation of selected indolic products in cultures of different *Malassezia* species

To expand the significance of indole synthesis by *Malassezia* species, we screened type strains of species that are mainly isolated from human skin. At least one of these AhR ligands was detected in 9 out of 12 *Malassezia* species strains studied (Table 3). Overall, the *M. furfur* pathogenic strains were the most efficient producers of these indoles (Tables 2 and 3). However, with the exception of *M. sympodialis* and *M. restricta*, all other isolates from humans (*M. furfur, M. obtusa, M. globosa, M. slooffiae, M. japonica, and M. yamatoensis*) in addition to the animal isolates, *M. pachydermatis* and *M.nana,* synthesize at least some AhR ligands.

Identification of indolic *Malassezia* metabolites in human skin scale samples

The identification of indirubin and FICZ, along with the previously known *Malassezia* metabolites malassezin, pityriacitrin, and ICZ, in EtOAc extracts of human skin scales was performed by LC/MS/MS. FICZ and ICZ were identified in 2 out of 7 patients' samples, pityriacitrin in 3 out of 10, malassezin in 4 out of 10, whereas indirubin was found in 5 out of 10 samples. The concentration of the identified metabolites ranged between 0.2 and 260 pmol mg^{-1} of

Table 1. Quantitation (mol per mg of extract) of malassezin, indirubin, ICZ, FICZ, and pityriacitrin by HPLC/MS/MS in the extracts of skin originating from patients with SD or PV, and healthy subjects (healthy1–3: elbows, palms, and soles, healthy4–6: facial skin)

	Malassezin, mol per mg skin extract	ICZ, mol per mg skin extract	FICZ, mol per mg skin extract	Indirubin, mol per mg skin extract	Pityriacitrin, mol per mg skin extract	EC_{50} $\mu g\,ml^{-1}$
SD1	ND[1]	ND	ND	8.1×10^{-11}	ND	0.1
SD2	ND	ND	ND	1.5×10^{-10}	ND	0.08
SD3	2×10^{-13}	ND	1.1×10^{-11}	ND	7.5×10^{-11}	0.88
SD4	ND	ND	5×10^{-13}	4.2×10^{-12}	ND	0.67
SD5	ND	ND	ND	ND	ND	>100
SD6	2×10^{-13}	1.0×10^{-11}	ND	ND	4.1×10^{-12}	7.9
PV1	ND	2.1×10^{-11}	ND	ND	1.5×10^{-11}	4.9
PV2	2.5×10^{-12}	ND	ND	2.6×10^{-10}	ND	0.07
PV3	1.1×10^{-12}	ND	ND	1.3×10^{-11}	ND	0.09
PV4	ND	ND	ND	ND	ND	>100
Healthy1	ND	ND	ND	ND	ND	53
Healthy2	ND	ND	ND	ND	ND	>100
Healthy3	ND	ND	ND	ND	ND	100
Healthy4	ND	ND	ND	ND	ND	83
Healthy5	ND	ND	ND	ND	ND	>100
Healthy6	ND	ND	ND	ND	ND	>100

Abbreviations: FICZ, 6-formylindolo[3,2-b]carbazole; ICZ, indolo[3,2-b]carbazole; MS/MS, tandem mass spectrometry; ND, not detected; PV, pityriasis versicolor; SD, seborrheic dermatitis.
Values are means of two independent measurements. The EC_{50} for stimulating aryl hydrocarbon receptor–dependent luciferase activity of each skin extract has been measured by the chemical activated luceferase gene expression cell bioassay.
[1]Limits of detection ($mol\,mg^{-1}$): malassezin: 4×10^{-14}; ICZ: 8×10^{-12}; FICZ: 2.9×10^{-13}; indirubin: 6×10^{-13}; pityriacitrin: 3×10^{-13}.

lesional skin scale extract (Table 1). None of these compounds was found in the control skin samples.

Relative AhR activation potency of chemically pure indolic *Malassezia* metabolites

Using the human hepatoma CALUX cell bioassay, the *Malassezia* metabolites were evaluated for their ability to stimulate AhR-dependent reporter gene expression at 6 hours of incubation. All tested compounds were potent AhR activators, with ICZ and FICZ equipotent to the prototypical AhR agonist TCDD; malassezin and tryptanthrin were 20- and 200-fold less potent than TCDD, respectively. Interestingly, indirubin was 20-fold more potent than TCDD as an activator of the AhR in the human cell line (compare EC_{50}s of 26 pM to 523 pM, respectively; EC_{50}s: FICZ at 348 pM, ICZ at 600 pM, malassezin at 11 nM, and tryptanthrin at 107 nM; Figure 1).

Activation of AhR pathway by *M. furfur* culture extracts in HaCaT cells

The significant variability of *Malassezia* culture extracts in their overall composition in indolic compounds with AhR activity complicates comparison of their relative potency. For this reason, in order to compare culture extracts of different *M. furfur* strains, we normalized extract dilutions for testing to 100 nM ICZ. This was selected because besides being identified in all tested strains, it is one of the most active AhR-inducers. However, as ICZ was only found in *M. furfur* strains, comparisons are restricted to *M. furfur* lesional skin isolates. Alterations in the expression of AhR-related genes (*Cyp1a1*, *Cyp1b1*, *AhRR*, *Aldh3a1*) as well as AhR-responsive Phase II genes (*Gstp1*, *Gstt1*; Yu *et al.*, 2009) by these indoles were assessed by reverse transcriptase real-time PCR. The extracts tested did not produce any obvious signs of toxicity in HaCaT cells when used at the equivalent of 100 nM ICZ (Supplementary Figure S2 online). Extracts at this ICZ equivalent concentration significantly induced expression of two prototypical markers of the AhR signaling pathway, namely *Cyp1a1* and *Cyp1b1*, with the level of CYP1A1 mRNA approximately sevenfold greater than that of CYP1B1 (Figure 2a and b). With the exception of WCH106, these extracts significantly downregulated the level of AhR repressor (AhRR) mRNA (Figure 2c) and both GSTP1 and GSTT1 mRNAs ($P<0.05$ compared with control; Supplementary Figure S3 online).

Differential potency of *Malassezia*-produced indoles to induce AhR pathway in HaCaT cells

A concentration–response protocol was applied to expose HaCaT cells to different concentrations of ICZ, malassezin, indirubin, FICZ, and tryptanthrin. A clear concentration-dependent increase in mRNA levels of the AhR-related genes *Cyp1A1* and *Cyp1b1* was observed in addition to *Aldh3a1* (Figure 3 and Supplementary Figure S4 online). FICZ, ICZ, and indirubin proved to be more potent AhR agonists than

Table 2. Quantitation (μg per mg of extract) of malassezin, indirubin, ICZ, tryptanthrin, and FICZ by HPLC/UV in the extracts of clinical *Malassezia furfur* strains originating from SD, PV, folliculitis, and HSs

Strain	Skin origin of the sample	Malassezin ($\mu g\,mg^{-1}$)	Indirubin ($\mu g\,mg^{-1}$)	ICZ ($\mu g\,mg^{-1}$)	Tryptanthrin ($\mu g\,mg^{-1}$)	FICZ ($\mu g\,mg^{-1}$)	Pityriacitrin ($\mu g\,mg^{-1}$)
M. furfur Bul19	SD	4.08 ± 0.35	0.084 ± 0.010	0.26 ± 0.01	0.59 ± 0.03	1.33 ± 0.15	4.82 ± 0.33
M. furfur Bul22	SD	3.33 ± 0.38	3.90 ± 0.33	2.50 ± 0.11	1.52 ± 0.09	1.14 ± 0.10	3.62 ± 0.30
M. furfur Bul23	SD	3.52 ± 0.29	0.25 ± 0.02	0.36 ± 0.02	0.38 ± 0.03	0.45 ± 0.04	3.63 ± 0.28
M. furfur Bul412	SD	2.02 ± 0.18	3.36 ± 0.34	0.95 ± 0.07	0.53 ± 0.03	0.72 ± 0.05	2.76 ± 0.27
M. furfur CBS9585	SD	2.02 ± 0.20	1.40 ± 0.13	0.40 ± 0.02	0.24 ± 0.02	0.73 ± 0.06	4.93 ± 0.35
M. furfur CBS9596	SD	4.12 ± 0.25	2.66 ± 0.21	1.60 ± 0.12	0.58 ± 0.03	0.68 ± 0.06	2.31 ± 0.23
M. furfur GS19A	SD	2.16 ± 0.15	0.022 ± 0.01	0.38 ± 0.02	0.05 ± 0.01	ND	2.49 ± 0.25
M. furfur WCH114	SD	4.21 ± 0.40	1.37 ± 0.15	1.47 ± 0.11	0.66 ± 0.05	0.58 ± 0.03	0.75 ± 0.35
M. furfur WCH100	PV	3.17 ± 0.37	1.58 ± 0.14	0.82 ± 0.05	1.65 ± 0.11	0.67 ± 0.04	3.07 ± 0.28
M. furfur WCH106	Folliculitis	5.99 ± 0.51	1.48 ± 0.14	3.27 ± 0.29	4.50 ± 0.38	0.57 ± 0.05	8.27 ± 0.45
M. furfur GS1B	HS	0.38 ± 0.03	0.006 ± 0.001	ND	0.029 ± 0.005	ND	0.41 ± 0.04
M. furfur GS2A	HS	0.40 ± 0.03	0.006 ± 0.001	ND	0.031 ± 0.005	ND	0.57 ± 0.03
M. furfur GS2B	HS	0.39 ± 0.04	0.009 ± 0.002	ND	0.094 ± 0.007	ND	0.55 ± 0.03
M. furfur GS4A	HS	0.39 ± 0.04	0.009 ± 0.002	ND	0.16 ± 0.01	ND	1.18 ± 0.09
M. furfur GS46A	HS	0.45 ± 0.02	0.010 ± 0.001	ND	0.19 ± 0.01	ND	0.77 ± 0.06
M. furfur GS9A	HS	0.56 ± 0.04	0.026 ± 0.002	ND	0.37 ± 0.02	ND	0.35 ± 0.05
M. furfur GA9B	HS	0.43 ± 0.04	0.008 ± 0.002	ND	0.088 ± 0.07	ND	0.68 ± 0.05

Abbreviations: FICZ, 6-formylindolo[3,2-b]carbazole; HS, healthy subject; ICZ, indolo[3,2-b]carbazole; ND, not detected; PV, pityriasis versicolor; SD, seborrheic dermatitis.
Values are means ± standard deviations of three independent measurements.

malassezin and tryptanthrin (Figure 3 and Supplementary Figure S4 online). This was also applicable for ALDH3A1 mRNA. Interestingly, *Cyp1a1* induction by FICZ, ICZ, and indirubin was higher at 24 hours as compared with 3 and 6 hours, a situation that is typically reversed for these compounds given that they are readily metabolized in intact cells (Supplementary Figure S5 online).

DISCUSSION

The data presented herein demonstrate the identification of an array of indolic metabolites possessing potent AhR activity in skin scales of patients with SD and PV and attribute their origin to the skin commensal and pathogen *Malassezia*. In addition to the previously found ICZ and malassezin, three more compounds, indirubin, FICZ, and tryptanthrin, were identified as *Malassezia* metabolites. Up to now, only tryptanthrin has been shown to be produced by the yeast species *Yarrowia (Candida) lipolytica* (Schrenk *et al.*, 1997) and *Candida glabrata* (Mayser *et al.*, 2007). Both FICZ (Wincent *et al.*, 2009; 2012) and indirubin (Prochazkova *et al.*, 2011) are proposed as endogenous AhR ligands and have multiple biological functions. Indirubin inhibits CDKs as well as the GSK-3 (Hoessel *et al.*, 1999) and can modulate key cellular signaling pathways like Wnt and NF-kB (Tian *et al.*, 1999). Also, bacteria can cause purple urine syndrome through the synthesis of indirubin (Kang *et al.*, 2011). FICZ has drawn attention as it has been implicated in the mediation of UV damage through AhR activation (Fritsche *et al.*, 2007). However, both could also cause indirect skin damage by the local leak of free radicals after AhR-induced hyperactivation of CYP enzymes (Park *et al.*, 1996). Presently, the identification of malassezin and pityriacitrin exclusively in lesional skin scale extracts points to *Malassezia* as the source of origin. Malassezin is uniquely produced by *Malassezia* species, and can be considered as a chemical marker of this genus. In humans, pityriacitrin has been shown to be produced also by *Candida glabrata* (Mayser *et al.*, 2007), the colon yeast equivalent of *Malassezia*. Cross-contamination only of the SD and PV samples with pityriacitrin originating from *Candida* strains residing in the colon is highly improbable and thus isolation of this indole from skin scales further corroborates toward its *Malassezia* origin.

Regarding the clinical significance of AhR ligand production by *Malassezia* yeasts, we have two important complementary findings: (1) these ligands are produced *in vitro* by the majority of *Malassezia* species and in particular by the ubiquitous

Table 3. Quantitation (μg per mg of extract) of malassezin, indirubin, ICZ, tryptanthrin, and FICZ by HPLC/UV in the extracts of 13 type and reference *Malassezia* species strains

Malassezia species	Malassezin ($\mu g\,mg^{-1}$)	Indirubin ($\mu g\,mg^{-1}$)	ICZ ($\mu g\,mg^{-1}$)	Tryptanthrin ($\mu g\,mg^{-1}$)	FICZ ($\mu g\,mg^{-1}$)	Pityriacitrin ($\mu g\,mg^{-1}$)
M. furfur CBS1878	2.02 ± 0.18	0.062 ± 0.005	0.42 ± 0.03	0.49 ± 0.04	0.84 ± 0.02	0.75 ± 0.05
M. furfur CBS6001	3.52 ± 0.25	ND	ND	ND	ND	ND
M. pachydermatis CBS6534	0.28 ± 0.02	0.002	ND	Traces	ND	ND
M. sympodialis CBS7222	ND	ND	ND	ND	ND	ND
M. obtusa CBS7876	Traces[1]	0.006 ± 0.001	ND	Traces	ND	ND
M. globosa CBS7966	Traces	0.006 ± 0.001	ND	0.074 ± 0.04	ND	ND
M. slooffiae CBS7971	Traces	ND	ND	ND	ND	ND
M. restricta CBS7991	ND	ND	ND	ND	ND	ND
M. dermatis CBS9170	Traces	ND	ND	ND	ND	ND
M. japonica CBS9432	0.30 ± 0.02	0.005 ± 0.001	ND	0.017 ± 0.02	ND	0.33 ± 0.05
M. nana CBS9557	0.34 ± 0.03	ND	ND	ND	ND	ND
M. yamatoensis CBS9726	0.29 ± 0.02	0.002 ± 0.001	Traces	Traces	ND	0.24 ± 0.04
M. caprae CBS10434	ND	ND	ND	ND	ND	ND

Abbreviations: FICZ, 6-formylindolo[3,2-b]carbazole; ICZ, indolo[3,2-b]carbazole; ND, not detected.
Values are means ± standard deviations of three independent measurements.
[1]Trace: detectable but not quantifiable.

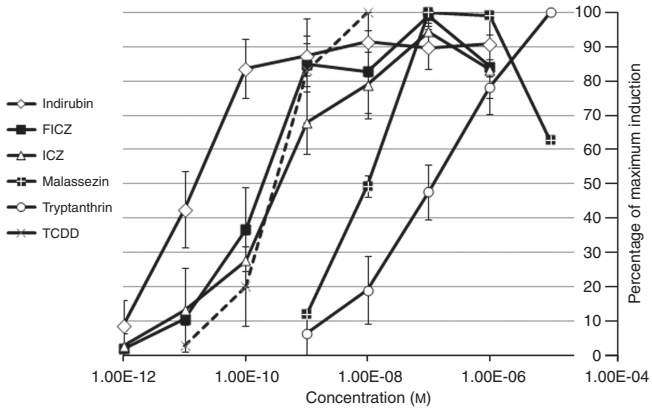

Figure 1. Aryl hydrocarbon receptor induction in recombinant human hepatoma (HG2L7.5c1) cells after incubation for 6 hours measured with the chemical activated luceferase gene expression cell bioassay. The results are normalized to the maximal activity of each compound. FICZ, 6-formylindolo[3,2-b]carbazole; ICZ, indolo[3,2-b]carbazole; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

M. globosa (Tajima *et al.*, 2008) and (2) their presence was confirmed on the skin scales of PV and SD by both analytical and biological assays. The quantitative differences in the identified indoles from the skin scales could represent variations in the activity of the yet unknown biosynthetic pathway or *in loco* modification by environmental factors as is the skin pH and/or solar radiation (Fritsche *et al.*, 2007). Thus, ICZ has been proposed to be easily produced through transformation of malassezin (Wille *et al.*, 2001), yet in our samples, we could find either one or both of these two substances. We cannot attribute the production of the AhR ligands in the skin scales solely to *M. furfur,* as it was isolated only in two of our present SD patients (data not shown). Probably, as shown from our results, additional *Malassezia* species, as is *M. globosa* (Tajima *et al.*, 2008), participate in the *in vivo* production of these ligands in SD and PV skin. For *M. furfur,* the ability to produce or not these ligands *in vitro* is a stable biological trait, as we found out by repeated subcultures of our clinical strains and is exemplary highlighted by the reference strain *M. furfur* CBS 6094. The latter is a healthy skin isolate and the inability to assimilate L-tryptophan for indole production was noted in two independent studies with at least 4 years difference (Mayser *et al.*, 2004; Gaitanis *et al.*, 2008).

The biological effects of the *Malassezia*-associated ligands found in skin scale extracts were evaluated in the human hepatoma cell line (CALUX assay) and correlated well with the respective indole quantification. The potency order of the pure indoles and TCDD when incubated for 6 hours was measured as follows: indirubin > FICZ > TCDD > ICZ > malassezin > tryptanthrin. Regarding skin scale extracts, the lowest EC_{50}s for gene induction were recorded for those containing the highest concentrations of indirubin with or without FICZ. For example, in samples SD1 and SD2 where only indirubin was found, the AhR activity could satisfactorily be explained by the levels of indirubin. In the case of SD2, the concentration of indirubin corresponding to the EC_{50} of the extract is 1.2×10^{-11} M, which is very close to the EC_{50} of pure indirubin (2.6×10^{-11} M). Overall, the EC_{50} for gene induction from the lesional skin extracts was significantly lower than that measured for the control skin extracts. The observed notable activation of the AhR from some healthy skin extracts, without identification of *Malassezia* indoles in the chemical analysis, could be attributed to environmental pollutants or inherent chemical factors with AhR activity found in human skin.

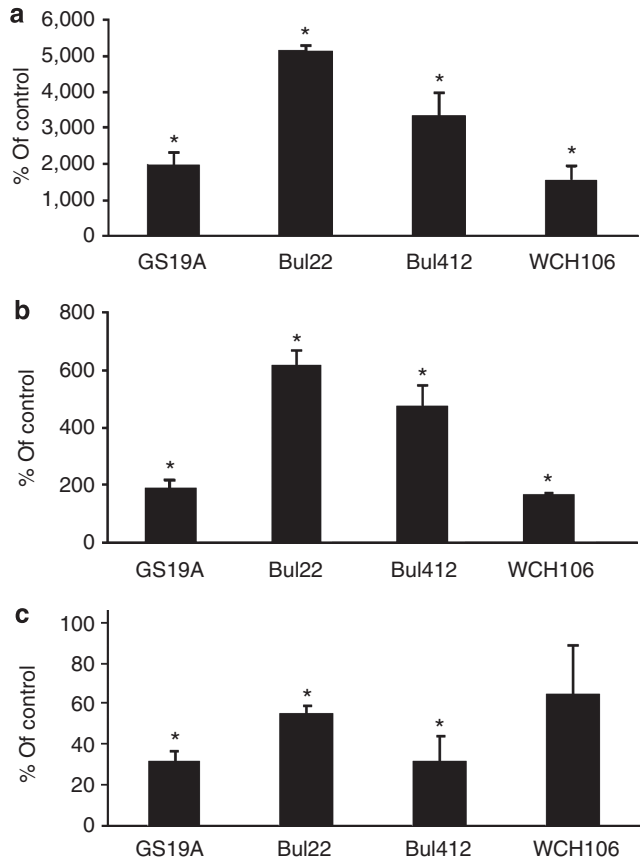

Figure 2. Effects of *M. furfur* extracts on aryl hydrocarbon receptor-dependent genes expression. Alterations in the relative levels of mRNA expression of the *Cyp1a1* (**a**), *Cyp1b1* (**b**), and *AhRR* (**c**) genes are shown (mean values ± standard deviations (bars) of three independent experiments). The extracts were normalized to their indolo[3,2-b]carbazole content. The asterisk denotes statistically significant differences compared with control, which is expressed as 100% ($P<0.05$; see also Figure 3). Bul22, Bul412, and GS19 are strains isolated from seborrheic dermatitis and WCH106 from *Malassezia* folliculitis.

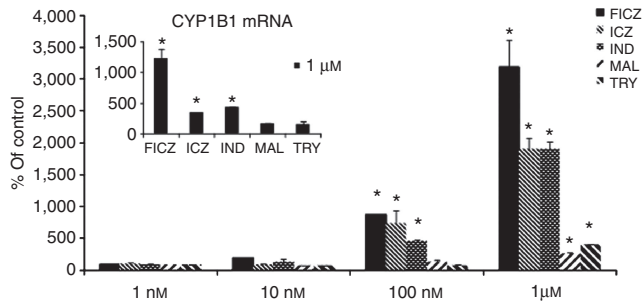

Figure 3. Comparative induction of CYP1A1 and CYP1B1 (inset) mRNA by 6-formylindolo[3,2-b]carbazole (FICZ), indolo[3,2-b]carbazole (ICZ), indirubin (IND), malassezin (MAL), and tryptanthrin (TRY) at 24 hours (mean values ± standard deviations (bars) of three independent experiments). Statistically significant induction of *Cyp1a1* is noted at 100 nM for FICZ, ICZ, and indirubin. At 1 μM, FICZ is a significantly more potent *Cyp1a1* inducer than ICZ or indirubin. This also applies for *Cyp1b1* at 1 μM (inset). The asterisk denotes statistically significant differences compared with control ($P<0.05$).

The clinical significance of the *Malassezia* produced indoles was further confirmed in the immortalized epidermal cell HaCaT by examining the AhR-inducing ability either of the pure indoles or of the *M. furfur* culture extracts. The tested AhR agonists showed clear ligand-, time-, and dose-dependent effects on CYP1A1, CYP1B1, and ALDH3A1 mRNA expression (Figure 3 and Supplementary Figure S4 online). FICZ, in addition to ICZ and indirubin, were found to be the most potent AhR agonists, as suggested from previous reports (Flaveny *et al.*, 2009; Wincent *et al.*, 2009). The culture extracts induced statistically significant alterations in AhRR, CYP1A1, CYP1B1, and ALDH3A1, but also in GSTT1 and GSTP1 mRNA expression (Figure 2 and Supplementary Figure S3 online). Interestingly, AhRR and GSTs mRNA expressions were decreased by the extracts, whereas *Aldh3a1* showed variable expression. AhRR downregulation points toward the existence of additional bioactive substances in the extracts, whereas downregulation of *Gstp* could be attributed to tryptanthrin (Yu *et al.*, 2009).

Our findings do not demonstrate any causal associations, yet this the next step, as the existence of potent AhR ligands should be complemented in future research with the assessment of the *in vivo* AhR activation level in the epidermis of *Malassezia*-associated skin diseases, as in SD and PV. Indeed, in an experimental model in mice, constitutively activated AhR resulted in inflammatory skin lesions (Tauchi *et al.*, 2005). In PV and SD, these lipophilic ligands could cross the defective epidermis, reach the granular and spinous layers, and activate the AhR (Swanson HI, 2004) and cause the respective downstream effects. The corresponding AhR-mediated local metabolic and immune aberrations have been proposed as the pathophysiological background of the suggested *Malassezia* indoles and basal cell carcinoma association (Gaitanis *et al.*, 2011). It is noteworthy that ICZ, like TCDD, has been considered as a tumor promoter (Herrmann *et al.*, 2002). Another interesting issue that rises from our findings is the impact these indoles could have on the local induction of P450 enzymes that metabolize antifungal azoles. Although the main azole-metabolizing enzyme CYP3A4 is not directly controlled by AhR (Swanson HI, 2004), there is increasing evidence that there is a cross talk between AhR, PXR, and CYP3A4 (Gerbal-Chaloin *et al.*, 2006) that could affect the skin metabolism of the commonly used antifungal azoles. Currently, AhR is also under intense investigation as it can modulate the function of regulatory T cells and T helper type 17 lymphocytes (Quintana *et al.*, 2008) as well as dendritic cells (Vlachos *et al.*, 2012; Voorhis *et al.*, 2012) in a ligand-dependent manner. Morevorer, the *Malassezia*-produced indirubin and ICZ have been recently shown to inhibit Toll-like receptor–induced maturation of dendritic cells, a finding that coincides with the need of a skin commensal to ameliorate local immune reactions in its niche (Vlachos *et al.*, 2012).

In conclusion, this article describes the synthesis of bioactive indoles by the majority of the currently accepted *Malassezia* species and the preferential production of all the most active known natural indolic AhR ligands in culture extracts of *M. furfur* pathogenic strains. These findings coupled by the presence of the AhR ligands in skin scales of

Malassezia-associated diseased skin corroborates indole production as a virulence factor in this yeast. Although the role of *Malassezia* yeasts in human skin is commonly neglected or underestimated, based on the observed strong activation of AhR in HaCaT cells by *Malassezia* extracts—and the pure indolic constituents—we can propose that the presence of a microorganism able to continually synthesize highly potent AhR ligands on the human skin has a crucial role in skin homeostasis and in the development of skin diseases, like SD and PV.

MATERIALS AND METHODS

Skin sample collection and extraction

Skin scales (50–100 mg) from 10 patients (6 SD and 4 PV) were collected and stored at −80 °C until extraction. For control samples, scraping of healthy facial skin ($N=3$) and normally thicker skin from the elbows, palms, and soles ($N=3$) was performed. Institutional Ethical Committee was granted and recruiting and sampling of volunteers was performed according to the Declaration of Helsinki. All patients gave a written informed consent. All samples were extracted with EtOAc (10 ml) under sonication (30 min). The resulting solution was filtrated and evaporated under reduced pressure. The residue was dissolved in DMSO to give a stock solution (100 mg per ml skin extract). Subsequently, a series of diluted solutions to 1 μg ml^{-1} were prepared for the AhR test.

Malassezia growth conditions

Type strains of 12 *Malassezia* species and clinical strains of *M. furfur* were grown in L-tryptophan agar as previously described (Gaitanis *et al.*, 2008).

Chemical synthesis

Malassezin, FICZ, ICZ, indirubin, and tryptanthrin were synthesized following literature methods described in Supplementary Information online.

Extraction and isolation of *Malassezia* metabolites from *M. furfur* cultures

Indirubin, FICZ, and tryptanthrin were isolated from the *M. furfur* Bul22 strain employing column chromatography with Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and preparative HPLC (column: Nucleosil 100-7, RP-18, 7 μm, 250 × 21 mm; Macherey-Nagel, Düren, Germany) with a gradient system from 100% H_2O to 100% acetonitrile for 180 minutes, with a flow rate of 5 ml min^{-1}.

Extraction and HPLC screening

The tested *Malassezia* strains (Tables 2 and 3) were grown, extracted, and submitted to analytical HPLC as previously described (Gaitanis *et al.*, 2008). The newly identified substances were detected with UV as follows: indirubin at 540 nm, ICZ and malassezin at 331 nm, FICZ at 454 nm, and tryptanthrin and pityriacitrin at 390 nm.

LCMS analysis

The LC/MS/MS analysis was performed using an Agilent 6460 QQQ (Agilent Technologies, Palo Alto, CA) operating on ESI mode. A portion of the stock solution (1 μl) was submitted to LCMS (RP Poroshell 120; Agilent Technologies; 150 × 2.1 mm^2) using a gradient system from 95% H_2O/5% acetonitrile to 100% acetonitrile in 10 minutes with a flow rate of 0.3 ml min^{-1} (Supplementary Figure S1 online).

HG2L7.5c1 CALUX cell bioassay

HG2L7.5c1 cells were trypsinized and resuspended in 20 ml α-MEM. An aliquot (100 μl) of the suspension was added into 96-well tissue culture plates and incubated for 24 hours. Subsequently, they were incubated with controls and test compounds for 6 or 24 hours at 37 °C. Luciferase activity was measured as previously described (Baston and Denison, 2010).

HaCaT cell analysis

HaCaT cells were grown in DMEM supplemented with 10% fetal bovine serum, L-glutamine, and streptomycin/penicillin. The extracts from the clinical *M. furfur* strains GS19A, Bul22, Bul412, and WCH106 were normalized at 100 nM ICZ. HaCaT cells were grown to nearly confluence and treated with the respective extract for 24 hours. The AhR ligands were also tested for dose- and time-response effects on HaCaT cells accordingly. Total RNA was isolated employing the Nucleospin kit (Macherey-Nagel).

Quantitative real-time reverse-transcriptase–PCR

mRNA of the AhR, AhRR genes, and AhR-regulated genes (CYP1A1, CYP1B1, ALDH3A1, GSTT1, and GSTP1) was measured with a CFX-96-quantitative RT-PCR system (Bio-Rad Lab, CA) with TaqMan Universal PCR master mix (4304437, Applied Biosystems, Hammonton, NJ) using inventoried 20 × assay mixes of the respective primer/probe sets as detailed, also for quantitative real-time reverse-transcriptase–PCR conditions in Supplementary Material online. Relative quantification was performed using the ΔCt method, which results in ratios between the target genes and the house-keeping reference gene *β-actin* (4352935E, Applied Biosystems).

Statistical analysis

For statistical analysis Mann–Whitney *U*-test and Student's *t*-test were employed using SPSS17 (IBM, Chicago, IL) for analysis. Two-sided $P<0.05$ values were considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Alyson Mitchell and Susan Ebeler for providing EM the opportunity to run the LCMS experiments in the Food Safety Laboratory at UC Davis. We also thank Stravroula Kritikou for expert technical assistance in *Malassezia* cultures, Guochun He for assistance in CALUX assays, and Nikos Lemonakis and Evagelos Gikas for their help in some LCMS experiments. This work was supported in part by the General Secretariat of Research and Technology of Greece (Program PENED), the National and Kapodistrian University of Athens (Program Kapodistrias), and the National Institutes of Environmental Health Sciences (ES007685 and ES04699).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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