Malassezin, a Novel Agonist of the Aryl Hydrocarbon Receptor from the Yeast *Malassezia furfur*, Induces Apoptosis in Primary Human Melanocytes

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Pityriasis versicolor is the most common skin mycosis in humans worldwide. Yeasts of the genus Malassezia*, particularly* M. furfur*, a saprophyte occurring widely on human skin, are generally regarded as the causative agents. Pityriasis versicolor is often accompanied by a long-lasting depigmentation that persists even after successful antimycotic therapy.* M. furfur *is able to convert tryptophan into a variety of indole alkaloids, some of them showing biological properties that correlate well with certain clinical features of pityriasis versicolor. This suggests a possible role for these compounds in the depigmentation process. We now report that human melanocytes undergo apoptosis when exposed to the crude mixture of tryptophan metabolites from* M. furfur*. The active compound was identified as malassezin, previously isolated by us from the same source and characterized as an agonist of the aryl hydrocarbon (Ah) receptor. The compound could, therefore, contribute to the marked depigmentation observed during the course of pityriasis versicolor.*

Introduction

Malassezia furfur is a lipophilic yeast occurring on the skin of nearly every human being as part of their residential flora. Members of the genus *Malassezia*, currently represented by nine species, are implicated as the pathogenic organisms involved in skin affections known as pityriasis versicolor.[1,2] This benign but irksome skin disease, causing mainly cosmetic damage (Figure 1), has several characteristic clinical features.

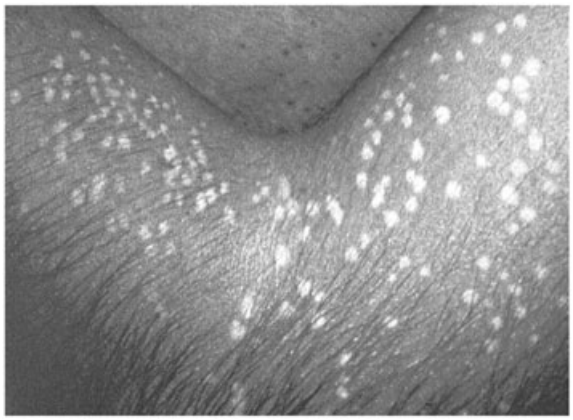

Figure 1. *Pityriasis versicolor alba with marked depigmentations.*

For example, the yellow-green fluorescence observed under UV light at 366 nm is used in diagnosis. Furthermore, skin depigmentations observed in the course of the disease are not accompanied by significantly increased sensitivity towards UV irradiation and the disease occurs with only minimal signs of inflammation. *M. furfur* produces a great variety of indole derivatives when cultured on a minimal agar medium with tryptophan as sole nitrogen source.[3] Interestingly, within the genus *Malassezia*, only *M. furfur* possesses this ability.[4] We recently isolated several new indole alkaloids from the cultural extract, and the biological properties of these compounds correlate well with the clinical features of pityriasis versicolor. Thus, pityriacitrin, a pale yellow solid, could contribute to the decreased UV sensitivity of the depigmented areas in this disease.[5] In solution, pityrialactone exhibits a strong green-to-blue fluorescence on irradiation with UV light at 366 nm, and

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this might contribute to the green fluorescent layer present in the skin affections.[6] Structurally speaking, the orange-to-red bis(indolyl)spiran alkaloids pityriarubin A, B and C resemble bisindolylmaleimides such as arcyriarubin A, a potent but nonspecific inhibitor of protein kinase C.[7] The pityriarubins may act as anti-inflammatory agents, thus explaining the modest levels of inflammation observed in affected skin areas. This thus suggests a role for these compounds in the pathogenesis of pityriasis versicolor. Chemical structures of the compounds are given in Figure S1 (Supporting Information).

As yet unexplained is the influence of *Malassezia* on the pigmentary system of human skin. Depigmentation occurs frequently in affected areas, causing a pronounced loss of melanin even after successful antimycotic therapy. Histopathological and ultrastructural examinations of the lesions have led to somewhat contradictory findings, such as decreased numbers of melanosomes[8] and melanocytes,[9] disturbed transport of melanosomes to keratinocytes,[10] and morphological changes in melanocytes that were ascribed to toxic influences.[11,12] This prompted us to investigate the influence of *Malassezia* alkaloids on cultured primary human melanocytes.

Results

On exposure to the crude extract of the yeast culture, the melanocytes exhibited marked morphological changes such as retraction of pseudopodia and rounding, accompanied by loss of adherence to the culture plates (Figure 2a, b). In order to identify the active principle, an activity-guided separation of the crude extract was carried out. Column chromatography afforded seven fractions (Figure 3), the third of which had the same effect on melanocytes as the crude extract. Further separation by thin layer chromatography yielded 12 fractions, and those with R_f values between 0.38 and 0.44 exhibited a melanocytotoxic effect. Preparative HPLC of these fractions afforded a single active compound, which was identified as malassezin, a metabolite previously isolated by us from *M. furfur*. The compound is easily accessible through total synthesis.[13]

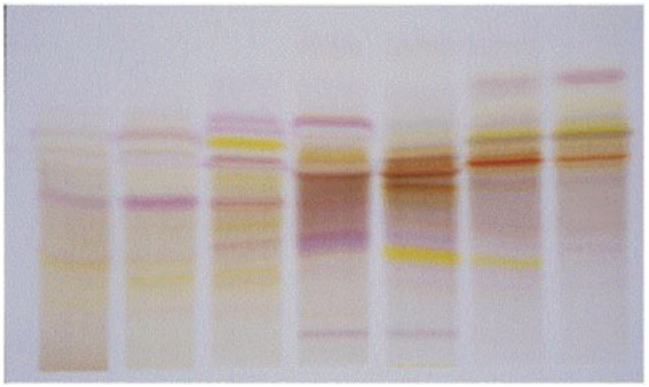
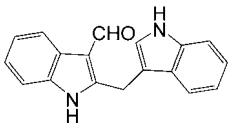


Figure 3. *Thin-layer chromatography of seven fractions obtained by Sephadex column chromatography of the crude extract.*



Since the cellular changes resembled apoptosis, further experiments with synthetic malassezin were performed on melanocytes. We found a dose-dependent induction of apoptotic markers [Fluorescence-activated cell sorting (FACS) analysis (Figure 4a, b), detection of caspase 9 (Figure 5), and DNA fragmentation by single-cell gel electrophoresis assay (Figure 6) and staining with bis-benzimide HOE 33342 (Figure 7)]. Caspase 8 was not activated. LDH (lactate dehydrogenase) can be taken as a sign of toxic influence on the cells and was detected in a similar dose-dependent way (Figure 8). Decreases in vitality, as measured by the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Figure 9), were also observed. Similarly, melanin synthesis, as determined by the surrogate parameter of [*U*-^{14}C]-tyrosine uptake into acid-insoluble particulate cell components,[14] was diminished in a comparable dose-dependent way (Figure 10).

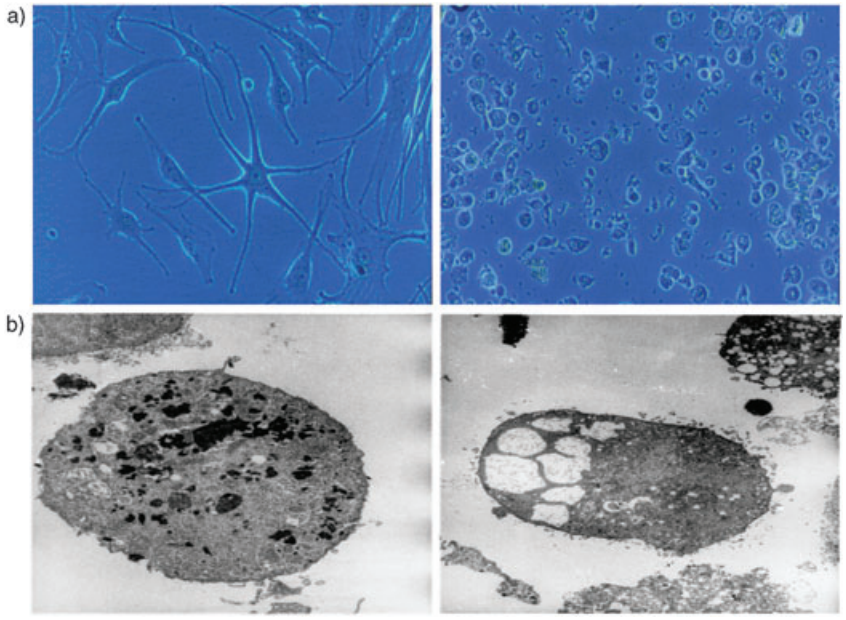


Figure 2. *a) Morphology of normal melanocytes (left) and melanocytes exposed to crude yeast extract after 4 h (400×). b) Electron microscopy of melanocytes exposed to crude yeast extract after 4 h: retraction of pseudopodia (left) and formation of vacuoles (right).*

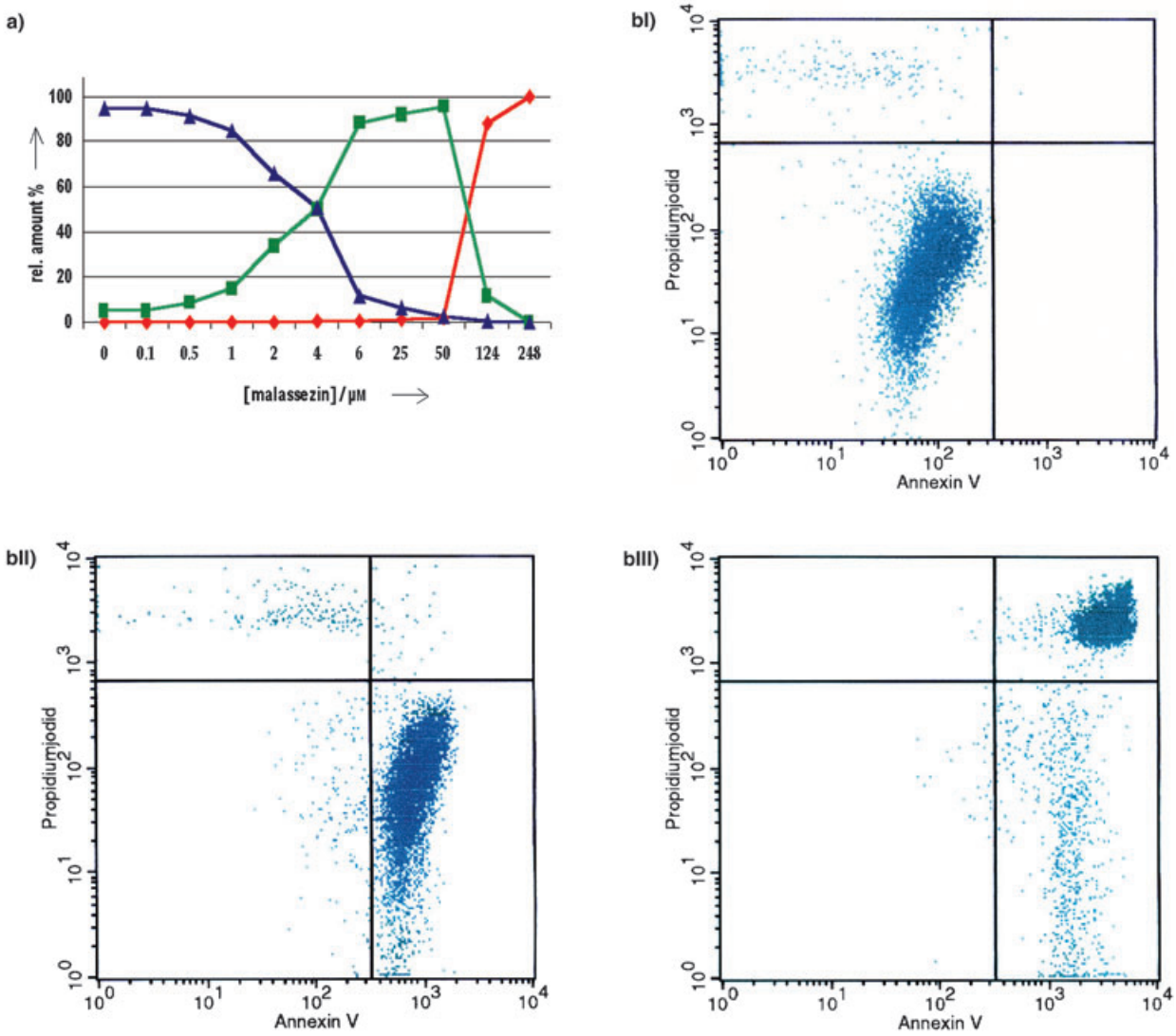


Figure 4. *FACS analysis. a) sorting of normal (blue), apoptotic (green) and necrotic cells (red) after incubation with malassezin for 24 h. Results obtained from the corresponding histograms with counting of 10000 cells. b) Histograms of selected concentrations of malassezin after incubation of cells for 24 h; lines indicating the thresholds for relative intensity of staining. I) control, II) 50 μM (apoptotic cells), III) 248 μM malassezin.*

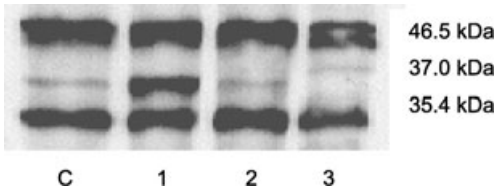


Figure 5. *Activation of caspase 9—cells treated with malassezin for 24 h: C) control cells, 1) 25 μM, 2) 6 μM, 3) 4 μM.*

Furthermore, under the influence of malassezin a decay of cytoskeletal actin filamentation was observed (Figure 11).

Discussion

The biological activities of some indole metabolites from *Malassezia furfur* seem to be related to certain clinical features of pityriasis versicolor, although we have not yet succeeded in direct detection of them in lesions or dandruff from pityriasis versicolor, due to the complex nature of these analytes. One typical symptom of the disease is the development of depigmentation in affected skin areas, although this is not accompanied by any increase in UV susceptibility. The pathogenesis of this depigmentation has not been elucidated thus far. At present, either a selective toxic influence on the skin's pigmentary system, namely on melanocytes, or an inhibition of tyrosinase by metabolites of the yeast, such as azelaic acid, are considered possible causes.[12,15] Reports of histopathological light and electron microscopic examinations have been somewhat controversial and demonstrated signs of selective toxic degeneration of melanocytes in affected areas and a disturbed distribution of melanosomes. We now describe, for the first time, a toxic influence of the *Malassezia* indoles on cultured melanocytes. Since the cellular changes induced both by the crude extract and by the purified metabolite malassezin are in accord

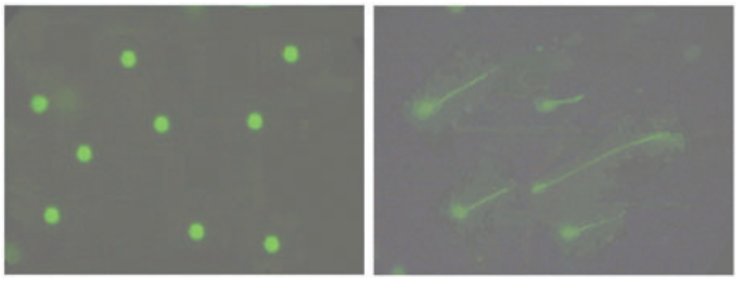


Figure 6. *Single-cell gel electrophoresis—visualisation of DNA damage after exposure to malassezin [fragmented DNA is extruding from the cells under the influence of electric field: left) control cells, right) cells treated with 25 μM malassezin for 24 h].*

with most reports on the histological and ultrastructural effects of pityriasis versicolor in lesions, these findings suggest that tryptophan metabolism is an important feature of the pathogenesis of this disease. The melanocytotoxic effect might be involved in the long-lasting depigmentations caused by the disease. We found a decrease in melanin production, which is probably caused by the toxic decay of the cells rather than by a specific influence on melanin synthesis. Malassezin has no tyrosinase-inhibiting effect.[13] The breakdown of cytoskeletal actin, found in other apoptotic processes,[16] might play an additional role in inhibiting melanin transport from melanocytes to keratinocytes, thus enhancing the depigmenting effect. The Ah receptor is widely distributed in mammalian tissues,[17] but has not yet been ascribed to melanocytes. It mediates the effects of polycyclic (halogenated) aromatic hydrocarbons—2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls and polycyclic aromatic hydrocarbons, for example[18]—in such tissues. Ah receptor activation results in a battery of transcriptional activation including induction of cytochrome P450 proteins such as CYP1A1 and the activation of caspases,[19,20] terminating in apoptotic cell death.[21] We have found characteristics of apoptosis induced by the crude yeast cultural extract in primary cultured human melanocytes. FACS analysis revealed extracellularly exposed phosphatidyl-serine by binding of annexin-V-fluos. DNA fragmentation was detected by single-cell gel electrophoresis, and chromatin condensation and formation of apoptotic bodies was seen by staining with HOE 33342. Caspase 9 was found to be activated and was taken as a marker of the cell-intrinsic apoptotic pathway mediated by mitochondrial disruption and subsequent release of cytochrome c.[20] By using an effect-guided separation of the extract, malassezin was identified as responsible for the induction of apoptotic cell death in a dose-dependent manner ($EC_{50} \approx 4$ μM, as seen by FACS analysis). The Ah receptor agonistic potency of malassezin has previously been reported to have an $EC_{50} = 1.57$ μM for the induction of CYP1A1-dependent 7-ethoxyresorufin-*O*-dealkylase (EROD) activity in hepatocytes.[13] Acid catalysis easily transforms malassezin into the more potent ($EC_{50} = 2.6 \times 10^{-7}$ M) Ah receptor agonist indolo[3,2*b*]carbazole,[22] which has been discussed

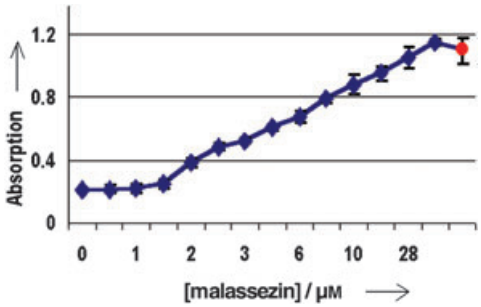


Figure 8. *Release of lactate dehydrogenase (LDH) after exposure to malassezin (n = 6) for 24 h (red point: maximum LDH release by cell lysis).*

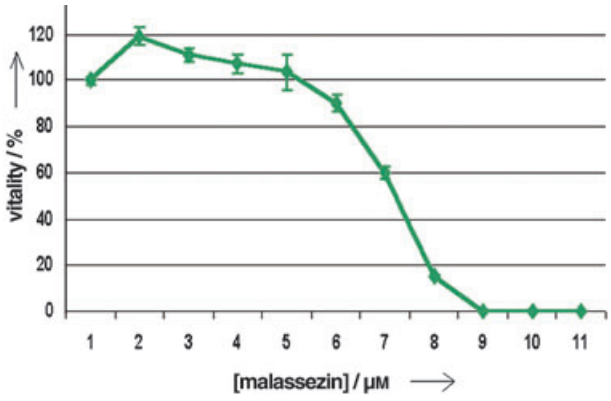


Figure 9. *MTT assay: vitality of cells treated with malassezin (n = 6) after 24 h.*

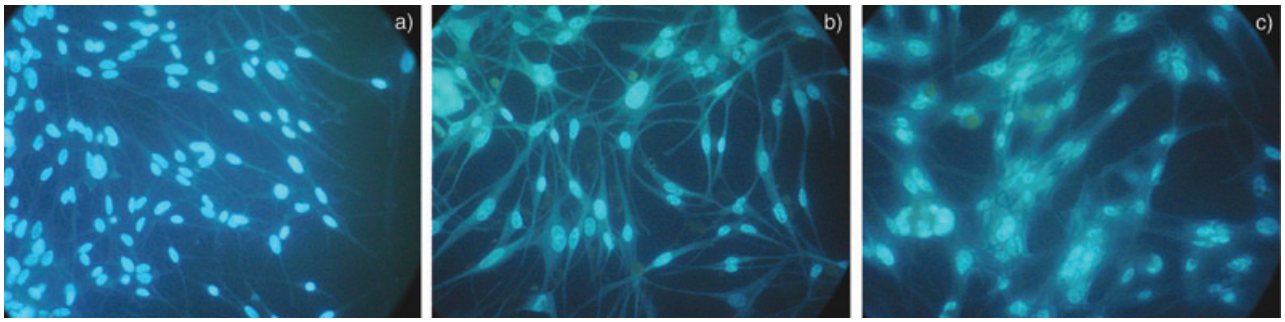


Figure 7. *Staining with bis-benzimide dye HOE 33342—induction of chromatin condensation and apoptotic bodies after treatment with malassezin (25 μM): a) control, b) 4 h treatment, c) 5 h treatment.*

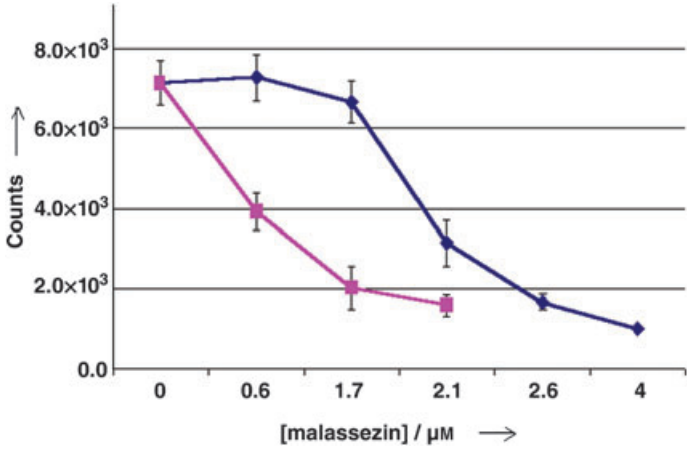


Figure 10. *Melanin synthesis measured as uptake of ^{14}C-(U)-tyrosine (n = 4); comparison of the tyrosinase inhibitor kojic acid (pink) and malassezin (blue) after 48 h incubation.*

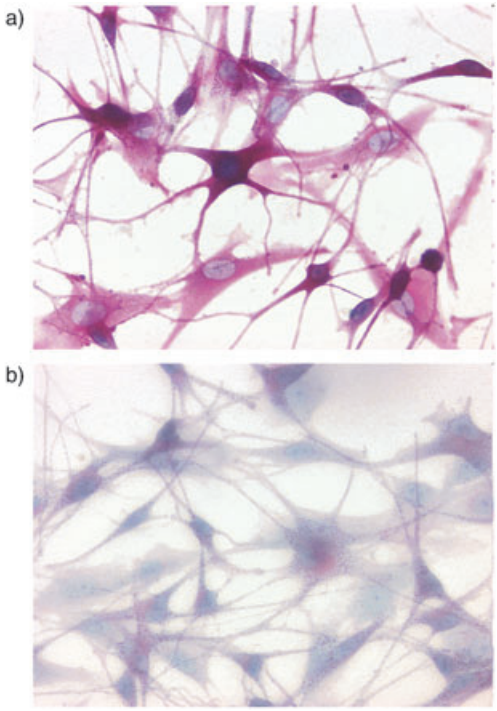


Figure 11. *Visualisation of cellular actin by immunohistological staining with mouse anti-human actin (HAF 35, MO 635 DAKO)—loss of immunoreactivity (red) in cells treated with malassezin. a) control cells, b) cells treated with 4.46 μM malassezin for 4 h.*

as final intracellular agent.[13] As this is the first Ah receptor agonist-mediated apoptosis reported in melanocytes, it may give new insight into the role of the Ah receptor in pathological processes. The carcinogenic effect of polyaromatic hydrocarbons has been discussed in terms of Ah receptor-mediated induction of xenobiotic enzymes of the P450 system with subsequent production of active carcinogenic epoxides.[21] A number of agonists of the Ah receptor either act as direct tumour promoters[23] or play a role in the selection of malignant cells by preferential apoptosis of normal cells.[24] In contrast, agonists of the Ah receptor inducing apoptosis in malignant cells have been discussed as possible therapeutic agents for selective induction of cell death in malignant cells.[25] Interestingly, in this context, no malignancies have ever been described in association with pityriasis versicolor. Many potent anticancer drugs—daunomycin in melanoma cells, for example—are effective apoptosis-inducing agents with marked selectivity for malignant cells over melanocytes.[26] Some malignant cells, such as pancreatic tumour cells, have enhanced expression of the Ah receptor and this has been identified as a potential therapeutic target.[27] Apoptosis-based therapeutics are being discussed as a promising new approach to cancer therapy,[25,27–30] and it is possible that these findings may have future implications for malignant melanoma therapy. The influence of malassezin on melanoma cell lines is currently under investigation.

Experimental Section

Organism and pigment production: The *M. furfur* reference strain CBS 1878T was used for pigment production, according to a previously published procedure.[31] The strain was maintained at 32 °C on modified Dixon agar (mDixon) as described in ref. [3] Additional information is available as Supporting Information.

Preparation and effect-guided separation of the crude extract: Suspensions of CBS 1878 were smeared on the agar medium with a swab. After 14 days of incubation, the contents of 200 Petri dishes were pureed and extracted with ethyl acetate (Merck) for 12 h. The extract was filtered through glass wool, the filtrate was evaporated to dryness, and the residue was dissolved in methanol. The resulting crude extract was divided into two parts. One part (50 μL) was used directly: it was dissolved in DMSO (100 μL), and aliquots (10 μL) of the resulting solution were added to MGM2 medium (2 mL), which was used, after sterile filtration, for the melanocyte experiments. The other part was separated under UV light (254 nm) into seven fractions by chromatography on Sephadex LH-20 (Sigma) with methanol as eluent. Each fraction was evaporated and redissolved in DMSO (1 mL). Aliquots (50, 25, 12.5, 6.25, 3, 1.5, 0.8 μL) were taken from each solution, and were each dissolved in DMSO (100 μL). Aliquots (10 μL) from these solutions were dissolved in MGM2 medium (2 mL) and sterile-filtered for the melanocyte experiments. After overnight exposure, the cells were analysed by light microscopy. Effects were seen to the fourth dilution (6.25 μL) of fraction 3, below which normal cells were found. Thin layer chromatography (TLC) was performed with fraction 3 on silica gel 60 plates (Merck) with toluene/ethyl formate/formic acid 10:5:3 (*v*/*v*) elution. After drying of the plates, 15 bands were marked under visible and UV light (366 nm), scraped out and partitioned between H_2O and EtOAc. The EtOAc layer was dried over anhydrous Na_2SO_4, evaporated and dissolved again in DMSO (1 mL). Aliquots of each band (amounts as above) were taken for melanocyte experiments after sterile filtration. As the cytotoxic effect was seen only in the DC bands with R_f values between 0.384–0.440, further purification of these combined fractions was achieved by preparative HPLC over a gradient with a LiChrospher-RP-8 column (30× 250 mm; Merck) and a LiChrosorb RP-18 precolumn. An M305 master pump combined with a 302 model pump (Gilson, Villiers le Bel, France) were used, each with a preparative 50 mL min^{-1} pump head, controlled by a Gilson 802 module. Detection was performed with a UV detector (Holochrome, Gilson) at 220 nm. The linear gradient used (180 min) ranged from 0–100% acetonitrile to

100–0% water (chromatography grade solvents; Merck), and the flow rate was 5 mL min^{-1}. The compounds were collected with a fraction collector (Super Frac, Pharmacia Biotech, Uppsala, Sweden; 180 fractions, 5 mL each), combined according to the resulting peaks (10 fractions), and then lyophilized by using a Lyovac GT2 (Leybold–Heraeus, Hanau, Germany) machine. To test their biological activity, they were treated as described above. The active component was identified as eluting from the preparative column in fraction numbers 80–88. It was repurified by means of an extended gradient (40–70% acetonitrile and 60–0% water, 180 fractions of 5 mL each). Elution occurred from fraction 10 to fraction 64, from which six groups of subfractions were combined and exposed to melanocytes after evaporation and dissolution of an aliquot (0.5 mg) of each fraction in DMSO (100 μL). The active compound was eluted in the subfraction 47–52 as single peak (by analytical HPLC) and was subjected to structural analysis.

The purity of the isolates was checked by analytical HPLC on a reversed-phase column (RP-18, 4×250 mm, stationary phase Shandon ODS Hypersil 3 μm, Life Science International Ltd., Cheshire, England). Elution was performed with a high-pressure gradient system (Gynkotek Gradientenpumpe 480, Gynkotek, Germering). Mobile phase: acetonitrile/water (linear gradient profile ranging from 100% water to 100% acetonitrile over 100 min, flow rate 1 mL min^{-1}). Detection of the components was achieved at 220 nm (UV detector 785, Bai, Bensheim), chromatographic monitoring being carried out with a C-R 6A Chromatopac integrator from Gynkotek (Germering). Application of specimens was achieved by using an Alcott Modell 738 autosampler from Bischoff Analysentechnik (Leonberg) at a volume of 100 μL.

The purity was about 99.5% in all samples. The active principle was identified as malassezin by its NMR and MS spectra and direct comparison with an authentic sample.[13]

Cell culture and biological evaluation: A primary human melanocyte culture was established according to the literature.[32] Detailed information on cell culture conditions and methods used for characterization of the melanocytotoxic effect of malassezin are provided as Supporting Information.

Acknowledgements

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Keywords: amino acids · apoptosis · *Malassezia furfur* · melanocytes · natural products · receptors

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