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Repurposing of auranofin and honokiol as antifungals against *Scedosporium* species and the related fungus *Lomentospora prolificans*

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ABSTRACT

The slowing-down *de novo* drug-discovery emphasized the importance of repurposing old drugs. This is particularly true when combating infections caused by therapy-refractory microorganisms, such as *Scedosporium* species and *Lomentospora prolificans*. Recent studies on *Scedosporium* responses to oxidative stress underscored the importance of targeting the underlying mechanisms. Auranofin, ebselen, PX-12, honokiol, and to a lesser extent, conoidin A are known to disturb redox-homeostasis systems in many organisms. Their antifungal activity was assessed against 27 isolates belonging to the major *Scedosporium* species: *S. apiospermum, S. aurantiacum, S. boydii, S. dehoogii, S. minutisporum*, and *Lomentospora prolificans*. Auranofin and honokiol were the most active against all *Scedosporium* species (mean MIC_{50} values of 2.875 and 6.143 µg/ml, respectively) and against *L. prolificans* isolates (mean MIC_{50} values of 4.0 and 3.563 µg/ml respectively). Combinations of auranofin with voriconazole or honokiol revealed additive effects against 9/27 and 18/27 isolates, respectively. Synergistic interaction between auranofin and honokiol was only found against one isolate of *L. prolificans*. The effects of auranofin upon exposure to oxidative stress were also investigated. For all species except *S. dehoogii*, the maximal growth in the presence of auranofin significantly decreased when adding a sublethal dose of menadione. The analysis of the expression of genes encoding oxidoreductase enzymes upon exposure of *S. apiospermum* to honokiol unveiled the upregulation of many genes, especially those coding peroxiredoxins, thioredoxin reductases, and glutaredoxins. Altogether, these data suggest that auranofin and honokiol act via dampening the redox balance and support their repurposing as antifungals against *Scedosporium* species and *L. prolificans*.

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Introduction

Over the last few decades, the increased frequency of infections caused by *Scedosporium* spp. and the related taxon *Lomentospora prolificans*, and the limited efficacy of current antifungals on these filamentous fungi have expanded concerns about finding new drugs to treat these infections. This is partly due to the increased number of immunocompromised patients, but also to the increase in life expectancy in another predisposed population, that are patients with cystic fibrosis (CF) [1–3]. Invasive infections often occur in patients with hematological malignancy and solid organ transplant recipients, leading to very high mortality rates [4]. However, some cases are also reported in healthy individuals [5]. In CF, the pretransplant colonization of lungs by these fungi is the main predictor of disseminated infections in case of immunosuppressive therapy, with prophylactic antifungal therapy having a poor influence on the outcome [6,7]. The issue is particularly worrying since *Scedosporium* and *Lomentospora* species are the second most common molds colonizing the CF lungs after *Aspergillus fumigatus* [1,3], and they exhibit limited susceptibility to all current antifungals, including the first-line therapy voriconazole [8,9].

Alongside their resistance to antifungals, *Scedosporium/Lomentospora* species have also adopted many pathogenic mechanisms that are responsible for their ability: (i) to adhere to the host tissues; (ii) to acquire the extracellular

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iron; and (iii) to cope with host defense-induced oxidative burst [3]. This last property is due, at least in part, to the presence of more than 30 genes encoding putative antioxidant enzymes in *S. apiospermum* genome [10]. Interestingly, transcriptional analysis of the given genes following exposure of *S. apiospermum* to chemically- or naturally-induced oxidative stresses revealed overexpression of many of them, especially those encoding thioredoxin reductases (TrxRs) and peroxiredoxins (Prxs) [11]. The thioredoxin system is known as the key component of redox homeostasis in eukaryotes. Thioredoxin reductases can be found in two variants: high molecular weight-thioredoxin reductases (HMW-TrxRs) occur only in higher eukaryotes, while low molecular weight-thioredoxin reductases (LMW-TrxRs) are found in prokaryotes and lower eukaryotes [12]. Thus, fungal TrxRs could constitute a selective therapeutic target.

Since the development of novel antifungals is a time-consuming and expensive process, alternative therapeutic approaches may help combat antifungal resistance. Although antifungal combinations are believed to deliver such alternatives, there is still limited information regarding the clinical efficacy of such combinations against *Scedosporium/Lomentospora* species [13–15]. Therefore, the repurposing of old drugs targeting the antioxidant machinery could be an economically and clinically attractive way to overcome antifungal resistance.

Several off-patent drugs are known to target antioxidant enzymes (Figure 1), especially TrxR, like auranofin (ARF) [2,3,4,6-tetra-*O*-acetyl-L-thio-β-D-glycopyrano-sato-*S*-(tri-ethyl-phosphine)-gold] which is a gold compound first approved against rheumatoid arthritis for its anti-inflammatory properties. Auranofin inhibits the HMW-TrxR via irreversible

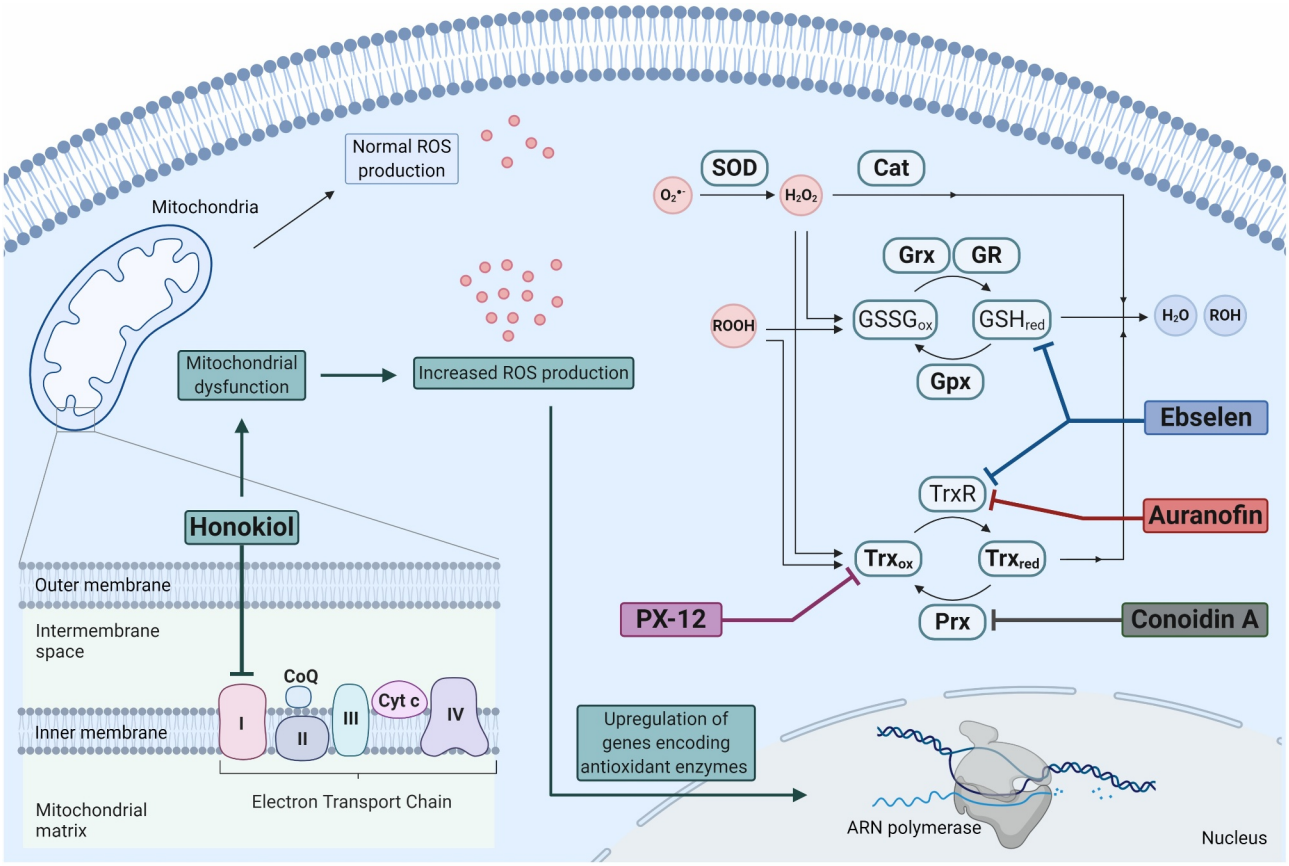

Figure 1. Mode of action of the drugs tested. Honokiol reduces the activity of mitochondrial respiratory chain complex I, leading to mitochondrial dysfunction and increased production of reactive oxygen species (ROS). Increased ROS content triggers the expression of antioxidant enzymes. Enzymes indicated in bold font are those for which overexpression has been reported in fungi exposed to honokiol. Ebselen can inhibit thioredoxin reductases but also deplete cellular glutathione content. Auranofin, conoidin A, and PX-12 inhibit thioredoxin reductases, peroxiredoxin, and thioredoxin, respectively. I–IV, Complexes of the electron transport chain; CoQ, Ubiquinone; Cyt c, Peripheral cytochrome c; ROS, Reactive oxygen species; $O_2^{\bullet-}$, Superoxide anions; H_2O_2, Hydrogen peroxide; ROOH, Hydroperoxide radicals; ROH, Alcohol; H_2O, Water; SOD, Superoxide dismutases; Cat, Catalases; Grx, Glutaredoxins; GR, Glutathione reductases; $GSSG_{ox}$, Oxidized glutathione; GSH_{red}, Reduced glutathione; Gpx, Glutathione peroxidases; TrxR, Thioredoxin reductases; Trx_{ox}, Oxidized thioredoxin; $Trxr_{ed}$, Reduced thioredoxin; Prx, Peroxiredoxins.

binding to its selenocysteinyl residue [16], but its inhibitory activity was also reported against LMW-TrxR lacking selenocysteine [17,18]. Therefore, it has been proposed for many infectious diseases, including bacterial, parasitic, and fungal infections [19–24]. In fungi, ARF demonstrated promising *in vitro* activity against several *Candida* species or *Cryptococcus (Cr.) neoformans*, but also against opportunistic molds such as *A. fumigatus*, as well as some *Scedosporium/Lomentospora* isolates [25,26].

Selenium is also found in another drug targeting antioxidant enzymes, called ebselen (EBS; 2-phenyl-1,2-benzisoselenazol-3(2 H)-one). EBS is a multifunctional selenoorganic compound inhibiting the inducible nitric oxide synthase (NOS) [27]. The drug also exhibits a glutathione peroxidase-like activity, and can act as a substrate for mammalian TrxR [28]. Recently, EBS demonstrated its inhibitory potential against bacterial TrxRs, with gram-negative bacteria being the least susceptible [29,30]. In some clinically relevant *Candida* species and *Cr. neoformans*, EBS was able to deplete cells in glutathione (GSH), leading to an increased level of reactive oxygen species (ROS) and a marked decrease in fungal load in murine models of infection [31]. A wide spectrum of filamentous fungi are also susceptible to EBS, but to a lesser extent than yeasts [32–34].

First identified as inhibiting the extension of a unique tubulin-based structure at the anterior of tachizoites of the parasite *Toxoplasma gondii* (the conoid) and therefore host cell invasion [35], conoidin A (CoA) (2,3-bis-(bromomethyl)-1,4-dioxide-quinoxaline) is one of the few reported peroxiredoxin inhibitors. The drug can bind covalently to the cysteine active site of some parasite PrxIIs, but also of human PrxI, PrxII, and PrxIV [36,37]. Due to its binding to mammalian Prxs, conoidin A was proposed as a host-directed drug against malaria, treated red blood cells being improper for development of *Plasmodium falciparum* while the parasite remained capable of invading the erythrocytes [38]. Likewise, PX-12 (2-[(1-methylpropyl)dithio]-1 H-imidazole) can prevent the reduction of thioredoxin molecules by TrxRs via an irreversible binding to a cysteinyl residue located outside the active site [39]. The drug completed phase I and phase II trials in cancer therapy, but its insufficient efficacy has rolled it out of trials [40,41]. Nevertheless, PX-12 increased the sensitivity of *A. fumigatus* hyphae to H_2O_2 and enhanced *in vitro* and *in vivo* neutrophil-mediated killing [42].

Honokiol (HOK) (5,5′-diallyl-2,4′-dihydroxybiphenyl) is a biphenolic compound derived from *Magnolia officinalis*. The drug was reported to affect the activity of the respiratory chain complex I, leading to mitochondrial dysfunction, increase in the cellular content in superoxide anions and subsequent apoptosis in *Candida albicans* and *Saccharomyces cerevisiae* [43,44]. In *S. cerevisiae*, the copper/zinc superoxide dismutase (cu/Zn-SOD) SOD1 and the High osmolarity glycerol (HOG) pathway were essential for protecting against HOK-induced oxidative stress [45]. Consistently, the HOG pathway is considered a common virulence trait since it is the leading contributor to stress-related responses in fungi [46]. Taking advantage of its biphenolic nature and its safety properties, HOK has been extensively investigated in many clinical fields, and its antifungal activity against a wide range of pathogenic fungi is now ascertained [47–49].

Considering the crucial role of the fungal antioxidant machinery played during evasion to the host immune response, we hypothesized that (i) targeting thiol-redox enzymes such as TrxRs and Prxs by auranofin, ebselen, conoidin A, or PX-12, or (ii) triggering the HOG pathway or disturbing the redox hemostasis status by honokiol could impair growth of *Scedosporium/Lomentospora* species. Here, we present the *in vitro* susceptibility to these drugs of 27 *Scedosporium/Lomentospora* isolates belonging to the main clinically relevant species (*S. apiospermum, S. aurantiacum, S. boydii, S. minutisporum*, and *L. prolificans*) or the environmental species *S. dehoogii*. The activities of voriconazole (VRC) and posaconazole (POS) were assessed in parallel.

Materials and methods

Fungal isolates, media, and chemicals

This study was conducted on 27 fungal isolates belonging to the main *Scedosporium* species (n = 21) or *L. prolificans* (n = 6), which were collected from different CF patients in France, or recovered from soil samples (Table 1). Isolates were preserved in our culture collection at the University of Angers (UA), France, or deposited in the Institute of Hygiene and Epidemiology – Mycology section (IHEM) culture collection at Sciensano, Brussels, Belgium. The quality control strain *Candida parapsilosis* ATCC 22,019 was purchased from the American Type Culture Collection (ATCC). All cultures were grown for nine days at 37°C on yeast extract-peptone-dextrose-agar plates, apart from *S. dehoogii* which was cultivated at 25°C. Spores were sterilely harvested with distilled water, then filtrated through Miracloth membranes, and enumerated by hematocytometer counts.

Voriconazole, posaconazole, and conoidin A were obtained from Cayman Chemical Company (MI, USA). Auranofin and ebselen were purchased from Enzo Life Sciences (NY, USA). PX-12 and RPMI 1640

Table 1. *Scedosporium* and *Lomentospora* isolates used in this study.

Species	Isolate	Origin
S. apiospermum	IHEM 14,268	CF patient, Giens, France
	IHEM 14,462	CF patient, Tours, France
	IHEM 23,580	CF patient, Toulouse, France
	UA120217796/2	Soil, Meknes, Morocco
	UA120218482/4	Garbage dump, Meknes, Morocco
S. aurantiacum	IHEM 23,578	CF patient, Tours, France
	UA100353192-01	CF patient, Rouen, France
	UA110349103-01/1	River sediment, Angers, France
	UA110349103-01/3	River sediment, Angers, France
	UA120008824-01/1	Waste-water treatment plant, Angers, France
S. boydii	IHEM 15,555	CF patient, Tours, France
	IHEM 23,574	CF patient, Angers, France
	IHEM 23,837	CF patient, Angers, France
	UA120217796/1	Cultivated soil, Meknes, Morocco
	UA120218478/1	Garbage dump, Meknes, Morocco
S. dehoogii	UA110354521-01/2	Soil, Meknes, Morocco
	UA110350905-01/1	Cow dung contaminated soil, Angers, France
	UA110354504-01/1	Fallow land soil, Angers, France
	UA120008799-01/1	Waste-water treatment plant, Angers, France
S. minutisporum	IHEM 23,833	CF patient, Angers, France
	UA110350824-01/3	Electrical transformer station, Angers, France
L. prolificans	IHEM 19,435	Soil, Belgium
	IHEM 22,180	CF patient, Giens, France
	UA40604032	CF patient, Lyon, France
	IHEM 22,177	CF patient, Giens, France
	IHEM 14,076	CF patient, Pontivy, France
	IHEM 21,157	CF patient, Angers, France

CF, cystic fibrosis; IHEM, Institute of Hygiene and Epidemiology – Mycology section (Scientific Institute of Public Health, Brussels, Belgium); UA, University of Angers (Angers, France).

medium were purchased from Sigma–Aldrich (MO, USA), and honokiol (99% HPLC) from AK scientific (CA, USA). All drugs were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions at concentrations 100-fold higher than the highest concentration tested. Stock solutions were kept at – 20°C until used.

In vitro susceptibility testing and determination of minimal inhibitory concentration (MIC)

The *in vitro* susceptibility testing was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference procedure with slight modifications [50]. Briefly, freshly prepared conidia were suspended in 0.165 M RPMI 1640 culture medium buffered at pH 7.2 with MOPS and supplemented with 0.1% Tween 80 to prevent the formation of fungal tufts on the surface of wells. Conidial suspensions (2×10^6 conidia/ml) were then incubated with an equal volume of serial two-fold dilutions of each drug to give final concentrations ranging from 15.625 ng/ml to 16 µg/ml.

Experiments were performed in 96 wells flat-bottom microplates with a final volume of 200 µl per well. After an incubation of 72 h at an adequate temperature, growth was monitored at 405 nm. The MIC_{50} and MIC_{80} were defined as the lowest concentrations of the drugs inhibiting 50% and 80% of growth, respectively, compared to that observed in the drug-free control well. Drug susceptibilities were compared using the MIC_{50} values, while the activity of drugs at the residual level was assessed at MIC_{80} endpoints. To calculate the geometric mean for resistant isolates, the MIC was considered as twice the highest concentration tested.

Candida parapsilosis ATCC 22,019 was used as quality control strain in each run. Controls also included wells receiving DMSO alone (1% final concentration) to check the lack of effect of the drug vehicle on fungal growth.

Antifungal combinations and fractional inhibitory concentration index (FICI)

Combination assays between ARF and VRC and between ARF and HOK were also performed in a checkerboard design with concentrations up to 8 µg/ml. Technical procedures and results monitoring were done as mentioned above. The interactions between drugs were analyzed using the commonly used non-parametric model of Loewe additivity. It consists of measuring the fractional inhibitory concentration index (FICI) defined as:

$$\mathrm{MICA}^{\mathrm{combined}}/\mathrm{MICA}^{\mathrm{alone}} + \mathrm{MICB}^{\mathrm{combined}}/\mathrm{MICB}^{\mathrm{alone}},$$

where A and B are the combined drugs.

The interaction indexes $FICI_{50}$ and $FICI_{80}$ were determined at two set points, corresponding to 50% and 80% of growth inhibition, respectively. Interactions were considered synergistic when FICI was ≤ 0.5, additive for 0.5 < FICI ≤ 1, indifferent for 1< FICI ≤ 4, and antagonistic when FICI was > 4 [51]. The median and the range of FIC reported in each matrix were used as output parameters.

Fungal susceptibility to auranofin under menadione-induced oxidative stress

The activity of ARF on fungal growth was also determined under oxidative stress. We first evaluated the effect of menadione on the growth of five randomly selected isolates representing each species. A sublethal dose of menadione (SDM) was defined as the highest concentration causing no decrease in fungal growth. SDM was estimated at 31.25 µM for the five tested isolates. The activity of ARF at the concentration range used above (15.625 ng/ml to 16 µg/ml) was then studied in the presence of SDM for all the isolates except *S. minutisporum* isolates. For each isolate, two concentration-growth curves were generated (ARF alone and ARF + SDM). Data from these curves were then grouped by species, and fitted to a four-parameter logistic model defined in the equation:

$$E = E_{min} + (E_{max} - E_{min}) / (1 + 10^{(\mathrm{LogIC_{50}} - X)\mathrm{slope}})$$

where X is the $\log_{10}$ of ARF concentration, and E is the percentage of fungal growth observed at X. The four parameters are: E_{min} and E_{max} corresponding to the minimal and maximal growth (%), respectively; the slope (Hill coefficient) describing the curve steepness; and the IC_{50} corresponding to the 50% inhibitory concentration.

The shift in the IC_{50} of ARF between the control curve (ARF alone) and the SDM-treated curve (ARF + SDM) was accepted only when it was ± four-fold the scale. For some species, the three-parameter logistic model (with slope fixed at −1) fitted better; thus, it was chosen over the four-parameter model. The coefficient of determination (R^2) was used to assess the goodness of fit, and the deviation from models was checked using the run test. Modeling was performed using the GraphPad Prism software (version 5).

Effects of honokiol and auranofin on fungal oxidoreductase activity

The reference strain *S. apiospermum* IHEM 14,462 was cultivated in 30 ml of yeast extract-peptone-dextrose broth (10^6 conidia/ml). After incubation for 24 h at 37°C with moderate shaking, the resulting hyphae were harvested by centrifugation, and then treated with different concentrations of HOK (8, 16, and 32 µg/ml) for 4 h at 37°C. A negative control group was carried out by treating hyphae with 1% DMSO alone (the drug vehicle). Afterward, hyphae were filtered on Miracloth membrane, washed once with distilled water, ground in liquid nitrogen, and kept at −20°C before RNA extraction. Total RNAs extraction and reverse transcription (RT)-quantitative PCR (qPCR) were done as described previously [11]. Primers used in qPCR experiments are listed in Supplemental table S1. Twenty-five genes encoding oxidoreductase enzymes were studied and the actin gene was used for normalization. The relative expression of each gene was calculated from the threshold cycle (Ct) values as previously described [11].

The thioreductase activity was also quantified after preincubation with auranofin. To do this, 24-h old hyphae were ground in liquid nitrogen, and the homogenate resuspended in cold 50 mM phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Total proteins were extracted through repeated bead-beating steps (3 x 1 min) and two centrifugation steps at 10 000 g for 15 min. Protein concentration was determined using the Bradford assay. The total protein extract was preincubated with or without ARF (4, 8, 16, and 32 µg/ml) for 30 min at room temperature before measuring the thioredoxin reductase activity using the Thioredoxin Reductase Assay Kit (Sigma–Aldrich).

Statistical analysis

All experiments were done in triplicate. McNemar test was used to evaluate the difference in the number of additive interactions between the two combination sets (ARF with VRC *vs.* ARF with HOK). For each species, the differences in the best-fit values between the two growth curves (SDM-treated cells and control) were analyzed using the extra sum-of-squares F test. The two-way ANOVA tests followed by Bonferroni posttests were run to check significant differences in gene expression levels between HOK-treated and non-treated fungal cells. All tests were performed using GraphPad Prism (version 5).

Table 2a. MIC values (in μg/ml) of tested drugs against *Scedosporium* species and the related fungus *Lomentospora* prolificans.

	VRC		POS		ARF		EBS		PX-12		CoA		HOK	
Isolate	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}
S. apiospermum														
IHEM 14,268	0.5	> 16	1	> 16	8	> 16	16	16	> 16	> 16	8	> 16	8	16
IHEM 14,462	0.5	1	2	4	2	4	4	16	> 16	> 16	16	> 16	16	> 16
IHEM 23,580	0.125	> 16	> 16	> 16	2	> 16	> 16	> 16	> 16	> 16	8	16	8	16
UA120217796/2	0.5	> 16	0.5	4	1	4	16	16	> 16	> 16	16	> 16	4	> 16
UA120218482/4	0.25	2	1	> 16	4	8	4	> 16	> 16	> 16	16	16	4	> 16
S. aurantiacum														
IHEM 23,578	0.0625	> 16	1	16	4	8	16	> 16	> 16	> 16	8	16	4	16
UA100353192-01	0.25	> 16	0.5	8	4	8	16	> 16	> 16	> 16	8	8	4	8
UA110349103-01/1	0.25	16	0.5	> 16	4	4	16	> 16	> 16	> 16	8	8	8	16
UA110349103-01/3	0.125	1	0.5	> 16	4	4	16	> 16	> 16	> 16	8	16	8	8
UA120008824-01/1	0.125	2	1	2	4	4	16	> 16	> 16	> 16	8	8	8	16
S. boydii														
IHEM 15,155	4	> 16	2	> 16	2	2	> 16	> 16	> 16	> 16	16	16	8	8
IHEM 23,574	0.5	> 16	0.5	16	2	> 16	> 16	> 16	16	> 16	16	16	4	8
IHEM 23,837	0.0625	> 16	0.0625	0.5	2	> 16	16	> 16	> 16	> 16	8	16	8	16
UA120217796/1	0.25	2	0.5	1	2	4	16	> 16	> 16	> 16	16	16	16	> 16
UA120218478/1	0.5	> 16	1	> 16	2	8	> 16	> 16	> 16	> 16	16	16	16	> 16
S. dehoogii														
UA110354521-01/2	0.25	> 16	0.5	> 16	2	> 16	> 16	> 16	1	4	8	16	4	> 16
UA110350905-01/1	1	> 16	1	> 16	4	8	> 16	> 16	> 16	> 16	16	> 16	4	8
UA110354504-01/1	0.25	> 16	0.25	> 16	4	> 16	> 16	> 16	> 16	> 16	8	> 16	4	16
UA120008799-01/1	0.25	> 16	0.5	> 16	2	4	> 16	> 16	16	> 16	> 16	> 16	4	8
S. minutisporum														
IHEM 23,833	0.25	> 16	16	> 16	4	16	>16	> 16	16	> 16	4	> 16	4	8
UA110350824-01/3	0.25	1	0.25	4	4	8	16	> 16	8	16	8	> 16	4	16
L. prolificans														
IHEM 19,435	> 16	> 16	> 16	> 16	4	8	> 16	> 16	> 16	> 16	16	16	4	16
IHEM 22,180	16	> 16	> 16	> 16	4	8	> 16	> 16	> 16	> 16	16	16	2	8
UA40604032	> 16	> 16	> 16	> 16	4	8	> 16	> 16	> 16	> 16	16	16	4	8
IHEM 22,177	> 16	> 16	> 16	> 16	4	8	> 16	> 16	> 16	> 16	16	16	4	16
IHEM 14,076	> 16	> 16	>16	> 16	2	8	> 16	>16	> 16	> 16	16	16	4	8
IHEM 21,157	> 16	> 16	> 16	> 16	8	> 16	> 16	>16	> 16	> 16	16	16	4	> 16

MIC, Minimal inhibitory concentration; MIC_{50}, Minimal concentration inhibiting 50% of fungal growth; MIC_{80}, Minimal concentration inhibiting 80% of fungal growth; VRC, Voriconazole; POS, Posaconazole; ARF, Auranofin; EBS, Ebselen; CoA, Conoidin A; HOK, Honokiol.

Results

Auranofin and honokiol demonstrate antifungal activity

Overall, ARF and HOK showed the most potent activities among the drugs tested (Tables 2a and 2b). ARF exhibited high antifungal activity against all *Scedosporium* species with MIC_{50} ranging between 1 and 8 μg/ml (GM_{50} = 2.875 μg/ml), followed by HOK with an active range of 2 to 16 μg/ml (GM_{50} = 6.143 μg/ml). The standard antifungals VRC and POS inhibited the growth of *Scedosporium* species at low MIC_{50} values (GM_{50} = 0.285 and 0.848 μg/ml, respectively). However, at 80% of growth inhibition, ARF was more active than VRC and POS with GM_{80} of 9.129 μg/ml for ARF and 12.699 μg/ml for both VRC and POS. Among *Scedosporium* species, *S. aurantiacum*, *S. boydii*, and *S. dehoogii* were the most susceptible to ARF. ARF was less active against VRC- and POS-resistant *L. prolificans* with GM_{50} of 4.0 μg/ml, but surprisingly, among all tested species, *L. prolificans* was the most susceptible to HOK (GM_{50} of 3.564 μg/ml).

CoA impaired the fungal growth at only high concentrations (8 or 16 μg/ml), although one *S. minutisporum* isolate was found to be inhibited at 4 μg/ml. The tolerance of all tested isolates to high doses of EBS proved the lack of antifungal activity of this compound except against two *S. apiospermum* isolates (MIC_{50} = 4 μg/ml). Only two isolates belonging to *S. dehoogii* and *S. minutisporum* showed some susceptibility to the least effective drug PX-12 with MIC_{50} of 1 μg/ml and 8 μg/ml, respectively.

Combinations of auranofin with voriconazole or honokiol show additive effects

The results of the interactions are given in Table 3. Based on FIC index analysis, additive effects were significantly more frequent with ARF + HOK than with ARF + VRC (66.7% *vs* 33.3%, $P < 0.05$): the combination of ARF with VRC showed additive interactions only against a limited number of isolates (9 out of 27 isolates). By contrast, additivity was revealed for the combination of ARF with HOK against 18 out of 27 isolates at either or both monitored endpoints (50% and 80% of inhibition). Among the different species tested, *L. prolificans* and *S. aurantiacum* were the most susceptible to both combination sets, whereas *S. apiospermum* was the least responsive species.

Table 2b. Geometric mean (GM) of MIC values (in µg/ml) of tested drugs at 50% and 80% of growth inhibition endpoints.

	VRC		POS		ARF		EBS		PX-12		CoA		HOK	
Species	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}
S. apiospermum	0.330	9.190	2.000	13.929	2.639	10.556	10.556	21.112	32.000	32.000	12.126	24.251	6.964	24.251
S. aurantiacum	0.144	8.000	0.660	12.126	4.000	5.278	16.000	32.000	32.000	32.000	8.000	10.556	6.063	12.126
S. boydii	0.435	18.379	0.500	6.063	2.000	9.190	24.251	32.000	27.858	32.000	13.929	16.000	9.190	16.000
S. dehoogii	0.354	32.000	0.500	32.000	2.828	13.454	32.000	32.000	11.314	19.027	13.454	26.909	4.000	13.454
S. minutisporum	0.250	5.657	2.000	11.314	4.000	11.314	22.627	32.000	11.314	22.627	5.657	32.000	4.000	11.314
GM for *Scedosporium* species	0.285	12.699	0.848	12.699	2.875	9.129	18.871	28.983	23.004	28.042	10.767	18.871	6.143	15.481
L. prolificans	28.509	32.000	32.000	32.000	4.000	10.079	32.000	32.000	32.000	32.000	16.000	16.000	3.564	12.699

MIC, Minimal inhibitory concentration; MIC_{50}, Minimal concentration inhibiting 50% of fungal growth; MIC_{80}, Minimal concentration inhibiting 80% of fungal growth; VRC, Voriconazole; POS, Posaconazole; ARF, Auranofin; EBS, Ebselen; CoA, Conoidin A; HOK, Honokiol.

Table 3. Interactions between auranofin and voriconazole or honokiol against *Scedosporium* species and the related fungus *Lomentospora prolificans*.

	ARF-VRC		ARF-HOK	
Isolate	$FICI_{50}$	$FICI_{80}$	$FICI_{50}$	$FICI_{80}$
S. apiospermum				
IHEM 14,268	I	I	I	0.757 (0.515–1.0)
IHEM 14,462	0.816 (0.750–1.0)	I	1.0 (1.0)	I
IHEM 23,580	I	I	I	I
UA120217796/2	I	I	I	I
UA120218482/4	I	I	I	I
S. aurantiacum				
IHEM 23,578	0.664 (0.516–1.0)	0.693 (0.531–1.0)	0.515 (0.515)	I
UA100353192-01	I	I	0.825 (0.750–1.0)	I
UA110349103-01/1	0.625 (0.5–0.75)	0.693 (0.531–1.0)	0.622 (0.503–1.0)	I
UA110349103-01/3	I	I	I	I
UA120008824-01/1	1.0 (1.0)	I	0.716 (0.562–1.0)	I
S. boydii				
IHEM 15,555	I	I	0.75 (0.75)	0.659 (0.515–1.0)
IHEM 23,574	I	I	I	0.693 (0.531–1.0)
IHEM 23,837	I	I	0.776 (0.625–1.0)	1.0 (1.0)
UA120217796/1	I	I	I	I
UA120218478/1	I	I	I	0.501 (0.5004–0.503)
S. dehoogii				
UA110354521-01/2	I	I	0.693 (0.531–1.0)	1.0 (1.0)
UA110350905-01/1	I	0.582 (0.508–0.75)	I	I
UA110354504-01/1	0.687 (0.625–0.75)	I	0.531 (0.531)	I
UA120008799-01/1	I	I	0.687 (0.625–0.75)	I
S. minutisporum				
IHEM 23,833	I	I	I	0.75 (0.75)
UA110350824-01/3	I	I	0.710 (0.531–1.0)	1.0 (1.0)
L. prolificans				
IHEM 19,435	0.791 (0.625–1.0)	I	1.0 (1.0)	0.687 (0.625–0.75)
IHEM 22,180	I	I	I	I
UA40604032	I	I	I	I
IHEM 22,177	I	I	0.75 (0.75)	0.781 (0.625–1.0)
IHEM 14,076	0.833 (0.750–1.0)	I	0.75 (0.75)	0.833 (0.750–1.0)
IHEM 21,157	0.875 (0.750–1.0)	I	I	0.607 (0.375–1.0)

Interactions were monitored at 50% and 80% of growth inhibition endpoints. Values correspond to the average FICI, and the range is indicated between parentheses.

FICI, Fractional inhibitory concentration index; ARF, Auranofin; VRC, Voriconazole; HOK, Honokiol; I, indifferent interaction.

Isolates belonging to *S. boydii* showed responsiveness only to the combination of ARF with HOK. More additive interactions were found against *S. dehoogii* with the combination ARF + HOK. For only one isolate of *L. prolificans* (IHEM 21,157), $FICI_{80}$ revealed both synergistic and additive interactions with values ranging between 0.375 and 1. Antagonism was not obtained in any case.

Addition of a sublethal dose of menadione decreases the maximal growth of the fungus exposed to auranofin

The two concentration-growth curves (ARF alone and ARF with SDM) of each species fitted to the sigmoid models are represented in Figure 2. Data obtained for each isolate were first grouped by species because isolates belonging to a same species proved similar growth patterns. Although susceptibility to ARF vary within some species, the sigmoid models were still able to effectively fit grouped curves as demonstrated by the high R^2 values (0.9617 to 0.9990; median = 0.9934) and the nonsignificant deviation from models ($P > 0.1$). For each species, except *S. dehoogii*, growth tended to a lower plateau level in the presence of SDM when compared with the control curve; this correlated with significantly lower E_{max} values ($P \leq 0.0005$) (Figure 2). This shift appeared to occur when the concentrations of ARF approached or surpassed the MIC_{50} of the species. The presence of SDM resulted in significant shifts toward lower best-fit IC_{50} values of ARF for *S. apiospermum, S. aurantiacum*, and *S. dehoogii*; however, shifts did not exceed two-fold dilutions. Therefore, differences were only attributed to the inter-experimental variations. No significant differences were observed in the other best-fit values between the two concentration-growth curves for any species.

Honokiol upregulates genes encoding antioxidant enzymes

Among the 25 genes studied encoding oxidoreductases, 17 were upregulated in *S. apiospermum* IHEM 14,462 upon exposure to HOK (Figure 3a and 3b). Significant fold changes ranged between 1.84 and 26.06. The upregulated genes included: two out of the four genes encoding catalases

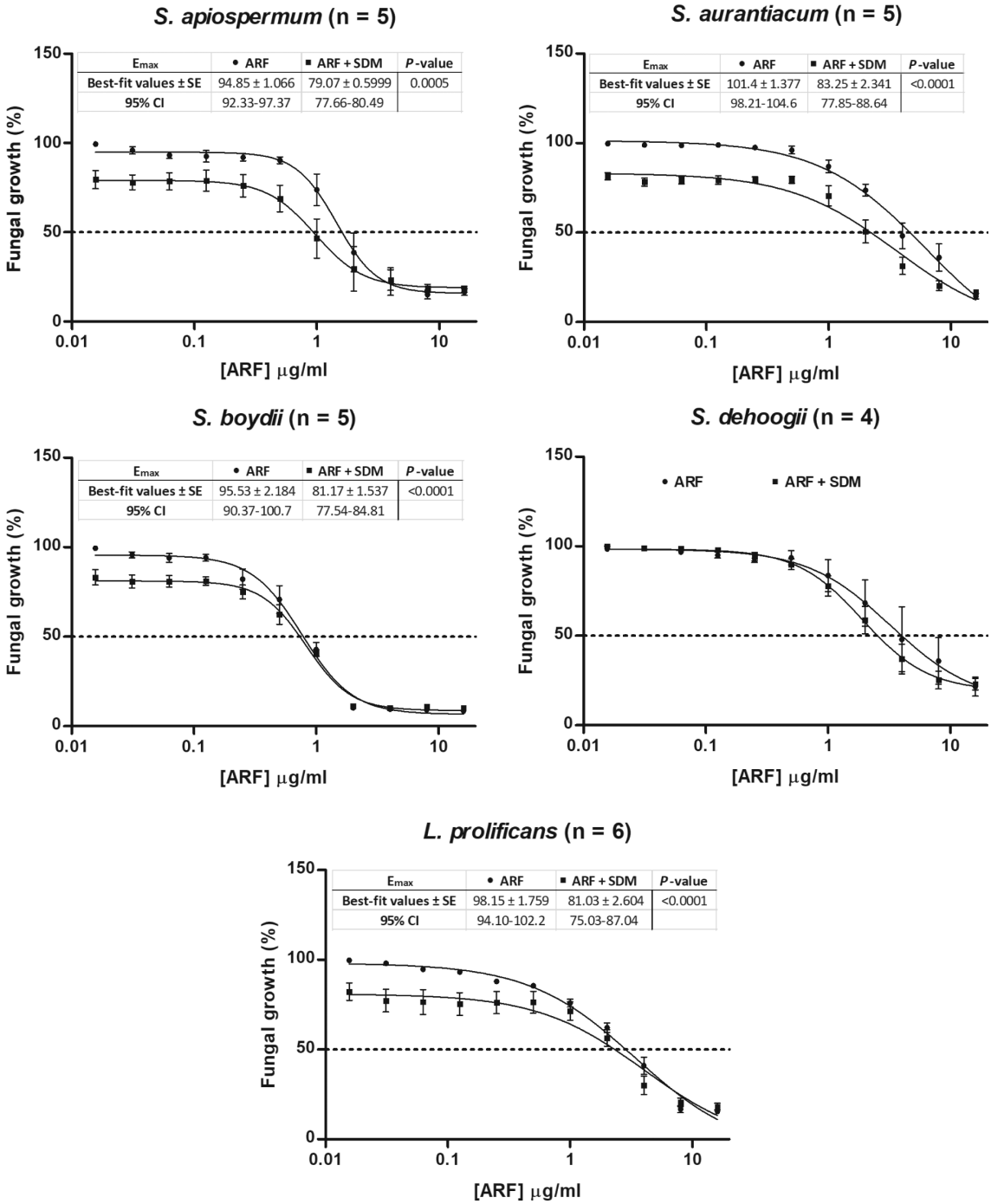

Figure 2. Concentration-growth curves of auranofin alone (ARF) or in the presence of a sublethal dose of menadione (ARF + SDM). Lines were generated by fitting data to a four-parameter logistic model, except for *S. aurantiacum* and *L. prolificans*, where the slope was constrained at −1 (three-parameter logistic model). The coefficients of determination R^2 ranged between 0.9699 and 0.9990 (median = 0.9934). The E_{max} values represent the maximal growth values generated using the models. The extra sum-of-squares F test was used to assess the difference in the best-fit values of E_{max} between the two curves for each species. The dashed line represents 50% growth. The error bars indicate standard errors of the means. SE, standard error; 95% CI, 95% confidence interval.

(Cat), the three genes encoding copper/zinc superoxide dismutases (Cu/Zn-SODs), two of the three genes encoding manganese-superoxide dismutases (Mn-SODs), two of the three genes encoding Prxs, one gene encoding TrxR (gene encoding the second TrxR was not tested), the only gene encoding thioredoxin (Trx), the two genes encoding glutathione peroxidases (Gpxs), three of the five genes encoding glutaredoxins (Grxs), and one of the three genes encoding

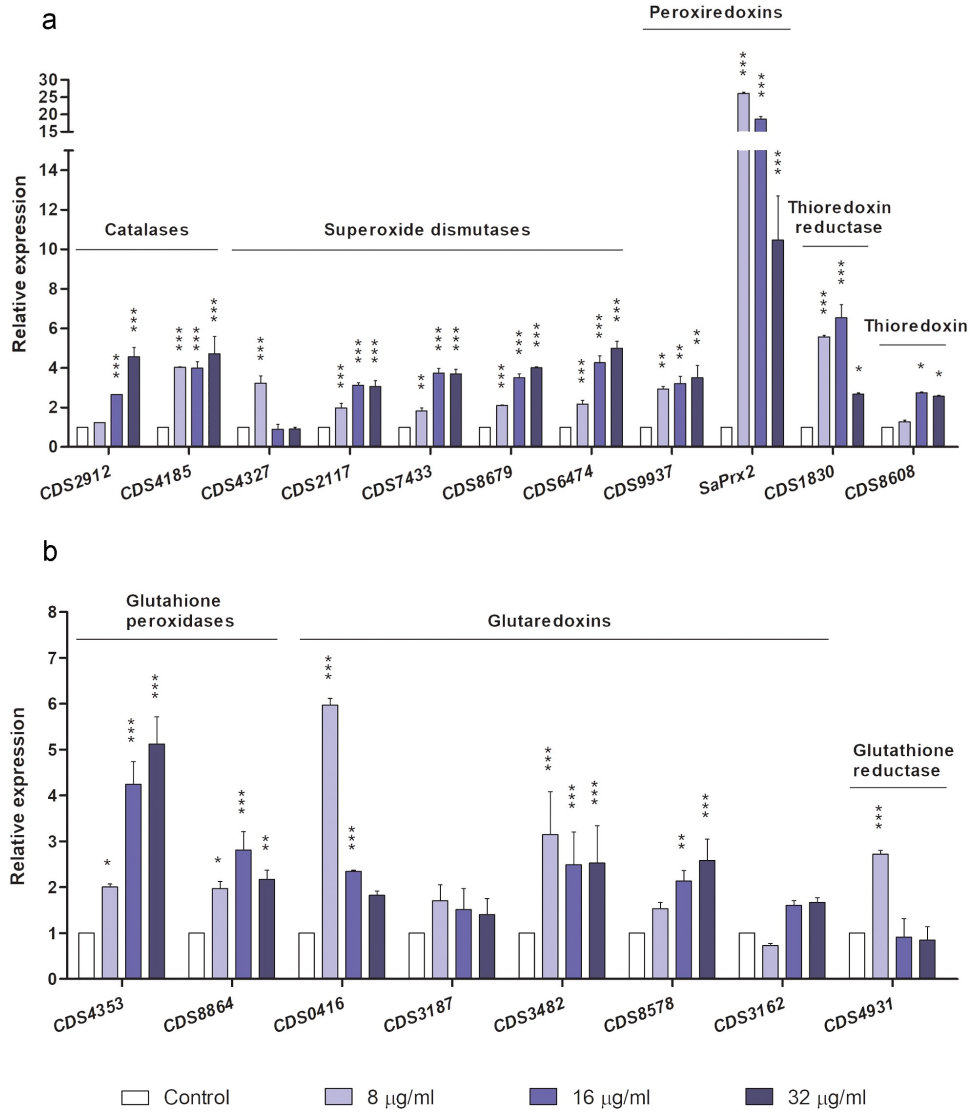

Figure 3. Relative expression of genes encoding oxidoreductase enzymes in *Scedosporium apiospermum* IHEM 14,462 treated with 8, 16, and 32 μg/ml of honokiol. (a), relative expression of genes encoding catalases, superoxide dismutases (SODs), peroxiredoxins (Prxs), thioredoxin reductase and thioredoxin; (b) relative expression of genes encoding glutathione peroxidases, glutaredoxins, and glutathione reductases (GRs). Two-way ANOVA tests and Bonferroni posttests were run to compare expressions in the untreated (control) and treated fungus. The error bars indicate standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. No change was seen in the expression level of the other genes studied, CDS4198 and CDS10583 which encode catalase peroxidases, CDS3426 encoding another Mn-SOD, CDS4142 encoding another Prx, and CDS6039 and CDS4534 encoding the two other GRs.

glutathione reductases (GRs). The significant increase in expression of these genes occurred at least following exposure to 16 and 32 μg/ml HOK, except one gene encoding Mn-SOD (CDS4327) and the gene encoding GR (CDS4931), which were upregulated only in fungal cells treated with 8 μg/ml HOK. Notably, overexpression was the highest for *SaPrx2* gene in all treatment conditions, with a fold change progressively decreasing with the increase in HOK concentration (26.06, 18.68, and 10.46 when treating the hyphae with 8, 16, and 32 μg/ml HOK, respectively). In addition to *SaPrx2*, genes showing maximum overexpression were those encoding TrxR (CDS1830) and Grx (CDS0416) with mean fold changes of 6.54 and 5.97, respectively. No significant downregulation was observed.

The preliminary investigation of potential targets of ARF in *S. apiospermum* 14,462 showed that preincubation of the total protein extract with 16 and 32 μg/ml of ARF decreased the thioredoxin reductase activity by 9% and 26.5%, respectively (data not shown).

Discussion

In the current study, we investigated the antifungal activity of a set of drugs against the major

Scedosporium species and *L. prolificans*. These drugs (auranofin, ebselen, PX-12, conoidin A, and honokiol), which have been previously reported to possess antimicrobial activity, were selected based on their known capacity to impair the cellular redox hemostasis. The selection was stemmed from very recent findings on the behavior of *S. apiospermum* under oxidative stress, where the genes encoding TrxRs and Prxs were not only the most upregulated among core oxidative-stress genes but also the fastest expressed upon exposure to neutrophils [11].

Among tested drugs, ARF and HOK were the most effective against *Scedosporium* and *Lomentospora* species with MIC_{50} ranging from 2.00 to 4.00 µg/ml and from 3.564 µg/ml to 9.190 µg/ml, respectively. Of note, both were active against the VRC- and POS-resistant *L. prolificans* isolates, with the latter species being the most susceptible to HOK. To the best of our knowledge, here we present the lowest MIC_{50} values ever reported for HOK acting as antibacterial or antifungal. Regarding ARF activity, our findings are consistent with those reported in a recent study conducted on a limited number of *Scedosporium* isolates [26]. At 80% of inhibition, ARF was also more effective than VRC and POS on several isolates, perhaps in relation with differences in their modes of action.

Given the lack of specificity of clinical signs and symptoms, the relative rarity of these infections, the lack of commercial diagnostic kits and the slow growth of the causative agents, the diagnosis of *Scedosporium/Lomentospora* infections is often delayed, suggesting high fungal loads in the organs. Our experiments, therefore, were carried out using a more concentrated inoculum than recommended. Because of the potential dependence of drug activities upon fungal inoculum, our MIC values should reflect more potent activities. For this reason, we reassessed the activity of ARF and HOK on the reference strain *S. apiospermum* IHEM 14,462 at a recommended inoculum size (2×10^4 CFU/ml). Results revealed a two- to four-fold decrease in MIC_{50} and MIC_{80} for both ARF and HOK (data not shown), thus confirming this assumption.

ARF displays well-established pharmacological properties that compare quite favorably to azole antifungals, especially VRC. This triazole drug may induce visual disturbances, as well as hepatotoxicity and phototoxicity events in CF patients [52,53]. In addition, its concomitant use with immunosuppressants in solid organ transplant recipients needs careful evaluation of serum level of the immunosuppressant [54]. Importantly, the demonstrated MIC values for ARF mirror the achievable blood levels following an oral or parenteral dose of ARF (maximum plasma concentration: 0.68 ± 0.45 µg/ml). Besides, similar ARF concentrations can be reached in the central nervous system [55], which is of importance for treatment of *Scedosporium/Lomentospora* infections with regard to the neurotropism of these fungi [1]. According to the manufacturer, the toxicity of ARF is only reached at very high concentrations and the side effects are quite rare.

Although TrxRs are likely the primary target of ARF, the exact mode of action of this drug is still disputable. Extensive studies reported its strong anti-parasitic activity, which correlates with a marked decrease in TrxR activity upon exposure to the drug, possibly because most parasites (i) lack the glutathione system or (ii) rely on a single TrxR for their antioxidant defense [19,24]. This is consistent with similar findings on bacteria since only species lacking the glutathione system were found susceptible [56]. The antifungal potential of ARF has been reported against several yeast species, including fluconazole-resistant *C. albicans, Candida glabrata, Candida tropicalis*, and *Cr. neoformans* with MIC values ranging from 0.25 to > 16 µg/ml [25,26,57]. However, ARF as targeting fungal TrxR is only assumed by the fact that the addition of glutathione antagonized the antifungal effect of the drug on *Cr. neoformans* [25]. To address the question of whether ARF targets TrxR, we reevaluated the susceptibility of *Scedosporium* and *Lomentospora* isolates to ARF in the presence of a sublethal dose of menadione (SDM). We also measured the thioredoxin reductase activity in a total protein extract pre-incubated or not with ARF. While high levels of menadione-induced ROS can cause oxidative stress-mediated cell death, low levels could act only as redox-signaling messengers without contributing to growth defects. In the presence of SDM, all species except *S. dehoogii* exhibited a significant decrease in their maximal growth in response to ARF. Given that the presence of SDM did not decrease further the MIC_{50} of ARF against any isolate, our results may reflect increased sensitivity of *Scedosporium* and *Lomentospora* isolates to the SDM-induced ROS following TrxR inhibition by ARF rather than sensitization of the isolates to low concentrations of ARF. Consistent with this finding, decreased MIC_{50} of ARF was reported when only high concentrations of ROS inducers were applied [25,58]. Our preliminary experiments showing a slight decrease in TrxR activity in ARF-treated protein extracts, also suggest the targeting of TrxR by ARF, but further investigations are needed to definitely support TrxR targeting. However, the antifungal effect of ARF seems to depend on weakened fungal redox statuses, and since the oxidative stress induced within the host may itself result in fungal damage, the *in vitro* evaluation of ARF activity of ARF could underestimate its *in vivo* activity, and thereby

in vivo investigations may assume of greater importance. In addition, intact growth curves in the presence of ARF, when tested with SDM, were only seen with *S. dehoogii*, which is mainly an environmental species that may have evolved differently from the pathogenic *Scedosporium* species. Comparative genomic studies are currently being performed in order to probe the virulence-related traits of the clinically relevant *Scedosporium* species in patients with CF.

Little information is available regarding pharmacokinetics of honokiol in humans. Recently, a phase I clinical trial on the use of liposomal-HOK for anticancer therapy was conducted which showed very low plasma concentrations (245 ± 49.6 ng/ml) following the administration of a very low single dose of HOK [59]. Nevertheless, HOK elicits minimal mutagenic and genotoxic effects in mice and rats independently of administered doses [60] and toxicity on human cell lines was only achieved at high concentrations (11.8–13.3 µg/ml) [61]. In addition, the drug pharmacokinetics have been extensively studied in mice and rats administered with liposomal-HOK. The steady-state concentration of HOK remained up to 30 µg/ml for 24 hours following 25 mg/kg IV injection in mice [62], which is several folds higher than most of our MIC values. Moreover, HOK is subject to enterohepatic circulation, allowing very rapid distribution into organs, including the brain [60]. Antifungal activity of HOK has been investigated in many species, including dermatophytes, phytopathogenic fungi, *A. fumigatus, Cr. neoformans*, and fluconazole-resistant *C. albicans* with relatively high MIC values [47,49,63]. HOK underwent successful *in vivo* trials for several fungal species exhibiting very high MIC values *in vitro* [48,49,64]. Therefore, considering the very low MIC values found in the present study, *in vivo* investigations should be conducted on murine models of *Scedosporium* and *Lomentospora* infections.

In fungi, HOK was found to trigger the accumulation of superoxide anion through mitochondrial dysfunction [43,44], resulting in a transcriptional response similar to that induced by H_2O_2 [65]. The cellular signature included increasing production of oxidoreductase enzymes, such as SODs, catalases, Trxs, Prxs, and Gpx [43,45,66,67]. Here we demonstrated that 17 out of 25 genes related to oxidoreduction functions were significantly upregulated in response to HOK in *S. apiospermum* IHEM 14,462. Upregulated genes included genes encoding SODs, catalases, and components of the glutathione (Gpx, Grx, GR) or thioredoxin (Trx, TrxR, and Prx) systems. Apart from genes encoding TrxRs which have never been reported before to be overexpressed in response to HOK, our findings are consistent with previous studies performed on other fungal species [43,45,66,67]. Interestingly, many of our upregulated genes were also found previously to be overexpressed in the same strain exposed to H_2O_2 [11], and *SaPrx2*, CDS1830 and CDS0416 genes (encoding one Prx, one TrxR and one Grx, respectively) which were the most overexpressed in response to HOK, also belonged to the most upregulated upon exposure to H_2O_2 [11].

Combining old drugs with front-line medication is common, but such studies are still limited regarding ARF and HOK. Additive interactions, but rarely synergistic, were reported when combining ARF with antibiotics against pathogenic bacteria [20,21,68,69]. Similar findings were reported when combining ARF with fluconazole or amphotericin B against pathogenic yeasts [23,25], which is consistent with the few additive interactions observed for the combination of ARF with VRC. The low frequency of additive effect observed for this combination compared to ARF with HOK may be related to the fact that VRC is already quite effective against most of tested isolates. Supporting this hypothesis is the greater frequency of additive effects observed with ARF + VRC against *S. aurantiacum* and *L. prolificans* isolates which usually have reduced susceptibility to VRC among the species studied. However, considering the overexpression of TrxR in *S. apiospermum* following exposure to HOK, one may also hypothesize that the higher frequency of the additive effects demonstrated by the combination of ARF with HOK may be related to the inhibition of TrxR. In the presence of HOK, genes encoding several antioxidant enzymes including TrxR are overexpressed to break down HOK-induced ROS, but at the same time, ARF prevents TrxR from fulfilling its function, which results in accumulation of ROS in the fungal cells and in major cell damage. Considering the double-edged nature of ARF and HOK, *in vivo* studies should also focus toward finding the balance between their antifungal and their anti-inflammatory potentials when combatting infections. Likewise, the activity of the combination of HOK with VRC should be evaluated since HOK was showed recently to act synergistically with fluconazole against *C. albicans* isolates [48].

Finally, the low activity of EBS, PX-12, and CoA, which also trigger components of the thioredoxin system, may be due to compensatory reactions of the glutathione system; this included ten redundant genes recently depicted in *S. apiospermum* genome [10]. Nevertheless, one cannot disregard that, depending on the target enzyme within the thioredoxin system, the blockage of ROS degradation and the subsequent antifungal activity will be more or less pronounced. It is worth mentioning that the considerable susceptibility

of *S. dehoogii* UA110354521-01/2 to PX-12 (MIC_{50} = 1 µg/ml) could be isolate-dependent as it is the only one recovered from a soil sample collected outside France (*i.e.* Morocco).

Conclusion

ARF and HOK display potent *in vitro* activity against clinical and environmental *Scedosporium* isolates. More interestingly, ARF and HOK are active against isolates of *L. prolificans* that shows resistance to the first-line therapy antifungals VRC and POS. Therefore, our results support their repurposing for treatment of *Scedosporium/Lomentospora* infections and suggest the targeting of redox components by these drugs in the fungal cells. *In vivo* investigations of their effectiveness in animal models of these infections are still to be explored.

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Disclosure statement

No conflicts of interest to declare.

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