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Title: Identification of repositionable drugs with novel antimycotic activity by screening Prestwick Chemical Library against emerging invasive molds.

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Abstract

The incidence of severe filamentous fungi infections has increased over the past decade. Some of these filamentous fungi are resistant to available antifungals; it is thus urgent to find new compounds that are active against such life-threatening pathogens. Here, 1280 drugs (Prestwick Chemical Library) were tested against six multidrug-resistant filamentous fungi including, *Aspergillus*, *Fusarium*, *Scedosporium*, *Rhizopus* and *Lichtheimia* species. We identified several hits that induce fungal growth inhibition $\geq 70\%$. Clioquinol, alexidine dihydrochloride, hexachlorophene and thonzonium bromide, displayed a broad activity against all strains tested. This study enriches the potential antifungal options that can be used against multidrug-resistant invasive fungal diseases.

Introduction

While more than 400 fungal species have been implicated as opportunistic pathogens in human infection [1], only 4 classes of systemic antifungal agents, namely polyenes, azoles, echinocandins and flucytosine, are mainly used in clinical practices [2]. Although these antifungal drugs are usually active against the major clinical fungi, there are limitations to their routine use, including off-target toxicity and drug-drug interaction. Moreover, a poor clinical response has been reported, especially against emerging resistant fungal isolates [3]. Antifungal resistance is a major concern that has been described in most clinical fungal genera. The major human fungal pathogens, including *Candida* spp., *Cryptococcus* spp. and *Aspergillus* spp. were commonly sensitive to antifungal therapy. However, nowadays, they display a relatively high-acquired resistance rate and constitute a serious public health concern. Additionally, rare life-threatening and emerging fungal pathogens, including opportunistic multidrug-resistant (MDR) pathogens, are increasingly being reported [4]. These latter include *Scedosporium/Lomentospora* spp., *Fusarium* spp. and other Mucorales [5]. Thus, this study aimed at determining available off-label drugs with potential antifungal activity against emerging multidrug-resistant molds. This promising strategy, which is commonly referred to as a “repurposing approach”, is particularly useful to elude drug-resistance expansion and prevent treatment failure [6].

Testing of Prestwick Chemical Library against MDR-filamentous fungi

We screened the Prestwick Chemical Library (Prestwick, Illkirch graffenstaden, France), which is a molecule-library containing 1,280 compounds, belonging to 291 different therapeutic classes, which mostly have been approved by the Food and Drug Administration (FDA), to identify molecules that inhibit the growth of a panel of multidrug-resistant fungi. The chemical library was initially provided in DMSO at a concentration of 10 mM for each drug. Here, all molecules were tested, following serial dilutions, at a fixed concentration of 10

70 μM (the final DMSO concentration was $\leq 0.5\%$). This low and fixed concentration (i.e. 10
71 μM) was used according to previous screening studies in order to find the drugs which display
72 antifungal activities at low concentrations and to avoid the toxicity and the adverse effect that
73 these tested drugs may induce at high concentrations. The fungal inoculum was prepared in
74 RPMI-1640 medium (Sigma Aldrich, St Louis, France) according to the Clinical and
75 Laboratory Standards Institute (CLSI) protocol (M38-A, Vol. 22 No. 16). The fungal growth
76 inhibition rate was calculated derived from Optical Density (OD) values measured with the
77 plate reader spectrophotometer (Multiskan spectrum, Thermo Scientific, France) at a
78 wavelength = 405 nm compared to the same untreated strain. The tested strains' incubation
79 time with drugs varied from 48 to 72 hours according to the optimal fungal growth time
80 observed in positive control wells. The six fungal strains that were isolated from various
81 clinical samples were tested twice; these include; *Aspergillus calidoustus* (bronchial
82 aspiration), *Fusarium oxysporum* (nails), *Fusarium solani* (eye), *Rhizopus oryzae* (sinus
83 biopsy), *Lomentospora prolificans* (blood) and *Lichtheimia corymbifera* (eye). All strains
84 were recovered from La Timone University Hospital in Marseille and were *in vitro* resistant
85 to several available systemic antifungal agents. *Aspergillus flavus* ATCC 204304 and
86 *Aspergillus niger* ATCC 200930 were used as internal quality controls. The minimum
87 inhibitory concentration (MIC) of posaconazole (POS), itraconazole (ITR), voriconazole
88 (VRC), isavuconazole (ISA), and amphotericin B (AMB) were determined using the E-test
89 method for each strain.

90 High AMB MICs ($> 32 \text{ mg/l}$) were recorded for all tested clinical strains, especially for
91 *Rhizopus oryzae*, *Lomentospora prolificans* and *Fusarium solani*, while MICs of azoles were
92 variable but remained higher than 32 mg/l for *Fusarium*, *Lomentospora* and *Rhizopus* species
93 [7]. Based on the primary drugs-screening, we identified 3 compounds that were active
94 against *R. oryzae*; 11 that were active against *F. oxysporum*, 12 that were active against *F.*

95 *solani*, 15 that were active against *L. prolificans* and *A. niger* ATCC, 13 that were active
96 against *A. calidoustus* and, finally, 14 that were active against both *A. flavus* ATCC and *L.*
97 *corymbifera* (Table 1). All retained compounds induced $\geq 70\%$ fungal growth inhibition and
98 were classified, in Table 1, according to the annotated database provided along with the
99 Prestwick chemical library. The identified hits belong to six different therapeutic classes,
100 including antifungals, antibacterials, antiseptics, anthelmintics, antineoplastics and other
101 miscellaneous drugs (Table 1). Not surprisingly, most of the hits were among antifungals (
102 47%). Nevertheless, there were some discrepancies between the E-test MIC results obtained
103 for AMB and VRC and the fungal growth inhibition measured for these compounds against
104 *Aspergillus* and *Fusarium* spp. strains. It is worth mentioning that the study by Lamoth et al.
105 2015 showed that VRC MICs for *Aspergillus* spp. tend to be lower with the E-test method as
106 compared to the broth micodilution method [8]. This may explain the absence of 70% of
107 fungal growth inhibition of *Aspergillus* spp. even with concentrations higher than VRC E-test
108 MICs. Furthermore, the aforementioned study considered that a variation, within a range of \pm
109 2 dilutions, of AMB and triazole MICs between E-test and broth microdilution methods
110 against *Aspergillus* and non-*Aspergillus* strains is acceptable. Among the non-antifungal
111 compounds that were effective against the clinical molds tested, four, namely clioquinol,
112 alexidine dihydrochloride, hexachlorophene, and thonzonium bromide, displayed a broad
113 antifungal spectrum against at least five of the six tested strains (Figure 1). Although these
114 compounds are mainly used as antiseptics, which may limit their systemic uses, our results
115 indicate how difficult it is to identify a drug with a broad spectrum of activity against fungal
116 pathogens.

117 Clioquinol (5-chloro-7-iodo-8-quinolinol) was initially used as a topical antiseptic and
118 for the treatment of intestinal amoebiasis [9]. In the early 1970s, the systemic administration
119 route of clioquinol was restricted or discontinued because it had been associated with

subacute myelo-optic neuropathy in Japanese patients [9]. Currently, topical clioquinol administration is used to treat skin infections [9]. Recently, You *et al* reported a significant activity of 3% clioquinol cream against a large number of fungal species [10]. Using modified agar diffusion assay, the authors showed that the inhibition zone was the largest for *Candida tropicalis*, *Candida guilliermondii*, *Aspergillus terreus* and *F. solani* [10]. In our study, clioquinol activity testing was performed using the microdilution broth method and showed significant growth inhibition of all tested mold strains except *R. oryzae*.

Alexidine dihydrochloride (AXD), a bis-guanide molecule, initially identified for its antibacterial properties, also has anti-inflammatory and anti-cancer effects [11]. In this study, AXD exerted a broad antifungal activity against *A. calidoutus* (81%), *F. solani* (82%), *F. oxysporum* (81%), *L. prolificans* (77%) and *L. corymbifera* (77%). Recently, in accordance with our results, Mamouel *et al*, reported an antifungal activity of AXD against a wide range of MDR-fungi, including the yeast *C. albicans*, *Candida auris*, *Cryptococcus neoformans* and the filamentous fungi *Scedosporium apiospermum*, *Aspergillus fumigatus*, *L. corymbifera* and also *R. oryzae*. Moreover, they reported a synergistic interaction between AXD and fluconazole against fluconazole-resistant *C. albicans* [12]. Intriguingly, we found no effect of 10 μ M (*i.e.* 5.8 mg/l) AXD against *R. oryzae* (Table 1), whereas the aforementioned study found AXD MIC₈₀ = 3 mg/l against *R. oryzae* strain. This discrepancy may be due to the multi-drug resistance profile (including posaconazole MIC >32 mg/l) of the *R. oryzae* strain used in our study, which contrasts with the one used in Mamouel *et al*'s study that displayed a posaconazole MIC = 0.25 mg/l. To our knowledge, the activity of AXD against *Fusarium* species and *L. prolificans* has not been previously reported.

Hexachlorophene (HCP), a chlorinated bisphenol, has significant bacteriostatic properties against several Gram-positive bacteria (including *Staphylococcus*) and is used as antiseptic in dermatological topical preparations [13]. HCP showed significant fungal growth

inhibition against all the tested strains, except *A. calidoustus* and *A. niger* (Table 1). The antifungal activity of HCP against difficult-to-treat fungal strains is reported here for the first time to our knowledge. Importantly, the clinical use of HCP has been restricted because studies on mice and human stem cells have demonstrated its chronic deleterious effect and oral or vaginal administration of high HCP doses has been shown to be embryotoxic and teratogenic in rats [13].

Thonzonium bromide (TB), a monocationic detergent, helps the penetration of active ingredients (antibacterial and anti-inflammatory drugs) through topical corticosteroid ear drops. Its antifungal activity against planktonic *C. albicans* strain has been highlighted in previous Prestwick chemical library screening [12]. It has been identified by Chan et al as a specific *C. albicans* ATPase inhibitor [14]. The significant activity of TB against all difficult-to-treat mold species tested in our study has not been described; it prompts for further study of its antifungal activity.

Conclusion

Despite the current advances in the diagnosis and prevention of invasive fungal infections, the incidence of mycosis, treatment failure and mortality remain excessively high in immunocompromised patients. This study results point out several off-label drugs that may be considered in the treatment of difficult-to-treat fungal infections. Further investigations of the antifungal properties of the compounds identified in this study, as monotherapy or in combination with other antifungal drugs, will enhance the management of MDR mold infections.

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Ethical Approval

169 Not required

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177 **Transparency declarations**

178 These is no conflict of interest or financial disclosure to declare for all authors.

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Inhibitors of V-ATPase proton transport reveal uncoupling functions of tether linking

216 cytosolic and membrane domains of V 0 subunit a (Vph1p). J Biol Chem
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218

219 **Table 1:** List of compounds showing activity (+) after the screening of the Prestwick Chemical Library against the 8 difficult-to-treat filamentous
220 fungi tested. (%): the proportion of fungal growth inhibition. (-): no or < 70% activity.

Compound name	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Aspergillus calidoustus</i>	<i>Fusarium solani</i>	<i>Fusarium oxysporum</i>	<i>Lomentospora prolificans</i>	<i>Rhizopus oryzae</i>	<i>Lichtheimia corymbifera</i>
<u>Antifungals</u>								
Tiabendazole	-	+ (70%)	-	+ (81%)	-	-	-	-
Voriconazole	-	-	+ (79%)	+ (80%)	+ (78%)	-	-	-
Butenafine Hydrochloride	-	-	-	+ (72 %)	+ (76%)	-	-	-
Econazole nitrate	+ (79%)	-	+ (83%)	+ (72%)	-	-	-	-
Amphotericin B	-	+ (74%)	-	+ (88%)	+ (70%)	-	-	-
Enilconazole	+ (81%)	+ (70%)	+ (79%)	-	+ (82%)	-	-	-
Terbinafine	-	-	-	-	+ (80%)	-	-	-
Haloprogin	+ (71%)	-	-	-	-	+ (80%)	-	+ (80%)
Liranaftate	+ (80%)	-	+ (82%)	-	-	+ (72%)	-	+ (72%)
Amorolfine hydrochloride	-	-	-	-	-	+ (80%)	-	+ (80%)
Itraconazole	+ (81%)	+ (74%)	+ (80%)	-	-	-	-	-
Oxiconazole Nitrate	-	-	+ (79%)	-	-	-	-	-
Butoconazole nitrate	-	+ (73%)	-	-	-	-	-	-
Sulconazole nitrate	-	+ (74%)	-	-	-	-	-	-
Posaconazole	-	+ (71%)	-	-	-	-	-	-
Miconazole	+ (81%)	-	-	-	-	-	-	-
Naftifine hydrochloride	+ (75%)	-	-	-	-	-	-	-
<u>Antibacterials</u>								
Chloroxine	-	-	+ (77%)	+ (87%)	+ (80%)	-	-	-
Alexidine dihydrochloride	+ (70%)	+ (76%)	+ (81%)	+ (82%)	+ (81%)	+ (77%)	-	+ (77%)
Dequalinium dichloride	-	-	-	-	-	+ (81%)	-	+ (81%)
Methyl benzethonium chloride	-	+ (73%)	-	-	-	+ (81%)	-	+ (81%)

Chlorhexidine	-	-	+ (70%)	-	-	+ (71%)	-	+ (70%)
Clotrimazole	+ (74%)	-	+ (78%)	-	-	+ (72%)	-	+ (71%)
Sertaconazole nitrate	-	-	+ (77%)	-	-	-	-	-
<u>Anthelmintics</u>								
Tiabendazole	-	+ (70%)	-	+ (81%)	-	-	-	-
Fenbendazole	-	+ (71%)	-	-	-	-	-	-
Albendazole	-	+ (74%)	-	-	-	-	-	-
Parbendazole	+ (73%)	+ (70%)	-	-	-	-	-	-
<u>Antiseptics</u>								
Thonzonium bromide	+ (72%)	+ (78%)	+ (73%)	+ (88%)	+ (83%)	+ (83%)	+ (72%)	+ (83%)
Hexachlorophene	+ (80%)	-	-	+ (79%)	+ (81%)	+ (81%)	+ (77%)	+ (81%)
Clioquinol	+ (71%)	+ (75%)	+ (73%)	+ (85%)	+ (77%)	+ (82%)	-	+ (82%)
<u>Antineoplastics</u>								
Azaguanine-8	-	-	-	-	-	+ (73%)	-	+ (74%)
Floxuridine	-	-	-	+ (92%)	+ (79%)	+ (73%)	-	+ (72%)
Camptothecine	+ (71%)	-	-	-	-	-	-	-
<u>Miscellaneous drugs</u>								
Pentetic acid (Chelator)	-	-	-	-	-	+ (73%)	+ (73%)	+ (73%)
Disulfiram (Alcohol addiction)	-	-	-	-	-	+ (74%)	-	+ (70%)

Figure legend

Figure 1: Venn diagram of non-traditional antifungal compounds active against clinical emerging filamentous fungi, with growth inhibition $> 70\%$.

Aspergillus calidoustus
(8)

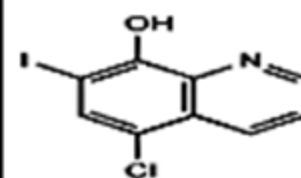
Lichtheimia corymbifera
(10)

Lomentospora prolificans
(11)

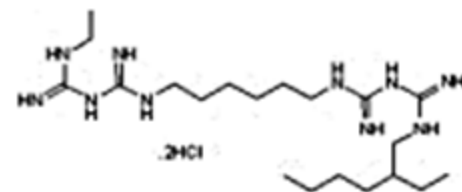
Rhizopus oryzae
(3)

Fusarium spp
(6)

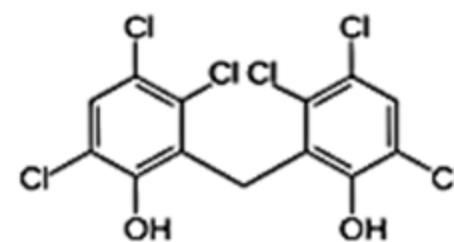
Clioquinol



Alexidine dihydrochloride



Hexachlorophene



Thonzonium bromide

