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 ≥ 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

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

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

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

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

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

127 Alexidine dihydrochloride (AXD), a bis-guanide molecule, initially identified for its
128 antibacterial properties, also has anti-inflammatory and anti-cancer effects [11]. In this study,
129 AXD exerted a broad antifungal activity against *A. calidoutus* (81%), *F. solani* (82%), *F.*
130 *oxysporum* (81%*), L. prolificans* (77%) and *L. corymbifera* (77%). Recently, in accordance
131 with our results, Mamouel et *al*, reported an antifungal activity of AXD against a wide range
132 of MDR-fungi, including the yeast *C. albicans*, *Candida auris*, *Cryptococcus neoformans* and
133 the filamentous fungi *Scedosporium apiospermum*, *Aspergillus fumigatus*, *L. corymbifera* and
134 also *R. oryzae*. Moreover, they reported a synergistic interaction between AXD and
135 fluconazole against fluconazole-resistant *C. albicans* [12]. Intriguingly, we found no effect of
136 10 µM (*i.e*. 5.8 mg/l) AXD against *R. oryzae* (Table 1), whereas the aforementioned study
137 found AXD MIC_{80} = 3 mg/l against *R. oryzae* strain. This discrepancy may be due to the
138 multi-drug resistance profile (including posaconazole MIC >32 mg/l) of the *R. oryzae* strain
139 used in our study, which contrasts with the one used in Mamouel *et al*'s study that displayed a
140 posaconazole MIC = 0.25 mg/l. To our knowledge, the activity of AXD against *Fusarium*
141 species and *L. prolificans* has not been previously reported.

142 Hexachlorophene (HCP), a chlorinated bisphenol, has significant bacteriostatic
143 properties against several Gram-positive bacteria (including *Staphylococcus*) and is used as
144 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 corticosporin-TC 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

Not required

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Transparency declarations

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

References

[1] De Pauw BE. What are fungal infections? Mediterr J Hematol Infect Dis 2011;3(1):e2011001.

[2] Scorzoni L, de Paula e Silva AC, Marcos CM, Assato PA, de Melo WC, de Oliveira HC, *et al*. Antifungal therapy: New advances in the understanding and treatment of mycosis. Front Microbiol 2017;8:36.

[3] Nicola AM, Albuquerque P, Paes HC, Fernandes L, Costa FF, Kioshima ES, *et al*. Antifungal drugs: New insights in research & development. Pharmacol Ther 2018;195:21-38.

[4] Enoch DA, Ludlam HA, Brown NM. Invasive fungal infections: A review of epidemiology and management options. J Med Microbiol 2006;55:809–18.

[5] Geddes-Mcalister J, Shapiro RS. New pathogens, new tricks: Emerging, drug-resistant fungal pathogens and future prospects for antifungal therapeutics. Ann N Y Acad Sci

2018;1435:57–78.

[6] Corsello SM, Bittker JA, Liu Z, Gould J, Mccarren P, Hirschman JE, et al. HHS Public Access. Nat Med 2017;23:405–8.

[7] Yousfi H, Ranque S, Rolain JM, Bittar F. *In vitro* polymyxin activity against clinical multidrug-resistant fungi. Antimicrobial Resistance and Infection Control 2019; 24;8:66.

[8] Lamoth F, D. Alexandera B. Comparing Etest and Broth Microdilution for Antifungal Susceptibility Testing of the Most-Relevant Pathogenic Molds.Journal of clinical Microbiology 2015;53: 3176–3181.

[9] Bareggi SR, Cornelli U. Clioquinol: Review of its mechanisms of action and clinical uses in neurodegenerative Disorders. CNS Neurosci Ther 2012;18:41–6.

[10] You Z, Ran X, Dai Y, Ran Y. Clioquinol, an alternative antimicrobial agent against common pathogenic microbe. J Mycol Med 2018;28:492–501.

[11] Zhu X, Gao J, Ng PY, Qin A, Steer JH, Pavlos NJ, *et al.* Alexidine Dihydrochloride Attenuates Osteoclast Formation and Bone Resorption and Protects Against LPS-Induced Osteolysis. J Bone Miner Res 2016;31:560–72.

[12] Mamouei Z, Alqarihi A, Singh S, Xu S, Mansour MK, Ibrahim AS, *et al*. Alexidine Dihydrochloride Has Broad-Spectrum Activities against Diverse Fungal Pathogens. MSphere 2018;3:1–11.

[13] Leśniak M, Zdanowski R, Suska M, Brewczyńska A, Stankiewicz W, Kloc M, *et al*. Effects of Hexachlorophene, a Chemical Accumulating in Adipose Tissue, on Mouse and Human Mesenchymal Stem Cells. Tissue Eng Regen Med 2018;15:211–22.

[14] Chan CY, Prudom C, Raines SM, Charkhzarrin S, Melman SD, De Haro LP, *et al*. Inhibitors of V-ATPase proton transport reveal uncoupling functions of tether linking

cytosolic and membrane domains of V 0 subunit a (Vph1p). J Biol Chem 2012;287:10236–50.

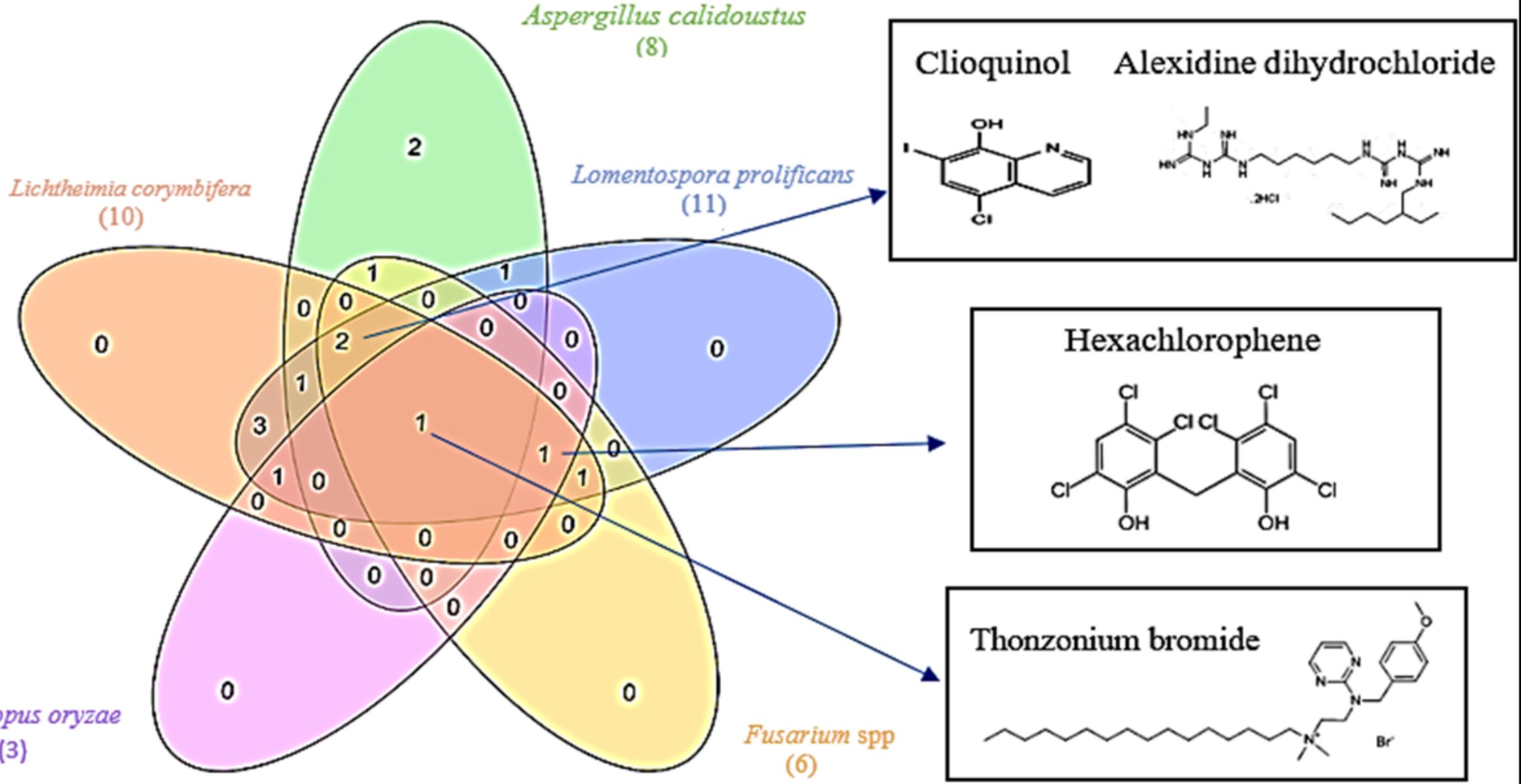
Table 1: List of compounds showing activity (+) after the screening of the Prestwick Chemical Library against the 8 difficult-to-treat filamentous fungi tested. (%): the proportion of fungal growth inhibition. (-): no or < 70% activity.

Compound name	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus calidoustus*	*Fusarium solani*	*Fusarium oxysporum*	*Lomentospora prolificans*	*Rhizopus oryzae*	*Lichtheimia corymbifera*
Antifungals								
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%)	-	-	-	-	-	-	-
Antibacterials								
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%)	-	-	-	-	-
Anthelmintics								
Tiabendazole	-	+ (70%)	-	+ (81%)	-	-	-	-
Fenbendazole	-	+ (71%)	-	-	-	-	-	-
Albendazole	-	+ (74%)	-	-	-	-	-	-
Parbendazole	+ (73%)	+ (70%)	-	-	-	-	-	-
Antiseptics								
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%)
Antineoplastics								
Azaguanine-8	-	-	-	-	-	+ (73%)	-	+ (74%)
Floxuridine	-	-	-	+ (92%)	+ (79%)	+ (73%)	-	+ (72%)
Camptothecine	+ (71%)	-	-	-	-	-	-	-
Miscellaneous drugs								
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