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Artemisinins, new miconazole potentiators resulting in increased activity against *Candida albicans* biofilms

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Running title: Artemisinins potentiate miconazole

Abstract

Mucosal biofilm-related fungal infections are very common and the incidence of recurrent oral and vulvovaginal candidiasis is significant. As resistance against azoles (preferred treatment) is occurring, we aimed at identifying compounds that increase the activity of miconazole against *Candida albicans* biofilms. We screened 1600 compounds of a drug repositioning library in combination with a sub-inhibitory concentration of miconazole. Synergy between the best identified potentiators and miconazole was characterized by checkerboard analyses and fractional inhibitory concentration indices. Hexachlorophene, pyrvinium pamoate and artesunate act synergistically with miconazole in affecting *C. albicans* biofilms. Synergy was most pronounced for artesunate and structural homologues thereof. No synergistic effect could be observed between artesunate and fluconazole, caspofungin or amphotericin B. Our data reveal enhancement of the antibiofilm activity of miconazole by artesunate, pointing to potential combination therapy consisting of miconazole and artesunate to treat *C. albicans* biofilm-related infections.

Introduction

Multiple fungal species possess the capacity to form biofilms, characterized by increased resistance against commonly used antimycotics, on both biotic and abiotic surfaces (1, 2). The population of people susceptible to this type of infection is growing, mainly as a consequence of the extended life span, increasing numbers of immunocompromised individuals and use of indwelling medical devices, which can serve as a substrate for biofilm formation (3–6). Therefore, the occurrence of biofilm-associated infections has expanded over the last decennia and the extent to which they impact the health of human hosts is enormous (7, 8).

The genus *Candida* predominates this type of fungal infection occurring in the oral cavity, upper and lower airways, gastro-intestinal and urinary tract, on wounds and on medical devices. Such a *Candida* biofilm infection can be of a rather restricted superficial mucosal type or can evolve to hazardous invasive candidiasis (1, 7, 9). Mucosal fungal infections are very common and can often be treated adequately using azoles. However, the incidence of recurrent oral and vulvovaginal candidiasis is significant and resistance against azoles is occurring (10–13). Vaginal infections caused by *Candida* spp. affect 70–75% of women at least once during their lives, and 40–50% of them will experience at least one recurrence (14). Also immunocompromised persons like HIV patients are susceptible to this type of recurrent candidiasis, mostly concerning the oral cavity (15, 16).

Mechanisms underlying the increased resistance of biofilm cells to antimycotics are still not fully understood. However, it has been reported that biofilm formation typically induces several stress response pathways that impair the activity of azole drugs, such as the induction of drug efflux pumps (17). Consequently, cells in a biofilm are up to 1000-fold more azole resistant than their planktonic counterparts (1, 18), supporting the need for new treatments.

Despite the considerable impact on human health and the problems with resistance related to fungal biofilms, the antimicrobial drug pipeline contains few novel agents that can be used against such biofilm-related infections (19). One approach to overcome the need for new antifungal and antibiofilm compounds is to enhance the activity of existing antimycotics by combining it with another compound, a strategy termed "potentiation". Such so-called potentiators can have multiple modes of action, including the inhibition of tolerance pathways in the biofilm or induction of increased uptake of the antimycotics.

In this study, we employed the concept of repurposing/repositioning existing market drugs. This concept has recently gained a lot of attention, also in the anti-*Candida* research (20–25). Repurposing of known drugs is favourable from an economic perspective. As these molecules have a safe toxicity profile and dosing regimens are known, the cost of performing new clinical trials, and possibly, reformulating the drug, are considerably less than for the development of a new drug from scratch (26). For example, toremifene citrate (a selective oestrogen receptor modulator used in the treatment of breast cancer) has been reported to be a good potentiator of amphotericin B and caspofungin, but not of azole-type antifungals, against *C. albicans* biofilms (20). Potentiation of azole antifungals by 2-adamantanamine, a derivative of amantadine (anti-influenza A drug also used to treat some of the symptoms of Parkinson's disease), against *C. albicans* biofilms was recently demonstrated, suggesting the opportunity to repurpose (analogues of) other FDA-approved medications (22).

For the above-mentioned reasons, we opted to screen a repositioning compound library for compounds that can potentiate the activity of the azole miconazole against *C. albicans* biofilms. This concept of potentiation is often favoured over the application of single compounds as it may lead to (i) a widened spectrum of drug activity, (ii) a more rapid antifungal effect, (iii) synergy, (iv) lowered dosing of toxic drugs and (v) reduced risk of antifungal resistance (27).

Materials and methods

Strains and chemicals. *C. albicans* strains SC5314 (28) and B2630 (29), used in this study were grown routinely on YPD (1% yeast extract, 2% peptone (International Medical Products, Belgium) and 2% glucose (Sigma-Aldrich, USA)) agar plates at 30°C. Stock solutions of miconazole (Sigma-Aldrich) were prepared in DMSO (VWR International, Belgium). RPMI 1640 medium (pH 7.0) with L-glutamine and without sodium bicarbonate was purchased from Sigma-Aldrich and buffered with MOPS (Sigma-Aldrich). The Pharmakon 1600 repositioning library (Microsource discovery systems, USA) was supplied by the Centre of Drug Design and Discovery (Dr. Patrick Chaltin, KU Leuven, Belgium). Pyrvinium pamoate (salt hydrate) and hexachlorophene were purchased from Sigma-Aldrich. Artesunate, artemisinin, dihydroartemisinin and artemether were purchased from TCI Europe (Belgium).

Antibiofilm screening assay. A *C. albicans* SC5314 overnight culture, grown in YPD, was diluted to an optical density of 0.1 (approximately 10^6 cells/mL) in RPMI medium and 100 µL of this suspension was added to the wells of a round bottomed microplate (TPP Techno Plastic Products AG, Switzerland) (30, 31). After 1 h of adhesion at 37°C, the medium was aspirated and biofilms were washed with 100 µL phosphate buffered saline (PBS) to remove non-adherent cells, followed by addition of 100 µL RPMI 1640 medium. Biofilms were allowed to grow for 24 h at 37°C. Afterwards, 5 µM of miconazole was added in combination with 20 µM of a compound from the Pharmakon 1600 library (2 mM stock solution in DMSO) in RPMI, resulting in 1.1 % DMSO background. Biofilms were incubated for an additional 24 h at 37°C. Finally, biofilms were washed and quantified with Cell-Titre Blue (CTB; Promega, USA) (32) by adding 100 µL CTB diluted 1/10 in PBS to each well. After 1 h of incubation in the dark at 37°C, fluorescence was measured with a fluorescence spectrometer (Synergy Mx multi-mode microplate reader, BioTek, USA) at λ_{ex} 535 nm and

λ_{em} 590nm. Fluorescence values of the samples were corrected by subtracting the average fluorescence value of CTB of uninoculated wells (blank). Percentage of metabolically active biofilm cells was calculated relative to the control treatment (1.1 % DMSO). Compounds were considered for retesting when their application in the presence of 5 µM miconazole resulted in less than 60% residual metabolic activity of *C. albicans* biofilm cells compared to the control and when the main reported application was not because of antifungal activity.

BEC-2 determination assay. To determine the Biofilm Eradication Concentration-2 value (BEC-2; the minimal concentration of the compound that causes a 2-fold decrease in biofilm metabolic activity) for the respective compounds, *C. albicans* SC5314 biofilms were grown in a round bottomed microplate as described above. Afterwards, biofilms were washed with 100 µL PBS and 100 µL of a concentration series of compound in RPMI was added to the biofilm, resulting in 0.5 % DMSO background. Biofilms were incubated for 24 h at 37°C after which they were washed and quantified with CTB as described above.

Biofilm checkerboard assay. In order to determine possible synergistic interactions between antifungal agents on one hand and identified potentiators on the other hand against *C. albicans* SC5314, checkerboard analysis was used. *C. albicans* biofilms were grown as described above. A combination of antifungal compound and potentiator, two-fold diluted across rows and columns of a microplate respectively, was added (DMSO background 0.6%). After 24 h of incubation at 37°C, biofilms were quantified with the CTB method. Synergism was determined by FICI (fractional inhibitory concentration index) calculations (20, 33). The FICI was calculated by the formula $FICI=[C(BEC\text{-}2_A)/ BEC\text{-}2_A]+[C(BEC\text{-}2_B)/BEC\text{-}2_B]$, in which $C(BEC\text{-}2_A)$ and $C(BEC\text{-}2_B)$ are the BEC-2 values of the antifungal drugs in combination, and $BEC\text{-}2_A$ and $BEC\text{-}2_B$ are the BEC-2 values of antifungal drugs A and B alone. The interaction was defined as synergistic for a value of $FICI\leq 0.5$, indifferent for $0.5<FICI<4$ and antagonistic for $FICI\geq 4.0$ (33).

Planktonic checkerboard assay. Synergistic action on growth of planktonic cells was determined by FICI calculations as described above. MIC-2 values (Minimal Inhibitory Concentration-2 value; the minimal concentration of the compound that causes a 2-fold reduction of planktonic cell growth) were used instead of BEC-2 values. To determine the MIC-2 for the respective compounds we applied similar conditions as Kaneko *et al.* (24). Briefly, an overnight culture of *C. albicans* SC5314 was diluted to an optical density of 0.1 in Synthetic Complete (SC) medium (1% CSM, complete amino acid supplement mixture (MP Biomedicals, USA), 1% YNB, yeast nitrogen base; 2% glucose (Sigma-Aldrich) in combination with a concentration series of the compounds, 2-fold diluted across the rows of the microplate. After 24 h of growth in the presence of the compounds at 37°C, growth was quantified by measuring the OD at 490 nm. Percentage growth reduction was calculated relative to the control treatment (0.5% DMSO). In the checkerboard assay, a combination of antifungal compound and potentiator, two-fold diluted across rows and columns of a microplate respectively, was added (DMSO background 0.6%) to the diluted overnight culture. After 24 h of incubation at 37°C, OD was measured at 490 nm.

ROS detection assay. *C. albicans* biofilms, grown as described above, were incubated for 2-24 h with artesunate, miconazole or a combination of both compounds in RPMI at 37°C. After washing the biofilm cells with PBS, biofilms were incubated in the presence of 10 μM H2DCFDA (Invitrogen, USA) in PBS for 1 h at 37°C. Fluorescence was measured at λ_{ex} 470 nm and λ_{em} 525 nm.

Statistical analysis. Results were analysed for statistical significance by unpaired two-tailed student's *t* test. Values were considered to be statistically significant when the *P* value was < 0.05.

Results and discussion

Screening for potentiators of the antibiofilm activity of miconazole against *C. albicans* biofilms

We screened 1600 off-patent drugs and other bioactive agents (Pharmakon 1600 repositioning library) to identify compounds that can enhance the antibiofilm activity of miconazole against mature biofilms. First we determined the effect of a concentration series of miconazole alone on mature *C. albicans* biofilms and found that BEC-2 of miconazole is 100 µM (Figure 1). We opted to add a combination of library-compound (20 µM) and a sub-antibiofilm concentration of miconazole (5 µM, resulting in 90-100 % remaining biofilm activity). We identified 8 compounds that resulted in less than 60% residual metabolic activity of the *C. albicans* biofilm cells when applied in the presence of 5 µM miconazole and with a main reported application other than antifungal activity. These are listed in Table 1 in which we additionally list their known medical application.

This initial screening strategy did not discriminate between compounds that affect the biofilm on their own or compounds that enhance the antibiofilm activity of miconazole. To discriminate between these two hypotheses, we examined the antibiofilm activity of 20 µM of these 16 compounds in the presence and absence of 5 µM miconazole. In Table 1 the compounds are sorted based on the ratio between the residual metabolic activity of the biofilm cells after treatment with the compound in combination with miconazole and after treatment with the compound alone. For compounds with a ratio > 1 there seems to be an increased antibiofilm effect of the combination with miconazole compared to the compound alone. However, many of the top compounds in this list are also very active on their own against *C. albicans* biofilms as illustrated by their BEC-2 value.

Hexachlorophene, pyrvinium pamoate and artesunate act synergistically with miconazole to diminish *C. albicans* biofilm activity

The top 3 compounds, based on the ratio between their effect in combination with miconazole and alone (hexachlorophene, pyrvinium pamoate and artesunate), were selected to determine whether they act synergistically with miconazole against *C. albicans* biofilms. Hexachlorophene is a topical anti-infective drug, often used in soaps, liquid detergents and cosmetics during the 1960's, but its use has been questioned because of toxicity (34). Pyrvinium pamoate is an antihelmintic drug that, when taken orally, is safe even at high doses, but systemic absorption from the gut is minimal (35). Artesunate, a semi-synthetic derivative of artemisinin extracted from *Artemisia annua* (sweet wormwood), is one of the most widely applied antimalarial drugs, recommended by the World Health Organisation (36). We performed checkerboard analysis and calculated the corresponding FICI to determine synergy (FICI $\leq$ 0.5) for each of these compounds in combination with miconazole (Table 2). Note that only one strain was used in this study and clinical strains may behave differently.

Although all three compounds establish a synergistic interaction with miconazole, artesunate is the only potentiator which has almost no antibiofilm activity against *C. albicans* when used alone (according to the BEC-2 value in Table 1), resulting in the lowest FICI values (Table 2). Additionally, combinations with this compound resulted in the highest reduction of the BEC-2 value of miconazole, as illustrated in Figure 1 and quantified in Table 2 (fold change). Therefore, this compound was selected for further extensive characterization.

The mechanism of action of artesunate (and structural homologs thereof, collectively called artemisinins) in the treatment of malaria is complex and only partially understood (37). Many effort is still put in the elucidation of the mode of action both in *Plasmodium falciparium*

(38–40) and yeast (41–43). A yeast model uncovered a role of mitochondria during the action of artemisinins with an important function for the electron transport chain and subsequent damage by locally generated ROS (41). We only observed slightly increased ROS accumulation in *C. albicans* biofilm cells treated with artesunate concentrations starting from 20 µM and a more pronounced ROS accumulation upon incubation with miconazole alone (as reported previously (44, 45)), but no increased ROS accumulation due to the combination of both compounds could be observed (data not shown).

Artesunate does not increase the activity of fluconazole, amphotericin B and caspofungin against *C. albicans* biofilms

We assessed whether artesunate could also increase the activity of other types of azoles like fluconazole against *C. albicans* biofilms. Fluconazole showed no significant antibiofilm activity on its own (BEC-2 > 500 µM) nor in combination with 20 µM artesunate (BEC-2 > 500 µM).

Kaneko et al. (2013) recently showed that artesunate could enhance the activity of the azole fluconazole against planktonic *C. albicans* SC5314 cultures (24). Therefore we checked whether artesunate could increase the antifungal activity of miconazole or fluconazole on planktonic cells by checkerboard analyses (data not shown). A concentration of 5 µM artesunate reduced the MIC-2 values of miconazole and fluconazole (0.11 ± 0.01 µM and 3.25 ± 0.43 µM, respectively) by 2-fold and 1.6-fold, respectively, in line with the previous reported data (24). However, artesunate did not act synergistically with miconazole or fluconazole on planktonic *C. albicans* cells as the resulting FICI was higher than 0.5.

Next, we determined whether artesunate could increase the activity of other types of antimycotics like amphotericin B and caspofungin against *C. albicans* biofilms (Supplemental Figures 1 and 2). However, the BEC-2 values of amphotericin B (1.8 ± 0.4

μM) and caspofungin (0.4 ± 0.06 μM) remained almost unaffected by the presence of all tested concentrations of artesunate, resulting in FICI values between 0.5 and 2. Consequently, the interaction of artesunate with amphotericin B and caspofungin is defined as indifferent.

Structural homologues of artesunate act synergistically with miconazole against *C. albicans* biofilms

Table 1 suggests that a structural homologue of artesunate, namely dihydroartemisinin, is also capable of increasing the activity of miconazole against *C. albicans* biofilms. Interestingly, one of the compounds that is on the border of the cut-off for the top 16 was another homologue of these compounds, namely artemisinin (resulting in 61% biofilm metabolic activity in the combination screen; data not shown). One other structural homologue was available to us during this study, namely artemether. Via checkerboard analyses we could show that the observed synergy between artesunate and miconazole against *C. albicans* biofilms is not only specific for artesunate but also applies to artemisinin, dihydroartemisinin and artemether (collectively called artemisinin derivatives or artemisinins, BEC-2 values for all single compounds > 200 μM) (
Figure 2). A concentration of 5 μM of these compounds with miconazole resulted in a 6.2-fold, 8.3-fold and 11.4-fold reduction of the BEC-2 value of miconazole (resulting in maximal FICI values of 0.186, 0.145 and 0.112) for artemisinin, dihydroartemisinin and artemether, respectively. These data suggest that the synergistic action with miconazole is a characteristic of all artemisinins, implying that the activity is probably attributed to the core chemical structure (a sesquiterpene scaffold with an endo-peroxide bridge) of this family.

We conclude that combinations of miconazole with artesunate or other artemisinins could be a novel therapeutic strategy to treat *C. albicans* biofilm-related infections.

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References

1. **Ramage G, Rajendran R, Sherry L, Williams C**. 2012. Fungal biofilm resistance. Int. J. Microbiol. **2012**:528521.

2. **Lewis K**. 2008. Multidrug tolerance of biofilms and persister cells. Curr. Top. Microbiol. Immunol. **322**:107–131.

3. **Lebeaux D, Fernández-Hidalgo N, Chauhan A, Lee S, Ghigo J-M, Almirante B, Beloin C**. 2013. Management of infections related to totally implantable venous-access ports: challenges and perspectives. Lancet Infect. Dis. **3099**:13–17.

4. **Tumbarello M, Fiori B, Trecarichi EM, Posteraro P, Losito AR, De Luca A, Sanguinetti M, Fadda G, Cauda R, Posteraro B**. 2012. Risk Factors and Outcomes of Candidemia Caused by Biofilm-Forming Isolates in a Tertiary Care Hospital. PLoS One. **7**: e33705.

5. **Rautemaa R, Ramage G**. 2011. Oral candidosis--clinical challenges of a biofilm disease. Crit. Rev. Microbiol. **37**:328–36.

6. **Alves MJ, Barreira JC, Carvalho I, Trinta L, Pereira L, Ferreira IC PM**. 2014. Propensity for biofilm formation by clinical isolates from urinary tract infections: developing a multifactorial predictive model to improve the antibiotherapy. J Med Microbiol. **63**:471-7.

7. **Ramage G, Williams C**. 2013. The clinical importance of fungal biofilms.Advances in applied microbiology. **84**:27-83.

8. **Harriott MM, Noverr MC**. 2011. Importance of Candida–bacterial polymicrobial biofilms in disease. Trends Microbiol. **19**:557-63.

9. **Lim CS-Y, Rosli R, Seow HF, Chong PP**. 2012. Candida and invasive candidiasis: back to basics. Eur. J. Clin. Microbiol. Infect. Dis. **31**:21–31.

10. **Chopra V, Marotta F, Kumari A, Bishier MP, He F, Zerbinati N, Agarwal C, Naito Y, Tomella C, Sharma A SU**. 2013. Prophylactic strategies in recurrent

vulvovaginal candidiasis: a 2-year study testing a phytonutrient vs itraconazole. J Biol Regul Homeost Agents. **27**:875–82.

11. **Marchaim D, Lemanek L, Bheemreddy S, Kaye KS, Sobel JD**. 2012. Fluconazole-resistant Candida albicans vulvovaginitis. Obstet. Gynecol. **120**:1407–14.

12. **Gallè F, Sanguinetti M, Colella G, Di Onofrio V, Torelli R, Rossano F, Liguori G**. 2011. Oral candidosis: characterization of a sample of recurrent infections and study of resistance determinants. New Microbiol. **34**:379–89.

13. **Marcos-Arias C, Eraso E, Madariaga L, Carrillo-Muñoz AJ, Quindós G**. 2012. In Vitro Activities of New Triazole Antifungal Agents, Posaconazole and Voriconazole, Against Oral Candida Isolates from Patients Suffering from Denture Stomatitis. Mycopathologia. **173**:35-46.

14. **Rosa MI, Silva BR, Pires PS, Silva FR, Silva NC, Souza SL, Madeira K, Panatto AP, Medeiros LR**. 2013. Weekly fluconazole therapy for recurrent vulvovaginal candidiasis: a systematic review and meta-analysis. Eur. J. Obstet. Gynecol. Reprod. Biol. **167**:132–6.

15. **Vazquez J**. 2010. Optimal management of oropharyngeal and esophageal candidiasis in patients living with HIV infection. HIV/AIDS - Res. Palliat. Care. **2**:89–101.

16. **Vazquez J.A.** 2000. Therapeutic options for the management of oropharyngeal and esophageal candidiasis in HIV/AIDS patients. HIV Clin. Trials **1**:47–59.

17. **Taff HT, Mitchell KF, Edward JA, Andes DR**. 2013. Mechanisms of Candida biofilm drug resistance. Future Microbiol. **8**:1325–37.

18. **Delattin, N., Cammue, B., Thevissen K**. 2014. Reactive oxygen species-inducing antifungal agents and their activity against fungal biofilms. Future Med. Chem. **6**:77–90.

19. **Butler MS, Cooper MA**. 2011. Antibiotics in the clinical pipeline in 2011. J. Antibiot. (Tokyo). **64**:413–425.

20. **Delattin N, De Brucker K, Vandamme K, Meert E, Marchand A, Chaltin P, Cammue BP a, Thevissen K**. 2013. Repurposing as a means to increase the activity of amphotericin B and caspofungin against Candida albicans biofilms. J. Antimicrob. Chemother. **69**:1035-44.

21. **LaFleur MD, Lucumi E, Napper AD, Diamond SL, Lewis K**. 2011. Novel high-throughput screen against Candida albicans identifies antifungal potentiators and agents effective against biofilms. J. Antimicrob. Chemother. **66**:820–6.

22. **Lafleur MD, Sun L, Lister I, Keating J, Nantel A, Long L, Ghannoum M, North J, Lee RE, Coleman K, Dahl T, Lewis K**. 2013. Potentiation of azole antifungals by 2-adamantanamine. Antimicrob. Agents Chemother. **57**:3585–92.

23. **Siles S a, Srinivasan A, Pierce CG, Lopez-Ribot JL, Ramasubramanian AK**. 2013. High-throughput Screening of a Collection of Known Pharmacologically Active Small Compounds for the Identification of Candida albicans Biofilm Inhibitors. Antimicrob. Agents Chemother. **57**:3681-7.

24. **Kaneko Y, Fukazawa H, Ohno H, Miyazaki Y**. 2013. Combinatory effect of fluconazole and FDA-approved drugs against Candida albicans. J. Infect. Chemother. **19**:1141-5.

25. **Spitzer M, Griffiths E, Blakely KM, Wildenhain J, Ejim L, Rossi L, De Pascale G, Curak J, Brown E, Tyers M, Wright GD**. 2011. Cross-species discovery of syncretic drug combinations that potentiate the antifungal fluconazole. Mol. Syst. Biol. **7**:499.

26. **Sekhon BS**. 2013. Repositioning drugs and biologics : Retargeting old / existing drugs for potential new therapeutic applications **4**:1.

27. **Bink A**. 2011. Anti-Biofilm Strategies: How to Eradicate Candida Biofilms? Open Mycol. J. **5**:29-38.

28. **Fonzi WA, Irwin MY**. 1993. Isogenic strain construction and gene mapping in Candida albicans. Genetics **134**:717–728.

29. **De Wit K, Paulussen C, Matheeussen A, van Rossem K, Cos P, Maes L**. 2010. In vitro profiling of pramiconazole and in vivo evaluation in Microsporum canis dermatitis and Candida albicans vaginitis laboratory models. Antimicrob. Agents Chemother. **54**:4927–4929.

30. **Van den Driessche F, Rigole P, Brackman G, Coenye T**. 2014. Optimization of resazurin-based viability staining for quantification of microbial biofilms. J. Microbiol. Methods **98**:31–34.

31. **Iraqui I, Garcia-Sanchez S, Aubert S, Dromer F, Ghigo JM, D'Enfert C, Janbon G**. 2005. The Yak1p kinase controls expression of adhesins and biofilm formation in Candida glabrata in a Sir4p-dependent pathway. Mol. Microbiol. **55**:1259–1271.

32. **O'Brien J, Wilson I, Orton T, Pognan F**. 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. **267**:5421–5426.

33. **Odds FC**. 2003. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother **52**:1.

34. **Kimbrough RD**. 1973. Review of the toxicity of hexachlorophene, including its neurotoxicity. J. Clin. Pharmacol. **13**:439–44.

35. **Smith TC, Kinkel AW, Gryczko CM, Goulet JR**. 1976. Absorption of pyrvinium pamoate. Clin. Pharmacol. Ther. **19**:802–806.

36. **World Health Organization**. 2010. Guidelines for the treatment of malaria, 2nd editionWHO. 194p.

37. **O'Neill PM, Barton VE, Ward SA**. 2010. The molecular mechanism of action of artemisinin--the debate continues. Molecules **15**:1705–1721.

38. **Klonis N, Creek DJ, Tilley L**. 2013. Iron and heme metabolism in Plasmodium falciparum and the mechanism of action of artemisinins. Curr. Opin. Microbiol. **16**:722-7.

39. **Wang J, Huang L, Li J, Fan Q, Long Y, Li Y, Zhou B**. 2010. Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation. PLoS One **5**: e9582.

40. **Krishna S, Pulcini S, Moore CM, Teo BH-Y, Staines HM**. 2014. Pumped up: reflections on PfATP6 as the target for artemisinins. Trends Pharmacol. Sci. **35**:4–11.

41. **Li W, Mo W, Shen D, Sun L, Wang J, Lu S, Gitschier JM, Zhou B**. 2005. Yeast model uncovers dual of mitochondria in the action of artemisinin. PLoS Genet. **1**:329–334.

42. **Moore CM, Hoey EM, Trudgett A, Timson DJ**. 2011. Artemisinins act through at least two targets in a yeast model. FEMS Yeast Res. **11**:233–237.

43. **Alenquer M, Tenreiro S, Sá-Correia I**. 2006. Adaptive response to the antimalarial drug artesunate in yeast involves Pdr1p/Pdr3p-mediated transcriptional activation of the resistance determinants TPO1 and PDR5. FEMS Yeast Res. **6**:1130–1139.

44. **Vandenbosch D, Braeckmans K, Nelis HJ, Coenye T**. 2010. Fungicidal activity of miconazole against Candida spp. biofilms. J. Antimicrob. Chemother. **65**:694–700.

45. **Bink A, Vandenbosch D, Coenye T, Nelis H, Cammue BP a, Thevissen K**. 2011. Superoxide dismutases are involved in Candida albicans biofilm persistence against miconazole. Antimicrob. Agents Chemother. **55**:4033–7.

Table 1. Hits from the miconazole potentiator screen. The ratio of the antibiofilm effect of 20 µM of the repurposed compounds alone and in combination with 5 µM miconazole, as well as the BEC-2 value of each compound alone is indicated (mean ± SEM of at least 3 independent biological replicates).

Compound	Application[a]	Ratio effect combination/alone	BEC-2[b] (µM)
Hexachlorophene	Antiinfective (topical)	2.29	9.3 ± 0.65
Pyrvinium pamoate	Antihelmintic	2.10	3.9 ± 0.13
Artesunate	Antimalarial	1.69	> 200
Broxyquinoline	Antiinfectant, disinfectant	1.42	1.2 ± 0.31
Dihydroartemisinin	Antimalarial	1.31	> 200
Gentian violet	Antibacterial, antihelmintic	1.18	1.5 ± 0.08
Bithionate disodium	Anthelmintic, antiseptic	1.09	6.0 ± 1.56
Nitroxoline	Antibacterial	0.94	3.1 ± 1.67

[a]as stated by the Pharmakon 1600 information sheet

[b]BEC-2 = Biofilm Eradication Concentration-2

388 **Table 2**. Synergistic activity of the potentiators hexachlorophene, pyrvinium pamoate and
389 artesunate with miconazole against *C. albicans* biofilms. The BEC-2 values are the mean ±
390 SEM of at least 3 independent biological replicates and were analysed for statistical
391 significance by unpaired two-tailed student's *t* test (against control treatment of miconazole
392 alone).

Combination	Concentration potentiator (µM)	BEC-2[a] miconazole (µM) ± SEM	P-value	Fold change[b]	FICI[c]
Miconazole alone	/	92.3 ± 13.0	NA	NA	NA
Miconazole + hexachlorophene	2.5	9.3 ± 3.4	0.0158	9.9	0.370
	1.25	15.7 ± 6.1	0.0228	5.9	0.305
	0.625	18.5 ± 4.8	0.0265	5.0	0.268
	0.3125	26.0 ± 8.0	0.0397	3.6	0.315
Miconazole + pyrvinium pamoate	1.25	9.3 ± 4.1	0.0164	10.0	0.421
	0.625	18.9 ± 7.7	0.0284	4.9	0.365
	0.3125	28.7 ± 11.6	0.0482	3.2	0.391
Miconazole + artesunate	5	4.0 ± 0.9	0.0050	22.9	<0.069
	2.5	5.2 ± 0.7	0.0055	17.7	<0.069
	1.25	12.2 ± 4.3	0.0194	7.5	<0.139
	0.625	15.2 ± 3.6	0.0234	5.9	<0.171

393 [a]BEC-2 = Biofilm Eradication Concentration-2, the BEC-2 value of Hexachlorophene,
394 Pyrvinium pamoate and Artesunate is 9.3, 3.9 and > 200 µM, respectively.
395 [b]Fold change = fold increase of biofilm activity of miconazole due to the combination,
396 calculated as (BEC-2 of miconazole alone)/(BEC-2 of miconazole in combination)
397 [c]FICI = Fractional Inhibitory Concentration Index, NA = Not Applicable.

FIGURE LEGENDS

Figure 1. Metabolic activity of *C. albicans* biofilms treated with a combination of artesunate and miconazole (checkerboard assay). The control curve of miconazole alone without artesunate is represented by diamonds, whereas combinations of miconazole with 0.625, 1.25, 2.5 or 5 μM artesunate are indicated by circles, crosses, squares and triangles, respectively. Values are the mean ± SEM of at least 3 independent biological replicates.

Figure 2. Metabolic activity of *C. albicans* biofilms treated with combinations of miconazole and artesunate, artemisinin, dihydroartemisinin or artemether. The control curve of miconazole alone is represented by diamonds, whereas combinations of miconazole with 5 μM artesunate, artemisinin, dihydroartemisinin or artemether are indicated by triangles, circles, crosses or squares, respectively. Values are the mean ± SEM of at least 3 independent biological replicates.