The emergence of drug-resistant fungi has prompted an urgent threat alert from the Centers for Disease Control. Biofilm assembly by these pathogens further impairs effective therapy. We recently identified an antifungal, turbinmicin, that inhibits the fungal vesicle-mediated trafficking pathway and demonstrates broad-spectrum activity against planktonically growing fungi. During biofilm growth, vesicles with unique features play a critical role in the delivery of the biofilm extracellular matrix components. As these components are largely responsible for the drug resistance associated with biofilm growth, we explored the utility of turbinmicin in the biofilm setting. We found that turbinmicin disrupts extracellular vesicle delivery during biofilm growth, and this impairs the subsequent assembly of the biofilm matrix. We demonstrated that elimination of the extracellular matrix renders the drug-resistant biofilm communities susceptible to fungal killing by turbinmicin. Furthermore, the addition of turbinmicin to otherwise ineffective antifungal therapy potentiated the activity of these drugs. The underlying role of vesicles explains this dramatic activity and was supported by phenotype reversal with the addition of exogenous biofilm extracellular vesicles. This striking capacity to cripple biofilm assembly mechanisms reveals a new approach to eradicating biofilms and sheds light on turbinmicin as a promising anti-biofilm drug.

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Title: Turbinmicin inhibits Candida biofilm growth by disrupting fungal vesicle-mediated trafficking

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ABSTRACT

The emergence of drug-resistant fungi has prompted an urgent threat alert from the Centers for Disease Control. Biofilm assembly by these pathogens further impairs effective therapy. We recently identified an antifungal, turbinmicin, that inhibits the fungal vesicle-mediated trafficking pathway and demonstrates broad-spectrum activity against planktonically growing fungi. During biofilm growth, vesicles with unique features play a critical role in the delivery of the biofilm extracellular matrix components. As these components are largely responsible for the drug resistance associated with biofilm growth, we explored the utility of turbinmicin in the biofilm setting. We found that turbinmicin disrupts extracellular vesicle delivery during biofilm growth, and this impairs the subsequent assembly of the biofilm matrix. We demonstrated that elimination of the extracellular matrix renders the drug-resistant biofilm communities susceptible to fungal killing by turbinmicin. Furthermore, the addition of turbinmicin to otherwise ineffective antifungal therapy potentiated the activity of these drugs. The underlying role of vesicles explains this dramatic activity and was supported by phenotype reversal with the addition of exogenous biofilm extracellular vesicles. This striking capacity to cripple biofilm assembly mechanisms reveals a new approach to eradicating biofilms and sheds light on turbinmicin as a promising anti-biofilm drug.
INTRODUCTION

Microorganisms exist predominantly in surface-associated communities called biofilms (1, 2). Microbes growing in this state are notorious for their resistance to antimicrobials (2-4). Candida albicans, the most common hospital-acquired fungal infection, frequently forms biofilms on implanted medical devices leading to lethal disseminated disease (5-7). Treatment recalcitrance is so significant that treatment guidelines recommend removal of Candida-infected devices (5, 8). The intrinsic resistance to therapeutics of these biofilms is multifactorial, but largely attributable to the extracellular matrix shielding the biofilm cells (2, 9-11).

We have reported that Candida biofilm extracellular matrix components critical for biofilm cell drug resistance are delivered by extracellular vesicles (12-14). Recent discovery of molecules produced by the microbiome of a marine animal identified turbinmicin as an antifungal that targets the vesicle delivery pathway (15). We hypothesized this therapeutic would subvert the protection afforded by the vesicle-delivered biofilm matrix. Here, we present evidence that turbinmicin inhibits biofilm vesicle production and, in turn, eliminates the extracellular matrix assembly. We find that vesicle reduction renders biofilm communities susceptible to the antifungal effects of turbinmicin itself, as well as clinically available antifungal agents. Our findings argue that fungal vesicle trafficking pathways represent promising fungal biofilm targets.
RESULTS/DISCUSSION

Role of turbinmicin in extracellular vesicle delivery of biofilm matrix

We recently identified a broad-spectrum antifungal, turbinmicin (15). The drug exhibits activity against Candida and Aspergillus species during planktonic growth and demonstrates safety and efficacy in animal models of infection. Mode of action investigations identified Sec14p as the likely target. Sec14p is a phosphatidylinositol/phosphatidylcholine transfer protein required for correct trans-Golgi network dynamics in the vesicle trafficking pathway (16, 17).

Extracellular matrix production, a canonical feature of biofilms, is necessary for community fortification and protection from antifungals (10, 18). Recent studies demonstrate that extracellular vesicles deliver much of the Candida biofilm matrix, and this process is critical for drug resistance (13). Given the activity of turbinmicin on fungal vesicle production, we theorized that treatment would subvert vesicle delivery and subsequent matrix assembly, ultimately leaving biofilm cells vulnerable to killing. To test this hypothesis, we initially assessed the impact of turbinmicin on C. albicans biofilm vesicle production (Figure 1A). Following exposure to turbinmicin we observed a more than 500% reduction in vesicle delivery at the lowest dose examined (2 µg/ml). We found a dose-dependent increase in inhibition with nearly complete abrogation of biofilm vesicles at the highest dose (16 µg/ml). Follow-on studies with three additional Candida species (C. tropicalis, C. glabrata, and C. auris) and a strain of Aspergillus fumigatus demonstrate the broad-spectrum impact of this biofilm observation (Figures S1A-D).

Given the role of biofilm vesicles in matrix production, we next used complementary assays to assess the impact of turbinmicin on C. albicans biofilm matrix (Figure 1B–E). Examination of biofilms by scanning electron microscopy (SEM) yielded a striking observation: exposure to turbinmicin (2.5 µg/ml) resulted in barely visible extracellular matrix compared to control biofilms (Figure 1B). Consistent with SEM findings, quantitative analyses of biofilm matrices revealed a
dose-dependent reduction in biomass upon turbinmicin treatment (Figure 1C). The antifungal protective qualities of the extracellular matrix have been linked to proteins and polysaccharides, including a unique mannan-glucan complex (11). Turbinmicin treatment similarly depleted each of these matrix components, with reductions of nearly 300% at the highest turbinmicin concentration examined (40 μg/ml; Figure 1D–D). Our results support a biofilm-relevant action for turbinmicin via eradication of the protective extracellular matrix.

**Turbinmicin biofilm efficacy**

We next sought to determine if turbinmicin exhibited efficacy against *C. albicans* biofilms. We propagated biofilms and subsequently treated them with turbinmicin over a 64-fold concentration range (0.5–64 µg/ml) (Figure 2A). We found that at between 2 and 4 µg/ml turbinmicin reduced the biofilm burden by 50% (biofilm MIC), based on an XTT assay measurement. This concentration for biofilm inhibition is approximately 4- to 6-fold higher than the amount required to inhibit planktonic *C. albicans* growth (MIC = 0.5 µg/ml). The need for higher concentrations to inhibit biofilm over planktonic cells is uniform across available antifungal classes (3, 19). However, the MIC ratio (planktonic:biofilm) observed for turbinmicin is lower than that described for other antifungals, which range from 10- to 100-fold for the echinocandins and polyenes, and to >1000-fold for the triazoles (2, 19, 20). This highlights the potential of turbinmicin as an anti-biofilm agent. Furthermore, increased turbinmicin concentrations eliminated ~ 80% of viable biofilm cells. The planktonic and biofilm activity of turbinmicin against other fungal species was also explored for three additional *Candida* species (*C. tropicalis, C. glabrata*, and *C. auris*) and a strain of *Aspergillus fumigatus*. Turbinmicin planktonic MICs for this group were relatively similar, 0.5, 0.5, 0.125, and 0.5 µg/ml, respectively. In the biofilm assay, turbinmicin likewise exhibited dose-dependent efficacy for these phylogenetically disparate fungal species (Figures S2A-D).

To further elucidate the potential clinical value of turbinmicin biofilm activity, we utilized a rat central venous catheter model that mimics an important clinical biofilm infections (Figure 2B and
Following the establishment of biofilms, we instilled turbinmicin for a 24 h treatment. We then removed catheters and assessed turbinmicin efficacy by measuring the remaining *Candida* viable burden and imaging the luminal biofilms. The lower concentration of turbinmicin (2.5 µg/ml) reduced the viable plate counts nearly 2 log\_10 compared to controls whereas the higher concentration (10 µg/ml) sterilized the catheter (below the detection limit). Using SEM, we observed nearly complete elimination of turbinmicin-treated biofilms, congruent with culture endpoints. These experiments show the utility of turbinmicin as a *Candida* biofilm therapeutic in a clinically relevant model.

We reasoned that if turbinmicin disrupted the assembly of protective biofilm matrix, the remaining biofilm cells would be vulnerable to the activity of other antifungals. To test this idea we elected to examine turbinmicin in conjunction with fluconazole, as *Candida* biofilms tolerate concentrations of fluconazole 1000-fold greater than planktonic cells. We used a checkboard format to investigate the impact of drug combinations. As expected, monotherapy with turbinmicin demonstrated activity while fluconazole exerted little impact at the highest concentration. Analyses of the two drug combinations revealed enhanced efficacy compared to either drug alone over a wide range of concentrations; these data are depicted as yellow and orange in the surface response plot (Figure 2D). Analysis of the combination of the two drugs demonstrated Bliss synergy with $\Delta E$ and its 95% confidence interval (CI) > 0 (Figure 2D). A large number of the drug combination exposures resulted in nearly complete biofilm elimination. We speculate the enhanced activity of fluconazole in the presence of turbinmicin is due to elimination of the matrix shield that otherwise renders antifungals ineffective.

Previous investigations have found that addition of exogenous extracellular vesicles to matrix-depleted *Candida* biofilms can restore matrix function (13). Therefore, to test the theory that the activity of the turbinmicin-fluconazole combination therapy was due to vesicle inhibition of matrix
delivery, we performed vesicle add-back experiments (Figure 2f). Remarkably, the addition of extracellular vesicles to the treated biofilms returned the majority of the community toward the drug-resistant state. The fact that some degree of efficacy was observed despite exogenous vesicles suggests the possibility of an additional, undefined turbinmicin effect. The sum of these findings is consistent with our proposal that turbinmicin biofilm efficacy is linked to subversion of vesicle matrix delivery. Our observations suggest that EV-based therapeutics may be a useful platform for anti-biofilm strategies and that turbinmicin is a promising broad-spectrum anti-fungal biofilm agent.
METHODS

Strains and media. *C. albicans* SN250, *C. tropicalis* 98-234, *C. glabrata* 4720, *C. auris* B11220, *Aspergillus fumigatus* 293 were utilized. *Candida* species were sustained on yeast extract-peptone-dextrose (YPD) medium with uridine. *A. fumigatus* was grown on glucose minimal medium (GMM; 6 g/liter NaNO₃, 0.52 g/liter KCl, 0.52 g/liter MgSO₄·7H₂O, 1.52 g/liter KH₂PO₄, 10 g/liter D-glucose, 15 g/liter agar supplemented with 1 ml/liter trace elements). Biofilms were grown in RPMI 1640 buffered with 4-morpholinepropanesulfonic acid (MOPS).

In vitro biofilm models. Three in vitro biofilm models were used, including a 96-well and 6-well polystyrene plate, or glass coverslip. Biofilm drug susceptibility and vesicle production were assessed using the 96-well polystyrene plate assay (22). Matrix composition assessment utilized the 6-well plate assay. Biofilm architecture was imaged using SEM of coverslip.

Extracellular Vesicle Isolation. EVs were isolated from biofilms grown in 6-well polystyrene plates. Media was removed from the plates, filter sterilized, and concentrated using a Vivaflow 200 unit (Sartorius AG, Goettingen, Germany) equipped with a Hydrosart 30 kDa cut-off membrane. The sample was centrifuged at 10,000 × g for 1 h at 4°C to remove cellular debris. The pellets were discarded, and the supernatant was centrifuged again as described above. This supernatant was then centrifuged at 100,000 × g for 1.5 h at 4°C. The supernatants were discarded, and the pellet was resuspended in phosphate-buffered saline (PBS) (pH 7.2). Next, the sample was subject to size exclusion chromatography on a HighPrep 16/60 Sephacryl S-400 HR column (GE Life Sciences) pre-equilibrated with PBS (pH 7.2) containing 0.01% NaN₃. All chromatographic separations were performed at room temperature on the high-performance liquid chromatography ÄKTA-Purifier 10 system (Amersham Biosciences AB, Uppsala, Sweden).
Quantitative extracellular vesicle analysis. EVs were quantified using a combination of imaging flow cytometry, image confirmation, and fluorescence sensitivity in low-background samples, as previously described (23–25). Prior to analysis, samples were stained with carboxyfluorescein succinimidyl ester (CSFE) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) at 37°C for 90 min. Excessive dye particles were removed using illustra microspin G-50 columns (GE Healthcare). Samples were analyzed on the ImageStreamX Mk II flow cytometry system from Amnis Corporation (Seattle, Washington, United States) at ×60 magnification, with default low flow rate/high sensitivity using the INSPIRE software.

In vitro and in vivo biofilm SEM imaging. In vitro biofilms were grown on coverslips in 6-well plates. Ten microliters of fetal calf serum was placed on each coverslip and dried for 1 h. Forty microliters of an inoculum of 10^8 cells/ml was placed on the coverslip and incubated at 37°C for 24 h. Following incubation, the cells were fixed with 4% formaldehyde and 1% glutaraldehyde at 4°C overnight. Coverslips were then washed with PBS and treated with 1% osmium tetroxide for 30 min at 22°C. Samples were subsequently washed with a series of increasing ethanol dilutions (30 to 100%), followed by critical point drying and coating with platinum. SEM of samples was performed using a LEO 1530 microscope.

In vivo biofilms were propagated in a rat (female, 400 g Sprague-Dawley) central venous catheter biofilm model as previously described (21). After a 48-h biofilm formation phase, the devices were removed, sectioned to expose the intraluminal surface, and processed for SEM imaging as described above.

Biofilm matrix isolation and analysis. Biofilms were grown in 6-well plates as described above, and extracellular matrix was collected from mature 48-h biofilms (11). Briefly, biofilms were removed with a spatula in sterile water. Biofilms were then sonicated for 20 min, and matrix was
separated from the biomass by centrifugation of the samples at 2,880 × g for 20 min at 4°C. To determine the concentration of matrix mannan and glucan, sugars were quantified by gas-liquid chromatography–flame ionization detector (GLC-FID) on a Shimadzu GC-2010 system after conversion to alditol acetate derivatives (22). A Crossbond™ 50% cyanopropylmethyl/50% phenylmethyl polysiloxane column was used (15 m × 0.25 mm with 0.25 μm film thickness, RTX-225, Restek). The GLC conditions were as follows: injector at 220°C, FID detector at 240°C, and a temperature program of 215°C for 2 min, then 4°C/min up to 230°C before holding for 11.25 min, run at constant linear velocity of 33.4 cm/sec and split ratio of 50:1. Data for these monosugars are presented as micrograms of matrix per milligram of biofilm biomass.

**In vitro biofilm and planktonic antifungal susceptibility assay.** In vitro biofilm drug susceptibility to was assessed using a tetrazolium salt XTT reduction assay (22). The percent reduction in biofilm growth compared to untreated controls is reported. The CLSI M27 A3 and M38-A2 broth microdilution susceptibility methods were used to determine activity against planktonic *Candida* and *Aspergillus* strains, respectively (26, 27).

**In vivo rat central venous catheter biofilm model.** A jugular vein rat catheter infection model was utilized for in vivo biofilm assessments (Envigo, Indianapolis, IN) (21). Quantitative cultures of *C. albicans* after 24 h of in vivo growth were utilized to measure viable cell burden. For drug treatment, turbinmicin (2.5 or 10 μg/ml) was instilled and dwelled in the catheter over a 24-h period. The post treatment viable burden was compared to untreated controls.

**Combination therapy analysis.** Bliss independence is described by the equation $E_{\text{IND}} = E_A + E_B - E_A \times E_B$ for a certain combination of $x$ μg/ml of drug A and $y$ μg/ml of drug B. $E_A$ is the % biofilm growth inhibition at $x$ μg/ml of drug A alone, $E_B$ is the % biofilm growth inhibition at $y$ μg/ml of drug B alone, and $E_{\text{IND}}$ is the expected % biofilm growth inhibition of a noninteractive (independent)
theoretical combination of x µg/ml of drug A with y µg/ml of drug B. The difference (\(\Delta E = E_{OBS} - E_{IND}\)) between the expected % growth inhibition, \(E_{IND}\), and the experimentally observed % growth inhibition, \(E_{OBS}\), describes the interaction for each combination of the two drugs. If \(\Delta E\) and its 95% confidence interval (CI) were >0 (i.e., \(E_{OBS} > E_{IND}\), and hence, more growth inhibition was observed than if the two drugs were acting independently), Bliss synergy was concluded for that particular combination. If \(\Delta E\) and its 95% CI were <0 (i.e., \(E_{OBS} < E_{IND}\), and hence, less growth inhibition was observed than if the two drugs were acting independently), Bliss antagonism was concluded for that particular combination. In any other case where the 95% CI of \(\Delta E\) would include 0, the conclusion was Bliss independence (28, 29). In each of the independent replicate experiments, % growth inhibition values for fluconazole and turbinmicin alone were obtained using the Emax model parameters. For each combination of x µg/ml of fluconazole with y µg/mL of turbinmicin, the \(\Delta E\) (\(E_{OBS} - E_{IND}\)) was calculated and the interaction was assessed as described above and plotted in a three-dimensional plot, an interaction surface plot was obtained, with peaks above and below the 0 indicating synergistic and antagonistic combinations, respectively, while the 0 itself indicated no statistically significant interactions.

**EV add-back assay.** Biofilms were propagated in the wells of 96-well plates. After a 6-h biofilm formation period, the biofilms were washed with PBS twice. Biofilms were then treated with either, turbinmicin (8 µg/ml), fluconazole (1 µg/ml), or the combination of turbinmicin and fluconazole. Exogenous purified EVs collected from 48h biofilms were administered at a concentration of 21804±1711 EVs/ml 1 hour before antifungal therapy for one series of wells. Following another 24h of incubation, the quantity of *Candida* biofilm cells was assessed using the XTT assay. The results are presented as a percent reduction by comparing untreated biofilms with those that were treated.
**Statistical Analysis.** Data sets were analyzed using the one-way analysis of variance (ANOVA) and the post-hoc Tukey HSD test.

**Ethics Statement.** Animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin (protocol DA0031).


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References

Figure 1. Turbinmicin disrupts biofilm extracellular vesicle matrix delivery. A. Escalating concentrations of turbinmicin reduced *C. albicans* SN250 biofilm extracellular vesicle quantity from in vitro biofilms over 24 hours (N=5) based upon imaging flow cytometry. B. SEM imaging of *C. albicans* in vitro coverslip biofilms demonstrate visible matrix reduction following turbinmicin exposure compared to untreated control samples. Scale bar indicates 10 µm. C. Escalating concentrations of turbinmicin reduced biofilm matrix weight in 6-well in vitro assays (N=3). Values are expressed as percent of the untreated biofilm matrix values. D. Dose dependent reduction of biofilm matrix protein in 6-well in vitro assays following turbinmicin exposure (N=3). E. Dose dependent reduction of biofilm matrix carbohydrate components in 6-well in vitro assays following turbinmicin exposure (N=3). Differences among treatments in panels A, C, D, and E were assessed by one-way analysis of variance (ANOVA) with post-hoc Tukey's HSD.
Figure 2. Turbinmicin exhibits vesicle matrix-linked Candida biofilm efficacy alone and in combination with fluconazole. A. Escalating concentrations of turbinmicin reduced the C. albicans SN250 biofilm burden using an XTT assay in an in vitro biofilm format (N=3). Biofilm activity is expressed as percent biofilm reduction compared with untreated biofilms. B. Increasing intraluminal doses of turbinmicin eliminated viable C. albicans SN250 in the rat central venous catheter model compared to buffer treated control animals (N=3). C. SEM imaging of the intraluminal surface of the rat central venous catheter C. albicans SN250 model demonstrates visibly reduced biofilm in a dose-response manner follow turbinmicin exposures compared to buffer treated control animals (N=3). Scale bar 300 µm D. Three-dimensional surface response-plot illustrates efficacy enhancement with the combination of turbinmicin and fluconazole compared to monotherapy in a 96-well C. albicans SN250 biofilm assay using an XTT assay
The vertical axis represents the percent reduction in biofilm growth compared to untreated control biofilms. Areas in yellow and orange represent enhanced activity due the drug combination. E. Interaction surfaces obtained from response surface analysis of Bliss independence drug interaction model for the in vitro combination of turbinmicin and fluconazole against \textit{C. albican} biofilm. The X- and Y- axis are the concentrations of turbinmicin and fluconazole, respectively. The Z- axis is the $\Delta$E. F. Addition of exogenous vesicles with the antifungal drugs reduces the efficacy of the combination of turbinmicin (8 µg/ml) and fluconazole (1 µg/ml) in a 96-well \textit{C. albicans} SN250 biofilm assay using an XTT assay (N=3). Differences among treatments in panels A, C, D, and E were assessed by ANOVA with post-hoc Tukey's HSD.