Poster #1522

Fungal Cytological Profiling of *Candida albicans* Exposed to Diverse Antifungal Agents Including the Novel Gwt1 inhibitor APX001A

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ABSTRACT

Background: Bacterial cytological profiling accelerates drug discovery efforts by rapidly revealing the mechanism of action (MOA) of newly developed antibacterial agents. We sought to adapt this technology, which uses a combination of fluorescent compounds to stain specific subcellular structures including DNA and membranes, to identify and study the MOA of antifungals. This Fungal Cytological Profiling (FCP) allowed us to study the characteristic morphological changes in *C. albicans* caused by 6 antifungal agents with unique MOA. Included in the analysis was the novel broad spectrum Gwt1 inhibitor APX001A, the active moiety of the prodrug APX001 which is currently in clinical trials for invasive fungal infections.

Methods: MICs of 6 antifungals were determined by CLSI methodology. For FCP, antifungals were added to cultures (1x10^5 cells/mL) in RPMI 1640 (buffered with MOPS) at concentrations near MIC: flucytosine (2 µg/mL); fluconazole (2 µg/mL); caspofungin (1 µg/mL); nikkomycin (3.33 µg/mL); amphotericin B (1 µg/mL); and APX001A (0.064 µg/mL); and incubated at 35˚ C with shaking. At 4 hr and 24 hr, samples were stained for 15 minutes with fluorescent dyes (FM 4-64 (membranes), DAPI (DNA), and Sytox Green (cell viability)) and observed by high resolution fluorescence microscopy. Image analysis and quantitation of cytological parameters (*e.g.* cell shape, length, width, DNA content) were used to create a cytological profile for each growth condition.

Results: Unique cytological signatures strongly correlated with antifungal MOA: FCZ resulted in rounded cells that lacked hyphal forms; APX001A resulted in abundant intracellular membrane labeling at 4 hr, consistent with an endoplasmic reticulum stress response, with cell death (Sytox Green Staining) at 24 hr.

Conclusions: FCP is a rapid and accurate method to establish MOA and distinguish between antifungals that inhibit specific biosynthetic pathways (e.g., cell wall) and sub-pathways (glucan vs chitin synthesis). In addition, this technology can be useful in drug discovery programs to determine on-target vs off-target activity of newly synthesized molecules.

METHODS

Reagents. Drug stocks were prepared in 100% dimethyl sulphoxide and aliquots stored at -20˚ C. Fluorescent dye stock aliquots were prepared in 100% dimethyl sulphoxide (FM 4-64 and Sytox Green) or water (DAPI) and stored at -20˚ C.

Strains. Wild-type *C. albicans* (ATCC90028) was obtained from American Type Culture Collection.

Antifungal testing. Broth MIC values were determined by CLSI methodology[1] and read at 50% growth inhibition[3]. The broth MIC values were used to determine the drug concentration ranges to use to pour a series of SDA plates to determine the minimum concentration of drug that would prevent the growth of macroscopically visible colonies of *C. albicans*.

Fungal Cytological Profiling. A *C. albicans* (ATCC90028) overnight culture was inoculated into 12-mL RPMI 1640 (buffered with MOPS) to a final concentration of 1x10^5 cells/mL and incubated at 35˚ C with shaking. Antifungals were added at the following concentrations: flucytosine (2 µg/mL); fluconazole (2 µg/mL); caspofungin (1 µg/mL); nikkomycin (3.33 µg/mL); amphotericin B (1 µg/mL); and APX001A (0.064 µg/mL). The DMSO (vehicle) concentration was held at 0.03% across all treatments, which was previously determined to have no effect on cell morphology. The 12-mL cultures were split into two tubes and incubated at 35˚ C with shaking.

At 4 hr and 24 hr one tube of cells for each treatment condition was concentrated to 1-mL and incubated for 15 minutes with a mixture of FM 4-64 (10 µg/mL), DAPI (10 µg/mL) and Sytox Green (10 µM). The cells were then washed once with an equal volume of RPMI and concentrated by centrifugation to ~20-30 µL. Samples of these cells were then placed on an agarose pad in a chambered slide and images were collected at 100x magnification on a DeltaVision microscope. For all images FM 4-64 membrane staining is shown in red, DNA staining with DAPI is shown in blue and loss of cell integrity/viability is shown with green Sytox Green staining.

RESULTS

We conducted studies with *C. albicans* to demonstrate the feasibility of FCP with fungal pathogens that grow as either elongated hyphae or as yeast like cells. *C. albicans* cells treated with different compounds have distinct morphologies that change over time. By comparing the results from various time points, we can identify unique cytological signatures that correlate with antifungal MOA.

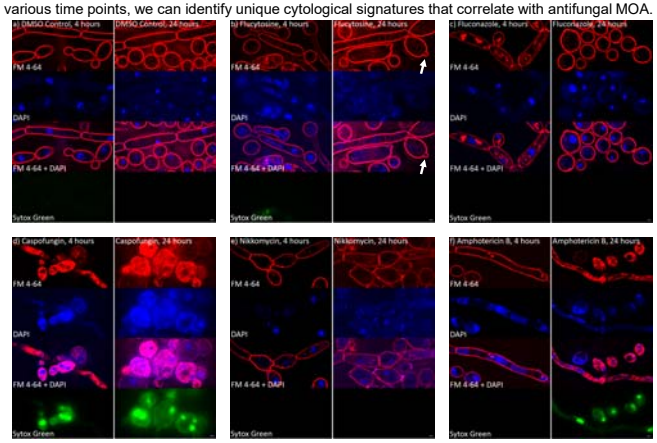

Fig. 1. Images of *C. albicans* after 4 or 24 hours of exposure to: a) DMSO (0.03%); b) flucytosine (2 µg/mL); c) fluconazole (2 µg/mL); d) caspofungin (1 µg/mL); e) nikkomycin (3.33 µg/mL); f) amphotericin B (1 µg/mL).

Caspofungin inhibits cell wall glucan synthesis, causing apoptosis and necrosis. Consistent with this MOA, caspofungin treated cells show a cell shape defect with substantial Sytox Green staining, indicating cell death (Fig. 1b). The fungistatic azoles target lanosterol demethylase and inhibit the transition from yeast to hyphae, consistent with the absence of hyphae and the accumulation of yeast cells after 24 hr of fluconazole treatment (Fig. 1c). Cells treated with flucytosine, which interferes with fungal nucleic acid synthesis, appear normal at 4 hr of treatment, but by 24 hr, they swell (compare Fig. 1a and d) and accumulate arrested buds (Fig. 1d arrow). Cells treated with amphotericin B, which binds ergosterol and disrupts fungal membranes, show extensive membrane permeability at 24 hr (Fig. 1e). Finally, the chitin synthase inhibitor nikkomycin causes accumulation of abnormal cell branches at 4 hr and an abundance of misshaped yeast-like cells at 24 hr (Fig. 1f).

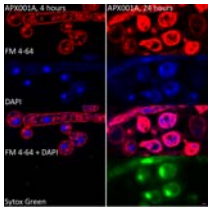

Fig. 2. Images of *C. albicans* exposed to APX001A (0.064 µg/mL) for 4 or 24 hours.

Treatment with the novel Gwt1 inhibitor APX001A results in abundant intracellular membrane labeling at 4 hr, consistent with an endoplasmic reticulum stress response (Fig. 2). By 24 hr, APX001A treated cells show substantial cell shape defects, lysis and death.

We also observed that the population of yeast-like cells in cultures treated with APX001A grow larger over time (Fig. 3).

The effect of APX001A also appears epistatic to certain other antifungal compounds. As shown in Fig. 4, combining APX001A with either Caspofungin or Fluconazole appears to mask the effects of those compounds alone in *C. albicans*.

RESULTS (cont'd)

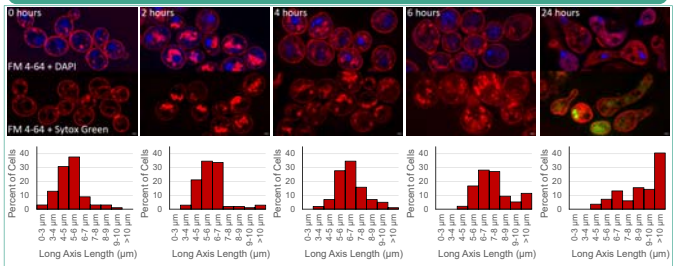

Fig. 3. The diameter of yeast-like cells in cultures of *C. albicans* treated with 0.064 µg/mL APX001A increases over time.

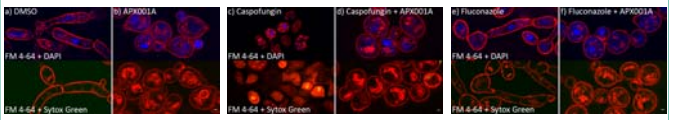

Fig. 4. APX001A treatment masks the effect of caspofungin or fluconazole: a) DMSO (0.03%); b) APX001A (0.016 µg/mL); c) caspofungin (0.25 µg/mL); d) caspofungin (0.25 µg/mL) + APX001A (0.016 µg/mL); e) fluconazole (0.5 µg/mL); f) fluconazole (0.5 µg/mL) + APX001A (0.016 µg/mL). Cells were treated for 4 hr.

CONCLUSIONS

FCP quickly and accurately establishes MOA and distinguishes between antifungals that target specific biosynthetic pathways (e.g., cell wall vs. nucleic acid) and sub-pathways (glucan vs chitin synthesis).

Our image analysis process can generate quantitative cytological profiles comprised of over 60 different parameters. We are in the process of testing additional conditions, exposure times, and staining procedures to optimize FCP and expand its utility in tracking and differentiating between even more antifungal targets.

This technology can be used in a drug discovery program to determine on-target vs off-target activity of newly synthesized molecules and to rapidly track structure-activity relationships.

REFERENCES

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