Antifungal Activity of Tamoxifen and its Analogs Against the Opportunistic Pathogen, *Candida albicans*

Julie M. Tabroff, Louis P. DiDone, Kristy Koselny

Advisers: Melanie Wellington, M.D., Ph.D. & Damian J. Krysan, M.D., Ph.D.

Departments of Biochemistry, Pediatrics, and Microbiology & Immunology

In recent a that TAM and its analogs have been used to create drug resistance in *Candida* species. Therefore, the goal of this study was to determine whether TAM and its analogs would inhibit the growth of *Candida* species in vitro.

**Table 1. Compounds tested for antifungal activity.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspofungin (CAS)</td>
<td>0.4</td>
</tr>
<tr>
<td>Toremifene (TOR)</td>
<td>0.8</td>
</tr>
<tr>
<td>Prochlorperazine (PRO)</td>
<td>4.0</td>
</tr>
<tr>
<td>Antipsychotics:</td>
<td></td>
</tr>
<tr>
<td>Clozapine (CLO)</td>
<td>0.4</td>
</tr>
<tr>
<td>Clozapine (CLO) + DMSO</td>
<td>1.8</td>
</tr>
<tr>
<td>Clozapine (CLO) + DMSO + 7% DMSO</td>
<td>3.0</td>
</tr>
</tbody>
</table>

**Materials and Methods**

**Yeast Strains, media and culture conditions**

*C. albicans* strain SC5314 was used for most experiments. *C. albicans* were grown overnight at 37°C in yeast extract-peptone-dextrose medium or RPMI 1640. Yeast peptone dextrose (YPD) was prepared with published recipes. RPMI 1640 medium containing 1X E.coli MOPS (pH 7) was prepared according to Clinical Laboratory Standards Institute (CLSI) procedures. 20μg/mL uridine was added to YPD agar plates.

**Minimum Inhibitory Concentration (MIC)**

The MIC of a drug is defined as the lowest concentration of an antifungal agent needed to visibly prevent growth of the initial inoculum of yeast cells after 48 hours. MICs of 0.25 μg/mL or less are considered active. The MIC was determined for TAM, its analogs, and other compounds using the CLSI broth microdilution method.

**Fractional Inhibitory Concentration (FIC)**

The FIC of drug A = MIC of drug A in combination / MIC of drug A alone

**Calcium Suppression Assay**

Two different YPD mixtures were made. From 2X-YP, a solution of 2% dextrose YPD and a solution of 2% dextrose YPD with 30μM of calcium were made. The solutions were divided into 2 flasks of equal volume. MIC concentrations of drug and the vehicle (DMSO) were added to the YPD and YPD + Ca++ media. SC5314 were grown overnight at 37°C and the optical density (OD) was recorded in the morning. The concentration of cells was adjusted to 0.1 OD (log phase) in all flasks. The growth was measured with a spectrophotometer for 6 hours using the corresponding media (YPD or YPD Ca++) as a blank.

**Budding assay**

SC5314 was grown overnight at 37°C in YPD to stationary phase. The stationary phase culture was diluted 1:200 in YPD 1, 2, and 5X of the diluted cells were added to 50 mL flasks and grown overnight at 37°C. This protocol ensured that the cells were in log phase in the morning. The cells were then adjusted to 0.1 OD in the morning. The cells were treated with the drug for 4 hours at 37°C. The number and type of buds were then counted using a light microscope.

**Actin staining**

SC5314 was grown according to the budding protocol described above. After counting the number and size of buds, the cells were then fixed in 10% formaldehyde and incubated at room temperature for 30 min. The cells were then washed and resuspended in 1mL PBS with 25μg/mL of a 200 U/mL FITC-phallolidin diluted in methanol and left to sit for 10 min. The cells were then washed in PBS and observed with a FITC filter.

**Filamentous growth assay**

SC5314 was grown overnight at 37°C in YPD to stationary phase. A 1:50 dilution of SC5314 was incubated for 2hrs with rotation in medium M199 at 37°C in the presence or absence of sub-MIC concentrations of drug. The cells were then cultured with light microscopy and the filamentous growth was counted.

**Immunofluorescence Assay (IFA)**

SC5314 was grown overnight at 37°C in YPD to stationary phase. 5μl of the overnight culture were added to 5mL YPD. The diluted cultures were treated with 16μg/mL of TAM, TOR, CLO, PRO, and TEP, 125μg/mL CAS (positive control) and DMSO (negative control) overnight. After treating the cells overnight, the cells were washed three times in PBS. The cells were suspended in block solution (3% ISA in PBS) for 15min at rotation of 4°C. They were then resuspended in anti-glucan primary antibody (Biospeples Inc., Parkville, Australia) diluted in block solution for 2 hours at 4°C with rotation. The cells were then washed in PBS and resuspended in a secondary Goat anti-mouse IgG with Texas red in the dark for 60-90 minutes at 4°C with rotation wrapped in aluminum foil. The cells were then imaged using a microscope.
Antifungal Activity of Tamoxifen and its Analogs Against the Opportunistic Pathogen, Candida albicans

Julie M. Tabroff, Louis P. DiDone, Kristy Koselny
Advisers: Melanie Wellington, M.D., Ph.D. & Damian J. Krysar, M.D., Ph.D.
Departments of Biochemistry, Pediatrics, and Microbiology & Immunology

In infections in immunocompromised patients binding to its target protein Myo2 in S. cerevisiae. PLWRVLVSURWHLQWUDIÀFNLQJH[RFWRVLVFWRVNHODVWUXFWXUHDQG plays an important role in several critical cellular processes such as in vivo activity toward its analog clomiphene have good in vitro activity toward pathogenic GUXJVZHLGHQWLÀHGDVHWRIFDOPRGXOLQLQKLELWRUVLQFOXGLQJWKH PHQWLVWRLGHQWLI\DQGH[SORUHWKHHIÀFDF\RIGUXJVFXUUHQWO\LQXVH IXQJLDQGKXPDQV7KLVSUHVHQWVGLIÀFXOWLHVLQGHYHORSLQJWUHDW treatments for invasive fungal infections, there are complications needed in order to prevent and treat these invasive fungal infections. Unfortunately, the treatment options for life-threatening fungal infections is as high as 20-35%. Accordingly, TAM was shown to inhibit new many in vitro synergistic interaction. The number and type of buds were then counted using a light microscope. Filamentous growth assay SC5314 was grown overnight at 37°C in YPD to stationary phase. The stationary phase culture was diluted 1/200 in YPD 1.2, and 5µl of the diluted cells were added to 50mL YPD, and grown overnight at 37°C. This protocol ensured that the cells were in log phase in the morning. The cells were then adjusted to 0.1 OD in the morning. The cells were treated with the drug for 4 hours at 37°C. The number and type of buds were then counted using a light microscope. calcium binding domains found in human calmodulin. These structural differences may explain why TAM targets molecularly may be attractive antifungal drugs. Two cellular processes that are CaM dependent in fungi are hyphae formation and cell wall biosynthesis. Both of these processes are relevant to antifungal therapy because the ability of C. albicans to form hyphal is required for virulence and the cell wall is required for cell integrity. We hypothesized that TAM analogs and other CaM inhibitors would block hyphae formation and affect cell wall architecture in C. albicans. To test this hypothesis, we (1) confirmed that TAM displaced phenotypes consistent with CaM inhibition in C. albicans, (2) assessed the ability of these compounds to block filamentous growth formation; and (3) probed the effects of TAM and CaM inhibitors on 1.5,8-glucan architecture. In addition, (4) we tested TAM in combination with the known antifungal, caspofungin, for any in vitro synergetic interaction. MATERIALS AND METHODS Yeast Strains, media and culture conditions C. albicans strain SC5314 was used for most experiments. C. albicans were grown overnight at 37°C in yeast extract-peptone-dextrose medium or RPMI 1640. Yeast peptone dextrose (YPD) was prepared using published recipes. RPMI 1640 medium containing 1165 M MOPS (pH 7) was prepared according to Clinical and Laboratory Standards Institute (CLSI) procedures. 30µg/ml uridine was added to YPD agar plates. Minimum Inhibitory Concentration (MIC) The MIC of a drug is defined as the lowest concentration of an antifungal agent needed to visibly prevent growth of the initial inoculum of yeast cells after 48 hours. 67 µL of an initial inoculum (final concentration 5 x 102 CFIU/mL) of SC5314 was plated in a 96 well clear bottom plate. A two-fold serial dilution of the drug of choice was added in successive columns along the 96 well plates. 67 µL of RPMI/MOPS + DMSO was added to keep final DMSO concentration at 1.28%, along with 67 µL of drug for a total volume of 201 µL per well. The CaM inhibitors and antifungal drugs were tested in the following ranges: 128 µg/mL to 1µg/mL for tamoxifen and its analogs and 250 ng/mL to 1.8 ng/mL for caspofungin (all diluted in RPMI/MOPS + DMSO). The plates were incubated at 37°C for 24 and 48 hours. Figure 1 is a schematic drawing of an MIC plate. Fractional Inhibitory Concentration (FIC) Once the MIC’s were determined for CAS and CaM inhibitors, a checkerboard experiment was performed. 67 µL of an initial inoculum (final concentration 5 x 102 CFIU/mL) of SC5314 was plated on a 96 well plate. Drug A was added in decreasing concentrations across the columns, while drug B was added in decreasing concentrations along the rows. The plate was incubated at 37°C and the results were recorded at 24 and 48 hrs. The fractional inhibitory concentration (FIC) was calculated using the following formula: FIC index = FIC of drug A + FIC of drug B FIC of drug A = MIC of drug A in combination / MIC of drug A alone FIC of drug B = MIC of drug B in combination / MIC of drug B alone Synergy is defined as an FIC index of ≤0.5. Indifference is defined as an FIC index of ≥0.5 but <4.0. Antagonism is defined as an FIC index of >4.0. Figure 2 is a schematic drawing of an FIC plate. Calcium Suppression Assay Two different YPD mixtures were made. From 2X-YP, a solution of 2% desfer YPD and a solution of 2% dextrose YPD with 30mM of calcium were made. The solutions were divided into 2 flasks of equal volume. MIC concentrations of drug and the vehicle (DMSO) were added to the YPD and YPD + Ca+ media. SC5314 were grown overnight at 37°C and the optical density (OD) was recorded in the morning. The concentration of cells was adjusted to 0.1 OD (log phase) in all flasks. The growth was measured with a spectrophotometer for 6 hours using the corresponding media (YPD or YPD + Ca+) as a blank.

Budding assay SC5314 was grown overnight at 37°C in YPD to stationary phase. The stationary phase culture was diluted 1/200 in YPD 1.2, and 5µl of the diluted cells were added to 50mL YPD, and grown overnight at 37°C. This protocol ensured that the cells were in log phase in the morning. The cells were then adjusted to 0.1 OD in the morning. The cells were treated with the drug for 4 hours at 37°C. The number and type of buds were then counted using a light microscope.

Actin staining SC5314 was grown according to the budding protocol described above. After counting the number and size of buds, the cells were fixed in 10% formalin, washed at room temperature for 30 min. The cells were then washed and resuspended in 1mL PBS with 25µl of a 200 U/ml FITC-phallidin diluted in methanol and left to sit for 10min. The cells were then washed in PBS and observed with a FITC filter.

Filamentous growth assay SC5314 was grown overnight at 37°C in YPD to stationary phase. A 150µl dilution of SC5314 was incubated with 2µl of a filamentation medium M199 at 37°C in the presence or absence of sub-MIC concentrations of drug. The cells were then enumerated by light microscopy and the percent filament formation was counted.

Immunoﬂuorescence Assay (IFA) SC5314 was grown overnight at 37°C in YPD to stationary phase. 5µl of the overnight culture was added to 5ml YPD. The diluted cultures were treated with 16µg/mL of TAM, TOR, CLM, PRO, and TEP, 1.25µg/mL CAS (positive control) and DMSO (negative control) overnight. After treating the cells overnight, the cells were washed three times in PBS. The cells were suspended in block solution (3% BSA in PBS) for 15min with rotation at 4°C. They were then resuspended in anti-glucan primary antibody (Bioseps Inc., Parkville, Australia) diluted into block solution for two hours at 4°C with rotation. The cells were then washed in PBS and resuspended in a secondary Goat anti-mouse IgG with Texas red in the dark for 60-90 minutes at 4°C with rotation wrapped in aluminum foil. The cells were then imaged using a microscope.
Calcium Suppression Assay in Candida albicans

![Graph showing calcium suppression assay results]

Figure 3: TAM may act as a CaM inhibitor in Candida albicans. Growth of yeast in the presence of extracellular calcium suppresses phenotypes due to CaM antagonism. To determine whether TAM acts through CaM in Candida, calcium suppression assays were completed. Candida albicans were grown overnight and diluted in Medium 199. The treatment was added and the growth was monitored with a spectrophotometer for 6 hours. 10ug/mL of TAM was used for this assay. The TAM-treated yeast showed no growth, while the TAM plus calcium-treated yeasts grew like the untreated control because the extracellular calcium suppressed TAM toxicity.

RESULTS
TAM-treated C. albicans display phenotypes consistent with CaM inhibition.

These phenotypes include defects in proper actin polarization and budding. First, to determine whether TAM acts through CaM in C. albicans as it has been previously shown in S. cerevisiae, calcium suppression assays were completed with MIC concentrations of TAM. Figure 3 displays the results of this experiment. The DMSO (untreated) yeast showed significant growth. The DMSO plus calcium treated yeast grew as the untreated yeast. The toxicity of TAM can be suppressed by growing C. albicans in high extracellular calcium, which is consistent with a calmodulin-based mechanism of activity.

In order to test TAM’s effect on proper actin localization, C. albicans were treated with sub-MIC concentrations of TAM and stained with a FITC-phalloidin antibody. During budding, actin cables are formed along the axis of the yeast which is important for establishing cell polarity. CaM is required for proper actin localization as well as new bud formation. Figure 4 shows the effect of increasing doses of TAM on actin localization in C. albicans. In the untreated sample, the actin is localized to areas of polarized growth. As the dosage of TAM is increased, the actin becomes less localized. This can be seen as increasing green spots throughout the cell. TAM therefore disrupts actin localization. Proper actin polarization is required for normal bud formation. Therefore, if TAM causes actin depolarization, it can cause a defect in budding. C. albicans were treated with increasing doses of TAM and the number of buds was enumerated by light microscopy. The bud size was categorized as no-bud, small bud and other. A small bud is one that is less than half the size of the mother cell. Buds that were larger were characterized as other. The mean from three separate experiments is presented in Figure 5. As the concentration of TAM was increased, there was an increase in small buds and a decrease in larger buds.

Figure 4: TAM may cause actin depolarization in C. albicans by disrupting the CaM signaling pathway. During budding, actin cables are formed along the axis of the yeast which are important for establishing cell polarity. CaM is required for proper actin localization. At high doses, the actin becomes less localized. This can be seen as increasing green spots throughout the cell. TAM therefore disrupts actin localization.

Budding Assay of TAM treated Candida albicans

![Diagram showing budding assays]

Figure 5: TAM inhibits the transformation from small to large bud in C. albicans. SC5314 cells were cultured overnight with the respective drug concentrations, fixed with formalin in the morning and observed under a light microscope. The bud size was categorized as no-bud, small bud and other. A small bud is one that is less than half the size of the mother cell. Buds that are larger were characterized as other. The mean from three separate experiments is presented. Error bars indicate the standard deviation.

Calmodulin inhibitors block filament formation in C. albicans.

After showing that TAM appears to act through the CaM signaling pathway in C. albicans, we also tested the other CaM inhibitors for antifungal activity in previous experiments. The structures of these CaM inhibitors are shown in Figure 6. They include toremifene (TOR), clomiphene (CLM), prochloroperazine (PRO), and trifluoperazine (TFP). The MIC for all the CaM inhibitors is 32ug/mL in C. albicans. It was important to determine the MIC of these drugs since we want to perform experiments at sub-MIC concentrations of drugs to prevent cell death and allow for the formation of filaments.

After C. albicans were incubated in Medium 199 for 2 hours, filaments were enumerated with a light microscope. Sub-MIC concentrations of TAM were used to determine the effects of different concentrations on the number of filaments formed. Figure 7 shows the average results of 3 experiments with error bars of the number of filaments formed with the differing concentrations of TAM. TAM-treated yeast showed a decrease in filament formation. There was a significant decrease in filament formation (20% filaments) at 16ug/mL, which is half the MIC.

Similar experiments were done with the TAM analogs, TOR, PRO, CLM, and TFP 16ug/mL of each drug was used since that was the concentration of TAM at which there was a significant decrease in filament formation. Figure 8 is a graph of the results of these experiments. All the TAM analogs showed decreased filament formation. PRO and TFP were more effective at preventing filament formation than TOR or CLM.

Calmodulin inhibitors after cell wall architecture in C. albicans.

The cell wall is composed primarily of mannoproteins and β-glucans. The mannoproteins span the outside and inside of the cell wall and are important in tethering the cell wall to the plasma membrane. The outer mannoproteins protect the inner β-glucan structure, which comprises the core structural component of the cell wall along with chitin microtubules. If the cell wall structure is disrupted by a cell wall targeting drug, then the β-glucans will become exposed. The primary antibody binds to the exposed 1,3-β-glucans on the cell wall. The extent of 1,3-β-glucan exposure can be enumerated using a secondary red fluorescent antibody. This experiment was done to determine whether TAM analogs would increase the β-glucan exposure (disrupt the cell wall) as TAM has already been shown to do.

We chose 1.25 ng/mL of CAS and 16ug/mL of TAM and its analogs, both sub-MIC concentrations, to see if the drug disrupted the cell wall without killing the cells. If the cells were killed, then all the β-glucans in the cell walls would be exposed, causing the whole cell to fluoresce red.

SC5314 in log phase were used. The caspofungin-treated C. albicans showed significant red staining along the periphery of the cells compared to the negative control (DMSO) which was barely with a Texas red filter set.
RESULTS

TAM-treated C. albicans display phenotypes consistent with CaM inhibition. These phenotypes include defects in proper actin polarization and budding. First, to determine whether TAM acts through CaM in C. albicans as it has been previously shown in S. cerevisiae, calcium suppression assays were completed with MIC concentrations of TAM. Figure 3 displays the results of this experiment. The DMSO (untreated) yeast showed significant growth. The DMSO plus calcium treated yeast grew like the untreated sample; these both acted as controls. The TAM-treated yeast showed no growth as expected. The yeast treated with TAM plus calcium showed significant growth similar to the untreated sample. Extracellular calcium suppresses TAM toxicity since the TAM plus calcium treated yeast grew like the untreated yeast. The toxicity of TAM can be suppressed by growing C. albicans in high extracellular calcium, which is consistent with a calmodulin-based mechanism of activity.

In order to test TAM’s effect on proper actin localization, C. albicans were treated with sub-MIC concentrations of TAM and stained with a FITC-phalloidin antibody. During budding, actin cables are formed along the long axis of the yeast which is important for establishing cell polarity. CaM is required for proper actin localization as well as bud formation. Figure 4 shows the effect of increasing doses of TAM on actin localization in C. albicans. In the untreated sample, the actin is localized to areas of polarized growth. As the dosage of TAM is increased, the actin becomes less localized. This can be seen as increasing green spots throughout the cell. Therefore, if TAM causes actin depolarization, it can cause a defect in budding, which is also consistent with a CaM inhibitory mode of action.

Calmodulin inhibitors block filament formation in C. albicans.

After showing that TAM appears to act through the CaM signaling pathway in C. albicans, we also tested the other CaM inhibitors for antifungal activity in previous experiments. The structures of these CaM inhibitors are shown in Figure 6. They include toremifene (TOR), clomiphene (CLM), prochlorperazine (PRO), and trifluoperazine (TFP). The MIC for all the CaM inhibitors is 32μg/mL in C. albicans. It was important to determine the MIC of these drugs since we want to perform experiments at sub-MIC concentrations of drugs to prevent cell death and allow for the formation of filaments.

After C. albicans were incubated in Medium 199 for 2 hours, filaments were enumerated with a light microscope. Sub-MIC concentrations of TAM were used to determine the effects of different concentrations on the number of filaments formed. Figure 7 shows the average results of 3 experiments with error bars of the number of filaments formed with the differing concentrations of TAM. TAM-treated yeast showed a decrease in filament formation. There was a significant decrease in filament formation (20% filaments) at 16μg/mL, which is half the MIC.

Similar experiments were done with the TAM analogs, TOR, PRO, CLM, and TFP 16μg/mL of each drug was used since that was the concentration of TAM at which there was a significant decrease in filament formation. Figure 8 is a graph of the results of these experiments. All the TAM analogs showed decreased filament formation. PRO and TFP were more effective at preventing filament formation than TOR or CLM.

Calmodulin inhibitors after cell wall architecture in C. albicans.

The cell wall is composed primarily of mannoproteins and β-glucans. The mannoproteins span the outside and inside of the cell wall and are important in retethering the cell wall to the plasma membrane. The outer mannoproteins protect the inner β-glucan structure, which comprises the core structural component of the cell wall along with chitin microtubules. If the cell wall structure is disrupted by a cell wall targeting drug, then the β-glucans will become exposed. The primary antibody binds to the exposed 1,3-β-glucans on the cell wall. The extent of 1,3-β-glucan exposure can be enumerated using a secondary red fluorescent antibody. This experiment was done to determine whether TAM analogs would increase the β-glucan exposure (disrupt the cell wall) as TAM has already been shown to do.

We chose 1.25 ng/mL of CAS and 16μg/mL of TAM and its analogs, both sub-MIC concentrations, to see if the drug disrupts the cell wall without killing the cells. If the cells were killed, then all the β-glucans in the cell wall would be exposed, causing the whole cell to fluoresce red.

SC3514 in log phase were used. The caspofungin-treated C. albicans showed significant red staining along the perimeter of the cells compared to the negative control (DMSO) which was barely

Figure 3: TAM may act as a CaM inhibitor in C. albicans. Growth of yeast in the presence of extracellular calcium suppresses phenotypes due to CaM antagonism. To determine whether TAM acts through CaM in C. albicans, calcium suppression assays were completed. C. albicans were grown overnight and then diluted to 0.1 OD in the morning. The treatment was added and the growth was measured with a spectrophotometer for 6 hours. 25μg/mL of TAM was used for this assay. The TAM-treated yeast showed no growth, while the TAM plus calcium-treated yeasts grew like the untreated control because the extracellular calcium suppressed TAM toxicity.

Figure 4: TAM may cause actin depolarization in C. albicans by disrupting the CaM signaling pathway. During budding, actin cables are formed along the axis of the yeast which are important for establishing cell polarity. CaM is required for proper actin localization. This can be seen as increasing green spots throughout the cell. TAM therefore disrupts actin localization. Thus, TAM

Figure 5: Bud size was categorized as no-bud, small bud and other. A small bud is one that is less than half the size of the mother cell. Buds that were larger were characterized as other. The mean from three separate experiments is presented in Figure 5. As the concentration of TAM was increased, there was an increase in small buds and a decrease in larger buds.

Figure 6: Similar experiments were done with the TAM analogs, TOR, PRO, CLM, and TFP 16μg/mL of each drug was used since that was the concentration of TAM at which there was a significant decrease in filament formation. There was a significant decrease in filament formation (20% filaments) at 16μg/mL, which is half the MIC.

Figure 7: Similar experiments were done with the TAM analogs, TOR, PRO, CLM, and TFP 16μg/mL of each drug was used since that was the concentration of TAM at which there was a significant decrease in filament formation. There was a significant decrease in filament formation (20% filaments) at 16μg/mL, which is half the MIC.

Figure 8: Similar experiments were done with the TAM analogs, TOR, PRO, CLM, and TFP 16μg/mL of each drug was used since that was the concentration of TAM at which there was a significant decrease in filament formation. There was a significant decrease in filament formation (20% filaments) at 16μg/mL, which is half the MIC.

Figure 9: Similar experiments were done with the TAM analogs, TOR, PRO, CLM, and TFP 16μg/mL of each drug was used since that was the concentration of TAM at which there was a significant decrease in filament formation. There was a significant decrease in filament formation (20% filaments) at 16μg/mL, which is half the MIC.

Figure 10: Similar experiments were done with the TAM analogs, TOR, PRO, CLM, and TFP 16μg/mL of each drug was used since that was the concentration of TAM at which there was a significant decrease in filament formation. There was a significant decrease in filament formation (20% filaments) at 16μg/mL, which is half the MIC.
S. cerevisiae have diverged evolutionarily from C. albicans and therefore have some structural and biochemical differences. In addition, there are differences in antifungal activity between these two species. For example, the MIC of TAM in S. cerevisiae is 12.5 μg/mL, while the MIC in C. albicans is 3.125 μg/mL. Despite this divergent evolution, they do share some similar phenotypes. We therefore experimented to see if the mode of action of TAM in C. albicans was similar to that in S. cerevisiae. Through calcium suppression assays, we showed that TAM works as an antifungal by disrupting the CaM signaling pathway similar to S. cerevisiae.

As described above, proper actin localization is important for cellular function and morphology. TAM has been shown to disrupt proper actin localization within the cell. Cells undergo actin rearrangement to regulate such critical processes as endocytosis, cytokinesis, cell polarity, and cell morphogenesis. Early in G1, unbound cells select a future bud site, and assemble polarity factors as well as actin cable to the site of new bud formation. When the cells are exposed to TAM, this process is disrupted as a result of TAM's interaction with CaM. As a CaM inhibitor, TAM disrupts the normal signaling leading to proper actin localization. The cells are not able to properly form their actin cytoskeleton, new bud formation and filament formation processes are also affected. We have shown that CaM inhibitors block filament formation and new bud formation at concentrations below MIC. Blocking filament formation has been proposed as a way to treat fungal infections because such filaments are required for virulence. By themselves these CaM inhibitors have the potential to be effective antifungals since they are relatively non-toxic. In addition, these CaM inhibitors have the potential to be effective antifungals since their relative activities may be different. These drugs may have similar characteristics to the known cell wall stressors such as CAS. This provides insight into the potential antifungal mechanism of these CaM inhibitors. We have also shown that CaM inhibitors are additive with cell wall inhibitors. The CaM inhibitors may sensitize the cell to further inhibition of β-glucan cell wall architecture by CAS.

DISCUSSION

1. Tamoxifen (Tam)

2. Toremifene (Tor)

3. Prochlorperazine (Pro)

4. Clomiphene (Clom)

5. Trifluoperazine (Trif)


TAM enhances the activity of the cell-wall-targeted antifungal caspofungin (CAS).

TAM was tested in combination with the known antifungal, CAS, to see if there was any synergistic interaction between the two drugs. Figure 10 shows the FIC value calculated for the combination of TAM and CAS. As described above, an FIC index of 0.5 indicates synergy, while an FIC index of 2.0 indicates indifference. Antagonism is defined as an FIC index of > 4.0. The combination of TAM and CAS had an FIC index of 0.75. This combination showed a potential interaction in vitro, although, according to this calculation, 0.75 is considered indifferent. A value of 0.75 indicates a possible additive effect since the addition of an additional drug to CAS lowered its MIC four-fold at half the MIC of TAM. Since TAM has been shown to disrupt the cell wall, TAM treatment sensitizes the yeast cell to further inhibition of the β-glucan architecture by CAS.

ACKNOWLEDGMENTS

I want to thank everyone in the Krysan and Wellington Labs for all their support and friendship over the past two years. I have learned so much and grown tremendously as a scientist. Thank you to Lou for helping me with my experiments and being a great lunch buddy. Thank you to Damian and Melanie for being great mentors. I want to also thank Dr. Butler and Dr. Dumont for agreeing to serve on my thesis defense committee.

REFERENCES


TAM treatment sensitizes the yeast cell to further inhibition of the MIC of TAM. Since TAM has been shown to disrupt the cell wall, an additional drug to CAS lowered its MIC four-fold at half the dose of 0.75 μg/mL indicates a possible additive effect since the addition of 2 drugs. Figure 10 shows the FIC value calculated for the combination of TAM and CAS, to see if there was any synergistic interaction between the 2 drugs. The vehicle (DMSO) treated cells showed minimal red staining for all the drugs. Figure 9 shows the normal signaling leading to proper actin localization. Since the cells are not able to properly form their actin cytoskeleton, new cell wall architecture is disrupted by CAS, thus suggesting that combination therapy may be feasible.

Overall, this research has direct clinical relevance. Since caspofungin and the CaM inhibitors are FDA approved, if we identify significantly active compounds, these results could be rapidly translated into clinical studies. These drugs are from anti-fungal drugs, they could also be put into rapid use because information about their safety and pharmacology is already known. It is our hope that these experiments will help improve the outcome for patients with these opportunistic infections.

ACKNOWLEDGMENTS

I want to thank everyone in the Keynan and Wellington Labs for all their support and friendship over the past two years. I have learned so much and grown tremendously as a scientist. Thank you to Lou for helping me with my experiments and being a great buddy. Thank you to Damian and Melanie for being great mentors. I want to also thank Dr. Butler and Dr. Dumont for agreeing to serve on my thesis defense committee.

REFERENCES

8. Pfaller MA, Sheehan DJ, Rex JH (2004). Determination of Fungicidal Activities Against Various Doses of TAM and Quantified by light microscopy. The data presented in the mean of three independent experiments and the error bars represent the SD. Propidium iodide staining indicated that >95% of cells were viable at the time of the filaments growth assay.

Figure 7: Filament formation is inhibited by TAM treatment, which may lead to decreased virulence. Filament formation is a key factor in C. albicans virulence. Filament formation was induced in C. albicans treated with various doses of TAM and quantified by light microscopy. The data presented in the mean of three independent experiments and the error bars represent the SD. Propidium iodide staining indicated that >95% of cells were viable at the time of the filaments growth assay.

Figure 8: Filament formation was induced in C. albicans at 1μg/ml talinol, tetrafilin, prelinnofilin, chalimargine and trichophytonerine and quantified by light microscopy. The data presented is the mean of three independent experiments and the error bars represent the SD. Filament formation was induced in C. albicans at different doses of TAM and quantified by light microscopy.

Figure 6: Molecular structures of CaM inhibitors.
The C. albicans cell wall architecture is disrupted by TAM and TOR treatment, which may lead to their decreased virulence. CaM inhibitors disrupt yeast cell wall. 1,3-β-glucan exposure was evaluated in CaM inhibitor-treated Candida albicans by IFA. C. albicans yeast were treated with 16 μg/mL of drug. Following treatment, the samples were probed with anti-1,3-β-glucan antibody; primary antibody was detected by a Texas Red-labeled secondary antibody. Caspofungin is a known cell wall inhibitor; therefore, it was included as a positive control. Compared to the untreated (DMSO) cells, the TAM, TOR, PRO and TFP-treated cells have increased 1,3-β-glucan exposure.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC&lt;sub&gt;TAM&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;CAS&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;TAM&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;CAS&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;TAM + CAS&lt;/sub&gt;</th>
<th>FIC Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>0.5 μg/mL</td>
<td>0.5 μg/mL</td>
<td>0.5 μg/mL</td>
<td>0.5 μg/mL</td>
<td>0.5 μg/mL</td>
<td>0.5 μg/mL</td>
</tr>
</tbody>
</table>