Small Molecules Inhibit *C. albicans* Biofilm Growth on Medical Substrates

Vanessa A. Perez

University of Vermont

Thesis Advisor: Dr. Douglas I. Johnson

Abstract

In this present investigation, biofilm inhibition of *Candida albicans* through the use of three small molecules was studied. *C. albicans*, an opportunistic yeast, has caused and continues to cause fungal infections in immunocompromised individuals, mainly through indwelling medical devices. Current fungal treatments cause serious side effects within patients and thus new specific antifungal treatments must be created. Small molecules, ETYA, clozapine and CGP-37157, through microplate-based experimentation were concluded to have inhibitory effects on biofilms utilizing medical substrates silicone and polyvinyl chloride (PVC) at specific concentrations. Utilizing silicone as a substrate and a small molecule soaking method, ETYA and CGP-37157 inhibitory effects were concentration dependent, while clozapine's inhibition took effect after increasing time parameters. On PVC, inhibition occurred only when soaking of small molecules was eliminated and a direct inoculation of small molecule with microorganisms was introduced. Although *in vitro* experimentation resulted in small molecules that can inhibit *C. albicans* biofilm formation on two medical substrates, many more experiments must be done before safe antifungal treatments can be utilized.

Literature Review

Candida albicans is an opportunistic fungus that problematically causes infections within immunosuppressed humans. Of importance here are the infections caused through indwelling medical devices. Medical devices in which *C. albicans* infections have occurred on include central and peripheral venous catheters, urinary catheters, endotracheal tubes, neurosurgical shunts, voice prostheses, dentures and intrauterine devices (Singh, Agrawal, Pemmaraju, Panwar, & Pruthi, 2011). Current antifungal drugs such as echinocandins function by inhibiting the synthesis of fungal cell walls (Denning, 2002) therefore lysing *C. albicans* cells but these drugs unfortunately, also cause serious side effects for humans. Due to the prevalence of biofilm infections and lack of safe antifungals, there is a need to discover new antifungal treatments that target specific virulence factors as opposed to just targeting cell growth.

C. albicans Infections

As mentioned *C. albicans* is an opportunistic fungus which causes a variety of diseases and/or infections in the human host. This fungus inhabits the gastrointestinal and genitourinary tract as normal flora in healthy individuals but in cases of immune system suppression, an overgrowth of *C. albicans* can occur, leading to a wide range of infections (Nobile & Mitchell, 2006). Of particular interest are the infections that occur on medical devices. Medical device infections can cause serious consequences that include life-threatening infections within the host, damage and removal of the medical device as well as subsequent tissue damage to the host after device removal (Kojic & Darouiche, 2004). From Kojic and Darouiche’s (2004) research, the various devices which have and continue to succumb to *C. albicans* infection include but are not limited to vascular catheters, joint prostheses, pacemakers, urinary catheters, central nervous system devices and prosthetic valves.

Biofilm Formation

The history of biofilms was first described among aquatic bacteria but the science interest surrounding biofilm-causing-organisms became prevalent only when they were discovered to cause infection within human hosts (Donlan, 2002). A biofilm is a community of maturing cells, pseudo-hyphae and hyphae all covered by an extracellular matrix. The focus here is on biofilms produced by the fungus *Candida albicans.*

Planktonic vs. Biofilm Cells

Biofilm formation is a complex process through which signaling pathways alter cells making them different from planktonic (free-floating) cells. (Donlan, 2002). Cells within a biofilm gain protection and are able to live in environments where planktonic cells cannot, such as in the presence of antibiotics (Donlan, 2002).

Steps in Biofilm Formation

Candida albicans biofilm formation requires several steps and internal signals (Blankenship & Mitchell, 2006). Steps include early, intermediate and maturation stages (Richard, Nobile, Bruno, & Mitchell, 2005). In the early phase, there is the presence of adhered yeast cells on a surface. During the intermediate stage, there is continued cell growth and production of hyphae and lastly during maturation, the extracellular matrix forms (Richard *et al.,* 2005).

Adherence. *C. albicans* adherence to surfaces and as well to other cells is an important first step in biofilm formation. It is necessary for colonization and disease (Blankenship & Mitchell, 2006). Blankenship and Mitchell (2006) stated that the ALS (agglutinin-like sequence) proteins are excellent candidates for biofilm adhesion. This statement however, was not expanded on in the article due to the researchers' lack of testing tools. With the availability of

genetic and molecular tools, Liu and Filler (2011) and Green *et al*., (2004) have expanded on this area where Blankenship and Mitchell could not.

The ALS gene encodes a family of cell wall glycoproteins (Green, Cheng, Chandra, Mukherjee, Ghannoum, Hoyer, 2004).Through an ALS gene RT-PCR assay, Green *et al*., (2004) determined that Als1, Als2, Als3, Als4, Als5, and Als9 were actively present in *C. albicans* cells during early biofilm creation. The Als3 protein encoded by the *ALS3 gene,* is crucial in adherence to surfaces (Liu & Filler 2011). The findings reported by Liu and Filler (2011) portrays this protein is able to adhere to various substrates within the host such as endothelial cells, oral epithelial cells, fibronectin, fibrinogen, laminin, and type IV collagen with the potential for additional host cell targets for Als3. It was determined that *C. albicans* mutants that lack Als3 produce disorganized and scant biofilms on catheter material *in vitro* (Liu and Filler 2011).

Hyphal formation. Hyphae play an important role in maturation. Hyphal formation is not necessary for a biofilm to be created but biofilms made of yeast alone aren't sturdy therefore, are easily removed from surfaces which suggest that the hyphal filaments serve to strengthen the structure (Blankenship & Mitchell, 2006). Richard *et al*., (2010) through utilizing homozygous insertion mutants identified genes required for the development of biofilms. Richard *et al*., (2010) mutants' resulted in defective hyphae strains that produced defective biofilms. This research thus portrays the crucial role hyphae play in building a strengthen biofilm.

Hyphae in matured biofilms have the capability to invade underlying surfaces. In the mucosa, it can disrupt the epithelial layer and cause an inflammatory response (Leonhard, Moser, Reumueller, Mancusi, Bigenzahn, &Schneider-Stickler, 2010). Hyphae are also able to penetrate

the soft medical silicones found in prosthetic devices that cause the silicone to expand and stiffen, as shown in Figure 1 (Leonhard *et al*, 2010).

Figure 1: Hyphae penetrate the silicone of a voice prosthesis, causing it to expand and stiffen (Leonhard *et al.*, 2010.)

Extracellular matrix. The last stage of biofilm maturation is the creation of an extracellular matrix. The extracellular matrix is mainly composed of carbohydrates and proteins (Richard *et al*., 2005). The main carbohydrate is β-1,3 glucan, when in elevated levels is characteristic of biofilm cells as compared to planktonic cells (Tobudic, Kratzer, Lassnigg, & Presterl, 2011). Blankenship and Mitchell (2006), suggested that biofilms function to defend against phagocytic cells, to maintain integrity, and/or to limit diffusion of toxic substances. The extracellular matrix indeed acts as a support and barrier that prevents antimicrobials to reach cells embedded within the biofilm (Donlan & Costerton, 2002).

Budded-to-Hyphal Transition

Candida albicans has the ability to transform from budded and hyphal states called budded-to-hyphal transition (BHT). BHT plays an important role in virulence and biofilm formation. Different environmental signals and biofilm processes such as BHT, are controlled by various signaling pathways (Midkiff, Borochoff-Porte, White, & Johnson, 2011). These

pathways work through activating different transcription factors that ultimately conduct a message down to induce the expression of hyphal-specific genes.

Budded-to-Hyphal Transition Inhibitors

Toenjes, Munsee, Ibrahim, Jeffrey, Edwards, & Johnson (2005) conducted a study with the focus of finding antifungal treatments through identifying small molecule inhibitors of the budded-to-hyphal transition. Since present treatments target broad *C. albicans* growth, a more specific tactic is needed. Toenjes *et al*., (2005) through the use of a microplate-based assay found 5 of 72 tested molecules (obtained from a Chembridge small molecule library) that inhibit BHT. In a later study conducted by Toenjes, Stark, Brooks, & Johnson (2009), the focus slightly shift to small molecules that where cytotoxic or direct BHT blockers. It was learned that the inhibition of BHT could be also be due to cytotoxicity of the molecule as well (Toenjes *et al*., 2009). From this study 480 molecules from the BIOMOL—Institute of Chemistry and Cell Biology Known Bioactives Collection were screened and resulted in findings of 53 cytotoxic molecules and 16 BHT inhibitors.

Clinical Isolates and Small Molecule Inhibitors

In a study performed by Grald, Yargosz, Case, Shea, & Johnson (2012), various clinical isolates of *C. albicans* were tested to determine their biofilm formation abilities along with the amount of hyphae produced. The effect BHT inhibitors had on the wild-type clinical isolate was also tested (Grald *et al*., 2012). A BHT assay and light microscopy were utilized to quantify averaged hyphae cell amount of each isolate and a microplate-based biofilm assay assessed their biofilm forming capabilities. Figure 2 portrays the results of this experiment and interestingly enough the study revealed the amount of hyphae formation does not correlate with biofilm formation (Grald *et al*.,2012).

Isolate	**Biofilm formation**	**% Hyphae**
Ca-WT*	++	78
Ca-141	–	1
Ca-123	+	1
Ca-136	–	5
Ca-122	++	6
Ca-112	+	11
Ca-124	+	15
Ca-137	++	15
Ca-138	–	16
Ca-126	++	19
Ca-132	+	21
Ca-116	++	21

Figure 2: Biofilm formation and hyphae production do not correlate (Grald *et al*., 2012)

In the Grald *et* al., 2012 study, three of seventeen inhibitory molecules used were able to successfully inhibit biofilm formation of *C.albicans*—buhytrinA, ETYA and CGP-37157 (Figure 3). The three BHT inhibitors varied in their capabilities of inhibition with ETYA being the most effective (Grald *et al*., 2012).

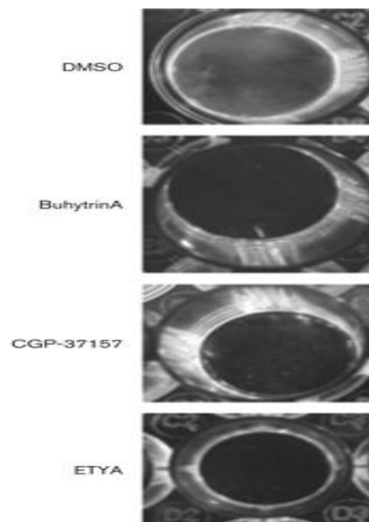

Figure 3: Negative biofilm inhibitor is conveyed by DMSO control, while Buhytrin, CGP-37157 and ETYA convey inhibition (Grald *et al*.,2012)

Small Molecules ETYA, CCG-37157 and Clozapine

Small molecules that inhibit a variety of mammalian signaling pathways and enzymes are detrimental to *C. albicans*. One small molecule, 5,8,11,14-eicosatetraynoic acid (ETYA) is a lipooxygenase inhibitor (Noverr, Erb-Downward, & Huffnagle, 2003). Lipooxygenases and cyclooxygenases play the role of converting arachidonic acid to eicosanoids (Noverr *et al.,* 2003). *C. albicans* cells produce many eicosanoids that impact cell growth, morphogenesis, and biofilm formation. ETYA, thus has a main role in inhibiting BHT.

Small molecule CGP-37157 or 7-Chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one is commonly used as a blocker of the mitochondrial Na^{+}/Ca^{2+}exchanger (Neumann, Diaz-Sylvester, Fleischer, & Copello, 2011). Lastly, small molecule clozapine, a key drug in the history of treatment of psychosis (Vera, Rezende, Molina, & Sanz-Fuentenebro, 2012), has also been identified as a BHT inhibitor.

Biofilm Growth on Synthetic Media

In order to achieve the goal of developing new therapeutic drugs, *in vitro* biofilm experiments that simulate the environment within a human host must be conducted. Due to *C. albicans'* ability to cause fungal urinary tract infections, Uppuluri, Dinakaran, Thomas, Chaturvedi, and Lopez-Ribot (2009) studied an *in vitro* Synthetic Urine (SU) model to mimic *in vivo* biofilms on urinary catheters. The SU model was used to copy the scenario of a catheter that is periodically covered in patient urine, where urine is the only nutrient source. SU biofilm growth was compared with growth within RPMI medium. Results conveyed biofilms in both RPMI and SU media followed similar developmental stages but cell growth was at least two fold slower in the SU medium and architecturally less complex (Uppuluri *et al*, 2009).

When comparing articles, results from Uppuluri *et al*., (2009) differed from experiments performed by Jain, Kohli, Cook, Gialanella, Chang, & Fries (2007) who observed an increase of biofilm formation in artificial urine (AU). Jain *et al*., (2007) had a similar experiment but instead utilized an AU medium with an 8% glucose concentration, fourfold higher than the concentration of glucose in the SU medium. This experimental design, although not optimal for simulating actual events in the human body, was beneficial in a sense by providing data with a different glucose concentration used.

In an *in vitro* experiment conducted by Ramage, Wickes, & Lopez-Ribot (2008) a seed and model was designed to mimic the constant exposure to biological fluids that a biofilm normally encounters within a host. This seed and feed model used the substrate poly metyl methacrylate and "fed" *C. albicans* cells with RPMI. Biofilms were visualized with scanning electron microscopy (SEM) and confocal laser scanning microscopy (Ramage *et* al., 2008). This *in vitro* study provided a reproducible methodology for future researchers to test biofilm growth on various biomaterials.

An example of a study conducted *in vivo* was performed by Chandra, Long, Ghannoum, & Mukherjee, (2011) utilizing a rabbit model. The study provided visualization, through scanning electron microscopy, of an *in vivo* central venous catheter biofilm infection as well as a model that can be applied to test other biomaterials (Chandra *et al*., 2011).

Models have provided and continue to provide critical information of biofilm formation and pathogenesis. Several experimental variables have been and must be incorporated into experimental systems to mimic patient conditions. Types of models could include, and are not limited to, simulation of blood, salivary proteins, variations in nutrient medium as well as uses of common device materials. Common medical device materials that will be the substrates used in

this experiment include silicone rubber and polyvinyl chlorine (PVC). Silicone rubber is a major polymer used to compose medical devices such as urinary catheters, and PVC is the most susceptible biomaterial for *C. albicans* biofilm formation (Singh *et al*., 2011).

In the process of creating this literature review, both recently published literature and older literature were examined. Examining both forms of research—old and new—prevents misinformation of concepts, and the presentation of skewed and inconsistent data today. As this review centers around *C. albicans'* ability to form biofilms and strategies taken to inhibit biofilms, keywords used to search the literature were primarily "*C. albicans*: biofilm formation, inhibition, morphology, and pathogenesis." If primary literature did not directly pertain to mentioned topics or focused on similar but, for the purposes of this paper irrelevant topics, majority, if not all was discarded. Cited articles were primarily chosen by analysis of abstracts in order to determine relevance. Once approved, articles were complete read, useful information was highlighted and limitations were determined.

From the information presented it is evident much work and research must continue in order to develop an efficient type of drug therapy against C. *albicans.* Many gaps of knowledge do still remain. For instance, will molecular tools further aid in filling gaps? Are there other small molecules that inhibit biofilm formation through the BHT inhibition? Can the small molecule inhibitors found in Toenjes *et al*., studies also inhibit biofilm formation on *in vitro* catheter simulated environments? If these molecules do indeed work *in vitro*, is this the same case for *in vivo*? Most importantly in regards to the focus of this paper; can the small molecules ETYA, clozapine and CGP -37157 inhibit *C. albicans* biofilm growth on medical substrates such as silicone and PVC?

Materials

Growth Media and Strains

The *Candida albicans* strains used in this study are: wildtype SC3514 and mutant delta Efg1. Strain SC3514 produces hardy biofilms so it was utilized for the positive control and as the strain tested against the small molecules. Efg1 produces weak biofilms so it was used as the negative control. Both strains were grown on rich medium yeast extract peptone dextrose (YEPD) composed of 1% yeast extract, 2% peptone and 2% dextrose (Midkiff *et al*., 2011).

Small Molecule BHT Inhibitors

All small molecule budded-to-hyphal transition inhibitors were dissolved in DMSO to a stock concentration of 40mM and stored at 0°C. The molecules clozapine and ETYA were purchased from BIOMOL International/Enzo Life Sciences. Molecule CGP-37157 was purchased from Tocris Bioscience (Midkiff *et al*., 2011).

Methods

Impregnating Silicone and PVC

1) The amount of small molecule(s) to be used for the experiment was determined. The appropriate number of microcentrifuge tubes were removed and thawed for 15 minutes before intended use.

Each tube contained 40mM of small molecule.

a) For ETYA, the 1X Minimal Inhibitory Concentration (MIC) in a 48 well plate = 100µM

b) For CGP-37157, the 1X MIC in a 48 well plate = 40µM

c) For Clozapine, the 1X MIC in a 48 well plate = 100µM

2) In the hood, the workspace was sterilized using ethanol. Silicone and PVC were cut into workable pieces of approximately 1cm by 1cm using sterilized instruments (*i.e.*, flamed forceps, scissors) and allowed to cool. Substrate (silicone or PVC) was then added to all wells being used

in the experiment. The substrate was pushed down into the well so it would not float once the media was added.

3) While in the hood, 0.5mL of 1xPBS was added to each well pertaining to the first time point (*i.e.,* 6 hours). Then, the appropriate amount of small molecule was added to each corresponding well based on the experimental design *(i.e.,* 100 µM of ETYA).

4) During the same time point preparation, 0.5ml of PBS + 2.5uL of DMSO were also added to a well that was marked for a DMSO 6hr control.

5) All substrates were visually examined to be stuck to the bottom of their wells, if not a gentle push with sterile forceps was required. The 48-well microtiter plate was put in a bag and incubated at 37℃. The substrate was soaked for an hour and then PBS and small molecule were added to subsequent wells at indicated time points, (*i.e*., 5hr, 4hr, 3hr, 2hr, 1hr) following the same procedure as in step 3.

6) After soaking the substrate for the desired amount of time, the solution was aspirated off from wells and substrate was transferred to unused wells. The substrate was then washed three times with 0.5ml of ddH_2O.

a) 0.5ml of ddH_2O was added to each well. The cover of each plate was replaced and the plate was shaken gently for 5 seconds. The cover was removed and the solution was ddH_2O solution was aspirate off. This washing step was repeated 2 more times.

7) The washed substrate was again moved to other unused wells. The procedure outlined below pertaining to preparing cultures to inoculate wells with cells was then followed.

Preparing Cultures

1) *C. albicans* cells SC3514 and delta Efg1 were grown on YEPD plates using a 3 zone streak.

2) After >24hrs, sterilized sticks were used to take an isolated colony of both *C. albicans* strains and inoculate 5ml test tubes of YEPD. The two test tubes were placed in a 30℃ roller drum and strains were grown overnight (14-18hrs).

3) Test tubes were vortexed for a few seconds and 0.5ml of each culture was added to its own cuvette. 0.5ml of YEPD was also added to a cuvette to be used as a blank. The absorbencies of the cells in the cuvette were read on a spectrophotometer and recorded.

4) The test tubes containing 4.5ml of YEPD and cells were centrifuged for 5 minutes at 1000rpm. The supernatant was aspirated off and the cells were resuspended in 4.5ml of PBS. The test tubes were centrifuged again for 5 minutes at 1000 rpm. The supernatant was again aspirated and 4.5ml of PBS was added.

5) New test tubes were labeled and appropriate amounts of RPMI media was added to each tube, using a little over the desired amount.

a) The appropriate amount of RPMI was determined by counting how many wells were going to be inoculated. Each well required 0.5ml of RPMI media. If 6 wells were need, then 3ml of media/cell mixture would be needed. To ensure enough media/cells, 4ml instead of 3ml were added.

6) The following materials were brought to the hood:

a) 1000, 200 pipettes + tips

b) Test tubes

c) parafilm

d) forceps

e) 48 well microtiter plates used

7) Culture dilutions of 1:100 were made and vortexed.

a) If 4ml of RPMI media was added to a tube then, 40ul of cell suspension were added.

8) 0.5ml of cells/RPMI media mix were added to each pertinent well. Two extra wells with unsoaked substrate were added to each plate to serve as the positive and negative controls.

9) Each plate was parafilmed, placed in a bag and incubated at 37℃ for 48 hours.

10) After 48 hours, plates were removed from the incubator and placed in a sterilized hood. Parafilm was removed and cell/media were aspirated from each well.

11) The substrates were placed into unused wells and washed with ddH_2O three times. Final washed substrates were then observed for positive or negative biofilm growth.

Results

Determination of biofilm growth was based on macroscopic growth. Figure 7 below shows the criteria used to differentiate between negative biofilm growth or inhibition (on the left) from positive biofilm growth (on the right). Biofilm inhibition is the absence of all visible *C. albicans* biofilms on the substrate used. Biofilm growth is the presence of any *C. albicans* biofilm growth whether the growth is rich as depicted in Figure 7 or scant.

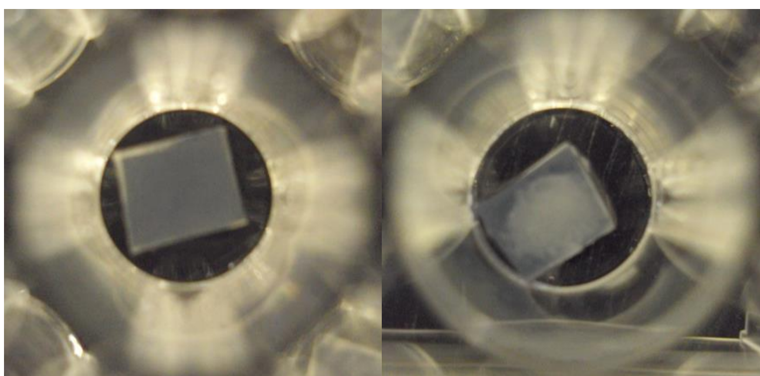

Figure 7: Biofilm inhibition versus Biofilm growth, respectively

Silicone: ETYA, Clozapine and CGP-37157

As determined through previous experiments, the minimum inhibitory concentration of BHT for ETYA and clozapine are both 100 µM using a 48-well microtiter plate. The minimum inhibitory concentration of CGP-37157 is 40µM using a 48-well microtiter plate. These concentrations were thus initially used to test if soaking the substrate (silicone) for a set amount of hours with small molecule would cause biofilm inhibition. Both positive and negative controls were run in each experiment to ensure accurate and readable results. A DMSO control was also run to show that DMSO, the solvent in which the small molecules were diluted and dissolved, does not cause inhibition. Each experiment was performed in triplicate.

Molecule	[Conc]	Positive Control	Negative Control	DMSO Control	Hour 6	Hour 5	Hour 4	Hour 3	Hour 2	Hour 1
ETYA	**100µM**	+	-	+	+	+	+	+	+	+
	200µM	+	-	+	-	-	-	-	-	-
Clozapine	**100µM**	+	-	+	+	+	+	+	+	+
	200µM	+	-	+	+	+	+	+	+	+
	300µM	+	-	+	+	+	+	+	+	+

Table 1: Inhibitory actions of small molecules, ETYA and Clozapine. “-” indicates no growth and “+“ is indicative of biofilm growth.

As portrayed by Table 1, soaking the silicone with ETYA at a concentration of 100µM results in C. *albicans* biofilm growth for all hours. When a concentration of 200µM was tested, inhibition of biofilms resulted. Soaking silicone with clozapine at concentrations of 100µM, 200µM and 300µM, using the same time parameters did not result in biofilm inhibition. As shown in Table 2, the time variable was changed to 24 hours of soaking clozapine as opposed to a maximum of 6 hours. Soaking clozapine at a concentration of 100µM for 24 hours resulted in biofilm growth. For clozapine concentrations of 200µM, 300µM, and 400µM, however, biofilm inhibition occurred.

Molecule	[Conc]	Positive Control	Negative Control	DMSO Control	Hour 24
Clozapine	**100µM**	+	-	+	+
	200µM	+	-	+	-
	300µM	+	-	+	-
	400µM	+	-	+	-

Table 2: Inhibitory action of Clozapine using 24 hours of soaking

Table 3 conveys the inhibitory actions of small molecule CGP-37157. By testing 40µM, its minimum inhibitory concentration, complete biofilm inhibition resulted. Using a concentration of 20 µM however, resulted in complete biofilm growth.

Molecule	[Conc]	Positive Control	Negative Control	DMSO Control	Hour 6	Hour 5	Hour 4	Hour 3	Hour 2	Hour 1
CGP-37157	**40µM**	+	-	+	-	-	-	-	-	-
	20µM	+	-	+	+	+	+	+	+	+

Table 3: Small Molecule CGP-37157 inhibitory action.

A further set of experiments included soaking individual substrates with two small molecules as opposed to one. As portrayed in Table 4, 40µM of CGP-37157 and 200µM of ETYA were first combined since these were the lowest concentrations that initially resulted in biofilm inhibition. Biofilm inhibition still occurred by mixing these two small molecules. Next, 40µM of CGP-37157 and 100µM of ETYA were used since CGP-37157 is known to inhibit *C. albicans* biofilm growth but ETYA at this concentration does not. The experiment also resulted in biofilm inhibition. Lastly, 20µM of CGP-37157 and 100µM of ETYA were combined and resulted in biofilm growth.

Molecules	[Conc]	Positive Control	Negative Control	DMSO Control	Hour 6	Hour 5	Hour 4	Hour 3	Hour 2	Hour 1
CGP-37157 ETYA	**40µM 200µM**	+	-	+	-	-	-	-	-	-
CGP-37157 ETYA	**40µM 100µM**	+	-	+	-	-	-	-	-	-
CGP-37157 ETYA	**20µM 100µM**	+	-	+	+	+	+	+	+	+

Table 4: Combinatorial actions of ETYA CGP-37157 and ETYA

PVC: ETYA, Clozapine and CGP-37157

The second medical substrate used in the set of experiments was polyvinyl chloride (PVC). The first experiment with this substrate was to determine whether *C. albicans* was able to produce biofilms or not. Results conveyed that biofilms were macroscopically visible on this substrate. The small molecules ETYA and CGP-37157 were then tested to determine whether they were capable of biofilm inhibition through soaking. As shown in Table 5, the same concentrations that inhibited biofilm growth of silicone did not inhibit growth on PVC.

Molecule	[Conc]	Positive Control	Negative Control	DMSO Control	Hour 6	Hour 5	Hour 4	Hour 3	Hour 2	Hour 1
CGP-37157	40µM	+	-	+	+	+	+	+	+	+
ETYA	200µM	+	-	+	+	+	+	+	+	+

Table 5: Small Molecules CGP-37157 and ETYA do not inhibit biofilm growth on PVC substrate

Experimental parameters were then altered to maintain all the same variables except the soaking stage of small molecules. Small molecules CGP-37157, ETYA and clozapine were incubated along with *C. albicans* microorganisms to determine if inhibition was still possible on PVC substrate. Table 6 shows that inoculating and incubating substrate with small molecules does indeed inhibit biofilm growth at 40µM of CGP-37157, 100µM and 200µM of ETYA and 100µM of clozapine.

Molecule	[Conc]	Positive Control	Negative Control	DMSO Control	48 Hour Incubation
CGP-37157	40µM	+	-	+	-
ETYA	100µM	+	-	+	-
	200µM	+	-	+	-
Clozapine	200µM	+	-	+	-

Table 6: Inhibitory activities of small molecules without soaking using PVC substrate.

Discussion

Silicone

Taking all the data into account allows for several conclusions. The small molecule clozapine does not inhibit biofilm growth when left soaking in silicone for a maximum of 6 hours. However, when left soaking in the substrate for 24 hours, inhibition did occur. This result reveals the difference in clozapine behavior compared to the other two small molecules, ETYA and CGP-37157. This result also reveals the importance of altering experimental design when appropriate in order to obtain clinically significant data.

Independently, ETYA was not able to inhibit *C. albicans* biofilm growth at a concentration of 100µM. At a concentration of 200µM, however, inhibition did occur. CGP-37157 was also found to inhibit biofilm formation at a concentration 40µM. While inoculated together, ETYA and CGP-37157 did inhibit biofilms at experimental concentrations of 200µM and 40µM as well as with concentrations of 100µM and 40µM, respectively. Biofilm inhibition did not occur, however, at concentrations of 100µM of ETYA and 20µM of CGP-37157. These data suggests that while ETYA and CGP-37157 can still have inhibitory properties within the same soaking substrate, their inhibition does not occur through synergistic effects.

Method	Substrate	Molecule	[Conc]	48 Hour Incubation	Hour 24	Hour 6	Hour 5	Hour 4	Hour 3	Hour 2	Hour 1
Soaking	Silicone	ETYA	200µM			-	-	-	-	-	-
		CGP-37157	40µM			-	-	-	-	-	-
		Clozapine	200µM		-	+	+	+	+	+	+
W/O Soaking	PVC	ETYA	100µM	-							
		CGP-37157	40µM	-							
		Clozapine	200µM	-							

Table 7: Important results highlighted from experimental runs for all small molecules on both substrates, silicone and PVC.

PVC

Conclusions from the PVC studies first include the fact that *C. albicans* is able to produce biofilms on this newly used substrate within the laboratory. It was also determined that soaking the substrate with small molecules is an ineffective tactic to inhibit biofilm formation. Inhibition occurred when the method of soaking was eliminated and instead inoculation of small molecule with microorganism was introduced. ETYA, CGP-37157 and clozapine were found to have inhibitory effects at concentrations of 100µM, 40µM and 200µM, respectively.

Conclusion

C. albicans is an opportunistic pathogen which is a causative agent of systemic fungal infections in humans. Due to its virulence factors, *C. albicans* problematically adheres and develops biofilms on medical devices. Current antifungal treatments unfortunately also target host cells leading to adverse side effects. Thus, there is a need to find drugs that target specific virulence factors as opposed to broad cell growth.

The three small molecules used in this experiment ETYA, clozapine and CGP-37157 through varying concentrations and time parameters have exhibited a means to inhibit *C. albicans* biofilm growth. Although the outcome of this experiment shows hope, this is just a preliminary stride before real medical strides in devising new antifungal medications can be made. A major limitation in this experiment includes an *in vitro* approach. To test the safety and efficacy of antifungal drugs, an in vivo experiment will have to be conducted. The small molecules tested do show inhibitory potential but this in vitro experiment does not allow one to know the effects of these small molecules within a human host.

References

Blankenship, J. R., & Mitchell, A. P. (2006). How to build a biofilm: a fungal perspective. *Current Opinion in Microbiology, 9*, 588-594. Retrieved from: http://www.ncbi.nlm.nih.gov/pubmed/17055772

Chandra, J., Long, L., Ghannoum, M., & Mukherjee, P. (2011). A rabbit model for evaluation of catheter-associated fungal biofilms. *Virulence*, *2*(5), 466-474.

Denning, D. (2002). Echinocandins: a new class of antifungal. *Journal of Antimicrobial Chemotherapy*, *49*, 889-891.

Donlan R. M. (2002). Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases, 8*(9), 881-890. Retrieved from http://wwwnc.cdc.gov/eid/article/8/9/pdfs/02-0063.pdf

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology, 15*, 167-169. Retrieved from http://cmr.asm.org/content /15/2/ 167.full.pdf+html

Grald, A., Yargosz, P., Case, S., Shea, K., & Johnson, D. I. (2012). Small-molecule inhibitors of biofilm formation in laboratory and clinical isolates of *Candida albicans. Journal of Medical Microbiology, 61.* 109-114.

Jain, N., Kohli, R., Cook, E., Gialanella, P., Chang, T., & Fries, B. C. (2007). Biofilm Formation by and Antifungal Susceptibility of *Candida* Isolates from Urine. *Applied and Environmental Microbiology*, *73*(6), 1697-1703. Retrieved from http://aem.asm.org/ content/73/6/1697.full.pdf+html

Kojic, E., & Darouiche, R. (2004). *Candida* Infections on Medical Devices. *Clinical Microbiology*, *17*(2), 255-267.

Leonhard, M., Moser, D., Reumueller, A., Mancusi, G., Bigenzahn, W., & Schneider-Stickler, B. (2010). Comparison of biofilm formation on new phonax and provox 2 voice prostheses—a pilot study. *Head and Neck*, *32*(7), 886–895.

Liu, Y., & Filler, S. G. (2011). *Candida albicans* Als3, a multifunctional adhesion and invasion. *Eukaryotic Cell, 10*(2), 168-173. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3067396/pdf/zek168.pdf

Midkiff, J., Borochoff-Porte, N., White, D., & Johnson, D.I. (2011). Small Molecule Inhibitors of the *Candida albicans* Budded-to-Hyphal Transition Act through Multiple Signaling Pathways. *PLoS ONE, 6*(9), e25395. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3180447/pdf/pone.0025395.pdf

Neumann, J., Diaz-Sylvester, P., Fleischer, S., & Copello, J. (2011). CGP-37157 Inhibits the Sarcoplasmic Reticulum Ca^{2+}ATPase and Activates Ryanodine Receptor Channels in Striated Muscle. *Molecular Pharmacology, 79*(1), 141-147. Retrieved from http://molpharm.aspetjournals.org/content/79/1/141.full.pdf+html

Nobile, C. J., & Mitchell, A. P. (2006). Genetics and genomics of *Candida albicans* biofilm formation. *Cellular Microbiology, 8*(9), 1382-1391. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1462-5822.2006.00761.x/pdf

Noverr, M., Erb-Downward, J., & Huffnagle, G. (2003). Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes. *Clinical Microbiology Reviews, 16*(3), 517-533. Retrieved from http://cmr.asm.org/content/16/3/517.full.pdf+html

Ramage, G., Saville, S. P., Wilckes, B. L., & Lopez-Ribot, J. L. (2002). Inhibition of *Candida*

albicans biofilm formation by farnesol, a quorum-sensing molecule. *Applied and Environment Microbiology, 68*(11), 5459-54637. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC129887/pdf/0413.pdf

Ramage, G., Wickes, B. L., Lopez-Ribot, J. L. (2008). A seed and feed model for the formation of *Candida albicans* biofilms under flow conditions using an improved modified Robbins device. *Revista Iberoamericana de Micologíal*, *25*(1), 37-40. Retrieved from http://www.reviberoammicol.com/2008-25/037040.pdf

Richard, M., Nobile, C. J., Bruno, V., & Mitchell, A. (2005). *Candida albicans* Biofilm-Defective Mutants. *Eukaryotic Cell, 4*(8), 1493-1502. Retrieved from http://www.ncbi.nlm.nih.gov/ pmc/articles/PMC1214533/pdf/0110-04.pdf

Sheppard, D. C., Yeaman, M. R., Welch, W. H., Phan, Q. T., Fu, Y., Ibrahim, A. S., Filler, S. G., Zhang, M., Waring, A. J., & Edwards, J .E. (2004). Functional and Structural Diversity in the Als Protein Family of *Candida albicans*. *The Journal of Biological Chemistry, 279*(29), 30480-30489. Retrieved from http://www.jbc.org/content/279/29/30480

Singh, N., Agrawal, V., Pemmaraju, S., Panwar, R., & Pruthi, V. (2011). Impact of infectious *Candida albicans* on biomaterials. *Indian Journal of Biotechnology. 10*, 417-422. Retrieved from: http://nopr.niscair.res.in/bitstream/123456789/12978/1/IJBT%2010 (4)%20417-422.pdf

Tobudic, S., Kratzer, C., Lassnigg, A., & Presterl, E. (2011). Antifungal susceptibility of *Candida albicans* in biofilms. *Mycoses, 55*(3), 199-204.

Toenjes, K. A., Munsee, S. M., Ibrahim, A. S., Jeffrey, R., Edwards, J. E., & Johnson, D. I. (2005). Small-Molecule Inhibitors of the Budded-to-Hyphal Form Transition in the

Pathogenic Yeast *Candida albicans*. *Antimicrobial Agents and Chemotherapy, 49*(3), 963-972.

Toenjes, K. A., Stark, B. C., Brooks, K. M., & Johnson D. I. (2009). Inhibitors of cellular signaling are cytotoxic or block the budded-to-hyphal transition in the pathogenic yeast *Candida albicans*. *Journal of Medical Microbiology*, *58*(6), 779-790. Retrieved from http://jmm.sgmjournals.org/content/58/6/779. full.pdf+html

Uppuluri, P., Dinakaran, H., Thomas, D .P., Chaturvedi, A. K., & Lopez-Ribot, J. L. (2009). Characteristics of *Candida albicans* Biofilms Grown in a Synthetic Urine Medium. *Journal of Clinical Microbiology, 47*(12), 4078-4083. Retrieved from http://jcm.asm.org/content/47/12/4078.long

Vera, I., Rezende, L., Molina, V., & Sanz-Fuentenebro, J. (2012). Clozapine as treatment of first choice in first psychotic episodes. What do we know? Actas Espanolas dePsiquiatria, *40*(5):281-289. Retrieved from http://www.actaspsiquiatria.es/repositorio/14/79/ENG/14-79-ENG-281-289-638437.pdf