SCREENING AND CHARACTERISATION OF INHIBITORS FOR GLYOXYLATE CYCLE ICL ENZYME ACTIVITY IN *Candida albicans*

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BY

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LIST OF SYMBOLS AND ABBREVIATIONS

ABCD	Amphotericin B colloidal dispersion
ABLC	Amphotericin B lipid complex
ADMET	Absorption, disposition, metabolism, excretion, and toxicity
AMB-DC	Amphotericin B deoxycholate
cDNA	Complementary DNA
cfu/mL	Colony forming unit per millilitre
CGD	*Candida* Genome Database
C_t	Cycle threshold
CYP450	Cytochrome P450
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetate
GPI	Glycosylphosphatidylinositol
HTS	High throughput screening
ICL	Isocitrate lyase
L-AMB	Liposomal Amphotericin B
MCL	Methylisocitrate lyase
MIC	Minimum inhibitory concentration
miLogP	Lipophilicity, octanol-water partition coefficient

mL	Millilitre
M-MLV	Moloney murine leukemia virus
mM	Millimolar
mm	Millimetre
$MgCl_2$	Magnesium chloride
mg/L	Milligram per litre
mRNA	Messenger RNA
MS	Malate synthase
ng/µL	Nanogram per microlitre
nM	Nanomolar
nm	nanometre
PAMP	Pathogen-associated molecular pattern
P-gp	P-glycoprotein
RNA	Ribonucleic acid
RNases	Ribonucleases
ROCT	Renal organic cation transporter
RO5	Rule-of-five
rpm	Rotation per minute
PRR	Pattern recognition receptor
SAP	Secreted aspartyl proteases
SAR	Structure-activity relationship
SMILES	Simplified molecular-input line-entry system

TCA	Tricarboxylic acid
v/v	Volume per volume
w/v	Weight per volume
YNB	Yeast nitrogen base
YPD	Yeast-peptone-dextrose
5-FU	5-fluorouracil
α	Alpha
β	Beta
^{o}C	Degree Celsius
≥	Greater or equal to
µL	Microlitre
%	Percentage
x *g*	Relative centrifugal force

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PENYARINGAN DAN PENCIRIAN PERENCAT UNTUK AKTIVITI ENZIM KITARAN GLIOKSILAT ICL DALAM *Candida albicans*

ABSTRAK

Candida albicans adalah patogen oportunis yang menyebabkan kandidiasis pada manusia. Laluan metabolik telah diterokai sebagai sasaran antimikrob yang berpotensi, iaitu kitaran glioksilat yang membolehkan *C. albicans* hidup dalam keadaan nutrien terhad. Kitaran glioksilat dan enzim utamanya merupakan sasaran antikulat yang menarik bagi *C. albicans*. Dalam kajian ini, pendekatan penyaringan alternatif berasaskan sel yang lebih mencerminkan fisiologi sistem manusia, digunakan untuk mengenalpasti sebatian yang merencat kitaran glioksilat. Sebatian yang dikenalpasti adalah asid kafeik, asid rosmarinik, dan apigenin, yang sebelum ini diabaikan, tetapi menunjukkan aktiviti antikulat terhadap *C. albicans* apabila dikaji dalam keadaan fisiologi yang sesuai, dan merencat aktiviti enzim isositrat liase (ICL). Kesan senarai sebatian terhadap transkripsi ICL1 menunjukkan keputusan yang menarik iaitu asid kafeik dan asid rosmarinik telah meningkatkan transkripsi ICL1. Sebaliknya, kesan daripada apigenin menunjukkan penyusutan transkripsi ICL1. Analisis *in silico* bagi struktur molekul untuk menilai persamaan dadah dan keselamatan melalui Lipinski's RO5 dan ADMET juga dipaparkan. Kesimpulannya, tiga sebatian yang mensasarkan *C. albicans* ICL telah dikenalpasti melalui penyesuaian saringan dan seterusnya telah digambarkan keadaan fisiologi yang lebih tepat.

SCREENING AND CHARACTERISATION OF INHIBITORS FOR GLYOXYLATE CYCLE ICL ENZYME ACTIVITY IN *Candida albicans*

ABSTRACT

Candida albicans is an opportunistic pathogen that causes candidiasis in human. Metabolic pathways have been recently explored as potential antimicrobial targets, for example glyoxylate cycle that enables the *C. albicans* to survive nutrient-limited niches. Glyoxylate cycle and its key enzymes would be an attractive antifungal drug target for *C. albicans*. In this study, an alternative cell-based screening approach that better reflect the physiological environment in host system was employed to identify lead compounds that inhibit the glyoxylate cycle. The lead compounds identified are caffeic acid, rosmarinic acid, and apigenin, which were previously undervalued, but demonstrated to have antifungal activity against *C. albicans* when assayed under physiologically relevant conditions, and have inhibitory property on isocitrate lyase (ICL) enzyme activity. The lead compounds were further characterized their effects on ICL1 transcriptions, which yielded interesting results that caffeic acid and rosmarinic acid demonstrated increased ICL1 transcriptions; while apigenin demonstrated down-regulation of ICL1 transcriptions throughout the drug exposure. The molecular structures were also analysed *in silico* to assess their drug-likeness and safety via Lipinski's rule-of-five (RO5) and ADMET properties. Taken together, three lead compounds targeting *C. albicans* ICL enzyme were identified by adjusting the screening condition to better reflect the physiological condition.

CHAPTER 1. INTRODUCTION

Candida albicans and other medically relevant *Candida* species are mainly commensal yeasts commonly found on mucosal surfaces, gastrointestinal and genitourinary tracts of human (Pfaller & Diekema, 2010). These yeasts are opportunistic pathogens that are most of the time benign, but can be infectious when host immune system becomes impaired or an environmental niche becomes available (Pappas *et al.*, 2009). The infections caused by *Candida* species are generally known as candidiasis, which can be further classified into superficial infections such as oral thrush and vaginal candidiasis, and invasive candidiasis (Perlroth *et al.*, 2007). Invasive candidiasis is one of the most common nosocomial invasive infections with high mortality rate even with first-line antifungal treatments (Perlroth *et al.*, 2007; Pfaller & Diekema, 2010; Mayer *et al.*, 2013).

Choices of antifungal drugs for candidiasis are limited. Those that are in routine clinical use include the polyenes, azoles, and echinocandins (Pappas *et al.*, 2009). Most of them are either targeting the membrane or the cell wall of fungi. Polyenes (Amphotericin B) cause membrane disruption by binding to ergosterol in the plasma membrane; azoles inhibit ergosterol synthesis; and the echinocandins inhibit the glucan synthesis (Pappas *et al.*, 2009; Chandrasekar, 2011). The limited choices of antifungal drugs for candidiasis treatments encourage the search for new antifungal drugs, but the progression is slow mainly due to difficulties caused by the limited

options of antifungal targets. Firstly, the protein-based targets are prone to the emergence of resistant strains. The latest antifungal echinocandins that inhibit the β-1,3-glucan synthase for instance, the clinically resistant strains were isolated shortly after their launch into the market (Walker *et al.*, 2010; Alexander *et al.*, 2013; Fekkar *et al.*, 2013). Secondly, fungi and animals have highly conserved and similar cellular pathways and structures further limits the target options for drug discovery, for example, the structural similarity between ergosterol in fungi and cholesterol in mammals limits the usage of drugs that targeting ergosterol such as polyenes, as it leads to toxicity in recipients (Carrillo-Munoz *et al.*, 2006; Cowen, 2008).

Recently, metabolic pathways have been explored as potential antifungal drug targets, due to their importance for the yeast cells to effectively assimilate various carbon sources and survive the nutrient-limited host niches (Brock, 2009; Mayer *et al.*, 2013). Metabolic pathways such as glycolysis, gluconeogenesis, tricarboxylic acid (TCA) cycle, and glyoxylate cycle, are all thought to be important for survival and pathogenicity of *C. albicans* during infection, although their specific roles are still remain to be fully explored (Ene *et al.*, 2012; Mayer *et al.*, 2013). The potential of glyoxylate cycle as antimicrobial drug target has been studied more than other metabolic pathways. The glyoxylate cycle is a modified version of tricarboxylic acid (TCA) cycle that bypasses the CO_2-generating steps to conserve carbons for gluconeogenesis (Lorenz & Fink, 2002; Dunn *et al.*, 2009). The key enzymes for this pathway: isocitrate lyase (ICL) and malate synthase (MS), are highly conserved

across wide range of organisms such as bacteria, plants, fungi, and nematodes, but lack in human systems (Kondrashov *et al.*, 2006; Dunn *et al.*, 2009). Besides, glyoxylate cycle also enables the utilization of alternative carbon sources such as lactate, ethanol, and glycerol (Lorenz & Fink, 2002; Dunn *et al.*, 2009).

Taken together, the conservation of carbons and utilization of alternative carbon sources enables the *C. albicans* to survive in the glucose-depleted environment in most niches of human body systems (Fernández-Arenas *et al.*, 2007; Marcil *et al.*, 2008; Dunn *et al.*, 2009). Previous studies suggested that *C. albicans* cells rely on glyoxylate cycle to survive nutrient-limited niches like inside macrophages and neutrophils (Fradin *et al.*, 2003; Prigneau *et al.*, 2003; Lorenz *et al.*, 2004; Fernandez-Arenas *et al.*, 2007; Marcil *et al.*, 2008; Dunn *et al.*, 2009). Besides, mutant strains incapable of glyoxylate cycle in mouse model experiments from previous studies also indicated that this metabolic pathway is essential for full virulence of *C. albicans* (Lorenz & Fink, 2001). Therefore, glyoxylate cycle is an attractive antifungal drug targets to be explored for *C. albicans*. Glyoxylate cycle has also been previously explored as potential antimicrobial drug target in other pathogens such as *Mycobacterium tuberculosis* (Gengenbacher *et al.*, 2010; Marrero *et al.*, 2010; Kratky & Vinsova, 2012) and *Burkholderia* species (Van Schaik *et al.*, 2009; Van Acker *et al.*, 2013). More importantly, glyoxylate cycle is not conserved in human systems, making it a more promising antifungal target for drug discovery in *C. albicans* with minimal toxicity potentials to recipients.

1.1 Objectives of Study

In this study, glyoxylate cycle or ICL of *C. albicans* was selected as an antifungal drug target for screening and identification of lead compounds from a collection of plant reference compounds. Lead compounds are the compounds identified via certain screening strategies to posses desired therapeutic or biological activity that it binds selectively to the target and elicits the desired functional cellular response from the target (Deprez-Poulain & Deprez, 2004; Hefti, 2008). High quality lead compounds that are qualify as future drug candidates must have sufficient bioavailability to reach the target site, and posses minimal toxicity for clinical trials (Hefti, 2008).

The lead compounds in this study are the compounds that survive the cell-based alternative screening employed in this study in which the compound has to posses the at least the following two criteria: it must be an inhibitor of ICL enzyme that selectively binds to the protein in *C. albicans*; it must not posses any general toxicity and detergent or detergent-like properties that can non-specifically reduce the growth of *C. albicans.* In order to identify new lead compounds that inhibit glyoxylate cycle which previously was undervalued when tested in physiologically irrelevant rich media supplemented with glucose, a minimal defined medium supplemented with lactate as a sole carbon source was employed to mimic the host niches environments. Besides, the effects of lead compounds on ICL1 expression in *C. albicans* and their drug-likeness and drug safety also were studied.

The objectives of this study are:

i. To identify lead compounds that inhibit *C. albicans* growth by targeting the ICL enzyme activity,

ii. To study the effects of the identified lead compounds (ICL enzyme inhibitor) on ICL1 transcriptions in *C. albicans*, and

iii. To predict the drug-likeness and ADMET properties of the identified lead compounds by *in silico* analysis.

CHAPTER 2. LITERATURE REVIEW

2.1 *Candida albicans* and Candidiasis

C. albicans is opportunistic pathogenic yeast that can cause life-threatening invasive candidiasis. Indeed, *C. albicans* and some other members of *Candida* species are among the most common cause of nosocomial invasive infections with mortality rate as high as 50% even with first-line antifungal treatments (Pfaller & Diekema, 2010; Mayer *et al.*, 2013). *Candida* species are the commensal yeast virtually present in every individual, reside on mucosal surfaces and the gastrointestinal and genitourinary tracts (Pfaller & Diekema, 2010). They are usually benign, but can establish infection if immune system of the host is impaired or an environment niche becomes available (Finkel & Mitchell, 2011). *C. albicans* can cause two major types of candidiasis in humans: mucocutaneous candidiasis - such as oral thrush, vaginal candidiasis, and potentially fatal invasive candidiasis (Calderone & Clancy, 2012).

2.1.1 General Characteristics of *C. albicans*

C. albicans is a diploid fungus with ability to mate without true sexual cycle. Genome sequences of *C. albicans* revealed many homologues with the model yeast *Saccharomyces cerevisiae* genes involved in sexual cycle, but the former lacks critical factors required for meiosis in budding yeast (Brown, 2006). Mating of two diploid *C. albicans* cells result in a tetraploid that divide to yield

recombinant diploid progeny (Finkel & Mitchell, 2011). *C. albicans* is polymorphic yeast that can grow either as unicellular yeast, as pseudo-hyphae, or as hyphae depending on the encountered environments and state of infections (Sudbery *et al.*, 2004; Brown, 2006; Mayer *et al.*, 2013). Further polymorphisms include white-opaque cells and chlamydospores, which are spore-like structures (Sudbery *et al.*, 2004). Yeast and hyphal forms are usually observed during infections with clear functions, but other morphologies are still remain to be further studied (Staib & Morschhauser, 2007; Soll, 2009). Besides, ability to form biofilms on surface is also an important characteristic of *C. albicans*. Like the biofilms formed by bacterial pathogens, *C. albicans* biofilms are highly resistant to antifungal drugs (Finkel & Mitchell, 2011). Besides, the CUG codon is translated as serine in *C. albicans*, not leucine as usually in other organisms (Brown, 2006).

2.1.2 Interactions with Human Hosts

The interaction between pathogens and their host are complex and dynamic, especially for pathogens like *C. albicans* that can establish recurrent and persistent candidiasis in both latent and active forms (Cottier & Pavelka, 2012; Tsai *et al.*, 2013). In order to establish an infection, *C. albicans* cells must adopt various strategies to survive the nutrient-limited host niches, host immune systems, and proliferate within the host. The host-pathogen interactions of *C. albicans* begins with cell-cell recognition, where the cell wall components of

the yeast act as pathogen-associated molecular patterns (PAMPs); while the host cells have pattern recognition receptors (PRRs) that can recognize the PAMPs of pathogens and activate host immune defense system (Tsai *et al.*, 2013). Furthermore, different sets of PRRs can be expressed on different cell types within host, plus phenotypic switching and polymorphism may also change *C. albicans* cell wall PAMPs within distinct host niches (Tsai *et al.*, 2013).

2.2 Carbon Metabolism of *C. albicans*

In order to survive and grow in a wide variety of host niches, *C. albicans* must be able to assimilate various carbon sources to generate new biomass, where it assimilates six-carbon sugars (glucose) via the glycolytic pathway, and two-carbon compounds such as lactate via glyoxylate cycle and gluconeogenesis. Besides, the yeast must rapidly regulates its metabolic pathways as it encounters new micro-environments within the host, depends on the carbon sources available. The metabolic pathways of glycolysis, gluconeogenesis, tricarboxylic acid (TCA) cycle, and glyoxylate cycle are summarized in Figure 2.1.

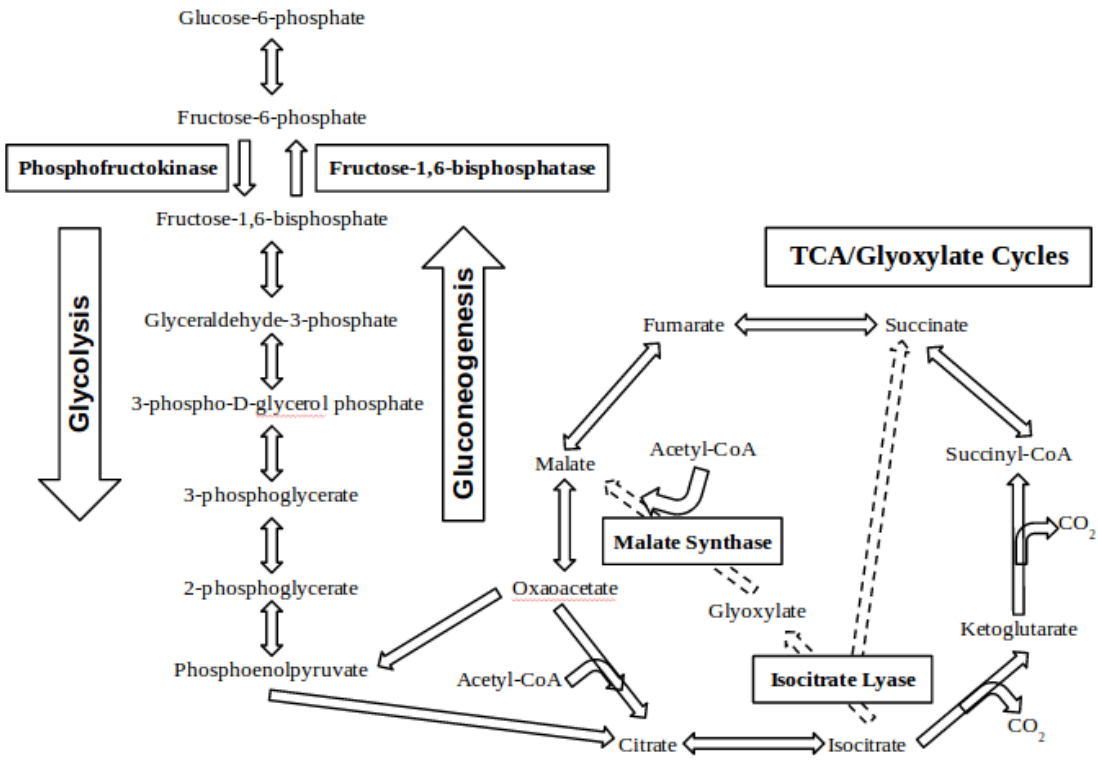


Figure 2.1. Metabolic pathways of glycolysis, gluconeogenesis, TCA cycle, and glyoxylate cycle. Gluconeogenesis is the enzymatically reversible pathway of glycolysis catalysed by the same enzymes except phosphofructokinase (glycolysis) and fructose-1,6-bisphosphatase (gluconeogenesis). Glyoxylate cycle (dashed arrow) is a modified TCA cycle that bypasses the CO_2-generating steps to conserve carbons, differentiated from TCA cycle by the two key enzymes: ICL and MS. Adapted from Lorenz & Fink (2002).

2.2.1 Glycolysis and Gluconeogenesis

Glycolysis is a central and highly conserved carbon metabolic pathway consists of the anaerobic conversion of glucose to pyruvate, plus the net production of two molecules of ATP. Pyruvate generated by glycolysis may be either anaerobically fermented to ethanol, or enter the TCA cycle under aerobic conditions. The genes encoding glycolytic enzymes and their expressions in yeast pathogens like *C. albicans* have all been identified and studied extensively at transcriptional and proteomic levels (Swoboda *et al.*, 1994; Fradin *et al.*, 2003; Barelle *et al.*, 2006; Kusch *et al.*, 2007). Generally, glycolysis is repressed during the early infection when glucose may not be readily available, and is expressed later during tissue colonization and invasion (Lorenz & Fink, 2002; Barelle *et al.*, 2006).

Gluconeogenesis is the reverse form of glycolysis, which allows the synthesis of glucose-6-phosphate from non-sugar compounds when glucose is not readily available. Gluconeogenesis may be an important source of carbohydrates building block monomers (glucose) for cell wall synthesis in *C. albicans* under certain conditions during infection (Eschrich *et al.*, 2002). Since gluconeogenesis and glycolysis are enzymatically reversible, they share most of the enzymes; only two key enzymes are specific to gluconeogenesis: fructose-1,6-bisphosphatase (FBP1) and phosphoenolpyruvate carboxykinase (PCK1). Gluconeogenesis might be crucial to *C. albicans* virulence as mutant

strains with deletion of either the genes encoding the key enzymes display reduced virulence (Barelle *et al.*, 2006; Ramirez & Lorenz, 2007). The regulation of gene expression involved in gluconeogenesis in *C. albicans* has been previously studied at both the transcriptional and proteomic levels (Eschrich *et al.*, 2002; Lorenz *et al.*, 2004; Barelle *et al.*, 2006; Kusch *et al.*, 2007; Ramirez & Lorenz, 2007).

2.2.2 Tricarboxylic acid (TCA) Cycle and Glyoxylate Cycle

The TCA cycle also known as the citric acid cycle or Krebs cycle, is one of the central metabolic pathways taken place in mitochondria of *C. albicans*. In the aerobic utilization of glucose, pyruvate generated by glycolysis is oxidatively decarboxylated into acetyl-CoA, which is completely oxidized to carbon dioxide and water in the TCA cycle. Besides, TCA cycle also generates intermediates for biosynthesis of amino acids, fatty acids, and for gluconeogenesis (Lorenz & Fink, 2002). The expression of TCA cycle enzymes and their enzymatic activities has been studied previously at the proteomic level in *C. albicans* (Kusch *et al.*, 2007).

The glyoxylate cycle is a modified TCA cycle that bypasses the carbon dioxide-generating steps to conserve carbons for gluconeogenesis, where they are incorporated into new molecules of glucose (Dunn *et al.*, 2009). In glyoxylate cycle, two-carbon compounds such as ethanol, lactate, and acetate

can be assimilated and incorporated into glucose via gluconeogenesis. Therefore, glyoxylate cycle enables growth of *C. albicans* under glucose-depleted conditions but two-carbon compounds are available as carbon sources. The key enzymes that specific to glyoxylate cycle are isocitrate lyase (ICL) and malate synthase (MS), which are functionally conserved in wide variety organisms across kingdoms, including fungi, plants, and bacteria, but not in mammals (Kondrashov *et al.*, 2006; Dunn *et al.*, 2009).

2.3 Pathogenicity of *C. albicans*

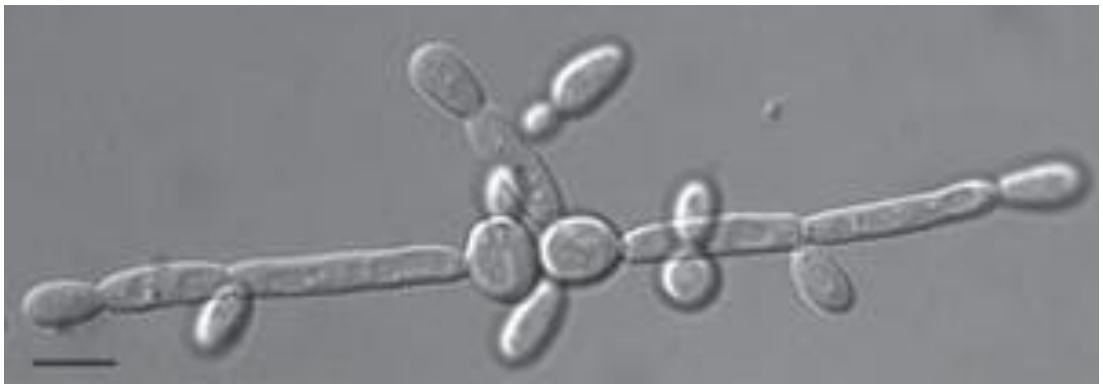
Candidiasis established by *C. albicans* are supported by combination of various pathogenicity mechanisms, which include virulence factors such as: polymorphism, adhesins and invasins, phenotypic switching, biofilm formation, hydrolytic enzymes secretion; and fitness attributes such as: rapid adaptation to environmental pH fluctuations, metabolic flexibility, and stress response (Nicholls *et al.*, 2011; Mayer *et al.*, 2013).

2.3.1 Polymorphism

C. albicans is a polymorphic yeast that can grows as unicellular budding yeasts, pseudo-hyphae, hyphae, white opaque cells, and chlamydospores depending on the environments it encounters (Sudbery *et al.*, 2004; Mayer *et al.*, 2013). The polymorphism of *C. albicans* is complex and dynamic, affected by wide variety of environmental factors such as pH, temperature, nutrient availability, and

quorum sensing molecules (Mayer *et al.*, 2013). The formation of pseudo-hyphae and hyphae by *C. albicans* from unicellular yeast (Figure 2.2) is affected by a numbers of factors, such as starvation, serum, temperature, carbon dioxide, and quorum sensing (Albuquerque & Casadevall, 2011; Sudbery, 2011).

Quorum sensing is the communication tool of *C. albicans* among themselves and with other microbes surrounding them in order to successfully survive in a niche (Hall *et al.*, 2011). The quorum-sensing molecules are secreted into the environment, generally to control processes that involve cell-cell interactions and the formation of multicellular structures such as hyphae and biofilm formation in *C. albicans* (Hogan, 2006). The well-studied quorum sensing molecules that influence the morphology of *C. albicans* include farnesol, tyrosol and dodecanol (Hornby *et al.*, 2001; Chen *et al.*, 2004; Hall *et al.*, 2011). Previous studies suggested that polymorphism of yeast to hyphal growths (Figure 2.2) are important for pathogenicity of *C. albicans* and potentially be antifungal drug targets (Jacobsen *et al.*, 2012). The hyphae form of *C. albicans* has been proven to be more invasive than that of the unicellular yeast from, which is believed to be involved in dissemination during infections (Berman & Sudbery, 2002; Jacobsen *et al.*, 2012).



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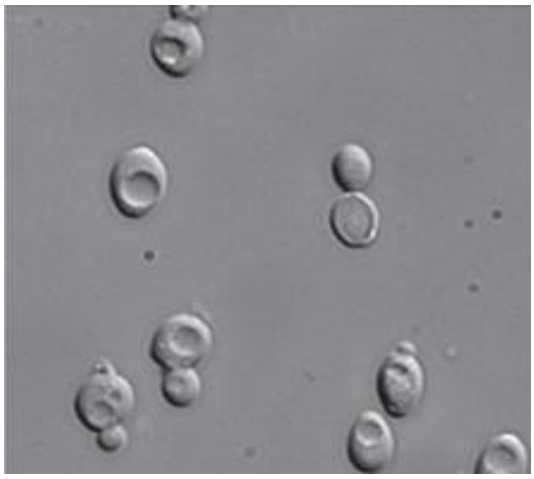

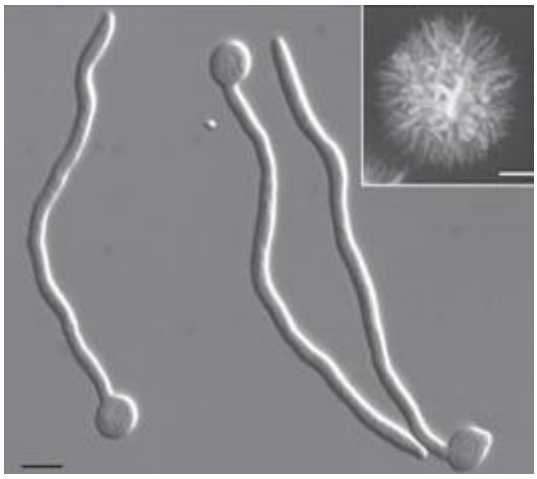

B C

Figure 2.2. Unicellular yeasts, pseudohyphae, and hyphae of *C. albicans*. A: pseudophyphae is resembling the true hyphae but have a constriction at the bud neck; B: unicellular yeasts appear similar to *Saccharomyces cerevisiae* diploid cells, some with budding daughter cells; and C: hyphae have a narrower and uniform filamentous germ tubes. Adapted from Sudbery (2011).

2.3.2 Adhesions and Invasions

The cell-cell adhesions among *C. albicans* cells, with other microorganisms and host cells, as well as to abiotic surfaces, are mediated by a set of cell surface glycoproteins, called adhesins (Garcia *et al.*, 2011). One of the best studied adhesins should be glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins, which are also among the most important adhesins that are up-regulated during various candidiasis (Sundstrom, 2002; Cheng *et al.*, 2005; Albrecht *et al.*, 2006; Naglik *et al.*, 2011). Besides, these adhesins were also shown to contribute to biofilm formation in *C. albicans* (Nobile *et al.*, 2008).

In terms of invasions, *C. albicans* employs two strategies: inducing endocytosis by the host cells, and active penetration, in order to invade the host cells and tissues (Wachtler *et al.*, 2012; Mayer *et al.*, 2013). For induced endocytosis, a set of cell surface proteins called invasins that bind to the host cell surface ligands such as E-cadherin and N-cadherin, are expressed by *C. albicans* cells to induce the engulfment by the host cells (Phan *et al.*, 2005; Wachtler *et al.*, 2012). There are two identified invasins: Als3 and Ssa1, which induce endocytosis by the host cells via binding to E-cadherin (Sun *et al.*, 2010).

In contrast, active penetration is initiated by hyphae of *C. albicans*, which penetrate by physical force into the host cells and tissues (Dalle *et al.*, 2010). The molecular mechanisms for active penetration is still remain to be fully

studied. However, it has been suggested that extracellular hydrolytic enzymes such as proteases and lipases play important roles to facilitate the active penetration into the host cells and tissues (Naglik *et al.*, 2003; Wachtler *et al.*, 2012).

2.3.3 Extracellular Hydrolytic Enzymes

Upon adhesion to host cell surfaces *C. albicans* secretes extracellular hydrolytic enzymes to enhance nutrient uptake and invasions by active penetration (Naglik *et al.*, 2003; Wachtler *et al.*, 2012). There are three types of extracellular hydrolytic enzymes being secreted by *C. albicans* during invasions: proteases, phospholipases, and lipases.

Secreted aspartyl proteases (SAPs) are extracellular proteases encoded by a gene family of ten members, namely SAP1 to SAP10; SAP1 to SAP8 are extracellular; whereas SAP9 and SAP10 are cell surface proteins (Naglik *et al.*, 2003; Taylor *et al.*, 2005; Albrecht *et al.*, 2006). These genes are differentially regulated depend on different growth stages of *C. albicans*-host interaction. However, the expression of other SAPs have been detected in various clinical infections, for instance expression of SAP7 has been detected in some clinical sample of human oral thrush, and SAP8 has been detected at high level in vaginal candidiasis (Naglik *et al.*, 2003).

Phospholipases of *C. albicans* consist of four different classes of proteins, namely A, B, C, and D, but only five members of class B are extracellular and involve in pathogenicity via disruption of host cell membranes (Mayer *et al.*, 2013). Mutant strains with deletion of any of these five genes have been shown to have attenuated virulence in mouse model invasive infection (Theiss *et al.*, 2006). Extracellular lipases of *C. albicans* is the least studied family of extracellular hydrolytic enzymes but the contribution of extracellular lipases to virulence has been proposed as they were observed to be differentially activated during the yeast-to-hyphae transition, in mouse model of invasive candidiasis, and from patient specimens (Hube *et al.*, 2000; Stehr *et al.*, 2004, Schofield *et al.*, 2005). Furthermore, extracellular lipases from *C. albicans* have been reported to exhibit cytotoxic effects on macrophages and liver cells via the production of reactive oxygen species (Paraje *et al.*, 2008; Paraje *et al.*, 2009).

2.3.4 Biofilm Formation

Many candidiasis established due to their ability to adhere to abiotic surface and form biofilm on implanted medical devices (Donlan & Costerton, 2002; Douglas, 2003). Biofilms is a unique form of growth for *C. albicans*, where the heterogeneous cells are encased in extracellular matrix (ECM) and shielded from host immunity and antimicrobial agents (Figure 2.3) (Chandra *et al.*, 2012).

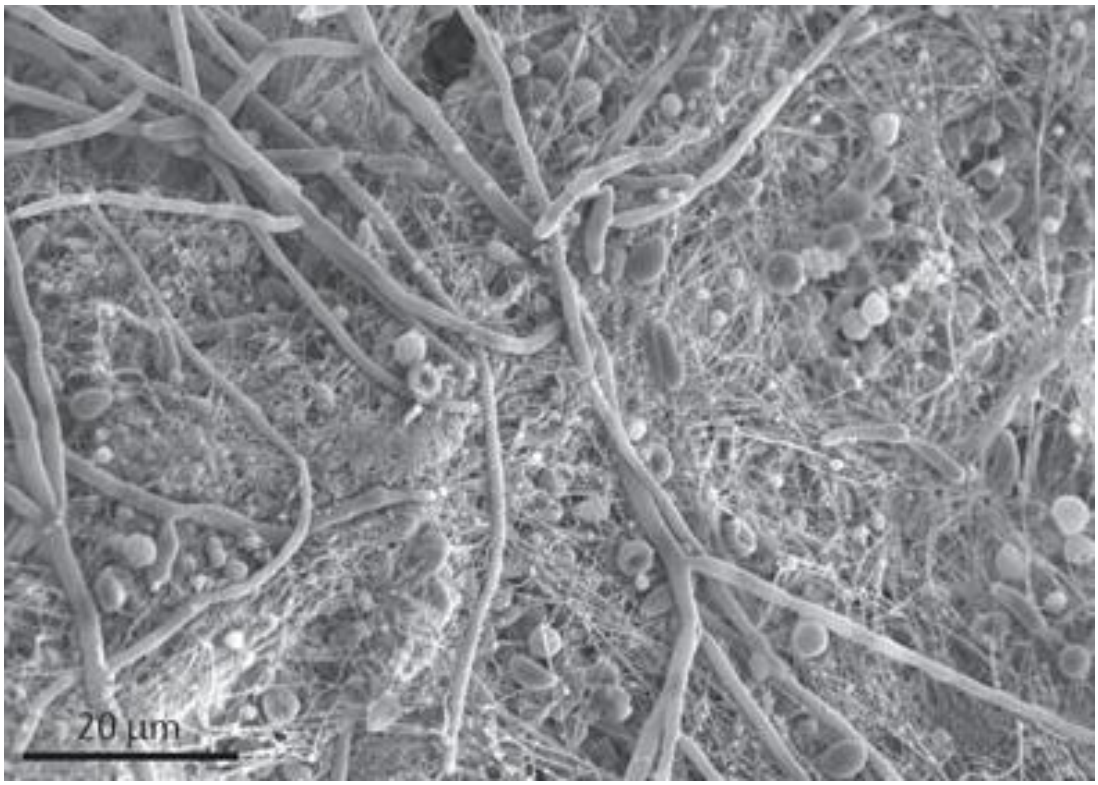

Figure 2.3. Scanning Electron Microscope (SEM) of an *in vivo C. albicans* biofilm. Unicellular yeasts, pseudohyphae, and hyphae are encased within the ECM in the biofilm. Adapted from Finkel & Mitchell (2011).

Implanted medical devices such as venous catheters, urinary catheters, pacemakers, and artificial joints, are commonly used with more than ten million recipients worldwide each year (Kojic & Darouiche, 2004; Taff *et al.*, 2012). Medical device-associated candidiasis are difficult to be eradicated, and like bacterial biofilms, *Candida* biofilms are highly resistant to antifungal drugs compared to their planktonic counterpart, even without accumulation of drug-resistance genes (Nett *et al.*, 2011; Taff *et al.*, 2012). Therefore, surgical replacement of implanted devices is mostly the only option for treatment of medical device-associated candidiasis.

Biofilm formation of *C. albicans* starts when the planktonic yeast cells attach to virtually any abiotic surfaces (implanted medical devices), followed by proliferation and mixed growth of unicellular yeast cells, pseudohyphae, and hyphae across the surface, ECM accumulates and shields the encased cells from host immunity attacks and high level of drug resistance is acquired as the biofilm matures (Figure 2.4) (Finkel & Mitchell, 2011). One important feature of *Candida* biofilm is ECM, which encases the yeast cells together, controlling disaggregation, and shields the yeast cells from antifungal drugs and host immune system (Taff *et al.*, 2012). The main component of ECM in *Candida* biofilm is β-1,3-glucan, where the genes related to glucan synthesis such as FKS1 (glucan synthase gene), are up-regulated during formation of biofilms (Nett *et al.*, 2007).

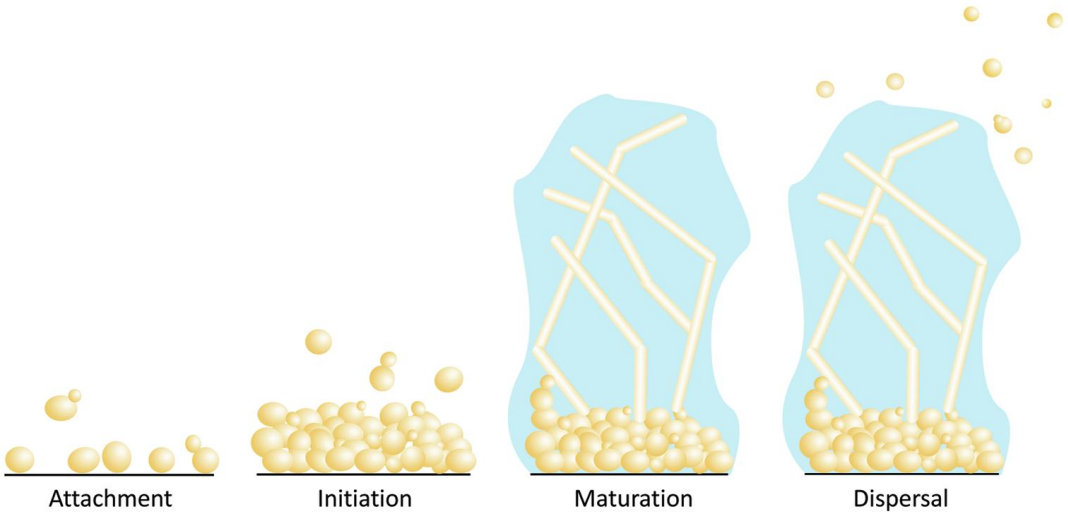

Figure 2.4. Biofilm development of *C. albicans*. Attachment: unicellular yeast cells adhere to abiotic surface; initiation: cell-cell adhesion and proliferation; maturation: mixed growth of yeast cells, pseudohyphae, and hyphae in ECM; dispersal: some of the cells detach from biofilm and migrate to new locations. Adapted from Fox & Nobile (2012).

Another important feature of *C. albicans* biofilm that contributes to resistance and recurrent candidiasis is presence of a population fraction called persister cells (Fauvart *et al.*, 2011). Persister cells are population fraction in the biofilm that are metabolically dormant and highly resistant to antifungals (Lewis, 2010). As described in Figure 2.5, antifungal drugs and host immune system kills most of the encased cells in the biofilm, except the persister cells which repopulate the biofilm with new population fraction of persister cells after discontinuation of antifungal therapy (Al-Dhaheri & Douglas, 2008).

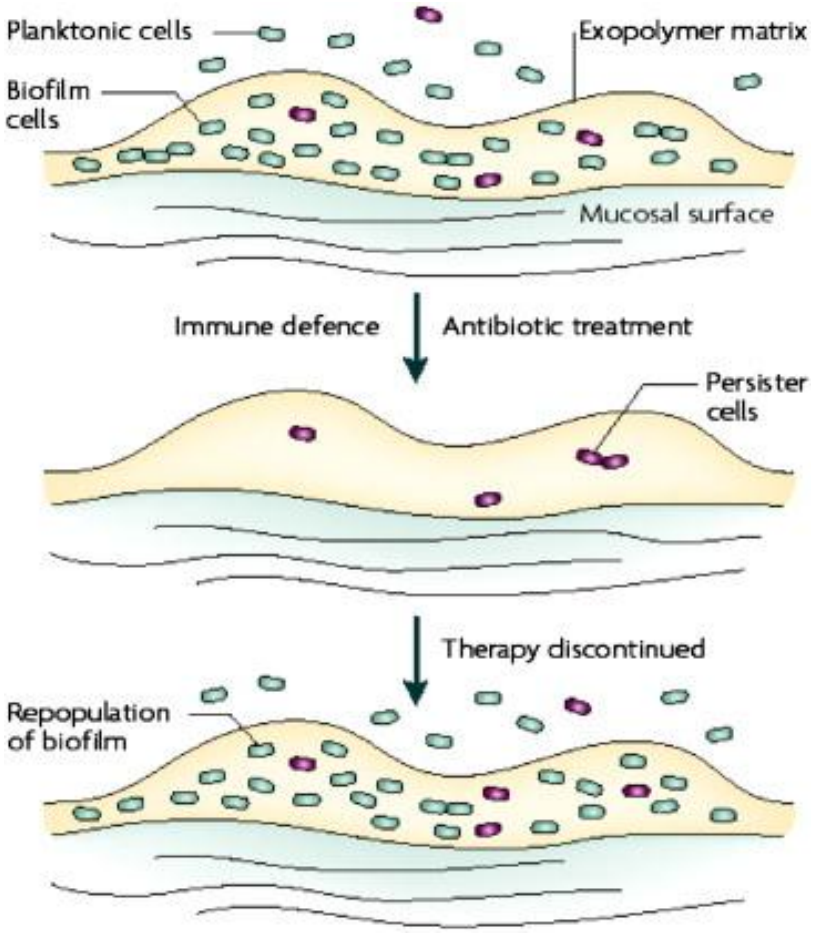

Figure 2.5. *C. albicans* biofilm and persister cells. Antimicrobial therapy and host immune defence system eliminates almost all the cells in the biofilm except persister cells which are highly resistance. The persister cells exit from dormancy and repopulate the biofilm after the therapy discontinued. Adapted from Lewis (2010).

2.3.5 Metabolic Flexibility

Carbon is central and fundamental element for survival and growth of all living things. The carbon sources for *C. albicans* in host are likely to be glucose, lipids, proteins, amino acids and other alternative carbon sources. In order to survive and establish candidiasis, *C. albicans* must be able to utilize these carbon sources and adapt rapidly to changes in nutrient availability (Mayer *et al.*, 2013). For instance, *C. albicans* in blood stream can utilize glucose, which is rich in the blood via glycolysis and TCA cycles, but the cells engulfed by phagocytic cells would suffer from starvation due to glucose-depletion environment inside the macrophages (Brock, 2009).

In response to glucose-depletion, *C. albicans* initially switches from glycolysis to gluconeogenesis, and activation of glyoxylate cycle in order to utilize lipids and amino acids available inside the phagocytic cells (Lorenz *et al.*, 2004). In other host niches, *C. albicans* also often encountered glucose-depletion niches and utilized host proteins, amino acids, lipids, and phospholipids as alternative carbon sources via glyoxylate cycle. It was previously shown that metabolic plasticity of *C. albicans* is not only crucial for survival and growth, but also affect pathogenicity (Brown, 2006; Ene *et al.*, 2012; Mayer *et al.*, 2013).

2.4 Current Drugs for Candidiasis

The antifungal therapy for candidiasis varies substantially depends on the *Candida* species, clinical situation of the patients, and the susceptibility of the isolate to specific antifungal drugs (Pappas *et al.*, 2009). Currently approved antifungal drugs for the treatment of candidiasis generally comprises four major categories: the polyenes (amphotericin B), the azoles (fluconazole, itraconazole, voriconazole, and posaconazole), the echinocandins (caspofungin, anidulafungin, and micafungin), and flucytosine. The current antifungal drugs for candidiasis and their mechanisms are summarized in Table 2.1.