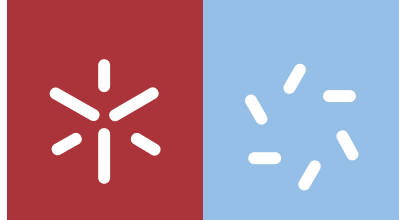


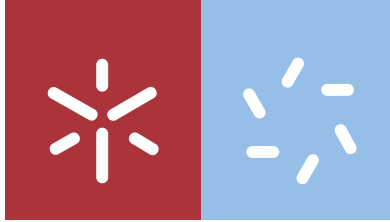


Ana Rita Santos Nunes

Molecular targets underlying benzo[a]phenoxazines antifungal activity: the role of ergosterol metabolism and endoplasmic reticulum stress

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benzo[a]phenoxazines antifungal activity:
the role of ergosterol metabolism and
endoplasmic reticulum stress**

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Trabalho efetuado sob a orientação de
**Professora Doutora Maria João Marques Ferreira Sousa
Moreira**
Professora Doutora Maria do Sameiro Torres Gonçalves

Abril de 2019

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Alvos moleculares subjacentes à ação antifúngica das benzo[*a*]fenoxazinas: o papel do metabolismo do ergosterol e do stress do retículo endoplasmático

Resumo

Com o passar dos anos, as infecções fúngicas têm-se tornado uma questão cada vez mais relevante, particularmente devido ao desenvolvimento de resistência contra as drogas antifúngicas em uso.

Recentemente, o nosso grupo tem contribuído para a elucidação do mecanismo de ação de um grupo de compostos químicos com atividade anti-proliferativa, as fenoxazinas e os seus derivados, nomeadamente as benzo[*a*]fenoxazinas. Trabalhos anteriores do nosso laboratório têm-nos fornecido um vasto conhecimento acerca do mecanismo de ação da benzo[*a*]fenoxazina BaP1. O BaP1 é capaz de induzir um processo de morte celular regulada, mediado pela permeabilização do vacúolo. As potencialidades exibidas por este composto, levaram o nosso laboratório a iniciar os estudos com um composto semelhante, o C9, que apenas difere do BaP1 numa substituição do sistema aromático nuclear da benzo[*a*]fenoxazina. Esta pequena diferença estrutural, contudo, teve um grande efeito na atividade do composto, uma vez que o C9 é um agente anti-proliferativo mais potente do que o BaP1. As primeiras abordagens mostram que este pode atuar de uma forma diferente do BaP1. Trabalhos desenvolvidos anteriormente no nosso laboratório já tinham demonstrado que este composto acumula não só na membrana vacuolar, mas maioritariamente na membrana do retículo endoplasmático. Tal como o BaP1, o C9 é capaz de induzir um processo de morte regulada, apesar de, no caso do C9, haver também o envolvimento mitocondrial. Não obstante, o(s) alvo/alvos deste composto continua/continuam por descobrir. De forma a contribuir para a elucidação dos alvos do C9 e, tendo em conta que o seu principal local de acumulação na célula é a membrana do retículo endoplasmático, colocamos a hipótese de que o metabolismo do ergosterol, nomeadamente, a sua via de biossíntese, pode estar envolvido no modo de ação do C9. Contrariamente ao que foi inicialmente proposto, observou-se que uma suplementação externa de ergosterol no meio durante o tratamento com o C9 poderá ter um papel efetor nos efeitos nocivos do C9 no crescimento celular, não tendo, no entanto, qualquer efeito na viabilidade das mesmas. Finalmente, mostramos que o mecanismo de ação do C9 não é mediado pelo stresse do retículo endoplasmático nem pela depleção do ergosterol membranar.

Palavras chave: antifúngico, benzo[*a*]fenoxazina; ergosterol; retículo endoplasmático; stress do retículo endoplasmático

Molecular targets underlying benzo[a]phenoxazines antifungal activity: the role of ergosterol metabolism and endoplasmic reticulum stress

Abstract

Over the years, fungal infections have become a great source of concern, especially due the development of resistance against antifungal drugs. In line with this, the necessity to develop new antimicrobial drugs that can maintain their potency and have a broad spectrum of targets arises.

Recently, our group has been making efforts to unravel the mechanism of action of a group of chemical compounds with antiproliferative activity, phenoxazines and their derivatives, namely benzo[a]phenoxazines. Previous work in our laboratory has provided us a vast knowledge regarding the mechanism of action of a benzo[a]phenoxazine called BaP1. This compound is able to induce a regulated cell death process, mediated by the permeabilization of the vacuole. Such an interesting information lead our laboratory to begin the studies on a similar compound called C9, that only differs from BaP1 on a substitution of the core benzo[a]phenoxazine aromatic system. Remarkably, this small structural difference has a big effect on the action of the compound, since C9 is a much more potent antiproliferative agent than BaP1. The first approaches showed that it may act in a different manner than BaP1. It has already been demonstrated that this compound accumulates not only in the vacuolar membrane, but mostly in the endoplasmic reticulum membrane. Like BaP1, C9 is able to induce a regulated cell death process, although in the case of C9 there is also a mitochondrial involvement. Nonetheless, the target/targets of this compound is/are still unknown.

Aiming to fully elucidate this compound's target(s), and bearing in mind that its main site of accumulation in the cell is the endoplasmic reticulum membrane, we hypothesized that ergosterol metabolism, namely ergosterol biosynthetic pathway, could be involved in this compound's antifungal action. Contrarily to what we first purposed, we observed that an external supplementation of ergosterol to the medium after C9's treatment may have an effector role on C9's deleterious effects on cell growth, while being harmless to the cell's viability. Finally, we demonstrated that C9's mechanism of action is not mediated by ER stress, nor through membrane ergosterol's deletion.

Keywords: antifungal; benzo[a]phenoxazine; endoplasmic reticulum; endoplasmic reticulum stress; ergosterol

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List of abbreviations and acronyms

Acetyl-CoA	Acetyl Coenzyme A
ANOVA	A nalysis of V ariance
ATP	A denosine T ri p hosphate
AUS1	A BC protein involved in U ptake of S terols 1
CFU	C olony F orming U nits
DIC	D ifferential I nterferential C ontrast
DMSO	D imethyl S ulfoxide
ECM22	E xtra C ellular M utant 22
ER	E ndoplasmic R eticulum
ERAD	ER - A ssociated D egradation
EtOH	E thanol
FPP	F arnesyl p yro p hosphate synthase
GFP	G reen F luorescent P rotein
GPI	G lycosyl p hosphatidyl i nositol
HMG-CoA	H ydroxymethylglutaryl- C o A
INM	I nnner N uclear M embrane
INS	I nter- N uclear Membrane S pace
MβCD	M ethyl- β -cyclodextrin
NADPH	N icotinamide a denine d inucleotide p hosphate
NE	N uclear E nvelope
O.D.	O ptical D ensity
ONM	O uter N uclear M embrane
PBS	P hosphate B uffer S aline
PDR11	P leiotropic D rug R esistance 11
RPM	R otations P er M inute
RT	R oom T emperature
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SD	S tandard D eviation
SRE	S terol R egulatory E lement

UPC2	Sterol U ptake C ontrol 2
WT	W ild T ype
YEPD	Y east E xtract P eptone D extrose
YEPDA	Y east E xtract P eptone D extrose A gar

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1. Introduction

1.1 Ergosterol, the major fungal sterol

Sterols are a class of lipids widely distributed in nature being important constituents of eukaryotic cell membranes and having an essential role on both their organization and function (Dupont *et al.*, 2012; Hu *et al.*, 2017)). There are three predominant forms of sterols found in eukaryotes: mammalian sterols, phytosterols and fungal sterols. In the case of mammalian sterols, cholesterol is often the main one, while phytosterols essentially include stigmasterol, sitosterol and campesterol. On the other hand, ergosterol is the predominant sterol found in fungi (Hannich, Umebayashi, & Riezman, 2011; Hu *et al.*, 2017). This fungal sterol is an important component of the fungal cell membrane and presents a variety of cellular functions. In structural terms, ergosterol binds to membrane phospholipids and modulates the thickness, fluidity and permeability of the membrane itself (Dupont *et al.*, 2012; Rodriguez *et al.*, 1985; Zhang & Rao, 2010; Zweytick *et al.*, 2000). Ergosterol is also able to promote the growth and proliferation of fungi, thus being considered a “fungal hormone” (Wangspa & Takemoto, 1998). Besides, ergosterol is also important in other fundamental processes such as cytoskeleton organization, mating, endocytosis, sporulation, among others (Kodedová & Sychrová, 2015; Zhang *et al.*, 2010). Moreover, it seems to be a key participant in yeast adaptation to a variety of environmental stresses. Ethanol, one of the main alcoholic fermentation products, can negatively impact many physiological mechanisms in yeast, namely their growth, viability, metabolism, transport processes, enzymatic activity, among several others (Aguilera *et al.*, 2006; Stanley *et al.*, 2010). Nevertheless, yeast have evolved to become more resistant to its toxicity. For instance, it has been shown that under an alcoholic environment, *S. cerevisiae* strains are able to increase the content of ergosterol in their cellular membranes, which minimizes membrane damage, maintains membrane permeability at a basal level and increases the fluidity of the membrane (Aguilera *et al.*, 2006; Vanegas *et al.*, 2012). Moreover, studies revealed that an increased content of ergosterol is involved in yeast resistance to freezing (Janković *et al.*, 2015; Villarreal *et al.*, 2018).

Besides being important for many cellular routines and responses, ergosterol has also a great value from the pharmaceutical industry’s point of view since it is a precursor of vitamin D2 and steroid hormone drugs, such as cortisone and progesterone (Francavilla *et al.*, 2013; Huang, Cai, & Xu, 2016; Karpova *et al.*, 2016).

1.1.1 Biosynthesis of ergosterol, its regulation and importance

The biosynthetic pathway that ultimately leads to the production of ergosterol is an energy-consuming process since it takes at least twenty-four ATP and sixteen NADPH molecules to produce this sterol. Interestingly, despite being a pathway that occurs in lower eukaryotes this process requires more energy than the biosynthetic process of mammalian cholesterol (Hu *et al.*, 2017; Kodedová & Sychrová, 2015). It is also an oxygen-requiring process. It is a complex pathway involving the participation of variety of enzymes (almost 30) known as Erg proteins, which, in turn, are encoded by *ERG* genes. Considering the intermediate products, this pathway can be roughly divided into three modules that involve numerous enzymatic steps and occur in the endoplasmic reticulum (ER) (Figure 1.1) (Alvarez-Vasquez *et al.*, 2011; Kodedová & Sychrová, 2015). The first module is conserved among all eukaryotes and it comprises the production of mevalonate from three molecules of acetyl-CoA, which is achieved by the enzymatic action of acetoacetyl-CoA thiolase, encoded by *ERG10* gene, hydroxymethylglutaryl-CoA synthase, encoded by the *ERG13* gene and the HMG-CoA reductases, encoded by the *HMG1* and *HMG2* genes (Hayakawa *et al.*, 2017; Lv *et al.*, 2016; Yuan & Ching, 2014). During the second section of the pathway, mevalonate is used to synthesize farnesyl pyrophosphate, which is a significant intermediate metabolite that participates in various metabolic pathways and in the production of numerous substances in the cells. This section involves the action of enzymes such as mevalonate kinase (Erg12p), phosphomevalonate kinase (Erg8p), mevalonate pyrophosphate decarboxylase (Erg19p), IPP isomerase and geranyl/FPP synthase (Erg20p) (Hu *et al.*, 2017). Finally, the third and last module comprises the synthesis of ergosterol from farnesyl pyrophosphate. It is a more complex part of the pathway, requiring enzymes such as squalene synthase (Erg9p) and squalene epoxidase (Erg1p), the latter being an enzyme that catalyses the conversion of squalene to squalene epoxide, in an oxygen-dependent reaction. It is an important step, leading to the synthesis of the first sterol-like structure that, upon cyclization catalysed by lanosterol synthase/oxidosqualene-lanosterol cyclase (Erg7p), results in the formation of lanosterol, the first sterol to appear during the whole biosynthetic process.

Afterwards, lanosterol is converted in ergosterol through a series of reactions that involve enzymes such as cytochrome P450-dependent lanosterol 14 α -demethylase (Erg11p), C-24 sterol methyltransferase (Erg6p), C24 (28) sterol reductase (Erg4p), among others (Klug & Daum, 2014).

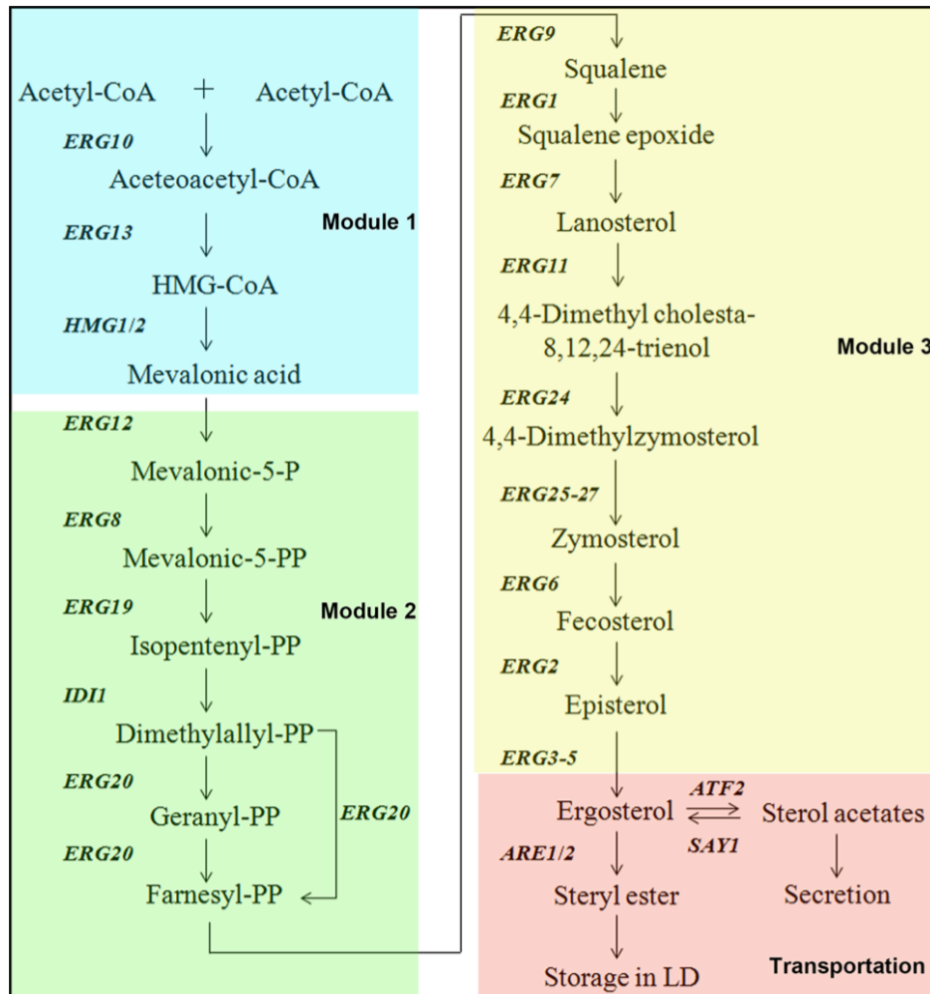


Figure 1.1.1-1: Schematic representation of the ergosterol biosynthetic pathway in *S. cerevisiae* (Zeng *et al.*, 2017)

As referred above, a variety of enzymes (almost 30) take part in this complex pathway. These proteins are encoded by genes that can either be essential or non-essential genes, depending on whether their function is or not required for yeast survival. For instance, *ERG9* and *ERG7* are two examples of essential genes (Hu *et al.*, 2017; Klug & Daum, 2014). Nonetheless, even though the non-essential genes are not critical to yeast survival, their absence can impair cellular processes. Deletion strains for non-essential genes can display a disrupted ergosterol biosynthesis, which can impair many basic mechanisms. For instance, previous studies have already demonstrated that mutants in *ERG6*, a non-essential gene, exhibit a slow growth as well as an impairment in membrane integrity, low tolerance to hyperosmotic stress, an

increased susceptibility to drugs, compromised tryptophan transport, among others (Kodedová & Sychrová, 2015; Parks & Casey, 2003). Another study reports abnormal mitochondrial structure and respiratory inability in strains harbouring deletions in the ergosterol pathway genes (Dimmer, 2002). On the other hand, deletions of ergosterol genes can also increase the resistance of the strains to therapeutically relevant antifungals (Bhattacharya, Esquivel, & White, 2018).

As mentioned before, ergosterol plays a role in a variety of cell functions and for each function, a specific ergosterol concentration is required. Thus, ergosterol levels must be rigorously regulated so that these cellular functions can occur but also to avoid accumulation of free sterols, which presents a source of toxicity to the cell. This regulation occurs mainly through feedback mechanisms at transcriptional, translational and post-translational levels (Klug & Daum, 2014). For instance, in conditions of excess of sterols, the degradation of HMG-CoA reductase, an enzyme participating in the ergosterol biosynthetic pathway, is induced, decreasing mevalonate synthesis and consequently leading to the down-regulation of sterol synthesis. This degradation occurs via the proteasome degradation pathway, mediated by an endoplasmic reticulum-associated degradation (ERAD) pathway, that will be discussed further on (Hu *et al.*, 2017). On the other hand, oxygen levels have also a regulatory role since ergosterol synthesis requires oxygen in order to occur. Under conditions of low oxygen, they can either try to increase its synthesis or they can uptake it from the external environment. Thus, in some cases, a transcriptional up-regulation of ergosterol pathway genes is induced when oxygen levels are low or reduced. In *S. cerevisiae*, two transcriptional factors called Upc2 and Ecm22 bind to the sterol regulatory element (SRE), activating the expression of ergosterol synthesis enzymes (Davies & Rine, 2006; Espenshade & Hughes, 2007; Yang *et al.*, 2015). In other cases, when external sterol uptake is needed, Upc2 is also necessary, as it induces the expression of *AUS1* and *PDR11* genes, that code for ATP-binding transporters, important for the uptake of exogenous sterols (Yang *et al.*, 2015; Zavrel, Hoot, & White, 2013).

Even with a diverse regulatory machinery, intracellular sterols can sometimes reach excessive levels under aerobic conditions and this poses a problem, since yeasts cannot degrade sterols. Thus, how can yeast cells maintain sterols at homeostatic levels, overcoming the toxic effects that arises from excessive intracellular sterols? They do it through the conversion of sterols to steryl esters, that can be stored in cytosolic lipid droplets. Moreover, they can also secrete the excess sterols as sterol acetates into the medium, which contributes not only for the detoxification but also to the removal of damaged lipids (Choudhary & Schneider, 2012; Klug & Daum, 2014; Kohlwein, 2017).

As already demonstrated, ergosterol plays a fundamental role in the overall growth and viability of yeast cells, as it is a major constituent of their cellular membrane. This turns ergosterol and its

biosynthetic pathway into an attractive target of antifungal drugs that are currently employed in the treatment of severe or not so severe fungal infections. The main antifungal drugs are sorted into three classes, namely polyenes, allylamines and azoles, that either target ergosterol itself or the enzymes that participate in its biosynthesis process (Alcazar-Fuoli & Mellado, 2013). For instance, the azoles will affect the activity of cytochrome P450-dependent lanosterol 14 α -demethylase (Erg11p), essential for the biosynthesis of ergosterol (Mast *et al.*, 2013), while the allylamines inhibit squalene epoxidase (Erg1p) (Mazu *et al.*, 2016). Regarding polyenes, they act through a different mechanism as they are able to bind to ergosterol present in the fungal membrane. Afterwards, they induce the formation of hydrophilic channels that will modify cell membrane permeability, leading to the leakage of intracellular contents to the extracellular medium (Pan, Hu, & Yu, 2018; Serhan *et al.*, 2014).

1.2 Endoplasmic reticulum in yeast

The endoplasmic reticulum (ER) is considered a very important structure in eukaryotic cells. It is a very large, membrane-bound and dynamic organelle (English & Voeltz, 2013; Schwarz & Blower, 2016). It possesses an extremely complex structure that is morphologically divided into two distinct structural domains, the nuclear envelope (NE) and the peripheral ER, which in yeast is localized near the plasma membrane, in a region called the cortex, being referred to as the cortical ER (Austriaco, 2012). Each of these sub-structures is involved in a specific function or functions. Two lipid bilayers constitute the NE, the inner nuclear membrane (INM) and the outer nuclear membrane (ONM). These two lipid bilayers are stacked over each other, separated by the inter-nuclear membrane space (INS) and they surround the nucleus, controlling the diffusion of molecules into and out of it. The two membranes merge at several points called nuclear pores and this is where the molecules can pass, whether they are coming in or out of the nucleus (De Magistris & Antonin, 2018; English & Voeltz, 2013). The peripheral ER is defined by an interconnected network of flat cisternal sheets and branched dynamic tubules, with a single lumen. Sheets consist of two closely apposed, moderately flat lipid bilayers with low membrane curvature. On the cytosolic flank, these sheets are defined by a high density of membrane bound ribosomes, which classifies them as rough sheets. The abundance of ribosomes in this cytosolic surface is easily explained by the fact that the synthesis, folding and post-translational modifications of secreted or membrane-bound proteins occurs mainly at this site. On the other side, ER tubules are elongated cylindrical units with a high membrane curvature in cross section. Contrarily to ER sheets, ER tubules possess fewer membrane

bound ribosomes, having a smoother membrane (Schwarz & Blower, 2016; Shibata *et al.*, 2010; Shibata, Voeltz, & Rapoport, 2006).

ER's architecture can suffer severe modifications in response to distinct cellular demands or conditions. The morphology and distribution of each of these ER domains is strictly regulated by integral membrane proteins. For instance, it has been shown that the shape of the lipid bilayer of ER tubules is regulated by two families of proteins, the reticulons (Rtns) and DP1/Yop1 proteins, and this is characteristic of many eukaryotes such as plants, animals and yeast (West *et al.*, 2011). On the other side, the mechanisms through which ER sheets are shaped are not fully understood. Some mechanisms have already been proposed and have been gaining acceptance. For instance, it seems that polyribosomes can bind the ER sheets membrane, flattening and stabilizing it (Shibata *et al.*, 2010). Climp63 is an integral ER membrane protein that also plays a role in shaping ER cisternal sheets as it maintains the interluminal space between them (Sandoz & van der Goot, 2015). Cell's cytoskeleton also shapes ER domains (Schwarz & Blower, 2016; Westrate *et al.*, 2015; Zhang & Hu, 2016).

This complex organelle is involved in a plethora of cellular processes, such as protein synthesis, folding and transport, regulation of calcium storage, lipids and steroid synthesis (Schwarz & Blower, 2016). Even though these functions are associated with this organelle in all eukaryotes, this present work will explore them in more detail through a yeast's perspective. One of its main functions is to harbour the synthesis of proteins for secretion, integration in the plasma membrane, as well as some cytosolic proteins. Ribosomes play a crucial role as they are localized to the cytosolic side of the ER upon protein synthesis and translation (Smith & Wilkinson, 2017). Afterwards, these ribosomes containing the mRNAs that are being translated, are recruited to the ER membrane and this occurs due to a signal sequence in the terminal part of the forming polypeptide that is recognized and bound by the signal recognition particle (SRP). After the signal recognition, this complex is targeted to the ER by anchoring on the SRP receptor. Next, translation proceeds and the nascent polypeptide can co-translationally enter the ER through the Sec61p translocon, a translocation pore complex on the ER membrane.(Brodsky & Morrow, 2010; Demirci *et al.*, 2013; Mandon, Trueman, & Gilmore, 2013). Generally, during translation process or even after it is finished, signal peptidase cleaves the signal sequence, which allows the free protein to go into the ER lumen (Naidoo, 2011). The destination and function of the proteins after they are synthesized is subsequently determined. If the newly formed protein is meant to function as an integral membrane protein, translocation will stop as the protein needs to be anchored within the membrane bilayer (High & Laird, 1997). On the other hand, if the protein is meant to enter the secretory pathway or go into the lumen of other organelles, the process of transport to the ER lumen continues and the protein can suffer

a series of modifications such as disulphide bond formation, oligomerization and N-linked glycosylation, but also folding actions, for which chaperones and folding enzymes are necessary. Molecular chaperones Hsp40p (J-proteins) and Hsp70p, protein disulphide isomerases (PDI) and oligosaccharyltransferases (OST) are among the group of proteins necessary for all of this protein alterations to occur (Aebi, 2013; Kleizen & Braakman, 2004; Schwarz & Blower, 2016). After suffering the proper alterations, the protein must travel through the Golgi apparatus through COPII transport vesicles where it will be sorted between various destinations depending on its function (Austriaco, 2012; Feyder *et al.*, 2015; Suda, Kurokawa, & Nakano, 2018). ER is also crucial for the synthesis of membrane lipids such as sterols, phospholipids and sphingolipids, as it is enriched in enzymes that participate in these biosynthetic pathways. As previously referred, biosynthesis of ergosterol takes place in the ER (Austriaco, 2012; Saheki & De Camilli, 2017). Another important aspect about this organelle is that it is a main depository of a well-known signalling molecule, calcium (Ca^{2+}). Moreover, it can also act as an intracellular source of Ca^{2+} ions, as they can be released through various calcium and calcium-related channels that exist in the ER and mobilized for various cellular processes (Liu, 2012; Schwarz & Blower, 2016).

1.2.1 Endoplasmic reticulum stress

As discussed above, protein synthesis is a main process occurring in the ER. Many molecules have their role in this complex process to ensure that a protein receives the right modifications and is properly folded. The concept of “quality control” is suitable to describe the control mechanism that operates on this process and is, indeed, what keeps protein synthesis in the ER at homeostatic levels. When a protein becomes misfolded or unfolded and cannot be re-folded in the correct way, it has to be targeted for degradation (Thibault & Ng, 2012). Nevertheless, alterations at a genetic level or environmental changes are amongst the factors that may negatively influence ER “quality control” (chaperones, folding enzymes, among others), making it unable to cope with an increasing occurrence of protein misfolding (Mizuno, Masuda, & Irie, 2015). This leads to misfolded proteins accumulation, contributing to ER stress, which is extremely harmful for the cell. Thus, eukaryotic cells have developed pathways that act to eliminate the stress and restore ER homeostasis. Either the cells decide to degrade the misfolded protein, employing, for this matter, the ER-associated degradation (ERAD) or they try to handle with the misfolded protein, employing the unfold protein response (UPR) (Brodsky & Skach, 2011).

In the ERAD pathway, molecular chaperones and other ER-resident proteins recognize the defective proteins, which are then conducted from the ER to the cytoplasm. Next, these proteins are poly-ubiquitinated in the cytoplasm, being then recognized and degraded by a large protease called the proteasome, a system usually referred to as ubiquitin-proteasome system. In yeast, the ubiquitylation is mainly carried out by two ubiquitin-ligases that exist in the ER membrane, Doa10 and Hrd1. Depending on the type of proteins that are going to be degraded, one of three sub-pathways is employed, ERAD-L, meant for misfolded ER-luminal proteins, ERAD-M, for proteins harbouring a misfolded membrane domains and ERAD-C, meant for proteins with a misfolded cytosolic domain (Berner, Reutter, & Wolf, 2018; Finley, 2009; Kaneko *et al.*, 2017; Zattas *et al.*, 2016).

Another cascade of events activated upon ER stress is, as mentioned above, the UPR. There are three independent branches that can sense ER stress in eukaryotes, but in yeast only one of them is found, the inositol-requiring enzyme-1 (Ire1) pathway (Wu, Ng, & Thibault, 2014). The accumulation of misfolded proteins in the ER or the release of a molecular chaperone called BiP triggers the oligomerization and autophosphorylation of a transmembrane kinase/nuclease protein Ire1 (Ire1p). Then, Ire1p will activate its RNase domain, that will, in turn, trigger the splicing of *HAC1* mRNA, that upon translation and synthesis gives rise to Hac1 protein (Hac1p). Hac1p is a transcription factor that will translocate into the nucleus to up-regulate the transcription of target genes of the UPR pathway, such as lipid biosynthesis enzymes or ER chaperones (Cox *et al.*, 2011; Hou *et al.*, 2014). The Ire1p pathway is represented in Figure 1.2.1.

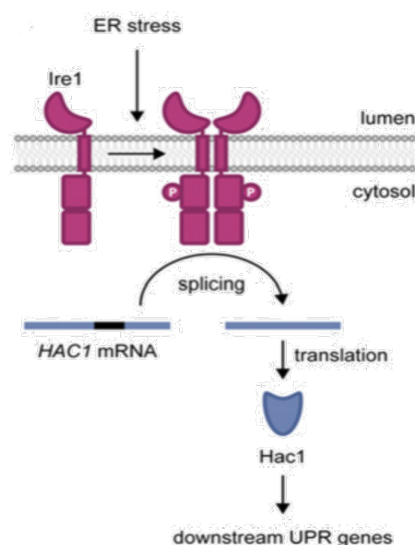


Figure 1.2.1-1: Schematic representation of the Ire1p pathway in yeast. Upon activation by ER stress, Ire1p suffers autophosphorylation and oligomerization. Then Ire1p will activate its RNase domain that will, in turn, induce the splicing of *HAC1* mRNA. Upon translation, this mRNA gives rise to Hac1p, a transcription factor that will translocate into the nucleus to up-regulate the transcription of target genes of the UPR pathway. Adapted from (Wu and Thibault, 2014).

It is, however, important to underline that the activation of the UPR by the molecular chaperone BiP is still a matter of debate, since there are reports stating that Ire1p is, in fact, activated by the release of BiP from its luminal domain. On the other hand, there are also reports stating that the interaction between BiP and Ire1 protein is not required to activate Ire1.(Wu *et al.*, 2014).

There is still another sub-pathway of the UPR called the cytosolic UPR. It is a more specific route meant for misfolded membrane or cytosolic proteins that are not proteins of the secretory pathway. This pathway induces a subcategory of heat shock response (HSR) target genes. Upon ER stress, the transcription factor Hsf1p up-regulates the HSR genes, involved in protein folding and cell wall organization, among others (Hou *et al.*, 2014).

1.3 Phenoxazines, benzo[a]phenoxazines and their derivatives

In life sciences, organic fluorophores are often useful tools that help to visualize compartments in cells or tissues or even to unravel the mechanisms underlying many biological processes. Over the past years, biotechnology has been evolving and more complex techniques are emerging, leading to a higher demand of labelling compounds that can cope with these emerging methods. In line with this, many researchers have dedicated their time to the design and synthesis of new organic fluorophores that can be useful in techniques such as DNA sequencing, proteomics or genetic analysis (Leitão *et al.*, 2014; Waggoner, 2006).

Cationic polycyclic benzo[a]phenoxazines and their derivatives are among those fluorescence markers. They are fluorescent oxazine dyes that absorb and emit at long wavelength light, in the 600-900 nm region of the spectrum. They can have a variety of biological applications since they have a low background interference from biological material and a high sensitivity (Barros *et al.*, 2006). Applications of these compounds include the covalent labelling of amino acids (Frade *et al.*, 2007), proteins (Salomi, Mitra, & Gorton, 2005) or DNA (Raju *et al.*, 2012). Besides, these heterocycles are also used in the non-covalent labelling of nucleic acids in a variety of contexts (Leitão *et al.*, 2016) or monitoring protein alterations (Nakanishi *et al.*, 2001).

Moreover, these oxazine heterocycles, such as benzo[a]phenoxazines and their derivatives have received great attention from the life sciences research community since they have important functions as antiproliferative agents, with potential uses both as antitumor and antimicrobial drugs (Abe, Yamane, & Tomoda, 2001; McLuckie *et al.*, 2011; Shimizu *et al.*, 2004; Verma *et al.*, 2009). Moreover, a variety of research studies show that these compounds indeed possess some important activities such as

antifungal (Frade *et al.*, 2008; Leitão *et al.*, 2016), anti-inflammatory (Silva *et al.*, 2004), antiviral (Hayashi *et al.*, 2010), among others.

The antiproliferative activity of these compounds is thought to potentially result from a strong interaction with DNA in the target cell, degrading the structure of the nucleic acids and compromising their biological functions. Specifically, their planar polycycles intercalate between DNA base pairs. Hydrogen bonds and π - π stacking interactions help to stabilize this binding (Frade *et al.*, 2008). Furthermore, these compounds may undergo enzymatic reduction, eventually producing free radical intermediates that can cause DNA damage due to oxidative stress, which may also account for their high antiproliferative activity (Alberti *et al.*, 2003).

Clearly, these compounds exhibit an increasing therapeutic potential, potentially acting as anticancer and antimicrobial drugs. Nevertheless, more insights about their mechanism of action are needed in order to fully understand their therapeutic potential.

1.3.1 Yeast as a model to understand the mechanism of action of benzo[a]phenoxazines

Yeasts, such as *Saccharomyces cerevisiae*, have been and are, undoubtedly, one of the preferred model organisms since they share their intracellular organization with higher eukaryotes. This has allowed the study of many physiological processes such as cell division, gene expression, signal transduction (Aitman *et al.*, 2011), intracellular trafficking (Feyder *et al.*, 2015; Hoffman-Sommer & Rytka, 2007), aging (Denoth *et al.*, 2014), oxidative stress (Farrugia & Balzan, 2012), among many others. Moreover, yeast cells are easy to handle and store in the laboratory and can be genetically modified very easily, which is very important for instance, for the study of important pathways occurring both in physiological and pathological mechanisms, because it allows for the expression of human genes involved in those processes or even the deletion of genes with an unknown function or that play important roles in many diseases or important cellular processes (Feyder *et al.*, 2015; Wloch-Salamon & Bem, 2013).

Recently, our group has had an increasing interest in phenoxazines and benzo[a]phenoxazines due to their various biological activities and their great potential in so many biological fields. Nile blue is a cationic dye that belongs to the benzo[a]phenoxazines family, and our group has been using this dye as a lead compound to produce a plethora of new benzo[a]phenoxazines, subsequently studying the most interesting ones recurring to yeast cells (Leitão, 2015; Martinez & Henary, 2016). N-(5-((4-ethoxy-4-

oxobutyl)amino)-10-methyl-9H-benzo[*a*]phenoxazin-9-ylidene) ethanaminium chloride, or BaP1, one of the benzo[*a*]phenoxazines synthesized by our group, has had increasing attention due to its antifungal properties and its action mechanisms have been uncovered using yeast cell as a model. It was already suggested that this compound accumulates at the vacuolar membrane and in the endoplasmic reticulum of *Saccharomyces cerevisiae* cells. It leads to vacuolar membrane damage and vacuolar permeabilization, ultimately leading to cell death (Carvalho, Gonçalves, & Sousa, 2011; Ferreira, 2017; Lopes, 2015). Pep4, which is a vacuolar protease, has also an effector role in the cell death induced by BaP1, as it is released from the vacuole to the cytosol due to the vacuolar permeabilization caused by the benzo[*a*]phenoxazine treatment (Ferreira, 2017; Lopes, 2015). However, the specific cell death process by which this compound acts remains to be clarified, as it was already suggested that neither autophagy nor the apoptotic mitochondrial pathway are involved.

A more recent study investigated another benzo[*a*]phenoxazine that was also synthesized by our group, N-(10-methyl-5-(propylamino)-9H-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride, or as it is also named, C9 (Sousa, 2018). This compound is much more potent than BaP1, since it presents a MIC of 6.25 μ M, whilst the BaP1 compound presents a MIC of 25 μ M. This may be due to the only structural difference between the two benzo[*a*]phenoxazines, which is a different single substituent in the position 5 of the compound's aromatic system. This substitution may potentiate the antifungal activity of C9, being a clear evidence of how structural differences influence the compound's activity Figure 1.3.1.1. (Leitão, 2015).

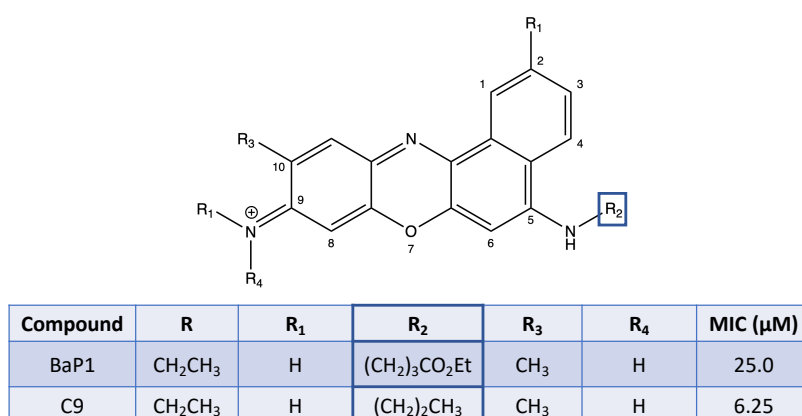


Figure 1.3.1-1: Structure of BaP1 and C9 and respective MICs against a *S.cerevisiae* strain called PYCC 4072. In this figure the small difference in the functional group R. of the core benzo[*a*]phenoxazine aromatic system of both BaP1 and C9 is demonstrated. Also demonstrated are the MICs of each compound.

Thus, this study aimed to characterize the mechanisms of action of C9, once more making use of the yeast *S. cerevisiae* as a model eukaryotic cell. Just as BaP1 compound, C9 also accumulates in the ER and in the vacuolar membrane. Contrarily to what was observed during BaP1 treatment, in this case, Pep4 had a protective role during the C9-induced cell death pathway. On the other hand, it seems that there is a mitochondrial involvement in the cell death process triggered by C9 (Sousa, 2018). However, there are still many questions regarding the mode of action of this compound. Since it is a more powerful benzo[*a*]phenoxazine than BaP1 and its mode of action is less understood, this present work continued to investigate this benzo[*a*]phenoxazine through a different perspective and considering other aspects about the compound.

2. Aim of the work

2.1 Aims of this work

Throughout the last few years, our group has been synthesizing a plethora of benzo[*a*]phenoxazines and exploring their potentialities (Leitão, 2015), aiming to discover promising compounds. In order to understand how these compounds exert their action, *S. cerevisiae* was extensively used as an eukaryotic cell model.

As previously denoted, one of the first benzo[*a*]phenoxazines to be studied was BaP1, a compound that was later discovered to exhibit a high antifungal activity. Later on, C9, another benzo[*a*]phenoxazine, began to be explored. Interestingly, the only difference between these two compounds is a single substitution in the aromatic system, but this minor difference confers a much higher antifungal activity to C9 in comparison to BaP1. It was already found that both compounds accumulate in the vacuolar membrane and in the endoplasmic reticulum membrane. Moreover, they both seem to induce a regulated cell death. However, they present some distinctions. Although they both lead to the permeabilization of the vacuolar membrane, a mitochondrial involvement is only seen in the case of C9's treatment. (Sousa, 2018).

Lately, we have been focusing our attention on C9, since it is much more potent than BaP1 and its mechanism of action is less understood. Even though we have already answered many questions about this compound, others arise that also need an answer. Therefore, the main goal of the present work is to continue to clarify the mechanism of action of this compound and, more specifically, try to uncover its target or targets. The research done by our group has suggested that a possible target of this compound could be located at the ER membrane: an enzyme belonging to the biosynthetic pathway of ergosterol (Leitão, 2015). For this matter, we have studied ergosterol's metabolism, since it could be affected by the compound and also the induction of ER stress. Yeast growth curves and viability assays were used to assess the effects of C9 in growth and viability of the cells, in the presence and in the absence of external ergosterol. An indirect UPR induction assay was employed to assess ER stress. Fluorescence microscopy was used to observe the intracellular localization of the compound but also to check its influence on the membrane lipid rafts, rich in ergosterol. To assess if the compound was having a direct influence on the expression of a specific *ERG* gene, a recently designed luminescence assay was employed (Carvalho, 2018).

3. Materials and Methods

3.1 Yeast strains and plasmids

The *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. BY 4741 and W303-1A were used as the wild type strains.

Table 1: List of *S. cerevisiae* strains used in the present work.

Yeast strain	Genotype	Source
BY4741	MATa, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i>	EUROSCARF
W303-1A	MATa, <i>ura3-52</i> , <i>trp1Δ2</i> , <i>leu2-3</i> , <i>112 his3-11</i> , <i>ade2-1</i>	EUROSCARF
BF264-15D	MATa, <i>ade1</i> , <i>his3</i> , <i>leu2-3</i> , <i>trp1</i>	(Costa, 2018)
BF264-15D pJT30	BF264-15D; MATa, <i>ade1</i> , <i>his3</i> , <i>leu23</i> , <i>trp1</i> ; pJT30 (URA3)	(Costa, 2018)
BY 4741 GFP-SEC66	MATa, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i>	Brian Zid
BY4741 pAM10	BY4741 harboring pAM10 (URA3)	(Carvalho, 2018)
BY 4743 Δ ERG11- yNluc	MATa/ α <i>his3Δ1</i> / <i>his3Δ1</i> <i>leu2Δ0</i> / <i>leu2Δ0</i> LYS2/ <i>lys2Δ0</i> <i>met15Δ0</i> /MET15 <i>ura3Δ0</i> / <i>ura3Δ0</i> Δ ERG11-yNluc- KanMX	(Carvalho, 2018)
W303-1A pDF01- VBA1-YEGFP	MATa, <i>ura3-52</i> , <i>trp1Δ2</i> , <i>leu2-3</i> , <i>112 his3-11</i> , <i>ade2-1</i> , pDF01-VBA1-YEGFP (URA3)	Josef Nosek

3.2 Media and growth conditions

Most *S. cerevisiae* cells of BY 4741 and W303-1A strains were grown in YEPDA (Yeast Extract Peptone Dextrose Agar) medium plates (1% yeast extract; 2% bactopectone; 2% glucose and 2% agar) at 30°C during 2 days. Afterwards, they were transferred to liquid SC (Synthetic Complete) medium (2% glucose; 0.5% (W/V) ammonium sulphate; 0.7% yeast nitrogen base without amino acids and ammonium sulphate; 0.2% dropout mix; 0.01% histidine, uracil; 0.02% leucine) and put in an orbital shaker at 200

rpm, at 30 °C and with a flask volume/medium ratio of 5:1. Then, they were allowed to reach the exponential phase ($OD_{640nm} \approx 0.5-0.7$).

Some of the strains transformed with plasmids (BF264-15D pJT30, BY4741 pAM10, W303-1A pDF01-VBA1-YEGFP and BY 4741 GFP-SEC66) were grown in SC medium plates without the appropriate amino acids, depending on the strain, at 30 °C during 2 days. After that, they were transferred to liquid SC (Synthetic Complete) medium (2% glucose; 0.5% (W/V) ammonium sulphate; 0.7% yeast nitrogen base without amino acids and ammonium sulphate; 0.2% dropout mix; appropriate amino acids) and put in an orbital shaker at 200 rpm, at 30 °C and with a flask volume/medium ratio of 5:1. Then, they were allowed to reach the exponential phase ($OD_{640nm} \approx 0.5-0.7$)

3.3 C9 stock solution preparation and treatment

N-(10-methyl-5-(propylamino)-9H-benzo[a]phenoxazin-9-ylidene)ethanaminium chloride (C9) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 4906 μ M.

As previously mentioned, yeast cells were grown in SC medium at 30 °C with agitation at 200 rpm, until they reached the exponential phase ($OD_{640nm} \approx 0.5 - 0.6$). After that, cells were collected and centrifuged for 3 minutes at 5000 rpm. Then, the pellet was washed with deionized sterile water, centrifuged again and resuspended in fresh SC medium or in another medium, depending on the assay in question.

C9 treatment was performed by adding C9 to the resuspended cells at different final concentrations: 2.5, 7.5, 12.5, 25 μ M. The same volume of DMSO (DMSO concentration was never superior to 1%) was added to another tube that served as a negative control. Incubation took place at 30 °C for a defined period of time, depending on the assay that was being performed. Also dependent on the assay were the time-points at which the cells were collected. In every assay the 0 minute sample was collected before adding the compound and DMSO.

3.4 Ergosterol stock solution preparation and treatment

Ergosterol stock solution was prepared by dissolving ergosterol in absolute ethanol to a final concentration of 4 mg/mL, using an ultrasonic bath. Yeast cells were grown as previously described.

Ergosterol treatment was performed by adding ergosterol to the resuspended cells at different final concentrations: 5, 10 and 20 $\mu\text{g}/\text{mL}$. The same volume of absolute ethanol was added to another tube that served as negative control. Incubation took place at 30 °C for a defined period of time, depending on the assay that was being performed. Also dependent on the assay were the time-points at which the cells were collected. In every assay the 0 minute sample was collected before adding the ergosterol and the absolute ethanol.

3.5 Cell growth assays

Yeast cells were grown in SC medium at 30 °C with agitation at 200 rpm and were allowed to reach the exponential phase ($\text{OD}_{640\text{ nm}} \approx 0.5 - 0.6$). Then, they were collected to a final $\text{OD}_{640\text{ nm}}$ of 0.1 and centrifuged for 3 minutes at 5000 rpm. Afterwards, the pellet was washed with deionized sterile water, centrifuged again and resuspended in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with the appropriate lacking aminoacids and buffered with 0.165 M of MOPS (3-(N-morpholino)propanesulfonic) acid. Before (Time zero) and after adding the C9, DMSO (negative control), ergosterol and absolute ethanol (negative control), samples of 100 μL of culture were collected every 90 minutes along 540 minutes and their OD was determined using a spectrophotometer. Cell growth was assessed through the construction of a growth curve and the determination of the specific growth rate of the cells in each condition.

3.6 Cell viability assays

Yeast cells were grown in SC medium at 30 °C with agitation at 200 rpm and were allowed to reach the exponential phase ($\text{OD}_{640\text{ nm}} \approx 0.5 - 0.6$). After that, cells were collected and centrifuged for 3 minutes at 5000 rpm. Then, the pellet was washed with deionized sterile water, centrifuged again and resuspended in RPMI medium supplemented with the appropriated lacking aminoacids. Next, cells were treated with various concentrations of C9, DMSO, ethanol, ergosterol and methyl- β -cyclodextrin (M β CD), depending on the conditions of the assays. Before (Time zero) and after adding the C9, DMSO (negative control), ergosterol and absolute ethanol (negative control), samples of 50 μL of culture were collected every 30 minutes along 120 minutes and diluted 10^{-4} in sterile deionized water. Seven drops of 30 μL from the 10^{-4} dilution were placed in YEPDA plates. The plates were incubated during 2-3 days at 30 °C

and CFU (colony-forming units) were counted. Cell viability was assessed considering that the number of CFU obtained in time zero corresponded to 100% survival.

3.7 β -Galactosidase activity assay

Yeast cells were grown in SC medium at 30 °C with agitation at 200 rpm and were allowed to reach the exponential phase ($OD_{640\text{ nm}} \approx 0.5 - 0.6$). Then, they were collected and centrifuged for 3 minutes at 5000 rpm. Afterwards, the pellet was washed with deionized sterile water, centrifuged again and resuspended in RPMI medium supplemented with adenine. Then, cells were treated with C9 (at various concentrations) or with DTT (positive control) and incubated at 30 °C for 1 hour. Afterwards, cells were centrifuged and washed with deionized water. Then, the pellets were resuspended in breaking buffer (100 mM Tris-HCl, 1 mM DTT, 10% Glycerol) and kept in ice. After this, cell beads were added to the samples, which were vortexed 6/7 times for 1 minute (with 1 minute pause in ice). Subsequently, samples were centrifuged, the supernatant was collected and protein quantification was performed through the Bradford method. From the remaining supernatant, the volume corresponding to 70 μ g of protein extract was added to Z buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 50 mM β -mercaptoethanol). After this, samples were placed in a water bath at 30 °C, during 5 minutes to stabilize the temperature. Then, 4 mg/mL of ONPG was added to the samples, which continued in the water bath at 30 °C during 1 hour. During this time a colour change should be observed. At two time points (30 and 60 minutes), Na_2CO_3 was added to the mixture to stop the reaction. Afterwards, samples were centrifuged and the $OD_{420\text{ nm}}$ of the supernatant was measured. Finally, β -galactosidase activity was determined using the following formula:

$$\beta\text{-gal. activity (nmol/min/mg)} = \frac{OD_{420} \times 0.7}{0.0045 \times [\text{protein}] \times V \times t}$$

3.8 Analysis of sterols distribution on cells exposed to C9

Fluorescence microscopy (Leica Microsystems DM-5000B epifluorescence microscope) was used to examine the distribution of cellular sterol-lipid through filipin staining. The procedure was

performed as described before (Beh & Rine, 2004; C. Ferreira & Lucas, 2008). Briefly, cells were grown overnight in adequate synthetic medium and allowed to reach the exponential phase ($OD_{640\text{ nm}} \approx 0.7$). Afterwards, cells were centrifuged, the pellet was washed and then, the washed pellet was resuspended in RPMI supplemented with the appropriate lacking aminoacids. Then, cells were treated either with different concentrations of C9, M β CD or the combination of both, for 2h at 30 °C with agitation at 200 rpm. After 2h of treatment, cells were centrifuged, resuspended in PBS 1x and stained with a fresh solution of filipin dissolved in methanol (5 mg/mL) and kept in the dark for 15 minutes, at room temperature. Finally, cells were mounted on slides with a drop of the anti-fading agent Vectashield (Vector Laboratories) in an attempt to overcome the instability of the filipin dye. Cells were then visualized in a fluorescence microscope (Leica DM-5000B, Leica Microsystems) with appropriate filter and objective settings. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

3.9 Luciferase treatment and luminescence measurements

The nano-Glo substrate (Promega GmbH, Germany) was diluted 1:100 with the supplied buffer. Reporter yeast strain BY 4743 (2N) *ERG11*-yNluc either treated or non-treated with the fungicide tebuconazole was used as positive and negative control, respectively. For this assay, cells were inoculated in adequate SC medium and grown overnight at 30 °C with orbital shaking at 200 rpm and allowed to reach the exponential phase ($OD_{640\text{ nm}} \approx 0.5$). Then, cells were centrifuged, the pellet was washed and resuspended in RPMI supplemented with the appropriate amino acids. Afterwards, cells were either treated with 25 μ M of C9 or 4 μ g/mL of tebuconazole. For the luminescence assay itself, at different time points (2h and 4h) cells at an $OD_{640\text{ nm}} = 1.5$ were centrifuged and then resuspended in SC-Glu medium (pH=8) to a final volume of 250 μ L, in order to maximize luciferase signal (Carvalho, 2018). Then, cells were mixed 1:10 with the luciferase substrate in white opaque 96-well microplates (OptiPlate-96), to a final volume of 100 μ L. After a 1 hour incubation, bioluminescence emissions were recorded using a Varioshkan Multimode reader (1000 ms integration time).

3.10 Epifluorescence microscopy

For the experiments requiring the use of epifluorescence microscopy, a Leica Microsystems DM-5000B microscope was used, with appropriate filter settings (red, green, blue and DIC (Differential Interference Contrast)) with a 100x objective that required the use of immersion oil. Images were obtained with a Leica DFC350 FX Digital Camera and processed with LAS AF Microsystems software. For phenotype quantification, at least 300 cells of at least three independent experiments were evaluated.

4. Results

4.1 Confirming intracellular distribution of C9

Preliminary studies using *S. cerevisiae* as a model have revealed that benzo[*a*]phenoxazines accumulate at the vacuolar and endoplasmic reticulum membranes. More specifically, it was possible to establish a correlation between the potency of each compound and its site of accumulation; benzo[*a*]phenoxazines with lower minimum inhibitory concentrations (MIC) tend to accumulate more in the membrane of the endoplasmic reticulum than in the membrane of the vacuole, while benzo[*a*]phenoxazines with a higher MIC accumulate mostly in the vacuolar membrane (Leitão, 2015)

In order to confirm that C9's distribution two yeast mutants were used. One expressed a GFP-tagged ER membrane protein called Sec66, a protein involved in the post-translational translocation of proteins to the ER (Lee & Heitman, 2012). The other mutant expressed a GFP-tagged vacuolar membrane protein called Vba1, a transporter protein essential for the uptake of basic amino acids in the vacuolar membrane (Jakubkova *et al.*, 2016; Sekito *et al.*, 2008). Below, in Figure 4.1.1 it is possible to observe the results from the observation of the mutant cells expressing the GFP-tagged ER membrane protein Sec66.

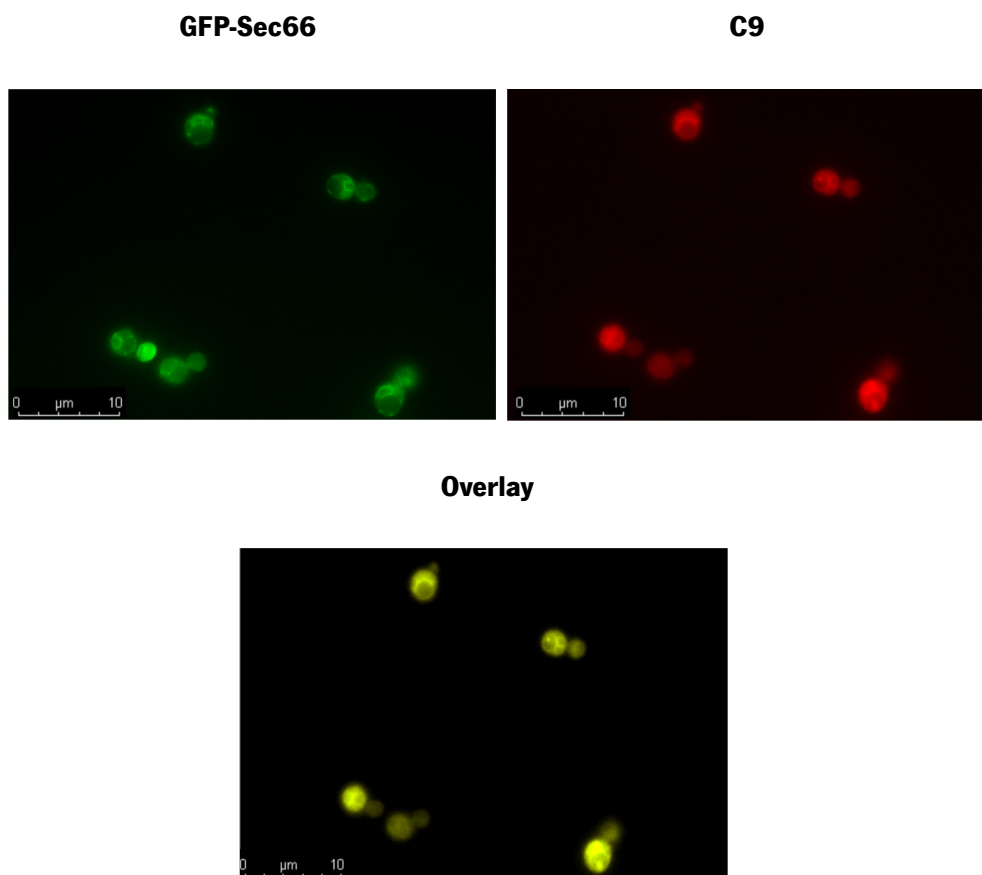


Figure 4.1-1: Intracellular distribution of C9 in the ER. Representative fluorescence microscopy images of BY 4741 GFP-SEC66 cells treated with C9. Cells were visualized by fluorescence microscopy with a 100x objective with immersion oil.

In terms of the ER, fluorescence microscopy images revealed that this compound co-localizes in the ER membrane, indeed. However, after observing the cells expressing the GFP-tagged vacuolar membrane protein, we were not able to conclude that the compound was in fact accumulated at the vacuolar membrane. This was probably due to some problems with the strain itself, since it was not even possible to observe any fluorescence emitted by the VBA1-GFP tagged cells.

4.2 External ergosterol seems to exacerbate the toxic effect of C9 on cell growth

As previously referred, preliminary studies have revealed that less active benzo[*a*]phenoxazines had a tendency to accumulate more in the vacuole membrane, while the most active compounds would accumulate simultaneously in the membrane vacuole and in the ER, with the latter being the major site of accumulation (Ferreira, 2017; Leitão *et al.*, 2015; Leitão, 2015). The first results of this present work have shown and confirmed that C9 seems to accumulate more in the ER membrane. Thus, it seems that the antiproliferative activity of this benzo[*a*]phenoxazine can correlate with their ability to accumulate in the ER and that the main target of these compounds could be located in this organelle, rather than the vacuole membrane.

The ER membrane contains several enzymes important for cell growth that could be potential targets of these compounds. One of those enzymes, oxidosqualene-lanosterol cyclase, is a key enzyme in the ergosterol biosynthesis pathway and has a substrate that shares with C9 the fact that it accommodates a 4 -ring system in his structure (Mo *et al.*, 2003). We hypothesized that if C9 would, somehow, be targeting this enzyme, cells would have their growth affected due to limiting ergosterol content. In line with this, we speculated that an external supplementation of ergosterol to the medium would render cells more resistant to C9's deleterious effect. To test this, growth assays with BY4741 WT cells treated with different concentrations of ergosterol, C9 or the combination of both, were performed during 9 h. Finally, the specific growth rate was calculated and the results are represented below in Figure 4.2.1.

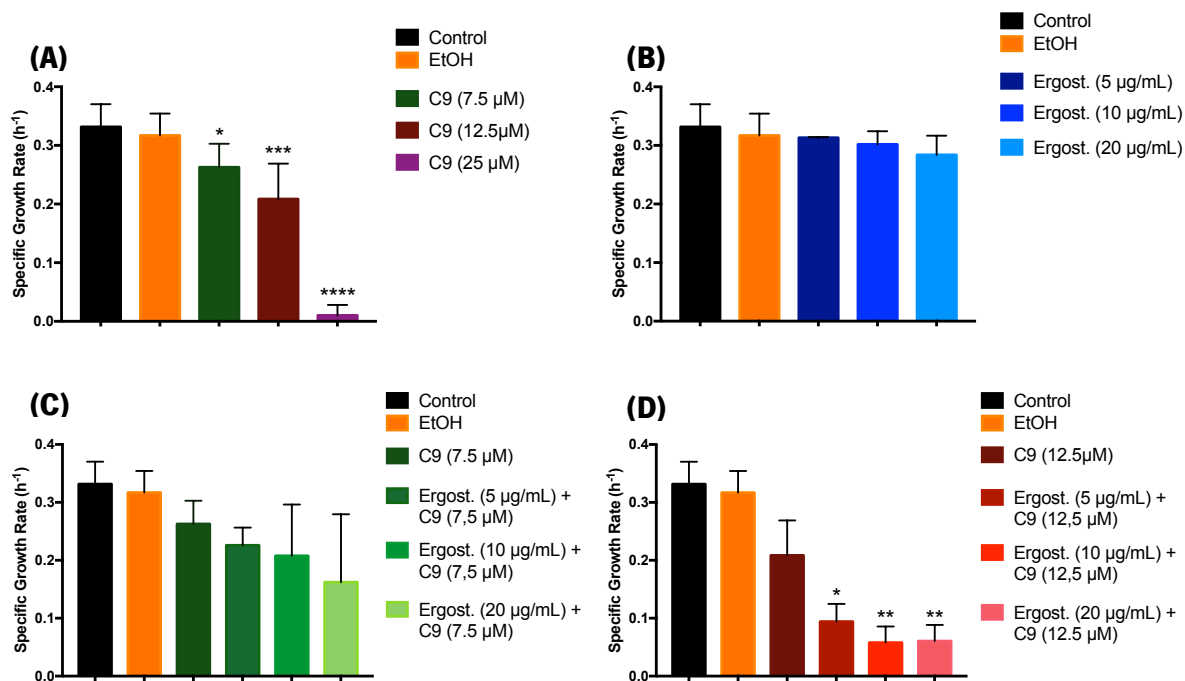


Figure 4.2-1: Effect of C9 in yeast growth with and without the addition of external ergosterol. Wild type BY4741 cells were incubated with C9 (2.5, 7.5 and 25 μM) and ergosterol (5, 10 and 20 $\mu\text{g/mL}$), either alone or in combination for 9h. At different time points (90, 180, 270, 360, 450 and 540 minutes) $\text{OD}_{600\text{nm}}$ was measured and the specific growth rate was posteriorly calculated.; **(A)** cells treated with different C9 concentrations were compared with cells with no treatment (control); **(B)** cells treated with various concentrations of ergosterol compared with untreated cells (control); **(C)** cells treated with 7.5 μM of C9 compared with cells treated with both 7.5 μM of C9 and different concentrations of ergosterol; **(D)** cells treated with 12.5 μM of C9 compared with cells treated with both 12.5 μM of C9 and different concentrations of ergosterol. The reported values represent means $\pm$ SD of at least three independent experiments. Statistical analysis was performed by One-way ANOVA test. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Considering the results, it is possible to observe that the inhibition of the cells' growth is proportional to the concentration of the compound; the higher the compound's concentration, the more inhibited the growth of the cells, and the lower the specific growth rate (Figure 4.2 1 A).

On the other hand, looking at the results regarding the treatment of the cells with increasing concentrations of ergosterol, we can observe that the specific growth rate of cells treated with increasing concentrations of ergosterol are similar to the specific growth rate obtained for untreated cells, suggesting that cell growth was not affected by the addition of external ergosterol to the medium (Figure 4.2 1 B).

However, in the case of cells treated with 7.5 μM of C9 and increasing concentrations of ergosterol simultaneously, it seems that, although not significant, there is a growth inhibition in comparison with cells treated only with 7.5 μM of C9. (Figure 4.2 1 C). Similar results were obtained for cells treated with 12.5 μM of C9 and increasing concentrations of ergosterol, simultaneously. However, in this case, the growth inhibition is significant (Figure 4.2 1 D).

Since for the tested concentrations, ergosterol, *per se* did not display any toxicity, the results suggest that the external ergosterol addition potentiates C9's toxicity. In fact, opposite to our hypothesis, the effect of C9 on cell growth does not seem to be due to a limiting ergosterol concentration, but rather due to the increase of ergosterol, that negatively affects cell growth in the presence of C9.

4.3 External supplementation of ergosterol has no protective effect on cell viability upon treatment with C9

Initially, we hypothesized that C9 could be targeting ergosterol biosynthetic pathway, more specifically, it could be targeting an enzyme that participates in this pathway and has a key role in the biosynthesis of ergosterol. Thus, if this was definitely C9's mechanism, then cellular ergosterol would probably decrease and the growth of the cells would be compromised, since ergosterol is very important for the maintenance of the cell membrane, contributing to its fluidity, permeability among other functions (Kohlwein, 2017; Zhang & Rao, 2010). Nevertheless, our results suggest that after exposing cells to C9, an external supplementation of ergosterol has an effector role rather than a protective one, since cells that receive an external supplementation of ergosterol during treatment with C9 have a lower specific growth rate than the ones who are exposed to C9 alone, indicating that external ergosterol could be increasing the compound's toxicity.

To further understand the role of increasing ergosterol concentration in C9 effects, we aimed to elucidate if, besides influencing -growth inhibition, an external supplementation of ergosterol could also have an effect in C9-induced cell death. In line with this, viability of cells treated with different concentrations of C9, either alone or in combination with different concentrations of ergosterol, was evaluated during a 2 h assay. The resulting percentages of CFU over time are represented below in Figure 4.3.1.

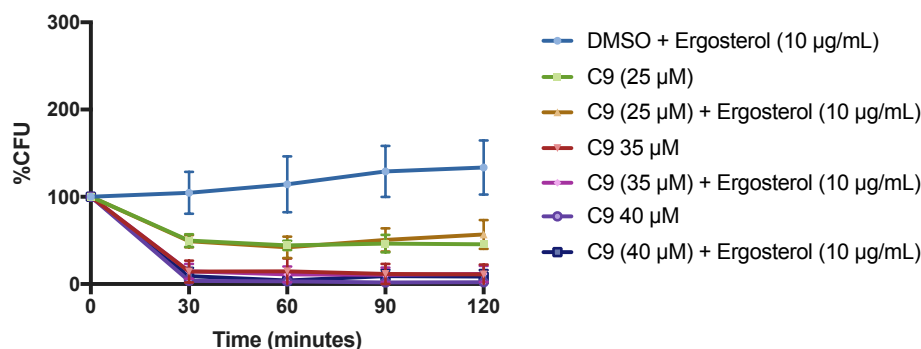


Figure 4.3-1: Effect of C9 alone or in combination with ergosterol on cell viability. Analysis of the viability of BY 4741 WT cells treated with different concentrations of C9 (25, 35 and 40 µM) and ergosterol (10 µg/mL). DMSO and ergosterol (negative control) were added 30 minutes before C9 in combination with ergosterol was added. Cell viability was evaluated by CFU counting at different time points (0, 30, 60, 90 and 120 minutes). The reported values represent means ± SD of at least three independent experiments. Statistical analysis was performed by the two-way ANOVA test to compare cells treated with C9 alone with cells treated with C9 and ergosterol simultaneously, for each time of the treatment.

It is possible to observe that after 120 minutes of treatment, as expected, there was a decrease in the viability of cells treated only with C9. This effect was enhanced as the concentration of C9 increased. The higher the concentration of C9, the bigger the decrease on the viability of the cells. When cells were exposed to the same concentration of C9 but also to ergosterol, there was a slight increase in cell's viability. However, the differences in the viability of cells treated only with C9 and cells treated with both C9 and ergosterol were not statistically significant.

4.4 C9 is not leading to ergosterol depletion

Very recently, and in a distinctive line of work, another group from our laboratory has developed a reporter yeast strain meant for detection of azole compounds, specifically, tebuconazole, in aqueous samples. Many research studies showed that, upon treatment with tebuconazole and other fungicides, transcripts of *ERG11* gene were amongst the most up-regulated genes, which is in agreement with the fact that, as previously referred in the introduction section, azole fungicides target the biosynthetic pathway of ergosterol, namely, the conversion of lanosterol to ergosterol, for which the cytochrome P450-dependent lanosterol 14 α -demethylase enzyme, encoded by *ERG11* gene, is strictly necessary (Prasad, Shah, & Rawal, 2016). Overall, this leads to ergosterol's depletion in the cells. In line with this, *ERG11* gene was selected to design the reporter biosensor for the detection of azole compounds. The *S.cerevisiae* strain used to design this biosensor was a diploid strain called BY 4743 with an *ERG11* deletion. Moreover, this strain was transformed with a cassette that contained the gene of a reporter protein called

nanoluciferase gene optimized for yeast expression (yNluc) (Masser *et al.*, 2016) under the control of the *ERG11* promoter (Carvalho, 2018). The correct functioning of this biosensor was successively achieved, as it was responsive to tebuconazole treatment. A simplified explanation of how this biosensor works is shown in Figure 4.4.1.

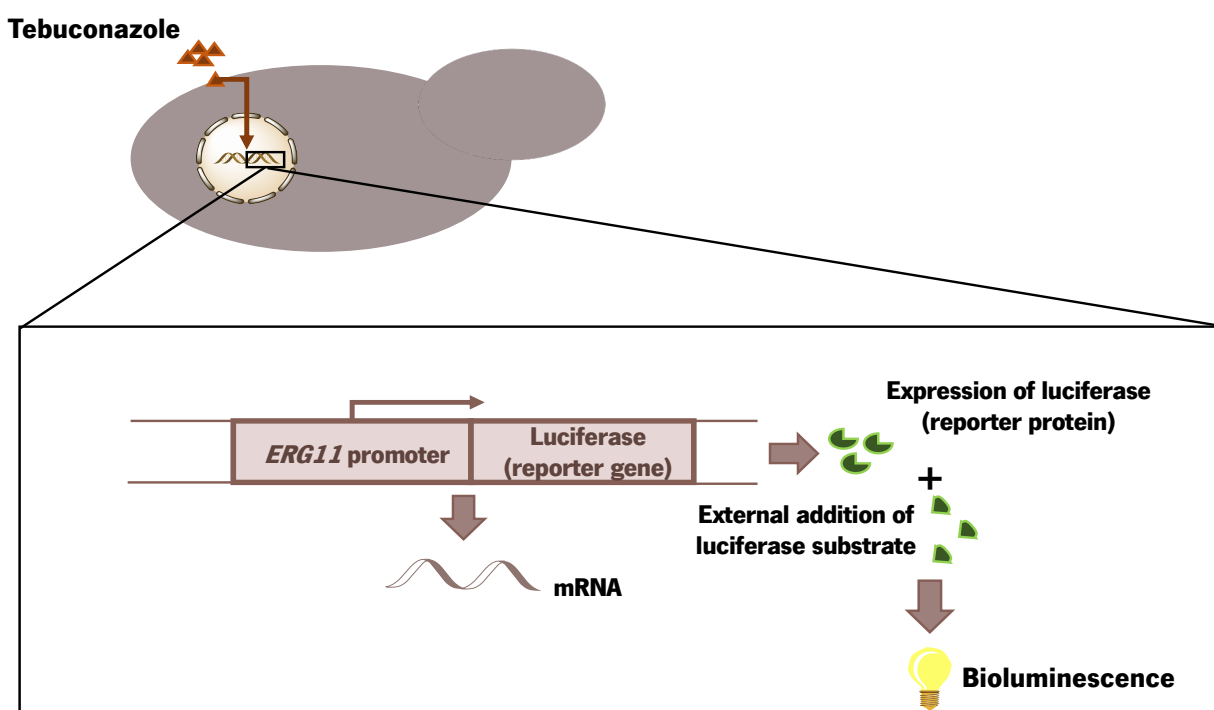


Figure 4.4-1: Schematic representation of the azole detection biosensor. When cells are treated with tebuconazole, ergosterol production is affected since tebuconazole will target cytochrome P450-dependent lanosterol 14 α -demethylase enzyme, which catalyses the conversion of lanosterol to ergosterol. Thus, *ERG11* promoter is activated, which, in turn, will lead to the transcription and translation of the luciferase protein. Then, after an external addition of the enzyme's substrate, there is the production of a bioluminescence signal.

In order to uncover if C9 could also be targeting ergosterol synthesis we employed this reporter strain. Firstly, control assays were performed to validate the functioning of the reporter strain. Untransformed BY 4741 and BY 4741 cells harbouring the pAM10 plasmid were used. This plasmid contained the yNluc gene that was under the control of heat-shock responsive promoter. For this assay we treated BY 4743 Δ ERG11- yNluc cells with C9 and tebuconazole during 4 hours. At 2h and 4h time points cells were mixed with luciferase substrate in white opaque 96-well microplates and after a 1h incubation, bioluminescence levels were recorded. The results of this assay are represented below in Figure 4.4.2.

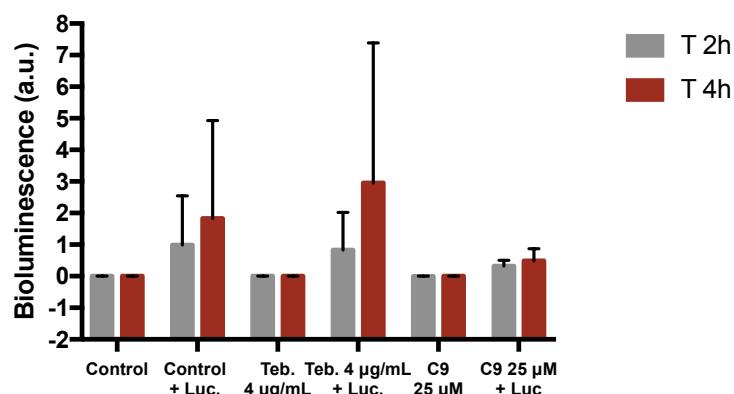


Figure 4.4-2: Effect of C9 on *ERG11* expression. BY 4743 Δ *ERG11*-yNluc cells were incubated with 25 μ M of C9 and 4 μ g/mL of tebuconazole during 4 hours. Untreated cells and cells treated with tebuconazole were used as negative and positive controls, respectively. At 2 and 4h of treatment, cells were collected, centrifuged and resuspended in SC-Glu pH 8 medium and then mixed 1:10 with the luciferase substrate in white opaque 96-well microplates (OptiPlate-96). Finally, after a 1h incubation with the substrate, luciferase signal was measured. The reported values represent means $\pm$ SD of at least three independent experiments. Statistical analysis was performed by the two-way ANOVA test to compare cells treated with C9 with cells treated with tebuconazole, for each time point of the treatment.

As expected, treating the cells with 4 μ g/mL of tebuconazole lead to a high luciferase signal, meaning that the fungicide is inducing the expression of *ERG11*. In the case of cells treated with 25 μ M of C9 it is possible to observe that there was a slight increase of luciferase signal in comparison with the negative control. Nonetheless, despite the levels of luciferase observed for C9-treated cells, it is possible to conclude that these bioluminescence levels are still very low when compared with the levels of luciferase signals observed in cells treated with tebuconazole. Additionally, there were no significant statistical differences between all of the conditions. Thus, the overall results suggest that C9 does not affect *ERG11* expression, thus not leading to a decrease in ergosterol content.

4.5 Depletion of ergosterol from the plasma membrane increases cell viability after treatment with C9

We next raised the question whether membrane ergosterol could be a key participant in the action of C9. To answer this question, we evaluated if decreasing ergosterol at the plasma membrane could be affecting the toxicity of this compound.

For such we exposed cells to two different concentrations of C9 (25 μ M and 40 μ M) and, at the same time, to 50 mg/mL of a sterol-depleting agent, methyl- β -cyclodextrin (M β CD), that extracts sterols from the plasma membrane of the cells. Then, the cell's viability was evaluated during a 2 h assay. The results are presented in Figure 4.5.1.

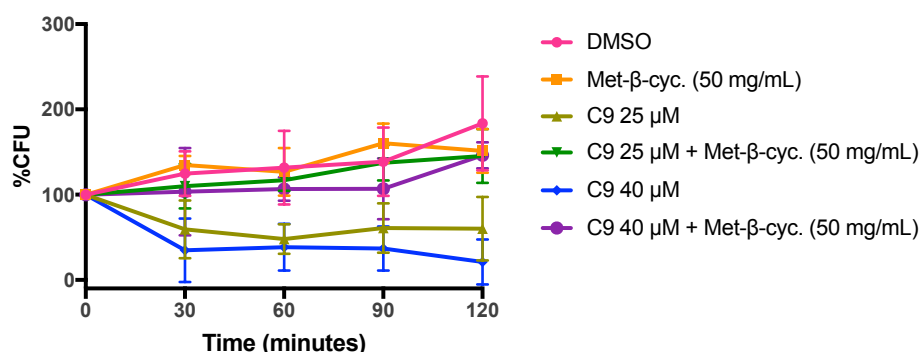


Figure 4.5-1: Effect of C9 in combination with Methyl- β -Cyclodextrin on cell viability Analysis of the viability of BY 4741 WT cells treated with two concentrations of C9 (25 40 μ M) and M β CD (50 mg/mL). DMSO (negative control) was added 30 minutes before C9 in combination with M β CD was added. Cell viability was evaluated by CFU counting at different time points (0, 30, 60, 90 and 120 minutes). 30 min: (C9 40 μ M vs C9 40 μ M + M β CD)*; 60 min: (C9 25 μ M vs C9 25 μ M + M β CD)*, (C9 40 μ M vs C9 40 μ M + M β CD)*; 90 min: (C9 25 μ M vs C9 25 μ M + M β CD)***, (C9 40 μ M vs C9 40 μ M + M β CD)*; 120 min: (C9 25 μ M vs C9 25 μ M + M β CD)***, (C9 40 μ M vs C9 40 μ M + M β CD)****. The reported values represent means $\pm$ SD of at least three independent experiments. Statistical analysis was performed by the two-way ANOVA test to compare cells treated with C9 alone with cells treated with C9 and M β CD simultaneously, for each time of the treatment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

Once more, as expected, cells treated with 25 μ M and 40 μ M of C9 presented a decrease in their viability during the 2 hours of treatment with the compound. When cells were exposed to the same concentrations of C9 but were also treated with M β CD, there was no decrease of viability, some growth being even observed during the course of the assay. Furthermore, treatment with M β CD seems to be even more effective on higher concentrations of C9, which is suggested by the results of the statistical analysis. Overall, these results strongly support that M β CD exerts a protective effect against C9 treatment.

4.6 Lipid rafts distribution upon C9's treatment

Disrupting membrane sterols is amongst the main antifungal mechanisms displayed by various compounds and drugs that have the ability to kill fungal cells. Specifically, these antifungal agents target ergosterol (fungal sterol) in different ways; depending on their type, they can either bind to key enzymes of the ergosterol biosynthetic pathway, ultimately leading to the inhibition of ergosterol production or they can also bind to ergosterol itself and form a pore-like complex that results in cell's membrane disruption

and the outflow of cytosolic contents (Prasad *et al.*, 2016). Normally, ergosterol exists in cellular membranes as a part of lipid rafts, which are membrane microdomains enriched also in sphingolipids and transmembrane proteins and important for the correct function of the yeast cell. These domains are involved in signal transduction pathways and membrane protein trafficking (Curwin *et al.*, 2013; Mollinedo, 2012).

In line with our hypothesis of ergosterol being a target of C9, we sought to monitor the sterol distribution, particularly in lipid rafts. For this assay, cells were stained with filipin, a polyene antibiotic that exhibits fluorescent properties and that bind to sterols, in this case, to ergosterol, one of the major constituents of lipid rafts (Van Leeuwen *et al.*, 2008). To validate our approach, we used methyl- β -cyclodextrin (M β CD) as positive control, a reagent known to extract sterols from membranes.

The main goal of this assay was to observe sterols distribution in cells either treated with C9 alone or C9 in combination with M β CD. Then, it was necessary to count at least 300 cells for each condition. Unfortunately, this was not possible due to the extreme instability of the filipin staining. During the course of the visualization of the cells under the microscope some of the conditions had no staining at all, which must have been due to some unwanted light that was entering the microscope room or even the excessive time that some of the slides took to be observed in the microscope. Therefore, the only analysis that can be made is a qualitative one, rather than a quantitative. Below in Figures 4.6.1 and 4.6.2. are representative images of each condition.

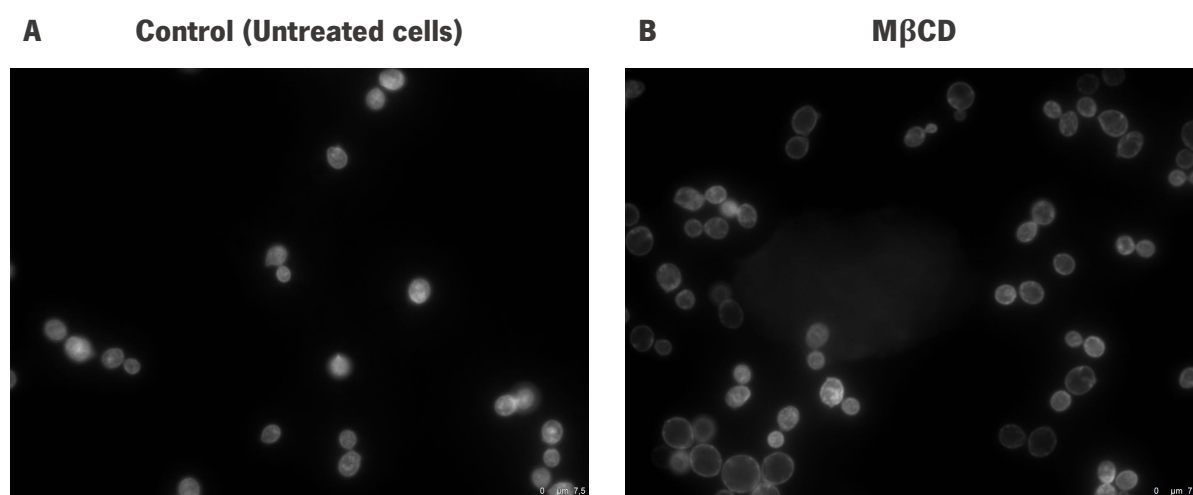


Figure 4.6-1: **Distribution of membrane ergosterol in untreated cells and cells exposed to methyl- β -cyclodextrin.** Representative images of untreated W303-1A WT cells and cells treated with 50 mg/mL of methyl- β -cyclodextrin, for 1 h. Then, they were stained with filipin during 15 minutes in the dark and finally, they were visualized using fluorescence microscopy. (A) untreated cells; (B) cells treated with M β CD.

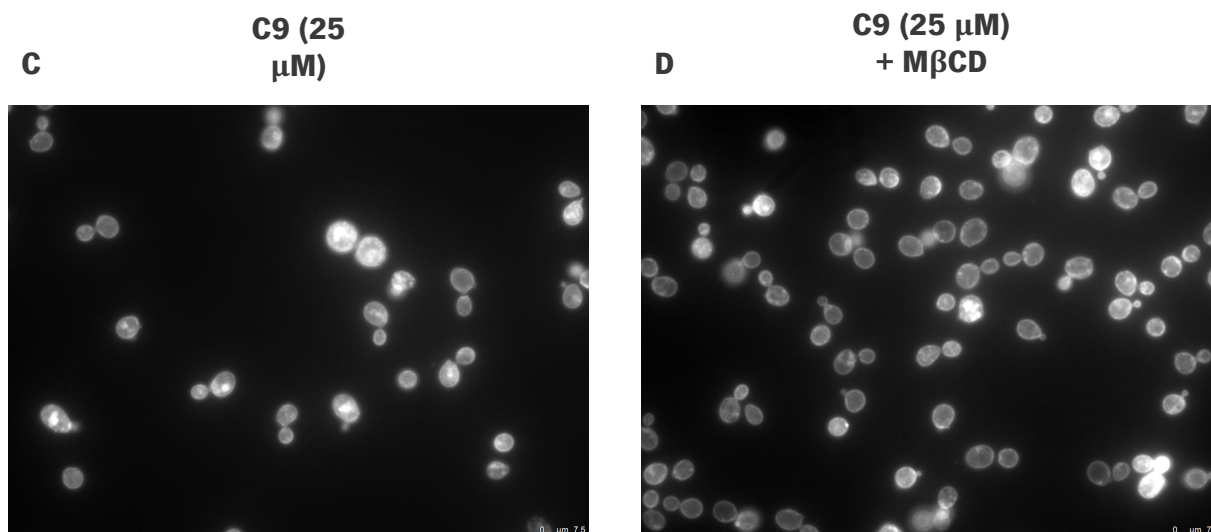


Figure 4.6-2: Distribution of membrane ergosterol in cells exposed to C9 alone and cells exposed to C9 and methyl- β -cyclodextrin simultaneously. Representative images of W303-1A WT cells that were incubated with 25 μ M of C9, 50 mg/mL of methyl- β -cyclodextrin, and the combination of the two compounds for 1 h. Then, they were stained with filipin during 15 minutes in the dark and finally, they were visualized using fluorescence microscopy. **(C)** cells treated with C9; **(D)** cells treated with C9 + M β CD.

If we observe cells from the positive control ([Figure 4.6.2 B](#)) it is possible to conclude that it did not work as expected and, as such, is not suitable for the validation of this assay. Normally, after treatment with M β CD, cells should have all or, at least, the majority of its membranal ergosterol depleted and localized in small aggregations inside the cell. However, looking at the representative images of positive control cells we see the majority of the cells still have ergosterol localized in the cellular membrane, almost like a ring-like structure around the cell. Moreover, looking at cells treated with C9 ([Figure 4.6.2 C](#)) it is possible to see some of the cells with cluster-like structures inside the cell, which could mean that the treatment with C9 lead to the formation of these structures. Finally, in the case of cells exposed to both C9 and M β CD ([Figure 4.6.2 D](#)), it is possible to observe that a vast number of cells presents the same ring-like structures visualized for cells treated only with M β CD. Thus, it seems that C9, in the presence of M β CD is no longer able to induce the formation of the cluster-like structures, which represent accumulations of ergosterol.

4.7 C9 does not seem to induce ER stress

As we have already shown, this compound accumulates mostly at the ER of the yeast cells. As such, we hypothesized that the compound could be impairing this organelle's function contributing to its

antiproliferative effects. Recently, it has been described that some antifungals seem to cause ER stress and that this was their main mode of action. This is the case of carvacrol, a monoterpene found in oregano and thyme oils that was found to be a potent antifungal against the pathogenic yeast *Candida albicans*. Its action causes ER stress, leading to the induction of a signaling pathway called unfolded protein response (UPR) but also to alterations on the morphology and integrity of the ER and it was already shown that mutants in the UPR pathway are more sensitive to the action of this compound (Chaillot *et al.*, 2015). Another example, is gepinacin. This drug-like compound inhibits a key step in the synthesis of ER glycosylphosphatidylinositol (GPI)-anchors of fungal cells. Specifically, this compound targets an acyltransferase, Gwt1, that acylates GPI-anchors. These GPI-anchors are essential for protein trafficking to the secretory pathway. Thus, the action of gepinacin inhibits the transport of GPI-anchored proteins and, as such, perturbs protein homeostasis within the ER, ultimately leading to ER stress and, in turn, to the induction of the UPR to cope with this stress (McLellan *et al.*, 2012).

As referred in the introduction section, when proteins do not fold correctly or do not fold at all, ER enters in a stress state and UPR is activated in order to cope with these complications (Pineau *et al.*, 2009). Ire1p pathway is activated by unfolded proteins that lead to its dimerization and autophosphorylation, leading to the activation of the substrate of Ire1p, called Hac1p. Hac1p is a transcription factor that will bind to the unfolded protein response elements (UPREs) that will, in turn, induce the transcription of certain target genes, such as ER chaperones or other proteins, like lipid biosynthesis enzymes.

Bearing this in mind, we thought to assess if C9 compound could also be targeting the ER and causing ER stress. For this, we assessed the induction of the unfolded protein response (UPR) upon treating the cells with C9. We used yeast cells that were transformed with a plasmid harboring an UPRE-*lacZ* gene fusion (Figure 4.7.1) and treated them with various concentrations of C9.

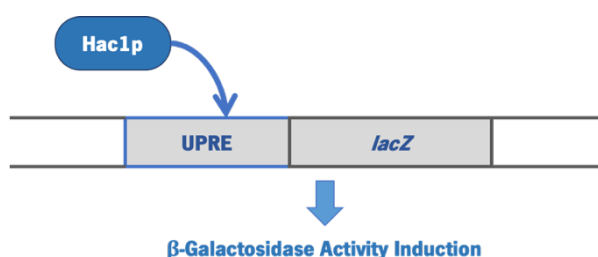


Figure 4.7-1: Schematic representation of UPRE-lacZ gene reporter. The used cells were previously transformed with a plasmid called pJT30, harbouring a UPRE-lacZ gene fusion, in which the UPRE region control lacZ reporter gene. Thus, upon ER stress, transcription factor Hac1p is activated, then binds to the UPRE region, which will, in turn, induce lacZ transcription, leading to the increase of β-galactosidase activity.

Being a known ER-stress inducer, DTT was used as positive control. After 1h of exposure, β -galactosidase activity was calculated and the final results are presented below in Figure 4.7.2

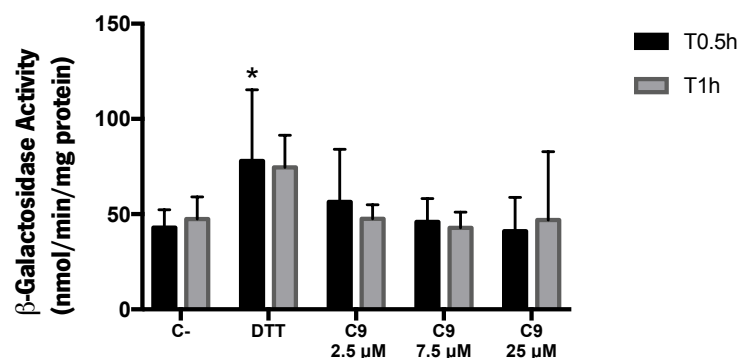


Figure 4.7-2: Assessment of unfolded protein response (UPR) machinery induction on endoplasmic reticulum (ER) in response to C9 treatment. BF264-15D cells harbouring an UPR::lacZ reporter (pJT30) were incubated with C9 (2.5, 7.5 and 25 μ M) or DTT (6 mM) as a positive control for 1h. For negative control untreated cells were used. After cell lysis and protein quantification, ONPG, the β -galactosidase substrate, was added to the samples. At two different time points (0.5 and 1 hour), OD_{405} was measured and the β -galactosidase activity was calculated. The reported values represent means $\pm$ SD of at least three independent experiments. Statistical analysis was performed by the two-way ANOVA test. * $P < 0.05$, cells treated with DTT compared with the negative control.

After treating the cells with various concentrations of C9 (2.5, 7.5 and 25 μ M) it was observed that the value of the β -galactosidase activity of cells treated with the compound were not significantly different from the values of cells from the negative control, in contrast to the positive control cells (treated with DTT). Thus, results suggest that C9 does not lead to the induction of the UPR, possibly meaning that it is not an ER-stress inducer.

5. Discussion and Future perspectives

5.1 Discussion and Future perspectives

Over the course of these last few years benzo[*a*]phenoxazines have proven to be remarkable compounds with many potentialities but still largely under-explored and insufficiently investigated. Interestingly, many compounds of this family have been synthesized and characterized, either chemically or biologically, by our group. As already mentioned, these compounds have a plethora of activities but our group has been focused on the ones that present an interesting potential as antifungal agents (Frade *et al.*, 2007). After many preliminary and screening studies of the many compounds that were synthesized, a specific compound emerged as the first one to have a great potential as an antifungal agent. BaP1, previously named MSG-111-cd3, exhibited a high antifungal activity (Carvalho *et al.*, 2011). After many studies on BaP1, it was reported that it accumulates in the membranes of the vacuole and of the ER, and it is able to induce a regulated cell death process with the involvement of vacuolar membrane, that becomes permeabilized upon treatment with this compound. Also, the protease Pep4 would later be revealed as an effector protein on this cell death process, since it was shown that strains lacking this protein were more resistant to BaP1 toxic effects (Ferreira, 2017; Lopes, 2015). Although this compound was very interesting, another one emerged, also with great potentialities. More potent and very similar to BaP1 in structural terms, C9 had only a difference in a functional group of its core aromatic system, in comparison with BaP1. Interestingly, this apparently small structural difference was responsible for an increase of the antifungal activity exhibited by this compound, when compared to BaP1. Thus, our group was now interested in uncovering the mechanism of action of this compound. Similarly, to BaP1, C9 was also accumulating at the vacuolar and ER membranes, which meant that, apparently, the structural difference between the compounds did not change their intracellular distribution. Studies conducted by our group showed that C9 was also able to induce vacuolar permeabilization, just like BaP1. Conversely, in the case of C9, Pep4 protease was having a protector role, rather than an effector one. Also, mitochondrial morphology was affected by this compound, which was not reported for BaP1. Moreover, the metacaspase Yca1p seemed to mediate the cell death process induced by this compound, since yeast strains deficient in this protein were more resistant to C9. Thus, it is clear that a single structural difference between the two compounds was making a big difference in their mode of action (Leitão, 2015; Sousa, 2018).

In this present work, we wanted to look at other aspects of C9 effect on cells in order to have a wider view of the structures, organelles or cellular processes that are being targeted by it.

First, we wanted to confirm the intracellular distribution of C9. It was indeed possible to demonstrate that this compound accumulates at the ER membrane. However, due to problems with the yeast strain expressing a GFP-tagged vacuolar membrane protein, we were not able to confirm that C9 was indeed accumulated at the vacuolar membrane. Possibly, the strain had already lost the plasmid carrying the GFP gene or there was a problem at the transcription level of the GFP protein itself. Thus, in the future, a new strain with a GFP-tagged vacuolar membrane protein must be constructed.

We next aimed to focus on the involvement that C9 could have with the ER rather than with the vacuole, which was the focus of previous studies. We believed that the accumulation of C9 and other potent benzo[*a*]phenoxazines in the ER membrane could not be harmless and maybe, one of its targets could, indeed, be located in the ER membrane. The base of our line of thought was a specific study conducted by our group which suggested that the benzo[*a*]phenoxazines with higher accumulation at the ER membranes seemed to be more toxic. As C9 distributes all over the ER membrane maybe it is targeting one of these enzymes, namely, lanosterol synthase/oxidosqualene-lanosterol cyclase, an essential enzyme involved in the ergosterol biosynthetic pathway. Molecular docking studies targeting this enzyme revealed that the more potent compounds were the ones which had a similar structure to the lanosterol synthase/oxidosqualene-lanosterol cyclase ligand, lanosterol. Thus, they have a higher affinity for the active site of the enzyme and, therefore, have high probabilities of being lanosterol synthase/oxidosqualene-lanosterol cyclase inhibitors (unpublished data). Although this was strictly an *in silico* study, it was a very interesting one and for that reason we tried to test this hypothesis. If this enzyme was, in fact, the target of the compound, then ergosterol production would be affected and the growth of the cells would be impaired. In line with this, we evaluated the growth of *S. cerevisiae* cells in the presence of externally supplemented C9 and ergosterol, alone or combination. As we expected, cells treated with C9 alone had their growth rate impaired due to the compound's toxicity. On the other hand, cells treated with ergosterol alone did not have their growth affected. Surprisingly, when we treated cells with C9 in combination with externally added ergosterol their growth rate was lower than when treated with C9 alone. This result suggests that, contrarily to what we had hypothesized, the addition of ergosterol to the medium exacerbates C9's toxicity. This can indicate that the negative effect of C9 on cell's growth is not due to a limiting ergosterol concentration, but rather aggravated with increased ergosterol concentration. It would be interesting to conduct the same assay but with lower concentrations of ergosterol. Moreover, it could also be interesting to construct a diploid strain deficient in, for instance, the *ERG7* gene, which codes for

lanosterol synthase/oxidosqualene-lanosterol cyclase enzyme and study the effect of C9 in the growth of this strain. However, since *ERG7* is an essential gene, the strain for this study would have to be diploid and an effect of *ERG7* deletion on growth may be unclear (Lees *et al.*, 1995).

As external ergosterol seemed to exacerbate the toxic effect of C9 on cell's growth, we next aimed to know what would be the role of ergosterol in C9-induced cell death. Thus, we assessed the viability of cells either treated with C9 alone, ergosterol alone or the combination of both. The results showed that, again, as expected, C9 alone induces loss of cell viability. Ergosterol alone does not seem to affect the viability of the cells. However, when cells were treated with the combination of C9 and ergosterol their viability had a slight increase over the course of the assay, when compared with treatment with C9 alone, but this increase was not statistically significant. This indicates that ergosterol supplementation is not able to protect the cells from C9-induced cell death as well. In the future, it would also be important to check the viability of strains lacking *ERG* genes and see if their viability would increase upon treatment with C9 alone and in combination with ergosterol.

Another group from our laboratory had recently constructed a reporter yeast strain for detection of azole compounds in aquatic samples. This reporter system is meant to detect the presence of tebuconazole and other azole fungicides whose target is already known: *ERG11* gene, that codes for cytochrome P450-dependent lanosterol 14 α -demethylase enzyme, an essential enzyme in the biosynthetic ergosterol pathway. Basically, when the reporter strain is in the presence of azole compounds, the *ERG11* promoter, which is regulating the expression of a reporter protein called luciferase, is activated, leading to the transcription and translation of this protein, that, when in contact with its substrate, emits bioluminescence. (Carvalho, 2018; Masser *et al.*, 2016). Although this reporter strain was constructed for ecologic applications, it can also be used to check if a specific compound is affecting the expression of *ERG11*, which could be suggestive of ergosterol depletion. In line with this, we have employed this reporter system to understand if C9 could be targeting *ERG11*. If so, this could indicate that C9's treatment was leading to ergosterol's depletion. The overall results seem to show that C9 is not leading to the depletion of ergosterol, since there was not an induction of *ERG11* gene expression. However, the results may not be as accurate as they could be because the luciferase substrate was very unstable and it is possible that, in some of the replicates, the substrate was not functioning correctly. Although the Nano-Glo substrate coupled with NanoLuc (the luciferase that is expressed by the reporter yeast strain) seems to be one of the best reporter assay systems nowadays, it

has to be employed very carefully (Brescia, Banks, & Landreman, 2016). Thus, in the future, and if this reporter assay system continues to be one of the most well-suited, it may be necessary to repeat this assay but with more restrictions. For example, for each independent assay, a fresh solution of luciferase substrate should be made in order to make sure that the substrate is unaltered.

We have also questioned if C9 could be targeting cellular membrane ergosterol. To evaluate if extraction of ergosterol from the cellular membrane could, somehow, play a protector role against C9-induced cell death, cells treated with two concentrations of C9 were exposed to a sterol-depleting agent, methyl- β -cyclodextrin (M β CD), that would extract the ergosterol from the plasma membrane. Then, the viability of such cells was evaluated. Interestingly, the results revealed that the extraction of ergosterol from the membrane renders the cells more resistant to C9's treatment. In literature, there are many reports stating that cyclodextrins, specifically, methyl- β -cyclodextrin, have been gaining attention due to their possible applications as drug delivery systems, since the interior of these cyclic oligosaccharides is hydrophobic and can encapsulate molecules that have low affinity with aqueous environments, thus having problems entering the cells (Gómez *et al.*, 2018; Jóhannesson, Stefánsson, & Loftsson, 2016). C9 is a relatively hydrophilic compound, thus having no difficulties in entering the cells. Therefore, C9 will not have a high affinity with methyl- β -cyclodextrin's lipophilic interior. In line with this, it is acceptable to think that treatment with M β CD would not exacerbate C9's effects, at least from this perspective. Thus, it seems reasonable to suggest that a possible reason why M β CD would play a protective role against C9-induced cell death is because, by extracting membrane ergosterols, it is, somehow, preventing C9 from targeting it. Thus, it would be interesting to further understand if it is possible to validate this hypothesis. For instance, this could be achieved through a fluorescence microscopy assay, in which the intracellular distribution of C9 should be observed, in order to understand if, after M β CD's treatment, C9 is located in the ER and vacuolar membranes, or not.

In line with the previous results, we went on to visualize the behaviour of cellular membrane lipid rafts after treating the cells with C9 alone and in combination with M β CD. In this fluorescence microscopy assay, cells were stained with filipin, a polyene antibiotic that exhibits fluorescent properties and that bind to sterols, in this case, to ergosterol, one of the major constituents of lipid rafts. This assay was supposed to be a quantitative assay, in order to understand which is the real phenotype displayed by the cells in each condition. However, filipin is an extremely unstable probe, suffering photobleaching very easily, even with the presence of Vectashield, a liquid medium that is applied to microscope slides in order to prevent the probes from suffering photobleaching caused by light exposure. We were not able to phenotypically

quantify all the conditions since some of them had already lost the filipin fluorescence at the time of the microscopic observation. Thus, in the future, it is mandatory to perform this assay during a lower period of time. Despite all of these problems, we still analysed the obtained images in order to, at least, understand what is the major phenotype displayed by the cells in each condition. The validation of the obtained results became impaired due to the fact that our positive control was not functioning correctly. It is important to have in mind that these results are preliminary. Thus, in the future, it is mandatory to repeat these assays. Probably, the positive control did not function correctly because it needed more time to have an effect on the cells, which is something to consider further on. A good example of how M β CD is supposed to act is reported in a very interesting assay published by Pacheco and co-workers (Pacheco *et al.*, 2013).

The fact that C9 accumulates mostly at the ER membrane can indicate that this organelle's function may be impaired after the cell's exposure to C9. In terms of the organelles' morphology, C9 does not seem to be disrupting its organization. Thus, C9 could be impairing cellular processes that take place in the ER and inducing ER stress. As no one had addressed this question before, we evaluated whether C9 could be causing ER stress. For this, we used an indirect assay that relates β -galactosidase activity with the induction of the UPR, a cellular process that is initiated upon ER stress. The obtained results seem to indicate that C9 is not an ER-stress inducer, since there is no induction of UPR in cells treated with the compound. To further confirm if C9 is not, indeed, an ER-stress inducer, viability of cells with deletions in *HAC1* and *IRE1* genes, which are very important for the UPR process, should be evaluated, upon treatment with C9. If C9 is not, in fact an inducer of ER stress, then these deficient strains should not be more resistant to the treatment. Another way to validate the hypothesis that C9 is not a stress inducer is to check if there is splicing of the transcription factor Hac1, using, for this matter, an RT-PCR assay. If there is no induction of the UPR, then there is no splicing of Hac1 (Chaillot *et al.*, 2015). Aside from ER stress, there are also other important cellular processes occurring in the ER that could be affected by the action of C9. For instance, a crucial process that occurs in the ER is protein N-linked protein glycosylation, a post-translational modification (Wang *et al.*, 2017). One of the assays that could be used to assess whether this important process is being affected or not by C9, is Western blotting, since glycosylation confers a higher molecular weight to the protein: the non-glycosylated proteins will migrate faster than glycosylated ones (Buentzel & Thoms, 2017). Tunicamycin would have to be used as a negative control, as it inhibits N-linked glycosylation (Samali *et al.*, 2010).

In essence, we demonstrate that, contrarily to what we first purposed, an external supplementation of ergosterol to the medium during C9's treatment does not act as a protection against C9's deleterious effects. Instead, it increases C9's ability to provoke growth impairment. However, this external supplementation of ergosterol is neither deleterious to the viability of the cells, nor protective. Furthermore, cytochrome P450-dependent lanosterol 14 α -demethylase does not seem to be a target of C9. Thus, it is possible that C9 is not targeting enzymes existing in the ER membrane and important for ergosterol synthesis, as we first proposed, although the induction of other enzymes of the pathway should be tested. On the other hand, it is possible that C9 is, instead, targeting cellular membrane ergosterol, since the extraction of ergosterol from the cell's membrane rendered the cells more resistant to C9-induced cell death.

Although C9 accumulation occurs mainly at the ER membrane there is no disruption of the morphology of the organelle, as observed by the first images of C9 localization in the ER membrane. Moreover, C9's mechanism of action is not mediated by ER stress, since we employed an assay to evaluate UPR induction after C9's treatment and the results show that UPR is not induced.

Hopefully, and although there are still many questions to be asked and answered about this compound, this work will lead us in new directions in order to fully understand how it is able to exert its antifungal activity. We hope that the different approaches employed in the present work will help us to achieve our ultimate goal, which is the use of benzo[*a*]phenoxazines as potent antifungal agents

6. References

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7. Supplementary data

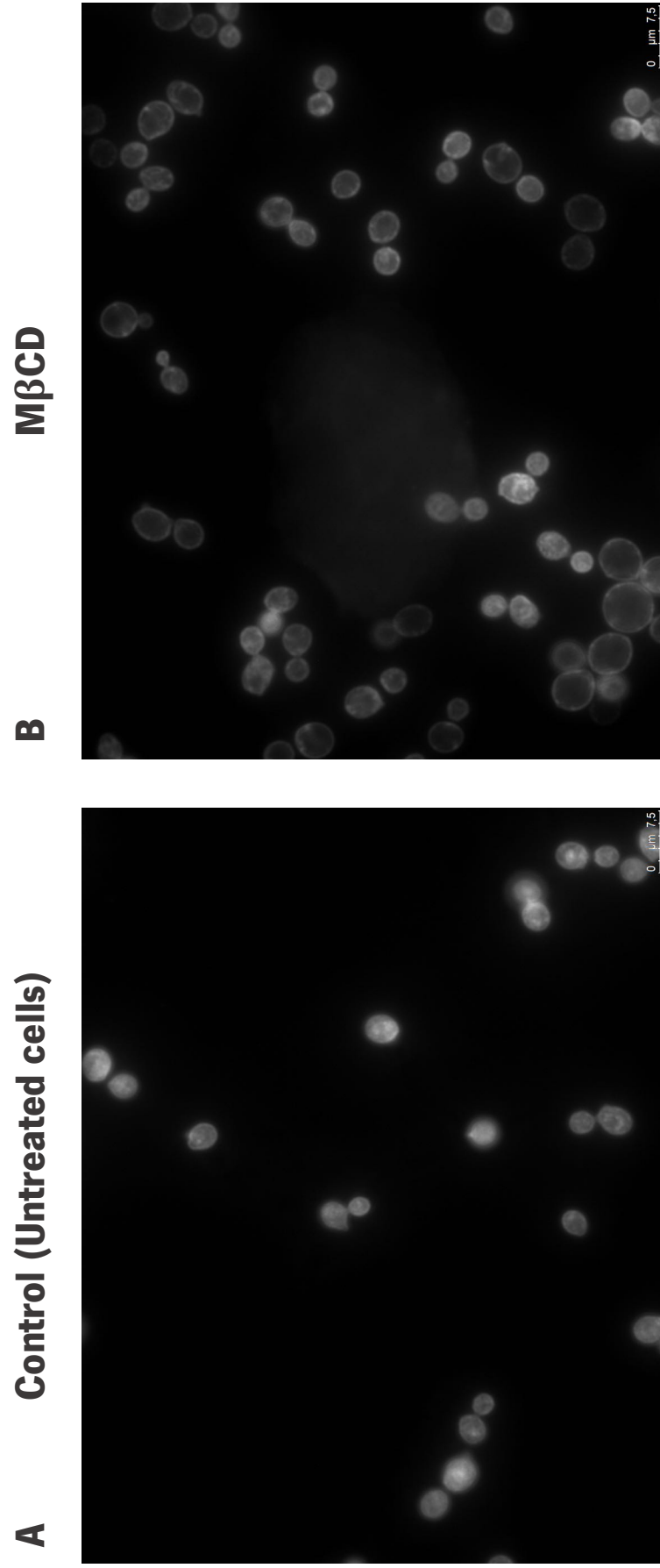
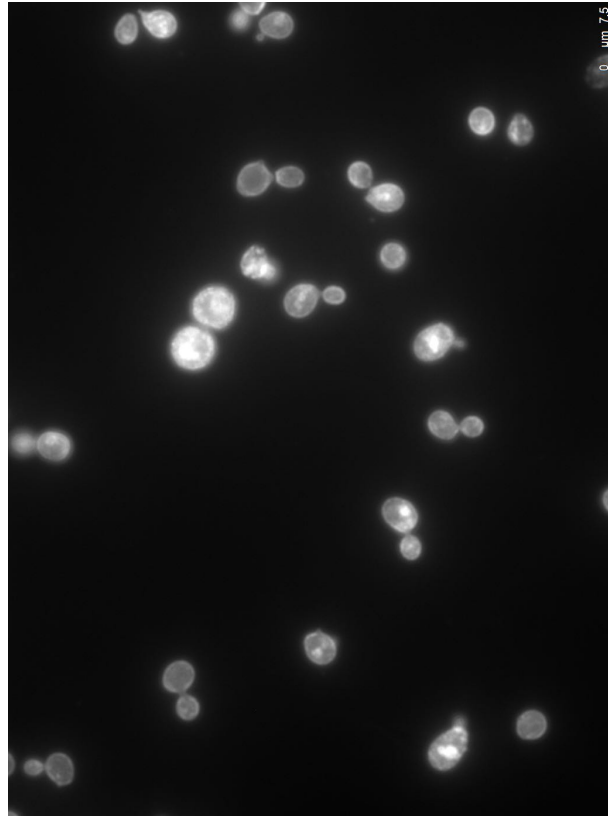


Figure 5.1-1: Distribution of membrane ergosterol in untreated cells and cells exposed to and methyl-β-cyclodextrin. Representative images of W303-1A WT cells that were incubated with 25 μM of C9, 50 mg/mL of methyl-β-cyclodextrin, and the combination of the two compounds for 1 h. Then, they were stained with filipin during 15 minutes in the dark and finally, they were visualized using fluorescence microscopy. **(A)** untreated cells; **(B)** cells treated with MβCD.

C C9 (25 μ M)



D C9 (25 μ M) + M β CD

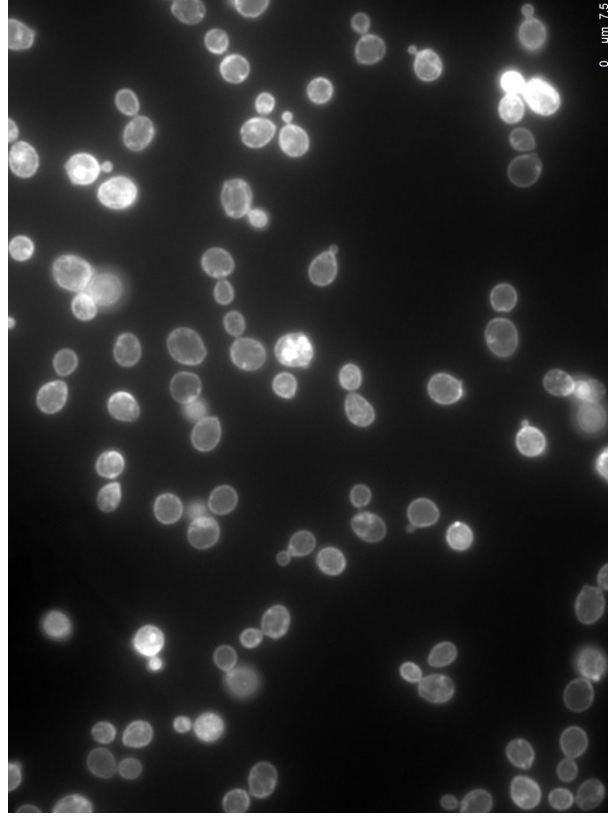


Figure 5.1-2: Distribution of membrane ergosterol in untreated cells and cells exposed to and methyl- β -cyclodextrin. Representative images of W303-1A WT cells that were incubated with 25 μ M of C9, 50 mg/mL of methyl- β -cyclodextrin, and the combination of the two compounds for 1 h. Then, they were stained with filipin during 15 minutes in the dark and finally, they were visualized using fluorescence microscopy. **(C)** cells treated with 25; μ M of C9 **(D)** cells treated with M β CD + 25 μ M of C9