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Antifungal Activity and/or Inhibition of Mechanisms of Resistance of a Small Library of Natural and Synthetic Compounds

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Atividade Antifúngica e/ou Inibição de Mecanismos de Resistência de uma Pequena Biblioteca de Compostos Naturais e Sintéticos

Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Química Farmacêutica

Trabalho realizado sob a orientação da Professora Maria Eugénia Ribeiro Pinto e da Professora Madalena Maria Magalhães Pinto

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"A admiração é filha da ignorância, porque ninguém se admira senão das coisas que ignora, principalmente se são grandes; e mãe da ciência, porque admirados os homens das coisas que ignoram, inquirem e investigam as causas delas até as alcançar, e isto é o que se chama ciência."

Padre António Vieira

Author's declaration

Under the terms of the Decree-Law nº 216/92, of October 13th, is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work and interpretation of the results included in this dissertation. Under the terms of the referred Decree-Law, is hereby declared that the following articles/communications were prepared in the scope of this dissertation.

Poster Communications - Original research

"Xanthone Derivatives with Antifungal Potential for Mycoses Treatment", *XX Encontro Luso Galego de Química*, 26-28 November 2014.

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Resumo

Nos últimos anos, tem-se assistido a um aumento da incidência de infeções fúngicas invasivas, particularmente as que são causadas por *Candida* spp., sendo estas frequentemente resistentes ao fluconazol e a outros azóis. O número de antifúngicos disponíveis é limitado, e além disso, embora existam já antifúngicos eficazes contra alguns fungos, a resistência intrínseca ou adquirida, exibida por alguns deles, obriga ao desenvolvimento de novos compostos com atividade antifúngica ou que sejam capazes de inibir alguns mecanismos de resistência. A resistência de *Candida albicans* aos azóis deve-se, muitas vezes, à reduzida acumulação dos fármacos no interior das células fúngicas devido ao efluxo ativo dos mesmos por bombas de efluxo.

Assim sendo, ao longo deste trabalho de investigação foi testada uma pequena biblioteca de compostos orgânicos naturais e sintéticos – chalconas e xantonas – para avaliar o seu potencial quer como antifúngicos, quer como inibidores das bombas de efluxo. Dos 15 compostos testados inicialmente, nenhum apresentou um valor de concentração mínima inibitória (MIC) relevante, sendo sempre superior ao que é observado com fluconazol. Assim, dos compostos testados, nenhum foi considerado como potencial antifúngico a desenvolver para o tratamento de micoses. Por outro lado, em termos de inibição das bombas de efluxo, dos 8 compostos testados, apenas a utilização de amiodarona em combinação com o fluconazol, a pH 5.0, apresentou valores de MIC muito inferiores aos obtidos com a utilização do fluconazol isoladamente.

Palavras-chave: *Antifúngicos; Inibidores de Bombas de Efluxo; Fluconazol; Mecanismos de Resistência.*

Abstract

In the last years, it has seen an increase in the incidence of invasive fungal infections, particularly caused by *Candida* spp., which are frequently resistant to fluconazole and others azoles. There are a limited number of antifungals available, and moreover, the intrinsic or acquired resistance exhibited by some of these fungi requires the development of new compounds with antifungal activity or those which are able to inhibit the mechanisms of resistance. Manifold mechanisms of azole resistance in *Candida albicans* including reduced accumulation of the drugs through active efflux by efflux pumps.

Therefore, throughout this work research, a small library of natural and synthetic compounds was tested, to assess their potential as antifungals or as efflux pump inhibitors (EPIs). From 15 compounds tested, none of them showed a minimum inhibitory concentration (MIC) relevant, and always higher than that observed with fluconazole. Therefore, none of these compounds were considered with antifungal potential to develop for the mycoses treatment. On the other hand, related to the efflux pump inhibition, from 8 compounds tested, only the use of amiodarone in combination with fluconazole, pH 5.0, exhibited a significant decrease in MICs.

Key words: *Antifungals; Efflux Pump Inhibitors; Fluconazole; Mechanisms of Resistance.*

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List of Abbreviations and Symbols

ABC – ATP-Binding Cassete

AmB – Amphotericin B

AMD – Amiodarone

ATCC – American Type Culture Collection

ATP – Adenosine Triphosphate

AZD – Sodium Azide

CEQUIMED-UP – *Centro de Química Medicinal da Universidade do Porto*

CFU – Colony-Forming Unit

DMSO – Dimethyl Sulfoxide

EPI – Efflux Pump Inhibitor

FLC – Fluconazole

GC – Growth Control

MDR – Multidrug Resistance

MFS – Major Facilitator Superfamily

MHB – Mueller-Hinton Broth

MIC – Minimum Inhibitory Concentration

MW – Microwaves

NBD – Nucleotide Binding Domain

NMR – Nuclear Magnetic Resonance

QC – Quality Control

RPMI – Medium "Rosewell Park Memorial Institute"

RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

SAR - Structure Activity Relationship

SC – Sterility Control

SDD – Sensitive Dose Dependent

TMS – Transmembrane Segment

VRP – Verapamil Hydrochloride

Outline of the Dissertation

The present dissertation is organized in five main parts:

Chapter I. - Introduction

In this chapter, a brief introduction of some key concepts about mycoses, the main microorganisms responsible for these diseases, as well as the therapy that is currently used. A brief description of the mechanisms of resistance to antifungals and possible ways to overcoming these mechanisms. In this chapter, it is also addressed the chemistry of xanthones and chalcones as well as their importance in Medicinal Chemistry.

Chapter II - Material and Methods

In this chapter it is explained the methods and techniques used throughout this work.

Chapter III – Results and Discussion

Here are presented all the results obtained and the discussion about them, as well as the explanation of the reasons why it may have occurred.

Chapter IV – Conclusion and Future Work

In this chapter it is done a consideration about the dissertation and the relevance of the issue addressed. It is also proposed some interesting new and different ideas and techniques to explore the results already obtained and to get better results and explanations.

Chapter V - References

It is described all the references that were used along this work, which were obtained from: PubChem (Open Chemistry Database), PubMed, Science Direct and EBSCO. All the chemical structures were designed by using ChemBioDraw®.

Chapter I

Introduction

Introduction

Infectious diseases represent one of the most important challenges due to the very high levels of morbidity and mortality they cause[1]. At the beginning of 20th century, bacterial infections were the most important cause of mortality, but around 1950s, when antibiotic therapies and cancer treatment were developed, a severe rise in fungal infections was observed[2-3]. Nowadays, treating human infectious diseases remain as a colossal health problem[1], despite the advances in clinical medical mycology since the beginning of 21st century[3].

Fungal infections are caused by two main types of microorganisms, primary or opportunistic pathogens. Primary pathogens are those which are able to establish an infection in the healthy immunocompetent individuals. In contrast, opportunistic pathogens, among them commensal microorganisms, are able to develop infectious colonization of the human body when particular criteria, undermentioned, exists[2].

Fungal pathogens can be divided into two main groups, filamentous fungi and yeasts. Filamentous fungi are formed by cylindrical and filamentous cells called *hyphae*, and a mass of *hyphae* is termed a *mycelium*. Yeasts are the simplest fungi and they are unicellular[3]. Most of the primary pathogens are filamentous fungi, while most of the opportunistic pathogens are yeasts and some species of filamentous fungi. Fungal infections can be also classified according to the tissue infected (Table 1). Superficial mycoses are limited to the most external part of the skin and hair; cutaneous mycoses, caused by dermatophytes, affect keratinized structures of the body, subcutaneous mycoses are caused by implantation of spores when, for example, occurs a puncture; mucosal infections are mostly caused by opportunistic yeasts, and systemic mycoses may involve any part of the body[3]. This last type of fungal infection with symptoms ranging from a simple fever to a severe and rapid septic shock, is very common in immunocompromised patients and is frequently associated with an elevated mortality rate[2]. *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* are the most common causes of invasive fungal infections[4], such as disseminated candidiasis, cryptococcal meningitis and invasive aspergillosis[3, 5].

Table 1 – Classification of mycoses based on the primary site of pathology[2-3]

Body Location	*Pathogen Type*	*Organ*	*Associated Genus*
Superficial	Primary	Skin and Hair	*Malassezia*
Cutaneous	Primary	Skin, Hair and Nails	*Trichophyton* *Epidermophyton* *Microsporum*
Mucosal	Opportunistic	Digestive Tract, Eye, Urinary Tract and Vagina	*Candida*
Systemic	Opportunistic	Any organ	*Candida* *Aspergillus* *Cryptococcus*

Candida albicans is a normal commensal of human mucosal surfaces and one of the most frequently observed opportunistic human fungal pathogen causing mucosal and systemic infections in individuals with compromised immune defenses[6-8]. Other non-*albicans Candida* species associated with biofilm formation and device-related infections include *C. glabrata* and *C. parapsilosis*. These types of infections can be superficial and affect the skin or mucous membranes, or can be disseminated by blood circulation with consequences. Therefore, systemic fungal infections are hard to diagnose, difficult to treat and may require both long-term antifungal therapy and the physical removal of the device to control the infection[9], contributing to their high morbidity and mortality[8].

In the last years, it has seen an increase in the incidence of invasive fungal infections, particularly caused by *Candida* spp., which are frequently resistant to fluconazole (FLC) and others azoles[7, 10-12]. Nowadays, invasive mycoses represent an exponentially growing threat due the difficult diagnosis and unavailability of effective antifungal drugs[13].

Paradoxically, medical progress has led to an expanding population of susceptible hosts with impaired immunological defenses against infection in the community and hospitals[14]. The use of broad-spectrum antibiotics, parenteral nutrition, indwelling catheters, the presence of immunosuppression, or disruption of mucosal barriers due to surgery, radiotherapy and chemotherapy, are among the most important predisposing factors for invasive fungal infection[9, 12].

The increasing resistance of pathogenic fungi to antifungal compounds and the reduced number of available drugs led to the search for therapeutic alternatives, also among natural products, including xanthones and chalcones[15]. Therefore, permanent drug discovery and research programs are still required[1].

Antifungal agents available

An ideal antifungal agent should be active and highly selective against a fungal-specific target, fungicidal rather than fungistatic due to its mechanism of action, with suitable ADME properties – absorption, distribution, metabolism and excretion – and proper for formulation by both oral and intravenous route[16].

There are a limited number of antifungals available, most of them only provide fungistatic but not fungicidal effects[6] (Table 2). Similarly, there are relatively few agents that can act only on targets not shared with human hosts, such as fungal cell wall because this is not present in human cells[17].

Table 2 – Topical and systemically active antifungal agents[3]

Antifungals		*Target Site*
Allylamines	Terbinafine	Ergosterol biosynthesis – squalene epoxidase
Azoles	Fluconazole, Itraconazole, Voriconazole, Posaconazole, Ketoconazole	Ergosterol biosynthesis – 1-4-α-demethylase
Echinocandins	Caspofungin, Anidulafungin, Micafungin	β-(1,3)-glucans synthesis in cell wall
Fluorinated Pyrimidine	5-Fluorocytosine	DNA, RNA and protein synthesis
Polyenes	Amphotericin B (lipid formulations) and Nystatin	Ergosterol in cell membrane

Allylamines

The class of allylamines acts by inhibition of the squalene epoxidase enzyme, resulting in a decrease in ergosterol and an increase in squalene concentration within the fungal cell membrane, damaging it[3, 18]. Squalene epoxidase is an enzyme of the ergosterol biosynthetic pathway that catalyzes the epoxidation of squalene to 2,3-oxidosqualene in fungi[19]. Terbinafine (Fig. 1, $C_{21}H_{15}N$) is an example of this class of antifungals, being a lipophilic agent that accumulates in skin, nails and fat tissues, from which is slowly released. It is an active agent in oral and topical formulations[3], and it has shown promise for the treatment of infections caused by FLC-resistant *Candida* strains when used in combination with FLC[18].

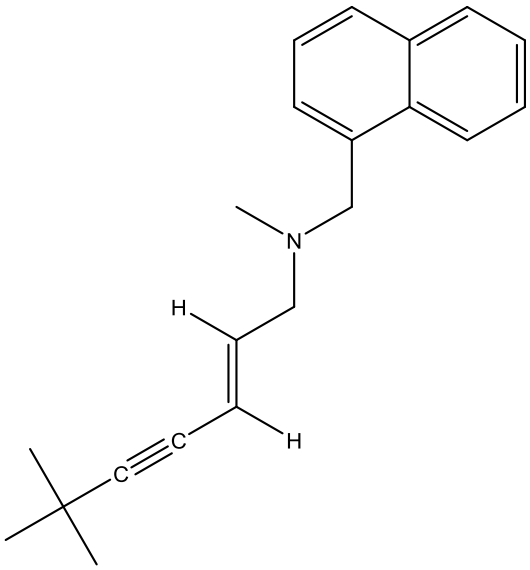

Figure 1 - Chemical structure of terbinafine

Azoles

Several azoles, which act by the inhibition of the enzyme lanosterol 14-α-demethylase, important to ergosterol biosynthesis pathway (Fig. 2), were widely used to treat *Candida* infections[18, 20].

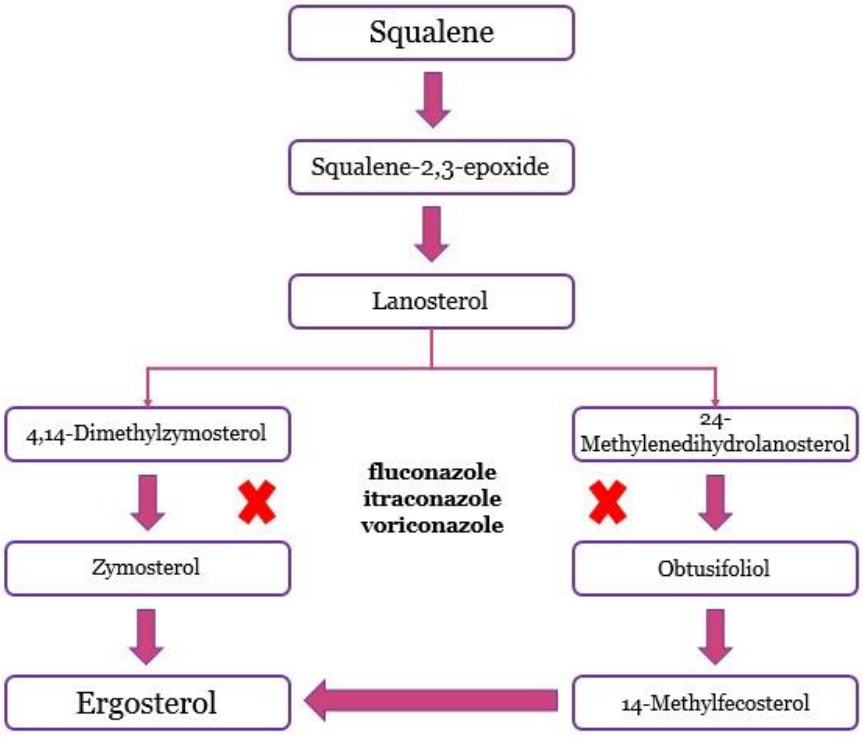

Figure 2 - Ergosterol biosynthetic pathway in fungi[3, 12, 21]

Ergosterol is a necessary sterol, important for maintaining the structural integrity of the fungal cell membrane. Inhibition of lanosterol 14-α-demethylase leads to depletion of ergosterol, which cause the formation of membranes with altered structure and function, and accumulation of sterol precursors[12], which can lead to the destruction of the fungal cell.

The azole class of antifungal agents can be divided based on the structure into imidazoles – two nitrogens in the azole ring – and triazoles – three nitrogens in the azole ring.

Among the imidazoles, the only antifungal agent used systemically is ketoconazole (Fig. 3, $C_{26}H_{28}Cl_2N_4O_4$).

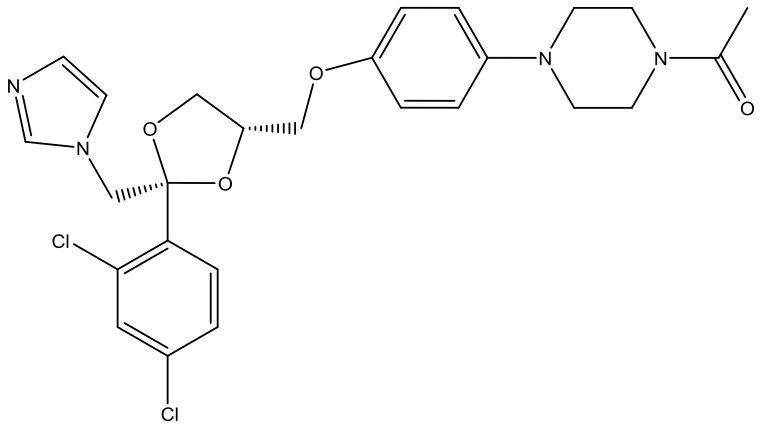

Figure 3 - Chemical structure of ketoconazole

The triazoles have systemic activity and include fluconazole (Fig. 4, $C_{13}H_{12}F_2N_6O$), itraconazole (Fig. 5, $C_{35}H_{38}Cl_2N_8O_4$), voriconazole (Fig. 6, $C_{16}H_{14}F_3N_5O$), and posaconazole (Fig. 7, $C_{37}H_{42}F_2N_8O_4$)[18].

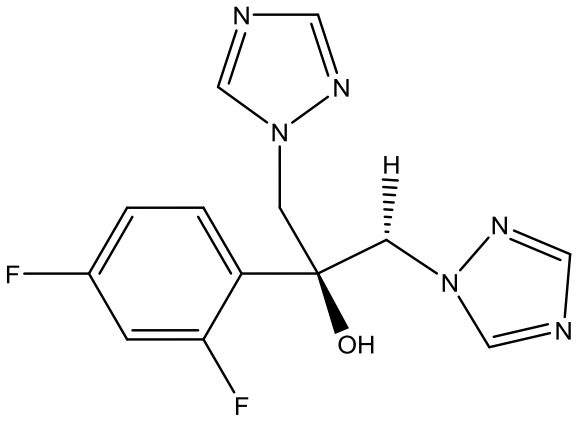

Figure 4 – Chemical structure of fluconazole

FLC is one of the most widely used antifungal agents, both for prophylaxis and therapy of *Candida* infection[6, 11]. The azole used as standard throughout this work was FLC, a first generation triazole with good oral bioavailability and low toxicity. This is a water-soluble agent and may be administered orally or intravenously, it has low protein binding and it is distributed to all organs and tissues, including the central nervous system[18].

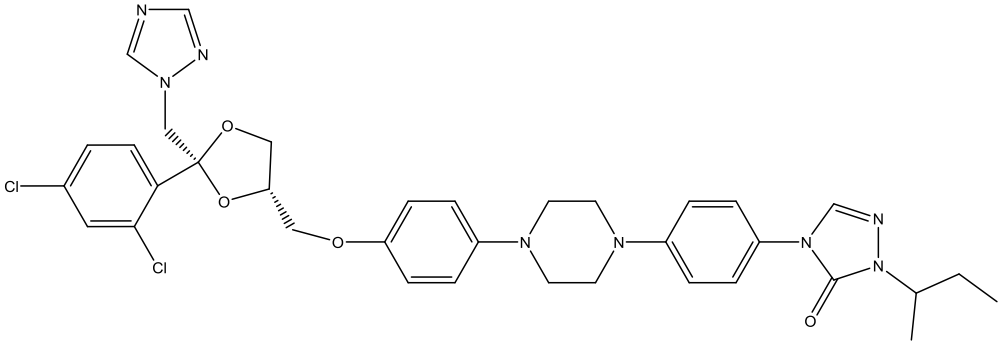

Figure 5 - Chemical structure of itraconazole

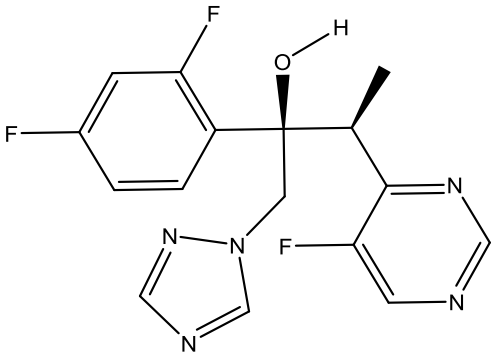

Figure 6 - Chemical structure of voriconazole

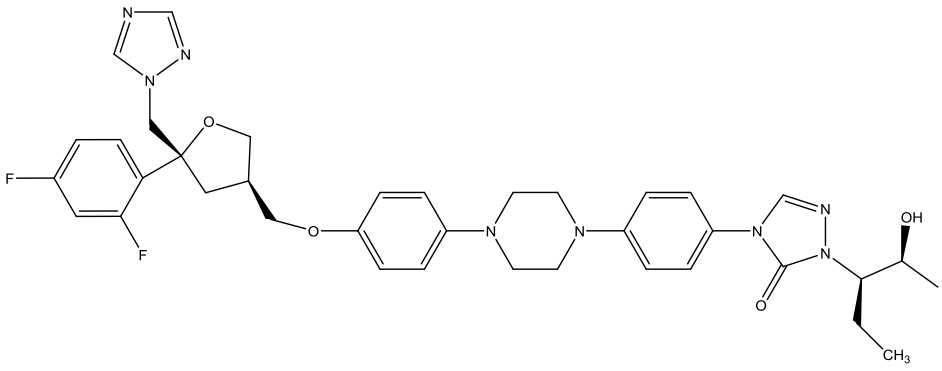

Figure 7 - Chemical structure of posaconazole

Isavuconazole (Fig. 8, $C_{22}H_{17}F_2N_5OS$) and albaconazole (Fig. 9, $C_{20}H_{16}ClF_2N_5O_2$) are recent azoles, undergoing clinical trials[22-23].

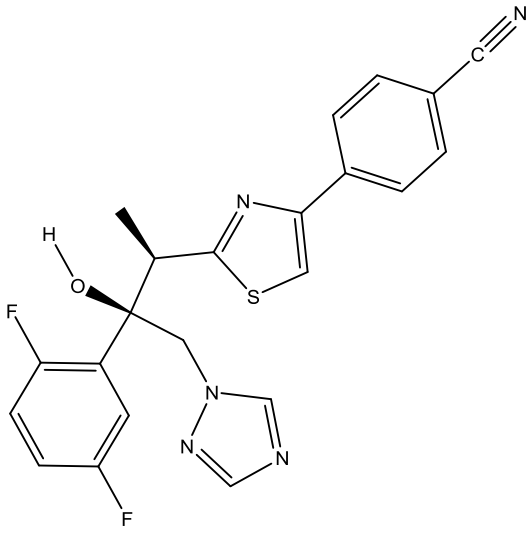

Figure 8 - Chemical structure of isavuconazole

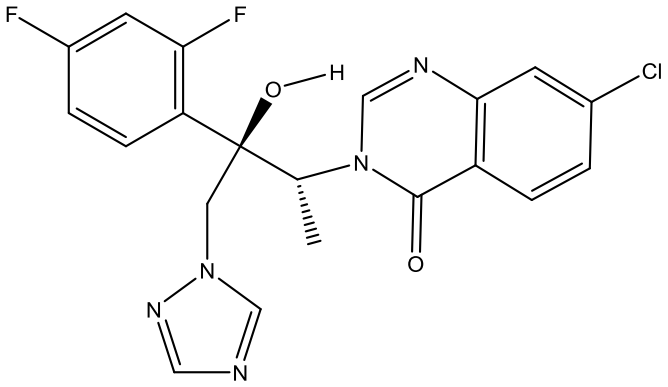

Figure 9 - Chemical structure of albaconazole

Echinocandins

Echinocandins are a high selective class of semisynthetic lipopeptides and its mechanism of action consists on non-competitive inhibition of synthesis of β-(1,3)-glucans, which are important constituents of the fungal cell wall. Since mammalian cells do not contain these constituents, this class of antifungal agents is selective and toxic only to fungi[4]. The glucans play an important role in maintenance of the osmotic integrity of the fungal cell, and are also needed to cell division and cell growth[18]. The first licensed echinocandin was caspofungin in 2001, followed by micafungin in 2005, and anidulafungin in 2006[4].

Caspofungin (Fig. 10, $C_{52}H_{88}N_{10}O_{15}$) is a water-soluble amphipathic lipopeptide with a molecular mass of 1213 kDa, being a semisynthetic derivative of the fermentation product of *Glarea lozoyensis*[3, 24].

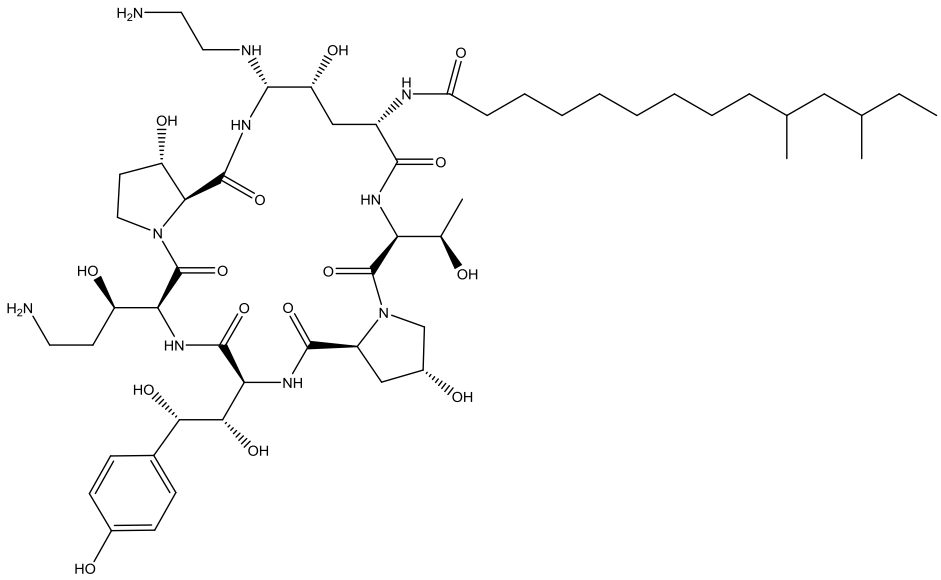

Figure 10 - Chemical structure of caspofungin

Micafungin (Fig. 11, $C_{56}H_{71}N_9O_{23}S$) is a promising echinocandin, a water-soluble antifungal agent which is derived from *Coleoptioma empedri,* a natural product of the fungi via enzymatic cleavage of a hexapeptide. The addition of a fatty N-acyl side chain improves its antifungal activity[25]. According to *Barrett, 2002*, and *Mikamo, 2000*, bearing a fatty acid acyl group on the N-terminal moiety lead to excellent water solubility by virtue of the presence of a sulfonate moiety on the homotyrosine residue. This molecular modification displayed more potent inhibition of β-1,3-glucan synthase from *C. albicans*[26-27].

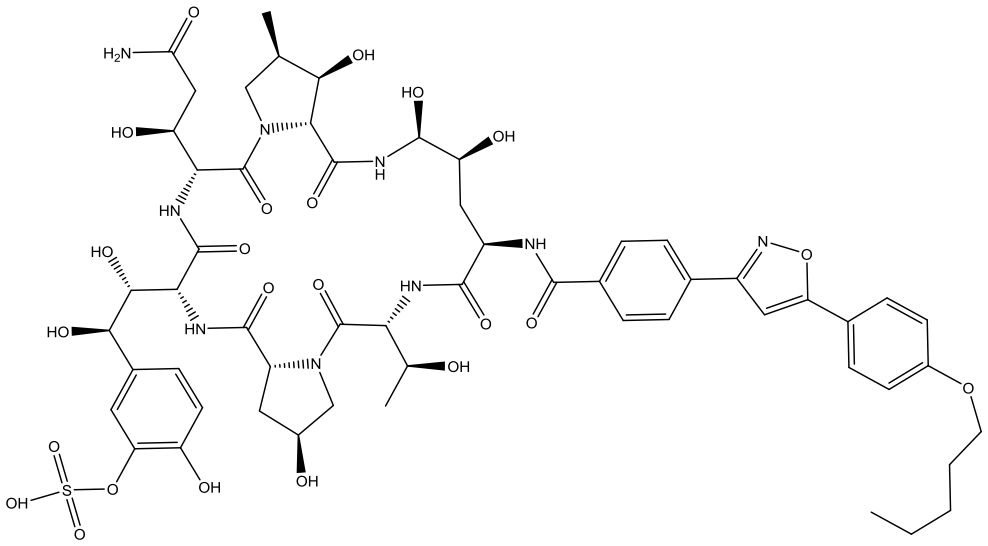

Figure 11 - Chemical structure of micafungin

Anidulafungin (Fig.12, $C_{58}H_{73}N_7O_{17}$) is a semisynthetic product from fermentation of *Aspergillus nidulans*[3].

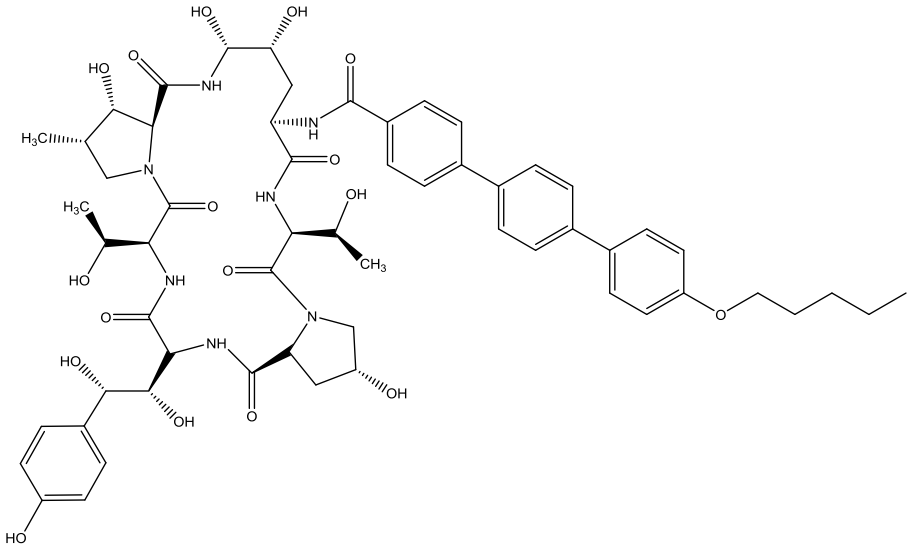

Figure 12 – Chemical structure of anidulafungin

Fluorinated Pyrimidine

5-Fluorocytosine (Fig.13, $C_4H_4FN_3O$) is the only available antifungal agent that acts as an antimetabolite. This molecule interferes with the DNA, RNA and protein synthesis in the fungal cell because it is a fluorinated pyrimidine analogue. This synthetic compound is water soluble and has an excellent bioavailability when administered orally. To avoid toxicity, it is important to monitoring serum concentrations of 5-fluorocytosine, because it can achieve high and toxic concentrations in the blood[18].

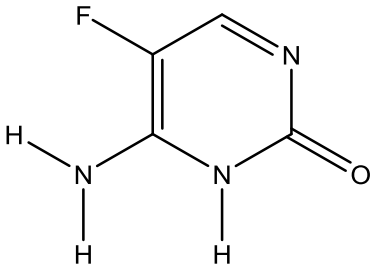

Figure 13 - Chemical structure of 5-fluorocytosine

Polyenes

Amphotericin B (AmB) and nystatin are examples of polyenes used in current therapy. AmB is a natural product from *Streptomyces nodosus*[3], and chemically, it is a heptaene – contains seven conjugated double bonds – which may be inactivated by extreme values of pH, heat and light[18]. The antifungal activity of AmB (Fig. 14, $C_{47}H_{73}NO_{17}$) involves the binding of this molecule to ergosterol, producing ion channels that destroy the integrity of the fungal cell membrane, and lead to damage on intracellular constituents and cell death. The selective toxicity for fungal cells exhibited by this drug, is due to the fact that AmB is more effective in permeabilizing fungal cell membranes with ergosterol than mammalian membranes with cholesterol[28]. AmB is also able to bind to cholesterol but with lower affinity[18]. This could be explained by the conformational difference between the cylindrical three-dimensional structure of the ergosterol and the sigmoid shape of the cholesterol. Due to the AmB molecular structure, there is higher affinity for the binding site with the ergosterol. However, this selectivity is low and suggests that AmB may have potential toxicity for mammalian cells, as could be seen in nephrotoxicity situations that occur with the use of this molecule[3]. AmB may also act by the generation of several oxidative reactions with formation of toxic free radicals[21]. Currently, there are AmB lipidic formulations more stable and soluble, with increased absorption and that allow to reduce the side effects, such as the nephrotoxicity aforementioned. These formulations are administered by intravenous route[3, 18]. Nevertheless, these have much higher associated costs, continuing to be used the traditional AmB formulations at hospitals of some countries.

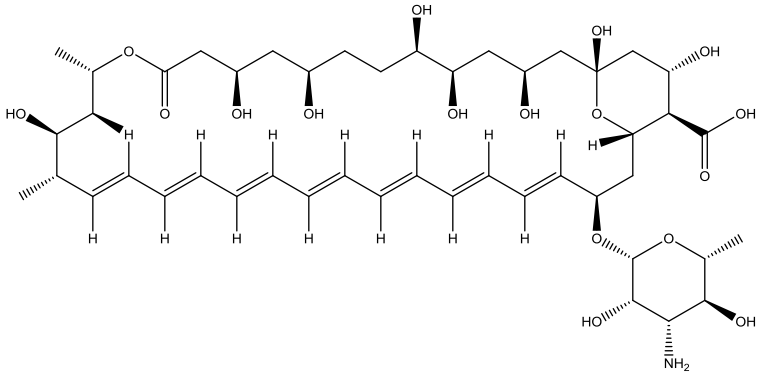

Figure 14 - Chemical structure of amphotericin B

Nystatin (Fig. 15, $C_{47}H_{75}NO_{17}$) is a polyene, currently used as a topical agent, considering the high toxicity when administered by systemic route. Nevertheless, this molecule is also being developed in a liposomal formulation for systemic use[29].

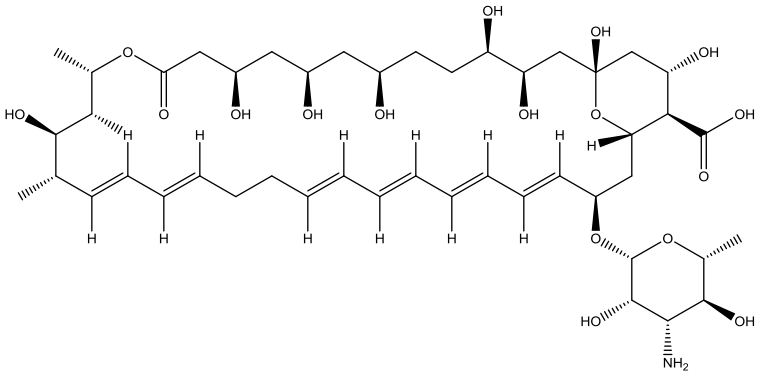

Figure 15 - Chemical structure of nystatin

Mechanisms of resistance to antifungals

For nearly 30 years, AmB was the sole drug available for the treatment of invasive fungal infections. Although AmB exhibited superior clinical effectiveness in the treatment of systemic candidiasis, its narrow therapeutic index and significant nephrotoxicity has limited its utility. The emergence of imidazoles and triazoles in the early 1990s was an important step to advance with the safe and effective treatment of local and systemic fungal infections. The high safety profile of the triazoles, particularly the FLC, its good bioavailability and clinical effectiveness, led to their extensive therapeutic and prophylactic use[12]. Although azole derivatives are the most widely used antifungal agents because of their high therapeutic index, they are fungistatic and not fungicidal against pathogenic yeasts, which leads to resistance to azoles in prolonged infections[4,12].

Nowadays, despite some antifungal agents being efficacious against some fungi, particularly the echinocandins and liposomal AmB formulations, and beyond the toxicity to the host, the intrinsic resistance exhibited by some of these fungi has led the researchers to detailed investigation. So, it is fundamental to find and to develop new compounds with some antifungal activity, or those which are able to inhibit some of the mechanisms of antifungal resistance[9, 30]. These mechanisms, some of them enumerated in Table 3, are complex and it can occur in response to a compound or an irreversible genetic change, including alterations or overexpression of target molecules, active extrusion through efflux pumps and tolerance, which are all characterized mechanisms utilized by fungi to resist to the antifungal treatment[9].

There has been a documented increase in fluconazole resistance among species of *Candida,* including *C. albicans, C. lusitaniae, C. tropicalis* and *C. dubliniensis*, which has been attributed to the use of fluconazole as empirical antifungal therapy since the 1990s[31-32].

As mentioned in Table 3, manifold mechanisms of azole resistance in *C. albicans*, including reduced accumulation of the drugs through active efflux (overexpression of genes *CDR1, CDR2* and *MDR1*), and alteration or overexpression of the target enzyme, have already been described[6-7].

Table 3 – Genetic mechanism of resistance in *Candida* spp.[14]

Decreased drug concentration (efflux pumps)	↑ *CDR* gene of ATP binding cassete (for all azoles) ↑ *MDR* gene of major facilitator class (for fluconazole) *C. albicans CDR1, CDR2, MDR1* *C. glabrata CgCDR1, PDH1, Snq2*
Target cell alteration	Mutation of *ERG11* ↑ ERG11p
Chromosomal aneuploidy or isochromosome	Chromosome 5 in *C. albicans*

Efflux pumps in resistance to azoles

The main molecular mechanism leading to high-level azole resistance in *C. albicans* is the efflux of drug mediated by the ATP-binding cassete (ABC) and the major facilitator superfamily (MFS) transporters[7, 9, 13, 32].

The ABC transporters use the energy from ATP hydrolysis to pump substrates out of the cell[20] (Fig. 16). This type of transporter is generally made up of two transmembrane domains (TMDs) and two cytoplasmic located nucleotide binding domains (NBDs). The TMDs comprise α-helices of 12 transmembrane segments (TMS), while the NBDs have α-helices and β-sheets. All this structure alone is probably not enough for substrate transport across the membrane bilayer, once the transport of these substrates requires energy from the hydrolysis of the ATP carried out at the NBDs[33].

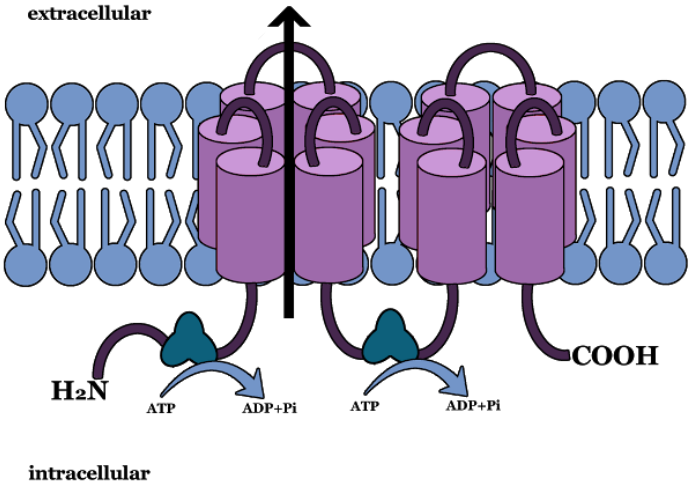

Figure 16 – Representation of ABC transporters of *Candida*[a]

The MFS transports substrates across a membrane in exchange for H^+ ions[20] (Fig.17). This class of major drug transporters was originally defined as a superfamily of permeases that are characterized by two structural units of six TMS α-helical segments, linked by a cytoplasmic loop[33]. Multiple antifungal agents can be substrates for these transporters, and thus, their overexpression can lead to cross-resistance among different drugs, particularly azoles[9].

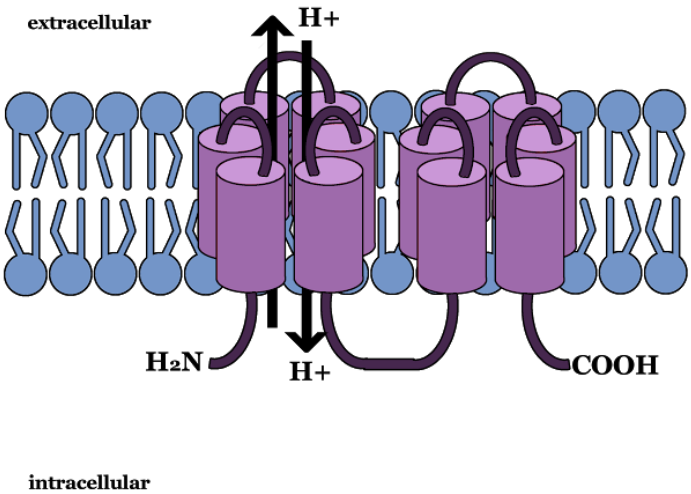

Figure 17 – Representation of MFS transporters of *Candida*[a]

The first multidrug efflux pump identified in a fungal pathogen was *MDR1* from *C. albicans*, which belongs to the MFS. A recent report showed that strong overexpression of *MDR1* in *C. albicans* resulted in increased resistance of the cells to compounds that are

[a] Figures 16 and 17 were based on figures from Prasad *et al.*, 2006

supposed to be substrates of this efflux pump, confirming that *MDR1* can mediate multidrug resistance (MDR) in *C. albicans*[5]. Similarly, the first described ABC transporter of a pathogenic fungus was also from *C. albicans*. The *CDR1* was found to confer cross-resistance to many structurally unrelated drugs, including various azoles[5, 34].

In *C. albicans,* both the ABC transporter genes *CDR1* and *CDR2* and the MFS gene *MDR1* can be upregulated, although not simultaneously, indicating the existence of separate regulatory pathways for the two transporter classes. Regulators of these transporters, *TAC1* and *MRR1*, which control the overexpression of *C. albicans CDR1/CDR2* and *MDR1*, respectively, have been identified. Mutations in these transcription factors are responsible for the overexpression of these transporters in clinical isolates[34].

Candida glabrata is a species that exhibits high intrinsic resistance to fluconazole, but its resistance can increase during FLC therapy[5]. In *C. glabrata*, increased levels of expression of the ABC transporters genes *CgCDR1* and *CgCDR2* have been also shown in azole-resistant isolates of *C. glabrata*. There is a genetic evidence supporting the role of multidrug transporters in the azole resistance of *C. glabrata*, similarly to *C. albicans*[35].

Candida krusei is intrinsically resistant to FLC, due to the low affinity of its sterol 14α-demethylase for this drug, and can acquire resistance to other azoles by reduced intracellular drug accumulation[5].

Modification of the ERG11 at molecular level

The fungal target of azole antifungals is a cytochrome P450 enzyme (encoded by *ERG11*) involved in 14-α-demethylation of lanosterol[14, 34]. Another azole resistance mechanism involves alteration of the target enzyme by amino acid substitutions caused by mutations in *ERG11*[34].

Some studies showed a decrease in fluconazole susceptibility, and by comparison of the DNA sequences of *ERG11* from azole-resistant isolates and sensitive *C. albicans* strains, it revealed a point mutation (R467K) that results in the replacement of arginine (R) for lysine (K) at position 467. It has been suggested that this mutation causes structural or functional changes in the heme cofactor of the enzyme. Preliminary studies indicate that R467K by itself can confer azole resistance by decreasing the affinity of the enzyme for fluconazole[12]. Overexpression of *ERG11* has also been documented but its contribution to azole resistance still is not clear[12].

Chromosomal aneuploidy or isochromosome

In *C. albicans* the only mechanism of gene amplification leading to azole resistance is the formation of aneuploidy or isochromosome. The chromosome arm bearing both transcription factor, regulating ABC transporter, and target of the azoles ERG11 is duplicated[14]

Antifungal resistance inhibition

Besides meeting pharmacological requirements, an ideal antifungal agent would not be susceptible to the development of resistance due to efflux mechanisms[8], or others. Several approaches have been proposed to block efflux-mediated antifungal drug resistance, including (1) the use of alternative antifungal drugs that are not efflux pump substrates (e.g. echinocandins and polyenes); protecting the efficacy of antifungals that are subject to efflux by developing treatments that prevent efflux, such as (2) give a drug in combination with a efflux pump inhibitor (EPI) or (3) use a drug that is a multifunctional inhibitor, affecting target, efflux pump activity, and efflux pump transcription; (4) deplete cells of energy required for drug efflux pump by inhibiting the plasma membrane H^+ ATPase; (5) design drugs with an enhanced rate of uptake and thus shift the balance between uptake and efflux, so that a high intracellular concentration of the drug in maintained, despite any upregulation of efflux[8, 13] (Fig. 18).

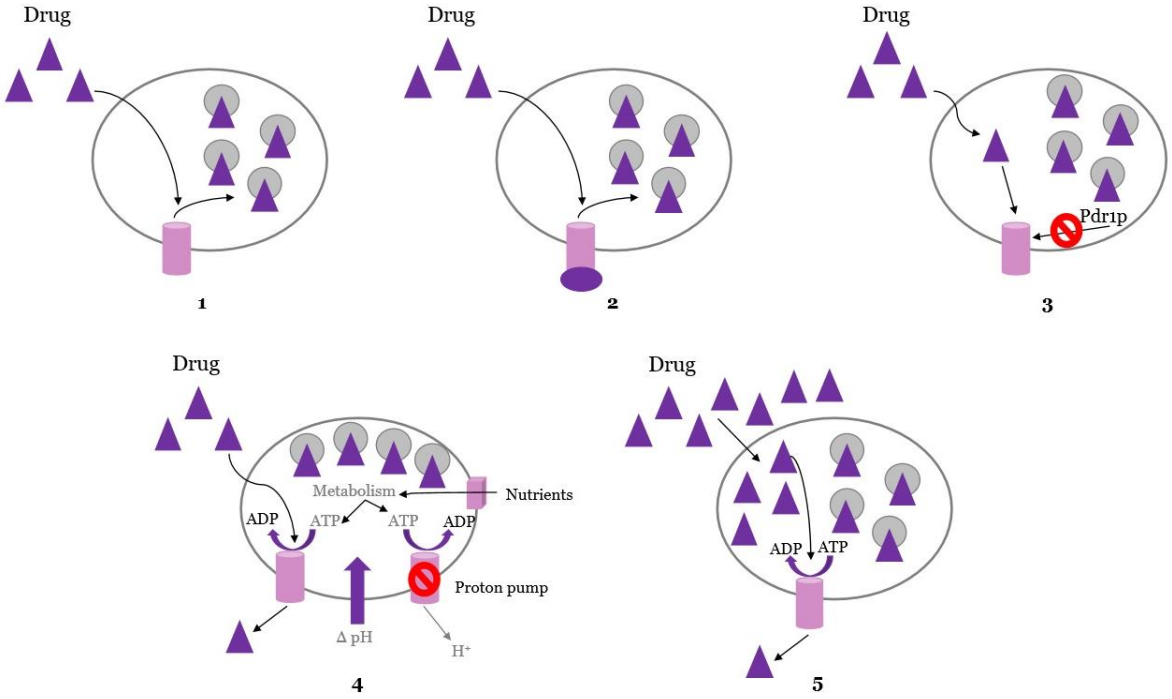

Figure 18 - Possible ways to overcoming efflux-mediated fungal drug resistance[b]

Thus, finding and developing new modulators that can block the efflux pumps, increasing the intracellular concentration of the antifungal drugs and reverting resistance, may be an innovative pharmaceutical strategy[6, 11]. The compounds used throughout this work can provide a great resource of antimicrobial activity or inhibition of the mechanisms of resistance.

Small library of natural and synthetic compounds

Plants produce an enormous array of secondary metabolites, and it is commonly accepted that a significant part of this chemical diversity helps to protect plants against microbial pathogens[36]. Therefore, xanthones and chalcones have been showed a potential as antifungals, as described in several work researches[1, 15, 37-38]. Antifungal agents based on xanthones and chalcones have important advantages such as the availability as natural compounds or the possibility to be synthesized easily and the potential to interact with some important targets in microorganisms. These compounds could consequently serve as a novel generation of antimicrobial agents as a strategy for responding to outbreaks of resistant fungal infections[1].

[b] The scheme (Fig.18) was adapted from Cannon *et al.*, 2009 and Prates *et al.*, 2011

Xanthones (Fig.19) are three ring oxygenated compounds which are mainly found as secondary metabolites in plants and microorganisms, with a large spectrum of biological and pharmacological activities[38-39]. The interest in these compounds is related with their structural scaffold and pharmacological importance, attracting many scientists to isolate or synthetize xanthone derivatives as an alternative to synthetic antifungal drugs[15, 39]. Many of them have proved to be important building blocks for the synthesis of new interesting compounds[40].

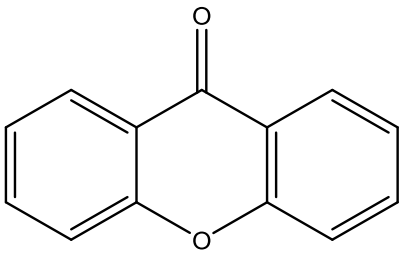

Figure 19 – Basic chemical scaffold of xanthones

Chalcones (Fig. 20) are intermediate precursors to all flavonoid compounds, and some of them have been reported for their growth inhibitory effect on human tumor cell lines[37]. It was decided to test such compounds in bacterial and fungal cells because, as eukaryotic cells, it could also affect their development.

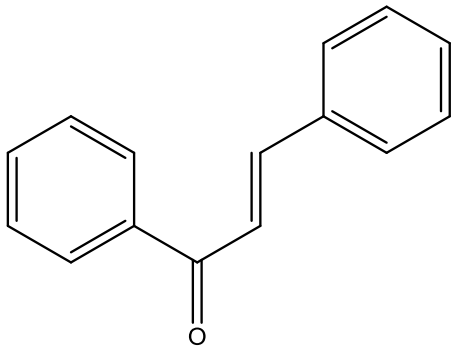

Figure 20 - Basic chemical scaffold of chalcones

Once efflux pump inhibitor (EPI)/antimicrobial combination drug should exhibit increased potency, enhanced spectrum of activity and reduced propensity for acquired resistance[41], three known EPIs (verapamil - VRP, amiodarone – AMD, and sodium azide - AZD) were used to first understand their mechanism of action and, thereby, it was possible to find a model to then test xanthones and chalcones.

Verapamil (Fig.21, $C_{27}H_{39}ClN_2O_4$), a known EPI, is widely used in the treatment of hypertension and angina pectoris and belongs to the phenylalkylamine class of calcium channel blockers[42]. As calcium channel blocker, it was decided to test this compound for inhibition of the efflux pump mechanism.

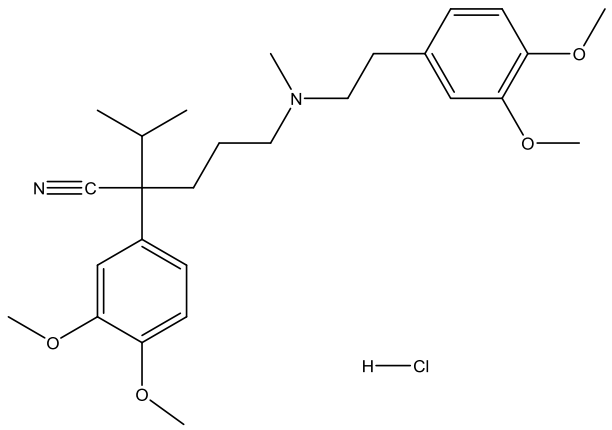

Figure 21 – Chemical structure of verapamil hydrochloride

Amiodarone (Fig.22, $C_{25}H_{29}I_2NO_3$) is an iodine benzo furan derivative that belongs to class III antiarrhythmic agents. It is wide used in the treatment of cardiac tachyarrhythmia due its capacity to block calcium, potassium and sodium channels. AMD is one promising new antifungal, once it has shown fungicidal activity against yeasts and other clinically important fungi. Furthermore, low doses of AMD have been reported to be synergistic with different azoles in resistant *Aspergillus fumigatus* strains. In *C. albicans,* it can be inferred that AMD induce calcium stress and modify the calcineurin pathway regulation, changing the tolerance to antifungal agents, cation homeostasis and virulence[43].

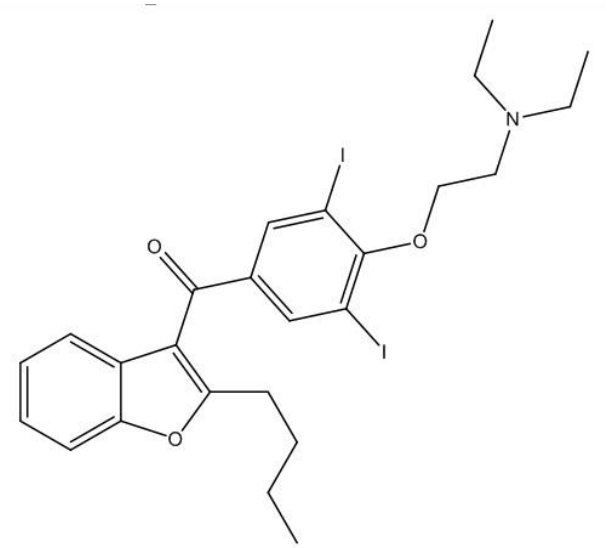

Figure 22 - Chemical structure of amiodarone

Sodium azide (Fig.23, N_3Na) was used to search FLC resistance by efflux pumps. This respiratory chain inhibitor decreases the energy potential of the cells and, consequently, block the efflux pumps depending on energy[6, 32].

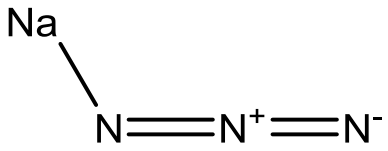

Figure 23 - Chemical structure of sodium azide

Thioxanthones are S-heterocycles with a dibenzo-γ-thiopyrone scaffold and they have interesting and important biological activities. The aminated thioxanthones derivatives, used in this work research, have dual activity as antitumor agents and P-glycoprotein (P-gp) inhibitors. The amine is described as an important pharmacophoric feature for P-gp inhibition. P-gp is a known efflux pump, from ABC super-family, that actively extrudes several structurally unrelated compounds out of the cells[44]. Therefore, thioxanthones were used to assess their potential as EPI.

Chapter II

Material and Methods

Material and Methods

Throughout this work, was screened a small library of 23 compounds to evaluate their potential antimicrobial activity, as well as to assay some potential EPIs in the reversion of resistance of strains of *Candida* spp. to azoles.

Xanthones and chalcones were used, in first place, to assess either the antifungal as the antibacterial activity.

Evaluating the antifungal activity of xanthones and chalcones

Library of natural and synthetic compounds

All the xanthones (Fig. 24-33) and chalcones (Fig. 34-38) tested were kindly provided by CEQUIMED-UP and were obtained *in house*. The chiral derivatives of xanthones tested throughout this work (Appendix A), were synthetized by coupling a carboxyxanthone or a carboxymethoxyxanthone with both enantiomers of commercially available chiral building blocks – amino alcohols, amines and amino esters[38]. Their structure elucidation was established by ^{1}H and ^{13}C NMR techniques[38].

These compounds were diluted in dimethyl sulfoxide (DMSO).

Xanthones

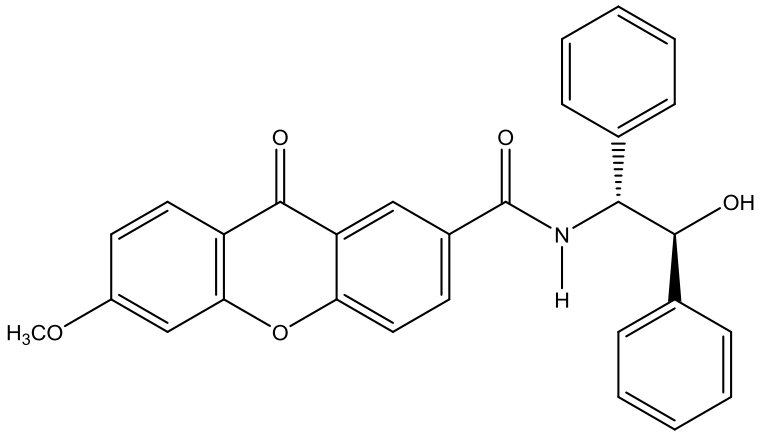

Figure 24 – Chemical structure of X2ADF-RS

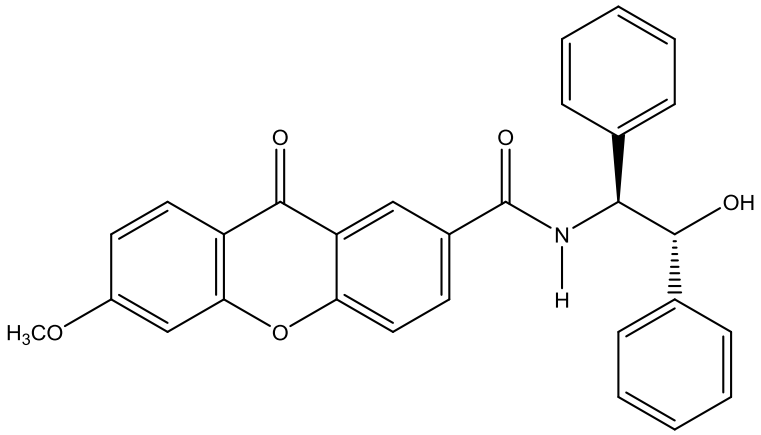

Figure 25 – Chemical structure of X2ADF-SR

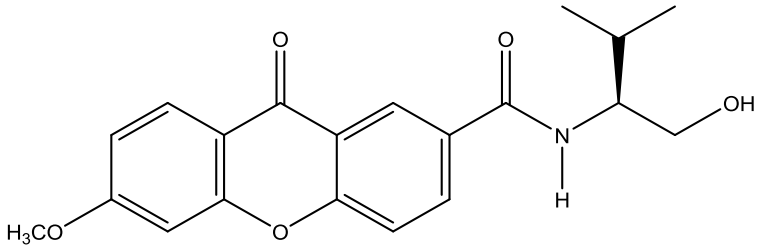

Figure 26 – Chemical structure of XEVOL-L

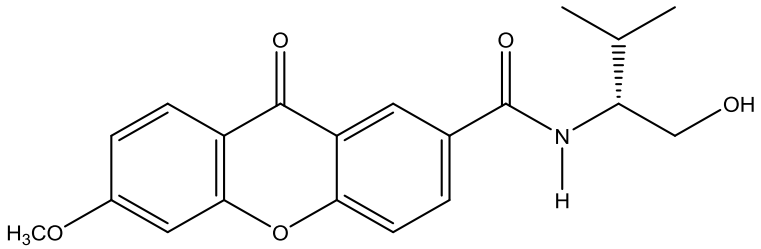

Figure 27 – Chemical structure of XEVOL-D

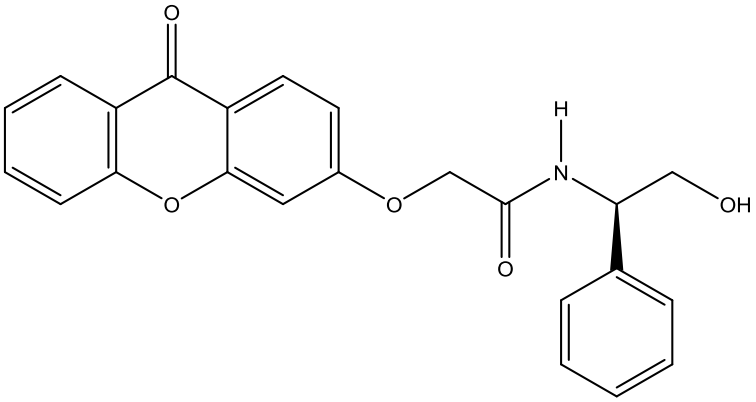

Figure 28 – Chemical structure of XEGOL-1D

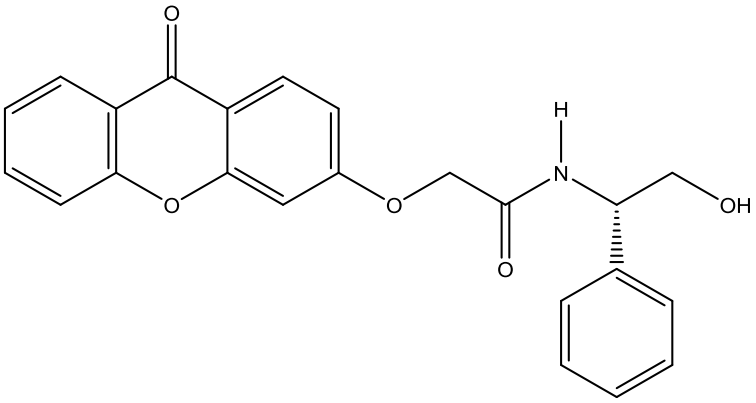

Figure 29 – Chemical structure of XEGOL-1L

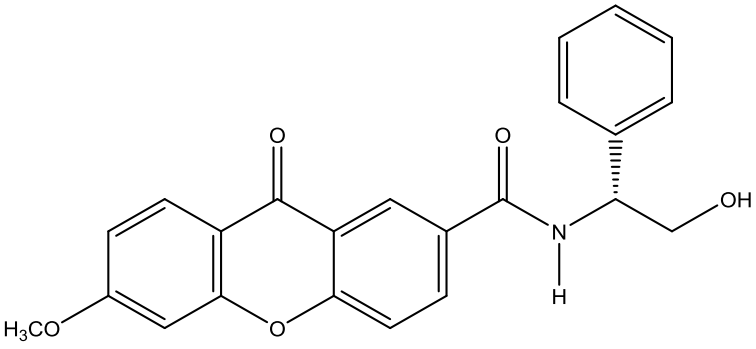

Figure 30 – Chemical structure of XEGOL-2D

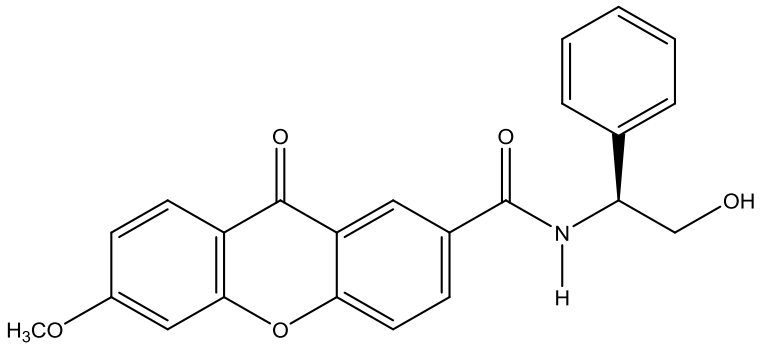

Figure 31 – Chemical structure of XEGOL-2L

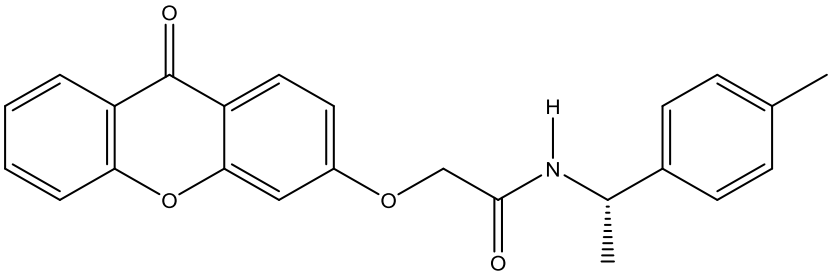

Figure 32 – Chemical structure of XEA-1S

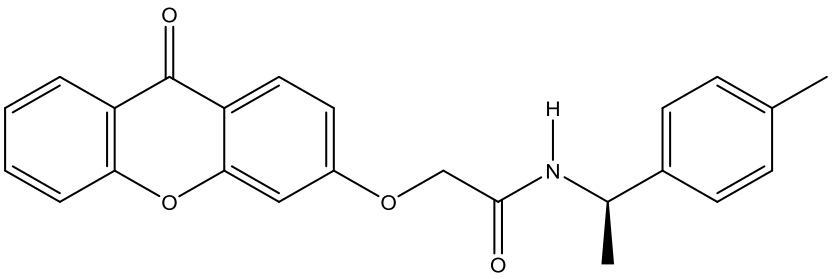

Figure 33 – Chemical structure of XEA-1R

Chalcones

Chalcones (Fig. 34-38) were prepared by base-catalyzed aldol condensation of properly substituted acetophenones and benzaldehydes under microwaves (MW) irradiation. Their structure elucidation was established by ^{1}H and ^{13}C NMR techniques[37].

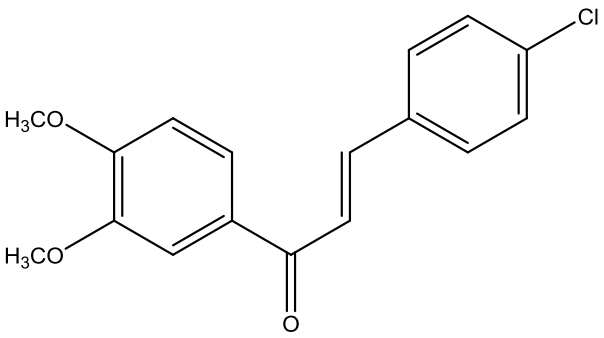

Figure 34 – Chemical structure of PB3

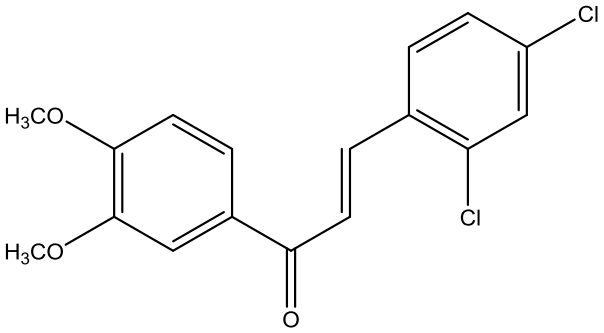

Figure 35 – Chemical structure of PB4

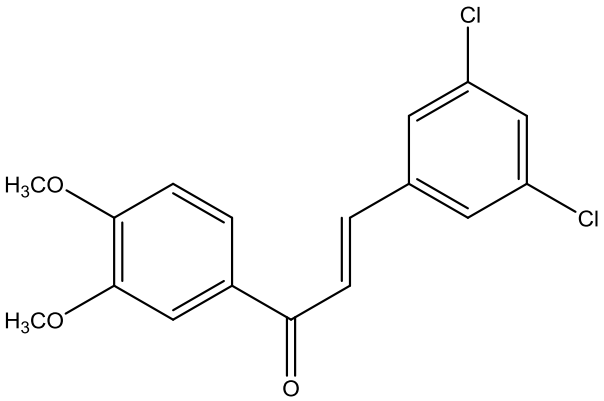

Figure 36 – Chemical structure of PB6

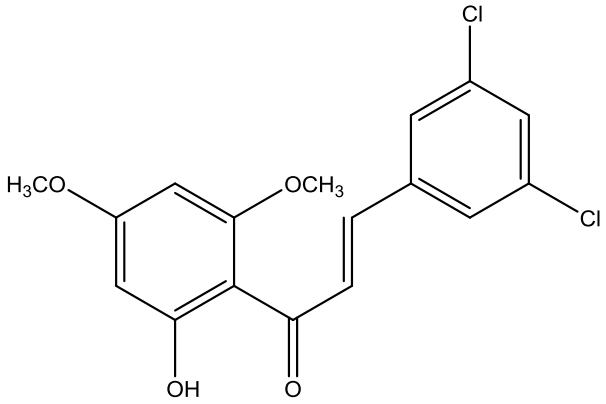

Figure 37 – Chemical structure of PB16

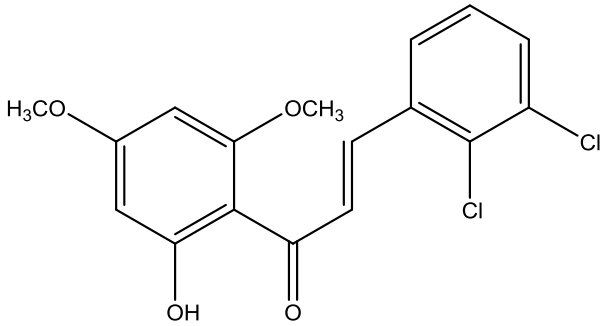

Figure 38 – Chemical structure of PB17

Microorganisms and media

In this work, the antifungal activity of compounds was evaluated against a yeast (*Candida albicans*), a filamentous fungus (*Aspergillus fumigatus*), and a dermatophyte (*Trichophyton rubrum)*. The microorganisms were sub-cultured on Sabouraud dextrose agar before use, to ensure purity and viability.

RPMI 1640 medium, with L-glutamate but without sodium bicarbonate (Biochrom), was supplemented with 3-(N-morpholino)-propanesulfonic acid (MOPS, Sigma Life Science), and pH was adjusted to 7.0 ± 0.2 (Appendix B). Minimum inhibitory concentrations (MICs) were obtained for all the microorganisms, according to the National Committee for Clinical Laboratory Standards Institute M27-A3, M27-S3 and M38-A2 protocols[45-47].

Antifungal susceptibility testing by broth microdilution[45-47]

All the aforementioned compounds were dissolved in DMSO, initial concentration of 25.6 mg/mL, diluted in RPMI 1640 and tested from 1024 μg/mL to 128 μg/mL on plates.

First of all, to evaluate the susceptibility of the microorganisms to the compounds, an assay in 96-well microtiter plates were performed. Sterility control (SC) received only 200 μL of RPMI 1640. A 100 μL volume of RMPI 1640 containing a twofold concentration of each compound tested, was added to a series of wells. Cultures were suspended in 2 mL of sterile 0.145 mol/L saline (8.5 g/L NaCl; 0.85% saline). The suspension was vortexed for 15 seconds and the cell density was adjusted with a spectrophotometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard at 530 nm wavelength. This procedure has yielded a yeast stock suspension of 1×10^6 to 5×10^6 cells/mL. A working suspension was made by 1:50 dilution followed by a 1:20 dilution of the stock suspension in RPMI 1640 to obtain the twofold inoculum concentration (1×10^3 to 5×10^3 CFU/mL). 100 μL of this suspension (1:1 dilution) was added to each well to give a final inoculum density of 0.5×10^3 to 2.5×10^3 CFU/mL and the final concentration of the compound. For filamentous fungi and dermatophytes, the spore suspension was prepared in approximately 1 mL of sterile 0.85% saline added of one drop (approximately 0.01 mL) of Tween 20 to facilitate the spore separation. The cell density was adjusted by spores counting at approximately 0.4×10^4 to 5×10^4 CFU/mL for *Aspergillus* spp. and 1×10^3 to 3×10^3 CFU/mL for dermatophytes. The growth control (GC) was prepared with 100 μL of RPMI 1640 with 2% DMSO and 100 μL of the inoculum. DMSO was added in the maximum concentration used in tested compounds. To ensure that the method used was being correctly performed, it was used a quality control. Quality control (QC) consists in execute the same procedures with a reference strain, which has already an expected result. The QC used was *Candida parapsilosis* ATCC 22019 with FLC. Plates were incubated for 24h to 48h, at 35ºC for *Candida* and *Aspergillus* species, and for five days, at 28ºC, for dermatophytes. MICs were interpreted regarding those concentrations that showed no growth compared with the growth control well; except for the QC with FLC, where the MIC should be interpreted as that concentration which showed a 50% decrease in fungal growth compared with de GC.

Evaluating the antibacterial activity of xanthones and chalcones

Library of natural and synthetic compounds

For this test were used the same compounds aforementioned (Fig. 24-38).

Microorganisms and media

Concerning to the antibacterial activity, were used three different bacteria - *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The microorganisms were sub-cultured on Mueller-Hinton Agar, for 24h, at 35ºC, to ensure purity and viability.

Mueller-Hinton Broth (MHB, Appendix B) was used. Minimum inhibitory concentrations (MICs) were obtained for all of the microorganisms, according to the National Committee for Clinical Laboratory Standards Institute M7-A9 and M100-S17[48-49].

Antifungal susceptibility testing by broth microdilution[48-49]

All the aforementioned compounds were dissolved in dimethyl sulfoxide (DMSO), initial concentration of 25.6 mg/mL, diluted in MHB and tested from 1024 µg/mL to 128 µg/mL on plates.

The method used of broth microdilution was exactly the same that was mentioned for the antifungal activity assessment. The QC used was *Pseudomonas aeruginosa* ATCC 27853 with Gentamicin. Plates were incubated for 24h at 35ºC and MICs were interpreted as those concentrations which showed no growth compared with the growth control well.

Assessment of the effect of thioxanthones and chalcones in efflux pumps inhibition

Library of natural and synthetic compounds

For this test were used eight compounds, 7 thioxanthones and 1 chalcone (Fig. 39-46). These compounds were diluted in DMSO or in distilled water (Appendix A).

Thioxanthones

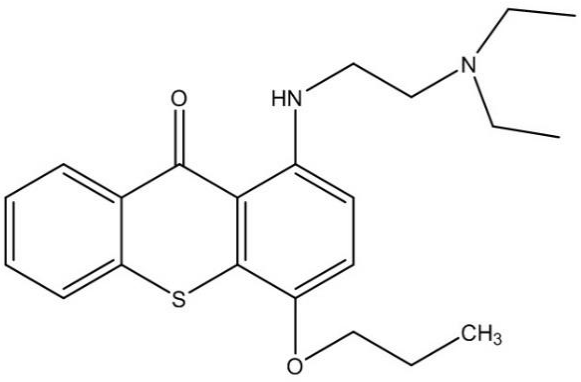

Figure 39 – Chemical structure of TXA1

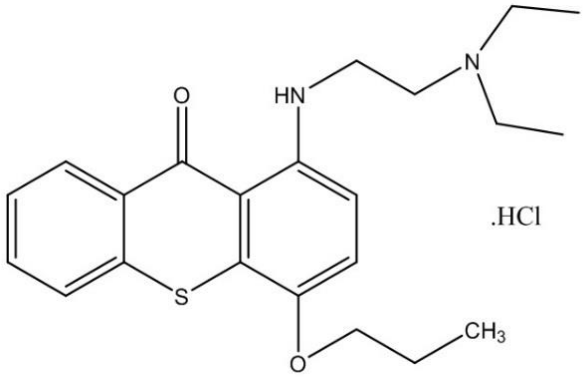

Figure 40 – Chemical structure of TXA1.HCl

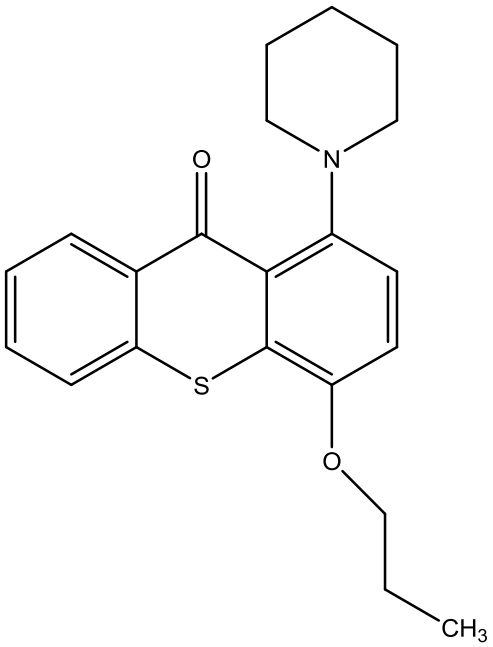

Figure 41 – Chemical structure of TX34

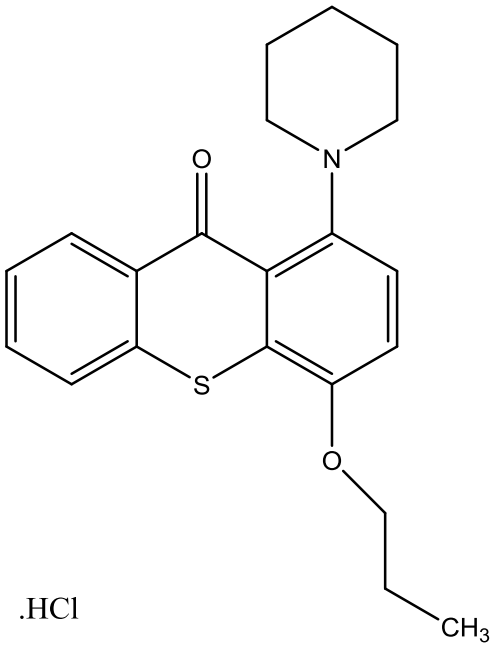

Figure 42 – Chemical structure of TX34.HCl

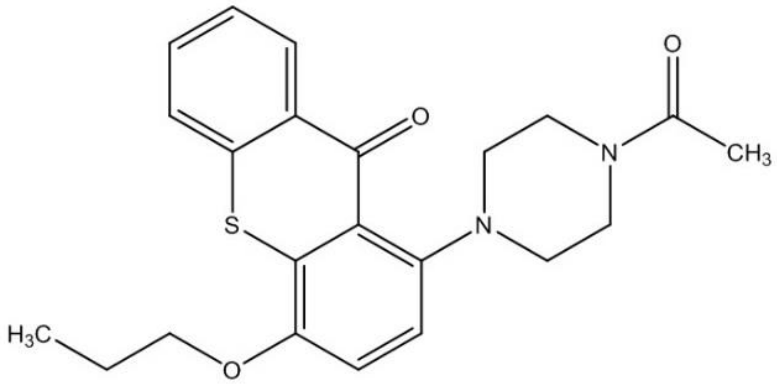

Figure 43 – Chemical structure of TX53

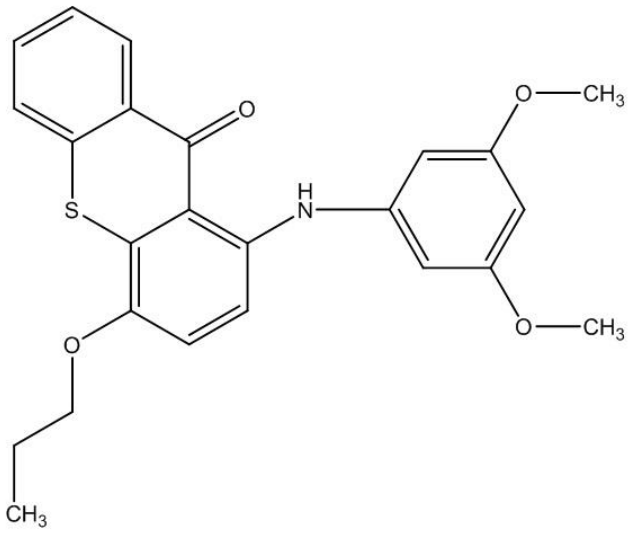

Figure 44 – Chemical structure of TX128

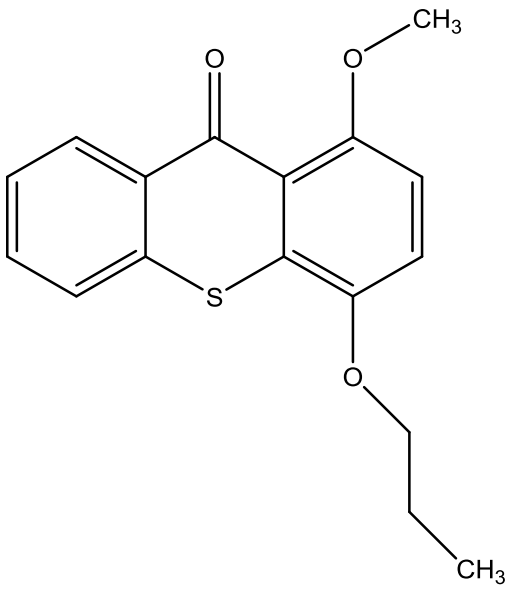

Figure 45 – Chemical structure of TXOMe

Chalcones

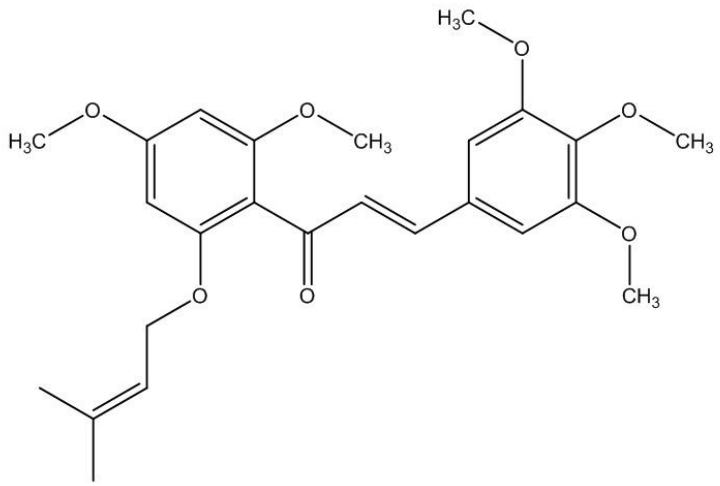

Figure 46 – Chemical structure of FP10

Microorganisms and media

Four *Candida* strains *(two C. albicans, DSY 294 and DSY 296,* and two C. *glabrata, DSY 562 and DSY 565)* were used in the present study, including two azole-susceptibility strains (DSY 294 and DSY 562) and two azole-resistant strains (DSY 296 and DSY 565). These strains were kindly provided by Prof. D. Sanglard (University of Lausanne, Lausanne, Switzerland). MICs of each compound were obtained for all of the strains according to the National Committee for Clinical Laboratory Standards Institute M27-A3 and M27-S3 protocols[45, 47], as described.

Antifungal agents

The commercial antifungals used over this work, to evaluate the susceptibility of *Candida* strains, were obtained from Sigma-Aldrich: Fluconazole, Itraconazole, Voriconazole and Posaconazole. These drugs were dissolved in distilled water or dimethyl sulfoxide (DMSO) according to NCCLS protocols, and were diluted in RPMI 1640.

Efflux pumps inhibitors

To test the inhibition of the efflux pumps were used known EPIs (Fig. 21-23): Verapamil hydrochloride, Amiodarone and Sodium azide, from Sigma Aldrich.

Efflux pump inhibition testing by broth microdilution[45, 47]

This assay was also accomplished using the broth microdilution already described for yeasts. It was also performed in a 96-well microtiter plates, using a SC, a GC and a QC to validate the results obtained. 24h cultures of *C. albicans* and *C. glabrata* strains were used to obtain the inoculum with 0.5 McFarland standard at 530 nm wavelength. This procedure has yielded a yeast stock suspension of 1×10^6 to 5×10^6 cells/mL. A working suspension was made by 1:50 dilution followed by a 1:20 dilution of the stock suspension to obtain the twofold inoculum (1×10^3 to 5×10^3 CFU/mL). 100 µL of this suspension (1:1 dilution) was added to each well to give a final density of 0.5×10^3 to 2.5×10^3 CFU/mL. In the first two rows, a 100 µL volume of yeast suspension, a 50 µL volume of RMPI 1640 containing a fourfold concentration of FLC (1 µg/mL -128 µg/mL) and a 50 µL volume of medium containing a fourfold concentration of each compound tested in different concentrations, was added to a series of wells. From third to sixth row, a 100 µL volume of RMPI 1640 containing a twofold concentration of FLC was added to a 100 µL of a mixture of yeast and compound tested in different concentrations, placed in contact 1 hour and 2 hours before. In the seventh row, it was only added the FLC to the culture to evaluate if there was or not a decrease in the MIC.

There was also a well to test each individual compound, to confirm that these compounds by themselves were not responsible by the growth inhibition. Plates were incubated for 24h at 35°C and MICs were interpreted as those concentrations which showed a 50% decrease in growth compared with the growth control well, due to the trailing effect of azoles.

Drug interaction evaluation

In vitro drug interactions were evaluated with a two dimensional, two agent broth microdilution checkerboard technique, to understand the interaction between the azoles and VRP, AMD, AZD and the compounds from CEQUIMED-UP.

Chapter III

Results and Discussion

Results and Discussion

Evaluating the antifungal activity of xanthones and chalcones

The results obtained on the evaluation of the antifungal activity of 10 xanthones and 5 chalcones, tested against *C. albicans*, *A. fumigatus and T. rubrum*, are present in Table 4.

Table 4 – Antifungal effect of the compounds tested

Compound		*C. albicans*	*A. fumigatus*	*T. rubrum*
Xanthones	X2ADF-RS	NI	NI	NI
	X2ADF-SR	NI	NI	NI
	XEVOL-L	NI	Growth inhibition at 512-1024 µg/mL concentrations	Growth inhibition at 512-1024 µg/mL concentrations
	XEVOL-D	NI	NI	NI
	XEGOL-1D	NI	NI	NI
	XEGOL-1L	NI	NI	NI
	XEGOL-2D	NI	NI	NI
	XEGOL-2L	NI	NI	NI
	XEA-1S	NI	NI	NI
	XEA-1R	NI	NI	NI
Chalcones	PB3	NI	NI	NI
	PB4	NI	NI	NI
	PB6	NI	NI	NI
	PB16	NI	NI	NI
	PB17	NI	NI	NI

NI – No growth inhibition

Foremost, concerning to the antifungal activity exhibited by the compounds from CEQUIMED-UP – xanthones and chalcones – almost none of the compounds tested has shown results that must be considered. The exception is XEVOL-L, a xanthone that seems to have some effect against *A. fumigatus* and *T. rubrum* between 512 µg/mL and 1024 µg/mL concentrations.

Such as many compounds existing in nature, XEVOL-L and XEVOL-D are enantiomers. In other words, the two molecules are related as object and mirror image. Usually, the chiral molecules contain an atom that is connected to four different substituent groups, which is called an asymmetric atom or a stereocenter[50]. The different biological and pharmacological activities of enantiomers, amongst the advances in synthetic and analytical methodologies allowed to increase the interest in this field[38]. Therefore, despite the similarity between the two molecules, only XEVOL-L was able to induce some reduction in

fungal growth, possibly due to its facility to bind on binding site. This result shows that has occurred enantioselectivity, because only one of these two molecules has showed some antifungal effect.

Concerning to XEVOL-L, , which has demonstrated some fungal growth inhibition in filamentous fungi, but no antifungal activity against yeasts, it would be also interesting to suppose that this is due to the presence of chitin in filamentous fungi cell wall. However, this mechanism should be studied and evaluated to understand exactly the reason why it occurs.

According to a study developed by Pinto *et al.*, 2014, the dermatophytes were found to be more sensitive to 8 of 16 active compounds tested, than *Candida* and bacteria species[51].

A study performed by CEQUIMED-UP, Faculty of Pharmacy, University of Porto[15], has tested some synthetic and naturally occurring xanthone derivatives to evaluate the influence of the nature and position of the substituents on structure-activity relationship (SAR) of antifungal xanthones and to investigate the effect of the most active compounds on sterol biosynthesis. From 27 simple oxygenated xanthones, their antifungal profile suggests that hydroxyl groups are important for activity. However, because of some limitations concerning biosynthesis and synthetic methods, the pattern of oxygenation is frequently restricted to positions 1, 3, 5, 6 for simple oxygenated and prenylated xanthones, and to 1, 4, 8 for polycyclic and dehydroxanthones.[15].

Hereafter, it is important to apply some molecular modifications to improve the potential antifungal activity of this compound and to understand exactly how the compound is recognized in the binding site. After further studies to uncover the molecular mechanism of action of these compounds, if the target will be elucidated, a door it will be open for design of new and more potent molecules.

Evaluating the antibacterial activity of xanthones and chalcones

The antibacterial activity was evaluated using 15 xanthones and chalcones, against three different bacteria – *E. coli, S. aureus* and *P. aeruginosa*. The selection of these bacteria was made to observe if there was some selectivity for a particular group, once *E. coli* is a Gram negative bacteria, *P. aeruginosa* is a Gram negative bacteria resistant to most antibiotics, and *S. aureus* is a Gram positive bacteria.

However, no antimicrobial activity was observed at 1024 μg/mL, the higher concentration tested.

Several previous studies, such as Pinto *et al.,* 2011, have reported some xanthones as important antifungal agents, and it seemed interesting to test this class of compounds against bacteria also, to evaluate its action spectrum[15].

In some studies performed with xanthones and many other natural compounds, antibacterial activity has showed a variable spectrum and potency depending on the bacteria. Some of these compounds showed to be selective to Gram positive bacteria, such as *S. aureus*, being inactive for Gram negative bacteria[51-52]. In general, *S. aureus* showed higher sensitivity than *E. coli* and *P. aeruginosa*, which could be related to the presence of an outer membrane in Gram negative bacteria or even to the cell wall composition. However, along this study, the tested compounds showed no activity, even for Gram positive bacteria.

Assessment of the effect of thioxanthones and chalcones in efflux pump inhibition

In order to evaluate the capability of some compounds to inhibit efflux pumps, known as a mechanism of resistance, were tested several azoles (fluconazole, itraconazole, voriconazole and posaconazole) against four *Candida* strains (showed in Table 5), to understand which were sensitive or resistant to this group of antifungals. To furthermore test the effect in EPIs, FLC was the only antifungal used against the resistant strains.

DSY 296 and DSY 565 showed MIC values representing resistance to all the azoles tested, a feature frequently associated with cross resistance to these antifungals[5, 35]. The overexpression of *CDR* genes confers resistance to different azoles, while overexpression of *MDR1* seems to confer specific resistance to FLC[14].

Table 5 – *Candida* strains used and their antifungal susceptibility to azoles

Strain Name	MIC (categorization according breakpoints)[45, 47]			
	Fluconazole	**Itraconazole**	**Voriconazole**	**Posaconazole**
C. albicans DSY 294	4 μg/mL (Sensitive)	2 μg/mL (Resistant)	0.03 μg/mL (Sensitive)	1 μg/mL (No Breakpoints)
C. albicans DSY 296	≥ 128 μg/mL (Resistant)	4 μg/mL (Resistant)	2 μg/mL (Sensitive Dose Dependent)	4 μg/mL (No Breakpoints)
C. glabrata DSY 562	8 μg/mL (Sensitive)	4 μg/mL (Resistant)	0.25 μg/mL (Sensitive)	4 μg/mL (No Breakpoints)
C. glabrata DSY 565	≥ 128 μg/mL (Resistant)	≥ 16 μg/mL (Resistant)	4 μg/mL (Resistant)	≥ 32 μg/mL (No Breakpoints)

Regarding to what was described in section *1. Introduction - Efflux pumps in resistance to azoles*, DSY 296[34] and DSY 565[35] have acquired resistance to FLC due to their overexpression of efflux pumps (*CDR1*, *CDR2* and *MDR1* for *C. albicans,* and CgCdr1 and

CgCdr2 for *C. glabrata)*. These strains, kindly provided by D. Sanglard, were previously investigated and compared by other groups research, and it was concluded, for example, that the transcription factor *TAC1* in the resistant isolate DSY296 contains a mutation (not present in sensitive strain DSY294), which results in high expression of *CDR1* and *CDR2*[34].

To verify if the resistance to azoles exhibited by DSY 296 and DSY 565, was due to an increase of the drug efflux, mediated by the higher expression of the genes that regulate efflux pumps, were first tested some compounds with known inhibitory efflux pump activity. The EPIs were used in sub inhibitory concentrations, in order to guarantee that EPIs by themselves were not responsible for inhibit fungal growth (Table 6).

Table 6 - Determination of the MIC of the commercial EPIs

Compound	MIC – pH 5.0		MIC – pH 7.0	
	DSY 296	*DSY 565*	*DSY 296*	*DSY 565*
VRP	250 μM	500 μM	500 μM	1000 μM
AMD	25 μM	25 μM	500 μM	1000 μM
AZD	100 μM	100 μM	500 μM	1000 μM

Based on a study developed by Gamarra *et al.*, 2010, it was decided to evaluate the effect of the FLC and commercial EPIs combination, at pH 7.0 and pH 5.0. According to that study, the MIC values for AMD against *C. albicans* showed significant variation as a function of pH with the lowest values observed at pH 5.0 for all strains examined[43]. In fact, it was observed that if the pH of the medium decreases, in general, there is a reduction in the FLC MIC, as well as the concentration of the commercial compound necessary to use in association (Table 7).

Table 7 – FLC MIC in combination with commercial EPIs

Compound	pH 5.0		pH 7.0	
	DSY 296	*DSY 565*	*DSY 296*	*DSY 565*
Fluconazole (FLC)	FLC 128 µg/Ml			
Verapamil (VRP)	VRP 100 µM + FLC 128 µg/mL	VRP 100 µM + FLC 128 µg/mL	VRP 100 µM + FLC 64 µg/mL	VRP 100 µM + FLC 128 µg/mL
Amiodarone (AMD)	AMD 15 µM + FLC 8 µg/mL	AMD 15 µM + FLC 8 µg/mL	AMD 100 µM + FLC 128 µg/mL	AMD 100 µM + FLC 128 µg/mL
Sodium Azide (AZD)	AZD 50 µM + FLC 128 µg/mL	AZD 50 µM + FLC 128 µg/mL	AZD 100 µM + FLC 128 µg/mL	AZD 100 µM + FLC 128 µg/mL

For AMD, a pH 5.0 medium allows to reduce the concentration of the compound from 100 µM to 15 µM, and FLC is able to inhibit fungal growth at 8 µg/mL, instead of 128 µg/mL (Fig. 47). For AZD, there is a decrease of its concentration from 100 µM to 50 µM, but the FLC concentration needed stills the same.

As aforementioned, this result for AMD probably is due to the capacity of this compound to induce calcium stress and modify the calcineurin pathway regulation, changing the tolerance to antifungal agents[43].

The use of lower concentrations to obtain the inhibition of fungal growth could help to decrease the probability of occurring side effects and toxicity to the human host.

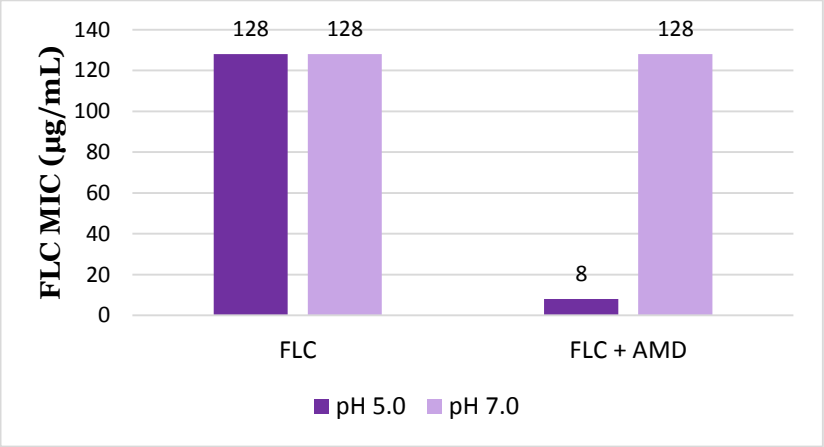

Figure 47 - Influence of the pH of the medium in FLC MIC

Toward the inhibition of efflux pumps, responsible for the resistance to antifungals, it was possible to observe the potential of AMD as EPI, at pH 5.0. In contrast, for pH 7.0 medium, none of the EPIs were able to revert the phenotype of resistance of these strains.

A study developed by *Pina-Vaz, C.*, 2000, using different strains of *C. albicans, C. glabrata* and *C. krusei,* showed significant reduction of FLC MIC when associated to VRP, allowing the reversal of the resistant (R) phenotype to sensitive (S) phenotype in 6 of the 8 strains tested[53]. However, this was not observed along this work, probably because it was performed with different strains with other features.

For pathogenic fungi, ambient pH is important because it determines the ability of the pathogen to successfully colonize and invade the targeted host. Therefore, modulation of the pH may induce alterations in the way pathogenic fungi act in the host, as well as it may affect the efflux pumps function and the secretion of pathogenicity factors[54].

During the period of time of this dissertation, the study was performed only with pH 7.0 medium. Further studies are needed to assess the feasibility of administering antifungal drugs with EPIs, in an action site with an acidic pH, lower than 7.0.

Using EPIs already known, it was found a method to then test the samples from CEQUIMED-UP, employing new compounds (Fig.39-46). Thioxanthones and chalcones were tested in order to assess their capacity to inhibit antifungal mechanisms of resistance.

Table 8 represents the MIC for these compounds when alone, in order to find the sub-inhibitory concentration that should be used when in combination with FLC.

Table 8 – Determination of the MIC of thioxanthones and chalcones

Compound	MIC - pH 7.0
TXA1	32 μg/mL
TXA1.HCl	32 μg/mL
TX34	> 128 μg/mL
TX34.HCl	> 128 μg/mL
TX53	> 128 μg/mL
TX128	> 128 μg/mL
TXOMe	> 128 μg/mL
FP10	> 128 μg/mL

TXA1 and TXA1.HCl seems to possess some antifungal activity by themselves, since their MICs were 32 μg/mL for both. Further studies are also needed to uncover the exact mechanism of action of this type of xanthones against DSY 296 and DSY 565 and other fungi.

Table 9 indicates the result for the *in vitro* activity of FLC alone and in combination with the compounds tested.

Table 9 – FLC MIC in presence of tioxanthones and chalcones

	MIC (μg/mL) – pH 7.0								
Strain	*FLC*	*FLC + TXA1 16μg/mL*	*FLC + TXA1.HCl 16μg/mL*	*FLC + TX34 128μg/mL*	*FLC + TX34.HCl 128μg/mL*	*FLC + TX53 128μg/mL*	*FLC + TX128 128μg/mL*	*FLC + TXOMe 128μg/mL*	*FLC + FP10 128μg/mL*
DSY 296	128	64	64	64	128	64	128	128	128
DSY 565	128	64	64	64	128	64	128	128	128

Associating FLC with one of these EPIs, MIC reduced only from 128 μg/mL (FLC alone) to 64 μg/mL. In some cases, the FLC MIC remained the same, with EPI or not.

Thus, it can be concluded that none of these thioxanthones was able to inhibit this mechanism of resistance, when associated to FLC. Hereafter, it should be interesting to test the same thioxanthones in different conditions – different azoles, different pH or different fungi, or bacteria.

It was expected some activity of FP10, once it has an aliphatic chain in the ortho-position of the phenyl residue adjacent to the C=O group. This feature seems to contribute significantly to increase its inhibitory activity in tumor cell lines, described by Neves *et al.*,2012[37]. Furthermore, the pharmacological activity of chalcones may be also related to the presence, number and position of OH and MeO groups in both A and B rings[37]. For these reasons, this compound was tested against fungi to understand if its mechanism was able to inhibit the fungal growth also.

Ultimately, along this work, both the evaluation of the antifungal activity and the assessment of the effect of a library of compounds in efflux pump inhibition, were performed using an *in vitro* susceptibility testing by broth microdilution, according to the NCCLS Institute M27-A3, M27-S3, M38-A2 and M7-A9 protocols. The main objective of all *in vitro* susceptibility testing, antifungal or antibacterial, is to predict the likely impact of administration of the tested agent on the outcome of infection caused by the tested organism or similar organisms[55].This is a simple visual method that allows observing the fungal growth. For azoles, there is a prominent trailing effect, which results in growth at all drug concentrations regardless of susceptibility. Therefore, determinations of the MIC depends on a difficult visual assessment of 50 to 80% reduction in growth relative to the drug-free control[12].

Hence, there is a need for more rapid test procedures or “real time” antifungal susceptibility testing[14]. Moreover, not always the test conditions through the broth microdilution are the same, and cause differences, being necessary to use methods of detection of the fungal growth that allows more accurate and reproducible results.

Concerning the screening of antifungal activity, flow cytometric methods have been adapted for testing some antifungals against *Candida* spp. This approach uses a standard flow cytometer and fluorescent DNA binding dyes to detect fungal cell damage following exposure to an antifungal agent. The flow cytometric method produces results within 6h that agree very well with MICs determined by the M27 reference method[56].

In addition, to evaluate the efflux pump inhibition, it is possible to appraise the intracellular concentration of FLC by liquid chromatography-tandem mass spectrometry method, described by Sun *et al.*[57], to measure the Rh123 uptake and glucose-induced efflux, or to measure the expression of *CDR1* using RT-PCR analysis[11].

A study developed by a group research of University of Otago, used two novel strategies to identify new candidate compounds that block efflux pumps in fungi. In this study, the yeast cell is incubated with a fluorescent substrate and glucose, allowing to quantify pump activity. They used control cells, without efflux pumps, producing high fluorescence; resistant yeast cells with ABC efflux pumps, emitting low fluorescence; and resistant yeast cells with EPIs that have also produced high fluorescence[58].

Chapter IV

Conclusion and Future Work

Conclusion and Future Work

Concerning to the antimicrobial activity of all compounds tested, only XEVOL-L has showed some antifungal activity against filamentous fungi, such as *A. fumigatus* and *T. rubrum*. None of the compounds tested have exhibited some antibacterial potential, in contrast to what is described for many natural compounds. Moreover, related to the efflux pump inhibition, none of the compounds have revealed some activity when associated to FLC. Only AMD and AZD seems to provide some advantage when associated to FLC, once the concentration of the commercial EPIs, at pH 5.0, showed a substantial reduction, and AMD allowed to decrease FLC MIC too.

Furthermore, it would be interesting to test more compounds from the same nature, as long as their chemical diversity and the possibility to be synthesized easily, could allow to find other compounds with potential to interact with some important targets in bacteria and fungi. Using other azoles and other resistant strains, such as *C. krusei* strains, which exhibit also resistance to azoles, could be also a challenging question to explore. According to the results obtained in the susceptibility testing done with DSY 294, DSY 296, DSY 562 and DSY 565, all these strains were resistant to itraconazole, so it would be interesting to test this particular azole against these 4 strains.

Additionally, it would be important to prove the potential of AMD to reverse antifungal resistance by inhibition of efflux pumps or, eventually, as antifungal agent, once there are some studies referring its antifungal potential. The results obtained at pH 5.0, with AMD and AZD, can lead to interrogate and search the exact mechanism whereby the pH affect the efflux pump activity, and how it is possible for compounds to achieve the site of action with a low pH that improve its activity.

Moreover, despite the use of the susceptibility testing by broth microdilution, could be used flow cytometric methods to evaluate the activity of some antifungals against fungi. In addition, to evaluate the efflux pump inhibition, it is possible to use other techniques such as liquid chromatography-tandem mass spectrometry to measure the concentration of FLC, or RT-PCR analysis to assess the expression of *CDR1*.

With this work is possible to say that it was given an interesting contribution to deeper knowledge, concerning the research area of biological activities of chalcones and xanthones derivatives. Although many compounds have not expressed antifungal or efflux pump inhibitory activity, these data may be relevant for later studies of SAR to be made for these families of compounds, as well as paving the way for design new and more potent molecules.

All this study suggests that there are several possibilities to revert the phenotype of resistance to the antifungals, because this is a real healthcare problem which requires concern and attention when treating these conditions. This issue is also responsible for

increasing the healthcare costs in hospitals, as well as in the community. Beyond all these factors, the conditions leading to clinical resistance should be considered while managing invasive fungal infections in patients with some of the predisposing factors mentioned over the work.

Chapter V

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Chapter VI

Appendix

Appendix

Appendix A – Compounds from CEQUIMED-UP

Figure	*Compound*	*Molecular Weight (g/mol)*	*Solvent*
24	**X2ADF-RS**	465.51	DMSO
25	**X2ADF-SR**	465.51	DMSO
26	**XEVOL-L**	355.39	DMSO
27	**XEVOL-D**	355.39	DMSO
28	**XEGOL-1D**	389.41	DMSO
29	**XEGOL-1L**	389.41	DMSO
30	**XEGOL-2D**	389.41	DMSO
31	**XEGOL-2L**	389.41	DMSO
32	**XEA-1S**	387.44	DMSO
33	**XEA-1R**	387.44	DMSO
34	**PB3**	302.75	DMSO
35	**PB4**	337.20	DMSO
36	**PB6**	337.20	DMSO
37	**PB16**	353.19	DMSO
38	**PB17**	353.19	DMSO
39	**TXA1**	384	DMSO
40	**TXA1.HCl**	419.5	Water
41	**TX34**	353.5	DMSO
42	**TX34.HCl**	389	Water
43	**TX53**	396.5	DMSO
44	**TX128**	421.5	DMSO
45	**TXOMe**	300.37	DMSO
46	**FP10**	442.5	DMSO

Appendix B – Medium composition

RPMI 1640 Medium

	Concentration	*Mass*	*Solvent*	*Final Volume*	*Final pH*
RPMI 1640 (Biochrom)	5.2 g/500 mL	10.4 g	Distilled Water	1000 mL	7 ± 0.2
M1254 MOPS (Sigma Life Science)	17.27 g/500 mL	34.54 g			

Powdered medium should be dissolved in 900 mL distilled H_2O and MOPS (final concentration of 0.165 mol/L) added. While stirring, the pH must be adjusted to 7,0 ± 0,2 at 25°C using 1 mol/L sodium hydroxide. Add additional water to bring medium to a final volume of 1 L. Filter sterilize and store at 4 °C until use.

Mueller Hinton Broth (BD Difco)

	Concentration	*Mass*	*Solvent*	*Final Volume*	*Final pH*
Extrait de Bouef	22.0 g/1000 mL	3.0 g	Water	1000 mL	7.3 ± 0.1
Hidrolysat Acide de Caséine		17,5 g			
Fécule		1,5 g			

Appendix C – Communication Poster in *XX Encontro Luso-Galego de Química, Porto, Portugal*, 26-28 November 2014

Xanthone derivatives with antifungal potential for mycoses treatment

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Opportunistic bacterial and fungal infections have increased due to the growing number of immunocompromised patients. *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, are the most commonly involved bacteria [1]. Although fungal infections contribute substantially to human morbidity and mortality, the impact of these diseases in human health is not widely appreciated. *Candida* and *Aspergillus* species are the most frequent nosocomial fungal pathogens responsible for systemic infections which kill about one and a half million people every year [2]. Nevertheless, the most common fungal diseases in humans are superficial infections, which affect approximately 25% of the world's population. They are predominantly caused by dermatophytes and affect also healthy persons with a considerable impact on patient's life quality [3].

The main challenge in developing antifungal drugs is related to the similarity between fungi and their hosts. Antifungal drugs often exhibit therapeutic limitations with fungistatic mechanism of action, high toxicity, many drug interactions, insufficient bioavailability and development of resistance or innate resistance [4]. Thus, it is imperative to continue the discovery and development of new antimicrobial drugs, more effective and less toxic than those already in use, especially those with new mechanisms of action.

Based on these precedents, this project for the thesis for Masters in Pharmaceutical Chemistry aims to evaluate the antimicrobial activity of a library of xanthonic derivatives, especially chiral compounds, against clinically relevant bacteria and fungi (*Candida*, *Aspergillus* and dermatophyte species) using the broth microdilution method by Clinical and Laboratory Standards Institute. Considering the battery of compounds available, it will be expected the possibility of the establishment of some structure-activity relationships (SARs). For the compounds showing antifungal activity some mechanisms of action will be evaluated.

Acknowledgments: This work is funded through national funds from FCT—Fundação para a Ciência e a Tecnologia under the project CEQUIMED—PEst-OE/SAU/UI4040/2014.

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XX ENCONTRO LUSO-GALEGO DE QUÍMICA

Faculdade de Farmácia UP
26 a 28 de novembro de **2014**

XANTHONE DERIVATIVES WITH ANTIFUNGAL POTENTIAL FOR MYCOSES TREATMENT

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INTRODUCTION

Opportunistic bacterial and fungal infections have increased due to the growing number of immunocompromised patients. *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, are the most commonly involved bacteria [1]. Although fungal infections contribute substantially to human morbidity and mortality, the impact of these diseases in human health is not widely appreciated. *Candida* and *Aspergillus* species are the most frequent nosocomial fungal pathogens responsible for systemic infections which kill about one and a half million people every year [2]. Nevertheless, the most common fungal diseases in humans are superficial infections, which affect approximately 25% of the world's population. They are predominantly caused by dermatophytes and affect also healthy persons with a considerable impact on patient's life quality [3]. The main challenge in developing antifungal drugs is related to the similarity between fungi and their hosts. Antifungal drugs often exhibit therapeutic limitations with fungistatic mechanism of action, high toxicity, many drug interactions, insufficient bioavailability and development of resistance or innate resistance [4]. Thus, it is imperative to continue the discovery and development of new antimicrobial drugs, more effective and less toxic than those already in use, especially those with new mechanisms of action.

PLANNING

This master project aims to search for new xanthone (Figure 1) derivatives with antimicrobial activity against clinically relevant bacteria and fungi.

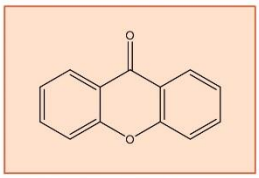


Figure 1. Chemical structure of xanthone

A library of compounds from CEQUIMED-UP will be tested against bacteria (Figure 2), and filamentous fungi, including dermatophytes (Figure 3), using the broth microdilution method by Clinical and Laboratory Standards Institute (CLSI).

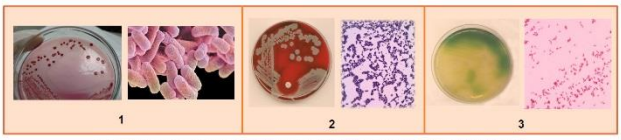


Figure 2. *Escherichia coli* (1), *Staphylococcus aureus* (2), and *Pseudomonas aeruginosa* (3)

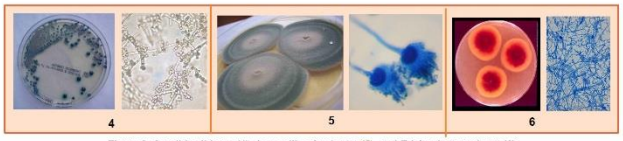
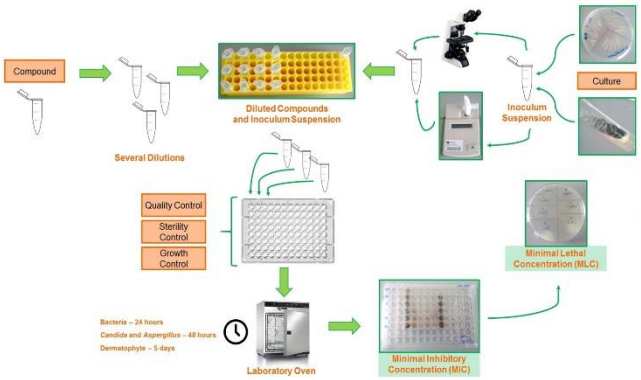


Figure 3. *Candida albicans* (4), *Aspergillus fumigatus* (5), and *Trichophyton rubrum* (6)

The screening will use the microorganisms above mentioned, but the spectrum of microorganisms will be extended to those compounds with more promising antimicrobial activity.

The broth microdilution method is more suitable once it uses a low amount of compound, allowing to determine the minimal inhibitory concentration (MIC) for bacteria and fungi. Once determined the MIC, it is possible to obtain and observe the minimal lethal concentration (MLC), which evidence if it is a fungicidal/bactericidal or a fungistatic/bacteriostatic compound (Scheme 1).



Scheme 1. Determination of MIC and MLC

PRELIMINARY RESULTS

At this time, it was only tested one xanthone derivative – X2ADF-RS. This compound was tested against *Candida albicans*, *Aspergillus fumigatus*, *Trichophyton rubrum*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. For this compound, the MIC was greater than 256 µg/mL.

FUTURE WORK

For compounds showing some antifungal activity, fungistatic or fungicidal, the possible mechanism of action will be evaluated. Considering the battery of compounds available, it is expected the possibility of the establishment of some structure-activity relationships (SAR).

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