Charles University in Prague
Faculty of Pharmacy in Hradec Králové

ANTIFUNGAL DRUG DISCOVERY: FOCUS ON INCRUSTOPORIN DERIVATIVES

Dissertation thesis

Luís André Vale Silva

Hradec Králové 2006

As a society, we need to be forever vigilant in maintaining the balance between research that advances our knowledge and research that offers practical benefits on the short- and medium-term.

Frank C. Odds

ACKNOWLEDGEMENTS

There are many people to whom acknowledgement is due in relation to this work and I would like to start by apologizing to all those whose names, although deserving, I did not include here. The fact that I made that mistake does not, definitely, minimize the importance of their contributions.

First I would like to acknowledge everybody at the Department of Biological and Medical Sciences of the Faculty of Pharmacy of Charles University in Hradec Králové. It has been my home for the last four years and it could not have been this satisfying without everybody's companionship. I specially thank Mrs. Marie Pancířová and Mrs. Ida Dufková for being always ready to help me in any way I needed.

I would also like to thank everybody at Life and Health Sciences Institute (ICVS) of the University of Minho in Braga, Portugal, especially at the Mycology laboratory. I felt at home from the first to the very last day of the six months I spent there! Thank you Assoc. Prof. Paula Ludovico for introducing me to flow cytometry techniques!

I could not forget PharmDr. Doris Vokurková, from the Institute of Clinical Immunology and Allergology of the University Hospital of Charles University in Hradec Králové. Her kindness made our work together extremely rewarding for me!

I am grateful to Prof. Maria da Conceição Montenegro, from the Faculty of Pharmacy of the University of Porto, Portugal, for encouraging me to take the step of moving to another country and promptly helping me whenever she could.

I could never forget my parents' support, without which it would have definitely not been possible for me to be here today. For that I thank my mother and father.

Finally, I whish to thank my supervisor, Assoc. Prof. Vladimír Buchta, for guiding me into, and through, the world of Mycology! It is his influence that is most noted in this work.

CONTENT

LIST OF ABBREVIATIONS

AC	*Absidia corymbifera*.
AF	*Aspergillus fumigatus*.
AFST-EUCAST	Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing.
AIDS	Acquired Immunodeficiency Syndrome.
AmB	Amphotericin B.
ATCC	American Type Culture Collection.
CA	*Candida albicans*
CFU	Colony Forming Units.
CG	*Candida glabrata*
CK	*Candida krusei*
CL	*Candida lusitaniae*.
CLSI	Clinical and Laboratory Standards Institute (formely NCCLS)
CP	*Candida* parapsilosis.
CT	*Candida tropicalis*.
$DiBAC_4(3)$	Bis-(1,3-dibutylbarbituric acid) trimethine oxonol.
DMSO	Dimethyl sulfoxide.
EUCAST	European Committee on Antimicrobial Susceptibility Testing.
FDA	Food and Drug Administration.
Flc	Fluconazole.
FUN-1	2-Choro-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1 phenylquinolinium iodide.
IC_{50}	Inhibitory concentration (50 %).
IC_{80}	Inhibitory concentration (80 %).
IC_{95}	Inhibitory concentration (95 %).
LNO6-22	3-(3,4-dichlorophenyl)-5-acyloxymethyl-2,5-dihidrofuran-2-one.
LNO15-22	3-(3-chlorophenyl)-5-acyloxymethyl-2,5-dihidrofuran-2-one.
LNO18	3-(4-bromophenyl)-5-hydroxymethyl-2,5-dihidrofuran-2-one.
LNO18-22	3-(4-bromophenyl)-5-acyloxymethyl-2,5-dihidrofuran-2-one.
MFC	Minimum fungicidal concentration.
MG	*Microsporum gypseum*.
MIC	Minimum inhibitory concentration.
MOPS	Morpholinepropanesulfonic acid.
NCCLS	National Committee for Clinical Laboratory Standards.
NYP	N-acetylglucosamine, yeast nitrogen base, and proline.
PBS	Phosphate buffered saline solution.
PI	Propidium iodide.
RPMI	Roswell Park Memorial Institute.
TB	*Trichosporon beigelii*.
TM	*Trichophyton mentagrophytes*.

1 INTRODUCTION

The incidence of invasive fungal infections caused by opportunistic fungi, frequently associated to high mortality rates, has been increasing steadily through the course of the last three decades. This tendency results from the growth of the population of immunocompromised patients, brought about by both the establishment of the Acquired Immunodeficiency Syndrome (AIDS) pandemic and the constant development of medical techniques, particularly involving oncology or transplantation patients.

On the other hand, the latest developments concerning systemic antifungal therapy have not resolved the need for new more effective systemic agents for the treatment of invasive mycoses. The available range of antifungal drugs is still very limited and there are mainly six drugs with use in the treatment of systemic infections: amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole, and caspofungin. Further more, in many cases they are associated with toxicity problems or low efficacy, particularly regarding infections by the emerging non-albicans *Candida* species, the *Zygomycetes*, and *Fusarium* and *Scedosporium* species.

This way, the development of new systemic antifungal agents is a timely challenge nowadays, with more relevance than ever before, conferring a strong motivation to the study of new natural or synthetic chemical agents for their antifungal potential. Our work is centred right on this goal, as we study new compounds for the development of their antifungal activity.

The goal of our work was the development of the previously tested incrustoporine derivatives. The first step was the broadening of the range of tested fungal species and strains, using adaptations of the reference CLSI (Clinical and Laboratory Standards Institute) protocols M27-A2 and M38-A. Later on, we conducted preliminary tests on their mechanism of antifungal action, including the study of the influence of subinhibitory concentrations on the morphogenetic transition in *Candida albicans*, the study of the kinetics of their growth inhibition using the Bioscreen method, and the study of the influence on various cellular parameters by flow cytometry.

Besides that, a wide number of other compounds from various different chemical groups were screened for their antifungal activity, again using adaptations of the reference CLSI protocols M27-A2 and M38-A, in search for new groups of potential antifungal agents to join the incrustoporin derivatives in the pursuit of further development. The 10 best among those groups, including a total of 152 compounds, were chosen to be included in this collection of results.

2 THEORETICAL PART

2.1 General considerations on fungi

Fungi are a wide and varied group of eukaryotic organisms with different shapes and sizes, including both macroscopic and microscopic forms. Although being originally classified as plants, they were eventually placed in their own of Whittaker's kingdoms of life as, unlike plants, they lack chlorophyll and are carbon heterotrophs. Fungi are, actually, more closely related to animals than plants, but they also differ from animals as, for example, they are saprotrophic (they absorb their food) and their cells are surrounded by cell walls.

Fungal cell walls are built from a majority of polysaccharides, mainly glucans and mannans with a lower percentage of chitin, also found in insects' exoskeletons. Glucans are β-2,6 and β-1,3-linked glucose units and mannans are present as a α-1,6-linked inner core with α-1,2 and α-1,3 side chains. Chitin, a homopolymer of β-1,4-linked N-acetylglucosamine units, always constitutes the basic structure in the architecture of fungal cell walls, even when present in very small amounts (typically in yeasts). Other components found in variable amounts are, for example, proteins, lipids, and inorganic phosphates.

The great majority of fungal species are free-living saprobes, usually the primary decomposer organisms in their habitats, allowing the continuous cycle of nutrients through ecosystems. Some others are biotrophs and can be found in symbiotic associations with plants, animals and prokaryotes (lichens and mykorrhizae are common examples). On the other end, only a few can be parasites. In fact, there are over one million known species of fungi (a lot more are estimated to exist), but only about 400 cause relevant diseases in man, animals or plants (Doctor Fungus, 2005a). Besides their pathogenesis, causing either human infections or economical losses in agricultural productions through animal and plant infections, fungi can also have important negative impacts through the spoilage of human products like food or medicines, for example. On the other hand, fungi are constant beneficial contributors to human activities as diverse as food preparation (like bread, cheese, beer or wine, etc.), production of chemical compounds (like some acids or alcohols, for example), production of pharmaceutical compounds (like antibiotics, cyclosporine A, enzymes, vitamins, etc.), biotechnology, and scientific research.

Regarding taxonomy, although some controversy and confusion is sometimes present, all fungi with medical importance, including veterinary pathogens, can be placed into one of five sexual groups: the phyla Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota, and

a further rather artificial group, Fungi Imperfecti or Mitosporic Fungi (also sometimes known as the phylum Deuteromycota) (Guarro *et al.*, 1999). The first four are true phyla, whose representatives are characterized by the production of sexual spores called ascospores, basidiospores, zygospores, and oospores, respectively. The last group, Fungi Imperfecti, includes all species for which only asexual or anamorph forms are known, species that can be included in the correspondent of the remaining phyla once teleomorph forms are discovered.

Importantly, fungi with relevance in human medicine can come only from four of the five referred groups, specifically the first three phyla plus the Mitosporic Fungi, and among these from some specific classes (Table 1). The flagellated organisms of the Chytridiomycota are not human pathogens and have relevance as infectious agents only in agriculture and in lower cold-blooded animals (Doctor Fungus, 2005b). Moreover, fungi for which sexual stadia are known receive different names, affecting both genus and species, to the teleomorph and the anamorph forms. Although the teleomorph form is the most important from the taxonomic point of view, allowing analysis of evolutionary relationships and inclusion into one of the true phyla, the corresponding anamorph forms are almost always found to be the infectious ones, reason why anamorphic names are the most commonly used in the clinical practice.

Table 1. Taxonomic system of medically relevant fungi.

Class	Order	Genera
Zygomycetes	Mucorales	*Mucor*, *Rhizopus*, *Rhizomucor*, *Absidia*
	Entomophthorales	*Basidiobolus*, *Conidiobolus*
Ascomycetes		*Talaromyces*, *Eurotium*, *Arthroderma*, *Ajellomyces, Claviceps*, *Saccharomyces*
Basidiomycetes	Agaricales	*Amanita*, *Boletus*, *Inocybe*, *Tricholoma*, *Filobasidiella*
Fungi Imperfecti[a] (Deuteromycetes)	Blastomycetes	*Candida*, *Cryptococcus*, *Malassezia*, *Rhodotorula*, *Trichosporon*
	Hyphomycetes	*Alternaria*, *Aspergillus*, *Blastomyces*, *Epidermophyton*, *Fusarium*, *Geotrichum*, *Histoplasma*, *Microsporum*, *Penicillium*, *Trichophyton*

[a] Artificial group including fungi for which no sexual stadia are known and, consequently, true taxonomic classification is not possible.

Fungi can also be subdivided into two broad groups, according to their morphology: moulds (filamentous fungi) and yeasts. Moulds are characterized by uni-, bi- or multinucleated hyphae that can be either septate or aseptate (coenocytic), the joint mass of which is the mycelium. Growth typically occurs in moulds by elongation of hyphal tips and they reproduce through the production of spores in specialized hyphae, the sporophores (referred to as co-

nidia and conidiophores, respectively, in the case of asexual reproduction). Yeasts, on the other hand, are reduced to unicellular forms which reproduce either by budding or fission. In some yeasts, the production of filamentous structures called pseudomycelium can be observed in certain conditions. However, these structures are not true hyphae but chains of elongated daughter buds that remain attached to the mother cell, as a result showing constrictions at the septa that are absent in true septate hyphae. A common example is the species *C. albicans*, for which pseudomycelium can be the predominant pathological form *in vivo* (Borgers *et al.*, 1979). Besides that, some species can alternate between both filamentous and yeast forms of growth, depending on relevant environmental conditions, a phenomenon referred to as dimorphism. In primary pathogenic fungi (e.g., *Histoplasma*, *Blastomyces*), budding yeast growth is generally found *in vivo*, while the filamentous forms develop in the environment (Gow *et al.*, 2002). Curiously, the referred pathogen *C. albicans* is an exception to these findings, as filamentous forms prevail *in vivo* (Gow *et al.*, 2002).

In a global sense, there are four types of mycotic diseases (Table 2). The focus of this thesis will be on infections only, however, to which the next section is devoted. The remaining types of mycotic diseases will not be discussed any further.

Table 2. Types of human mycotic diseases.

Mycotic disease	Description
Mycoallergy	Hypersensitive immune reaction to contact with fungal structures (typically after inhalation of spores).
Mycotoxicosis	Poisoning by ingestion of food products contaminated by toxin producing fungi (typically with grain products).
Mycetism	Poisoning by ingestion of toxin producing fungi as food products (typically with mushrooms).
Mycosis	Infection by pathogenic fungi involving host tissue or bodily fluid affection.

2.2 Human fungal infections

Like it was stated in the previous section, only a very small part of all known fungal species are human parasites. In fact, less than 0.5 % of all known species were ever found to cause infections. Human infections caused by these organisms are commonly organized according to the affected anatomical location into superficial, cutaneous, subcutaneous, and systemic (visceral, deep-seated, or invasive) infections.

Superficial mycoses are infections limited to the outermost layers of the skin and hair. Examples are pityriasis versicolor, tinea nigra, black piedra, and white piedra.

Cutaneous mycoses, also called dermatomycoses, are infections that extend deeper into the epidermis, as well as invasive hair and nail diseases, although being restricted to keratinized layers. They are typically caused by the so-called dermatophytes, referring to a group of keratinophilic species from the genera *Epidermophyton*, *Trichophyton*, and *Microsporum*. In a wider sense, other fungi, like *Candida* sp., *Aspergillus* sp., and *Cryptococcus neoformans*, are also recognized etiologic agents of dermatomycoses.

Subcutaneous mycoses refer to the affection of deeper layers of the skin, involving the dermis, subcutaneous tissue, or bone. They are usually initiated by trauma to the skin and have a chronic growth pattern, eventually extending to the epidermis. Examples are sporotrichosis, chromoblastomycosis, and mycetoma. These infections are difficult to treat and surgical intervention (excision or amputation) is frequently employed.

Finally, systemic mycoses are infections that may spread to deep organ systems (two and more by definition). These infections can be caused by a small group of primary pathogens, bearing specific adaptations for parasitic existence, or, nowadays more commonly, by secondary (opportunistic) pathogens causing disease mainly in predisposed individuals, particularly immunocompromised patients. Opportunistic systemic mycoses are frequently associated with nosocomial acquirement and are usually hard to diagnose, as these organisms are normally present in the environment and the natural colonizing microbiota. Empiric antimycotic therapy is usually employed and mortality rates are very high.

The groups of primary and secondary fungal pathogens are not restricted as etiological agents to systemic mycoses, however. The two classifications are independent and both primary and opportunistic fungi can cause infections in different anatomical locations. This way, among primary human pathogens two main groups, comprising a limited number of species, should be mentioned: the above referred dermatophytes and a group of dimorphic pathogens. Dermatophytes represent well-adapted pathogens including several true obligate parasites for humans, consisting of a few tens of species from the three referred genera that cause cutaneous mycoses. The dimorphic pathogens generally include normally soil-borne endemic fungi that have adapted to the environment of the human body, into which that enter through inhalation. Specifically, this group includes the thermally dimorphic species *Coccidioides immitis*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Penicillium marneffei*, etiological agents of systemic infections, plus *Sporothrix schenckii*, the causative agent of the referred subcutaneous infection sporotrichosis. The encapsulated yeast *C. neoformans* can be

considered both a primary and an opportunistic pathogen, depending on the specific variety and serotype in question (Speed and Dunt, 1995).

The group of secondary pathogens is more numerous, including a wide range of opportunistic saprobes led by *Candida* and *Aspergillus* species, and *C. neoformans*. In fact, and bearing in mind that infection is due in this case more to patient predisposition than specific fungal virulence, it can be said that no fungus can be safely considered non-pathogenic to the severely immunocompromised host.

Although medical mycology was traditionally a limited discipline, studying only the defined number of primary pathogens referred, some of which constrained to specific geographical regions, the last few decades brought about a rise in its importance, with the strong expansion in both number and variety of opportunistic fungal infections. This evolution has been following the growth in the population of debilitated and immunocompromised patients, resulting mainly from the acquired immunodeficiency syndrome (AIDS) pandemic and the evolution of more aggressive medical techniques. Modern medical interventions like bone marrow or solid-organ transplantation, chemotherapy for cancer patients, the use of invasive monitoring devices, parenteral nutrition, broad-spectrum antimicrobial therapy and assisted ventilation, for example, all contributed to the increase of the hospitalized population of patients at risk of developing invasive fungal infections. As a result, opportunistic systemic mycoses are now a common problem among susceptible patients. In fact, in a study in American hospitals in the late 90s *Candida* species were shown to be the fourth most commonly isolated nosocomial bloodstream pathogens and the ones with the highest crude mortality (Table 3), while fungi accounted for 8 % of the infections overall (Edmond *et al.*, 1999).

Table 3. Rank order of nosocomial bloodstream pathogens and the associated crude mortality in United States hospitals.*

Rank	Pathogen	Proportion of cases (%)	Crude mortality (%)
1	Coagulase-negative staphylococci	31.9	21
2	*Staphylococcus aureus*	15.7	25
3	Enterococci	11.1	32
4	*Candida* species	7.6	40
5	*Escherichia coli*	5.7	24

* Adapted from Edmond *et al.* (1999)

On the other hand, *Candida* species are known to lead the number of bloodstream infections due to fungi, with *C. albicans* always accounting for the great majority of the cases (Beck-Sague *et al.*, 1993; Fridkin and Jarvis, 1996; Pfaller *et al.*, 2000; Pfaller *et al.*, 1998a; Lewis and Klepser, 1999; Abi-Said *et al.*, 1997; Nguyen *et al.*, 1996). A good example is the report analyzing the trends in the epidemiology among nosocomial fungal infections in the United States of America (USA) from 1980 to 1990 (Beck-Sague *et al.*, 1993). It shows an elucidative picture of the general situation, with *Candida* species accounting for 88 % of all nosocomial fungal infections, *C. albicans* alone accounting for over 60 % of the cases (Table 4).

Table 4. Relative proportions of nosocomial fungal infections in United States hospitals, by pathogen, from 1980 to 1990.*

Fungal pathogen	Estimated proportion (%)
Candida albicans	61
Non-*albicans Candida* spp.	27
C. glabrata	
C. parapsilosis	
C. tropicalis	
C. krusei	
C. lusitaniae	
Aspergillus spp.	1
Other	11
Yeasts (*Malassezia* and *Trichosporon* spp.)	
Zygomycetes (*Mucor* and *Rhizopus* spp.)	
Hyalohyphomycetes (*Fusarium* and *Acremonium* spp.)	
Phaeohylahyphomycetes (*Alternaria*, *Bipolaris*, and *Curvularia* spp.)	

* Adapted from Fridkin and Jarvis (1996); data from Beck-Sague *et al.* (1993)

The global picture regarding invasive fungal infections has been changing, however, both among the genus *Candida* and involving other fungi. An emergence of species of *Candida* other than *C. albicans* (Fridkin, 2005; Pfaller and Diekema, 2004; Pfaller *et al.*, 1998b; Abi-Said *et al.*, 1997; Nguyen *et al.*, 1996; Pfaller, 1996; Price *et al.*, 1994), the so-called non-*albicans Candida* species, other yeasts like *Malassezia* and *Trichosporon*, and moulds like *Aspergillus* species, zygomycetes and *Fusarium* species (Brown, 2005; Torres *et al.*, 2005; Fridkin, 2005; Pfaller and Diekema, 2004; Nucci *et al.*, 2004; Nucci *et al.*, 2003; Torres *et al.*, 2003; Marr *et al.*, 2002; Fridkin and Jarvis, 1996) has been clearly detected. This trend poses serious problems, since most of these pathogens are typically less sensitive to the currently available antifungal drugs.

Fungal infections are now a recognized major cause of morbidity and mortality among seriously immunocompromised patients, with traditionally less common, difficult to diagnose, and hard to treat infections emerging. The epidemiology of invasive fungal infections is expected to continue to evolve in the future, driven by the rise in the number of immunocompromised patients, antifungal therapy pressure and environmental shifts, demanding a constant evolution in treatment standards.

2.3 Antifungal drugs

2.3.1 Short description of the evolution of antifungal therapy

The fact that up until the 1980s fungi were not globally recognized as important pathogens (Anaissie and Bodey, 1989), medical mycology being traditionally limited to the study of a limited number of endemic fungal pathogens (see section "2.2 Human fungal infections"), explains a general situation involving less pressure for the development of new drugs than in the case of bacteriology. As shown in the chronological representation of the development of the most important antifungal drugs introduced so far (Table 5), the development of new compounds was not traditionally intense, especially regarding systemic agents.

Until the 1950s the only antifungal therapy available was the oral administration of a saturated solution of potassium iodide for cutaneous sporotrichosis (Mercurio and Elewski, 1993). In fact, although griseofulvin, chlormidazole (the first antifungal azole), and nystatin (the first antifungal polyene) had been reported in 1939, 1944, and 1949, respectively, it was not until 1958 that oral griseofulvin and topical chlormidazole became available for clinical use (Sheehan, 1999).

More important was the discovery of the polyene amphotericin B, isolated from the actinomycete *Streptomyces nodosus* (Hazen and Brown, 1951). After a failed attempt with nystatin, because of its toxicity, amphotericin B was successfully tested and in 1960 it was introduced as a life-saving drug for systemic application in the treatment of serious fungal infections, evidently improving the poor prognostics previously prevailing in such cases (Gallis *et al.*, 1990). In fact, amphotericin B has resisted through time as the "gold standard" of antifungal therapy, and only since very recently its serious nefrotoxicity and availability of appropriate alternatives are causing some authors to reconsider its leading position (Ostrosky-Zeichner *et al.*, 2003).

Table 5 Chronological organization of important antifungal drug developments.*****

	Antifungal drug development
1930s	Griseofulvin reported.
1940s	Chlormidazole reported.
	Nystatin reported.
1950s	Amphotericin B reported.
	Griseofulvin marketed.
	Chlormidazole marketed.
1960s	Amphotericin B marketed.
	Flucytosine (antifungal activity) reported.
	Miconazole and clotrimazole (topical formulations) marketed.
1970s	Flucytosine (for antifungal therapy) marketed.
	Econazole marketed.
	Amorolfine reported.
	Miconazole (parenteral formulation) marketed.
1980s	Ketoconazole (oral formulation) marketed.
	Naftifine in clinical trials.
	Polyene lipid formulations in development.
	Caspofungin reported.
1990s	Fluconazole marketed.
	Itraconazole marketed.
	Voriconazole, posaconazole, and ravuconazole reported.
	Amphotericin B lipid complex (Abelcet®) marketed.
	Terbinafine and amphotericin B colloidal dispersion (Amphocil® and Amphotec®) marketed.
	Liposomal amphotericin B (AmBisome®) and itraconazole oral solution marketed.
2000s	Caspofungin marketed.
	Voriconazole marketed.
	Micafungin marketed.
	Posaconazole marketed.

* Data from Sheehan (1999); Krčméry (2005); Anonymous (2005).

The next development came about during the 1960s, with the discovery of the antifungal activity of the antimetabolite 5-fluorocytosine (flucytosine), originally developed as an anti-tumour agent. It was licensed in 1972 but, because of its limited spectrum of activity, bone marrow toxicity, and rapid development of resistance *in vivo*, it is now only used in combination therapy (Patel, 1998).

The period between the late 1960s and beginning of the 1980s witnessed the establishment of the group of azole antifungals, with the release of several imidazoles both for

topical and systemic application. The first generation included, for example, econazole, clotrimazole, and, as the most important representative, the systemic miconazole. The second generation included drugs like oxiconazole, terconazole, tioconazole, and the most successful imidazole, the orally administered ketoconazole (Bodey, 1992; Fromtling, 1988).

During the 1980s the number of opportunistic invasive fungal infections started to grow significantly (see section "2.2 Human fungal infections") and the need for the development of new antifungal agents became bigger. The result was the introduction, in the beginning of the 1990s, of the new triazoles fluconazole and itraconazole, two drugs that represented a real breakthrough in antifungal therapy, for their high therapeutic efficacy combined with low toxicity (Sheehan, 1999).

A further advance was introduced in the 1990s when three amphotericin B lipid formulations were approved: liposomal amphotericin B (AmBisome®), amphotericin B lipid complex (Abelcet®), and amphotericin B colloidal dispersion (Amphotec®) (Lemke, 2005). These preparations notably improved the drug's therapeutic index by avoiding its known nefrotoxicity and keeping its high antifungal activity.

Since then, recent considerable advances have been introduced: the development of a second generation of triazole drugs, with broad-spectrum activity and favourable pharmacokinetic profiles, and the development of a new class of drugs, the echinocandins, representing the first class of drugs acting on fungal cell wall approved for clinical use. The new triazoles include voriconazole, posaconazole, and ravuconazole, and the echinocandins are caspofungin, micafungin, and anidulafungin. Caspofungin and voriconazole were already approved by the Food and Drug Administration (FDA) in the USA and the European Medicines Agency (EMEA), the echinocandin in 2001 and the triazole in 2002 (Krcmery, 2005). More recently, in 2005, micafungin was approved by the FDA and posaconazole was approved by both the EMEA and the FDA.

2.3.2 Antifungal drugs and mechanisms of action

The fact that fungal cells are eukaryotic, therefore resembling mammalian cells much closer than bacteria, explains the difficulties in the development of highly active selective compounds with antifungal activity. Antifungal drug development is a very active field, however, driven by the current increase in invasive fungal infections, and new drugs with improved characteristics or different mechanism of action are under development. There are today several different groups of antifungal drugs acting in a considerable amount of different

cellular targets (Table 6 and Fig. 1), although the total number of available preparations is a lot lower than the number of antibacterial preparations.

Table 6 Most important chemical groups of clinically available and experimental antifungal agents, including examples, and their mechanisms of action.*

Chemical group and examples	Mechanism of action
Polyenes Amphotericin B Nystatin	Bind to cytoplasmic membrane ergosterol, creating hydrophilic pores that allow the leakage of vital cellular contents and consequently lead to cell death.
Azoles Imidazoles Miconazole Ketoconazole 1st generation triazoles Fluconazole Itraconazole 2nd generation triazoles Voriconazole Posaconazole Ravuconazole	Inhibit the cytochrome P-450 dependent 14α-demethylation of lanosterol in the ergosterol biosynthetic pathway, leading to ergosterol depletion and accumulation of aberrant sterols in the membrane.
Allylamines Terbinafine Naftifine	Inhibit the enzyme squalene epoxidase in the ergosterol biosynthetic pathway, leading to ergosterol depletion and accumulation of aberrant sterols in the membrane.
Thiocarbamates Tolnaftate Tolciclate	Inhibit the enzyme squalene epoxidase in the ergosterol biosynthetic pathway, leading to ergosterol depletion and accumulation of aberrant sterols in the membrane.
Morpholines Amorolfine	Inhibit the enzymes sterol Δ^{14} reductase and Δ^{7}-Δ^{8} isomerase in the ergosterol biosynthetic pathway, leading to ergosterol depletion and accumulation of aberrant sterols in the membrane.
Fluorinated pyrimidine 5-Fluorocytosine	Is deaminated to 5-fluorouracil, which (a) is converted to the nucleoside triphosphate and incorporated into RNA, causing premature chain termination, and (b) is converted to deoxynucleoside, which inhibits thymidylate synthase and thus DNA synthesis.
Benzofurane Griseofulvin	The precise mechanism is still unknown, but it is generally recognized to interfere with microtubule assembly, blocking mitosis.
Echinocandins Caspofungin Micafungin Anidulafungin	Inhibit a complex of proteins responsible for the synthesis of β-1,3 glucan polysaccharides, therefore inhibiting the synthesis of the fungal cell wall.
Sordarins	Block the function of fungal translation elongation factor 2, thus inhibiting protein synthesis.
Nikkomycins Nikkomycin Z	Inhibit the enzyme chitin synthase, this way inhibiting the synthesis of the fungal cell wall.

* Data from Odds *et al.*, (2003a,b); Georgopapadakou and Walsh (1996)

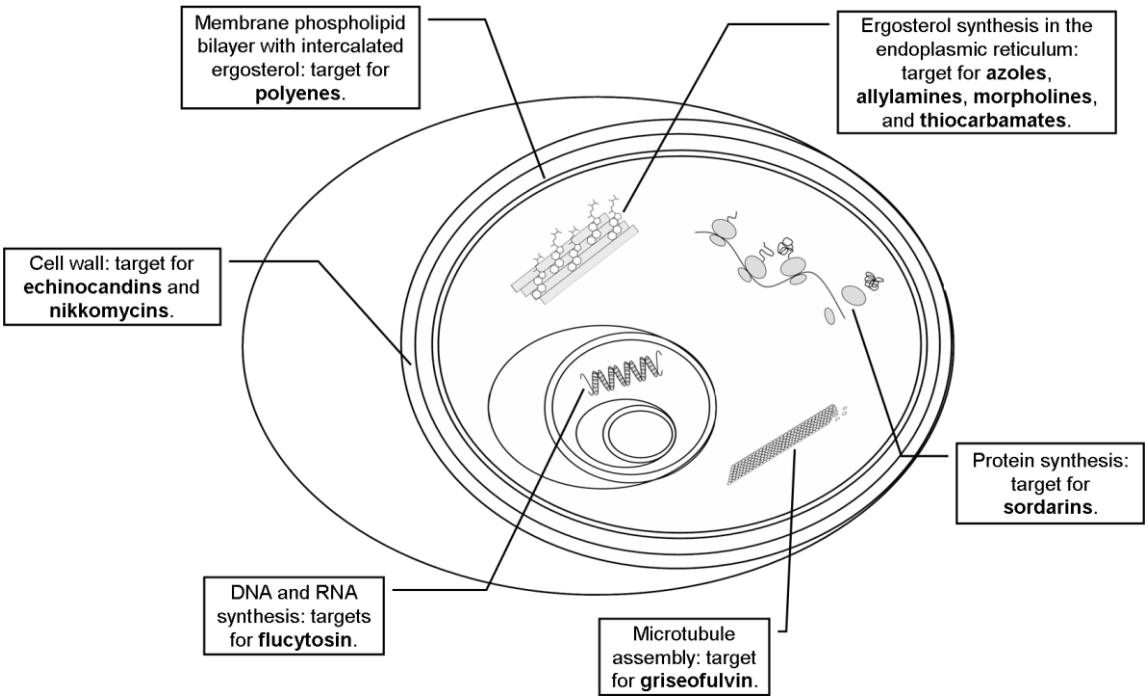


Figure 1 Cellular targets for the most important available and experimental antifungal agents.

Regarding their origin, currently licensed drugs come from three classes of natural products (griseofulvin, polyenes and echinocandins) and five classes of synthetic chemicals (azoles, allylamines, flucytosine, morpholines, and thiocarbamates). The great majority of them, coming from five of the different referred chemical groups, have mechanisms of action involving either direct binding to ergosterol, the major fungal membrane sterol, or inhibition of its biosynthesis (Table 6 and Fig. 1), although it can be argued that this is not the ideal target (see the sections on the individual groups of antifungal agents below). There are several other cellular targets for other classes of compounds, however, and the recent development of echinocandins has proven the dedication of research attention to fungal cell wall as a specific target for antifungal activity. The next few sections are dedicated to individual overviews of each one of the most important antifungal chemical groups available and under development.

2.3.2.1 Polyene antifungal agents

Polyene antifungals are macrocyclic molecules of microbial origin (isolated from *Streptomyces* species) with a very wide spectrum of activity. There are three compounds used in clinical practice: the topical agents nystatin and natamycin, and the systemic amphotericin B (Fig. 2).

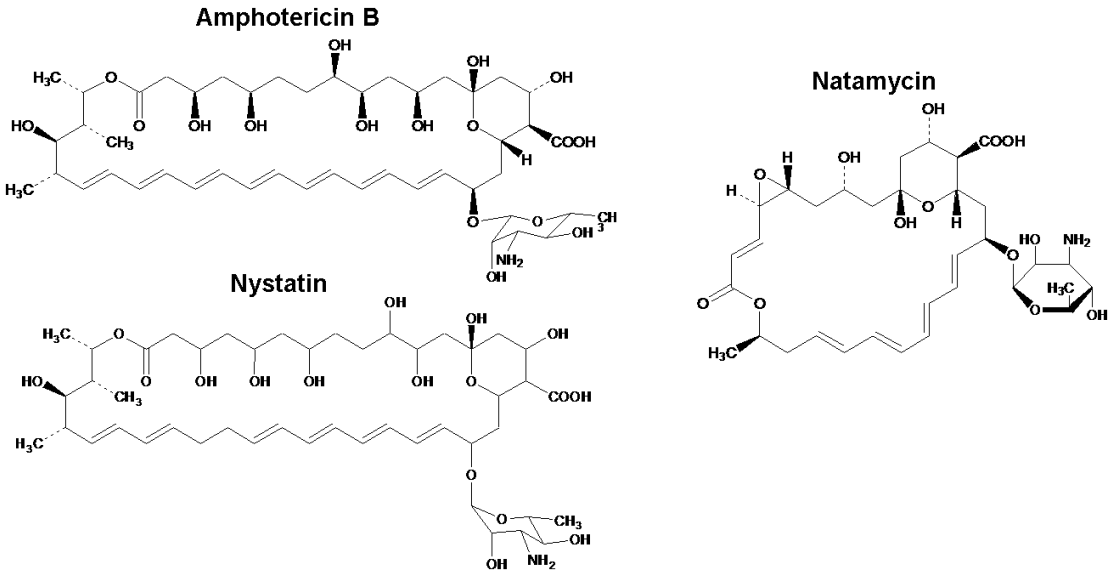


Figure 2 Chemical structures of clinically used polyene antifungal agents.

Their antifungal activity is based on a rather atypical mode of action: instead of inhibiting an enzyme, they directly bind to ergosterol, the main sterol in fungal cytoplasmic membranes, this way disrupting the membrane integrity and leading to the leakage of cellular contents. Concretely, the amphoteric amphotericin B molecule, for example, hydrofobically binds to ergosterol in the membrane phospholipid bilayer, leaving its hydrophilic edge (with the hydroxyl residues) unbalanced in relation to the hydrophobic portion of the complex. The complex then migrates within the membrane to align its hydrophilic face with aggregations of other complexes, giving rise to aqueous pores formed by an annulus of eight amphotericin B molecules. These pores, with the polyene hydroxyl residues facing inward, lead to altered membrane permeability, with leakage of vital cytoplasmic components and consequent cell death (Ghannoum and Rice, 1999). In addition to this mechanism, amphotericin B has also been implicated in causing oxidative damage to fungal cells (Ghannoum and Rice, 1999; Okamoto *et al.*, 2004).

As it was referred above, ergosterol is not the ideal target for antifungal agents, since the structure of ergosterol is not that different from the structure of cholesterol (Fig. 3), the major mammalian membrane sterol, consequently lowering the level of selective toxicity of polyenes. However, amphotericin B does have greater binding affinity for ergosterol over cholesterol, a difference explained by the fact that the ergosterol molecule has a cylindrical three-dimensional structure, unlike the closely chemically related cholesterol, which has a sigmoid shape. This difference in binding affinity, together with the higher ratio ergosterol:phospholipids in fungal membranes, explain the selectivity in the activity of ampho-

tericin B (Brajtburg *et al.*, 1974). In fact, and although not excluding the basis for its toxicity in clinical use, it has been shown that amphotericin B binds 10-fold more tightly to unilamellar vesicles containing ergosterol than to those containing cholesterol (Readio and Bittman, 1982).

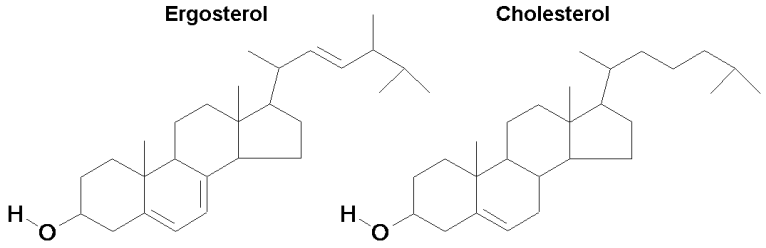


Figure 3 Chemical structures of the major fungal and mammalian membrane sterols: ergosterol and cholesterol.

Amphotericin B has also been proposed to have both immunostimulatory and immunosuppressive effects on the host. Immunostimulatory effects have been found on macrophages (Brajtburg *et al.*, 1990; Jahn *et al.*, 1998) and immunosuppressive effects were connected to the disruption of cell membranes of polymorphonuclear leucocytes (Jullien *et al.*, 1991). A further immunosuppressive property is the induction of lipid peroxidation in the mammalian cell membranes, leading to their fragility ad increased permeability (Brajtburg and Bolard, 1996; Barwicz *et al.*, 2000).

In spite of its serious toxicity, which is now leading some authors to reconsider its position (Ostrosky-Zeichner *et al.*, 2003), amphotericin B was considered ever since the 1950s and until recently the "gold standard" of therapy of invasive mycoses. It is available as a systemic drug for parenteral application and in a topical formulation for selective intestinal decontamination or the treatment of refractory mucosal infections (Lass-Florl *et al.*, 2003; Vazquez, 2000; Paterson *et al.*, 2001; Manson *et al.*, 1992). It is still very important for the treatment of several serious life-threatening infections, especially in deeply immunocompromised patients, although it is now being progressively replaced by newer less toxic drugs (Kontoyiannis, 2001; Patterson, 2005).

A significant recent advance was the development of the new lipid formulations of amphotericin B: liposomal amphotericin B (AmBisome®), amphotericin B lipid complex (Abelcet®), and amphotericin B colloidal dispersion (Amphotec®). These preparations greatly improved the drug's therapeutic index, by avoiding its serious toxicity while keeping the same activity levels (Barrett *et al.*, 2003). A serious limitation, however, currently limiting its practical application, is its extremely high cost, although it can be argued that what is spent above when choosing lipid formulations of amphotericin B could be neutralized by savings in hospital

costs associated with treatment of conventional amphotericin B deoxycholate nefrotoxicity (Cagnoni *et al.*, 2000).

It is also worth noticing the recent development of an inhalation powder formulation of amphotericin B (Nektar Therapeutics), approved in December 2005 by the FDA in orphan drug status for the prevention of pulmonary fungal infections in patients at risk for aspergillosis due to immunosuppressive therapy. The use of inhalable amphotericin B has been shown to yield a statistically significant improvement in the survival of immunosuppressed rabbits challenged with a pulmonary dose of *A. fumigatus* spores. In addition, it is very interesting to notice that little or no pulmonary toxicity was observed when animals were administered amphotericin B at doses 10-fold or greater than those expected for use in humans. Toxicology data revealed no systemic toxicity and serum drug concentrations were lower than those generally considered as toxic to humans, even at the 10-fold greater doses. This formulation is currently undergoing further clinical trials.

Nystatin and natamycin are agents for local application used in the treatment of mucosal yeast infections and fungal infections of the gastrointestinal tract. A liposomal nystatin formulation (Nyotran®) has also been developed (Wasan, 1997) for systemic application and indications similar to those of amphotericin B. It is now in late Phase III clinical trials.

2.3.2.2 Azole antifungal agents

Azoles are the largest group of antifungal agents in clinical practice. They are totally synthetic agents with broad-spectrum activity that have been developed throughout the last few decades (see section "2.4.1 Short description of the evolution of antifungal therapy"), starting from the appearance of the first imidazoles in the late 1960s, through a further generation of imidazoles, and finally evolving to two generations of triazoles.

Azole antifungals are one of the antimycotic classes acting on the ergosterol biosynthetic pathway (Fig. 4), where their main effect is the inhibition of the 14α-demethylation of lanosterol. Their specific target is the cytochrome P450-dependent enzyme 14α-demethylase which catalyses the oxidative removal of the 14α-methyl group of lanosterol by a typical P450 mono-oxygenase activity. This protein contains an iron protoporphyrin moiety located at the active site, and the antifungal azoles bind to the iron atom via a nitrogen atom in the imidazole or triazole ring. The rest of the azole molecule binds to the apoprotein in a different extent depending on the individual structures of the enzyme and the azole derivative in question (Vanden Bossche *et al.*, 1995). Ergosterol is a bioregulator of cytoplasmic membrane fluidity and asymmetry, and the final result of azole activity is a fungistatic effect through the replacement of ergosterol by its precursors (14α-methylated sterols) in the fungal membrane,

causing alterations of its normal permeability and fluidity (Ghannoum and Rice, 1999). Secondary consequences for membrane-bound enzymes, such as those involved in cell wall synthesis, are also detected, particularly with imidazole agents, which seem to have a more heterogenic activity (Sud and Feingold, 1981).

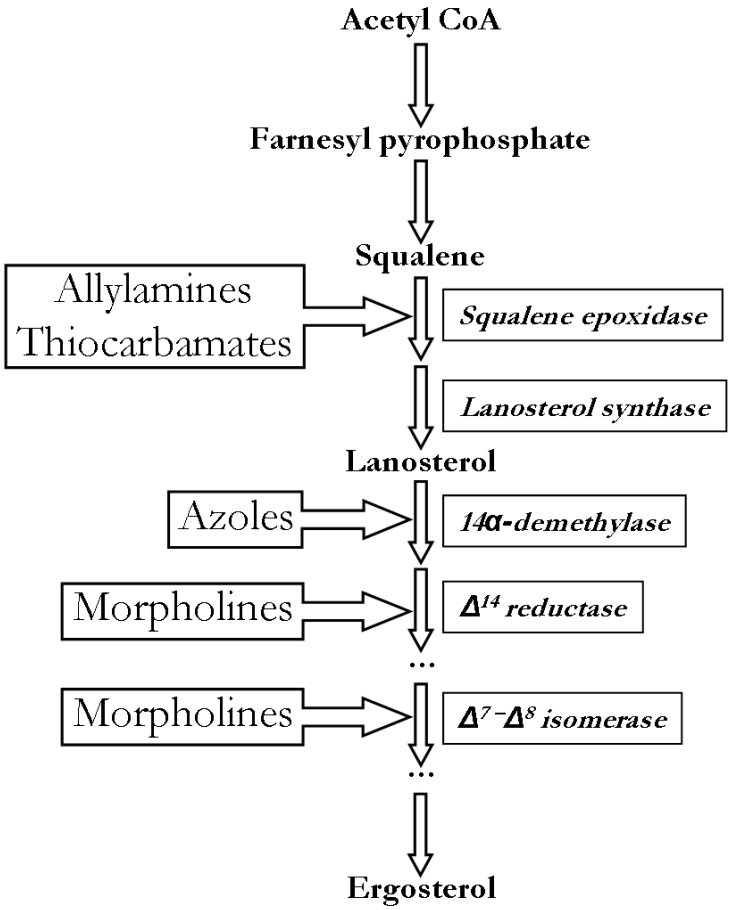


Figure 4 Simplified ergosterol biosynthesis pathway with indication of the sites of inhibition of different classes of antimycotic agents.

Among the agents integrating the group of first generation imidazoles, clotrimazole, econazole, and miconazole (Fig. 5) could be considered the most important. The first two are topical agents used in the therapy of dermatomycoses and miconazole represents the first systemic imidazole ever available, suitable for intravenous administration. Today it is not used in systemic therapy anymore, though.

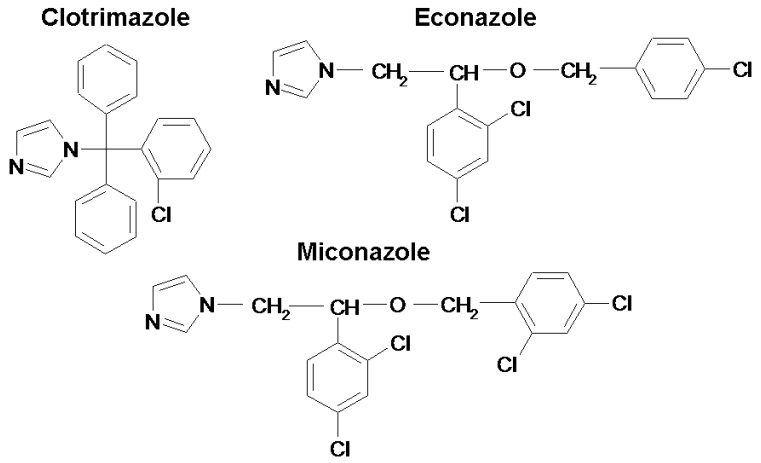


Figure 5 Chemical structures of some first generation imidazoles.

Some examples of second generation imidazoles are oxiconazole, bifonazole, isoconazole, and, most importantly, ketoconazole (Fig. 6). In fact, the development of ketoconazole represented a great advance for antimycotic therapy, since it was the first broad-spectrum systemic antifungal agent suitable for oral application. The other three agents have similar uses to the referred first generation imidazoles econazole and clotrimazole, while ketoconazole, which is now considered a second-line drug because of the availability of the safer triazoles (see below), is used today mainly in the treatment of superficial mycoses and infections caused by dimorphic fungi.

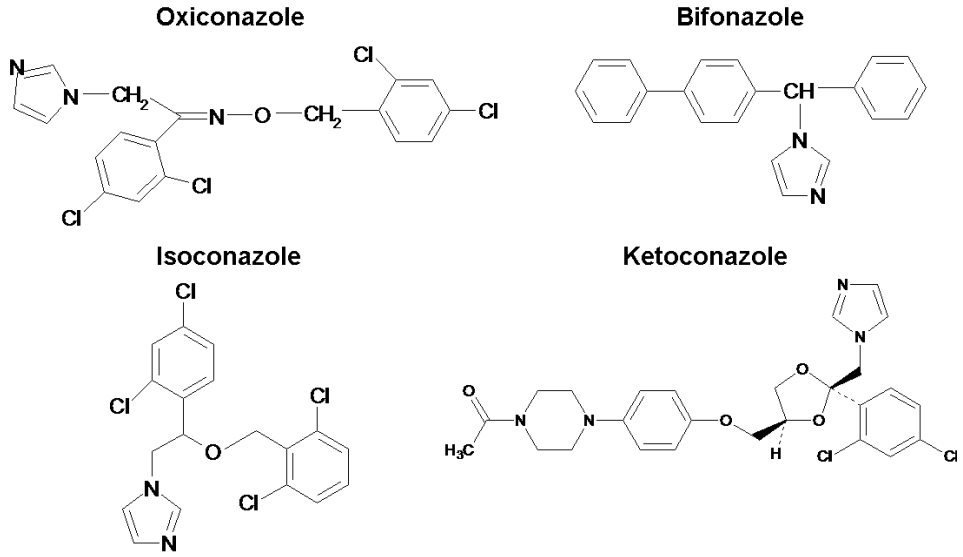


Figure 6 Chemical structures of some second generation imidazoles.

A real breakthrough for antifungal therapy was the structural substitution of the imidazolic ring by a triazolic ring, resulting in the development of third generation azole agents that are generally more active and have broader spectra of activity than imidazoles. Three

compounds are worth mentioning: fluconazole, itraconazole, and terconazole (Fig. 7). Fluconazole is a systemic antifungal agent especially valuable for its excellent pharmacokinetic profile, good tolerability, and minimal organ toxicity. It is very useful in the treatment of candidiasis and cryptococcosis in particular, but also some dermatomycoses and some infections caused by dimorphic fungi. It is also commonly used for prophylaxis in transplant patients. Itraconazole, on the other hand, is a per oral and intravenous systemic antifungal agent which is especially valuable, in comparison with fluconazole, for its extended spectrum of activity to *Aspergillus* species and some fluconazole-resistant *Candida* species. Finally, terconazole is a triazole derivative which is only used for local application in the treatment of vulvovaginal candidosis.

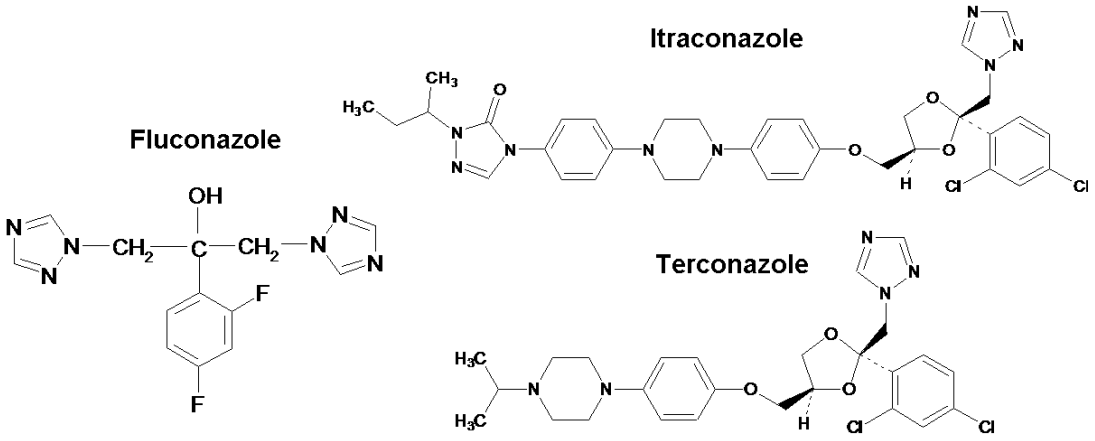


Figure 7 Chemical structures of some third generation azoles: the first triazole antifungal agents.

Since very recently, a second generation of extended-spectrum triazole drugs is under development. These drugs include the already approved voriconazole and posaconazole and the experimental ravuconazole (see section "2.3.1 Short description of the evolution of antifungal therapy"). Voriconazole and ravuconazole (Fig. 8) are structurally related to fluconazole (Fig. 7), while posaconazole (Fig. 8) is derived from the ketoconazole prototype and resembles itraconazole (Fig. 7). Voriconazole has a very broad spectrum of activity, showing effect even against very hard-to-treat infections like the ones caused by *Fusarium* or *Scedosporium*, and is even fungicidal against some isolates of filamentous fungi (Espinel-Ingroff *et al.*, 2001). Today it is assuming a dominant role in the treatment of invasive fungal infections, and it is already approved for therapy of invasive aspergillosis, *Fusarium* spp. infections, *Scedosporium apiospermum* infections, *Candida* esophagitis, and candidaemia, including infections with *C. krusei* and *C. glabrata*. Posaconazole has also been repeatedly shown to have high activity against aspergillosis and other invasive fungal infections (Patterson, 2005; Torres *et al.* 2005), but its

most interesting attribute is the favourable and promising clinical results against some zygomycetes (Brown, 2005; Greenberg *et al.* 2006; Chayakulkeeree *et al.* 2006), a group of emerging opportunistic pathogens which are not sensitive to other drugs. Ravuconazole, on the other hand, and although having shown activity against aspergillosis also, has been assessed in early clinical trials up to Phase II but appears not to be currently undergoing further development (Kirkpatrick *et al.*, 2002; Petraitis *et al.*, 2003).

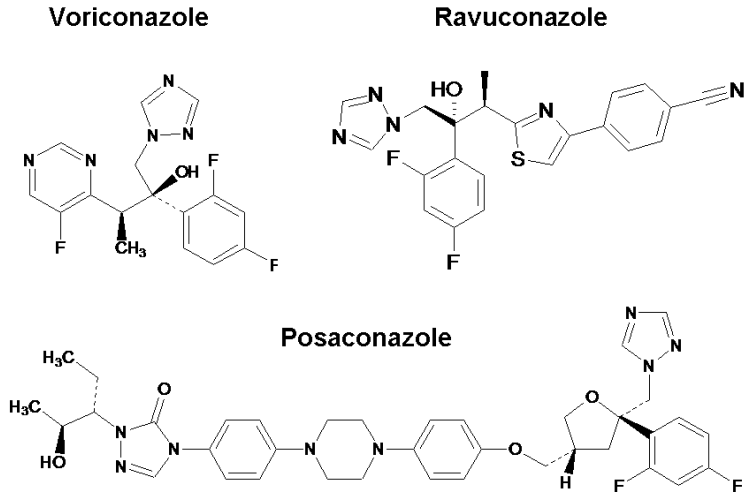


Figure 8 Chemical structures of the three newest triazole antimycotic agents.

In conclusion, analyzing the refinement of azole derivatives over the last four decades, since the first generation imidazoles up until the newest triazole agents, some structural changes could be highlighted for their consequences in the interaction of these antifungals with the target enzyme at the molecular level. This way, the replacement of the imidazole ring by a triazole ring in the active pharmacophore was undoubtedly crucial, leading to a higher specificity in binding to fungal P450 cytochrome. For molecules derived from the ketoconazole prototype, extension of the side chain, like in the cases of itraconazole and the newer posaconazole, enhances binding to the P450 apoprotein. On the other hand, regarding the molecules derived from the fluconazole prototype, the inclusion of an α-*O*-methyl group, like in the new voriconazole and ravuconazole, confers activity against *Aspergillus* species and many other filamentous fungi (Odds *et al.*, 2003). The global result, in clinical terms, was the development of a wide class of antifungal agents with relevance in the treatment of both local and visceral infections, with at least one representative deserving consideration almost anytime appropriate management of a fungal infection is under consideration.

2.3.2.3 Allylamines

Allylamines include the topical naftifine, the systemic terbinafine, plus the more recently developed topical butenafine (Fig. 9), a group of synthetical benzylamine derivatives.

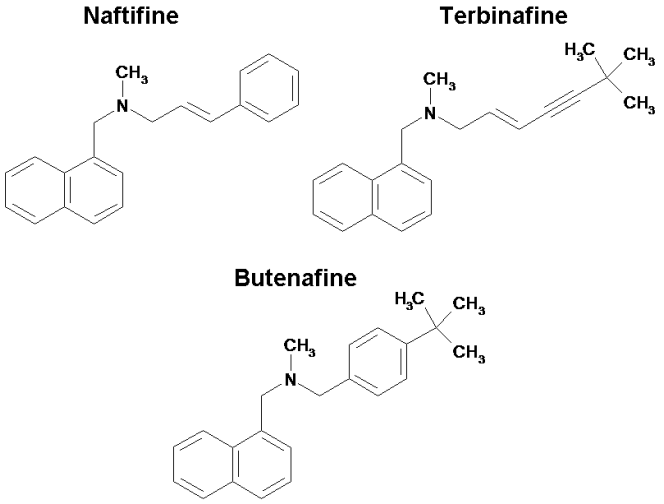


Figure 9 Chemical structures of allylamine antifungal agents in clinical use.

These drugs constitute another group of drugs acting in the ergosterol biosynthetic pathway, where they reversibly and noncompetitively inhibit squalene epoxidase, an early step in the pathway necessary to the conversion of squalene to lanosterol (Fig. 4). The resulting ergosterol depletion and squalene accumulation affect membrane structure and function, leading to a fungicidal effect. Fungal cell death is primarily related to squalene accumulation, rather than to ergosterol depletion, however (Ryder and Favre, 1997). In addition to these effects, butenafine also causes direct membrane effects in ergosterol-depleted cells (Iwatani *et al.*, 1993).

Allylamines have a wide-spectrum of antifungal activity, including mainly dermatophytes, *Candida* spp., and *Aspergillus* spp. They are only used in the treatment of dermatomycoses, however, especially dermatophytoses (including dermatophytic onychomycoses) for which terbinafine is a first choice agent. Not even the systemic agent terbinafine, available both for oral and topical administrations, is used in the treatment of systemic infections, although it has been suggested that its potential could justify such applications (Perez, 1999; Hay, 1999).

2.3.2.4 Thiocarbamates

The group of thiocarbamate antifungal agents includes the synthetic chemicals tolnaftate, tolciclate and liranaftate (Fig. 10), from which the first is the only licensed for human therapy, concretely for topical application.

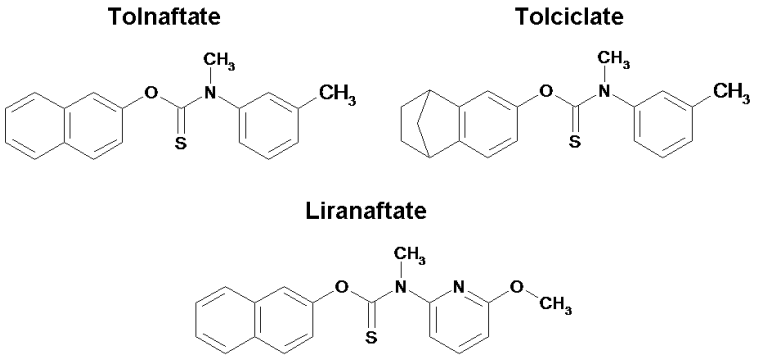


Figure 10 Chemical structures of three thiocarbamate antifungal agents.

Thiocarbamates represent a further group of antifungal agents acting on the ergosterol biosynthetic pathway. They have an inhibitory effect on the exact same target in the pathway as allylamines, the enzyme squalene epoxidase (Fig. 4). The result, as described in the previous section for allylamines, is the depletion of membrane ergosterol and the accumulation of squalene, affecting membrane structure and function.

These agents have a narrow spectrum of activity, practically only against dermatophytes, and the clinical importance of tolnaftate is very limited (Hart *et al.* 1999).

2.3.2.5 Amorolfine

The synthetic agent amorolfine (Fig. 11) is the sole representative of the morpholine, or phenylmorpholine, class of antifungal agents with clinical interest.

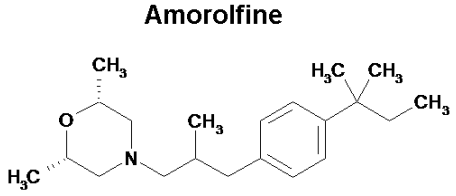


Figure 11 Chemical structure of the antifungal agent amorolfine.

Amorolfine represents the last class of agents acting on the ergosterol biosynthesis pathway, as it affects two targets late in the pathway. It inhibits the enzymes sterol Δ^{14} reductase and Δ^{7} Δ^{8} isomerase, leading to effects similar to the ones caused by the other ergosterol biosynthesis inhibitors: ergosterol depletion and accumulation of aberrant sterols in the membrane.

This agent has a narrow spectrum of activity, including dermatophytes and some yeasts. It is used for local application in the therapy of dermatomycoses, including onychomy-

coses, although it does not represent, today, first choice therapy for any infection (Hart *et al.* 1999).

2.3.2.6 Flucytosine

Flucytosine (5-fluorocytosine) (Fig. 12) is a fluorinated pyrimidine obtained by synthesis, whose antifungal activity was first reported in the 1960s.

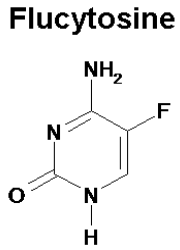


Figure 12 Chemical structure of flucytosine.

It is a pro-drug that works as an antimetabolite after conversion to 5-fluorouracil inside target cells. Fluorouracil is then converted both to the nucleoside triphosphate, which becomes incorporated into RNA and causes miscoding and consequent premature chain termination, and to the deoxynucleoside (5-fluorodeoxyuridine monophosphate), which inhibits DNA synthesis through effects on thymidylate synthase. For these effects to take place the target cells must possess the enzymes cytosine permease, for flucytosine to be taken up into the cells, cytosine deaminase, to convert it to 5-fluorouracil, and uracil phosphoribosyl tranferase, to convert 5-fluorouracil to the nucleoside triphosphate, the substrate for nucleic acid synthesis (Ghannoum and Rice, 1999). The fact that mammalian cells usually lack or have a very low level of activity of cytosine deaminase makes flucytosine relatively non-toxic to mammals, while flurouracil is a potent anticancer agent (Georgopapadakou and Walsh, 1996).

The spectrum of activity of flucytosine is restricted to pathogenic yeasts, particularly *Candida* species and *C. neoformans*, because most filamentous fungi lack the referred necessary enzymes for flucytosine to be active. Furthermore, it is traditionally considered to be associated with a high frequency of primary and secondary resistance, resulting from deficiencies in one or more of the three referred indispensable enzymes. For that reason, it is only used in combination antifungal therapy, mainly with amphotericin B in the treatment of some *Candida* invasive infections and cryptococcal meningitis (Patterson, 2005).

2.3.2.7 Griseofulvin

Griseofulvin (Fig. 13) is a natural benzofurane drug which was first isolated from a *Penicillium* spp. in 1939 and became the firs specific antimycotic agent ever reported (Table 5).

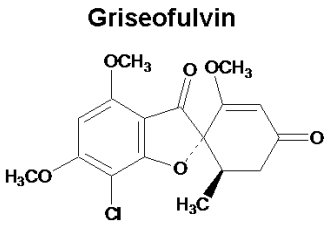


Figure 13 Chemical structure of griseofulvin.

The precise mechanism of action of griseofulvin is still doubtful and several different possibilities have been proposed (Develoux, 2001). The fact that it interferes with microtubule assembly (Gull and Trinci, 1973) is the favoured explanation, however. Microtubules are dynamic polymers of α- and β-tubulin dimmers that form a highly organized cellular skeleton and the mitotic spindle in all eukaryotic cells. Griseofulvin acts by interacting with the polymerized microtubules, one of the consequences being the disruption of the mitotic spindle and the consequent inhibition of mitosis in fungal cells (Gull and Trinci, 1973). The final result is fungistatic activity.

Griseofulvin is available as a systemic agent for oral administration, but it has a narrow spectrum of activity, mainly against dermatophytes. Nowadays its use is limited as it was replaced by newer, less toxic, and more active alternatives, namely terbinafine and the azoles.

2.3.2.8 Echinocandins

Echinocandins are natural compounds (fungal secondary metabolites), structurally consisting of a cyclic hexapeptide core with a lipid side chain (Fig. 14) responsible for their antifungal activity. The originally found echinocandins have been developed through semi-synthetic procedures, resulting in three compounds that entered clinical development in the late 1990s: caspofungin, anidulafungin, and micafungin (Fig. 14), all available for intravenous administration only.

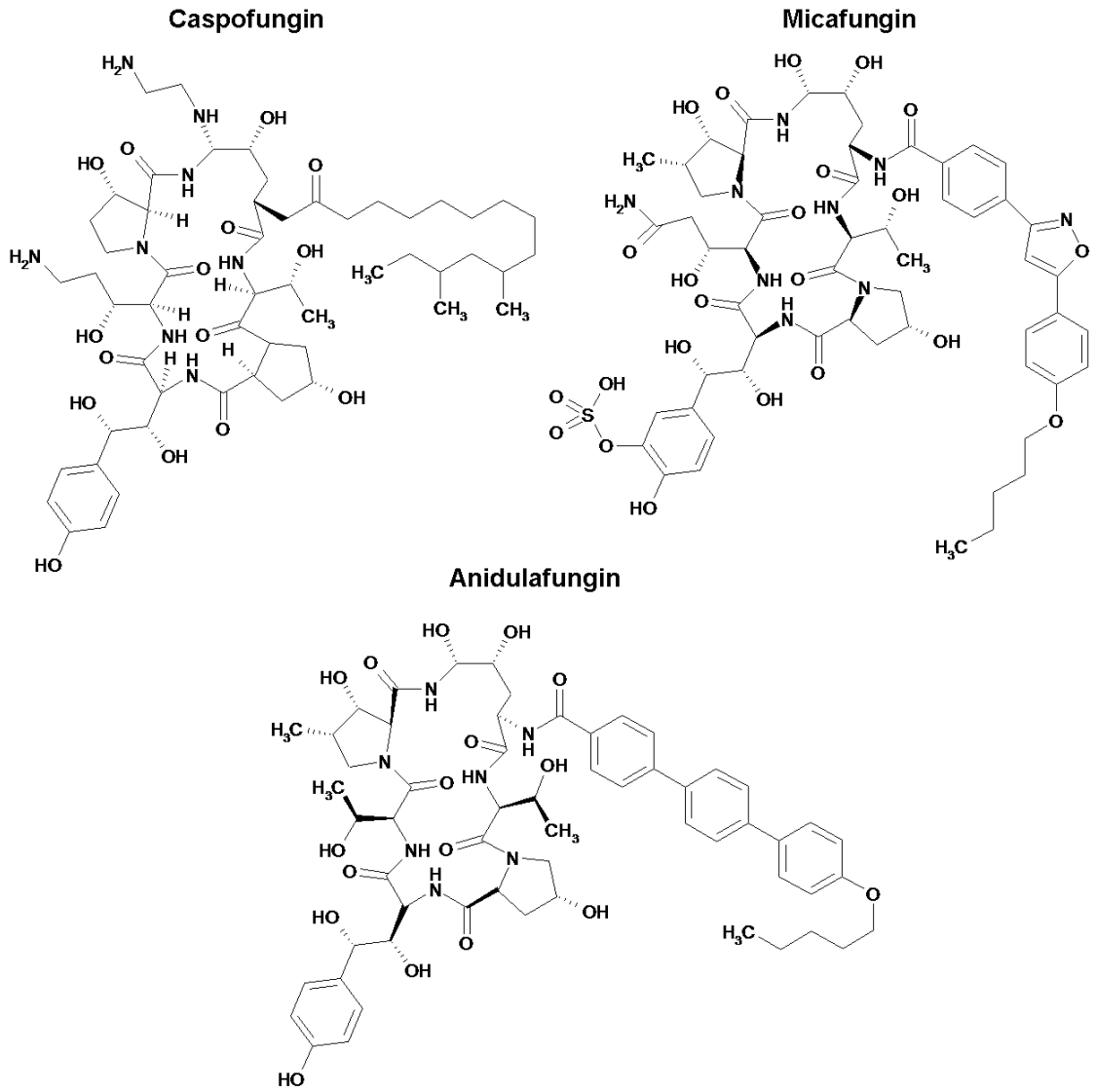


Figure 14 Chemical structures of three echinocandin antifungal agents.

Echinocandins are the first approved agents acting specifically on fungal cell wall, a theoretically ideal target as mammalian cells lack this structure (Denning, 1997). These agents are specific noncompetitive inhibitors of β-(1,3)-glucan synthase, a large integral membrane heterodimeric protein (Hector, 1993), inhibiting the synthesis of glucans in fungal cell walls.

These agents have a wide spectrum of activity, including all common fungal pathogens with the notable exception of *C. neoformans* (Letscher-Bru and Herbrecht, 2003). They are also active against *Pneumocystis jiroveci*, however. Furthermore, they are fungicidal against *Candida* but not *Aspergillus* (Bowman *et al.*, 2002). Just like *C. neoformans*, some hard-to-treat emerging pathogens like *Fusarium* spp. and *Scedosporium* spp. are resistant to echinocandins (Letscher-Bru and Herbrecht, 2003).

Caspofungin has already assumed an important role in the management of invasive fungal infections. It is approved for patients refractory to or intolerant of standard therapies for invasive aspergillosis and for primary therapy of *Candida* infections, as well as empirical therapy of fever with neutropenia (Patterson, 2005). It is recommended as first-line therapy for candidaemia (Pappas *et al.*, 2004). Micafungin has recently been approved (No authors listed, 2005) for oesophageal candidosis and *Candida* prophylaxis. Anidulafungin appears to have both activity and toxicity profiles similar to those of the other echinocandins (Krause *et al.*, 2004). It is currently undergoing Phase III clinical trials.

2.3.2.9 Antifungal drug classes currently under development

Nowadays, the priority in the development of new classes of antifungal agents is for dugs with a broad spectrum of susceptible species. In fact, the current emergence of uncommon and hard to diagnose fungal pathogens resistant to the available drugs is clearly stressing the need for new antimycotic agents (see section "2.2 Human fungal infections"). From the variety of drugs currently under development, two classes with new mechanisms of action are especially noteworthy: the sordarins and the nikkomycins.

Sordarins (see an example in Fig. 15) were discovered by routine screening and are now known to inhibit protein synthesis by blocking the function of fungal translation elongation factor 2 (Dominguez and Martin, 1998), a soluble protein required for the polypeptide chain elongation reactions both in fungi and in mammals. This mechanism is intriguing for researchers, since fungal elongation factor 2 is very similar to the human equivalent, making this a rather surprising target for an antifungal drug. Regarding their *in vitro* antifungal activity, it is especially important to refer their high fungicidal activity against dimorphic fungi.

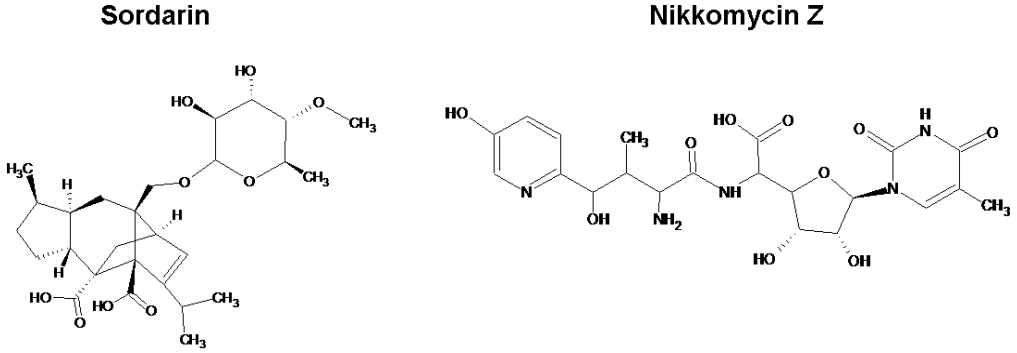


Figure 15 Chemical structures of two experimental antifungal agents: a sordarin derivative and nikkomycin Z.

The second class comprises the nikkomycins, nucleoside-peptide antibiotics produced by streptomycetes, the most important representative being nikkomycin Z (Fig. 15). Nikko-

mycins are a further class of antifungal agents targeting fungal cell wall, acting as analogs of the substrate of chitin synthase (Decker *et al.*, 1990), a membrane bound enzyme catalyzing the polymerization of the *N*-acetylglucosamine subunits that add up to constitute chitin. This way, they competitively inhibit the enzyme's function and, consequently, the synthesis of chitin, leading to the inhibition of septation and osmotic lysis in fungal cells. The antifungal activity of these agents has been demonstrated both *in vitro* and in animal models of disease, and the best results were obtained with combinations of nikkomycins with triazoles, resulting in activities higher than the ones of any of the agents when used alone (Neely and Ghannoum, 2000).

2.4 Antifungal susceptibility testing

In very simple terms, antifungal susceptibility testing represents the *in vitro* determination of the degree of susceptibility of a certain fungal strain to a given tested antifungal agent. It is, therefore, easily recognized as a very important subject, with essential applications in the areas of drug discovery (as a screening tool), epidemiology (to determine and monitor patterns of susceptibility and resistance in a given population), and, ultimately, clinical prediction of therapeutic outcome *in vivo*. In the context of this work, however, the most important practical application of antifungal susceptibility testing methodologies is the field of drug discovery, concretely in the screening of the *in vitro* antifungal potential of newly developed chemical compounds.

Until the 1980s antifungal susceptibility testing was not routinely used, as there was generally only one therapeutic option, amphotericin B, to treat most serious mycoses. Over the last two decades, however, the need for antifungal susceptibility testing has been growing steadily, due not only to the increased number of and resistance to the available antifungal agents, but also to the increased importance of fungal infections. On one side there is the evolution of the available battery of antifungal agents, recently boosted by the discovery of newer triazoles and the representatives of a new antifungal class, the echinocandins, (see section "2.4.2 Currently available antifungal preparations and mechanisms of action") together with the emergence of the problem of antifungal drug resistance. Simultaneously, the growing incidence of morbidity and mortality caused by fungal infections, a phenomenon that results from the increase in the number of immunocompromised patients vulnerable to opportunistic fungi (caused both by the modern developments of medical techniques, like the extending transplantation programs, and the ongoing AIDS pandemic), also has a direct influence on the need for new drugs (see section "2.2 Human fungal infections"). These factors concurrently

contribute to the increase in the need to choose the most appropriate among the available treatment alternatives for each particular patient and fungal infection. Although having benefited from the extrapolation from previously existing methods for antibacterial testing, antifungal susceptibility testing remains less developed and significantly less implanted in clinical laboratories than its bacterial counterpart.

Regarding the subject of this dissertation thesis, the above referred growing need for the development of new antifungal agents should be highlighted, directly justifying the need for antifungal susceptibility testing applied to drug discovery.

2.4.1 Traditional classification of antifungal susceptibility testing methods

Antifungal susceptibility testing methods are traditionally classified in different groups according to whether they are agar or broth-based and whether they rely on diffusion or dilution of the test antifungal agent (Table 7).

Table 7 Traditional classification of antifungal susceptibility testing methods.

Classification	Method
Agar diffusion methods	Disk diffusion test
	Gradient diffusion test - Etest (Epsilonmeter test)
Agar dilution methods	Agar dilution test
Broth dilution methods	Macrodilution broth test
	Microdilution broth test

Agar diffusion methods rely on the interpretation of growth inhibition zones resulting from the activity of the antifungal agent on the test fungal strain. The fungus is uniformly inoculated in the whole surface of a solid agar medium and a paper disk or strip impregnated with the drug is placed on the surface of the medium. The drug then diffuses through the medium, creating a gradient of decreasing concentrations. In the case of disk diffusion test, the result is a semiquantitative determination of the susceptibility through the measurement of the diameter of the round inhibition zone. With Etest, a quantitative determination of the minimum inhibitory concentration (MIC) is possible through the reading of the point of interception of the elliptical inhibition zone with the calibrated scale printed on the paper strip.

The agar dilution test is a quantitative test based on the preparation of different dilutions of the test antifungal agent in solid agar medium that are then inoculated with the test

fungal strain. MICs are read as the lowest antifungal concentration for which there is no fungal growth.

Finally, the broth dilution tests are quantitative tests based on the same principle behind the agar dilution test for the determination of the MIC, but in which the solid medium is substituted by a liquid medium. The macrodilution broth test is performed in test tubes, with a volume of two mL for each tube (each drug concentration), and in the microdilution test the tubes are substituted by the wells of microtiter plates, with a volume of 200 μL for each drug concentration. These are today the reference tests in antifungal susceptibility testing (see section "2.5.2 Main antifungal susceptibility testing methods", bellow).

A number of different tests not included in this traditional classification of methodologies are now available. The next section is dedicated to the separate allusion to the most important antifungal susceptibility testing methods, including the reference broth dilution methods.

2.4.2 Main antifungal susceptibility testing methods

2.4.2.1 CLSI reference method

The Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) in the United States published macro and microdilution broth reference methodologies for yeasts and moulds. These reference protocols are the result of extensive cooperative work between different laboratories (Espinel-Ingroff *et al.*, 1991; Espinel-Ingroff *et al.*, 1992; Espinel-Ingroff *et al.*, 1995; Espinel-Ingroff *et al.*, 1997; Barchiesi *et al.*, 1994; Rex *et al.*, 1997) beginning in the 1980s. Although it started by defining only the macrodilution broth-based method, it later included the microdilution variation that became the method of choice for its less cumbersome nature. The latest versions of the approved methods are protocols M27-A2 (NCCLS, 2002a) and M38-A (NCCLS, 2002b) for yeasts and filamentous fungi, respectively. Besides establishing rules for the control of most test variables (Table 8), they also include sets of quality control limits, both at 24 and 48 h of incubation, for determined drug-organism pairs. The great advantage of the methods is the standardization of a universal reproducible procedure that allows results to be readily compared between different laboratories all around the world. It is, therefore, a valuable tool for all researchers in the field.

Table 8 Comparison of the reference microbroth dilution antifungal susceptibility testing methods: CLSI method for yeasts (M27-A2) (NCCLS, 2002a), CLSI method for moulds (M38-P) (NCCLS, 2002a), and EUCAST method for yeasts (EUCAST, 2002).

Conditions	M27-A2	M38-P	EUCAST
Medium	Synthetic medium RPMI 1640 0.2% glucose, with glutamine, and without bicarbonate	Synthetic medium RPMI 1640 **2% glucose**, with glutamine, and without bicarbonate	Synthetic medium RPMI 1640 **2% glucose**, with glutamine, and without bicarbonate
Medium pH	7.0 at 25 ºC	7.0 at 25 ºC	7.0 at 25 ºC
Buffer	MOPS 0.165 M	MOPS 0.165 M	MOPS 0.165 M
Inoculum	0.5-2.5 × 10^3 cfu/mL	**0.4-5 × 10^4** cfu/mL	**0.5-2.5 × 10^5** cfu/mL
Incubation length	48 h 72 h for *C. neoformans*	**24 h-72 h / 120 h**	**24 h**
Incubation temperature	35 ºC	35 ºC	35-37 ºC
Concentrations tested			
Fluconazole, flucytosine	0.125-64 mg/L	0.125-64 mg/L	0.125-64 mg/L
Other antifungals	0.030-16 mg/L	0.030-16 mg/L	0.015-8 mg/L
Solvent	DMSO	DMSO	DMSO
Reading of MICs			
Visual			
Polyenes	Complete inhibition	Complete inhibition[a]	—
Other antifungals	Approx. 50%	Approx. 50%	—
Spectrophotometric			
Polyenes	100 % (IC_{95}) [b]	100 % (IC_{95})	100 % (IC_{95})
Other antifungals	80 % (IC_{80}) [b]	**50 %** (IC_{50})[a,b]	**50 %** (IC_{50})

[a] The criterion for itraconazole is also 100% inhibition (IC_{95}).

[b] $IC_{95/80/50}$ – Inhibitory concentration causing a reduction of growth in comparison with control (medium without drug) by 95/ 80/ 50%.

2.4.2.2 EUCAST method

Based on the CLSI M27-A protocol (at the time, NCCLS M27-A), the first edition of the approved CLSI method for yeasts (NCCLS, 1997), the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST) has also published a standardized antifungal susceptibility testing method (EUCAST, 2002). The most important modifications introduced by the EUCAST protocol, the definition of an inoculum size of 0.5-2.5 × 10^5 cfu/mL (up to 100 times higher than in the

NCCLS method) and the supplementation of the test medium to a final concentration of 2% D-glucose (10 times higher than in the CLSI method), were focused on the attempt to achieve better endpoint definition and reduction of the incubation times to 24 h (Table 8). A comparative study investigating the intra and inter-laboratory reproducibility of the EUCAST method reported excellent results (Cuenca-Estrella *et al.* 2003) and the comparison between the CLSI and the EUCAST protocols has been favourable to the latter (Chryssanthou and Cuenca-Estrella, 2002; Cuenca-Estrella *et al.* 2002). A recent collaborative study comparing MICs of triazole drugs for *Candida* spp. obtained by the CLSI and EUCAST methods refers generally good but variable agreement (Espinel-Ingroff *et al.*, 2005). Importantly, EUCAST results compared better with 24-h CLSI endpoints and, like it was concluded during its establishment (EUCAST, 2002), the EUCAST MICs were generally lower.

2.4.2.3 Etest

Etest (AB Biodisk, Sweden) is a quantitative agar diffusion method using antimicrobial gradient embedded strips available for several antifungal agents. It has been frequently reported as a possible alternative to the CLSI reference protocol, both for yeasts and moulds, although yielding variable results regarding the quality of the correlation to the same reference method (Martin-Mazuelos *et al.*, 1999; Arendrup *et al.*, 2001; Koc *et al.*, 2000; Matar *et al.*, 2003; Maxwell *et al.*, 2003a; Maxwell *et al.*, 2003b). It is particularly interesting to note reports of better detection of amphotericin B resistant yeast strains (Pfaller *et al.*, 1998; Lozano-Chiu *et al.*, 1998; Peyron *et al.*, 2001), a task in which the CLSI reference protocol traditionally performs poorly due to clustering of the determined MICs around a limited value of concentrations (NCCLS, 2002a).

2.4.2.4 Disk diffusion test

The disk diffusion test is a semi-quantitative agar diffusion method using antifungal-embedded disks. It is another alternative to the reference methods towards the clinical practice, although for the present only for fluconazole and voriconazole, and it has been shown to compare positively both with the M27-A protocol and with Etest (Matar *et al.*, 2003; Morace *et al.*, 2002; Barry *et al.*, 2002; Vandenbossche *et al.*, 2002; Pfaller *et al.*, 2004). The CLSI has also recently published an approved method for antifungal disk diffusion susceptibility testing of fluconazole against *Candida* species – protocol M44-A (NCCLS, 2004).

2.4.2.5 Commercial kits

There are several commercially available kits based on different variations of broth microdilution, colorimetric, and agar-based techniques. Candifast (International Microbio/Stago Group, Diagnostic International Distribution, Milan, Italy), Fungitest panel (Sanofi Diagnostics Pasteur, Paris, France), Integral System Yeasts (Liofilchem Diagnostics, L'Aquila, Italy), and Sensititre YeastOne (Trek Diagnostic Systems, Inc., Westlake, OH/Trek Diagnostic Systems Ltd., East Grinstead, England) are the most popular among them and have been compared to the CLSI reference protocol, although the results were not always ideal (Morace *et al.*, 2002; Chryssanthou, 2001; Posteraro *et al.*, 2000). ATB Fungus (API-bioMeriéux, Marcy l'Etoile, France), Mycostandard (Institut Pasteur, Paris, France), and Mycototal (Behring Diagnostic, Rueil-Malmaison, France) are similar systems that have not been so well studied (Rex *et al.*, 2001).

2.4.2.6 Bioscreen microdilution method

The Bioscreen microdilution method is a semiautomated broth microdilution test performed using computer-controlled equipment (Bioscreen; Labsystems, Helsinki, Finland) that allows automatic incubation, shaking, and turbidimetric reading of growth changes in specially developed 100 wells microtiter trays (honeycomb trays). Besides decreasing the preparation time for the tests, this system permits continuous measurement of sample turbidity, with automatic creation of the correspondent growth curves, having the potential to yield interesting information on the kinetics of fungal growth inhibition by antifungal agents. It has been shown to have excellent agreement with the CLSI reference protocol when testing both fluconazole and amphotericin B (Eldere *et al.*, 1996, Vale-Silva *et al.*, 2006).

2.4.2.7 Quantitation of ergosterol content

Arthington-Skaggs *et al.* (Arthington-Skaggs *et al.*, 1999) have described the measurement of ergosterol content in *C. albicans* as a way to determine susceptibility to fluconazole, based on the fact that azole antifungals act by inhibition of ergosterol synthesis. Conceptually, it is a very different method from all the previously referred, introducing the specific measurement of the influence on one particular yeast biosynthetic process, rather than a general growth inhibition. It relies on an incubation period of 18 h and has the limitation of being potentially useful to test the ergosterol biosynthesis inhibitors only. Nevertheless, it is interesting to note that it was shown to have very good correlation results both *in vitro* and *in vivo* (Arthington-Skaggs *et al.*, 1999; Arthington-Skaggs *et al.*, 2000).

2.4.2.8 Antifungal susceptibility testing by flow cytometry

Flow cytometry is an analytical method based on the measurement of illuminated particles (or cells, in the case of susceptibility testing) in suspension as they flow individually through a beam of light. Light scattering and fluorescence emission are acquired for each cell in the test sample suspension and the results are presented as sums of the individual characteristics of the whole analyzed population. The sizes of these populations can be in the order of a few tens of thousands of cells (Vale-Silva and Buchta, 2006 in press).

Table 9 Description of some fluorochromes used in flow cytometry protocols applied to antifungal susceptibility testing.*

Fluorochromes	Excitation maximum (nm)	Emission maximum (nm)	Mechanism of action in FC
PI	535	617	Permeate damaged membranes (dead cells) and bind to nucleic acids, enhancing fluorescence.
Ethidium bromide	510	595	Permeate damaged membranes (dead cells) and bind to nucleic acids, enhancing fluorescence.
Rose Bengal	540	550-600	Permeate live cells and suffer esterase activity, enhancing fluorescence.
FUN-1	508	525-590	Freely taken up and converted from a diffusely distributed stain into compact CIVS; the conversion requires plasma membrane integrity and metabolic capability.
AO	495	519	Interacts with DNA and RNA by intercalation or electrostatic attractions; in condensed chromatin, the bulk of DNA is packed in a way that does not allow AO intercalation.
$DiOC_5(3)$	484	500	Accumulates on hyperpolarized membranes, being translocated into the lipid bilayer.
$DiBAC_4(3)$	493	516	Permeates depolarized membranes and binds to proteins, enhancing fluorescence.
JC-1	514	529	Exists as a green-fluorescent monomer at low concentrations or at low membrane potential; at higher concentrations or higher potentials, forms "J-aggregates".

* data from Vale-Silva and Buchta (in press), CIVS – cylindrical intravacuolar structures.

The general practical procedure for antifungal susceptibility testing by flow cytometry includes the treatment of cell samples with twofold serial dilutions of a solution of the test

antifungal, a procedure shared with the standard procedures, and the subsequent staining of the samples and flow cytometric reading itself. The choice of the dye to use is a crucial step and several different have been used (Table 9). There is typically no cell growth involved, which is probably the most significant change between standard protocols and flow cytometric methodologies, and the activity of the test antifungal drug is detected by subtle dosage-response effects on specific cell parameters identified by changes in the measured cell fluorescence. The interpretation of such changes is based on the knowledge of the mechanisms of action of the specific fluorescent probes chosen (Table 9) and the MIC is the minimum concentration of the test antifungal for which there is a major rise in the percentage of cells with changed fluorescence.

3 MATERIAL AND METHODS

3.1 Fungal organisms

For the flow cytometry studies the American Type Culture Collection *C. albicans* strain ATCC 90028 was used. A constant series of eight fungal strains was used for regular screening of antifungal susceptibility. In some cases this list was supplemented with five more strains, including the referred *C. albicans* (Table 10).

Table 10 List of fungal strains used in routine screening of antifungal activity.

Fungal strain	Abbreviation
Candida albicans ATCC 44859*	CA1
Candida albicans ATCC 90028	CA2
Candida parapsilosis ATCC 22019	CP
Candida krusei ATCC 6258	CK1
Candida krusei E28*	CK2
Candida tropicalis 156*	CT
Candida glabrata 20/I*	CG
Candida lusitaniae 2446/I	CL
Trichosporon beigelii 1188*	TB
Aspergillus fumigatus 231*	AF
Absidia corymbifera 272*	AC
Microsporum gypseum 27339/01	MG
Trichophyton mentagrophytes 445*	TM

* Organisms included in the basic eight-strain set for screening of antifungal susceptibility.

Besides those, other strains were used in more extensive works. The total list, including the ones referred in Table 10, comprised a total of 191 strains (Table 11).

Table 11 Fungal species and total number of strains used in the experimental work.

Species	Number of strains	Species	Number of strains
Blastoschizomyces capitatus	8	*Candida utilis*	4
Candida albicans	25	*Cryptococcus* spp.	3
Candida famata	3	*Saccharomyces cerevisiae*	10
Candida glabrata	12	*Trichosporon beigelii* complex	10
Candida guilliermondii	6	*Absidia corymbifera*	1
Candida inconspicua	8	*Aspergillus fumigatus*	12
Candida kefyr	10	*Aspergillus flavus*	3
Candida krusei	9	*Aspergillus niger*	7
Candida lusitaniae	10	*Aspergillus terreus*	1
Candida norvegensis	6	*Aspergillus* sp.	1
Candida parapsilosis	11	*Geotrichum* spp.	4
Candida pelliculosa	10	*Microsporum gypseum*	1
Candida rugosa	4	*Trichophyton mentagrophytes*	1
Candida tropicalis	13	*Wangiella dermatitidis*	1

In addition, four other *C. albicans* strains were used in the studies of germ tube inhibition by subinhibitory concentrations of the drugs. These strains were *C. albicans* 26453/00 (CA4), *C. albicans* 26677/00 (VVC) (CA5), *C. albicans* 7137/02 (CA7), and *C. albicans* 26233/02 (RVVC) (CA8), from our laboratory's collection.

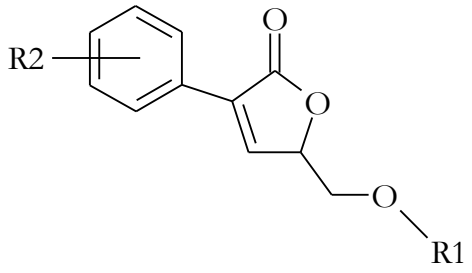
With the exception of the ones belonging to culture collections, all the fungal strains used were clinical isolates obtained from biological materials (blood, urine, sputum, bronchoalveolar lavage fluid, and oral and vaginal swabs) of patients with suspected or proven mycoses. They were identified by standard microbiology methods and stored in skim milk medium (Becton Dickinson) at –40°C before use. To ensure optimal growth characteristics and purity, each isolate was passaged on Sabouraud dextrose agar (Difco) before use in any experiment.

3.2 Drugs

Three semi-synthetic derivatives of (–)incrustoporin, the 3-(halogenated phenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-ones LNO6-22, LNO15-22, and LNO18-22, plus the pa-

rental compound 3-(4-bromophenyl)-5-hydroxymethyl-2,5-dihydrofuran-2-one (LNO18) (Table 12), referred to as the incrustoporin derivatives, were the main group of compounds studied. They have been previously synthesized at the Department of Inorganic and Organic Chemistry of the Faculty of Pharmacy of Charles University in Hradec Králové by Assoc.Prof. Milan Pour and developed by tuning of antifungal activity (Pour *et al.*, 2000; Pour *et al.*, 2001; Pour *et al.*, 2003; Skopalová-Kubanová, 2003).

Table 12 Chemical structures of the studied incrustoporin derivatives.



Code	R1	R2
LNO6-22	CH_3CO–	3,4–Cl_2
LNO15-22	CH_3CO–	3–Cl
LNO18	H–	4–Br
LNO18-22	CH_3CO–	4–Br

Other compounds, coming from a wide number of different chemical groups, were included in the regular screening of antifungal activity tests. The most important of those groups were the quinoline derivatives, including compounds 1 to 11 (Table 13); 4-substituted phenylguanidinium salts, including compounds 4a to 4u (Table 14); azo dye organotin(IV) compounds containing a C,N-chelating ligand, including compounds 1a to 6a and 1b to 6b (Table 15); quinaldine derivatives, including compounds 1 to 16 (Table 16); (*E*)-3-(nitrophenyl)-1-(pyrazin-2-yl)prop-2-en-1-ones (the chalcones), including compounds 7a to 7e, 8a to 8e, 9a to 9e, 10, 11, 12, and 13 (Table 17); salicylanilides and thiosalicylanilides, including compounds 1a to 1f and 2a to 2f (Table 18); 1-phenyl-5-benzylsulfanyltetrazoles, including compounds 1a to 1d, 2a to 2d, 3a to 3c, 4a to 4d, and 5a to 5d (Table 19); arylsulfanylpyrazine-2-carboxylic acid derivatives, including compounds 1 to 13 (Table 20); ring-substituted (*E*)-3-aryl-1-pyrazin-2-ylprop-2-en-1-ones, including compounds 1a to 1e, 2a to 2e, 3a to 3c, 4b and 4c, and, finally, 5c (Table 21); and quinazoline-4-thiones, including compounds 1a to 1k and 2a to 2g (Table 22).

Table 13 Chemical structures of the studied quinoline derivatives.

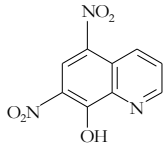
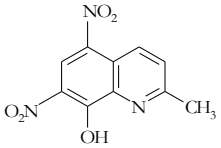
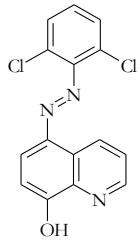
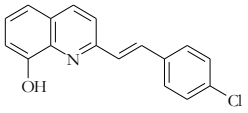
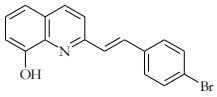
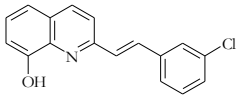
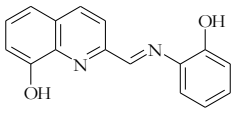
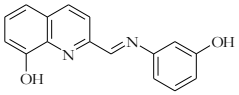
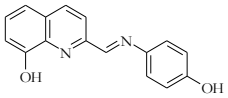
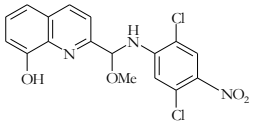
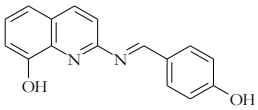
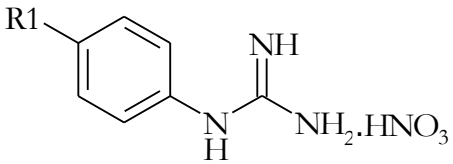
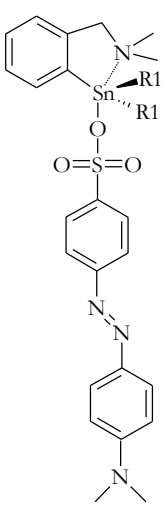
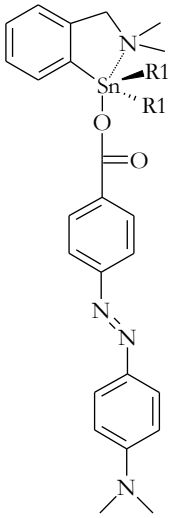
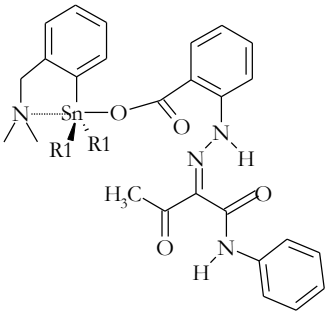
Code	1	2	3	4
Structure				
Code	5	6	7	8
Structure				
Code	9	10	11	
Structure				

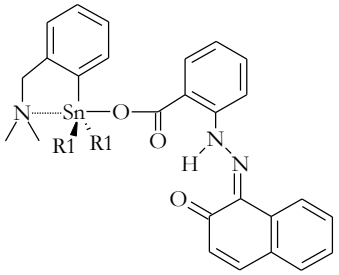
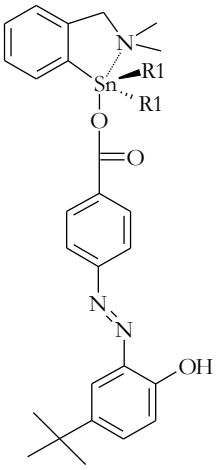
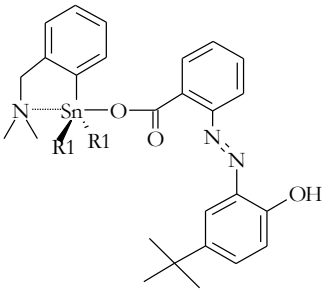
Table 14 Chemical structures of the studied 4-substituted phenylguanidinium salts.



Code	R1	Code	R1	Code	R1
4a	C_2H_5S–	4h	$C_9H_{19}S$–	4o	$C_{16}H_{33}S$–
4b	C_3H_7S–	4i	$C_{10}H_{21}S$–	4p	p-$CH_3C_6H_4S$–
4c	C_4H_9S–	4j	$C_{11}H_{23}S$–	4q	$C_6H_5CH_2S$–
4d	$C_5H_{11}S$–	4k	$C_{12}H_{25}S$–	4r	$C_6H_{11}S$–
4e	$C_6H_{13}S$–	4l	$C_{13}H_{27}S$–	4s	C_6H_5S–
4f	$C_7H_{15}S$–	4m	$C_{14}H_{29}S$–	4t	C_7H_{15}–
4g	$C_8H_{17}S$–	4n	$C_{15}H_{31}S$–	4u	$C_6H_{13}O$–

Table 15 Chemical structures of the studied azo dye organotin(IV) compounds.

Code	1	2	3
Structure			

Code	4	5	6
Structure			

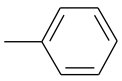
Substituent			
	A	R1	
	B	R1	—$CH_2CH_2CH_2CH_3$

Table 16 Chemical structures of the studied quinaldine derivatives.

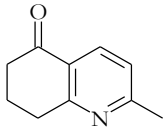
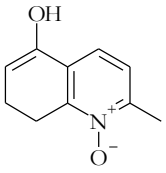
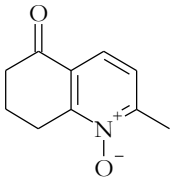
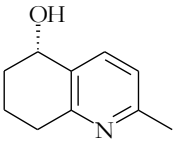
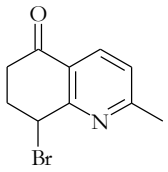
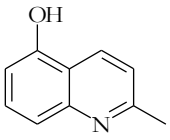
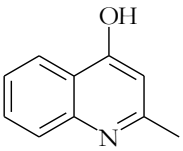
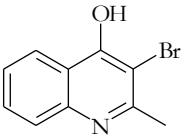
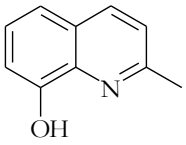
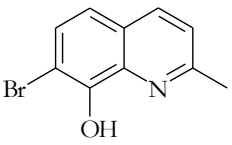
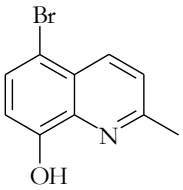
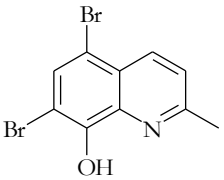
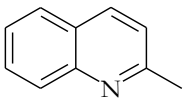
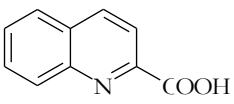
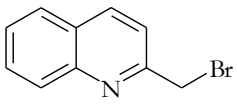
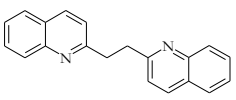
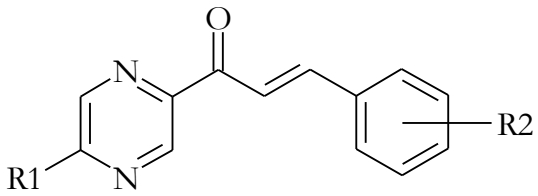
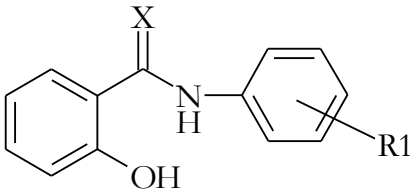
Code	1	2	3	4
Structure				
Code	5	6	7	8
Structure				
Code	9	10	11	12
Structure				
Code	13	14	15	16
Structure				

Table 17 Chemical structures of the studied (*E*)-3-(nitrophenyl)-1-(pyrazin-2-yl)prop-2-en-1-ones (the chalcones).



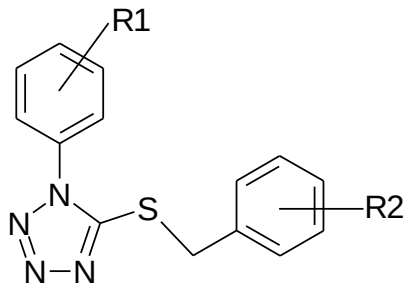
Code	R1	R2	Code	R1	R2
7a	H–	2–NO_2	9a	H–	4–NO_2
7b	*Tert*-butyl–	2–NO_2	9b	*Tert*-butyl–	4–NO_2
7c	Isobutyl–	2–NO_2	9c	Isobutyl–	4–NO_2
7d	Butyl–	2–NO_2	9d	Butyl–	4–NO_2
7e	Propyl–	2–NO_2	9e	Propyl–	4–NO_2
8a	H–	3–NO_2	10	Butyl–	2–OH
8b	*Tert*-butyl–	3–NO_2	11	*Tert*-butyl–	3–OH
8c	Isobutyl–	3–NO_2	12	Butyl–	4–OH
8d	Butyl–	3–NO_2	13	*Tert*-butyl–	2–OH
8e	Propyl–	3–NO_2			

Table 18 Chemical structures of the studied salicylanilide and thiosalicylanilide derivatives.



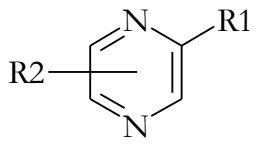
Code	X	R1	Code	X	R1
1a	O=	H–	2a	S=	H–
1b	O=	4–CH_3	2b	S=	4–CH_3
1c	O=	4–Cl	2c	S=	4–Cl
1d	O=	3,4–Cl_2	2d	S=	3,4–Cl_2
1e	O=	4–Br	2e	S=	4–Br
1f	O=	4–F	2f	S=	4–F
1g	O=	Isopropyl–	2g	S=	Isopropyl–

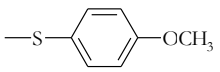
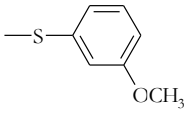
Table 19 Chemical structures of the studied 1-phenyl-5-benzylsulfanyltetrazole derivatives.



Code	R1	Sub-code	R2
1	H–	a	H–
2	4–Cl	b	4–Cl
3	3,4–Cl2	c	4–CH3
4	4–CH3	d	4–OCH3
5	4–OCH3		

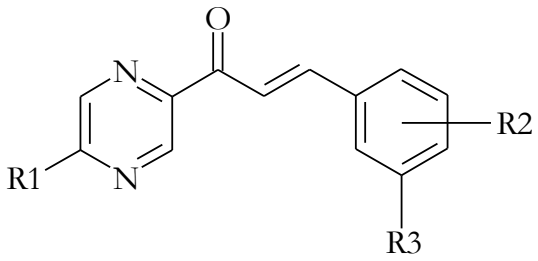
Table 20 Chemical structures of the studied arylsulfanylpyrazine-2-carboxylic acid derivatives.



R2: 4-CH_3OPhS–  3-CH_3OPhS 

Code	R1	R2
1	–$COOC_2H_5$	6-(4-CH_3OPhS–)
2	–CN	6-(4-CH_3OPhS–)
3	–$CONH_2$	6-(4-CH_3OPhS–)
4	–CN	6-(3-CH_3OPhS–)
5	–$CONH_2$	6-(3-CH_3OPhS–)
6	–CN	5-(4-CH_3OPhS–)
7	–$CONH_2$	5-(4-CH_3OPhS–)
8	–CN	5-(3-CH_3OPhS–)
9	–$CONH_2$	5-(3-CH_3OPhS–)
10	–CN	3-(4-CH_3OPhS–)
11	–$CONH_2$	3-(4-CH_3OPhS–)
12	–CN	3-(3-CH_3OPhS–)
13	–$CONH_2$	3-(3-CH_3OPhS–)

Table 21 Chemical structures of the studied ring-substituted (*E*)-3-aryl-1-pyrazin-2-ylprop-2-en-1-ones.



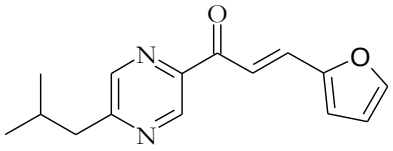
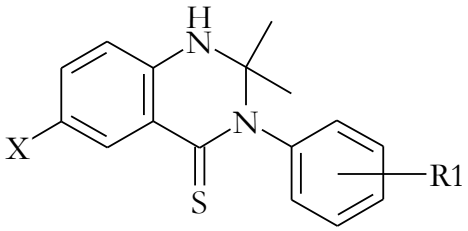
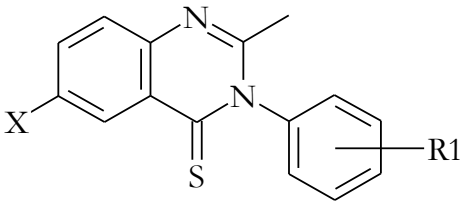
Code	R1	R2	R3
1a	H–	3–OH	H–
1b	*Tert*-butyl–	3–OH	H–
1c	Isobutyl–	3–OH	H–
1d	Butyl–	3–OH	H–
1e	Propyl–	3–OH	H–
2a	H–	4–Cl	H–
2b	*Tert*-butyl–	4–Cl	H–
2c	Isobutyl–	4–Cl	H–
2d	Butyl–	4–Cl	H–
2e	Propyl–	4–Cl	H–
3a	H–	H–	H–
3b	*Tert*-butyl–	H–	H–
3c	Isobutyl–	H–	H–
4b	*Tert*-butyl–	2–OH	$–NO_2$
4c	Isobutyl–	2–OH	$–NO_2$
5c			

Table 22 Chemical structures of the two studied series of quinazoline-4-thione derivatives.





Code	X	R1	Code	X	R1
1a	H–	H–	2a	Cl–	H–
1b	H–	4–Cl	2b	Cl–	3–Cl
1c	H–	3,4–Cl_2	2c	Cl–	4–Cl
1d	H–	4–CH_3	2d	Cl–	4–Br
1e	H–	4–C_2H_5	2e	Cl–	4–CH_3
1f	H–	4–IsoC_3H_7	2f	Cl–	4–IsoC_3H_7
1g	Cl–	H–	2g	Cl–	4–OCH_3
1h	Cl–	3–Cl			
1i	Cl–	3,4–Cl_2			
1j	Cl–	4–IsoC_3H_7			
1k	Cl–	4–C_4H_9			

Besides the experimental drugs, several commercial antifungal drugs were used in the tests for result control and comparison purposes. These reference drugs included the human antifungals amphotericin B (Sigma), fluconazole (Pfizer), and ketoconazole (Janssen-Cilag), plus the agricultural antifungal dodine.

3.3 Antifungal susceptibility testing

MIC determination was performed by the broth microdilution methods according to the CLSI (formerly NCCLS) reference protocols M27-A2 (NCCLS, 2002a) for yeasts and M38-A (NCCLS, 2002b) for moulds, with slight modifications, as explained below.

Inoculum preparation. The isolates of yeasts and filamentous fungi were grown in Sabouraud dextrose agar (Difco) for 1 and 4 to 14 days, respectively, at 37ºC. Yeast suspensions were prepared in sterile water and, for moulds, conidia suspensions were prepared in sterile water supplemented with 0.01% Tween 80. Suspension densities were determined mi-

croscopically using a Bürker's chamber in order to yield final test sizes ranging from 0.5×10^3 to 2.5×10^3 CFU/mL for yeasts and from 0.5×10^4 to 5×10^4 CFU/mL for moulds. In some experiments inoculum sizes were verified by plating 100 μL of serial dilutions of each suspension onto Sabouraud dextrose agar plates and incubating them until growth became visible.

Drug solutions. The test drugs, as well as the reference antifungal agents, were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma), with the exception of fluconazole, which was dissolved in sterile distilled water. These solutions were always prepared immediately before testing and then added to Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine but without sodium bicarbonate (Sevapharma, Prague) buffered to pH 7.0 ± 0.1 with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma), the final DMSO concentration being 1%. In some cases, due to poor solubility of the drugs, this concentration was raised to a maximum of 2% DMSO in the total final volume.

MIC determination. Twofold serial dilutions of the drug solutions were prepared in RPMI 1640 medium and dispensed in 200 μL aliquots into 96 well microtiter trays. Wells containing drug-free medium were included as the growth control for each test strain. The wells were then inoculated with the inoculum suspensions in volumes of 10 μL, in order to get the referred final cell densities, and the trays were incubated at 35ºC in humid atmosphere without agitation. The MICs were determined after 24 and 48 h for most yeasts, after 48 and 72h for *Cryptococcus* and *Aspergillus* spp., and after 72 and 120 h for dermatophytes, either visually or spectrophotometrically (iEMS reader MF; Labsystems). With spectrophotometrical reading, MICs were read as the lowest concentrations yielding 80% growth inhibition (IC_{80}) in comparison to the dug-free control wells, except in the cases of fluconazole and amphotericin B, for which IC_{50} and IC_{95}, respectively, were used.

MFC determination. For the determination of the minimum fungicidal concentration (MFC), 10 μL samples were taken, after agitation, from each well showing no growth after the MIC determination. These samples were inoculated in Sabouraud dextrose agar and incubated at 35ºC until growth became visible. The MFC was defined as the lowest concentration for which no colonies were visible after incubation.

3.4 Germ tube inhibition test

The *in vitro* antifungal effects of subinhibitory concentrations of some studied compounds were evaluated by the inhibition of the germ tube formation of *C. albicans* in NYP medium (N-acetylglucosamine [Sigma; 10^{-3} mol/L], yeast nitrogen base [Difco; 3.35 g/L], proline [Roanal; 10^{-3} mol/L]) with NaCl (4.5 g/L) adjusted to pH 6.7 ± 0.1 (Marichal *et al.*

1986). The suspensions of four *C. albicans* strains (CA4, CA5, CA7, and CA8) in the NYP medium were prepared from overnight cultures on Sabouraud dextrose agar to obtain a final density of (1.0 ± 0.2) × 10^6 CFU/mL. The compounds were dissolved and diluted in DMSO and added in a volume of 20 μL to the yeast suspension in NYP medium (1.98 mL) to obtain 1/10 and 1/50 of the MIC (MIC/10 and MIC/50, respectively). After a 3-h incubation at 37°C, the percentage of germ tube formation of 100 cells was determined by using a Bürker's chamber. The germ tubes were counted when they were at least as long as the diameter of the blastospores.

3.5 Flow cytometry test

Flow cytometry was used to study the mechanism of action of incrustoporin derivatives against *C. albicans* strain ATCC 90028. Compound LNO18-22 was studied in comparison with the parental compound LNO18 (Table 12) and the reference compounds amphotericin B and fluconazole.

Incubation of the cells with the drugs. C. albicans ATCC90028 was grown in Sabouraud dextrose agar for 24 h at 37ºC and cell suspensions were then prepared in RPMI 1640 medium, with L-glutamine but without sodium bicarbonate, buffered to pH 7.0 ± 0.1 with 0.165 M MOPS. The suspensions were determined microscopically using a Bürker's chamber to have (2.5 ± 0.1) × 10^5 CF/mL. Stock solutions of 10 mg/mL were prepared for LNO18-22, LNO18, and amphotericin B in DMSO, and 5 mg/mL for fluconazole in sterile distilled water, immediately before each experiment. They were then added to the cell suspensions in order to obtain final drug concentrations of 0.1, 1.0, 10, and 100 μg/mL in a total volume of 5 mL, with a final DMSO concentration of 1%. Growth control tubes containing drug-free cell suspensions in the test medium were included. Because of the formation of micelles by amphotericin B at high concentrations (Kirschbaum and Kahn, 1967), and the possibility of similar insolubility of the incrustoporine derivatives, drug control tubes with 100 μg/mL of each compound without cells were included. This allowed later flow cytometric confirmation of the discrimination between cells and the smaller compound micelles. All the tubes were later incubated for 22 h at 35ºC in humid atmosphere without agitation. The same samples were incubated in parallel for the Bioscreen broth test.

Staining of the samples. Appropriate concentrations of the fluorescent dyes for our experimental conditions were determined through preliminary tests. PI (Sigma) final concentration was retained from previous experience, being 0.01 μg/mL. For DiBAC4(3) (Molecular Probes) final concentrations of 0.2, 0.5 μg/mL, 2, and 5 μg/mL were tested, and, for FUN-1

(Molecular Probes) concentrations of 0.05, 0.1, 0.5, 1, and 5 μM were tested. Final dye concentrations of 0.2 μg/mL and 0.5 μM were chosen for DiBAC4(3) and FUN-1, respectively. This way, stock solutions were prepared for PI in PBS at a concentration of 1 μg/mL, for DiBAC4(3) in DMSO at 50 μg/mL, and for FUN-1 in DMSO at 100 μM and stored at –40ºC before use. Each dye was used alone and experiments with PI and DiBAC4(3) simultaneously were also performed. After the described incubations with the drugs, the yeast cell suspensions were harvested by centrifugation for 10 min at 5,000 rpm, washed, and ressuspended in 3 mL of saline solution. Controls and other samples without growth inhibition during the incubation period were diluted to get the originally prepared cell densities. Aliquots (500 μL) of each sample were transferred to flow cytometry tubes and the dyes were added in volumes of 5 μL for PI, 2 μL for DiBAC4(3), and 2.5 μL for FUN-1, getting final dye concentrations of 0.01 μg/mL, 0.2 μg/mL, and 0.5 μM, respectively. Flow cytometric readings were started immediately after adding the dye to the last flow cytometry tube for PI and DiBAC4(3), corresponding to waiting times of around 10 min at room temperature. For FUN-1, the readings were done after a previous incubation of the suspensions with the dye for 20 min at 35ºC away from incident light.

Flow cytometry reading. A Cytomics FC 500 Flow Cytometer (Coulter Corporation, Hielah, FL), equipped with a 15-mW argon-ion laser with excitation at 488 nm was used. Forward scatter (FS), side scatter (SS), and fluorescence in FL3 channel (log red fluorescence, 620 nm) for PI, FL1 channel (log green fluorescence, 525 nm) for DiBAC4(3), and FL2 channel (log yellow/orange fluorescence, 575 nm) for FUN-1 were acquired and recorded using a logarithmic scale. For all samples a gate that excluded debris, cell clusters, and compound micelles was established in forward-scatter versus side-scatter dot-plot cytograms and a minimum of 10,000 events (yeast cells) falling in the referred gate were acquired. The results were analyzed using WinMDI 2.8 software (Coulter Corporation, Hielah, FL). In order to obtain the percentage of positive cells regarding fluorescence in each sample, markers were adjusted in histogram representations of the number of events versus fluorescence of the controls to include a maximum of 5% of the events and then used in the analysis of all samples to define positive cells. In the experiments with PI and DiBAC4(3), dot-plot cytograms of FL3 versus FL1 channel fluorescence were analyzed in addition to the referred separate histogram analysis.

3.6 Bioscreen test

In every flow cytometry experiment, two aliquots (300 μL) of each incubation tube were distributed into wells of a flat-bottomed 100-well honeycomb microtiter plate and incu-

bated in the Bioscreen reader (Eldere *et al.* 1996). The incubation program was set for 24 h at 35ºC with turbidimetric readings at intervals of 1 h. The reading wavelength was 540 nm and shaking of the plate was set for a period of 30 s at medium speed before each turbidimetric reading.

4 RESULTS

For simplification, the results are organized in different sections according to the corresponding chemical groups of the tested drugs. Incrustoporine derivatives include results referring to several different kinds of studies, since this group has been developed during the whole period of the experimental work. For the remaining drugs the results refer to susceptibility studies only, as they constituted the object of the routine screening of antifungal activity performed at our laboratory among high number and wide structural variety of experimental drugs.

4.1 Incrustoporin derivatives

Three compounds from the group of incrustoporin derivatives previously discovered to have high antifungal activity were tested against a wide group of fungal species. The susceptibility results obtained for the three chosen compounds, LNO6-22, LNO15-22, and LNO18-22, plus the reference amphotericin B and fluconazole, against a set of yeasts and moulds are shown in Table 23.

The MICs of LNO6-22, LNO15-22, and LNO18-22 when tested against CLSI quality control strains were 1.0, 2.0, and 0.5 μg/ml, respectively, for *C. albicans* ATCC 90028; 2.0 μg/ml each for *C. parapsilosis* ATCC 22019; and 8.0, 2.0, and 2.0 μg/ml, respectively, for *C. krusei* ATCC 6258.

The same incrustoporin derivatives and reference drugs were also tested for the inhibition of germ-tube formation in four *C. albicans* strains at subinhibitory concentrations (1/10 and 1/50 of the correspondent MICs). Amphotericin B was the only showing significant interference at the lowest concentration (MIC/50). The results for the concentration MIC/10 can be seen in Table 24 and Fig. 16, LNO6-22 producing the highest inhibitions, with results similar to the ones of amphotericin B. Fluconazole had only a slight influence in germ tube production under these experimental conditions. The strain CA7 showed a tendency to produce pseudo-hyphae rather than true germ tubes, resulting in percentages of germ tube production lower than the values for the other strains.

Table 23 Antifungal susceptibilities of the tested fungal species to the incrustoporin derivatives determined by the microdilution broth method.*

Species (no. of isolates) and drug	Range of MICs (μg/ml)[a]	Species (no. of isolates) and drug	Range of MICs (μg/ml)[a]	Species (no. of isolates) and drug	Range of MICs (μg/ml)[a]
Blastoschizomyces capitatus (8)		*Candida lusitaniae* (10)		*Absidia corymbifera* (1)	
Amphotericin B	0031-0.125	Amphotericin B	0.031-0.25	Amphotericin B	2
Fluconazole	4-16	Fluconazole	0.5-32	Fluconazole	>64
LNO6-22	1-2	LNO6-22	1-8	LNO6-22	4
LNO15-22	0.5-1	LNO15-22	14	LNO15-22	2
LNO18-22	0.5-1	LNO18-22	1-4	LNO18-22	4
Candida albicans (25)		*Candida norvegensis* (6)		*Aspergillus fumigatus* (12)	
Amphotericin B	0.063-0.25	Amphotericin B	≤0.016-0.063	Amphotericin B	0.063-0.25
Fluconazole	0.125->64	Fluconazole	16-64	Fluconazole	>64
LNO6-22	1-16	LNO6-22	0.5->64	LNO6-22	1-2
LNO15-22	1-8	LNO15-22	0.25-4	LNO15-22	0.5-2
LNO18-22	0.5-4	LNO18-22	0.25-4	LNO18-22	0.5-1
Candida albicans Flc^s (16)[b]		*Candida parapsilosis* (11)		*Aspergillus flavus* (3)	
Amphotericin B	0.063-2	Amphotericin B	0.031-0.125	Amphotericin B	1-1
Fluconazole	0.125-16	Fluconazole	0.5-64	Fluconazole	>64
LNO6-22	1-4	LNO6-22	1-8	LNO6-22	2-2
LNO15-22	1-4	LNO15-22	1-4	LNO15-22	1-2
LNO18-22	0.5-2	LNO18-22	1-4	LNO18-22	1-1

Table 23 continued

Species (no. of isolates) and drug	Range of MICs (μg/ml)[a]	Species (no. of isolates) and drug	Range of MICs (μg/ml)[a]	Species (no. of isolates) and drug	Range of MICs (μg/ml)[a]
Candida albicans Flc^r (9)[c]		*Candida pelliculosa* (10)		*Aspergillus niger* (7)	
Amphotericin B	0.063-2	Amphotericin B	≤0.016-0.25	Amphotericin B	0.063-0.125
Fluconazole	64->64	Fluconazole	0.5->64	Fluconazole	>64
LNO6-22	1-16	LNO6-22	0.5-1	LNO6-22	1-2
LNO15-22	1-8	LNO15-22	0.5-1	LNO15-22	1-2
LNO18-22	0.5-4	LNO18-22	0.25-1	LNO18-22	0.5-2
Candida famata (3)		*Candida rugosa* (4)		*Aspergillus terreus* (1)	
Amphotericin B	1-2	Amphotericin B	1-2	Amphotericin B	1
Fluconazole	2->64	Fluconazole	4->64	Fluconazole	>64
LNO6-22	0.5-8	LNO6-22	2-4	LNO6-22	1
LNO15-22	0.5-4	LNO15-22	1-1	LNO15-22	2
LNO18-22	0.25-4	LNO18-22	0.5-1	LNO18-22	0.25
Candida glabrata (12)		*Candida tropicalis* (13)		*Aspergillus* sp. (1)	
Amphotericin B	0.125-0.25	Amphotericin B	0.031-0.25	Amphotericin B	0.125
Fluconazole	8->64	Fluconazole	0.5->64	Fluconazole	>64
LNO6-22	0.5-2	LNO6-22	2-8	LNO6-22	2
LNO15-22	0.25-1	LNO15-22	0.5-4	LNO15-22	1
LNO18-22	0.5-1	LNO18-22	1-4	LNO18-22	1
Candida guillier-mondii (6)		*Candida utilis* (4)		*Geotrichum* spp. (4)	
Amphotericin B	1-4	Amphotericin B	2-2	Amphotericin B	2-2
Fluconazole	0.5-32	Fluconazole	1-16	Fluconazole	0.5-32
LNO6-22	0.125-8	LNO6-22	0.5-2	LNO6-22	2-4
LNO15-22	0.125-4	LNO15-22	0.5-1	LNO15-22	0.5-2
LNO18-22	≤0.063-4	LNO18-22	0.5-2	LNO18-22	0.5-1

Table 23 continued

Species (no. of isolates) and drug	Range of MICs (μg/ml)[a]	Species (no. of isolates) and drug	Range of MICs (μg/ml)[a]	Species (no. of isolates) and drug	Range of MICs (μg/ml)[a]
Candida inconspicua (8)		*Cryptococcus* spp. (3)		*Microsporum gypseum* (1)	
Amphotericin B	0.063-0.125	Amphotericin B	1-2	Amphotericin B	2
Fluconazole	16-64	Fluconazole	0.5-2	Fluconazole	16
LNO6-22	0.5-4	LNO6-22	2-4	LNO6-22	0.25
LNO15-22	0.25-1	LNO15-22	0.25-2	LNO15-22	0.5
LNO18-22	0.25-1	LNO18-22	1-1	LNO18-22	0.5
Candida kefyr (10)		*Saccharomyces cerevisiae* (10)		*Trichophyton mentagrophytes* (1)	
Amphotericin B	0.063-0.25	Amphotericin B	0.031-0.125	Amphotericin B	1
Fluconazole	0.25-1	Fluconazole	2-64	Fluconazole	16
LNO6-22	0.25-1	LNO6-22	0.5-2	LNO6-22	0.5
LNO15-22	0.25-1	LNO15-22	0.25-1	LNO15-22	0.5
LNO18-22	0.5-1	LNO18-22	0.25-2	LNO18-22	0.5
Candida krusei (9)		*Trichosporon beigelii* complex (10)		*Wangiella dermatitidis* (1)	
Amphotericin B	0.125-0.25	Amphotericin B	1-4	Amphotericin B	0.5
Fluconazole	32->64	Fluconazole	0.25-8	Fluconazole	4
LNO6-22	2-16	LNO6-22	0.25-4	LNO6-22	0.5
LNO15-22	2-2	LNO15-22	≤0.063-2	LNO15-22	0.5
LNO18-22	1-2	LNO18-22	0.125-2	LNO18-22	0.5

[*] Buchta *et al.*(2004)

[a] Determined after 24 h for yeasts (48 h for *Cryptococcus* spp.), 48 h for *Aspergillus* spp. and *A. corymbifera*, and 72 h for *T. mentagrophytes* and *M. gypseum* (IC_{95} for amphotericin B and IC_{80} for the other four drugs).

[b] Fluconazole-susceptible and -intermediate isolates for which the MIC was <64 μg/ml.

[c] Fluconazole-resistant isolates for which the MIC was ≥64 μg/ml.

Table 24 Inhibition of germ tube formation in the *C. albicans* test strains by the incrustoporin derivatives and reference compounds at a concentration of MIC/10.[a]

	Germ tube formation by *C. albicans* strains (%)			
	CA4	CA5	CA7	CA8
LNO6-22	1.7 ± 0.9	1.7 ± 0.5	1.3 ± 1.2	3.0 ± 1.6
LNO15-22	58.7 ± 2.9	30.0 ± 2.2	5.7 ± 1.2	30.3 ± 2.1
LNO18-22	70.3 ± 3.7	62.7 ± 3.1	6.7 ± 1.2	75.0 ± 4.5
Amphotericin B	1.0 ± 0.8	2.0 ± 1.6	0.3 ± 0.5	1.0 ± 0.8
Fluconazole	74.3 ± 1.7	51.0 ± 2.2	29.7 ± 2.5	98.0 ± 0.8
Control	88.5 ± 5.3	78.5 ± 17.3	57.8 ± 8.9	95.0 ± 2.1
Control with DMSO[b]	87.8 ± 12.5	79.3 ± 14. 8	49.8 ± 22.6	97.5 ± 2.2

[a] All values are geometric means of the results of three experiments ± standard deviations.

[b] NYP medium with 1% DMSO.

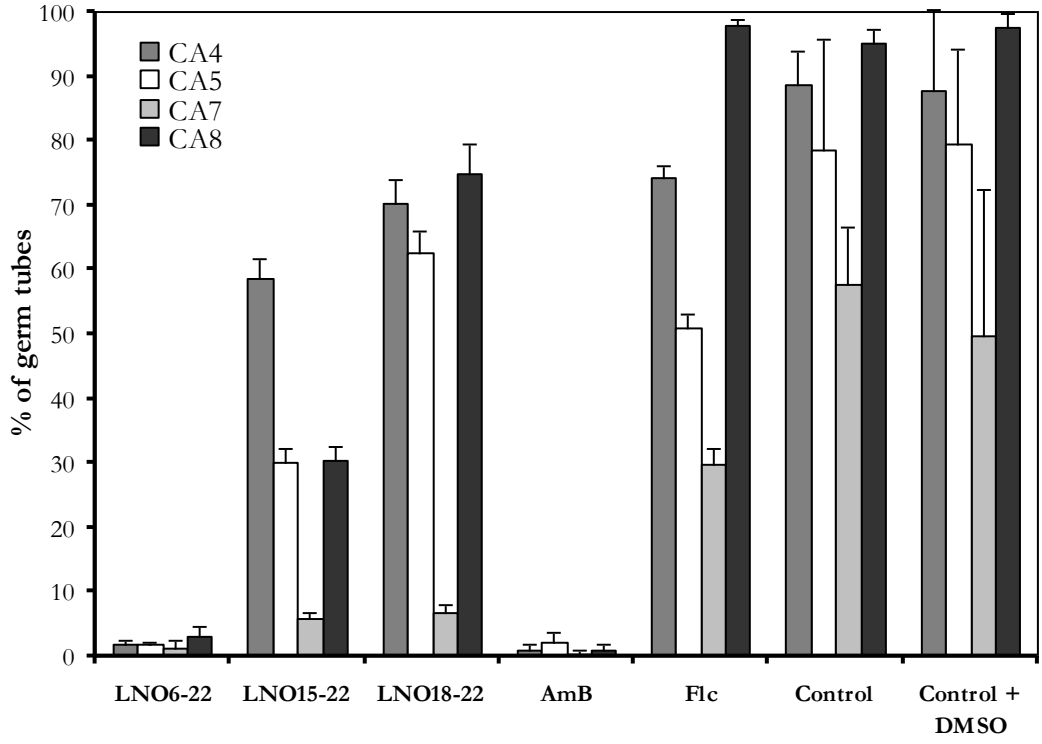


Figure 16 Inhibition of germ tube formation by the incrustoporin derivatives, amphotericin B, and fluconazole, represented as the % of germ-tube forming cells for each of the four *C. albicans* strains.

Notes: AmB, amphotericin B; Flc, fluconazole; Control + DMSO, cell suspension in NYP medium with 1% DMSO. See Table 24 for data.

In the sequence of these studies, LNO18-22, which appeared to be the most potent derivative after the susceptibility studies, was investigated in comparison with the parental compound LNO18 and the same reference drugs, amphotericin B and fluconazole, in an attempt to gather some preliminary information on its mechanism of antifungal action. For that purpose, the compounds were studied for their influence on the *C. albicans* strain ATCC 90028 by flow cytometry and simultaneous analysis of the kinetics of growth inhibition using the Bioscreen method. The growth curves determined by the Bioscreen method for the test strain incubated with four different concentrations of each of the test drugs are shown in Fig. 17.

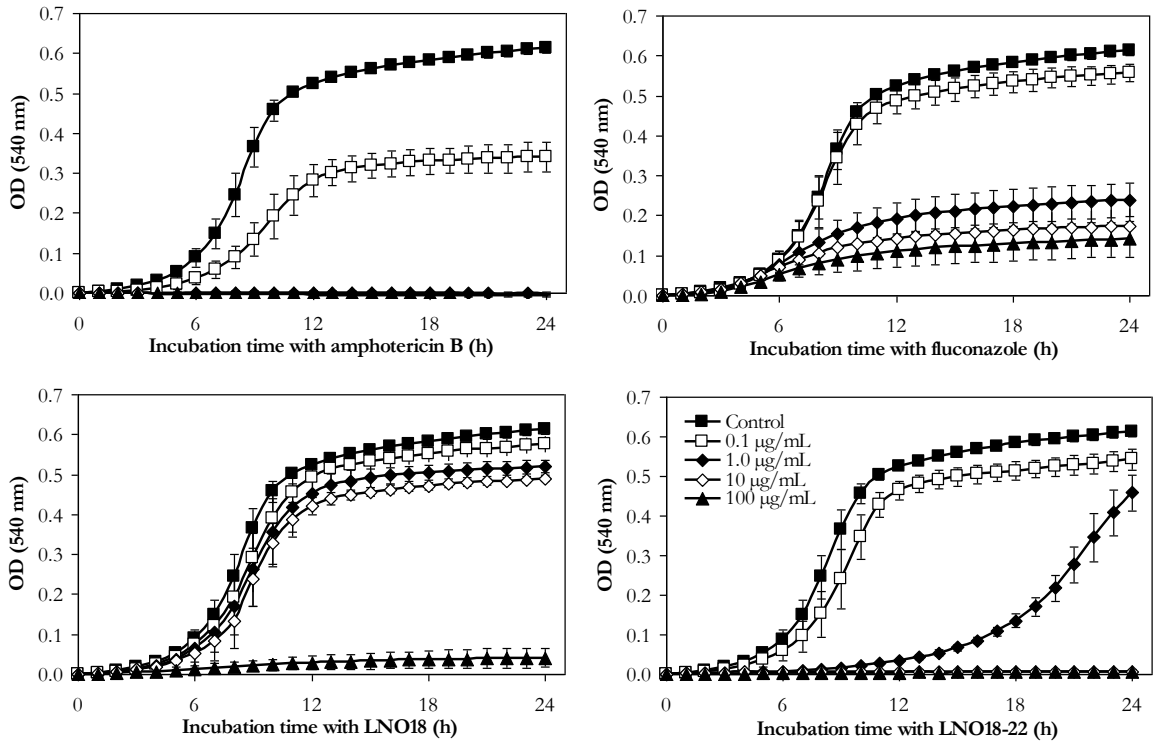


Figure 17 Growth curves of *Candida albicans* ATCC 90028 in control samples (closed squares) and samples treated with 0.1 (open squares), 1.0 (closed diamonds), 10 (open diamonds), and 100 µg/mL (closed triangles) of amphotericin B, fluconazole, LNO18, and LNO18-22.
Notes: Averages of a minimum of four values ± standard deviations. In some cases the standard deviation bars are smaller than the symbols and thus not seen.

Regarding the flow cytometric studies, and before addressing the final complete results, Fig. 18 exemplifies the use of the samples containing only the drug with no cells to discriminate between cells and compound micelles in dot-plot cytograms of the samples' physical characteristics. As the represented examples show, the inclusion of this extra control samples (samples containing the drugs only, without cells) allow the confirmation of the presence of compound micelles (the population of smaller and less complex particles). The same procedure was applied with the incrustoporin derivatives.

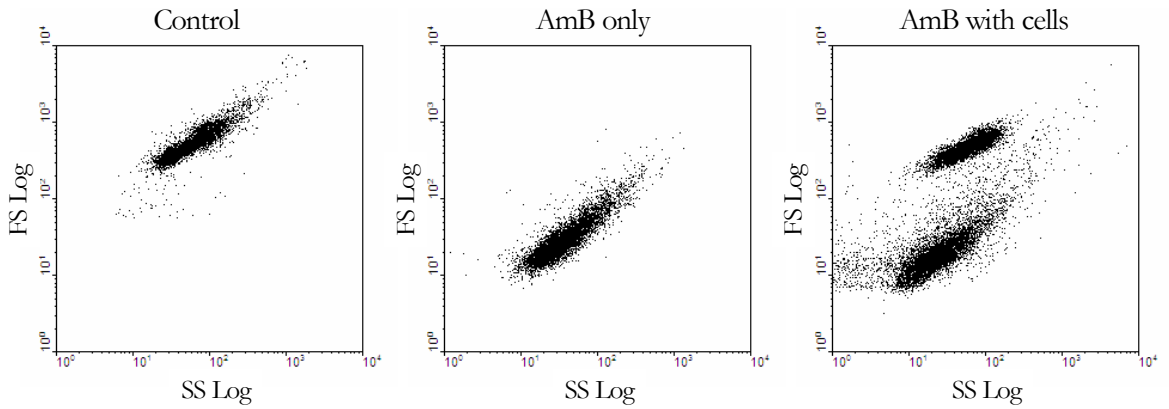


Figure 18 Biparametric representations (dot-plot cytograms) according to logarithmic scales of particle size (FS Log) and complexity (SS Log) showing a control sample, a drug control sample containing 100 μg/mL of amphotericin B and no cells, and a test sample containing cells treated with 100 μg/mL of amphotericin B.

Notes: FS Log, logarithmic scale of forward-scatter; SS Log, logarithmic scale of side-scatter; AmB, amphotericin B.

The analysis of the physical properties of the samples, in this case the forward scatter (FS, indication of cell size), revealed that treatment of the cells with inhibitory concentrations of amphotericin B and fluconazole caused opposite effects on cell size. While amphotericin B seems to have led to a slight shrinkage of the *C. albicans* cells (Fig. 19 A and B), fluconazole treatment appears to have induced an increase in cell size, evidenced by the development of a separate population of bigger cells (Fig. 19 C and D).

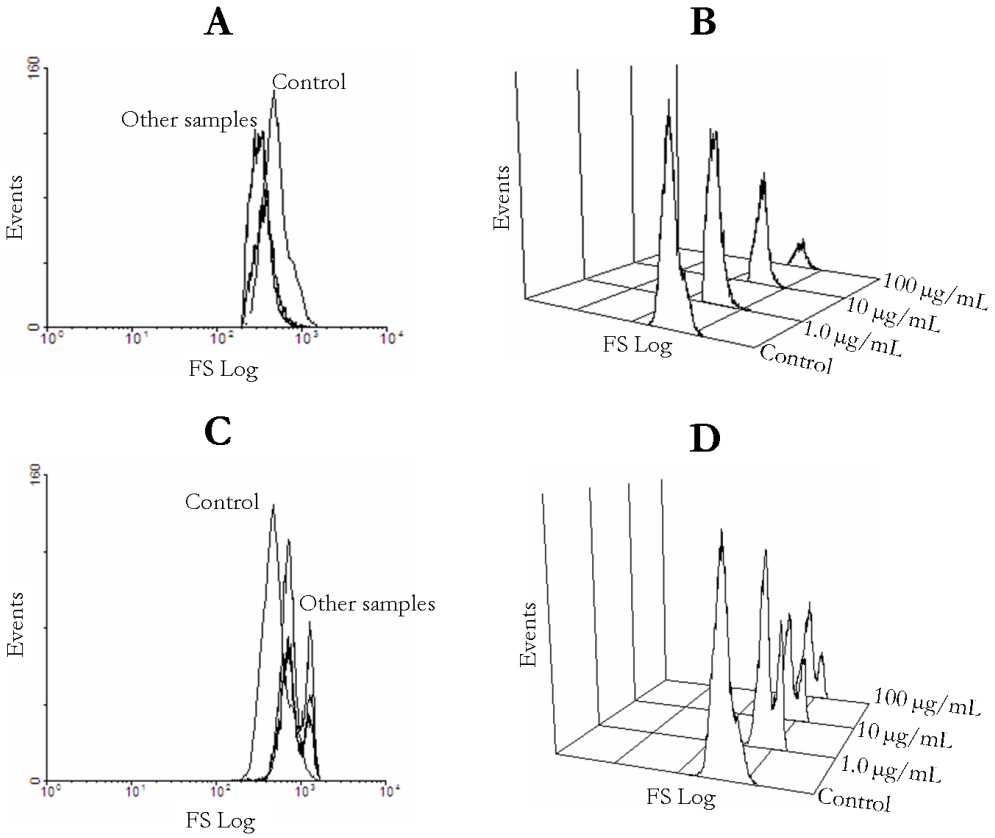


Figure 19 Overlayed (A and C) and sequential plotting histograms (B and D) of logarithmic scales of particle size (FS Log) versus the number of particles (Events) representing control samples and samples treated with inhibitory concentrations of amphotericin B (A and B) and fluconazole (C and D).

Fig. 20 illustrates an example of the analysis of the flow cytometric fluorescence results. In this particular case, a control sample and the samples treated with the array of concentrations of incrustoporin derivative LNO18-22 are represented after staining with PI using two different flow cytometry data displays.

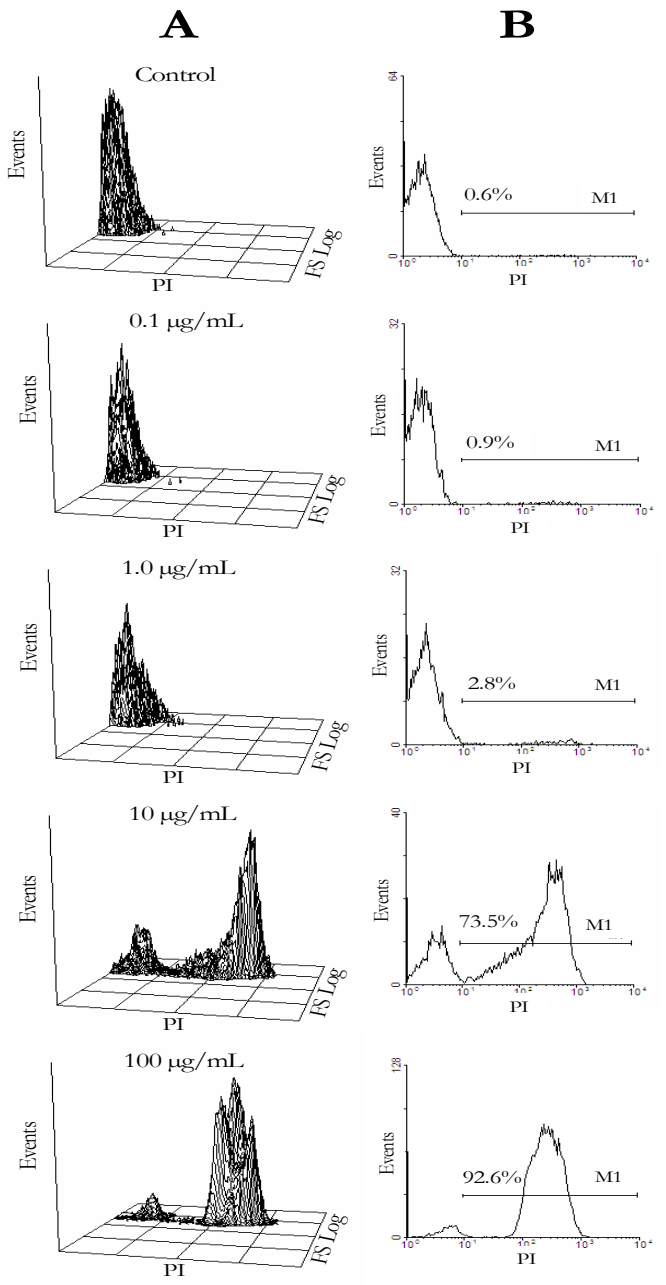


Figure 20 Two representations (A and B) of the flow cytometric results of a sequence of samples stained with PI, from the control, through the logarithmic array of test concentrations of incrustoporin LNO18-22, to the maximum test concentration.

Notes: (A) Three-dimensional representation of the distribution of the cells according to logarithmic scales of their size (FS) and FL3 channel red fluorescence (PI). (B) Monoparametric histograms of the analysed cells distributed according to the correspondent red fluorescence in FL3 channel (PI); the markers (M1) represent the area of positivity (elevated fluorescence in relation to the control) and include the indication of the percentage of positive cells (dead cells, in this case). Events, number of cells; FS Log, logarithmic scale of forward-scatter; PI, logarithmic scale of propidium iodide red fluorescence in FL3 channel; M1, marker 1.

The same histograms represented in Fig. 20 (B) can be seen in Fig. 21 in sequential plotting.

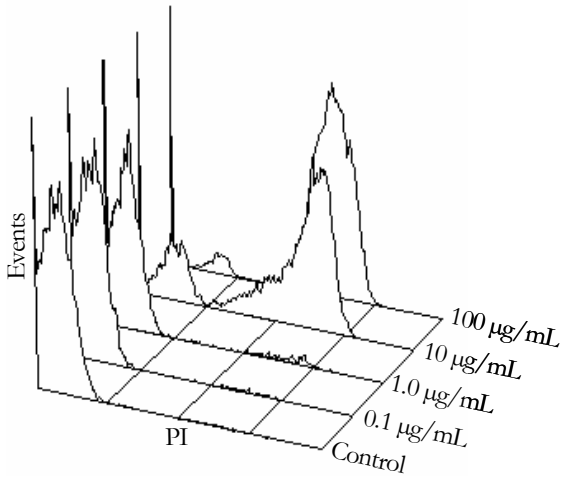


Figure 21 Sequential plotting of the histograms represented in Fig. 20 (B), showing results for the control and samples treated with the four concentrations of LNO18-22 after staining with PI.

Notes: Events, number of cells; PI, logarithmic scale of propidium iodide red fluorescence in FL3 channel.

All these results, plus the ones for the other fluorescent probes and tested compounds, can be seen in Fig. 22, a graphical representation of both the complete flow cytometric results and the values of the percentages of growth inhibition at the end of the incubation time for each antifungal agent tested.

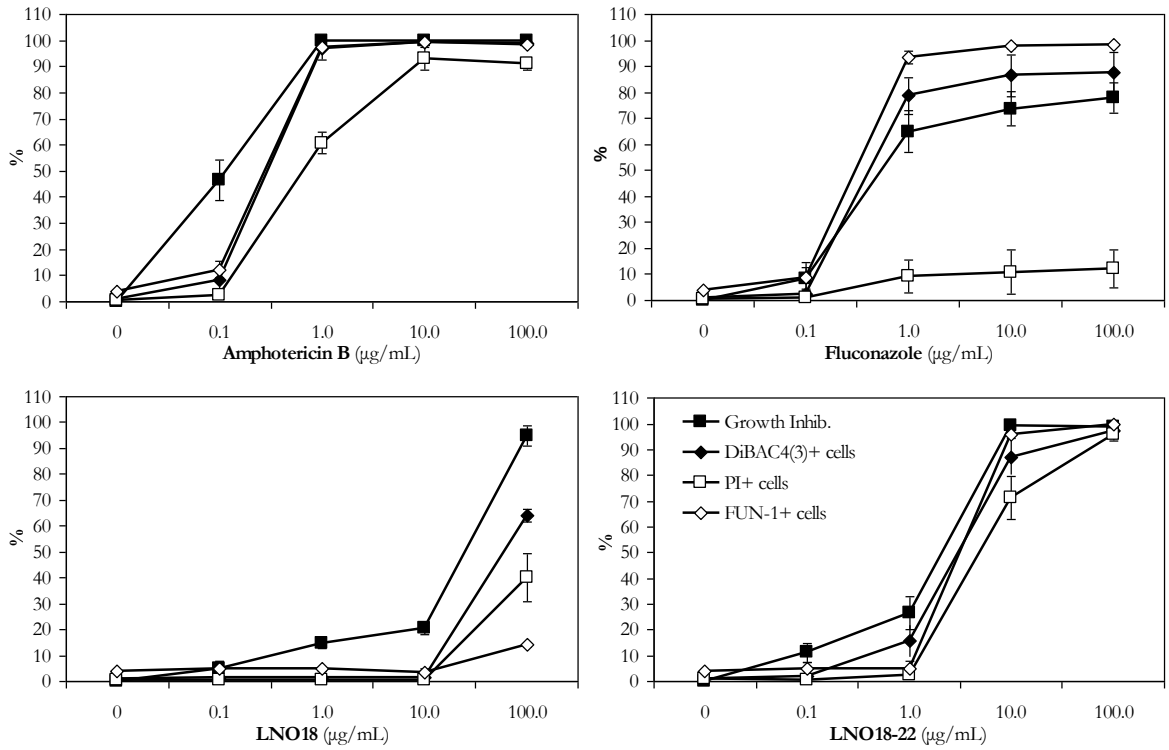


Figure 22 Percentages of growth inhibition in relation to the controls (closed squares) and $DiBAC_4(3)$ (closed diamonds), PI (open squares), and FUN-1 (open diamonds) positive cells after treatment of *Candida albicans* ATCC 90028 with the logarithmic array of concentrations of amphotericin B, fluconazole, LNO18, and LNO18-22.

Notes: Averages of a minimum of five values for the growth inhib., four values for PI and $DiBAC_4(3)$ and two values for FUN-1 ± standard deviations. In some cases the standard deviation bars are smaller than the symbols and thus not seen.

The persistence of PI negativity in contrast with the $DiBAC_4(3)$ results for fluconazole where confirmed by analyzing fluconazole and LNO18-22 treated samples according to their fluorescence properties in the experiments with simultaneous PI and DiBAC4(3) staining (an example is shown in Fig. 23). As it is shown in the example of Fig. 23, even when treated with the highest concentration of fluconazole the sample has a maximum of 3.26% PI positive cells, while LNO18-22 yields a total of 92.56% positives. With $DiBAC_4(3)$, however, both drugs yield high positivity.

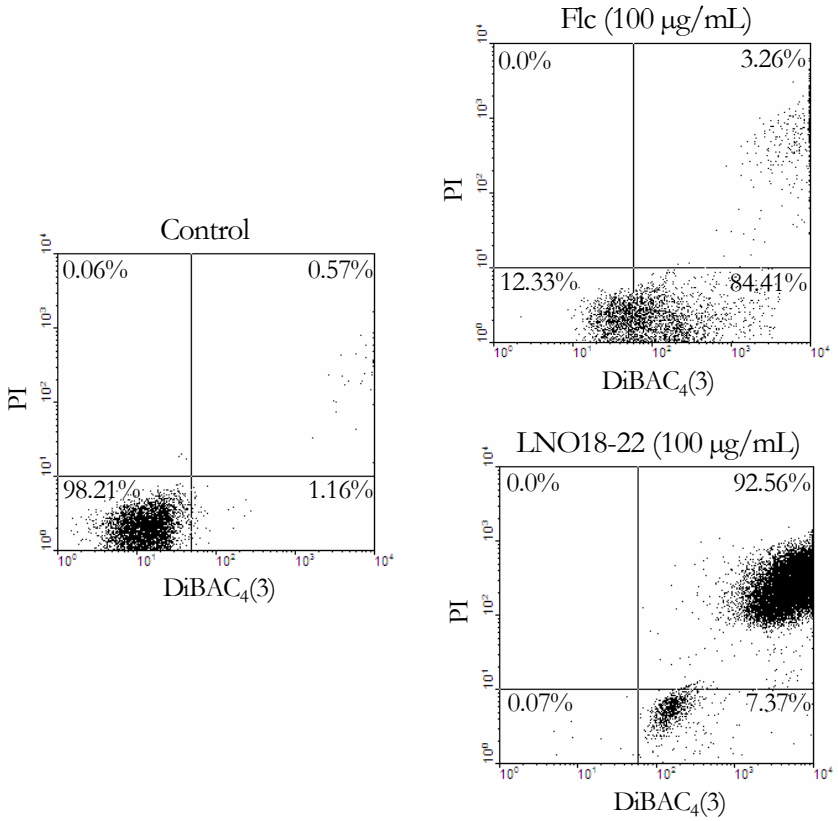


Figure 23 Biparametric representations (dot-plot cytograms) according to logarithmic scales of FL3 channel red fluorescence (PI) and FL1 channel green fluorescence [DiBAC4(3)] showing a control sample, a sample treated with 100 μg/mL of fluconazole, and a sample treated with 100 μg/mL of LNO18-22.

Notes: The percentages of cells in each quadrant are shown in the correspondent areas. PI, logarithmic scale of propidium iodide red fluorescence in FL3 channel; DiBAC4(3), logarithmic scale of DiBAC4(3) green fluorescence in FL1 channel; Flc, fluconazole.

The screening of antifungal activity results obtained with the remaining tested chemical groups of experimental compounds are shown in the following sections.

4.2 Quinoline derivatives

The studied quinoline derivatives could be divided into two groups, according to their chemical structures (Table 13), the first including compounds 1 to 3 and the second including compounds 4 to 11. Table 25 shows the results of the screening of antifungal activity of all the compounds and the reference drug fluconazole against the basic set of fungal species (for the descriptive list of the used strains see Table 10).

Table 25 *In vitro* antifungal activity (IC_{80}) of the tested quinoline derivatives in comparison with the standard fluconazole.

Compound	**MIC/IC_{80}** (μmol/L)							
	CA1	**CT**	**CK1**	**CG**	**TB**	**AF**	**AC**	**TM**
	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	72h 120h
1	1.95 7.81	15.63 31.25	15.63 31.25	15.63 31.25	7.81 15.63	3.91 3.91	31.25 62.5	7.81 15.63
2	62.5 250	500 1000	500 500	500 1000	500 500	125 250	500 500	250 250
3	31.25 125	31.25 62.5	62.5 62.5	15.63 31.25	125 >125	31.25 62.5	>125 >125	31.25 31.25
4	3.91 >62.5	7.81 >62.5	>62.5 >62.5	3.91 62.5	>62.5 >62.5	>62.5 >62.5	31.25 >62.5	>62.5 >62.5
5	3.91 >62.5	7.81 >62.5	125 >125	3.91 >62.5	125 >125	>62.5 >62.5	7.81 >62.5	>62.5 >62.5
6	7.81 15.63	15.63 15.63	15.63 31.25	7.81 15.63	31.25 62.5	15.63 31.25	7.81 7.81	15.63 15.63
7	31.25 62.5	125 125	62.5 125	62.5 125	125 250	62.5 125	31.25 62.5	62.5 125
8	31.25 125	15.63 62.5	125 250	3.91 15.63	>250 >250	125 >250	>250 >250	15.63 >250
9	1.95 31.25	0.49 3.91	15.63 31.25	0.24 0.49	62.50 250	7.81 62.5	31.25 62.5	7.81 15.63
10	1.95 62.5	7.81 31.25	62.5 125	1.95 7.81	>500 >500	3.91 >500	>500 >500	>500 >500
11	3.91 15.63	3.91 7.81	3.91 7.81	1.95 3.91	31.25 125	3.91 125	31.25 125	15.63 15.63
Fluconazole	0.06 0.12	0.12 125	3.91 15.62	0.98 3.91	0.24 0.48	>125 >125	>125 >125	1.95 3.91

4.3 4-Substituted phenylguanidinium salts

The results for this group of compounds are shown in the following table (Table 26).

Table 26 *In vitro* antifungal activity (IC_{80}) of the tested 4-substituted phenylguanidinium salts in comparison with the standards dodine and ketoconazole.

Compound	**MIC/IC_{80}** (µmol/L)							
	CA1	**CT**	**CK1**	**CG**	**TB**	**AF**	**AC**	**TM**
	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	72h 120h
4a	>1000 >1000	>1000 >1000	125 125	1000 >1000	500 >1000	>1000 >1000	>1000 >1000	125 500
4b	>1000 >1000	>1000 >1000	250 250	>1000 >1000	1000 >1000	>1000 >1000	>1000 >1000	250 500
4c	125 250	125 250	7.81 15.63	62.5 125	250 250	250 500	1000 1000	125 125
4d	125 250	125 250	7.81 15.63	62.5 125	250 250	250 500	1000 1000	125 125
4e	31.25 62.5	31.25 62.5	7.81 15.63	31.25 62.5	31.25 62.5	62.5 125	500 500	≤1.95 ≤1.95
4f	31.25 62.5	15.63 31.25	15.63 31.25	31.25 31.25	15.63 31.25	31.25 62.5	125 125	31.25 62.5
4g	7.81 15.63	≤7.81 ≤7.81	7.81 7.81	15.63 15.63	7.81 7.81	15.63 31.25	31.25 31.25	31.25 31.25
4h	7.81 15.63	7.81 7.81	7.81 7.81	7.81 7.81	3.91 7.81	7.81 15.63	62.5 62.5	7.81 7.81
4i	15.63 15.63	15.63 15.63	15.63 15.63	7.81 7.81	7.81 7.81	7.81 15.63	15.63 62.5	15.63 15.63
4j	7.81 7.81	3.91 15.63	3.91 7.81	3.91 3.91	3.91 3.91	15.63 15.63	62.5 62.5	3.91 3.91
4k	31.25 31.25	15.63 15.63	15.63 62.5	15.63 31.25	7.81 7.81	31.25 62.5	62.5 62.5	1.95 7.81
4k-acetate	31.25 31.25	31.25 31.25	7.81 15.63	15.63 15.63	3.91 7.81	7.81 62.5	15.63 125	≤1.95 ≤1.95
4k-sorbate	7.81 31.25	15.63 62.5	3.91 7.81	3.91 7.81	1.95 3.91	3.91 7.81	15.63 62.5	≤1.95 ≤1.95
4k-base	7.81 7.81	31.25 31.25	7.81 15.63	3.91 3.91	≤1.95 3.91	3.91 31.25	15.63 15.63	≤1.95 ≤1.95

Table 26. continued

Compound	**MIC/IC$_{80}$** (μmol/L)							
	CA1	**CT**	**CK1**	**CG**	**TB**	**AF**	**AC**	**TM**
	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	72h 120h
4l	31.25 125	31.25 31.25	31.25 62.5	7.81 7.81	31.25 62.5	31.25 >125	>125 >125	1.95 3.91
4m	125 250	250 >500	250 250	31.25 125	250 250	125 >500	>500 >500	3.91 3.91
4n	250 >250	>250 250	>250 125	250 >250	>250 >250	>250 >250	>250 >250	7.81 31.25
4o	125 >125	>125 >125	125 >125	>125 >125	125 >125	>125 >125	>125 >125	125 125
4p	250 250	250 250	62.5 125	500 500	125 250	500 1000	1000 1000	250 250
4q	250 250	250 500	15.63 31.25	62.5 125	250 250	62.5 500	500 500	15.63 125
4r	500 1000	1000 >1000	1000 1000	1000 >1000	500 500	500 500	1000 1000	500 500
4s	500 >500	500 500	31.25 62.5	250 500	250 250	125 >500	500 500	31.25 31.25
4t	15.63 31.25	7.81 15.63	3.91 7.81	15.63 31.25	15.63 15.63	31.25 62.5	250 250	7.81 15.63
4u	125 125	62.5 62.5	31.25 31.25	125 125	125 125	125 500	1000 1000	125 250
Dodine	7.81 7.81	3.91 3.91	3.91 3.91	7.81 7.81	7.81 7.81	7.81 15.63	625 62.5	15.63 15.63
Ketoconazole	≤0.24 ≤0.24	1.95 3.91	0.98 1.95	0.49 1.95	≤0.24 ≤0.24	7.81 7.81	31.25 31.25	0.98 1.95

4.4 Azo dye organotin(IV) compounds

Table 27 displays the results for this group of tested agents.

Table 27 *In vitro* antifungal activity (IC_{80}) of the most active of the tested azo dye organotin(IV) compounds.

Compound	**MIC/IC_{80}** (μmol/L)							
	CA1	**CT**	**CK1**	**CG**	**TB**	**AF**	**AC**	**TM**
	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	72h 120h
1a	7.81 15.63	15.63 15.63	3.91 3.91	31.25 62.5	7.81 15.63	15.63 15.63	1.95 3.91	3.91 7.81
2a	15.63 15.63	7.81 15.63	3.91 7.81	>62.5 >62.5	31.25 >62.5	15.63 31.25	7.81 7.81	7.81 15.63
3a	15.63 62.5	>125 >125	3.91 3.91	>125 >125	15.63 62.5	7.81 7.81	3.91 3.91	3.91 15.63
5a	3.91 7.81	15.63 62.5	1.95 3.91	15.63 62.5	15.63 62.5	7.81 15.63	3.91 3.91	3.91 7.81
1b	0.977 7.81	1.95 15.63	0.977 1.95	15.63 15.63	0.977 7.81	3.91 7.81	0.488 0.488	0.977 1.91
2b	1.91 7.81	0.488 7.81	3.91 3.91	>125 >125	1.95 >125	3.91 >125	0.122 0.977	1.91 3.91
4b	1.91 15.63	3.91 7.81	3.91 7.81	62.5 125	3.91 15.63	7.81 15.63	0.977 0.977	3.91 7.81
6b	1.91 3.91	7.81 15.63	1.91 1.91	7.81 15.63	7.81 15.63	3.91 7.81	1.91 3.91	1.91 3.91
Compound	**MFC** (μmol/L)							
	CA1	**CT**	**CK1**	**CG**	**TB**	**AF**	**AC**	**TM**
	48h	48h	48h	48h	48h	48h	48h	120h
1a	31.25	15.63	31.25	>250	>250	>250	3.91	31.25
2a	>62.5	15.63	31.25	>62.5	>62.5	>62.5	7.81	15.63
3a	>125	>125	62.5	>125	>125	>125	7.81	>125
5a	15.63	>125	7.81	>125	>125	>125	31.25	>125
1b	7.81	31.25	31.25	62.5	>500	>500	15.63	62.5
2b	15.63	15.63	>125	>125	>125	>125	3.91	31.25
4b	125	>125	>125	>125	>125	>125	7.81	125
6b	7.81	31.25	7.81	15.63	>125	>125	31.25	31.25

4.5 Quinaldine derivatives

The results for the tested quinaldine derivatives are presented in Table 28.

Table 28 *In vitro* antifungal activity (IC_{80}) of the most active of the tested quinaldine derivatives in comparison with the standard fluconazole.

Compound	**MIC/IC_{80}** (μmol/L)							
	CA1	**CT**	**CK1**	**CG**	**TB**	**AF**	**AC**	**TM**
	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	72h 120h
2	125 >125	125 >125	125 >125	125 >125	125 >125	125 >125	125 >125	31.25 31.25
3	>250 >250	>250 >250	>250 >250	>250 >250	>250 >250	>250 >250	>250 >250	250 250
4	125 >125	125 >125	125 >125	125 >125	125 >125	125 >125	125 >125	125 >125
6	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	125 125
9	>250 >250	>250 >250	>250 >250	>250 >250	>250 >250	>250 >250	>250 >250	250 250
10	3.91 7.81	31.25 31.25	7.81 7.81	31.25 31.25	15.63 62.5	15.63 31.25	31.25 62.5	31.25 62.5
11	3.91 3.91	15.63 31.25	3.91 7.81	15.63 15.63	31.25 31.25	15.63 15.63	15.63 15.63	15.63 15.63
12	≤0.45 0.9	7.81 15.63	7.81 15.63	7.81 15.63	31.25 62.5	15.63 15.63	62.5 62.5	7.81 15.63
15	>250 >250	>250 >250	>250 >250	>250 >250	>250 >250	>250 >250	>250 >250	>250 >250
16	125 >125	125 >125	125 >125	125 >125	125 >125	125 >125	125 >125	125 >125
Fluconazole	0.06 0.12	0.12 >125	3.91 15.63	0.98 3.91	0.24 0.48	>125 >125	>125 >125	1.95 3.91

4.6 (*E*)-3-(Nitrophenyl)-1-(pyrazin-2-yl)prop-2-en-1-ones (the chalcones)

The chalcones were generally more active against *T. mentagrophytes*. Their results are presented in Table 29.

Table 29 *vitro* antifungal activity (IC_{80}) of the tested (*E*)-3-(Nitrophenyl)-1-(pyrazin-2-yl)prop-2-en-1-ones (the chalcones) in comparison with the standard ketoconazole.

MIC/IC_{80} (μmol/L)			
Compound	**TM** 72h 120h	Compound	**TM** 72h 120h
7a	31.25 31.25	9a	15.63 15.63
7b	125 500	9b	31.25 >62.5
7c	15.63 31.25	9c	31.25 >62.5
7d	15.63 15.63	9d	15.63 31.25
7e	250 250	9e	7.81 15.63
8a	7.81 15.63	10	3.91[a] 15.63[a]
8b	>125 >125	11	31.25[b] 62.5[b]
8c	15.63 15.63	12	31.25[a] 62.5[a]
8d	7.81 15.63	13	>250[a] >250[a]
8e	7.81 15.63	Ketoconazole	0.24 1.95

[a] Previously determined (Opletalová *et al.*, 2002)
[b] Previously determined (Opletalová *et al.* 2005).

4.7 Salicylanilides and thiosalicylanilides

These compounds were prepared as two different series, salicylanilides and tiosalicylanilides (series 1 and 2, respectively), with the same R1 substituents (see Table 18), allowing us to compare them directly. The results of the susceptibility tests are shown in Table 30.

Table 30 *In vitro* antifungal activity (IC_{80}) of the most active salicylanilides and thiosalicylanilides.

Compound	**MIC/IC_{80}** (μmol/L)							
	CA1	**CT**	**CK1**	**CG**	**TB**	**AF**	**AC**	**TM**
	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	72h 120h
1a	62.5 125	125 250	125 125	125 250	125 250	125 125	125 125	31.25 62.5
1b	15.63 62.5	>500 >500	>500 >500	>500 >500	>500 >500	>500 >500	125 250	31.25 62.5
1c	15.63 31.25	62.5 62.5	15.63 15.63	31.25 62.5	31.25 62.5	31.25 62.5	31.25 31.25	7.81 15.63
1d	7.81 250	>125 >125	125 >125	>125 >125	>125 >125	>125 >125	31.25 62.5	1.95 3.91
1e	7.81 250	31.25 >125	15.63 15.63	15.63 >125	15.63 15.63	15.63 >125	15.63 15.63	3.91 7.81
1f	62.5 125	250 500	125 125	250 250	125 500	62.5 125	62.5 125	31.25 31.25
1g	31.25 62.5	>125 >125	62.5 62.5	>125 >125	>125 >125	31.25 62.5	31.25 31.25	7.81 7.81
2a	62.5 62.5	125 250	31.25 31.25	125 125	62.5 125	15.63 31.25	0.98 0.98	7.81 31.25
2b	62.5 62.5	125 125	31.25 31.25	125 125	62.5 62.5	31.25 31.25	≤0.49 ≤0.49	3.91 15.63
2c	7.81 15.63	31.25 31.25	7.81 7.81	15.63 31.25	15.63 15.63	7.81 15.63	≤0.49 ≤0.49	0.98 1.95
2d	3.91 3.91	15.63 31.25	<0.49 0.98	7.81 15.63	3.91 7.81	7.81 7.81	≤0.49 ≤0.49	≤0.49 ≤0.49
2e	7.81 15.63	31.25 31.25	3.91 3.91	15.63 31.25	7.81 15.63	3.91 15.63	≤0.49 ≤0.49	≤0.49 ≤0.49
2f	31.25 62.5	125 125	31.25 31.25	62.5 62.5	31.25 31.25	7.81 62.5	≤0.49 0.98	7.81 15.63
2g	15.63 31.25	>125 >125	7.81 7.81	62.5 >125	31.25 >125	62.5 >125	0.98 0.98	3.91 7.81

4.8 1-Phenyl-5-benzylsulfanyltetrazoles

The activity of the tested 1-phenyl-5-benzylsulfanyltetrazoles against most of the test fungal strains was negligible. The best results were recorded with *T. mentagrophytes* and *A. fumigatus* and are presented in Table 31.

Table 31 *In vitro* antifungal activity (IC_{80}) of the tested 1-phenyl-5-benzylsulfanyltetrazoles against *T. mentagrophytes* and *A. fumigatus*.

Compound	**MIC/IC_{80}** (μmol/L)		Compound	**MIC/IC_{80}** (μmol/L)	
	TM	**AF**		**TM**	**AF**
	72h	48h		72h	48h
1a	31.25	>250	4a	>250	>250
1b	62.5	>125	4b	31.25	250
1c	31.25	>250	4c	250	>250
1d	31.25	>250	4d	>250	>250
2a	31.25	>250	5a	62.5	>250
2b	>250	>250	5b	62.5	>250
2c	>250	>250	5c	15.63	>125
2d	>250	125	5d	>250	>250
3a	125	>250	3c	62.5	>250
3b	>250	>250			

4.9 Arylsulfanylpyrazine-2-carboxylic acid derivatives

The results for the tested arylsulfanylpyrazine-2-carboxylic acid derivatives are presented in Table 32.

Table 32 *In vitro* antifungal activity (IC_{80}) of the most active of the tested arylsulfanylpyrazine-2-carboxylic acid derivatives in comparison with the standard fluconazole.

Compound	**MIC/IC_{80}** (μmol/L)							
	CA1	**CT**	**CK1**	**CG**	**TB**	**AF**	**AC**	**TM**
	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	72h 120h
1	62.5 250	500 >500	500 >500	500 >500	500 >500	250 500	500 >500	125 250
2	31.25 125	125 250	125 250	125 250	125 500	62.5 250	250 500	31.25 62.5
3	125 250	250 500	250 500	250 500	500 500	125 250	250 500	125 250
4	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	125 125
5	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	250 500
6	62.5 250	500 >500	500 500	500 >500	250 250	125 125	250 500	62.5 125
7	250 500	>500 >500	>500 >500	>500 >500	500 >500	250 >500	>500 >500	125 250
8	>500 >500	>500 >500	>500 >500	>500 >500	>500 >500	>500 >500	>500 >500	>500 >500
9	125 500	>500 >500	>500 >500	>500 >500	500 >500	250 >500	>500 >500	250 500
10	31.25 125	250 500	125 250	125 250	125 500	62.5 >500	250 >500	62.5 62.5
11	125 250	500 >500	250 500	250 500	500 >500	125 250	>250 >500	125 250
12	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125
13	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125
Fluconazole	0.06 0.12	0.12 >125	3.91 15.62	0.98 3.91	0.24 0.48	>125 >125	>125 >125	1.95 3.91

4.10Ring-substituted (*E*)-3-aryl-1-pyrazin-2-ylprop-2-en-1-ones

The results for the tested ring-substituted (*E*)-3-aryl-1-pyrazin-2-ylprop-2-en-1-ones are presented in Table 33.

Table 33 *In vitro* antifungal activity (IC_{80}) of the most active of the tested ring-substituted (*E*)-3-aryl-1-pyrazin-2-ylprop-2-en-1-ones in comparison with the standard fluconazole.

Compound	**MIC/IC_{80}** (μmol/L)							
	CA1	**CT**	**CK1**	**CG**	**TB**	**AF**	**AC**	**TM**
	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	72h 120h
1a	62.5 62.5	125 125	125 125	125 125	125 125	62.5 125	125 250	15.63 31.25
1b	62.5 125	62.5 62.5	31.25 62.5	62.5 62.5	31.25 31.25	31.25 62.5	250 250	31.25 62.5
1c	31.25 62.5	62.5 125	125 125	125 125	125 125	125 >250	125 >250	15.63 31.25
1d	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	15.63 125
1e	62.5 62.5	62.5 125	>125 >125	62.5 62.5	62.5 >125	31.25 >125	>125 >125	15.63 15.63
2a	125 125	125 >250	125 125	125 250	>250 >250	125 125	>250 >250	≤125 ≤125
2b	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5
2c	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	≤62.5 ≤62.5
2d	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5
2e	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	>62.5 >62.5	7.81 7.81
3a	15.63 31.25	31.25 62.5	31.25 62.5	31.25 62.5	250 250	125 125	250 500	7.81 15.63
3b	62.5 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	15.63 31.25
3c	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	>125 >125	≤62.5 ≤62.5

Table 33 continued

Compound	**MIC/IC$_{80}$** (μmol/L)							
	CA1	**CT**	**CK1**	**CG**	**TB**	**AF**	**AC**	**TM**
	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	24h 48h	72h 120h
4b	>188 >188	>188 >188	>188 >188	>188 >188	>188 >188	>188 >188	>188 >188	≤94 ≤94
4c	62.5 125	500 500	500 500	500 500	125 500	125 125	125 250	15.63 15.63
5c	125 250	>250 >250	250 250	>250 >250	>250 >250	125 >250	>250 >250	62.5 62.5
Fluconazole	≤0.06 ≤0.06	3.91 7.81	1.95 1.95	0.49 1.95	≤0.06 ≤0.06	7.81 7.81	15.63 15.63	0.24 1.95

4.11 Quinazoline-4-thiones

The tested 2,2-dimethyl-3-phenyl-1,2-dihydroquinazoline-4(3*H*)-thiones (series 1) and 2-methyl-3-phenylquinazoline-4(3*H*)-thiones (series 2) were all inactive, with the exception of derivative 1 h. This compound showed a moderate activity against *A. fumigatus* (MIC = 62.5 μmol/L) and *A. corymbifera* (MIC = 62.5 μmol/L) after both 24 h and 48 h of incubation.

5 DISCUSSION

The 3-(halogenated phenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-ones are semi-synthetic compounds derived from the structure of (–)incrustoporin, a phytopathogenic compound isolated from the extract of fermentation of the basidiomycete *Incrustoporia carneola* (Zapf *et al.* 1995). They represent a new class of butenolide antifungal agents, developed over the last few years by semi-synthetic tuning of antifungal activity (Pour *et al.* 2000; Pour *et al.* 2001; Pour *et al.* 2003; Skopalová-Kubanová, 2003), as referred previously. The result was the refinement of the biological activity of the precursor (–)incrustoporin and the development of potent antimycotics (Fig. 24).

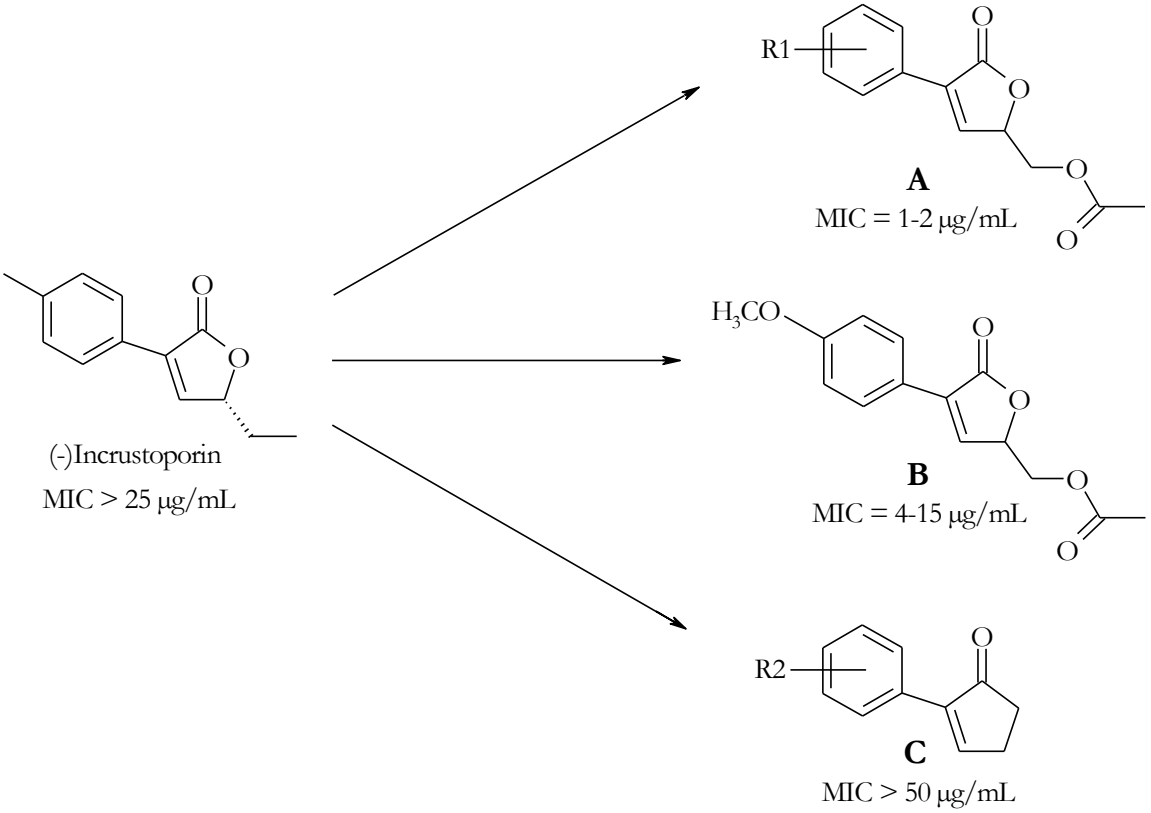


Figure 24 Chemical structure of (–)incrustoporin and three groups of its derivatives, including the range of determined MICs for most of the fungal strains tested.

Notes: (**A**) 3-(Halogenated phenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-ones. R1=3,4-Cl_2 (LNO6-22); 3-Cl (LNO15-22); 4-Br (LNO18-22). (**B**) 3-(4-Methoxyphenyl)-5-acetoxymethyl-2,5-dihydrofuran-2-one (Pour *et al.* 2001). (**C**) 2-(Substituted aryl)cyclopent-2-enones (Pour *et al.* 2003). R2= H; 4-CH_3; 4-OCH_3; 4-Cl; 3,4-Cl_2.

Specifically regarding our studies with the 3-(halogenated phenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-ones (Fig. 24A), the *in vitro* susceptibility results showed the incrustoporin de-

rivatives to have a broad spectrum of antifungal activity, yielding very low MICs against a variety of pathogenic yeasts and moulds (Table 23). Furthermore, the results against fluconazole-resistant fungi could be stressed as particularly relevant.

Among the three tested derivatives, the differences in antifungal activity were quite small. The 4-bromophenyl derivative (LNO18-22) seemed to be the most potent, however, particularly against some of the tested fungal species (Table 23 and Fig. 25). In the specific cases of *C. glabrata* and some strains of *C. tropicalis*, on the other hand, the 3-chlorophenyl derivative (LNO15-22) yielded the lowest MICs.

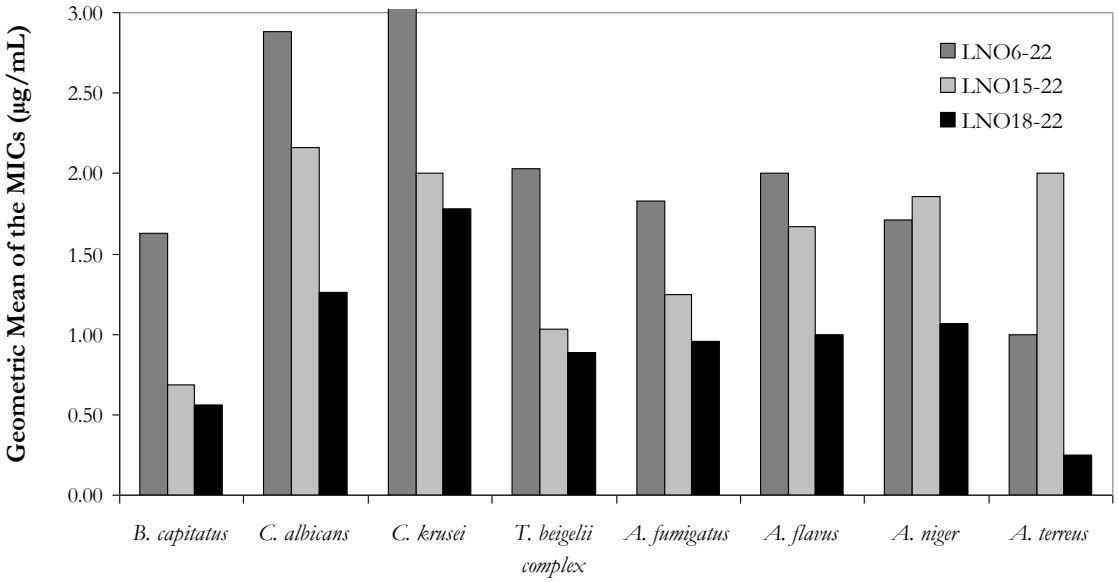


Figure 25 Comparison between the determined MICs in µg/mL (geometric mean of the results for the different species) of the three studied incrustoporin derivatives for some of the tested fungal species.

Note: the value for LNO6-22 against *C. krusei* is 6.0 µg/mL.

Further more, these compounds proved to be comparably active against fungi with decreased susceptibility to fluconazole, including the fluconazole-resistant *C. albicans* strains, *C. krusei*, *C. glabrata*, *S. cerevisiae*, and *Aspergillus* sp. A good example is the comparison between the MICs determined for the fluconazole-resistant and fluconazole-susceptible *C. albicans* strains. In fact, the MICs for fluconazole-resistant strains were only one or two dilutions higher than the ones for susceptible strains (Fig. 26).

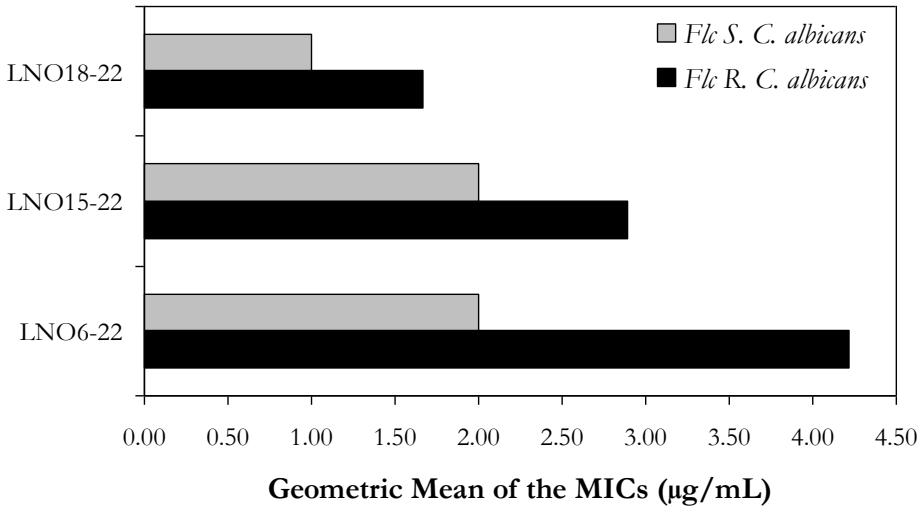


Figure 26 Comparison between the determined MICs in μg/mL (geometric mean of the results for the different species) of the three studied incrustoporin derivatives after 24 h of incubation for the tested fluconazole-susceptible (Flc S.) and fluconazole-resistant (Flc R.) *Candida albicans* strains.

Besides that, the incrustoporins appear to show higher activity against filamentous fungi. As we can see in Table 23, with the exception of the strain of the zygomycete *A. corymbifera*, the MICs for mould strains were never higher than 2.0 μg/mL. All *A. fumigatus* strains were inhibited at a concentration of 1 μg/mL of LNO18-22 or lower. In a time when fluconazole-resistant yeast infections, like the cases of refractory candidiasis in AIDS patients, and invasive aspergillosis in transplant recipients and neutropenic patients are growing problems, these results should be pointed out as particularly relevant.

In terms of structure-activity relationships, the substitutions of bromine or chlorine in positions 3 and 4 on the phenyl moiety at C-3 of the furanone core (Fig. 24A) appears to be the most favourable for the development of antifungal activity, as it was established during the development of these compounds (Pour *et al.* 2001). In fact, a derivative with a 4-methoxyphenyl group at C-3 is significantly less active (Fig. 24B). Besides that, it had also been previously established (Pour *et al.* 2003) that the five-membered lactone ring is essential for the activity, as no antifungal activity was found for cyclopent-2-enones (Fig. 24C).

Interestingly, the results of the inhibition of filamentation in *C. albicans* strains revealed an opposite tendency in comparison with the susceptibility results. In this case, on the contrary, it was LNO6-22 that produced the best results, with a practically complete inhibition of germ tube production at a concentration of 1/10 of the respective MIC, a result similar to the one of amphotericin B (Table 24 and Fig. 16). LNO15-22 followed and, in the order of activity, only then came LNO18-22, these last two both showing results comparable to the ones of fluconazole, with only slight influences on the number of positive cells in comparison to the

controls. These results have introduced some confusion in the definition of antifungal potency for the incrustoporin derivatives. In fact, as preliminary speculation, but considering that filamentation has normally been associated with virulence in *Candida albicans* (Lo *et al.* 1997; Kretschmar *et al.* 1999; Mitchell, 1998; Navarro-García *et al.* 2001; Sánchez-Martínez *et al.* 2001), it would be possible to argue that LNO6-22 could, in comparison with LNO15-22 and LNO18-22, be more effective in treatment when reaching the infection anatomical site in subinhibitory concentrations. There is, however, no confirmation for that fact and the susceptibility results still guide our view of the relative potency of the drugs.

Apart from that, the results obtained with the commercial drugs are in agreement with previous reports. For example, Ellepola *et al.* previously reported an almost complete inhibition of germ tube formation by the polyenes nystatin and amphotericin B, and only a negligible one by ketoconazole, fluconazole, and flucytosine (Ellepola *et al.* 1998).

Butenolides and structurally related compounds in general had been previously shown to have a range of biological activities, including antifungal activity (Kazmaier *et al.* 2002; Khan and Husain, 2002; Strobel *et al.* 2002; Nagata *et al.* 1998; Del Poeta *et al.* 1998; Gamard *et al.* 1997; Martín *et al.* 1990). The mechanisms of action of these compounds have not been thoroughly investigated, however, a task we have now initiated for the incrustoporin derivatives.

Concerning the results of the Bioscreen experiments, in most cases the kinetics of growth inhibition did not reveal any additional unexpected information that would not be disclosed by the simple turbidimetric analysis of the final growth inhibition after 24 h of incubation (Fig. 22 and Fig. 27). The exception is the case of the samples treated with 1.0 μg/mL of the incrustoporine derivative LNO18-22. This concentration caused a delay of about 8-10 h in the beginning of logarithmic phase, although by the end of the incubation time the optical density was already very close to the one registered in the control samples (Fig. 17).

In respect to the flow cytometric results, it is important to notice that the findings regarding the influence of amphotericin B and fluconazole on the yeast cell size, concretely referring to the reduction of cell size in samples treated with inhibitory concentrations of amphotericin B (Fig. 19 A and B) and the increase in cell size in samples treated with fluconazole (Fig. 19 C and D), are in agreement with previously described results. Ramani *et al.* found similar effects after treating *C. albicans* cells for only 2 h with high concentrations of amphotericin B and 4 h with fluconazole (Ramani *et al.* 1997).

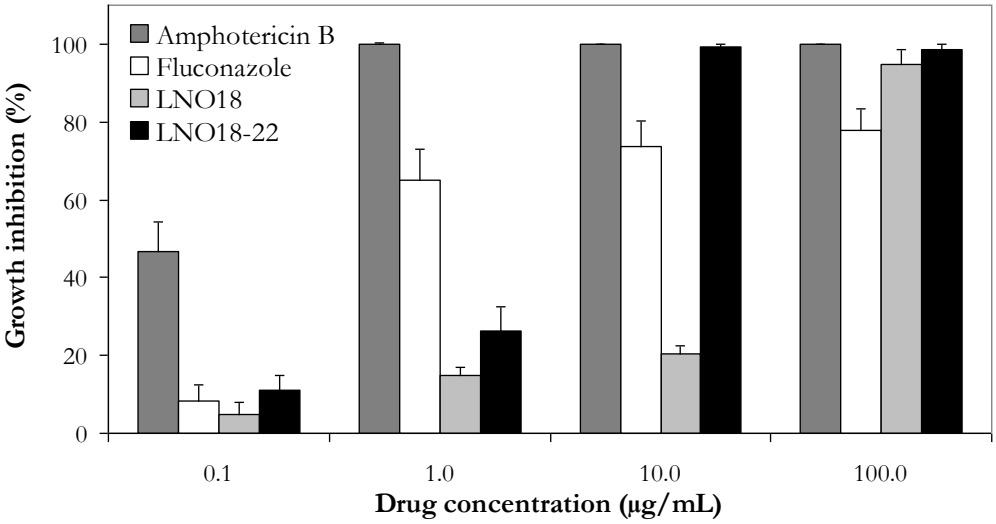


Figure 27 Percentages of growth inhibition in relation to the controls after treatment of *Candida albicans* ATCC 90028 for 24 h with the four test concentrations of amphotericin B, fluconazole, LNO18 and LNO18-22. Results expressed as averages (of a minimum of five results) + standard deviations.

The analysis of Fig. 22 discloses a number of different facts. Amphotericin B was the only agent to show influence on the cell growth at the lowest concentration (0.1 µg/mL), causing an inhibition of almost 50% (see also Fig. 27). At the three higher concentrations complete growth inhibition was accompanied by damage of cell membranes and cell death evidenced by PI positivity, particularly at 10 and 100 µg/mL (see also Fig. 28).

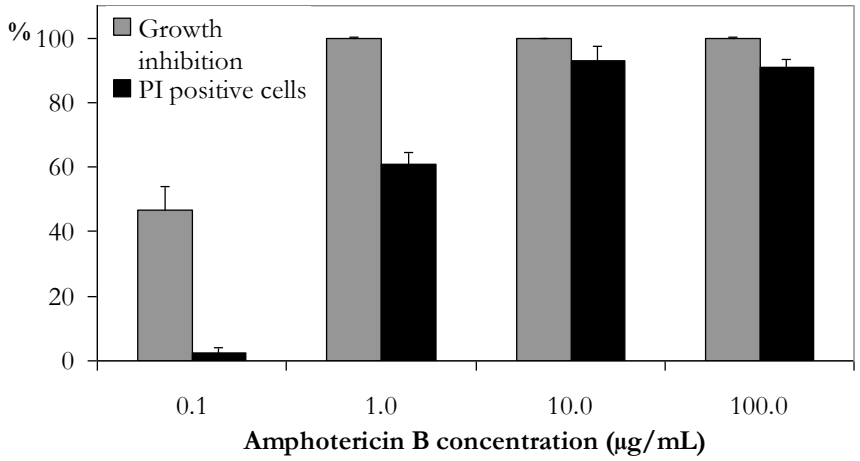


Figure 28 Percentages of growth inhibition and PI positive cells in relation to the controls after treatment of *Candida albicans* ATCC 90028 with the four test concentrations of amphotericin B.

Notes: Results expressed as averages (of a minimum of five results for the growth inhibition and four for PI positivity) + standard deviations.

With fluconazole, growth inhibition was never over 80% in comparison to the control, an effect clearly illustrated already by the growth curves in Fig. 17. Besides that, a persistence of PI negativity was observed through the whole range of test concentrations, whereas the membrane potential and the metabolic activity where deeply affected (as represented by the high levels of $DiBAC_4(3)$ and FUN-1 positivity, respectively) (Fig. 29).

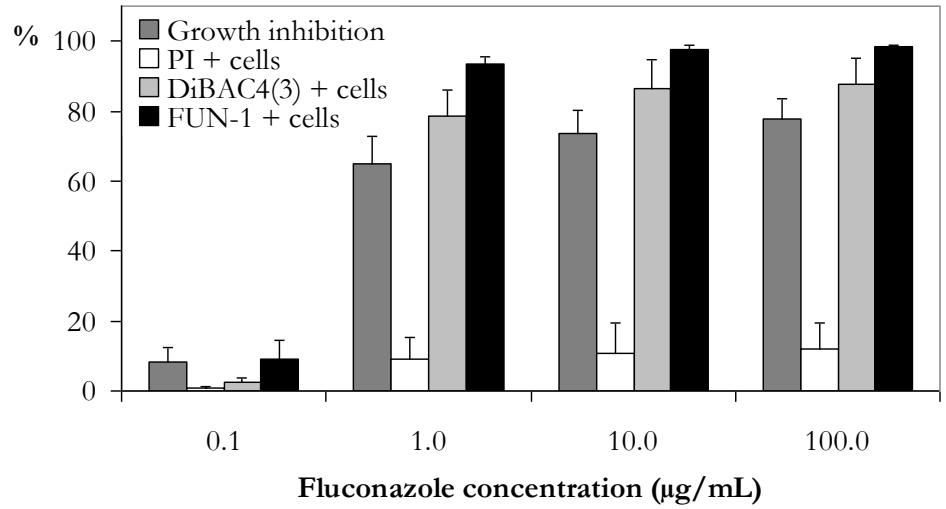


Figure 29 Percentages of growth inhibition and PI, $DiBAC_4(3)$, and FUN-1 positive cells in relation to the controls after treatment of *Candida albicans* ATCC 90028 with the four test concentrations of fluconazole.

Notes: Results expressed as averages (of a minimum of five results for the growth inhibition, four for PI and $DiBAC_4(3)$, and two for FUN-1) + standard deviations.

Regarding the incrustoporin derivatives, the parental compound LNO18 did not yield any flow cytometric positivity when tested at concentrations below 100 µg/mL, the one resulting in total growth inhibition. At this concentration, some degree of cell death and plasmatic membrane depolarization were recorded, corresponding to around 40 % of PI positive cells and over 60% of $DiBAC_4(3)$ positive cells, respectively (Fig. 30).

LNO18-22, on the contrary, caused complete *C. albicans* growth inhibition and high cell death, cell membrane depolarization, and metabolic inactivity at 10 µg/mL. PI positivity closely related to the growth inhibition results through the range of test concentrations (Fig. 31).

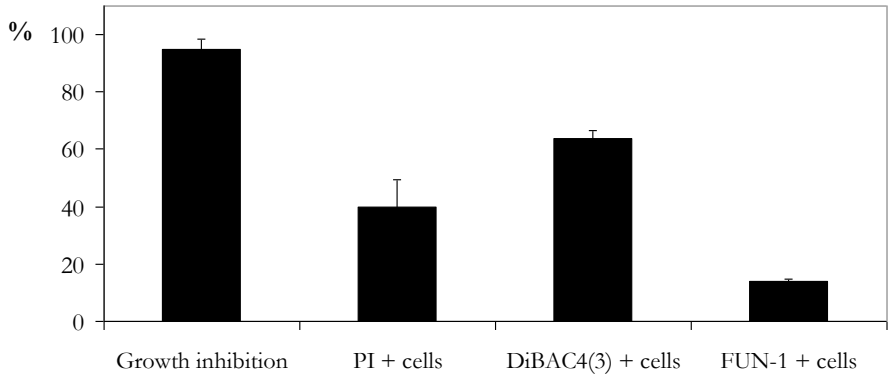


Figure 30 Percentages of growth inhibition and PI, $DiBAC_4(3)$, and FUN-1 positive cells in relation to the controls after treatment of *Candida albicans* ATCC 90028 with 100 μg/mL of LNO18.

Notes: Results expressed as averages (of a minimum of five results for the growth inhibition, four for PI and $DiBAC_4(3)$, and two for FUN-1) + standard deviations.

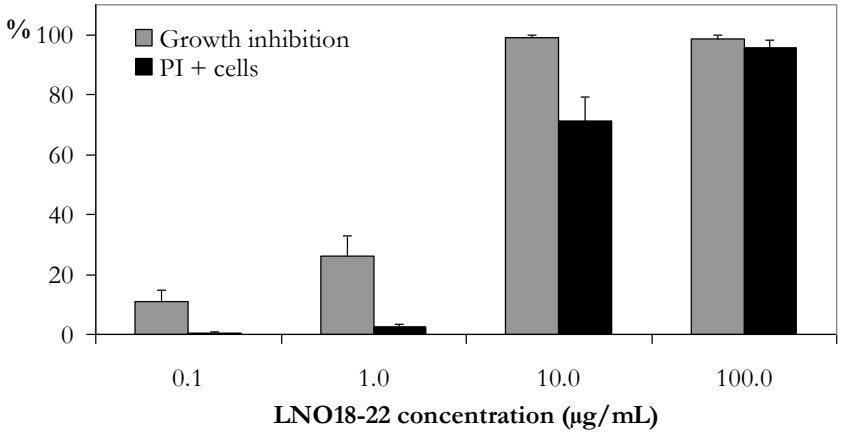


Figure 31 Percentages of growth inhibition and PI positive cells in relation to the controls after treatment of *Candida albicans* ATCC 90028 with the four test concentrations of LNO18-22.

Notes: Results expressed as averages (of a minimum of five results for the growth inhibition and four for PI) + standard deviations.

The flow cytometric results for amphotericin B and fluconazole were, then, in perfect agreement with their known mechanisms of action (Odds *et al.* 2003). The experiments with amphotericin B confirmed its typical fungicidal mechanism of action through direct influence on the cell membrane, resulting in high levels of cell death and impaired membrane integrity. On the contrary, PI negativity with fluconazole evidenced its fungistatic activity. Besides that, its characteristic trailing effect (NCCLS, 2002a) was demonstrated by the fact that there was

always at least 20% growth in comparison to the controls in every test sample, even in samples of cells treated with inhibitory concentrations of this drug.

Regarding the experimental compounds LNO18 and LNO18-22, they have been previously shown to have significantly different antifungal activities (Pour *et al.* 2001). It is interesting to notice that LNO18-22 is obtained from the simple esterification of LNO18, with a consequent rise in lipophilicity, a characteristic that may very well influence the ability of the compounds to penetrate fungal cells. Besides that, LNO18-22 is a chiral compound, but there is no significant difference in antifungal activity between the enantiomers, the racemic mixture having even been previously found to be more potent (Pour *et al.* 2001)

In conclusion, the flow cytometric and Bioscreen results have allowed us to conclude that the studied incrustoporin derivative (LNO18-22) displays a potent fungicidal activity against *C. albicans*, resulting in disruption of the plasmatic membrane integrity, either by a direct interaction or by secondary consequences of metabolic impairment, and leading to ion channel or pore formation and free diffusion of PI into the cytoplasm. Plasmatic membrane depolarization and metabolic activity arrest were obvious findings in cells found to be dead and disrupted by this derivative. The results obtained with the fungicidal amphotericin B and fungistatic fluconazole completely confirmed the known physiological consequences of their antifungal activities, further supporting the validity of the findings for the experimental derivative.

Regarding the findings for the remaining groups of compounds studied, they include, as mentioned before, susceptibility results only. The first referred is the group of quinoline derivatives, tested in comparison with fluconazole. It is important to refer that some of these compounds were poorly soluble in the test medium (RPMI 1640), reason why the maximum concentration tested was quite low and the respective MICs could not be determined accurately. An overall look through Table 25 reveals that compound 2 showed only a moderate *in vitro* antifungal activity, while the ten other compounds showed medium or high activity against all the test strains, with MICs ranging from 0.24 to 250 μmol/L.

In general, the three compounds composing the first structural group (1 to 3) showed medium or moderate activity. While compound 1, which is substituted by two nitro groups in the C-5 and C-7 positions of the quinoline core, showed medium activity against all tested strains, the methylation at C-2 presented in compound 2 resulted in loss of the antifungal effect. Compound 3, with a dichlorophenylazo moiety at the C-5 position of the quinoline group, again displayed moderate activity.

The whole of the second structural group of quinoline derivatives tested displayed generally high biological activity. The phenolic moiety at the C-4 position on the phenyl ring, which is conjugated with the quinoline nucleus (compounds 9 and 11), seems to yield the highest antifungal activity, however. The substitution by chlorine (compound 4) or bromine (compound 5) at C-4 resulted in decreased activity. It also led to a lower solubility in DMSO, allowing the maximum test concentration or these compounds to be 62.5 μmol/L only. Substitution by a phenolic or chlorine moieties (compounds 6 to 8) at the C-2 or C-3 positions of the phenyl ring resulted in considerably decreased activity. The results were also influenced by the inclusion and position of nitrogen in the olefinic linker, its presence providing the best results, and the position of the iminic nitrogen is important as well. 2-[(4-Hydroxyphenolimino)methyl]quinolin-8-ol (compound 9) appeared to be the most active compound overall from the studied group, particularly against the *Candida* species. Compounds 10 and 11 have also revealed a very promising antifungal activity (Fig. 32).

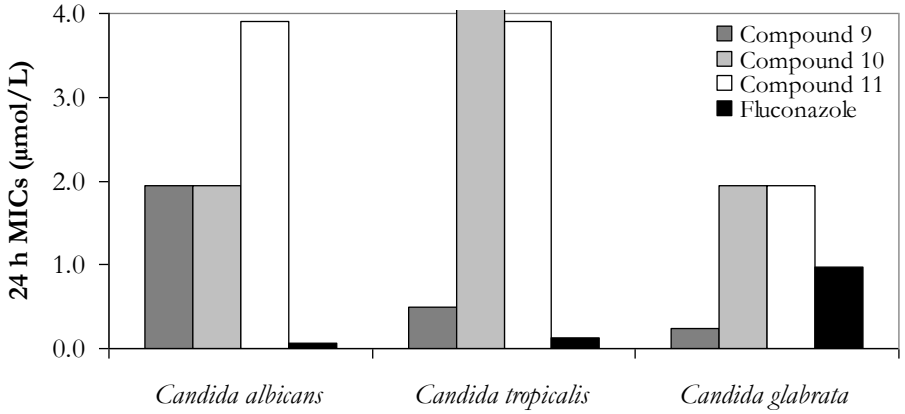


Figure 32 Comparison between the determined 24 h MICs of the most active quinoline derivatives in comparison with fluconazole against three of the tested *Candida* species.

Note: the value for the MIC of compound 10 against *C. tropicalis* is 7.81 μmol/L.

The analysis of the results of the next group of compounds, the 4-substituted phenylguanidinium salts, shows that this group displays generally high antifungal activity (Table 26). Lipophilicity seems to play a relevant role for the antifungal activity. It is clear from the analysis of the structure-activity relationship for these compounds that their potency rises with the length of the aliphatic chain until a maximum number of carbon atoms of about 13, after which it decreases again (Table 14 and Table 26). It is also important to notice that there are not big increases from 24 to 48 h MICs, an indication that the compounds have a fungicidal mechanism of action, as opposed to a fungistatic mechanism. The most active compounds of

the series appear to be 4j and 4k, including its acetate and sorbate salts and the basic form. The objective of the preparation of these forms of compound 4k was to study the potentially toxic effects of the nitrate ion and the possibility of potentiation of the activity by sorbic and acetic acids. No high differences in activity between the different forms of this compound were found, however (Fig. 33).

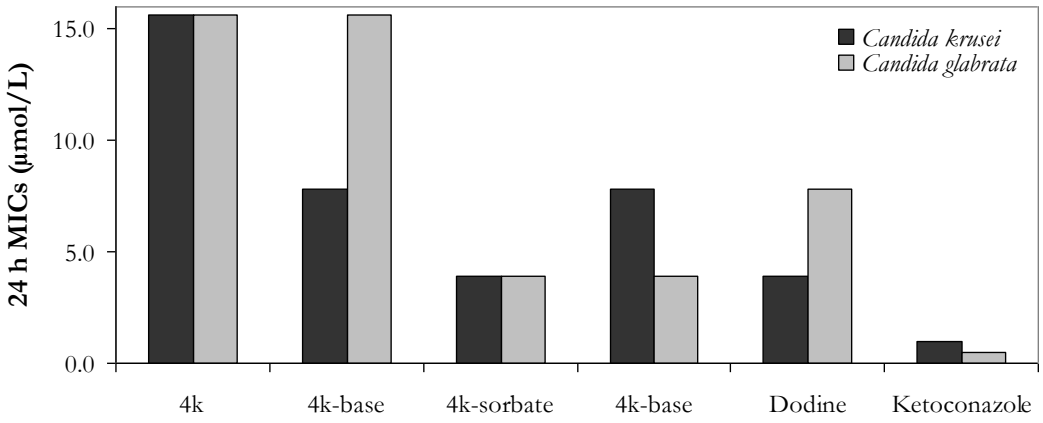


Figure 33 Comparison between the determined 24 h MICs of the different tested forms of 4-substituted phenylguanidinium compound 4k, in comparison with dodine and ketoconazole, against *Candida krusei* and *C. glabrata*.

In more general terms, it can be said that the tested 4-substituted phenylguanidinium salts are more active against *T. mentagrophytes*. In this case, compounds 4e, 4h, 4j, 4k (all forms), 4l, and 4m are more active than dodine and at least as active as ketoconazole. They are also highly active against *T. beigelii*, with some compounds being more active than dodine (4h, 4j, 4k-acetate, 4k-sorbate, and 4k-base). Beside these, some other examples of activity comparable to the one of the reference drugs can be found among the results (Table 26).

Regarding the tested azo dye organotin(IV) compounds, another group of studied compounds, some of them were not tested due to precipitation from the test medium. Analyzing the results displayed in Table 27, and comparing the two series of compounds tested, it can be concluded that the alkyl-substituted compounds (series b) are slightly more active than the aryl-substituted compounds (series a). Particularly the closely structurally related compounds 1b and 2b (Table 15) could be highlighted for their marked *in vitro* antifungal activity (Table 27 and Fig. 34).

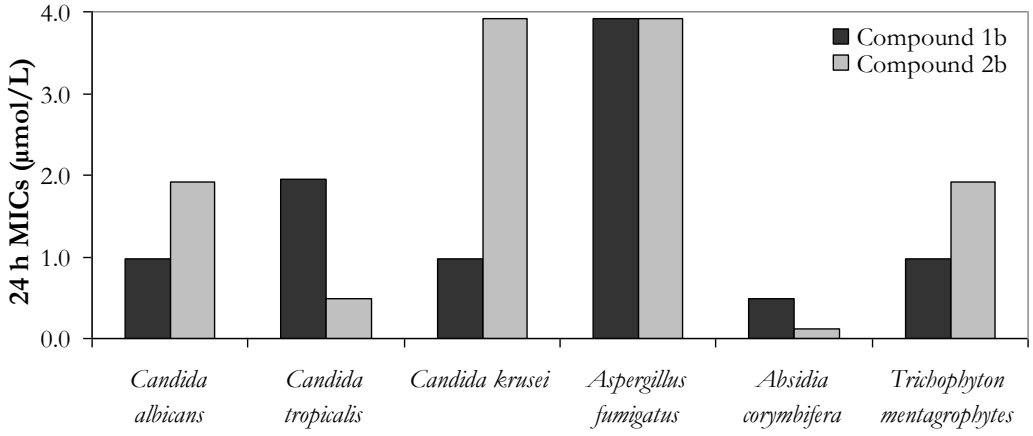


Figure 34 Determined 24 h MICs of azo dye organotin compounds 1b and 2b against some tested yeast and filamentous fungi strains.

Compounds 1b to 6b had been previously shown to yield susceptibility results comparable to the ones of commercial drugs like ketoconazole, fluconazole, and amphotericin B (Růžička *et al.* 2002), but they were less active than the previously published tributyltin(IV) compounds with four-coordinated tin atoms (Růžička *et al.* 2002). On the other hand, the analysis of the determined MFC values suggests that the compounds have a fungistatic, rather than fungicidal, mechanism of action, although in some cases the MFC values are pretty low (Table 27).

In the next group of studied compounds, the quinaldine derivatives, again some compounds were not soluble in the test medium, preventing us to obtain the correspondent susceptibility results. Among the tested compounds, some were found to be inactive, while compounds 2, 4, 10, 11, 12, and 16 showed moderate or higher antifungal activity (Table 28). Concerning structure-activity relationships, the presence of the hydroxylic group, which can be conjugated with the aromatic heterocyclic ring, appears to be essential for the activity (Table 16 and Table 28). Moreover, the presence of the bromine substituent, increasing the compound's lipophilicity, further increases the *in vitro* antifungal activity. This way, compound 10 (with bromine at the position C-7 of the quinaldine group), compound 11 (with the bromine atom at C-5 on the quinaldine group), and compound 12 (the dibromo derivative) were determined to be the most active from this group, yielding low MICs for all tested fungal strains (Table 28 and Fig. 35).

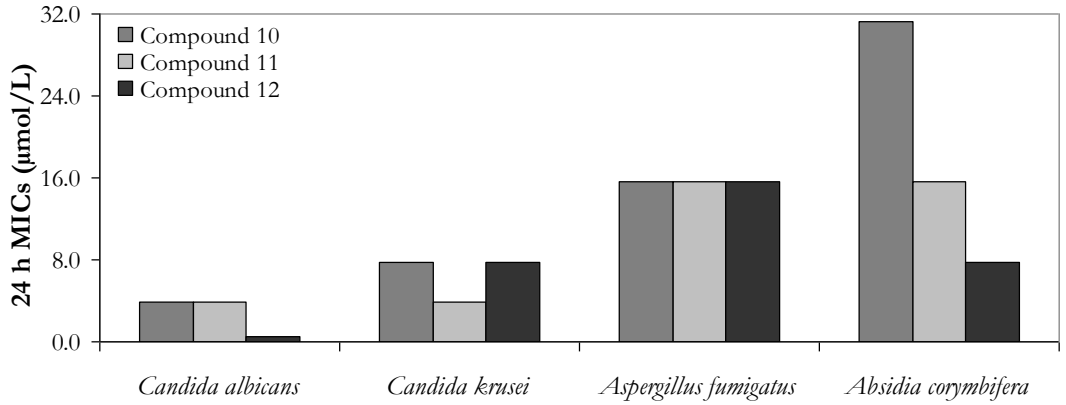


Figure 35 Comparison between the determined 24 h MICs of the most active quinaldine derivatives against three of the tested fungal species.

Note: the value for the MIC of compound 12 against *C. albicans* is in fact not determined precisely, as all the tested concentrations were inhibitory: it can only be concluded that the MIC is equal to or lower than 0.45 µmol/L.

The group of the (*E*)-3-(nitrophenyl)-1-(pyrazin-2-yl)prop-2-en-1-ones, the chalcones, was investigated not only for the presence of antifungal activity but also for antimycobacterial and photosynthesis-inhibiting activity, showing a range of biological activities. Regarding antifungal activity, they displayed moderate effects, particularly against *Candida* spp. and *T. mentagrophytes*. The importance of the presence of electron-withdrawing groups in the B-ring of the chalcones for their antifungal activity has been previously demonstrated by other authors (López *et al.* 2001; Boeck *et al.* 2005). In the specific case of our tested compounds, the influence of substitution on the antifungal activity was less evident. Nevertheless, in most cases the nitro derivatives were more potent than their hydroxylated analogues (Table 17 and Table 29). Besides being active against dermatophytes, like the previously referred chalcones (López *et al.* 2001; Boeck *et al.* 2005), these (*E*)-3-(nitrophenyl)-1-(pyrazin-2-yl)prop-2-en-1-ones showed also moderate activity against the tested *Candida* species. For example, the hydroxylated derivatives 10, 11, and 12 yielded MICs against *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. glabrata* between 7.81 and 125 µmol/L (Table 29). Furthermore, compound 11 showed activity against *T. beigelii* and *A. fumigatus*. The most sensitive species overall, however, was *T. mentagrophytes*. Among the different compounds, the most active were the (*E*)-3-(3-nitrophenyl)-1-(pyrazin-2-yl)prop-2-en-1-ones, compounds 8a, 8d, and 8e. Compound 8a in particular was effective not only against *T. mentagrophytes* but also against *Candida* spp., resulting in MICs between 7.81 and 15.63 µmol/L (Table 29).

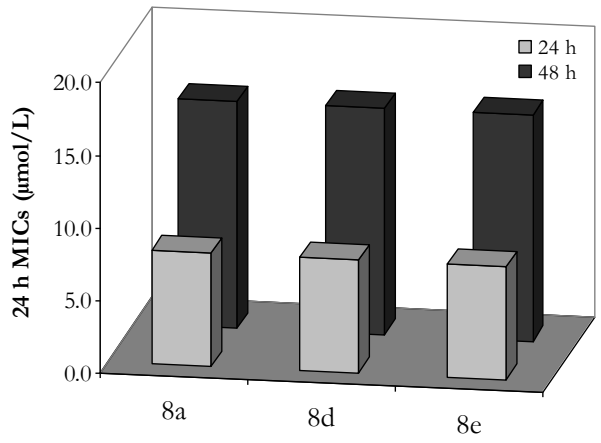


Figure 36 Determined 24 and 48 h MICs of the most active (*E*)-3-(nitrophenyl)-1-(pyrazin-2-yl)prop-2-en-1-ones (derivatives 8a, 8d, and 8e) against *Trichophyton mentagrophytes*.

The results presented in Table 30 clearly show that the thiosalicyanilides (series 2, Table 18) are pronouncedly more active than the correspondent salicylanilides (series 1, Table 18). Among the most active series, series 2, MICs are particularly low against *A. corymbifera* and *T. mentagrophytes* (Fig. 37). Some of the compounds, however, display interesting activity against all tested strains, the best of which are 2c, 2d, and 2e.

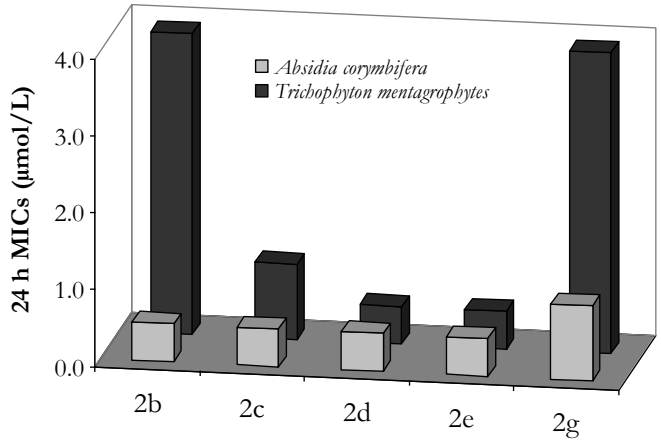


Figure 37 Comparison between the determined 24 h MICs of the most active thiosalicylanilides against *Absidia corymbifera* and *Trichophyton mentagrophytes*.

Note: when the values for the MICs of compounds 2b, 2c, 2d, and 2e are displayed as 0.49 μmol/L they in fact not determined precisely, as this was the lowest tested concentration: it can only be concluded that the MIC is equal to or lower than 0.49 μmol/L.

Another group of compounds studied were the 1-phenyl-5-benzylsulfanyltetrazole derivatives. The antifungal activities of all the tested 19 compounds were negligible, except

against *T. mentagrophytes* and *A. fumigatus* (Table 31). In the cases of these two species, some compounds showed moderate activity, especially regarding the first of them: *T. mentagrophytes*. The most active were the compounds from series 1 (Fig. 38), with a 4-methoxy group on the benzene ring in position 1 of the tetrazole (Table 19).

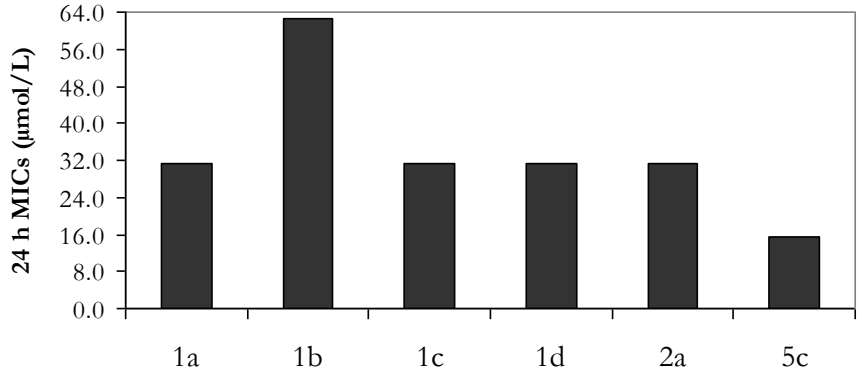


Figure 38 Comparison between the determined 24 h MICs of the most active 1-phenyl-5-benzylsulfanyltetrazole derivatives against *Trichophyton mentagrophytes*.

Among the 13 studied arylsulfanylpyrazine-2-carboxylic acid derivatives, we can find only moderate *in vitro* antifungal activity, with MICs ranging from 31.25 to 500 µmol/L, especially against *T. mentagrophytes* and *C. albicans* (Table 32). The analysis of the structure-activity relationship shows that the position of the methoxy group is very important for the antifungal activity. Importantly, during the tests it was verified that the methoxy group at C-3 on the benzene ring caused a loss of solubility of the compounds in the test medium, resulting in precipitation and, consequently decreased concentration of the compounds in solution. This way, since the real concentration tested is lower than the theoretical concentration, these derivatives showed lower activity than the ones with the methoxy group at C-4. The position of the methoxyphenylsulfanyl moiety at C-6, C-5, or C-3 of the pyrazine nucleus further influences the biological activity, with the C<6 substitution appearing to be the most favourable.

The C-2 substitution was also found to be important, with the presence of a carbonitrile moiety yielding higher activity than the presence of an amide group. Compound 1, showing an ester moiety at C-2, displayed some activity, but it was probably due to the favourable substitutions at C-6 in the pyrazine group and at C-4 in the benzene ring.

Overall, it can be concluded that the most active of the 13 studied arylsulfanylpyrazine-2-carboxylic acid derivatives were 6-[(4-methoxyphenyl)sulfanyl]-pyrazine-2-carbonitrile (compound 2) and 3-[(4-methoxyphenyl)sulfanyl]-pyrazine-2-carbonitrile (com-

pound 10), especially against *C. albicans*, *A. fumigatus*, and *T. mentagrophytes* (Table 32 and Fig. 39).

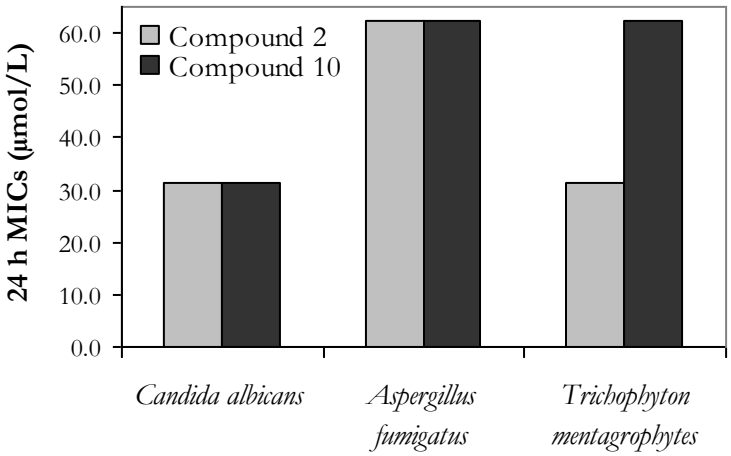


Figure 39 Comparison between the determined 24 h MICs of the most active arylsulfanylpyrazine-2-carboxylic acid derivatives against *Candida albicans*, *Aspergillus fumigatus*, and *Trichophyton mentagrophytes*.

The next group are the ring-substituted (*E*)-3-aryl-1-pyrazin-2-ylprop-2-en-1-ones, a second group of chalcones. These compounds presented serious solubility problems during antifungal susceptibility testing, posing difficulties to the accurate determination of the correspondent MICs. In fact, for compounds 2a, 2b, 2c, 2d, 3c, 4b only two concentrations were tested, in order to obtain at least an estimate of the order of their MICs. Most of the derivatives, however, displayed antifungal activity, especially against *T. mentagrophytes* (Table 33). Some further displayed moderate activity against other species, although not comparable to the one of the reference drug fluconazole.

Based on the results for this group of compounds, no structure-activity relationships can be safely deduced (Table 21 and Table 33). Other reports of antifungal activity found in compounds from this group synthesized at the same laboratory have been frequently produced (Opletalová *et al.* 2002; Opletalová *et al.* 2001; Opletalová *et al.* 2003a; Víchová *et al.* 2003; Jun *et al.* 2003; Chlupáčová *et al.* 2003; Opletalová *et al.* 2003b; Opletalová *et al.* 2004a; Opletal *et al.* 2004; Opletalová *et al.* 2004b). In this particular study, in conclusion, the best derivatives in terms of antifungal activity overall appear to be compounds 1b and 3a (Table 33 and Fig. 40).

The last of the groups of compounds tested referred to in this work is the group of the quinazoline-4-thiones. As stated in the "Results" section (section 4), from both series of tested quinazoline-4-thiones, the 2,2-dimethyl-3-phenyl-1,2-dihydroquinazoline-4(3*H*)-thiones (series

1) and 2-methyl-3-phenylquinazoline-4(3*H*)-thiones (series 2) (Table 22), only one compound showed *in vitro* antifungal activity.

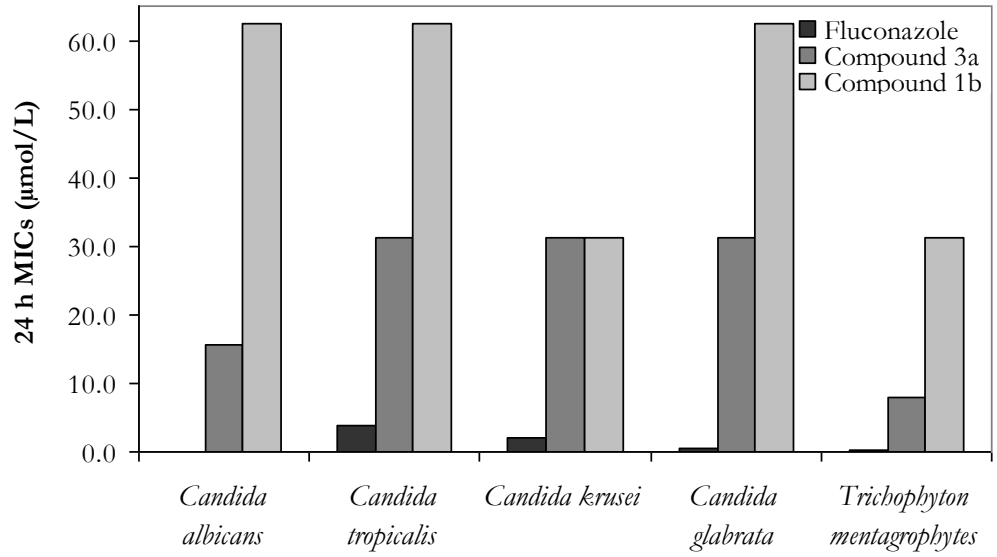


Figure 40 Comparison between the determined 24 h MICs of the most active ring substituted (E)-3-aryl-1-pyrazin-2-ylprop-2-en-1-ones against the four tested *Candida* species and *Trichophyton mentagrophytes*.

Note: the value for the MIC of fluconazole against *Candida albicans* was equal to or lower than 0.06 µmol/L (this was the lowest tested concentration).

Concretely, the active compound was derivative 1h, showing inhibitory activity against *A. fumigatus* and *A. corymbifera* (Fig. 41). However, the compounds displayed other biological activities, concretely, antimycobacterial activity, photosynthesis-inhibiting activity in spinach chloroplasts, and reduction of chlorophyll content in the green algae *Chlorella vulgaris* (Kubicová *et al.* 2003). Besides that, the active compounds were in majority not toxic according to a toxicological screening bioassay using the brine shrimp larvae (*Artemia salina*) (Kubicová *et al.* 2003). Further studies could, in the future, yield more active compounds from this group.

A global analysis of our complete results shows a number of chemical groups displaying a potential for antifungal activity. *In vitro* activity has been demonstrated and the justification exists for further studies to be pursued, either by refining the biological activity through structure-activity-oriented synthesis of more derivatives or by entering deeper studies in their development. The group of the incrustoporin derivatives is definitely the most important of all and has already been taken a step further, with our studies regarding the preliminary investigation of their mechanism of antifungal activity. Susceptibility results for a broad spectrum of fungal species are now available, some information regarding mechanisms of action was gath-

ered, and some introductory toxicological studies were carried out, including both cytostatic activity assays (on animal and human cell lines) and animal toxicity assays on mice (Buchta *et al.* 2004; Vale-Silva *et al* 2006). The next steps in the development of these agents shall be the

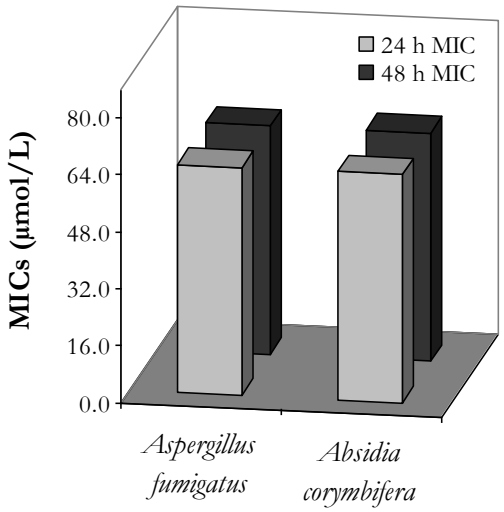


Figure 41 Determined 24 and 48 h MICs of the quinazoline-4-thione 1h against *Aspergillus fumigatus* and *Absidia corymbifera*.

deepening of the knowledge on their mechanism of action and toxicological profile. Besides that, further studies involving testing on animal models of mycotic infections are also being planned. Concretely, experiments involving mice models of invasive aspergillosis will be carried over in the future.

Among the remaining compounds tested the most active appear to come from the groups of the quinolines, the 4-substituted phenylguanidinium salts, the azo dye organotin(IV) compounds, and the quinaldine derivatives. These are the groups showing the highest *in vitro* antifungal activity, although the remaining groups could, with further studies, still yield more active compounds. In any case, however, there is no information regarding the *in vitro-in vivo* correlation of activity and no data is available to sustain a high potential for *in vivo* antifungal activity for these compounds, the incrustoporin derivatives included.

The way for the development of all these agents includes, right away, the refinement of the biological activity through the use of structure-antifungal activity relationship analysis to guide semi-synthetic modifications on the best of the already tested derivatives. Subsequently, a broadening of the number of fungal species and strains tested will be necessary and the study of the correspondent mechanisms of action will also be favourable, a stage the group of incrustoporin derivatives has already reached. The successful agents reaching this phase are then selected to complete the set of preclinical studies, including toxicological studies (cy-

tostatic activity using animal and human cell lines and animal toxicity tests), the study of the antifungal potential in animal models of fungal infection, and the study of the pharmacokinetic and pharmacodinamic profiles. Should all these tests and correspondent barriers be overcome, a successful compound could enter clinical trials, establishment of its real clinical value, appropriate dosage and formulation for administration and concomitant registration of detected side effects. It is obviously an extremely long and serious process a very limited number of all potential drugs ever complete.

6 CONCLUSIONS

In the context of Medical Mycology today, the development of new more effective antifungal agents is a priority. In fact, there are still only about six drugs in use to treat invasive fungal infections, concretely the polyene amphotericin B (including its new lipidic formulations), the antimetabolite flucytosine, the triazoles fluconazole, itraconazole, and voriconazole, and the echinocandin caspofungin. Simultaneously, the incidence of invasive opportunistic mycoses has been increasing steadily with the increasing number of immunocompromised patients, caused both by HIV (human immunodeficiency virus) infection and AIDS and the development of medical techniques, particularly referring to oncology or transplant patients.

In this setting, the relevance of the development of new antifungal agents and, hence, our work presented here, is easily understandable. The group of the acyloxymethylated incrustoporin derivatives is now in a higher stage of development, after being previously studied concerning structure-antifungal activity relationship and tuning of antifungal activity. Our work has shown the best derivatives from the group, compounds LNO6-22, LNO15-22, and LNO18-22, to have broad spectrum *in vitro* antifungal activity and high potency, inhibiting growth of a variety of pathogenic yeasts and moulds at very low concentrations, including, importantly, fluconazole-resistant strains. Although the differences in activity between the three compounds are quite small, it has also been concluded that LNO18-22 is the most potent of the three. Interestingly, however, the study of the inhibition of filamentation in *C. albicans* at subinhibitory concentrations has shown LNO6-22 to be the strongest inhibitor, yielding results comparable to the ones produced by amphotericin B. Besides that, the flow cytometric studies of LNO18-22 have shown this compound to have a potent fungicidal activity against *C. albicans*. It was found to cause plasmatic membrane depolarization, metabolic activity arrest, and, ultimately, disruption of the plasmatic membrane integrity, either by a direct interaction or by secondary consequences of metabolic impairment, leading to ion channel or pore formation. Simultaneously, the results with the reference drugs amphotericin B and fluconazole confirmed the expected results from the knowledge of their mechanisms of antifungal action.

Regarding the remaining 10 chemical groups of compounds tested, four could be highlighted for producing better results, including compounds with high activity against some of the eight test fungal strains. Concretely, these groups were the quinoline derivatives, the 4-substituted phenylguanidinium salts, the azo dye organotin(IV) compounds, and the quinaldine derivatives, yielding a group of about 10 to 15 compounds overall with very promising

potency. The remaining of the chosen groups showed usually moderate activity, justifying their presence in this report by the potential to yield, after tuning of their antifungal activity, more potent derivatives than the ones obtained so far.

In conclusion, the incrustoporin derivatives are the most promising class of agents, justifying a serious investment in further development. The remaining groups include a series of compounds that require further work in cooperation with the organic chemistry laboratory in order to produce agents with firmer potential.

7 LITERATURE

1. Abi-Said, D.; Anaissie, E.; Uzun, O.; Raad, I.; Pinzcowski, H.; Vartivarian, S. The epidemiology of haematogenous candidiasis caused by different *Candida* species. *Clin Infect Dis.* 1997, 24, 1122-1128.
2. Anaissie E. J.; Bodey G. P. Nosocomial fungal infections – old problems and new challenges. *Infect Dis Clin North Am.* 1989, 3, 867–882.
3. Arendrup, M.; Lundgren, B.; Jensen, I. M.; Hansen, B. S.; Frimodt-Moller, N. Comparison of Etest and a tablet diffusion test with the NCCLS broth microdilution method for fluconazole and amphotericin B susceptibility testing of *Candida* isolates. *J Antimicrob Chemother.* 2001, 47, 521-526.
4. Arthington-Skaggs, B. A.; Jradi, H.; Desai, T.; Morrison, C. J. Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. *J Clin Microbiol.* 1999, 37, 3332-3337.
5. Arthington-Skaggs, B. A.; Warnock, D. W.; Morrison, C. J. Quantitation of *Candida albicans* ergosterol content improves the correlation between *in vitro* antifungal susceptibility test results and *in vivo* outcome after fluconazole treatment in a murine model of invasive candidiasis. *Antimicrob Agents Chemother.* 2000, 44, 2081-2085.
6. Barchiesi, F.; Colombo, A. L.; McGough, D. A.; Rinaldi, M. G. Comparative study of broth macrodilution and microdilution techniques for *in vitro* antifungal susceptibility testing of yeasts by using the National Committee for Clinical Laboratory Standards' proposed standard. *J Clin Microbiol.* 1994, 32, 2494-2500.
7. Barrett, J. P.; Vardulaki, K. A.; Conlon, C.; Cooke, J.; Daza-Ramirez, P.; Evans, E. G.; Hawkey, P. M.; Herbrecht, R.; Marks, D. I.; Moraleda, J. M.; Park, G. R.; Senn, S. J.; Viscoli, C.; Amphotericin B Systematic Review Study Group. A systematic review of the antifungal effectiveness and tolerability of amphotericin B formulations. *Clin Ther.* 2003, 25, 1295-1320.
8. Barry, A. L.; Pfaller, M. A.; Rennie, R. P.; Fuchs, P. C.; Brown, S. D. Precision and accuracy of fluconazole susceptibility testing by broth microdilution, Etest, and disk diffusion methods. *Antimicrob Agents Chemother.* 2002, 46, 1781-1784.
9. Barwicz, J.; Gruda, I.; Tancrede, P. A kinetic study of the oxidation effects of amphotericin B on human low-density lipoproteins. *FEBS Lett.* 2000, 465, 83-86.
10. Beck-Sague, C. and Jarvis, W. R. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. National Nosocomial Infections Surveillance System. *J Infect Dis.* 1993, 167, 1247-1251.
11. Bodey, G. P. Azole antifungal agents. *Clin. Infect. Dis.* 1992, 14 (Suppl. 1), S161–S169.
12. Boeck, P.; Leal, P. C.; Yunes, R. A.; Filho, V. C.; López, S.; Sortino, M.; Escalante, A.; Furlán, R. L.; Zacchino, S. Antifungal activity and studies on mode of action of novel xanthoxyline-derived chalcones. *Arch Pharm (Weinheim)* 2005, 338, 87-95.
13. Borgers, M.; De Brabander, M.; Van Den Bossche, H.; Van Cutsem, J. Promotion of pseudomycelium formation of Candida albicans in culture: a morphological study of the effects of miconazole and ketoconazole. *Postgrad Med J.* 1979, 55, 687-691.
14. Bowman, J. C.; Hicks, P. S.; Kurtz, M. B.; *et al.* The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus in vitro*. *Antimicrob Agents Chemother.* 2002, 46, 3001–3012.

15. Brajtburg, J. and Bolard, J. Carrier effects on biological activity of amphotericin B. *Clin Microbiol Rev.* 1996, 9, 512-531.

16. Brajtburg, J.; Powderly, W. G.; Kobayashi, G. S.; Medoff, G. Amphotericin B: current understanding of mechanisms of action. *Antimicrob Agents Chemother.* 1990, 34, 183-188.

17. Brajtburg, J.K. *et al.* The molecular basis for the selective toxicity of amphotericin B for yeast and filipin for animal oils. *Antimicrob Agents Chemother.* 1974, 5, 377–382.

18. Brown, J. Zygomycosis: an emerging fungal infection. *Am J Health Syst Pharm.* 2005, 62, 2593-2596.

19. Buchta, V.; Pour, M.; Kubanová, P.; Silva, L.; Votruba, I.; Vopršálová, M.; Schiller, R.; Fáková, H.; Špulák, M. *In vitro* antifungal activities of 3-(halogenated phenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-ones against common and emerging yeasts and molds. *Antimicrob. Agents Chemother.* 2004, 48, 873-878.

20. Cagnoni, P. J.; Walsh, T. J.; Prendergast, M. M.; Bodensteiner, D.; Hiemenz, S.; Greenberg, R. N.; Arndt, C. A.; Schuster, M.; Seibel, N.; Yeldandi, V.; Tong, K. B. Pharmacoeconomic analysis of liposomal amphotericin B versus conventional amphotericin B in the empirical treatment of persistently febrile neutropenic patients. *J Clin Oncol.* 2000, 18, 2476-2483.

21. Chayakulkeeree, M.; Ghannoum, M. A.; Perfect, J. R. Zygomycosis: the re-emerging fungal infection. *Eur J Clin Microbiol Infect Dis.* 2006 (Epub ahead of print).

22. Chlupáčová, M.; Kubanová, P.; Opletalová, V.; Buchta V. Book of Abstracts of the 3rd International Symposium on Natural Drugs, Naples, October 2 – 4, 2003, p. 153.

23. Chryssanthou, E. Trends in antifungal susceptibility among Swedish *Candida* species bloodstream isolates from 1994 to 1998: comparison of the E-test and the Sensititre YeastOne Colorimetric Antifungal Panel with the NCCLS M27-A reference method. *J Clin Microbiol.* 2001, 39, 4181-4183.

24. Chryssanthou, E.; Cuenca-Estrella, M. Comparison of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing proposed standard and the E-test with the NCCLS broth microdilution method for voriconazole and caspofungin susceptibility testing of yeast species. *J Clin Microbiol.* 2002, 40, 3841-3844.

25. Cuenca-Estrella, M.; Lee-Yang, W.; Ciblak, M. A.; *et al.* Comparative evaluation of NCCLS M27-A and EUCAST broth microdilution procedures for antifungal susceptibility testing of *Candida* species. *Antimicrob Agents Chemother.* 2002, 46, 3644-3647.

26. Cuenca-Estrella, M.; Moore, C. B.; Barchiesi, F.; *et al.* Multicenter evaluation of the reproducibility of the proposed antifungal susceptibility testing method for fermentative yeasts of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST). *Clin Microbiol Infect.* 2003, 9, 467-474.

27. Decker, H.; Walz, F.; Borman, C.;Fiedler, H. P.; Zahner, H.; Heitsch, H. H.; Konig, W. A. Metabolic products of microorganisms. 255. Nikkomycins Wz and Wx, new chitin synthetase inhibitors from *Streptomyces tendae*. *J. Antibiot.* 1990, 43, 43–48.

28. Del Poeta, M.; Schell, W. A.; Dykstra, C. C.; Jones, S.K.; Tidwell, R. R.; Kumar, A.; Boykin, D. W.; Perfect, J. R. *In vitro* antifungal activities of a series of dication-substituted carbazoles, furans, and benzimidazoles. *Antimicrob Agents Chemother.* 1998, 42, 2503-2510.

29. Denning, D. W. Echinocandins and pneumocandins: a new antifungal class with a novel mode of action. *J Antimicrob Chemother.* 1997, 40, 611-614.

30. Develoux, M. Griseofulvin. *Ann Dermatol Veneréol.* 2001, 128, 1317-1325.

31. Doctor Fungus [Homepage on the internet]. Introduction to fungi. The fungi. New York, NY, USA, 2005a. http://www.doctorfungus.org/thefungi/index.htm

32. Doctor Fungus [Homepage on the internet]. Taxonomy and nomenclature. The Fungi. New York, NY, USA, 2005b. http://www.doctorfungus.org/thefungi/nomenclature.htm

33. Dominguez, J. M. and Martin, J. J. Identification of elongation factor 2 as the essential protein targeted by sordarins in *Candida albicans*. *Antimicrob Agents Chemother.* 1998, 42, 2279–2283.

34. Edmond, M. B.; Wallace, S. E.; McClish, D. K.; Pfaller, M. A.; Jones, R. N.; Wenzel, R. P. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin Infect Dis.* 1999, 29, 239-244.

35. Eldere, J. V.; Joosten, L.; Verhaeghe, V.; Surmont, I. Fluconazole and amphotericin B antifungal susceptibility testing by National Committee for Clinical Laboratory Standards broth macrodilution method compared with E-test and semiautomated broth microdilution test. *J Clin Microbiol.* 1996, 34, 842-847.

36. Ellepola, A. N.; Samaranayake, L. P. The effect of limited exposure to antifungal agents on the germ tube formation of oral *Candida albicans*. *J Oral Pathol Med.* 1998, 27, 213-219.

37. Espinel-Ingroff, A.; Barchiesi, F.; Cuenca-Estrella, M.; *et al.* International and multicenter comparison of EUCAST and CLSI M27-A2 broth microdilution methods for testing susceptibilities of *Candida* spp. to fluconazole, itraconazole, posaconazole, and voriconazole. *J Clin Microbiol.* 2005, 43, 3884-3889.

38. Espinel-Ingroff, A.; Bartlett, M.; Bowden, R.; *et al.* Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. *J Clin Microbiol.* 1997, 35, 139-143.

39. Espinel-Ingroff, A.; Boyle, K.; Sheehan, D. J. *In vitro* antifungal activities of voriconazole and reference agents as determined by NCCLS methods: review of the literature. *Mycopathologia.* 2001, 150, 101-115.

40. Espinel-Ingroff, A.; Kish Jr., C. W.; Kerkering. T. M.; *et al.* Collaborative comparison of broth macrodilution and microdilution antifungal susceptibility tests. *J Clin Microbiol.* 1992, 30, 3138-3145.

41. Espinel-Ingroff, A.; Rodriguez-Tudela, J. L.; Martinez-Suarez, J. V. Comparison of two alternative microdilution procedures with the National Committee for Clinical Laboratory Standards reference macrodilution method M27-P for *in vitro* testing of fluconazole-resistant and -susceptible isolates of *Candida albicans*. *J Clin Microbiol.* 1995, 33, 3154-3158.

42. Espinel-Ingroff, A; Kerkering, T. M.; Goldson, P. R.; Shadomy, S. Comparison study of broth macrodilution and microdilution antifungal susceptibility tests. *J Clin Microbiol.* 1991, 29, 1089-1094.

43. European Committee on Antibiotic Susceptibility Testing. Method for determination of minimal inhibitory concentration (MIC) by broth dilution of fermentative yeasts. Discussion document E. Dis. 7.1. Taufkirchen, Germany, 2002.

44. Fridkin, S. K. and Jarvis, W. R. Epidemiology of nosocomial fungal infections. *Clin Microbiol Rev.* 1996, 9, 499-511.

45. Fridkin, S. K. The changing face of fungal infections in health care settings. *Clin Infect Dis.* 2005, 41, 1455-1460.

46. Fromtling R. A. Overview of medically important antifungal azole derivatives. *Clin Microbiol Rev.* 1988, 1, 187–219.

47. Gallis, H. A.; Drew, R. H.; Pickard, W. W. Amphotericin B: 30 years of clinical experience. *Rev Infect Dis.* 1990, 12, 308-329.

48. Gamard, P.; Sauriol, F.; Benhamou, N.; Belanger, R. R.; Paulitz, T. C. Novel butyrolactones with antifungal activity produced by *Pseudomonas aureofaciens* strain 63-28. *J Antibiot* (Tokyo). 1997, 50, 742-749.

49. Georgopapadakou, N. H. and Walsh, T. J. Antifungal agents: chemotherapeutic targets and immunologic strategies. *Antimicrob Agents Chemother.* 1996, 40, 279-291.

50. Ghannoum, M. A. and Rice, L. B. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin Microbiol Rev.* 1999, 12, 501-517.

51. Gow, N. A.; Brown, A. J.; Odds, F. C. Fungal morphogenesis and host invasion. *Curr Opin Microbiol.* 2002, 5, 366-371.

52. Greenberg, R.N.; Mullane, K.: van Burik, J. A.; Raad, I.; Abzug, M. J.; Anstead, G.; Herbrecht, R.; Langston, A.; Marr, K. A.; Schiller, G.; Schuster, M.; Wingard, J. R.; Gonzalez, C. E.; Revankar, S. G.; Corcoran, G.; Kryscio, R. J.; Hare, R. Posaconazole as salvage therapy for zygomycosis. *Antimicrob Agents Chemother.* 2006, 50, 126-133.

53. Guarro, J.; Gené, J.; Stchigel, A. M. Developments in fungal taxonomy. *Clin Microbiol Rev.* 1999, 12, 454-500.

54. Gull, K. and Trinci, A. P. J. Griseofulvin inhibits fungal mitosis. *Nature.* 1973, 244, 292-294.

55. Hart, R.; Bell-Syer, S. E.; Crawford, F.; Torgerson, D. J.; Young, P.; Russell, I. Systematic review of topical treatments for fungal infections of the skin and nails of the feet. *BMJ.* 1999, 319, 79-82.

56. Hazen, E. L. and Brown, R. Fungicidin, antibiotic produced by soil actinomycete. *Proc Soc Exp Biol Med.* 1951, 76, 93-97.

57. Hector, R. F. Compounds active against cell walls of medically important fungi. *Clin Microbiol Rev.* 1993, 6, 1-21.

58. Iwatani, W.; Arika, T.; Yamaguchi, H. Two mechanisms of butenafine action in *Candida albicans*. *Antimicrob Agents Chemother.* 1993, 37, 785-788.

59. Jahn, B.; Rampp, A.; Dick, C.; Jahn, A.; Palmer, M.; Bhakdi, S. Accumulation of amphotericin B in human macrophages enhances activity against *Aspergillus fumigatus* conidia: quantification of conidial kill at the single cell level. *Antimicrob Agents Chemother.* 1998, 42, 2569-2575.

60. Jullien, S.; Capuozzo, E.; Salerno, C.; Crifo, C. Effects of polyene antibiotics on the activation of human polymorphonuclear leukocytes. *Biochem Pharmacol.* 1991, 41, 2037-2040.

61. Jun, D.; Chlupáčová, M.; Opletalová, V.; Hronek, M., Opletal, L. Book of Abstracts of the 3rd International Symposium on Natural Drugs, Naples, October 2 – 4, 2003, p. 152.

62. Kazmaier, U.; Pahler, S.; Endermann, R.; Habich, D.; Kroll, H. P.; Riedl, B. Straightforward syntheses of furanomycin derivatives and their biological evaluation. *Bioorg Med Chem.* 2002, 10, 3905-3913.

63. Khan, M. S. and Husain, A. Syntheses and reactions of some new 2-arylidene-4-(biphenyl-4-yl)-but-3-en-4-olides with a study of their biological activity. *Pharmazie* 2002, 57, 448-452.

64. Kirkpatrick, W. R.; Perea, S.; Coco, B, J.; Patterson, T. F. Efficacy of ravuconazole (BMS-207147) in a guinea pig model of disseminated aspergillosis. *J Antimicrob Chemother.* 2002, 49, 353–357.

65. Kirschbaum, J.; Kahn, S: G. Micelle size and stability of aqueous amphotericin B (polyene) systems. *J Pharm Sci.* 1967, 56, 278–279.

66. Koc, A. N.; Gokahmetoglu, S.; Oguzkaya, M. Comparison of Etest with the broth microdilution method in susceptibility testing of yeast isolates against four antifungals. Mycoses 2000, 43, 293-297.

67. Kontoyiannis, D. P. A clinical perspective for the management of invasive fungal infections: focus on IDSA guidelines. *Pharmacotherapy.* 2001, 21, 175S-187S.

68. Krause, D. S.; Reinhardt, J.; Vazquez, J. A.; *et al.* Phase 2, randomized, dose-ranging study evaluating the safety and efficacy of anidulafungin in invasive candidiasis and candidemia. *Antimicrob Agents Chemother.* 2004, 48, 2021–24.

69. Krčmery, V. Jr. Antifungal chemotherapeutics. *Med Princ Pract.* 2005, 14, 125-35.

70. Kretschmar, M.; Hube, B.; Bertsch, T.; Sanglard, D.; Merker, R.; Schröder, M.; Hof, H.; Nichterlein, T. Germ tubes and proteinase activity contribute to virulence of *Candida albicans* in murine peritonitis. *Infect Immun.* 1999, 67, 6637–6642.

71. Kubicová, L.; Šustr, M.; Králová, K.; Chobot, V.; Vytlačilová, J.; Buchta, V.; Silva, L.; Jahodář, L.; Vuorela, P.; Macháček, M.; Kuneš, J.; Kaustová, J. Quinazoline-4-thiones: synthesis, biological activity, and toxicological screening. Proceedings of ECSOC-7, The Seventh International Electronic Conference on Synthetic Organic Chemistry, ecsoc-7.htm; September 1-30, 2003 Section C. Bioorganic chemistry and natural products. Article C003. (http://www.mdpi.net/)

72. Lemke A.; Kiderlen A. F.; Kayser O. Amphotericin B. *Appl Microbiol Biotechnol.* 2005, 68, 151-162.

73. Letscher-Bru, V. and Herbrecht, R. Caspofungin: the first representative of a new antifungal class. *J Antimicrob Chemother.* 2003, 51, 513–521.

74. Lewis, R. E. and Klepser, M. E. The changing face of nosocomial candidemia: epidemiology, resistance, and drug therapy. *Am J Health Syst Pharm.* 1999, 56, 525-533.

75. Lo, H. J.; Köhler, J. R.; Didomenico, B.; Loebenberg, D.; Cacciapuoti, A.; Fink, G. R. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* 1997, 90, 939–949.

76. López, S. N.; Castelli, M. V.; Zacchino, S. A.; Domínguez, J. N.; Lobo, G.; Charris-Charris, J.; Cortês, J. C.; Ribas, J. C.; Devia, C.; Rodríguez, A. M.; Enriz, R. D. *In vitro* antifungal evaluation and structure-activity relationships of a new series of chalcone derivatives and synthetic analogues, with inhibitory properties against polymers of the fungal cell wall. *Bioorg Med Chem.* 2001, 9, 1999-2013.

77. Lozano-Chiu, M.; Paetznick, V. L.; Ghannoum, M. A.; Rex, J. H. Detection of resistance to amphotericin B among *Cryptococcus neoformans* clinical isolates: performances of three different media assessed by using E-test and National Committee for Clinical Laboratory Standards M27-A methodologies. *J Clin Microbiol.* 1998, 36, 2817-2822.

78. Marichal, P.; Gorrens, J.; Van Cutsem, J.; Vanden Bossche, H. Culture media for the study of the effects of azole derivatives on germ tube formation and hyphal growth of *C. albicans*. *Mykosen* 1986, 29, 76–81.

79. Marr, K. A.; Patterson, T.; Denning, D. Aspergillosis. Pathogenesis, clinical manifestations, and therapy. *Infect Dis Clin North Am.* 2002, 16, 875-894.

80. Martin, M. L.; San Roman, L.; Dominguez, A. *In vitro* activity of protoanemonin, an antifungal agent. *Planta Med.* 1990, 56, 66-69.

81. Martin-Mazuelos, E.; Gutierrez, M. J.; Aller, A. I.; *et al.* A comparative evaluation of Etest and broth microdilution methods for fluconazole and itraconazole susceptibility testing of *Candida* spp. *J Antimicrob Chemother.* 1999, 43, 477-481.

82. Matar, M. J.; Ostrosky-Zeichner, L.; Paetznick, V. L.; *et al.* Correlation between E-test, disk diffusion, and microdilution methods for antifungal susceptibility testing of fluconazole and voriconazole. *Antimicrob Agents Chemother.* 2003, 47, 1647-1651.

83. Maxwell, M. J.; Messer, S. A.; Hollis, R. J.; Diekema, D. J.; Pfaller, M. A. Evaluation of Etest method for determining voriconazole and amphotericin B MICs for 162 clinical isolates of *Cryptococcus neoformans*. *J Clin Microbiol.* 2003b, 41, 97-99.

84. Maxwell, M. J.; Messer, S. A.; Hollis, R. J.; *et al.* Evaluation of Etest method for determining fluconazole and voriconazole MICs for 279 clinical isolates of *Candida* species infrequently isolated from blood. *J Clin Microbiol.* 2003a, 41, 1087-1090.

85. Mercurio, M. G. and Elewski, B. Therapy of sporotrichosis. *Semin Dermatology.* 1993, 12, 285.

86. Mitchell, A. P. Dimorphism and virulence in *Candida albicans*. *Curr Opin Microbiol.* 1998, 1, 687–692.

87. Morace, G.; Amato, G.; Bistoni, F.; *et al.* Multicenter comparative evaluation of six commercial systems and the national committee for clinical laboratory standards M27-A broth microdilution method for fluconazole susceptibility testing of *Candida* species. *J Clin Microbiol.* 2002, 40, 2953-2958.

88. Nagata, K.; Hirai, K. I.; Koyama, J.; Wada, Y.; Tamura, T. Antimicrobial activity of novel furanonaphthoquinone analogs. *Antimicrob Agents Chemother.* 1998, 42, 700-702.

89. National Committee for Clinical Laboratory Standards. Method for antifungal disk diffusion susceptibility testing of yeasts; approved guideline. NCCLS document M44-A. Wayne, PA, USA, 2004.

90. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard. NCCLS document M27-A2. Wayne, PA, USA, 2002a.

91. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi. Approved standard M38-A. Wayne, Pa, USA, 2002b.

92. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard NCCLS document M27-A. Wayne, Pa, USA, 1997.

93. Navarro-García, F.; Sánchez, M.; Nombela, C.; Pla, J. Virulence genes in the pathogenic yeast *Candida albicans*. *FEMS Microbiol Rev.* 2001, 25, 245–268.

94. Neely, M. N.; Ghannoum, M. A. The exciting future of antifungal therapy. *Eur J Clin Microbiol Infect Dis.* 2000, 19, 897-914.

95. Nguyen, M. H.; Peacock, J. E. Jr.; Morris, A. J.; Tanner, D. C.; Nguyen, M. L.; Snydman, D. R.; Wagener, M. M.; Rinaldi, M. G.; Yu, V. L. The changing face of candidemia: emergence of non-*Candida albicans* species and antifungal resistance. *Am J Med.* 1996, 100, 617-623.

96. Nucci, M. Emerging moulds: *Fusarium*, *Scedosporium* and Zygomycetes in transplant recipients. *Curr Opin Infect Dis.* 2003, 16, 607-612.

97. Nucci, M.; Marr, K. A.; Queiroz-Telles, F.; *et al. Fusarium* infection in hematopoietic stem cell transplant recipients. *Clin Infect Dis.* 2004, 38, 1237-1242.

98. Odds, F. C. Antifungal agents: their diversity and increasing sophistication. *Mycologist* 2003, 17, 51-55.

99. Odds, F. C.; Brown, A. J. P; Gow, N. A. R. Antifungal agents: mechanisms of action. *Trends Microbiol.* 2003, 11, 272-279.

100. Opletal, L.; Jun, D.; Opletalová, V.; Chlupáčová, M.; Hronek, M.; Kuča, K. Book of Abstracts of the Joint Meeting of the Czech Pharmaceutical Society, German Pharmaceutical Society and Austrian Pharmaceutical Society, Regensburg, October 6 – 9, 2004, p. 121.

101. Opletalová, V.; Chlupáčová, M.; Buchta, V.; Silva, L. Book of Abstracts of the 3rd International Symposium on Natural Drugs, Naples, October 2 – 4, 2003, p. 154.

102. Opletalová, V.; Chlupáčová, M.; Buchta, V.; Silva, L. *Natural Drugs 2003. Proc. 3rd Int. Symp., Naples, October 2-4, 2003* (F. Borelli, F. Capasso, N. Milic and A. Russo, Eds), p. 259. Universita degli Studi di Napoli Federico II, Naples and Indena, Milano, 2005.

103. Opletalová, V.; Chlupáčová, M.; Posledníková, M.; Kuneš, J.; Silva, L.; Buchta, V.; Jun, D. Book of Abstracts of the Joint Meeting of the Czech Pharmaceutical Society, German Pharmaceutical Society and Austrian Pharmaceutical Society, Regensburg, October 6 – 9, 2004, p. 121.

104. Opletalová, V.; Chlupáčová, M.; Posledníková, M.; Silva, L.; Buchta, V.; Kráľová, K. Book of Abstracts of the 33rd Conference on Synthesis and Analysis of Drugs, Nitra, September 9 –10, 2004. In: Farm. Obzor 73, 2004, 250.

105. Opletalová, V.; Hartl, J.; Buchta, V.; Kráľová, K. Book of Abstracts of the 30th Conference on Synthesis and Analysis of Drugs, Brno, September 17 – 19, 2001, p. 94.

106. Opletalová, V.; Hartl, J.; Patel, A.; Palát, K. Jr.; Buchta, V. Ring substituted 3-phenyl-1-(2-pyrazinyl)-2-propen-1-ones as potential photosynthesis-inhibiting, antifungal and antimycobacterial agents. *Farmaco* 2002, 57, 135-144.

107. Opletalová, V.; Hartl, J.; Patel, A.; Palát, K. Jr.; Buchta, V. Ring substituted 3-phenyl-1-(2-pyrazinyl)-2-propen-1-ones as potential photosynthesis-inhibiting, antifungal and antimycobacterial agents. *Farmaco* 2002, 57, 135-144.

108. Opletalová, V.; Palát, K. Jr.; Kastner, P. Book of Abstracts of the 32nd Conference Synthesis and Analysis of Drugs, Velké Karlovice, September 16 – 19, 2003, p. 76.

109. Ostrosky-Zeichner, L.; Marr, K. A.; Rex, J. H.; Cohen, S. H. Amphotericin B: time for a new "gold standard". *Clin Infect Dis.* 2003, 37, 415-425.

110. Pappas, P. G.; Rex, J. H.; Sobel, J. D.; *et al.* Guidelines for treatment of candidiasis. *Clin Infect Dis.* 2004, 38, 161–189.

111. Patel, R. Antifungal agents. Part I. Amphotericin B preparations and flucytosine. *Mayo Clin Proc.* 1998, 73, 1205-1225.

112. Patterson, T. F. Advances and challenges in management of invasive mycoses. *Lancet* 2005, 366, 1013-1025.

113. Petraitis, V.; Petraitiene, R.; Sarafandi, A. A.; Kelaher, A. M.; Lyman, C. A.; Casler, H. E.; Sein, T.; Groll, A. H.; Bacher, J.; Avila, N. A.; Walsh, T. J. Combination therapy in treatment of experimental pulmonary aspergillosis: synergistic interaction between an antifungal triazole and an echinocandin. *J Infect Dis.* 2003, 187, 1834–1843.

114. Peyron, F.; Favel, A.; Michel-Nguyen, A.; *et al.* Improved detection of amphotericin B-resistant isolates of *Candida lusitaniae* by Etest. *J Clin Microbiol.* 2001, 39, 339-342.

115. Pfaller, M. A. and Diekema, D. J. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus funigatus*. *J Clin Microbiol.* 2004, 42, 4419-4431.

116. Pfaller, M. A. Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission. *Clin Infect Dis.* 1996, 22, S89-94.

117. Pfaller, M. A.; Jones, R. N.; Doern, G. V.; Sader, H. S.; Hollis, R. J.; Messer, S. A. International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and antifungal susceptibilities of isolates collected in 1997 in the United States, Canada, and South America for the SENTRY Program. The SENTRY Participant Group. *J Clin Microbiol.* 1998a, 36, 1886-1889.

118. Pfaller, M. A.; Jones, R. N.; Doern, G. V.; Sader, H. S.; Messer, S. A.; Houston, A.; Coffman, S.; Hollis, R. J. Bloodstream infections due to *Candida* species: SENTRY antimicrobial surveillance program in North America and Latin America, 1997-1998. *Antimicrob Agents Chemother.* 2000, 44, 747-751.

119. Pfaller, M. A.; Jones, R. N.; Messer, S. A.: Edmond, M. B.; Wenzel, R. P. National surveillance of nosocomial blood stream infection due to species of *Candida* other than *Candida albicans*: frequency of occurrence and antifungal susceptibility in the SCOPE Program. SCOPE Participant Group. Surveillance and Control of Pathogens of Epidemiologic. *Diagn Microbiol Infect Dis.* 1998b, 30, 121-129.

120. Pfaller, M. A.; Messer, S. A.; Bolmstrom, A. Evaluation of Etest for determining *in vitro* susceptibility of yeast isolates to amphotericin B. *Diagn Microbiol Infect Dis*. 1998; 32: 223-227.

121. Pfaller, M. A.; Messer, S. A.; Boyken, L.; *et al.* Evaluation of the NCCLS M44-P disk diffusion method for determining susceptibilities of 276 clinical isolates of *Cryptococcus neoformans* to fluconazole. J Clin Microbiol 2004; 42: 380-383.

122. Posteraro, B.; Romano, L.; Sanguinetti, M.; *et al.* Commercial systems for fluconazole susceptibility testing of yeasts: comparison with the broth microdilution method. *Diagn Microbiol Infect Dis*. 2000, 38, 29-36.

123. Pour, M.; Špulák, M.; Balšánek, V.; Kuneš, J.; Buchta, V.; Waisser, K. 3-Phenyl-5-methyl-2H,5H-furan-2-ones: tuning antifungal activity by varying substituents on the phenyl ring. *Bioorg. Med. Chem. Lett.* 2000, 10, 1893–1895.

124. Pour, M.; Špulák, M.; Buchta, V.; Kubanová, P.; Vopršalová, M.; Wsól, V.; Fáková, H.; Koudelka, P.; Pourová, H.; Schiller, R. 3-Phenyl-5-acyloxymethyl-2H,5H-furan-2-ones: synthesis and biological activity of a novel group of potential antifungal drugs. *J. Med. Chem.* 2001, 44, 2701–2706.

125. Pour, M.; Špulák, M.; Balšánek, V.; Kuneš, J.; Kubanová, P.; Buchta, V. Synthesis and structure-antifungal activity relationships of 3-aryl-5-alkyl-2,5-dihydrofuran-2-ones and their carbanalogues: further refinement of tentative pharmacophore group. *Bioorg. Med. Chem.* 2003, 11, 2843–2866.

126. Price, M. F.; LaRocco, M. T.; Gentry, L. O. Fluconazole susceptibilities of *Candida* species and distribution of species recovered from blood cultures over a 5-year period. *Antimicrob Agents Chemother.* 1994, 38, 1422-1424.

127. Ramani, R.; Ramani, A.; Wong, S. Rapid flow cytometric susceptibility testing of *Candida albicans*. *J. Clin. Microbiol.* 1997, 35, 2320-2324.

128. Readio, J. D. and Bittman, R. Equilibrium binding of amphotericin B and its methyl ester and borate complex to sterols. *Biochem Biophs Acta* 1982, 685, 219-224.

129. Rex, J. H.; Pfaller, M. A.; Galgiani, J.N.; *et al.* Development of interpretive breakpoints for antifungal susceptibility testing: Conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and *Candida* infections. *Clin Infect Dis.* 1997, 24, 235-247.

130. Rex, J. H.; Pfaller, M. A.; Walsh, T. J.; *et al.* Antifungal susceptibility testing: practical aspects and current challenges. *Clin Microbiol Rev.* 2001, 14, 643-658.

131. Růžička, A.; Dostál, L.; Jambor, R.; Buchta, V.; Brus, J.; Císařová, I.; Holčapek, M.; Holeček, J. Structure and *in vitro* antifungal activity of [2,6-bis(dimethylaminomethyl)phenyl] diphenyltin(IV) compounds. *Appl. Organometal. Chem.* 2002, 16, 315-322.

132. Ryder, N. and Favre, B. Antifungal activity and mechanism of action of terbinafine. *Rev Contemp Pharmacother.* 1997, 8, 275-287.

133. Sánchez-Martínez, C.; Pérez-Martín, J. Dimorphism in fungal pathogens: *Candida albicans* and *Ustilago maydis* – similar inputs, different outputs. *Curr Opin Microbiol.* 2001, 4, 214–221.

134. Sheehan, D. J.; Hitchcock, C. A.; Sibley, C. M. Current and emerging azole antifungal agents. *Clin Microbiol Rev.* 1999, 12, 40–79.

135. Skopalová-Kubanová, P. Hodnocení antifungální activity *in vitro* derivátů benzylsalicylamidu a inkrustoporinu mikrodiluční bujónovou metodou. Disertační práce, Farmaceutická fakulta, Univerzita Karlova, Hradec Králové, 2003.

136. Speed, B.; D. Dunt. Clinical and host differences between infections with the two varieties of Cryptococcus neoformans. *Clin Infect Dis.* 1995, 21, 28-34.

137. Strobel, G.; Ford, E.; Worapong, J.; Harper, J. K.; Arif, A. M.; Grant, D. M.; Fung, P. C.; Ming Wah Chau, R. Isopestacin, an isobenzofuranone from *Pestalotiopsis microspora*, possessing antifungal and antioxidant activities. *Phytochemistry* 2002, 60, 179-183.

138. Sud, I. J. and Feingold, D. S. Mechanisms of action of the antimycotic imidazoles. *J. Investig Dermatol.* 1981, 76, 428-441.

139. Torres, H. A.; Hachem, R. Y.; Chemaly, R. F.; Kontoyiannis, D. P.; Raad, I. I. Posaconazole: a broad-spectrum triazole antifungal. *Lancet Infect Dis.* 2005, 5, 775-785.

140. Torres, H. A.; Rivero, G. A.; Lewis, R. E.; Hachem, R.; Raad, I. I.; Kontoyiannis, D. P. Aspergillosis caused by non-*fumigatus Aspergillus* species: risk factors and *in vitro* susceptibility compared with *Aspergillus fumigatus*. *Diagn Microbiol Infect Dis.* 2003, 46, 25-28.

141. Vale-Silva, L. A.; Buchta, V.; Vokurková, D.; Pour, M. Investigation of the mechanism of action of 3-(4-bromophenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-one against *Candida albicans* by flow cytometry. *Bioorg Med Chem Lett.* 2006, 16, 2492-2495.

142. Vanden Bossche, H.; Koymans, L, Moereels, H. P450 inhibitors of use in medical treatment: focus on mechanisms of action. *Pharmacol Ther.* 1995, 67, 79-100.

143. Vandenbossche, I.; Vaneechoutte, M.; Vandevenne, M.; Baere, T. D.; Verschraegen, G. Susceptibility testing of fluconazole by the NCCLS broth macrodilution method, E-test, and disk diffusion for application in the routine laboratory. *J Clin Microbiol.* 2002, 40, 918-921.

144. Víchová, P.; O´Connor, R.; Opletalová, V. Book of Abstracts of the 32nd Conference Synthesis and Analysis of Drugs, Velké Karlovice, September 16-19, 2003, p.153.

145. Wasan, K. M. Diversity of lipid-based polyene formulations and their behavior in biological systems. *Eur J Clin Microbiol Infect Dis.* 1997, 16, 81-92.

146. Zapf, S.; Anke, T.; Sterner, O. Incrustoporin, a new antibiotic from *Incrustoporia carneola* (Bres.) Ryv. (*Basidiomycetes*). *Acta Chem Scand.* 1995, 49, 233-234.

8 LIST OF PUBLICATIONS

Articles

1. Buchta V., M. Pour, P. Kubanová, **L. Silva**, I. Votruba, M. Vopršálová R. Schiller, H. Fáková, M. Špulák. 2004. *In vitro* antifungal activities of 3-(halogenated phenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-ones against common and emerging yeasts and molds. *Antimicrob. Agents Chemother.* 48(3):873-878. [ISSN 0066-4804]

2. Braunerová G., Buchta V., **Silva L.**, Kuneš J.,Palát K. Jr. 2004. Synthesis and *in vitro* antifungal activity of 4-substituted phenylguanidinium salts. *Il Farmaco* 59(6): 443-450. [ISSN: 0014-827X]

3. Novák P., Lyčka A., Císařová I., Buchta V., **Silva L.**, Kolářová L., Růžička A., Holeček J. 2005. Structure of azo dye organotin(IV) compounds containing a C,N-chelating ligand, part II, and their *in vitro* antifungal activity. *Appl. Organometal. Chem.* 19(4): 500–509. [ISSN 0268-2605, IF 1.385]

4. Jampílek J., Doležal M., Kuneš J., Buchta V., **Silva L.**, Kráľová K. 2005. Quinaldine derivatives: Preparation and biological activity. *Med. Chem.* 1:591-599. [ISSN 1573-4064]

5. Jampílek J., Doležal M., Palek L., **Silva L.**, Buchta V. 2005. Antifungal properties of some arylsulfanylpyrazine-2-carboxylic acid derivatives. *Folia Pharm.Univ.Carol.* 33: 23-29. [ISSN 1210-9495]

6. Adamec J., Waisser K., **Silva L.**, Buchta V. 2005. Antifungal activity of 1-phenyl-5-benzylsulfanyltetrazoles. *Folia Pharm. Univ. Carol.* 33:13-17 . [ISSN 1210-9495]

7. Chlupáčová M., Opletalová V., Kuneš J., **Silva L.**, Buchta V., Dušková L., Kráľová K. 2005. Synthesis and biological evaluation of some ring-substituted (E)-3-aryl-1-pyrazin-2-ylprop-2-en-1-ones. *Folia Pharm. Univ. Carol.* 33: 31-43. [ISSN 1210-9495]

8. Musioł R., Jampílek J., Buchta V., **Silva L.**, Niedbala H., Podeszwa B., Pałka A., Majerz-Maniecka K., Oleksyn B., Polanski J. 2006. Antifungal properties of new series of quinoline derivatives. *Bioorg. Med. Chem.* 14:3592–3598. [ISSN 0968-0896]

9. Opletalová V., Pour M., Kuneš J., Buchta V., **Silva L.**, Chlupáčová M., Kráľová K., Meltrová D., Peterka M., Posledníková M. 2006. Synthesis and biological evaluation of (*E*)-3-(nitrophenyl)-1-(pyrazin-2-yl)prop-2-en-1-ones. *Collect. Czech. Chem. Commun.* 71:44-58. [IF_{2004} = 1,062; ISSN 0010-0765]

10. **Vale-Silva L. A.**, Buchta V., Vokurková D., Pour M. 2006. Investigation of the mechanism of action of 3-(4-bromophenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-one against *Candida albicans* by flow cytometry. *Bioorg. Med. Chem. Lett.* 16(9):2492-2495. [IF_{2004} =2,333; ISSN 0960-894X]

11. **Vale-Silva L. A**., Buchta V. Antifungal susceptibility testing by flow cytometry: is it the future? *Mycoses* (in press).

12. **Vale-Silva L. A.**, Buchta V., Valentová E. Effect of subinhibitory concentrations of some established and experimental antifungal compounds on the germ tube formation in *Candida albicans*. *Folia Microbiologica* (In press).

Posters and abstracts

1. Kubicová L., Šustr M., Králová K., Chobot V., Vytlačilová J., Buchta V., **Silva L.**, Jahodář L., Vuorela P., Macháček M., Kuneš J., Kaustová J. 2003. Quinazoline-4-thiones: Synthesis, biological activity, and toxicological screening. Proceedings of ECSOC-7, The Seventh International Electronic Conference on Synthetic Organic Chemistry; http://www.mdpi.net/ecsoc-7.htm; September 1-30, 2003 Section C. Bioorganic chemistry and natural products. Article C003

2. Opletalová V., Chlupáčová M., Buchta V., **Silva L.** 2003. Antifungal properties of chalcones and heterocyclic analogues. Abstract. In: 3rd International symposium on natural drugs. Naples, October 2-4, 2003, p.154. Article: Book of Proceedings in 2005: 259-261

3. Buchta V., **Silva L.**, Valentová E. 2003. Využití testu inhibice klíčních hyf *Candida albicans* při hodnocení antifungálních látek v podmínkách in vitro. Sborník abstrakt. 3.Česko-Slovenské mezioborové konference lékařské mykologie, Pardubice 6.-8.11.2003, poster P-2, str.52. [ISBN 80-239-1689-0]

4. Buchta V., E. Valentová, P. Kubanová, H. Jiroutová, M. Pour, K. Palát, **L. Silva.** 2003. Vyhodnocení spektra antifungální aktivity u nově syntetizovaných látek na Farmaceutické fakultě UK v Hradci Králové. Sborník abstrakt. 3.Česko-Slovenské mezioborové konference lékařské mykologie, Pardubice 6.-8.11.2003, poster P-5, str.55. [ISBN 80-239-1689-0]

5. Kubicová L., Šustr M., Veselý O., Silva L., Buchta V. 2003. 4-methylthiosacilicylanilidy. Syntéza a antifungální aktivita. Sborník 32. konference "Syntéza a analýza léčiv", 16. až 19. září 2003, Velké Karlovice, str. 139. [ISBN 80-900306-5-3]

6. Palát K, Braunerová G., Buchta V., **Silva L.** 2004. 4-substituted phenylquanidium salts. Synthesis and antifungal activity. 56.Sjezd chemických společností."Chemie uhlí a uhlíkatých materiálů pro 21.století" Ostrava, 6.-9.září 2004, 4P-23, str.668.

7. Buchta V., Štepánková M., Förstl M., **Silva L.**, Tomšová M.. 2004. Isolation of *Aspergillus* spp. from biological specimens in the teaching hospital, Hradec Králové (Czech Republic) in 1995-2003. Advances against Aspergillosis. San Francisco, September 9-11, 2004, Poster abstract 55, p.83.

8. Opletalová V., Chlupáčová M., Posledníková M., Kuneš J., **Silva L.**, Buchta V., Jun D. 2004. Synthesis and biological evaluation of substituted (e) 1 (5 isopropylpyrazin-2-yl)-3-phenylprop 2 en-1-ones. Book of Abstracts. Jahrestagung – Joint Meeting 2004 of the Czech Pharmaceutical Society, Deutsche Pharmazeutische Gesellschaft and Österreichische Pharmazeutische Gesellschaft, Faculty of Chemistry and Pharmacy, University of Regensburg, October 6-9, 2004, p.121. [ISBN 3-00-014723-3]

9. Chlupáčová, M., Opletalová, V., **Silva, L.**, Buchta, V., Kuneš, J. 2004 Biologická aktivita nové série aduktů chalkonu s thioly. Sborník XXXIII. konferencie „Syntéza a analýza liečiv", Nitra, 9.-10. září 2004. In: Farm. Obzor 2004, LXXIII, 242; [ISSN: 0014-8172]

10. Opletalová, V., Chlupáčová, M., Posledníková, M., **Silva, L.**, Buchta, V., Kráľová, K. 2004 Syntéza a biologické hodnocení 2´,5´-diazachalkonů IX. 3-nitroderiváty. Sborník XXXIII. konferencie „Syntéza a analýza liečiv", Nitra, 9.-10. září 2004. In: Farm. Obzor 2004, LXXIII, 250; [ISSN: 0014-8172]

11. Opletalová V., Pour M., Kuneš J., **Silva L.**, Buchta V., Jampílek J., Richardson D.R., Kalinowski D.S., Švandová M., Zobalová D. 2005. Thiosemicarbazones of acetylpyrazines as potential drugs. Poster PO-70. 11th Blue Danube Symposium on Heterocyclic Chemistry. August 28-September 1, 2005, Brno.

12. Doležel J., Opletalová V., **Silva L.**, Buchta V. 2005 Syntéza derivátů rhodaninu jako potenciálních léčiv. Sborník příspěvků XXXIV. konference Syntéza a analýza léčiv, Brno, 12.-14. září 2005. Veterinární a farmaceutická fakulta, Brno 2005, s. 54. [ISBN 80-7305-533-3]

13. Chlupáčová M., Opletalová V., Doseděl M., Švédová L., **Silva L.**, Buchta V. 2005. Syntéza a antifungální hodnocení (E)-3-(2-methoxyfenyl)-1-pyrazin-2-ylprop-2-en-1-onů. Sborník příspěvků XXXIV. konference Syntéza a analýza léčiv, Brno, 12.-14. září 2005. Veterinární a farmaceutická fakulta, Brno 2005, s. 66. [ISBN 80-7305-533-3]

14. Kubicová L., Macháček M., Šustr M., **Silva L.**, Buchta V. 2005. Antifungální aktivita 5-bromthiosalicylanilidů. In: Sborník 40. konference Pokroky v organické, bioorganické a farmaceutické chemii, Nymburk, 18.-20.11.2005, Chem.Listy, 99:857-858. [ISSN 0009-2770] Pozn.: v záhlaví špatně uveden rok 2004. http://chemicke-listy.vscht.cz/docs/full/2005_11_827-878.pdf > [ISSN 1213-7103]

15. Macháček M., Kubicová L., Šustr M., **Silva L.**, Buchta V. 2005. Aktivita 5-bromthiosalicylanilidů proti *Absidia corymbifera*. In: Nové směry ve výzkumu léčiv. Sborník 34. konference Syntéza a analýza léčiv. 12.-14.9.2005, Brno. Veterinární a Farmaceutická Univerzita, Poster, s. 88. [ISBN 80-7305-533-3]

9 LIST OF TABLES AND FIGURES

9.1 Tables

9.2 Figures