ROLE OF CURCUMIN IN ANTIFUNGAL RESISTANCE

Thesis submitted in partial fulfillment of the requirement for the award of degree of

Doctor of Philosophy

By

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Certificate

This is to certify that this thesis titled **"Role of curcumin in antifungal resistance"** submitted to Jawaharlal Nehru University, New Delhi, in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy, embodies original research work carried out by **Ms. Monika Sharma** at School of Life Sciences, Jawaharlal Nehru University, New Delhi under our guidance and has not been submitted in part or full for any degree or diploma of this or any other University.

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DEDICATED

TO.....

My Parents

&

Loving Family

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Monika

ABBREVIATIONS

AA	Ascorbic acid
ABC	ATP Binding Cassette
AIDS	Acquired immunodeficiency syndrome
CUR	Curcumin mixture
CUR-I	Pure curcumin-I
CDR	Candida drug resistance
CM	Crude membrane
CONC	Concentration
DCFH-DA	Dichlorofluorescein diactate
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetra acetic acid
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
GFP	Green fluorescent protein
H	Hour
IC_{50}	Inhibitory concentration 50
IAAP	Iodoarylazidoprazosin
MDR	Multidrug resistance
MFS	Major Facilitator Superfamily
MIC	Minimal Inhibitory Concentration
MIN	Minutes
PDTC	Pyrrolidine di thio carbamate
$P450_{14\alpha dm}$	P450 14α-demethylase
PAGE	Polyacrylamide gel electrophoresis
PM	Plasma membrane
ROS	Reactive oxygen species
SEC	Seconds
SYN	Synergism

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Introduction

1. INTRODUCTION

The incidence of fungal infections has increased dramatically over the past few decades due to increase in the members of population susceptible to such infections. This population includes individuals undergoing chemotherapy for cancer, those enduring long-term treatment with antibacterial agents, those receiving immunosuppressive drugs following transplantation or those immunosuppressed due to diseases, such as AIDS, or malignancies (Frosco and Barrett, 1998). The severity of the fungal infections varies from superficial infections to life-threatening systemic infections. Most of the infections are opportunistic because the infecting agent is found as a commensal or is ubiquitous in the environment and can easily gain access to debilitated patients. While infections due to *Candida* and *Aspergillus* species are most common, previously rarely encountered opportunistic fungi have emerged recently as significant pathogens (Prasad, 1991). As example *Trichosporon beigelii*, *Fusarium* species, *Pseudallescheria boydii* and moulds of the class zygomycetes can cause invasive infections. *Candida* spp., *Cryptococcus neoformans*, and *Aspergillus* spp. are among the leading fungi responsible for these invasive infections. In addition to the opportunistic pathogenic fungi, a limited number of fungi exist with a true pathogenic potential for healthy hosts and cause life threatening infections. These pathogens, e.g., *Histoplasma capsulatum, Paracoccodioides brasiliensis, Pencillium marneffei* and *Coccidioides* immitis, are therefore classified as biohazard class 3 (Marichal, 1999).

1.1 *Candida albicans* and Candidiasis

Candida albicans (Figure 1) is the species most frequently associated with fungal infections in humans (Vanden Bossche *et al.*, 1998). Although a potential pathogen, *Candida albicans* is present as a commensal organism in many, if not most, healthy individuals, where it may be found on the skin and in the oral cavity, gastrointestinal tract and vagina. When something perturbs the balance between the host and this yeast, notable when

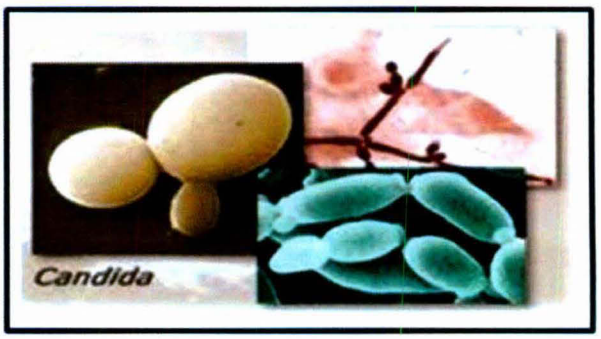


Figure 1. ***Candida albicans***

the host's immune system becomes compromised or when the microenvironment shifts to favor the growth of the yeast, this commensal organism really becomes pathogenic. For example, when the immune system of the HIV infected individuals begins to deteriorate, they frequently develop oral Candidiasis.

This condition also often develops among individuals with normal immune systems who are taking antibiotics because such drugs eliminate the normal, local bacteria and allow *C. albicans* to grow in that microenvironment. Similarly, changes in the hormone levels that affect the microenvironment of the vagina lead certain women to experience frequent episodes of vaginal Candidiasis. *Candida vulvovaginitis* is found in 10% women of childbearing age. Its prevalence increases to up to 30% during pregnancy. In AIDS patients, 90% have at least one episode of mycosis during their illness. About 50% of these infections are due to *C. albicans.* Although C. *albicans* is the most common cause of human infection, the genus *Candida* includes about 200 species. *C. albicans* is now listed as one of the top five most frequently isolated organisms from blood infections, as it is recognized as a major contributor to morbidity and mortality worldwide (Frosco and Barrett, 1998).

C. albicans, the principal infectious agent of human infection, is oval yeast of 2-6 μM in diameter. It exists as diploid, asexual polymorphic yeast with various biochemical abilities, both assimilative and fermentative, but lacks any proper sexual stage as well as carotenoid pigments. This medically significant fungus has the ability to undergo phenotypic switching and has 8 chromosomes (Odds, 1988; Prasad, 1991). One of the most remarkable aspects of *C. albicans* biology is its ability to assume a variety of cell morphologies (Figure 2). These range from yeast like cells to a variety of elongated growth forms, including a thread like hyphal growth form, germ tubes

Figure 2. *Candida albicans* cells in various morphological forms

during the transition between yeast and hyphae and pseudohyphae, which vary in shape from attached strings of yeast like cells to long filaments with constrictions at septae. The yeast form is the one most commonly found in the laboratory. The

transition to elongated growth is promoted by a wide range of environmental conditions, including growth at 37°C or exposure to serum (Ernst and Schmidt, 2000). Although both bud and hyphae are found in infected tissues, it seems likely that the elongated hyphae penetrate tissues, leaving its path in lateral colonies of budding cells that in turn give rise to new penetrating hyphae (Odds, 1985; Shepherd, 1985; Soll *et al.*, 1988; Scherer and Magee, 1990; Berger *et al.*, 1990). The etiological, biochemical and morphological attributes of *Candida* makes it a unique eukaryote.

1.2 Treatment of fungal infections

The therapeutic options for treating fungal infections, often caused by the emerging new pathogens whose incidence has increased due to the AIDS pandemic and use of immunosuppressive drugs in transplant and cancer patients, are limited by the relatively low number and structural variety of antifungals (Kolaczkowski & Goffeau, 1997). The discovery and development of the ideal antifungal drugs have been a long and difficult journey (Figure 3), beginning with FDA approval of amphotericin B (AMB) in 1957, the first azole in 1980, lipid formulations of AMB in 1989, and the first echinocandins in 2001 (Chapman *et al.*, 2008). The existing antifungal armamentarium contains four classes of drug- polyenes, nucleic acid synthesis inhibitors, ergosterol biosynthesis inhibitors (EBIs), and echinocandins. Clinical resistance has been observed for all classes of antifungal, and no single class of antifungal is effective against all invasive mycoses (Chapman *et al.*, 2008). Each class of drug has a specific mode of action and a distinct role in the treatment of fungal pathogens (Table 1). Figure 4 depicts the sites of action of some of the common antifungals. Most commonly used antifungals inhibit the ergosterol biosynthetic pathway and chiefly include azoles, allylamines and morpholines and others such as polyenes and 5-flucytosine impair membrane barrier function and macromolecule synthesis respectively (Vanden Bossche *et al.,* 1997).

1.3 Multidrug resistance

Multi **D**rug **R**esistance (MDR) is defined as the resistance of an organism towards a number of structurally and functionally unrelated compounds and is not a result of one mechanism but is caused by the synergistic action of a number of mechanisms. MDR can develop after sequential or simultaneous exposure to all the

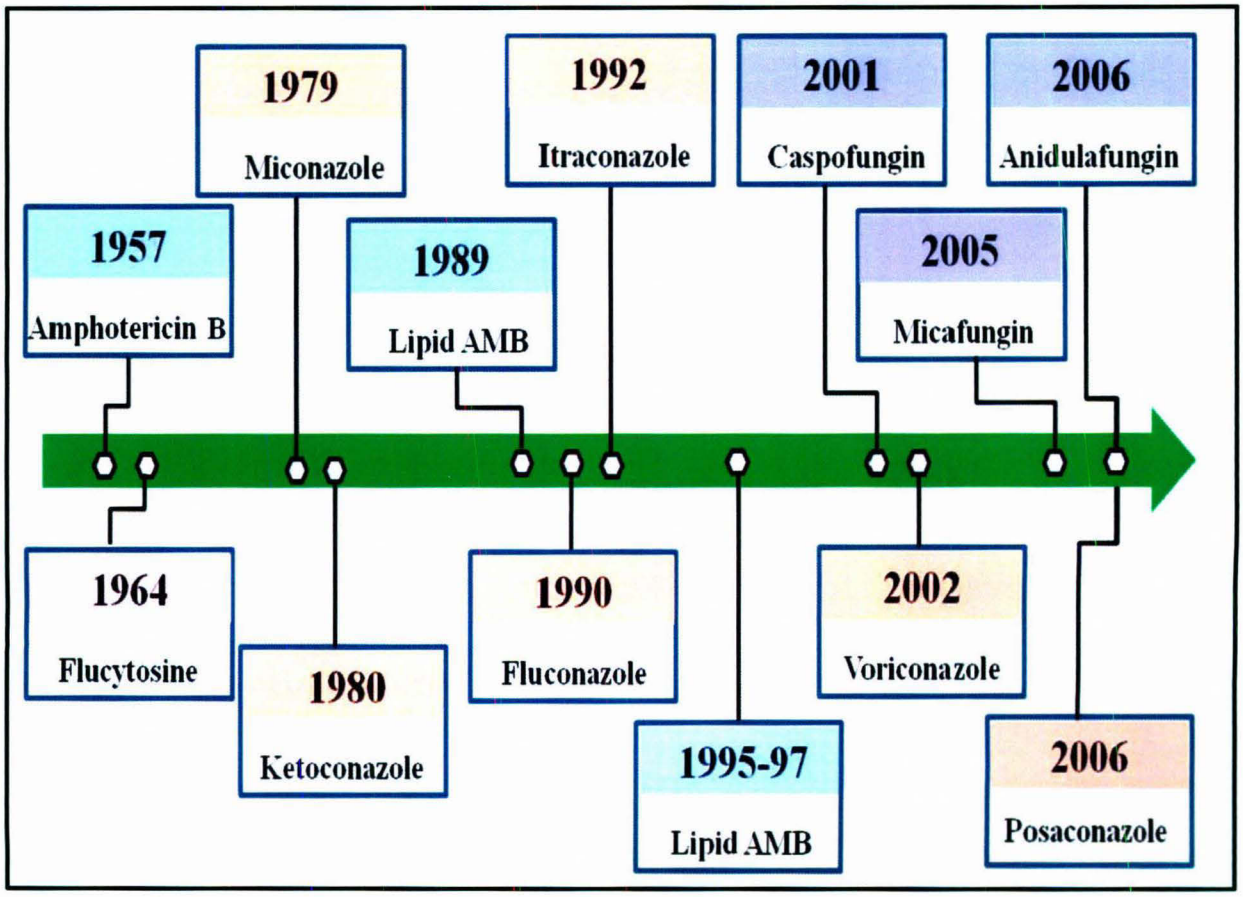


Figure 3. Timeline of development of antifungal agents
(Adapted from Chapman *et al.*, 2008).

Table 1: Commonly used antifungals and their mode of action

Antifungal	Spectrum/Comments	Mode of action
Polyenes		
Amphotericin B	Broad activity against *Candida* spp (except *C. lusitaniae*), *Cryptococcus neoformans* and filamentous fungi	Binding to ergoste-rol and destabiliz-ation of cell membrane Functions
Pyrimidines analogues		
5-fluorocytosine (5-FC)	Active against *Candida* spp and *Cryptococcus* spp; however, rapid emergence of resistance can appear when 5-FC is used as monotherapy	Impairment of nucleic acid biosynthesis by formation of toxic fluorinated pyrimidine anti Metabolites
Azoles		
Fluconazole	Active against *Candida* spp and *Cryptococcus* spp, less active against *C. glabrata* and no activity against *C. krusei*; no activity against filamentous fungi	Inhibition of cytochrome P450 14α-lanosterol demethylase
Itraconazole	Like fluconazole, but enhanced activity against filamentous fungi	
Voriconazole	Like fluconazole, but enhanced activity against filamentous fungi, including *Aspergillus* and *Fusarium* spp	
Posaconazole	Closely related to itraconazole, but more active	
Allylamines		
Terbinafine	Active against most dermatophytes, poor activity against*Candida* spp	Inhibition of squalene epoxidase
Morpholines		
Amorolfine	Active against most dermatophytes, poor activity against *Candida* spp	Inhibition of sterol Δ^{14} reductase and $\Delta^{7,8}$ isomerase
Echinocandins		
Caspofungin	Active against *Candida* spp with fungicidal activity, moderately active against *Aspergillus* spp, poor activity against *C. neoformans*	Inhibition of the cell wall synthesis enzyme β-1,3 glucan synthase

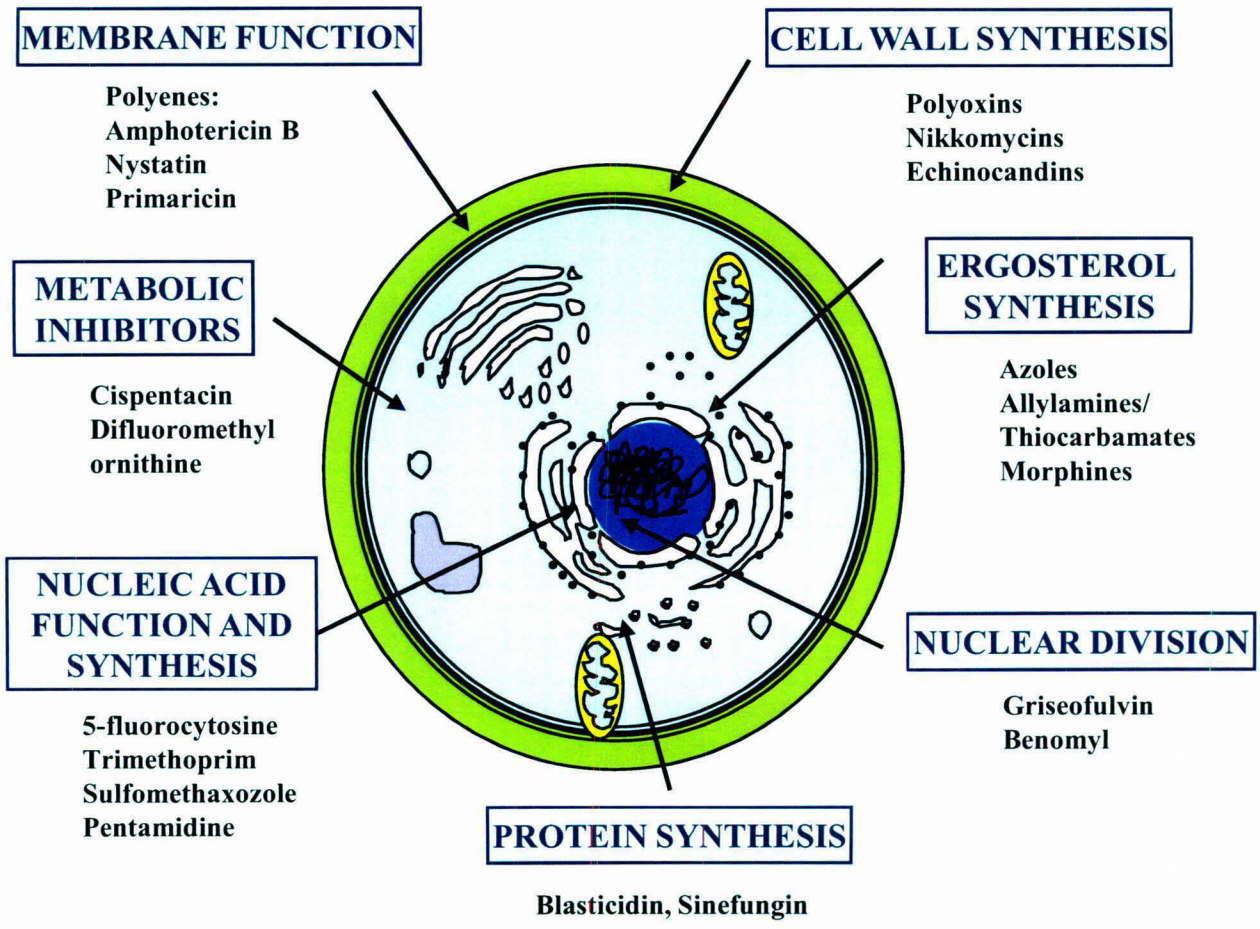


Figure 4. Site and mechanism of action of different classes of drugs on a typical fungal cell (Source: Adapted from Georgopapadakou and Walsh, 1994).

different drugs to which the cell or micro organism is resistant. MDR is a major concern in medical and agricultural developments. In medicine, the emergence of resistance to multiple drugs commonly used in the therapy is a major obstacle in the treatment of several tumors as well as of diverse diseases such as malaria (Chow and Volkman, 1998), tuberculosis and various bacterial and fungal infections (Balzi and Goffeau, 1995; Gottesman and Pastan, 1993) which often complicate major debilitating syndromes like AIDS. In agriculture, the control of resistance to plant pathogens towards natural plant defence toxins and towards common fungicides as well as the development of parasite-toxins resistant crops, are of major economic importance.

MDR generally involves a network of membrane associated transporters acting as multidrug efflux pumps and transcription factors regulating the expression of these pumps. These multidrug efflux systems present a disturbing clinical threat, since the acquisition of such a single system by a cell may decrease its susceptibility to a broad spectrum of chemotherapeutic drugs. MDR is one of the major obstacles for cancer therapy. One well documented mechanism underlying drug resistance in cancer cells implicates the over-expression of a membrane protein, the P-glycoprotein (P-gp or *MDR1*), functioning as an ATP-dependent extrusion pump for drugs and physiological substrates (Balzi and Goffeau, 1991). In another important field of health sciences, the resistance to drugs developed by *Plasmodium falciparum* is becoming a major obstacle for the treatment of malaria. The mechanism responsible for drug resistance in malaria seems analogous to that evoked for mammalian tumor cells. It involves the amplification and over-expression of a family of genes highly homologous to the mammalian P-gp encoding genes (Wilson *et al.*, 1989). Finally a understanding of a fatal and widespread hereditary disease was obtained by the isolation of the gene responsible for cystic fibrosis, and by the discovery that it encodes a putative membrane transport protein, remarkably similar to the mammalian multidrug resistance pump, and to its counterpart from other species (Schwiebert *et al.*, 1998).

In yeast studies on MDR received a recent impetus, not only because of the involvement of some yeast species in pathogenicity for men and plants, but also because yeast is a universal, easy-to-manipulate model system for the study of higher eukaryotic cells. Therefore mechanisms of fundamental importance for mammalian cells may sometimes be approached more efficiently by the study of similar yeast

functions. In this respect, the recent identification in yeast of genes homologous to mammalian MDR genes opens new prospects (Goffeau *et al.*, 1997; Decottignies and Goffeau, 1997; Paulsen *et al.*, 1998).

1.3.1 MDR in *Candida albicans*

Dimorphic, opportunistic and the most predominant human pathogenic yeast *C. albicans* is naturally more resistant to several drugs than *Saccaromyces cerevisiae.* In addition, the incidence of *C. albicans* cells acquiring resistance to antifungals like azoles has increased considerably in recent years which have posed serious problems towards the successful chemotherapy. The incidence of antifungal resistance has also increased in the non-*albicans* species such as *C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei. Candida* infections are treated with antifungal agents, particularly with the triazole derivatives fluconazole (FLC), itraconazole (ITR), ketoconazole (KTC), and voriconazole (VRC). In order to combat the attack of antifungals, evolution has equipped *Candida* with an exodus of protecting systems. The earliest known protecting system in *C. albicans* involved in the alteration or over-expression of the target enzymes $P450_{14\alpha dm}$. Recently, the characterization of efflux pumps which throw the drugs out of the cell in *C. albicans, C. dubliniensis* and *C. glabrata* have opened newer avenues and has provided impetus to dissect the mechanism of resistance employed by this pathogenic fungi to evade the toxic effects of the drugs.

1.4 Mechanism of antifungal drug resistance

Although the molecular basis of antifungal resistance in fungi are not very clear. Evidence accumulated so far suggests that MDR is a multifactorial phenomenon comprising a combination of several mechanisms (White, 1997; Ghannoum and Rice, 1999). A few of the well known molecular mechanisms of antifungal drug resistance in *C. albicans* are discussed below.

1.4.1 Mechanisms of azole resistance

Azole antifungal agents are by far the most commonly used drugs to treat infections caused by *C. albicans*. The target for azole antifungal agents in yeast is a cytochrome P-450. This enzyme is involved in the 14α-demethylation of lanosterol

which is an important step in the biosynthesis of ergosterol. Azole drugs interact in the active site of the target enzyme by binding to the heme moiety, acting as competitive inhibitors of the substrate. Figure 5 depicts molecular mechanisms of azole resistance that are commonly present in the pathogenic yeast *C. albicans* which include increased over-expression of multidrug transporters, mutations in lanosterol demethylase that reduce azole binding and increased expression of *ERG11* (Marichal, 1999; Vanden Bossche *et al.*, 1994; Vanden Bossche *et al.*, 1998; Bossche *et al.*, 1994). Additional known mechanisms have also been demonstrated to participate in azole resistance in *C. albicans* clinical isolates. These include decreased import, alterations in other enzymes in ergosterol biosynthetic pathway, chromosomal alterations, modification or degradation of the drug and transcriptional regulators. Nevertheless, other unknown mechanisms of azole resistance may still be found in clinical isolates. In the following sections these mechanisms have been discussed in detail.

1.4.1.1 Permeability resistance

Defects in drug import are a common mechanism of drug resistance. Many hydrophilic drugs, for example the anticancer antimetabolite methotrexate, cannot easily diffuse through the plasma membrane (PM) and have to use specific transporters for this purpose. Alterations in these transporters often lead to reduced influx of drugs. In the case of methotrexate (MTX), resistance is found to be associated with alterations in the folate transporter (Skovsgaard *et al.*, 1994). Decreased toxicity can also be caused due to changes in the lipid composition of the membranes leading to a decrease in permeability. The decreased permeability and fluidity of the membranes result from *cis-* to *trans*-isomerization of their saturated fatty acids. Resistance to the polyene systemic antifungal such as AMB, which interacts with membrane ergosterol to form pores in membrane, results in most cases from defects in the ergosterol biosynthetic pathway, leading to a decreased ergosterol levels in the PM (Kelly *et al.*, 1997). Several studies have demonstrated that when the ergosterol component of the plasma membrane is eliminated or reduced in favor of other sterol components such as 14α-methyl sterols, there are concomitant permeability changes in the PM and a lack of fluidity (Bossche *et al.*, 1987). These changes may lower the capacity of azole drugs to enter the cell. Most AMB resistant

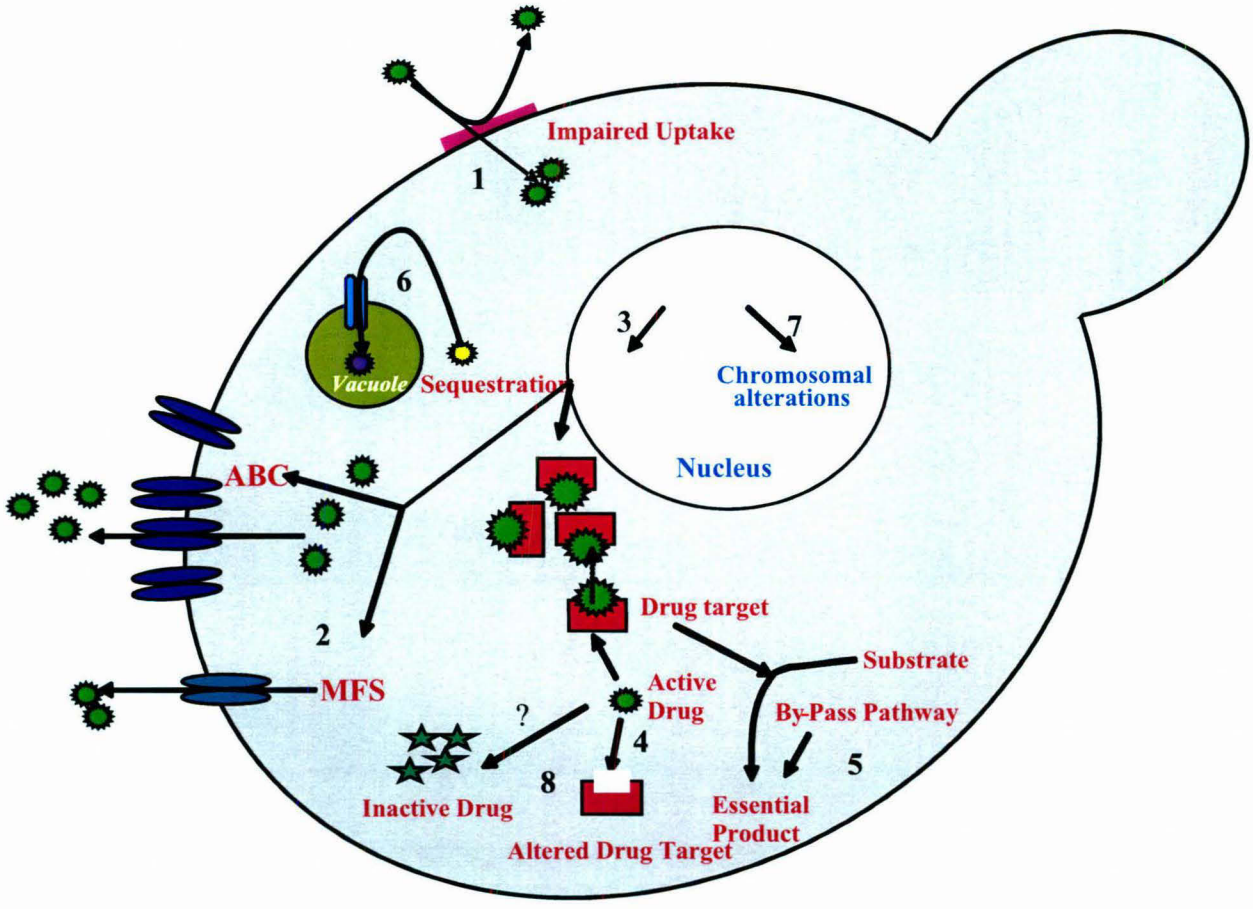


Figure 5. Mechanisms of azole resistance in fungi.

(1) changes in cell wall/plasma membrane leading to impaired azole uptake.
(2) efflux of drugs mediated by the ABC or the MFS class of efflux pumps.
(3) over-expression of the drug target or of the efflux pumps.
(4) mutation in the drug target (p45014DM) does not allow the drug to bind due to low affinity.
(5) activation of alternate pathways such as Δ5,6-desaturase.
(6) sequestration of the drug into an organelle-like vacuole by organellar pump.
(7) chromosome alterations or changes in chromosome number as a means to maintain more copies of the required gene.
(8) modification of azoles (CYP52) to an inactive form.
(**Courtesy**: modified from Prasad *et al.*, 2000).

isolates contain abnormally low number of ergosterol molecules in their PM thereby limiting the number of available binding sites for the polyene and thus preventing the membrane damage. Existence of other mechanisms of polyene resistance not linked to sterol alterations has also been suggested (Joseph-Horne and Hollomon, 1997). Another kind of permeability barrier in *C. albicans* involves formation of biofilms, which are large masses of cells. They have taken center stage with the increasing recognition of their role in human infections. In the protected microenvironment of biofilms, the pathogens are more resistant to antimicrobial therapies (Reynolds and Fink, 2001). However reduction of membrane permeability due to changes in the membrane biophysical properties is not a very efficient way of resistance unless it is accompanied by another resistance mechanism, such as active efflux or enzymatic activation, which often is the case.

1.4.1.2. Efflux mediated resistance

In addition to an alteration or an over-expression of 14α-lanosterol demethylase involved in sterol biosynthesis, azole resistance in *C. albicans* is also elicited by other mechanisms like the reduced intracellular drug accumulation of drugs due to drug efflux pumps. The most intriguing aspect of these transporters generally relates to their lack of specificity, wherein a single transporter can recognize a variety of unrelated xenobiotics. It is apparent from a host of studies that drug transporters of yeasts, similar to its homologues in mammalian system, are capable of transporting substrates that are structurally diverse. Recent studies indicate that fungi possess at least two efflux systems (Marger and Saier, 1993; Michaelis and Berkower, 1995) (i) ATP-binding cassette (ABC) superfamily of proteins (Figure 6). (ii) Proteins belonging to major facilitator superfamily (MFS) (Figure 7).

1.4.1.2.1 ABC transporters

A typical ABC transporter consists of four units, *viz.*, two membrane domains comprising of six transmembrane segments (TMS) and two nucleotide-binding domains (NBDs), which bind and hydrolyze ATP. These four modules can be expressed as separate polypeptides (Higgins, 1993). ABC transporters have been identified till now in species including bacteria, yeasts, insects, protozoa, plants and humans (Higgins, 1992; Fath and Kolter, 1993; van Veen and Konings, 1997) (Figure

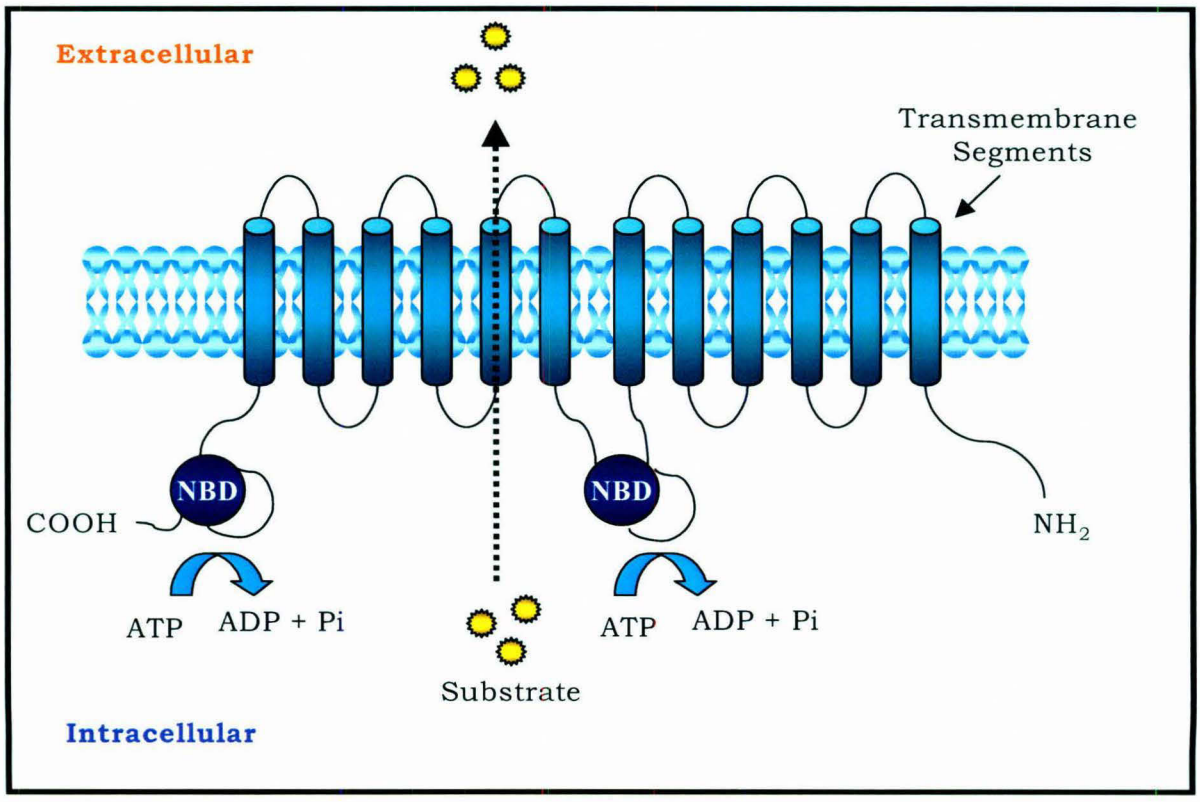


Figure 6. A diagrammatic representation of an ABC transporter of yeast.

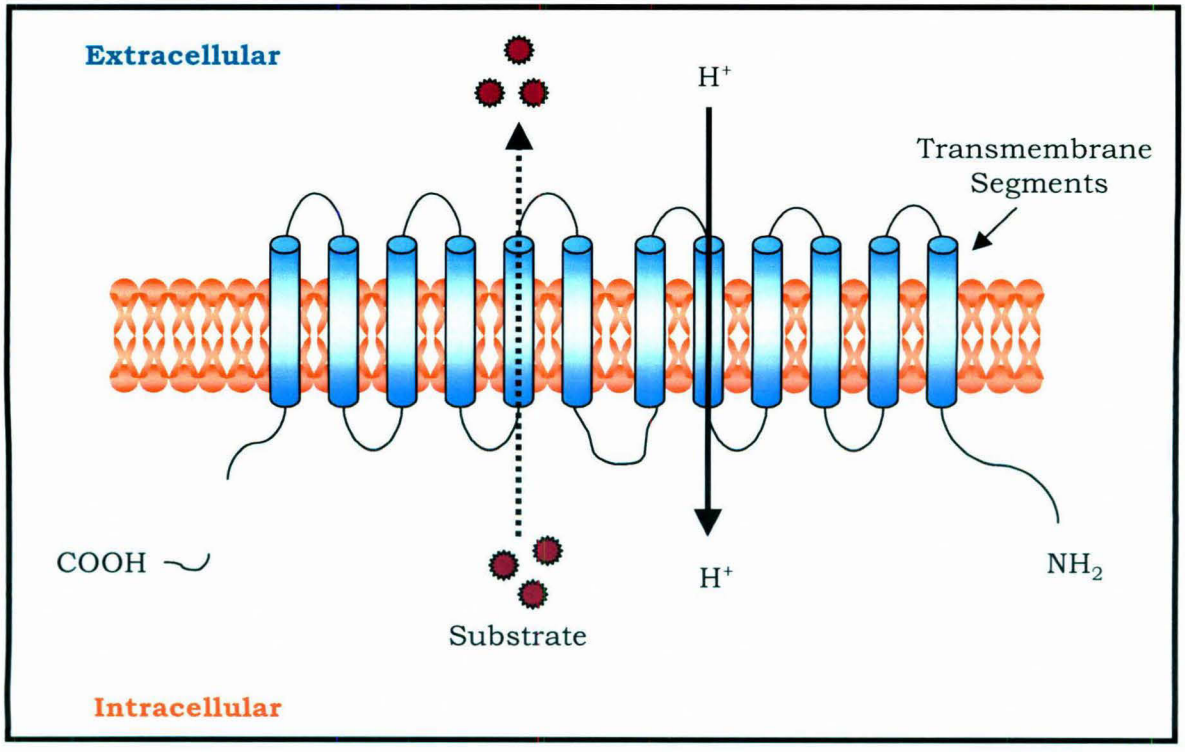


Figure 7. A diagrammatic representation of a MFS transporter of yeast.

6). They fulfil a remarkable variety of cellular functions. While most ABC proteins are purely ATP-driven membrane transporters, some of them act as ion channels, channel regulators, receptors, proteases and even sensing proteins (Higgins, 1995). As far as the transport of substrates is concerned, ABC transporters mediate translocation of ions, heavy metals, carbohydrates, anticancer drugs, amino acids, phospholipids, steroids, glucocorticoids, bile acids, mycotoxins, antibiotics, pigments, peptides through membranes and in some cases even whole proteins (Higgins, 1992; Dean and Allikmets, 1995; Ambudkar and Gottesman, 1998; Bevers *et al.*, 1999). However, each ABC transporter transports a large variety and size of substrates, but at the same time maintains selectivity for its particular substrate, this represents an intriguing and yet unsolved mystery. Several mammalian ABC proteins are medically important, because mutations in corresponding genes cause severe genetic diseases such as cystic fibrosis (Harris and Argent, 1993), adrenoleukodystrophy (Mosser *et al.*, 1993) and zeweller syndrome (Gartner and Valle, 1993), dubin-johnson syndrome (Paulusma *et al.*, 1996), familial hyperinsulinemic hypoglycemia of infancy, hepatic choleostasis and Stargardt's mascular dystrophy of the eye (Klein *et al.*, 1999).

The drug efflux pump encoded by *CDR1* of *C. albicans* was the first ABC efflux pump implicated in conferring resistance to cycloheximide in a *PDR5* disruptant hypersensitive strain of *S. cerevisiae* (Prasad *et al.*, 1995). *CDR1* encodes a 1501 amino acid long protein (169.9 kDa) whose predicted structural organization is characterized by two homologous halves, each comprising a hydrophobic region with a set of six transmembrane stretches, preceded by a hydrophilic nucleotide binding fold (Figure 6). The structure is identical to that of the *S. cerevisiae* ABC proteins Pdr5p and Snq2p. It mirrors the architecture of the yeast a-mating pheromone transporter Ste6, as well as mammalian drug resistance P-gp and cystic fibrosis factor CFTR (Dassa and Bouige, 2001). The significance of inversion of domains in some of the ABC drug transporters is not understood and may be related to their physiological functions. Cdr1p is remarkably similar to Pdr5p of *S. cerevisiae*. The similarity is not limited to only the ATP binding motif but is conserved along the entire length of the protein. Despite the high homology between *CDR1* and *PDR5*, and their encoded products, some functional features tend to distinguish them. For example, while single or low copies of *CDR1* are sufficient to increase drug resistance in *S. cerevisiae*, multiple copies of *PDR5* are required to yield a similar level of drug resistance. That efflux pumps other than *CDR1* could be contributing to drug resistance became

apparent after isolation of its close homolog *CDR2*. *CDR2* exhibits 84% identity with Cdr1p and confers resistance to FLC and several other drugs (Sanglard *et al.*, 1997).

Since azole resistance appears to be a multi-factorial phenomenon this led to search of more homologs of known *CDRs*. Using PCR-based cloning, other homologs of *CDR1* and *CDR2* namely *CDR3, CDR4*, and *CDR5*, were identified (Alarco *et al.*, 1997; Balan *et al.*, 1997; Franz *et al.*, 1998b, Sanglard *et al.*, 1998, Sanglard *et al.*, 1999). Cdr3p and Cdr4p show the highest homology to Cdr1p and Cdr2p; however, compared to Cdr1p and Cdr2p which are > 90% similar, Cdr3p and Cdr4p are only 75% similar to Cdr1p and Cdr2p. Interestingly over-expression of *CDR3* and deletion of *CDR3* and *CDR4* could only affect drug susceptibilities (Alarco *et al.*, 1997; Franz *et al.*, 1998b). Why some of the known Cdrp's are unable to elicit a multidrug resistance phenomenon is not yet clear.

1.4.1.2.2 MFS Transporters

Another class of transporters that are structurally quite similar to ABC pumps but do not contain ATP-binding domains, are known as MFS proteins. The MFS has originally been defined as a superfamily of permeases that are characterized by two structural units of six transmembrane spanning α-helical segments, linked by a cytoplasmic loop (Figure 7). MFS proteins that are involved in symport, antiport, or uniport of various substrates, have been found to be ubiquitously present from bacteria to higher eukaryotes. These proteins are proton motive force (PMF)-dependent antiporters which efflux out substrates in exchange of one or more H^+ ion. These transporters have been classified into five distinct clusters or families of membrane transport proteins within the MFS involved in (i) drug resistance, (ii) sugar uptake, (iii) uptake of Krebs cycle intermediates, (iv) phosphate ester/phosphate antiport and (v) oligosaccharide uptake (Saier and Reizer, 1991; Marger and Saier, 1993; Paulsen *et al.*, 1996). On the basis of hydropathy and phylogenetic analyses, MFS drug efflux proteins which have more than 100 members, can be divided into two distinct groups containing either 12 or 14 TMS (Paulsen *et al.*, 1996).

CaMDR1 (previously known as BEN^r) and *FLU1* are the two MFS genes identified in *C. albicans*. *CaMDR1* was initially identified as a gene, which conferred resistance to tubulin binding agent benomyl and tetrahydrofolate reductase inhibitor MTX (Fling *et al.*, 1991, Ben-Yaacov *et al.*, 1994). *FLU1* on the other hand, was initially picked up as a clone that could confer resistance to FLC. *CaMDR1*

expression in *S. cerevisiae* confers resistance to several unrelated drugs and its over-expression has been linked to azole resistance in *C. albicans*. Homologues of *CaMDR1* have been identified from *C. dubliniensis* and *C. glabrata* which are termed as *CdMDR1* and *CgMDR1*, respectively (Moran *et al.,* 1998, Sanglard *et al.,* 1999). It appears that increased expression of *CdMDR1* is the main mechanism of FLC resistance involved in *C. dubliniensis* clinical isolates (Moran *et al.,* 1998). Since *CgMDR1* confers specific resistance to FLC, its constitutive expression in *C. glabrata* may be responsible for the intrinsically low susceptibility of this yeast species to fluconazole (Sanglard *et al.,* 1999).

1.4.2 Molecular alterations of *ERG11*

As discussed above the predominant target site of the azole drugs is the heme protein, lanosterol demethylase also called *CYP51A1* (encoded by *ERG11*) which co catalyzes cytochrome P450 dependent 14α-demethylation of lanosterol (Ghannoum and Rice, 1999). Inhibition of 14α-demethylation leads to the depletion of ergosterol and accumulation of sterol precursors, including 14α-methylated sterols (lanosterol, 4, 14-dimethylzymosterol, and 24-methylenedihydrolanosterol), resulting in the formation of PM with altered structure and function. Several lines of evidence implicate a modification in the quantity or quality of 14α-demethylase in the expression of resistance to azole antifungal agents. Several genetic alterations like point mutations, over-expression of the gene, gene amplification and gene conversion have been identified in *ERG11* of *C. albicans*.

Point mutations

Alteration of the affinity of azole derivatives for *CYP51A1*, which has been described in different post-treatment yeast isolates of *C. albicans* (Bossche, 1994) is another important mechanism of resistance. A survey of resistant and sensitive clinical *C. albicans* isolates has identified different point mutations that are associated with azole-resistant isolates. Sequence data identified Y132H (tyrosine 132 is replaced by a histidine), T135A (threonine 315 is replaced by alanine) or R476K (arginine 476 is replaced by lysine) mutations that decrease the affinity of the target for an azole derivative. It has been shown that R467K alone is sufficient to cause azole resistance (Marichal, 1999).

Another significant change observed in the *ERG11* gene of the resistant isolate was reported by White (White, 1997b), namely loss of allelic variation in the *ERG11* promoter and in the downstream *THR1* gene (which encodes homoserine kinase, which is involved in threonine synthesis). Point mutations in *ERG11* have been developed in laboratory strains also that result in azole resistance. Although these changes may account for resistance development, they are not the only factors involved (Ghannoum and Rice, 1999).

Over-expression

Over-expression of *CYP51A1* as a resistance factor has been mentioned for a few *C. albicans* and *C. glabrata* isolates and has been implicated as a mechanism of resistance to azole antifungals (Vanden Bossche *et al.*, 1998; Ghannoum and Rice, 1999). Over-expression of *CYP51A1* in *C. albicans* resistant isolates has been measured and was up to three times higher than could be measured in azole susceptible isolates, thus probably accounting for a minor effect on the development of resistance. However, some *C. albicans* strains have up to a 10-fold increase in *CYP51A1* mRNA. Over-expression of *CYP51A1* can be achieved by gene amplification, as shown in a *C. glabrata* isolate resistant to azole derivatives (Marichal *et al.*, 1997). Hybridization experiments on chromosomal blots indicate that this increase in copy number is due to amplification of the entire chromosome containing the *CYP51A1* gene. Azole dependent upregulation of *ERG11* has been described in several different clinical isolates of *C. albicans* (Sanglard *et al.*, 1995; Albertson *et al.*, 1996; White *et al.*, 1997). But it is difficult to assess the contribution of *ERG11* over-expression to a resistant phenotype, since these limited cases of over-expression have always accompanied other alterations associated with resistance including mutations in *ERG11* and over-expression of efflux pumps (White, 1997a). Hence over-expression of the target enzyme plays only a limited role in clinical resistance to the azoles.

Azole dependent up-regulation is not limited to *ERG11* and also involves up regulation of other *ERG* genes upstream and downstream of *ERG11*. Likewise *ERG11* up regulation was also not limited to azole drugs and *ERG11* was also inducible by drugs that target other enzymes of the ergosterol biosynthetic pathway (Morita and Nozawa, 1985). These data suggest a common global *ERG* up regulation *e.g.* in response to ergosterol depletion.

1.4.3 Molecular alterations in other *ERG* genes

Another enzyme of ergosterol biosynthesis pathway, $\Delta^{5,6}$-desaturase (*ERG3*), has been shown to contribute to azole resistance. A defect in *ERG3* leads to the accumulation of 14α-methylfecosterol instead of 14α-methylergosta-8, 24 (28)-dien-3β,6-α diol. Accumulation of sufficient amounts of 14α-methylfecosterol compensates for ergosterol in the membranes and thus contributes to azole resistance in *C. albicans* (White *et al.*, 1998; Ghannoum and Rice, 1999). The lethality of *S. cerevisiae* disruptant of *ERG11* can be suppressed by $\Delta^{5,6}$-desaturase (Kelly *et al.*,1997). The decrease in ergosterol content due to a defect in $\Delta^{5,6}$-desaturase in FLC resistant clinical isolates of *C. albicans* also results in cross-resistance to AMB (Kelly *et al.*, 1997). Another cytochrome P450, Δ^{22}-desaturase (CYP61 and also *ERG5*) has been purified from an *ERG11* (P45014DM) disrupted strain of *C. glabrata* (Lamb *et al.*, 1999). The purified enzyme showed desaturase activity in a reconstituted system. Δ^{22}-desaturase and its homologues have also been identified in *C. albicans* and *Schizosaccharomyces pombe*. The spectral analyses obtained with azole antifungal compounds *viz.*, KTC, FLC and ITR in reconstituted Δ^{22}-desaturase suggests that these drugs directly interact with the cytochrome heme (Lamb *et al.*, 1999).

1.4.4 Mechanisms of non-azole mediated resistance

1.4.4.1 Resistance to 5-flucytosine

Primary resistance to 5-FC is a common phenomenon. Resistance may occur due to the deficiency or lack of enzymes involved in the uptake or metabolism of 5-FC, or may be due to the deregulation of the pyrimidine biosynthetic pathway, whose products can compete with the fluorinated metabolites of 5-FC (Kerridge and Whelan, 1984). Detailed investigations on the molecular mechanisms of resistance to 5-FC have shown that intrinsic resistance in fungi can be due to a defect in cytosine permease (with the exception of *C. albicans*), while acquired resistance results from a failure to metabolize 5-FC to 5-FUTP and 5-FdUMP, or from the loss of feedback control of pyrimidine biosynthesis.

1.4.4.2 Resistance to polyenes

Polyene resistance has not been a major clinical problem to date, although polyene resistant isolates have been isolated and characterized. Acquired resistance to

AMB is often associated with alteration of membrane lipids, especially sterols. Most polyene resistant clinical *Candida* isolates have a greatly reduced ergosterol content in their membranes (Hitchcock *et al.*, 1987; White *et al.*, 1998). There is evidence to suggest that alterations in the membrane structure or in the sterol-to-phospholipid ratio in the membrane may be associated with resistance. Recently, clinical isolates of *C. albicans* resistant to AMB were described lacking ergosterol and accumulating 3-β-ergosta-7,22-dienol and 3-β-ergosta-8-enol, typical for a defect in the sterol $\Delta^{5,6}$ desaturase system (Kelly *et al.*, 1997). Such a defect is known in laboratory yeasts (*S. cerevisiae*) harbouring a defect in the $\Delta^{5,6}$ desaturase gene *ERG3* (Kelly *et al.*, 1994; Geber *et al.*, 1995).

1.4.4.3 Resistance to allylamines

Squalene epoxidase (product of the *ERG1* gene) is the target enzyme of the allylamines naftifine and terbinafine (Favre *et al.*, 1999). Both are used mainly to treat dermatophytosis. The gene encoding squalene epoxidase (*ERG1*) has been cloned in *S. cerevisiae*. Deletion of this gene affected viability of *S. cerevisiae* during aerobic growth. Resistance of yeasts to allylamines has been reported only rarely however, the potential to develop resistance by the action of multidrug efflux transporters does exist. For example, the over-expression in *S. cerevisiae* of the *C. albicans CDR1* and *CDR2* genes, and of the *CaMDR1* gene, can confer resistance to terbinafine (Sanglard *et al.*, 1997), showing that this compound is a substrate for these transporters. Moreover, deletion of the *CDR1* gene in *C. albicans* renders cells hypersusceptible to the same drug (Sanglard *et al.*, 1996). Several *C. albicans* isolates resistant to azole antifungal agents are also less susceptible to terbinafine. Since such isolates are resistant to azole derivatives by the mechanism of multidrug efflux transporter gene over- expression, the cross-resistance to terbinafine could perhaps be explained by this phenomenon.

1.4.4.4 Resistance to morpholines

Amorolfine is the only morpholine derivative in clinical use. It inhibits at least two enzymes in the post lanosterol ergosterol biosynthesis pathway, Δ^{14}-reductase (product of the *ERG24* gene) and $\Delta^{8,7}$-isomerase (product of the *ERG2* gene). Acquired resistance to morpholine derivatives has not been reported so far in yeast

pathogens and this is probably due to the limited use of this antifungal for the treatment of superficial fungal infections. However, resistance to morpholine derivatives in *S. cerevisiae* can be by the over-expression of the *ERG24* or *ERG4* (sterol C-24 (28) reductase) genes (White *et al.*, 1998). Moreover, recent work has pointed out that amorolfine, like terbinafine, could be a substrate of multidrug efflux transporters of the ABC-family. This was concluded from results showing that: (i) over-expression of the *C. albicans CDR1* and *CDR2* genes in *S. cerevisiae* could render cells resistant to amorolfine (Sanglard *et al.*, 1997), (ii) *C. albicans* multidrug transporter mutants were hypersusceptible to this antifungal agent (Sanglard *et al.*, 1996), and (iii) *C. albicans* clinical isolates resistant to azole antifungal agents over-expressing the *CDR1* and *CDR2* genes were less susceptible to amorolfine (D. Sanglard, *unpublished results*). Therefore, the potential for developing resistance to this agent do exist.

1.4.4.5 Resistance to 1, 3-β-glucan synthase inhibitors

In *S. cerevisiae*, β-(1,3)-glucan synthase is a multienzyme complex with two subunits encoded by the *FKS1* and *FKS2* genes. Deletion of both genes in this yeast results in a lethal phenotype (Kelly *et al.*, 2000; Douglas *et al.*, 1997). Resistance to these compounds is possible, since spontaneous mutants resistant to the echinocandin L-733560 have been isolated *in vitro* in *S. cerevisiae* (Douglas *et al.*, 1997, Douglas, 2001) and in *C. albicans* (Kurtz *et al.*, 1996). The mechanism of resistance in these two yeast species is thought to be a lower affinity of echinocandin to the β-(1,3)-glucan synthase produced in these mutants. Resistance to echinocandin may not be relevant in clinical situations, since it was shown that *C. albicans* resistant mutants exhibited attenuated virulence in animal experiments (Kurtz *et al.*, 1996).

1.5 Natural products--the future scaffolds for novel antifungals

Prolonged usage of azoles and other popular drugs have led to the development of MDR in clinical isolates of *Candida*. Studies are underway to discover novel antifungal agents so that the resistant isolates can be dealt with. Natural products are attractive prototypes for novel antifungal agents due to their broad spectrum of biological activities. Various research groups have initiated antifungal screening programmes for plants used all over the world as anti-infectious

agents in traditional medicine. Table 2 lists some of the antifungal compounds obtained from plants. Turmeric (the common name for *Curcuma longa*) belongs to the Zingiberaceae family and is widely used as a spice, food flavoring and coloring agent as well as in traditional medicinal applications. Three main curcuminoids have been isolated from turmeric: curcumin I (CUR-I) or diferuloylmethane, curcumin II (CUR-II) or demethoxycurcumin and curcumin III (CUR-III) or bisdemethoxycurcumin. The commercially available curcumin (CUR) is a mixture of curcuminoids and contains predominantly CUR-I (~77%), followed by CUR-II (17%), and CUR-III (3%) which display a wide range of biological and pharmacological properties (Ammon and Wahl, 1991; Kuo *et al.*, 1996). Figure 8 summarizes the various properties of CUR.

1.5.1 Role of CUR in MDR of cancer cells

MDR of cancer cells is an obstacle to effective chemotherapy of cancer. ABC transporter including P-gp (ABCB1), MRP1 (ABCC1) and ABCG2, play an important role in development of this resistance. An attractive approach to overcoming MDR is the inhibition of the pumping action of these transporters.
Table 3 shows the clinically relevant drugs and other compounds that interact with ABCB1, ABCC1 and ABCG2.

P-gp is the most well-known and best characterized of the ABC transporters, it has been studied intensively since it was discovered in 1976 (Juliano and Ling, 1976). Despite 30 years of study, the nature of the physiological substrates of this pump is still not well understood. P-gp is expressed at the apical membranes of the intestinal epithelial cells, where it can prevent any amphipathic drugs from entering the body through the gut. P-gp in the gut mucosa, liver and kidney facilitates the process of eliminating toxins from the system. Thus, it confers resistance to a vast array of clinically and toxicologically relevant compounds, including anticancer drugs, HIV protease inhibitors, antibiotics, antidepressants, antipileptics and analgesics (Sarkadi *et al.*, 2006).

MRP1 was the first member of the MRP family (MRP1-9) to be identified, in 1992 (Cole *et al.*, 1992). In contrast to P-gp, which transports xenotoxins, MRP1 transports less toxic, but more water soluble, anionic glutathione (GSH), sulphate and glucuronate drug conjugates. Although MRP1 predominantly transports anionic drug conjugates, it can also transport neutral/basic drugs in the presence of GSH. These drugs and GSH are presumable co-transported (Loe *et al.*, 1996a; Renes *et al.*, 1999).

Table 2: Antifungal compounds obtained from plants

Compound	Source	Example	Antifungal activity against various sps.
Phenols	*Baseonema acuminatum, Lycium chinense, Croton hutchinsonianus, Artocarpus nobilis, Piper aduncum*	Tannins, Salicyclic acid	*C. albicans, S.cerevisiae, Cladosporium cladosporioides, C. sphaerospermum*
Flavonoids	*Hildegardia barteri, Artemisia giraldii, Alpinia officinarum, Blumea balsamifera, Camptotheca acuminate*	Scandenone, Trliroside, quercetin	*C. albicans, Trichophyton mentagrophytes, Aspergillus niger, Fusarium avenaceum*
Coumarins	*Baccharis pedunculata, Tordylium apulum, Amburana cearensis, Fsafetida foetida*	Angelicin, Clausenidin	*C. albicans, S.cerevisiae, C. neoformans, A. niger*
Quinones	*Cryptomeria japonica, Heliotropium ovalifolium*	Kigelinone, Isopinnatal,	*Pyricularia orizae, Alternaria alternata, C. cucumerinum, C. albicans*
Saponins	*Smilax medica, Phytolacca tetramera Tribulus terrestris, Ypsilandra thebetica, Medicago sativa*	Phytolaccosides B, E, Kalopanaxsaponins A, I	*C. albicans, C. glabrata, C. tropicalis, C. neoformans, Microsporum gypseum*
Xanthones	*Securidaca longepedunculata, Monnina obtusifolia, Xanthium macrocarpum,*	Caledonixanthone E, Toxyloxanthone, Wighteone,	*Staphylococcus aureus, A. niger, A. fumigatus,C. albicans, C. glabrata, A. Fumigates*
Alkaloids	*Aniba panurensis, Melochia odorata, Pleodendron costaricense*	Berberine, methylhydrasteine hydroxylactam, Frangulanine	*C. albicans, A. fumigatus, C. neoformans*
Polypeptides	*Astragalus mongholicus, Agrocybe cylindracea, Trigonells foenumgraecum, Cicer arietinum, Basella rubra*	Cucurmoschin, Cicerarin, Basrubrins	*C. albicans, Trichosporon beigelii, S. Cerevisiae*
Terpenoids and essential oils	*Agastache rugosa, Litsea cubeba, Alpinia galangal, Detarium microcarpum, Celastrus hypoleucus*	Pristimerin, Celastrol, Triterpenetetrol	*C. albicans, Cryptococcus sps. C. glabrata, A. fumigatus, C. cucumerinum*

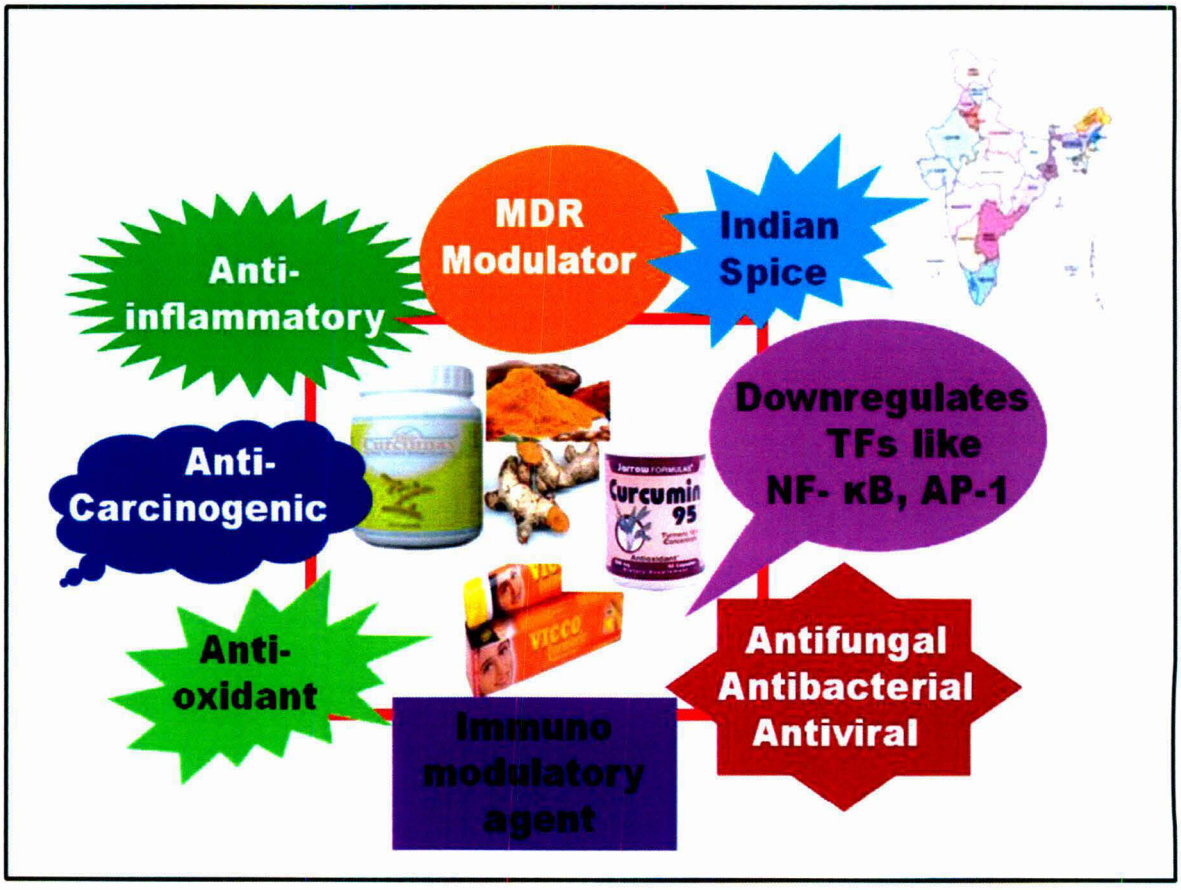


Figure 8. Spicy curcumin has multiple roles

Table 3: Clinically relevant drugs and other compounds that interact with ABCB1, ABCC1 and ABCG2.

Drugs and compounds interacting with ABCB1	
Anticancer Drugs	Vinca alkaloids (vinblastine and vincristine), Anthracyclines (Doxurubicin and daunorubicin), Taxanes (paclitaxel and docetaxel), Epipodophyllotoxins (etoposide and teniposide), Camptothecins (topotecan), Anthracenes (bisantrene and mitoxantrone)
HIV Protease inhibitors	Ritonavir, Saqvinavir, Nalfinavir
Analgesics	Morphine
Antihistamines	Terfinadine, Fexofinadine
H2 receptor antagonists	Cimetidine
Immunosuppressive agents	Cyclosporine A, Tacrolimus (FK5060)
Antiarrhythmics	Quinidine, Amiodarone, Propaphenone
Antiepileptics	Felbamate,Topiramate
Fluorescent Compounds	Calcein-AM, Hoechst 33342, Rhodamine 123
HMG-CoA reductase inhibitors	Lovastatin, Simavastatin
Antiemetics	Ondansetron
Tyrosine kinase inhibitors	Imatinib mesylate, Gifitinib
Cardiac glycosides	Digoxin
Antihelminthics	Ivermestin
Calcium-channel blockers	Verapamil, Nifedipine, Azidopine, Diltiazem
Calmodulin antagonists	Trifluparazine, Chlorpromazine, Trans-flupentixol
Antihypertensives	Reserpine, Propanoiol
Antibiotics	Erythromycin, Gramicidine A
Steroids	Corticosterone, Dexamethasone, Aldosteronen, Cortisol
Pesticides	Metylparathion, Endosulfan, Cypermethrin, Fenvalerate
Natural Products	Curcuminoids, Colchicine
Antialcoholism drug	Disufiram

Drugs and compounds interacting with ABCC1	
Anticancer drugs	Vinca alkaloids (Vinblastine and vincristine), Anthracyclines (doxorubicin and daunorubicin), Epipodophyllotoxins (etoposide and teniposide), Campothecins (topotecan and irinotecan), Methotrexate
Metalloids	Sodium arsenate, Sodium arsenite, Potassium antimonite
Peptides	Glutathione (GSH, GSSG)
Glutathione conjugates	Leucotrienes C4, D4 and E4, Prostaglandin A2-SG, Hydrxynonenal-SG, Aflatoxin B1-epoxide-SG, Melphalan-SG, Cyclophosphamide-SG , Doxorubicin-SG
Sulphate conjugates	Estrone -3-sulphate, Dehydroepiandrosterone-3-sulphate, Sulfatolithocholyl taurine
Pesticides	Fenitrothion, Methoxychlor
Toxins	Aflatoxin B1
Glucuronide conjugates	Glucuronosylbilirubin, Estradiol-17-ß D-glucuronide, Etoposide-glucuronide, NS-38-glucuronide
HIV Protease inhibitors	Ritonavir Saquinavir
Tyrisine kinase inhibitors	Imatinib mesylate, Gefitinib
Fluorescent compounds	Calcein, Fluo-3, BCECF
Antibiotics	Difloxacin, Grepafloxicin
Folates	Folic acid, L-leucovarin
Natural products	Curcuminoids

Drugs and compounds interacting with ABCG2	
Anticancer drugs	Mitoxantrone, Bisantrene (R482T mutant form), Epipodophyllotoxins (etoposide and teniposide), Flavopiridol, Anthracyclines (doxorubicin and daunorubicin ; R482T mutant form)
Antifolates	Methotrexate
Porphyrins	Pheophorbide a, protoporphyrin IX, Hematoporphyrin,
Tyrosine kinase inhibitors	Imatinib mesylate, Gefitnib
Flavonoids	Genestein, Quercetin
Carcinogens	Aflatoxin B, PhiP
Fungal toxins	Fumitremorgin, Ko143
Drug and metabolite conjugates	Acetomenaphen sulphate, Estrone-3-sulfate, Dehydroepiandrosterone sulfate, Estradiol-17-ß-D-glucuronide, Dinitrophenyl-S-glutathione
HMG CoA reductase inhibitors	Rosuvastatin, Pravastatin, Cerivastatin
Antihypertensives	Reserpine
Antibiotics	Ciprofloxacin, Norfloxacin
Fluorescent compounds	Hoechst 33342, BODIPY Prazosin, Rhodamine 123 (R482T/G)
Antiviral drugs	Zidovudine, Lamivudine
Natural products	Curcuminoids

MRP1 may also be involved in immuno-response sensitivity, given that leukotriene C_4 (LTC_4) is a high affinity substrate of MRP1. In addition to transporting drug conjugates, MRP1 confers resistance to anticancer drugs, including Vinca alkaloids, anthracyclins, epipodophyllotoxins, methotrexate and camphothecins (Loe *et al.*, 1996b; Allen *et al.*, 2000; Johnson *et al.*, 2001). In presence of GSH, MRP1 transports vincristine and other drugs (Renes *et al.*, 1999; Loe *et al.*, 1996b; Loe *et al.*, 1998).

ABCG2 is a relatively new member of the family, identified in 1998 (Allikmets *et al.*, 1998; Doyle *et al.*, 1998, Miyake *et al.*, 1999). It is mainly expressed at the apical membranes of placental syncytiotrophoblasts, in the mammary gland, intestine, colon, enterocytes, hepatocytes, erythrocytes and human brain microvessel endothelium (Maliepaard *et al.*, 2001; Cooray *et al.*, 2002). Similar to P-gp, ABCG2 seems to play an important role in the defense of the body against xenobiotics and protects specific body compartments such as the placenta (Mao and Unadkat, 2005) and the blood brain barrier (Cooray *et al.*, 2002). In addition to transporting physiological substrates, ABCG2 can actively efflux a substantial variety of compounds out of cells, ranging from fluorescent dyes to both anionic and cationic drugs (Doyle *et al.*, 1998; Sarkadi *et al.*, 2006). Reported ABCG2 substrates include doxorubicin, mitoxantrone, methotrexate, topotecan etoposide, prazosin, flavopiridol, Hoechst 33342 and anthracyclines (Sarkadi *et al.*, 2006). Inaddition to chemotherapeutics, ABCG2 also transports porphyrins and sterols (Krishnamurthy and Schuetz, 2006).

CUR-I was most potent in inhibiting/modulating the function of ABCB1, ABCC1 and ABCG2. Recent studies suggest that CUR can be used as a broad spectrum modulator of MDR. Unfortunately, CUR is reported to have low bioavailability when given orally and is also metabolized to dihydrocurcumin (DHC) and tetrahydrocurcumin (THC) by the endogenous reductase system. Therefore, one of the major metabolites, THC, which is easily absorbed in the gastrointestinal tract, was also evaluated for its inhibition of the three major drug transporters. It was observed that THC also inhibited these transporters (Limtrakul *et al.*, 2007), suggesting that metabolite produced from CUR biotransformation in the body can be used to sensitize MDR cells.

Apart from the modulator effects, CUR has been shown to have potent preclinical antitumor effects. The molecular basis of tumor inhibition by CUR is potentially attributable to its effects on transcription factors, apoptotic genes,

angiogenesis regulators and cellular signalling pathways (Aggarwal *et al.*, 2003). Inhibition of one of these pathways is considered one of the most important mechanism for preventing cancer initiation and progression (Aggarwal *et al.*, 2003). Therefore CUR represents an ideal compound that could be developed as a broad spectrum inhibitor, as it not only has an antitumor effect, but also has potent inhibitory effects on the ABC drug trasnporters.

1.5.2 Problems and Promises related to CUR

Various animal models (Shankar *et al.*, 1980; Qureshi *et al.*, 1992) and human studies (Lao *et al.*, 2006a; Lao *et al.*, 2006b; Cheng *et al.*, 2001; Shoba *et al.*, 1998b) have proved that CUR is extremely safe even at very high doses. For example, three different phase I clinical trials indicated that CUR, when taken as high as 12g per day, is well tolerated (Lao *et al.*, 2006b; Cheng *et al.*, 2001; Shoba *et al.*, 1998). Similarly, the efficacy of CUR in various diseases including cancer has been well established (Aggarwal *et al.*, 2007). Several clinical studies dealing with the efficacy of CUR in humans can also be cited (Hsu and Cheng, 2007). The pharmacological safety and efficacy of CUR makes it a potential compound for treatment and prevention of a wide variety of human disease. Inspite of its efficacy and safety, CUR has not yet been approved as a therapeutic agent, and the relative bioavailability of CUR has been highlighted as a major problem for this. Studies over the past three decades related to absorption, distribution, metabolism and excretion of CUR irrespective of the route of administration have revealed poor absorption, rapid metabolism and elimination of CUR that severely curtails its bioavailability.

To improve the bioavailability of CUR numerous approaches have been undertaken. Some of the possible ways to overcome these problems are discussed below.

Adjuvants

Piperine, a known inhibitor of hepatic and intestinal glucuronidation, was combined with CUR and administered in rats and healthy human volunteers by Shoba *et al* (Shoba *et al.*, 1998a). It was observed that concomitant administration of piperine produced 2000% increase in bioavailability (Shoba *et al.*, 1998b).

Nanoparticles

Recently, targeted and triggered drug delivery systems accompanied by nanoparticle technology have emerged as prominient solutions to the bioavailability of therapeutic agents. Nanoparticle based delivery systems will probably be suitable for highly hydrophobic agents like CUR circumventing the pitfalls of poor aqueous solubility. A recent study has reported the synthesis, physiochemical characterization and cancer related application of a polymer based nanoparticle of CUR namely "nanocurcumin" with less than 100 nm size (Bisht *et al.*, 2007). Nanocurcumin was found to have similar *in vitro* activity as that of free CUR in pancreatic cell lines.

Liposomes, Micelles and Phospholipid Complexes

Liposomes are excellent drug delivery systems since then can carry both hydrophilic and hydrophobic molecules. Li *et al* investigated the *in vitro* and *in vivo* antitumor activity of liposomal CUR against human pancreatic carcinoma cells and demonstrated that liposomal CUR inhibits pancreatic carcinoma growth and, in addition, exhibits antiangiogenic effects.

Miscelles and phospholipid complexes can improve the gastrointestinal absorption of natural drugs, thereby giving higher plasma levels and lower kinetic elimination resulting in improved bioavailablility. The intestinal absorption of CUR and micellar CUR formulation with phospho-lipid and bile salt was evaluated using an *in vitro* model consisting of everted rat intestinal sacs. This study suggested biological transformation of CUR during absorption. Further, the *in vitro* intestinal absorption of CUR was found to increase from 47% to 56% when the same was present in micelles (Bisht *et al.*, 2007). In a separate study about 1.5 fold increase in CUR half-life in rats was found for the CUR- phospholipid complex over free CUR.

Derivatives and Analogues

The chemical structure of CUR plays a pivotal role in its biological activity. For example isomerisation has been proved to have an influence on antioxidant activity of CUR. Numerous studies dealing with the enhanced biological activity of CUR derivatives and/or analogues can be found in the literature (Mosley *et al.*, 2007; Ohori *et al.*, 2006). A CUR analogue designated EF-24 was reported to be a lead compound displaying increased antitumor action *in vitro* and *in vivo* in comparison to CUR.

Another strategy to improve the biological activity of CUR was to chelate it with metals. The presence of two phenolic groups and one active methelene group in CUR molecule makes it an excellent ligand for any chelation. Several metal chelates of CUR are reported to possess biological activity over that of free CUR. Copper complexes of CUR and its derivatives were found to be better antitumor agents than were the parent compounds. Studies by Sui (Sui *et al.*, 1993), showed that the modest activity of CUR as an *in vitro* inhibitor of HIV-1 and HIV-2 proteases is enchanced more than 10 fold when CUR is complexed with boron. Similarly, it was demonstrated that CUR-manganese complex exhibited a more potent neuroprotective activity than CUR both *in vitro* and *in vivo*. A vanadyl CUR complex, a series of gallium and indium complexes were reported to show improved biological activity over CUR (John *et al.*, 2002; Vajragupta *et al.*, 2003).

Bioconjugates

Bioconjugates can increase the cellular uptake and hence better bioavailability of CUR. For example, BCM-95 (also called Biocurcumax) curcuminoids combined with turmeric oil (turmerons) in a specific proportion enhanced the bioavailability and showed better absorption into blood and had longer retention time compared to CUR. This product showed 700% more activity and 7-8 times more bioavailability over CUR as confirmed by human clinic trials (Mishra *et al.*, 2005).

1.6 Aims and objectives of the present study

The search for novel and new antifungal drugs continues since we still have a limited repertoire of effective antifungal drugs. Thus, the availability of a limited number of antifungals and prolonged usage of azoles has lead to the emergence of clinically resistant species. One of the most clinically significant mechanisms of azole resistance in pathogenic yeast *C. albicans* is the over-expression of multidrug transporter protein Cdr1p or CaMdr1p. This is well-established with the reports from FLC resistant clinical isolates of *C. albicans* where the enhanced expression of Cdr1p and CaMdr1p has been shown to help the pathogen to efflux this therapeutic azole and hence facilitate its own survival. Therefore, it is necessary to discover new classes of antifungal compounds that could either block these drug transporters more efficiently and specifically or there should to novel drug targets to cure the fungal infections.

In view of the above background and considering the success story of CUR as an effective modulator of ABC proteins involved in MDR of tumor cells and the pharmacological safety and efficacy which is associated with this natural polyphenol, in the present study, a considerable attention is being devoted towards assessment of the various properties of CUR against the pathogenic yeast *C. albicans*. The thesis embodies three sections dealing with the role of CUR in the drug resistance of the fungal pathogen *C. albicans*.

The first section of this study explores the potency of CUR in modulating the efflux activity of fungal transporters. Our results demonstrate that CUR is a specific modulator of ABC transporters and has no impact on efflux activity mediated by MFS transporters.

The second section of the study explores the antifungal effects of CUR against the various species of *Candida*. The mechanism of antifungal action is shown along with the effect of CUR on the morphogenesis of *Candida*.

Third and the last section deals with, the synergistic effect of CUR-I with azoles and polyenes in azole resistant clinical isolates of *C. albicans*.

The three sections of the thesis are titled as-

I. Curcumin modulates efflux mediated by yeast ABC multidrug transporters and is synergistic with antifungals.

II. Antifungal curcumin induces reactive oxygen species and triggers an early apoptosis but prevents hyphae development by targeting the global repressor *TUP1* in *Candida albicans*.

III. Synergistic anticandidal activity of pure polyphenol Curcumin I in combination with azoles and polyenes generates reactive oxygen species leading to apoptosis.

Materials & Methods

2. MATERIALS AND METHODS

2.1 Materials

Rhodamine 6 G (R6G), commercial grade mixture of curcuminoids, commonly known as curcumin/CUR, protease inhibitors (PMSF, leupeptin, aprotinin, pepstatin A, TLCK and TPCK), bicinchoninic (BCA) acid protein determination kit, miconazole (MCZ), ketoconazole (KTC), itraconazole (ITR), anisomycin (ANI), cycloheximide (CYH), FK520, oligomycin, dinitrophenol (DNP), deoxyglucose (DOG), 3-(4, 5- Dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), nystatin (NYS), amphotericin B (AMB), 2', 7'- dichlorofluorescin diacetate (DCFHDA), pyrrolidinedithiocarbamate (PDTC), ascorbic acid (AA), polyethylene glycol (PEG), piperine (PIP), RPMI 1640 and other molecular grade chemicals were obtained from Sigma Chemicals Co. (St. Louis, Mo). Fluconazole (FLC) and voriconazole (VRC) were kindly provided by Ranbaxy Laboratories Limited, India. [^{3}H]-FLC (specific activity 19 Ci/mmol) was custom synthesized from Amersham Biosciences, UK and [^{3}H]-methotrexate (MTX, specific activity 8.60 Ci/mmol) was procured from Amersham Biosciences, UK. The radiolabeled [^{125}I] iodoarylazidoprazosin (IAAP) (2,200 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). Annexin V-FITC apoptosis detection kit was obtained from BD Biosciences (USA). Zymolyase 100T was purchased from Seikagaku Corporation, Japan.

2.1.1 Strains and growth media

The strains used in this study are listed in Appendix I. The detail of the composition of the media used is given in Appendix II. All the yeast strains were maintained at 30°C. For agar plates, 2.5% (*w/v*) bacto agar (Difco, BD Biosciences, NJ) was added to the medium. All strains were stored as frozen stocks with 15% glycerol at -80°C. Before each experiment, cells were freshly revived on YEPD (Yeast Extract Peptone Dextrose) plates from the stock. Clinical *Candida* isolates were obtained from the All India Institute of Medical Sciences (AIIMS), New Delhi, India.

2.1.2 Oligonucleotides

Oligonucleotides used for probe preparation for Northern blotting were commercially custom synthesized from Sigma-Aldrich Company, USA. Appendix III shows a complete list of all the oligonucleotides used in this study.

2.2 Methods

2.2.1 Extraction and isolation of CUR

The rhizomes of *Curcuma longa* were obtained from Central Institute of Medicinal and Aromatic Plants (CIMAP) field gene bank (Accession no. CIMAP-1554). The rhizomes were dried at room temperature (25-35°C), pulverized and stored at 10-15°C until extraction. The powdered rhizomes (145 g) were first defatted with hexane (1000 ml.) in a soxhlet apparatus (4 hrs) and then extracted with chloroform (1000 ml.) for 5 hrs. The chloroform solution was filtered and evaporated under vacuum (50°C) to afford a curcuminoid rich extract (5.221g, 3.6%). The crude mass (5.1g) was charged on silica gel (180g, 60-120 mesh, 5x70cm glass) column and eluted successively with hexane (800 ml), chloroform: hexane (20% to 80%, 1000 ml each), chloroform (2000 ml) and acetone-chloroform (1% to 5%, 2000 ml each). The fractions were collected and spotted on thin layer chromatography (TLC) aluminium sheets coated with Silica gel. Fractions that showed the same pattern on TLC (200 ml each) were pooled [CUR-I (fractions 39-63), CUR-II (fractions 67-73) and CUR-III (fractions 78-82) were obtained successively] and the organic solvent was removed to obtain the powder form. The purity of CUR I, II and III was analysed by high pressure liquid chromatography (HPLC) and was found to be 98.4%, 98.1% and 97.6%, respectively. CUR I was the major component (~77%) of the curcuminoid mixture which was used in present study. The purity of CUR-I, II and III was further confirmed by Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-TOF- MS) analysis.

MALDI-TOF MS with a Bruker Autoflex II TOF/TOF mass spectrometer (Bruker Daltonics, Germany) was carried out. The MS was performed on the reflectron mode at an acceleration voltage of 20 kV. The MS was calibrated with the external standards Bradykinin 1-7 (757.39 Da), angiotensin II (1046.5 Da), angiotensin I (1296.6 Da), substance P (1347.7 Da), bombesin (1619.8 Da), Renin-substrate (1758.9Da), ACTH 1–17 (2093.0 Da), ACTH 18–39 (2465.1 Da) and

Somatostatin 28 (3147.4 Da). Monoisotopic peptide masses were recorded. The spectra were processed by the Flex control and data processing software (Flex Analysis Autoflex II TOF/TOF, Bruker Daltonics, Germany) and the peaks annotated automatically and checked manually. After calibration, the spectra for intact mass analysis of CUR mixture and pure curcuminoids *viz.* CUR-I, CUR-II and CUR-III was acquired (Figure 9).

2.2.2 Efflux of Rhodamine 6G

Efflux of R6G was determined essentially using a previously described protocol (Shukla *et al.*, 2003). Briefly, approximately 1×10^6 yeast cells from overnight grown culture were transferred into YEPD media and allowed to grow for 5 hrs. Cells were pelleted, washed twice with phosphate-buffered saline (PBS, without glucose) and resuspended as 2% cell suspension which corresponds to 10^8 cells (*w/v*) in PBS without glucose. The cells were then de-energised for 45 min. in DOG (5mM) and DNP (5mM) in PBS (without glucose). The de-energised cells were pelleted, washed and then resuspended as 2% cell suspension (*w/v*) in PBS without glucose to which R6G was added at a final concentration of 10 µM and incubated for 40 min. at 30°C. The equilibrated cells with R6G were then washed and resuspended as 2% cell suspension (*w/v*) in PBS without glucose. Samples of 1 ml. volume were withdrawn at indicated time and centrifuged at 9000×g for 2 min. The supernatant was collected and absorption was measured at 527 nm. Energy dependent efflux (at indicated time) was measured after addition of glucose (2%) to the cells resuspended in PBS (without glucose). Glucose free controls were included in all the experiments. For competition assays, CUR (100 µM) was added to the de-energized cells 5 min. before the addition of R6G and allowed to equilibrate.

2.2.3 Measurement of drug accumulation

The accumulation of [^{3}H]-FLC (specific activity, 19 Ci/mmol) and [^{3}H]-MTX (specific activity, 8.60 Ci/mmol) was determined essentially by the methods described previously (Pasrija *et al.*, 2007). Briefly, cells from mid log phase (5×10^6) were centrifuged at 3000×g for 3 min. and resuspended in PBS as 2% cell suspension. For accumulation studies, 100 nM FLC and 25 µM MTX were routinely used (Pasrija *et al.*, 2007). 100 µM CUR was added 5 min. before the addition of drugs and allowed to equilibrate. 100 µl of cell suspension containing drugs/drugs + CUR were incubated at

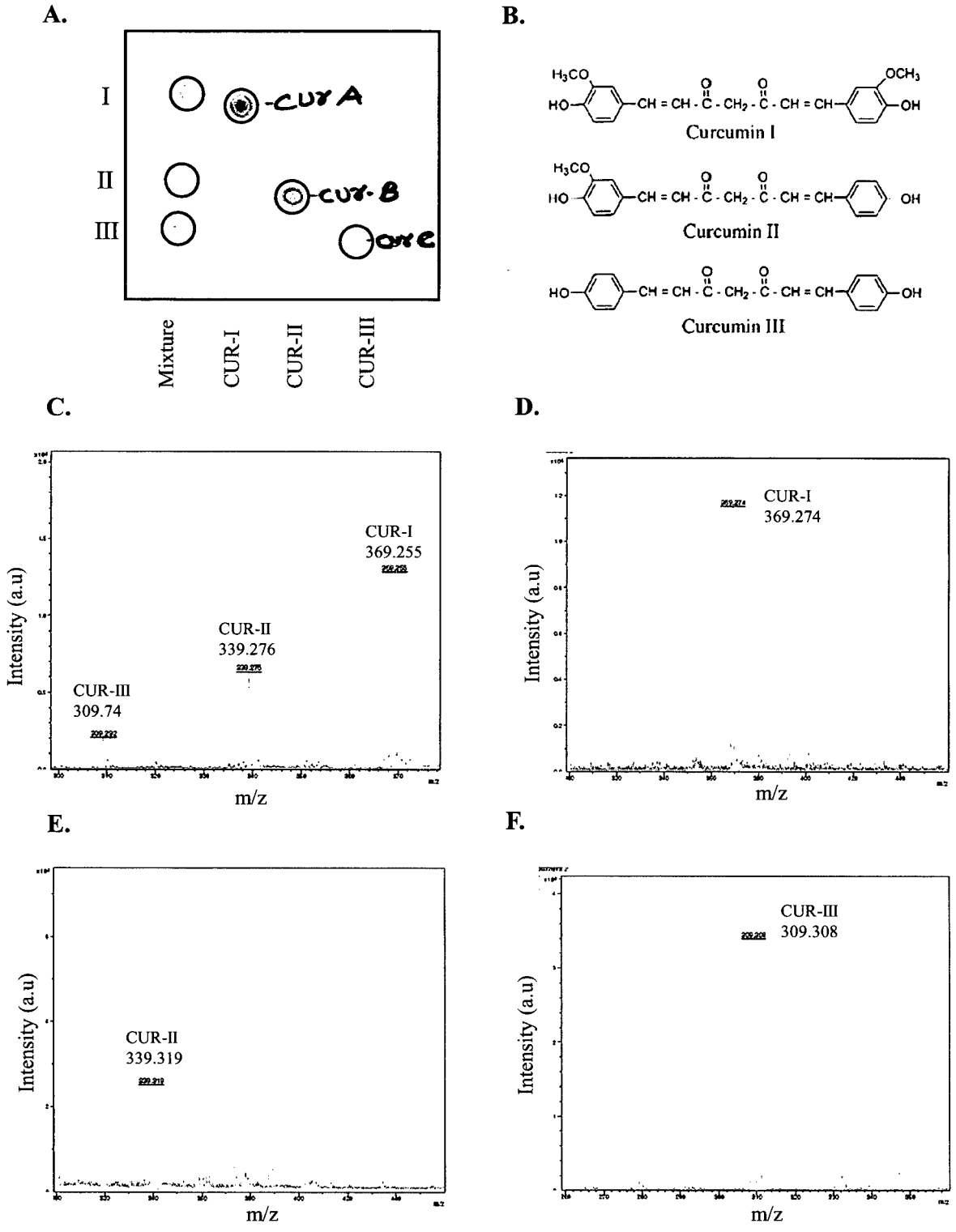


Figure 9. Determination of purity of curcuminoids.

(A) The crude ethanolic extract/ CUR mixture and pure form of curcuminoids collected from silica gel 60 column chromatography after eluting with $CHCl_3$ or $CHCl_3$/MeOH were subjected to TLC analysis. The extracts were spotted on Silica gel and conducted the TLC in solvent system of $CHCl_3$:Ethanol:acetic acid (94:5:1). I, II, and III indicates the position of CUR I, II and III, respectively. **(B)** The structures of CUR I, II, and III. **(C)** The histogram of CUR mixture and **(D)** Pure CUR-I **(E)** Pure CUR-II **(F)** Pure CUR-III as determined by MALDI TOF/TOF. The y-axis represents intensity of the peak and x-axis represents m/z ratio.

30^0C for 40 min, filtered rapidly and washed twice with PBS (pH 7.4) on Millipore manifold filter assembly using 0.45 μm cellulose nitrate filter (Millipore, USA). The filter discs were dried and put in cocktail 'O' and the radioactivity was measured in a liquid scintillation counter (Beckman, USA). The accumulation was expressed as pmoles/mg dry weight.

2.2.4 Transport of CUR

Briefly, approximately 1×10^6 yeast cells from overnight grown culture were transferred into YEPD media and allowed to grow for 5 hrs. Cells were pelleted, washed twice with phosphate-buffered saline (PBS, without glucose, pH 7.4) and resuspended as 2% cell suspension which corresponds to 10^8 cells (*w/v*) in PBS without glucose. The cells were then de-energised for 45 min. in DOG (5mM) and DNP (5mM) in PBS (without glucose). The de-energised cells were pelleted, washed and then resuspended as 2% cell suspension (W/V) in PBS without glucose to which CUR was added at a final concentration of 100 μM and incubated for 40 min. at 30°C. Samples of 1 ml. volume were withdrawn at indicated time and centrifuged at 9000×g for 2 min. The supernatant was collected and absorption was measured at 420 nm. Energy dependent efflux (at indicated time) was measured after addition of glucose (2%) to the cells resuspended in PBS (without glucose). Glucose free controls were included in all the experiments.

For measuring pH-mediated efflux, 2% cell suspension (in PBS, pH 7.4) was incubated with CUR (100 μM) for 40 min. in the presence of Carbonyl cyanide m-chloro phenyl hydrazone (CCCP, 100 μM) to allow accumulation of CUR by passive diffusion and then cells were pelleted and resuspended in MES buffer (pH 3.5) to initiate efflux. Samples of 1 ml. volume were withdrawn at indicated time and centrifuged at 9000×g for 2 min. The supernatant was collected and absorption was measured at 420 nm.

2.2.5 Isolation of crude and plasma membrane

Crude membrane (CM) from *S. cerevisiae* cells grown in YEPD (control or 100 μM CUR treated) to late exponential phase. The cells were broken with glass beads by vortexing the cells 4 times for 30 sec followed by 30-sec interval on ice. The homogenization medium contained 50 mM Tris pH 7.5 and 2.5 mm EDTA and the protease inhibitor cocktail (1mM PMSF, 1μg/ml leupeptin, pepstatin A and aprotinin).

The CM were recovered by centrifuging at 3500 rpm to remove unbroken cells and pelleting the CM by ultracentrifugation at 25,000 rpm for 1 hr. PM fractions were obtained from CM fractions by sucrose gradient centrifugation as described by Monk *et al.* (Monk *et al.*, 1991).

2.2.6 Immunodetection of ABC proteins

The PM protein concentration was determined by BCA using bovine serum albumin as the standard. The protein samples (20 µg) were separated on 8% SDS-PAGE gel (Appendix IV) and either stained with colloidal Coomassie G250 or electroblotted (40V, 1hr., 4°C) on to nitrocellulose membranes (Appendix IV). The Western blot analysis was conducted using anti-GFP monoclonal antibody (1:5000), as described previously (Shukla *et al.*, 2003). Proteins on immunoblots were visualized using the enhanced chemiluminescence assay system (ECL kit, Amersham Biosciences, Arlington Heights, IL.).

2.2.7 Confocal microscopy

The cells were grown in YEPD media to late log phase, washed and resuspended in appropriate volume of 50 mM HEPES pH 7.0 (Shukla *et al*, 2003). The cells were then directly viewed with 100X oil immersion objective on a Biorad confocal microscope (Radiance 2100, AGR, 3Q/BLD, Biorad, UK).

2.2.8 Flow cytometry and FACS analysis

Flow cytometric analysis of the Cdr1p-GFP and CaMdr1-GFP expressing cells was performed with a FACSort flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.). Cells were grown to mid-log phase, and 10^6 cells were harvested and washed with 50 mM HEPES (pH 7.0). Cells were resuspended in 500 µl of 50 mM HEPES (pH 7.0). Fifteen thousand cells were analyzed in acquisition. Analysis was performed with CellQuest software (Becton-Dickinson Immunocytomry Systems). The mean fluorescence intensity was calculated using the histogram stat program.

2.2.9. Photoaffinity labeling with IAAP

The crude membrane proteins (50 μg) prepared from AD-CDR1 cells (Shukla *et al.*, 2003) were incubated with CUR or with R6G for 10 min at 37°C in 0.1ml of 50 mM Tris-HCl (pH 7.5). The samples were brought to room temperature and 3 to 6 nM [^{125}I] Iodoarylazidoprazosin (IAAP) (2,200 Ci/mmol) was added and incubated for additional 5 min. under subdued light. The samples were then illuminated with a UV lamp assembly (PGC, Scientifics, Gaitherberg, MD) fitted with two black light (self filtering) UV-A long wavelength F15T8BLB tubes (365 nm) for 10 min at room temperature (21 to 23°C). 1 ml. of RIPA buffer was added to the samples and CaCdr1p cross linked with [^{125}I] IAAP was immunoprecipitated with 10 μg GFP monoclonal antibody (BD Biosciences, Palo Alto, CA) (Shukla *et al.*, 2003). The samples were then separated on 7% Tris-acetate gel at constant voltage, gels were dried and exposed to Bio-Max MR film (Eastman Kodak, Rochester, N.Y) at -80^{0}C for 12 to 24 hrs. The radioactivity incorporated into the CaCdr1p band was quantified using a STORM 860 phosphorimager system (Molecular Dynamics, Sunnyvale, Calif.) and the software Image QuaNT as described previously (Shukla *et al.*, 2003).

2.2.10 ATPase assay

The Cdr1p associated ATPase activity of the purified PM either alone or in presence of varying concentrations of ATP (0.5 mM to 7 mM) was measured as oligomycin sensitive release of inorganic phosphate. Membrane suspension (10 μg of PM protein) (Schaffner and Weissmann, 1973) was incubated at 30°C in 0.1 ml of a medium containing 59 mM Tris pH 7.5 and 7 mM $MgCl_2$ (ATPase assay buffer, Appendix IV) and 20 μM oligomycin where indicated. To eliminate possible contributions from non-specific phosphatases and vacuolar or mitochondrial ATPases, 0.2 mM ammonium molybdate, 50 mM KNO_3, and 10 mM NaN_3, respectively were included in the reaction mixture. The reaction was started by addition of ATP and was stopped by the addition of 0.1 ml of 5% SDS solution. The amount of inorganic phosphate released was determined immediately as described previously (Sarkadi *et al.*, 1992)

2.2.11. Susceptibility assays

The susceptibility of yeast cells to CUR alone or in combination with drugs was determined by two different methods. The drugs along with their respective solvents used in this study are listed in Appendix V.

2.2.11.1 Spot assay

The yeast cells were grown overnight on YEPD plates. The cells were then resuspended in normal saline to an A_{600} of 0.1. Five microlitres of five-fold serial dilutions of each strain was spotted on to YEPD plates either in the absence (control) or presence of range of CUR concentration. Growth was not affected by the presence of the solvents used. These assays with each drug were repeated at least 4-5 times.

2.2.11.2 Microdilution assay

Minimum Inhibitory Concentrations (MICs) of various drugs and CUR against the yeast cells was determined by broth microdilution using two fold serial dilutions in YEPD or RPMI 1640 medium, as described by the Clinical and Laboratory Standards Institute (CLSI, formely NCCLS) method M27-A. The test was carried out in 96-well flat-bottomed microdilution plates (100 µl/well) and different concentrations of drugs (Talibi and Raymond, 1999; Kohli *et al.*, 2002; Rukayadi *et al.,* 2009; Guo *et al.,* 2009). After agitation for 15 sec, the plates were incubated at 30°C without shaking and readings were performed after 48 hrs of incubation by visual reading and OD determination with a spectrophotometer set at 492 nm. Experiments were performed in triplicate, and the average MIC value was calculated. The susceptibility end point was defined as the lowest concentration of antifungal, which resulted in 80% inhibition of growth compared with that of the drug free control.

2.2.12 Cytotoxicity assay

The cytotoxic effect of CUR was determined by MTT assay (Chearwae *et al.*, 2004; Chearwae *et al.*, 2006a). Yeast cells (10^4) were seeded into 96 well plates in absence and in presence of varying concentrations of CUR (25-500 µM) and grown for 48 hrs at 30°C. 100 µl of MTT solution was added to each well and incubated for 3-4 hrs, 200 µl of isopropanol was added to stop the reaction. Absorbance was measured using a micro plate spectrophotometer at 570 nm with a reference

wavelength of 650 nm. Cell survival (% of control) = (mean absorbance in test well)/ (mean absorbance in control wells) × 100.

2.2.13 Checkerboard titer tests

The interaction of CUR-I with FLC/ KTC/ MCZ/ ITR/ VRC / NYS/ AMB was evaluated by the checkerboard method recommended by the NCCLS and expressed as the sum of the fractional inhibitory concentration (FIC) index for each agent. The FIC of each agent is calculated as the MIC of this agent in combination divided by the MIC of this agent alone (Rukayadi *et al.,* 2009; Guo *et al.,* 2009). In brief, serial double dilutions of the anticandidal compounds were prepared (μg/ml) ranging from 0.25-128 for FLC, 0.019-10 for KTC and ITR, 0.019-10 for MCZ and AMB, 0.078-2 for VRC, 0.029-15 for NYS, and 1.4-740 for CUR. After making drug dilutions, a 100 μl suspension of *Candida* strains adjusted to 5×10^5 CFU/ml was added to each well and cultured at 30°C for 48 hrs in RPMI 1640 medium. Then visual reading of MICs was performed, and OD_{492} values were measured. The background OD was subtracted from the OD of each well. Each isolate was tested in triplicate on different days. Each checkerboard test generates many different combinations and by convention the FIC value of the most effective combination is used in calculating the FIC index. FICI was calculated by adding both FICs: FICI=

FICA + FICB=$C_A^{comb}/MIC_A^{alone}+C_B^{comb}/MIC_B^{alone}$, where MIC_A^{alone} and MIC_B^{alone} are the MICs of drug A and B when acting alone and C_A^{comb} and C_B^{comb} are concentrations of drugs A and B at the isoeffective combinations, respectively. Off- scale MICs were converted to the next highest or next lowest doubling concentration. The FICI was interpreted as synergistic when it was ≤ 0.5, as antagonistic when > 4.0, and any value in between as indifferent (Rukayadi *et al.,* 2009; Guo *et al.,* 2009; Odds, 2003).

2.2.14 Filter disc assays

Filter disc assays were done as described earlier (Shukla *et al.,* 2003) in RPMI 1640 medium. 5 to 10 μl of either azole/ polyene alone or in combination with CUR were spotted on paper disks of 6mm. After incubation of the plates for 48 hrs at 30°C, the respective zones of inhibition were recorded.

2.2.15 Time kill assays

C. albicans cells at a concentration of 10^3CFU/ml were inoculated in YEPD/RPMI 1640 medium. The concentrations used are the MIC_{80} values used alone and in combination as mentioned in the results. At various pre-determined time points (0, 4, 8, 12, 16, 20 and 24 hrs, at 30°C incubation; agitation 200 rpm), a 100 µl aliquot was removed, serially diluted (10 fold) in 0.9% saline and spread on Sabourad dextrose agar plates. Colony counts were determined after incubation at 30°C for 48 hrs. The experiment was performed in triplicate. Synergism was defined as decrease of ≥ 2 $\log_{10}$ CFU/ml in antifungal activity produced by the combination compared with the more active agent alone after 24 hrs (Quan *et al.,* 2006).

2.2.16 *In vivo* antifungal susceptibility testing

C. albicans strain (ATCC 36082) was grown overnight on YEPD at 30°C and suspended in sterile normal saline to adjust the OD_{600} to 1.0. The final inoculum was prepared by 1:20 dilution of the original suspension. The CFU (colony forming units) per ml of inoculum was 5.5×10^7. CUR was dissolved in 20% PEG, FLC and PIP were dissolved in sterile water. Swiss albino mice (n=6) of either sex weighing 20±2g were procured from in house facility. Animals were taken 2-3 days prior to the start of experiments to acclimatize to the experimental environment. Feed and water were provided *ad libitum* during the entire study. We have complied with the ethical standards which were approved by the institutional ethical committee. All mice were infected with 200µl of the cell suspension (1×10^7 CFU) by intravenous (IV) route. Treatment started 1 hr post infection; in one group CUR alone (100mg/kg body weight) was intraperitoneally (IP) administered; a second group was dosed with CUR plus PIP (100 and 20mg/kg body weight, respectively) administered orally (PO), while in the third group reference standard FLC was given (50mg/kg body weight, PO). The fourth group was kept as untreated control and was administered vehicle (20% PEG, PO) only. In all the groups, a second dose was administered 6 hr. after the first dose and animals were treated for two days. The data was analyzed by Graph pad Prism version 5.1. The limit of detection for live *C. albicans* was 1.7 $\log_{10}$.

2.2.17 Measurement of Reactive Oxygen Species production

Endogenous amounts of reactive oxygen species (ROS) were measured by a fluorometric assay with 2', 7'-dichlorofluorescin diacetate (DCFH-DA) (Kobayashi *et al.*, 2002). Briefly, the cells were adjusted to an OD_{660} of 1 in 10ml of PBS and centrifuged at 2,500 g for 15 min. The cell pellet was then resuspended in PBS and treated with appropriately diluted pyrrolidinedithiocarbamate (PDTC) or ascorbic acid (AA) for 1 hr or was left untreated at room temperature (25°C). After incubation with CUR at 37°C for different time intervals as indicated, 10μM DCFH-DA in PBS was added. The fluorescence intensities (excitation and emission of 485 and 540nm respectively) of the resuspended cells were measured with a Spectrofluorometer (Varian, Cary Eclipse) and the images of DCF Fluorescence were taken by using a fluorescence microscope (Carl Zeiss, USA).

2.2.18 Analysis of apoptotic markers

After treatment the cells were harvested and washed in sorbitol/EDTA buffer (2 M sorbitol, 0.5 M EDTA, pH 8). Then, 6% cell suspension was made in this buffer and zymolyase added for spheroplasting (50mg zymolyase dissolved in 1ml sorbitol/EDTA buffer, Appendix IV). After two and a half hr, the spherolpasts were checked under microscope and then they were harvested, washed in binding buffer twice (10mM HEPES/NaOH, pH 7.4, 140 Mm NaCl, 2.5 mM $CaCl_2$ and 1.2M sorbitol). The cells were suspended in binding buffer to make the concentration 3×10^7 cells/ml. 100 μl of the cells were stained with propidium iodide (PI) and FITC labeled Annexin V by using the Annexin V-FITC apoptosis detection kit (BD Biosciences, USA) to assess cellular integrity and the externalization of phosphatidylserine (PS) as described earlier (Balzan *et al.*, 2004). The cells were analyzed by using a Fluorescence Activated Cell Sorter (FACS) caliber flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose , CA) using 488 nm excitation and a 515 nm band pass filter for FITC detection and a filter >560 nm for PI detection. A total of 10,000 events were counted at the flow rate. Data analysis was performed using Cell Quest software (Becton-Dickinson Immunocytometry Systems), (Balzan *et al.*, 2004).

2.2.19 Morphological studies

To check the hyphae status, the cells were grown in presence/absence of CUR alone or CUR+PDTC in liquid or solid (2.5% agar) YEPD with 10% fetal bovine serum (FBS) or in liquid spider media (Appendix II) and incubated for 6 hrs (in liquid media) or for 3 days (in case of solid media) at 37°C. Colony morphologies on solid plates and filamentation in liquid media were analysed microscopically (Carl Zeiss, USA) (Prasad *et al.*, 2005).

2.2.20 RNA isolation

Total RNA from the mid-logarithmically grown *C. albicans* cells was prepared in presence/absence of CUR. In a standard preparation (Manoharlal *et al.*, 2010), 10 ml of cells, optical density at 600 nm (OD_{600}) of 1.0, were pelleted and washed with 10 ml of ice-cold H_2O and spun at 5000 rpm. The pellet was resuspended in 1.0 ml of TRI® Reagent (Sigma) and 0.3 ml of ice-cold acid-washed 0·4-0·6 mm diameter glass beads (Sigma, St. Louis, MO, USA) were added and vortexed for 5 min. Chloroform (0.2 ml) without isoamyl alcohol was added and the tubes were shaken vigorously for 15 sec. The samples were incubated at room temperature for 15 min, followed by centrifuge at 12,000 × g for 15 min at 4°C. The upper colorless aqueous phase was transferred to a new tube and 0.5 ml of isopropanol was added. The tubes were incubated at room temperature for 10 min, followed by centrifugation at 12,000 × g for 10 min. and the pellet washed with 75% ethanol and recentrifuged. The pellet was air dried and resuspended in 100 µl of H_2O. For this preparation, all experiments were done with DEPC treated H_2O. DNA free RNA was prepared by treating total RNA with DNase RQ1 (promega). The OD_{260} and OD_{280} were measured, and the integrity of the total RNA was visualized by subjecting 2-5 µl of the sample to electrophoresis through a denaturing 1% agarose/2.2 M formaldehyde gel. The total RNA preparation isolated was stored at -80°C till further use.

2.2.21 Northern Blotting

For Northern analysis standard protocol was used (Sambrook *et al.*, 1989). The electrophoresed RNA was visualized on UV transilluminator. Northern transfer was performed overnight as given in standard laboratory protocol (Sambrook *et al.*, 1989) using Hybond^{TM} nylon membrane (Amersham). Briefly, RNA (25µg) was

electrophoresed in agarose gels, vaccum blotted onto a Hybond^{+} nylon membrane (Amersham Pharmacia Biotech New Zealand, Auckland, New Zealand), and fixed by UV irradiation. Membranes were hybridized with [α-^{32}P] dATP-labeled probes using Megaprime DNA labeling system (Amersham Pharmacia Biotech) for Northern blot hybridization under high-stringency conditions. Probes were derived from gene-specific sequences as mentioned in Appendix III. Relative intensities (RI) of mRNA signals in Northern hybridizations were developed by exposure of the hybridized membrane in a FLA5000 Fuji Phosphoimager.

Results & Discussion

Section-I

3.1 CUR Modulates Efflux Mediated By Yeast ABC Multidrug Transporters And Is Synergistic With Antifungals

3.1.1 Introduction

Over expression of ABC multidrug transporters, including P-glycoprotein (*ABCB1*), multidrug resistance protein (*ABCC1*) and mitoxantrone resistance protein (*ABCG2*) play a major role in the development of MDR in cancer cells (Litman *et al.*, 2001). Among the various strategies to combat MDR, blocking the functioning of MDR transporters represents an attractive approach (Gottesman *et al.*, 2002). Notably, several functional inhibitors of MDR proteins have been tested but thus far none are clinically successful due to the dose limiting toxic effect of the modulators.

To circumvent this problem in recent years, extensive efforts are underway in identifying natural inhibitors of MDR exporters, as natural products have the potential to yield a large number of new drugs. Curcuminoids, from rhizomes of *Curcuma longa* have been reported to reverse the drug resistance phenotype in cancer cells, over-expressing ABC transporters *viz. ABCB1, ABCG2* and *ABCC1* (Anuchapreeda *et al.*, 2002; Chearwae *et al.*, 2006a; Chearwae *et al.*, 2006b). Curcuminoids blocked the efflux of fluorescent substrates, calcein AM, rhodamine 123 and bodipy-FL-vinblastine in MDR cervical carcinoma cell lines over expressing *ABCB1,* and of mitoxantrone and pheophorbide A mediated by *ABCG2,* in HEK293 cells (Chearwae *et al.*, 2004; Chearwae *et al.*, 2006a).

In yeasts, including the pathogenic *Candida,* an up-regulation of multidrug transporter genes belonging to either ABC or MFS is frequently observed in the cells exposed to the drugs and leads to the phenomena of MDR (Smith and Edlind, 2002; Vermitsky and Edlind, 2004). In the case of clinical isolates of *C. albicans*, it has been established that ABC transporters CaCdr1p, CaCdr2p and MFS CaMdr1p are major MDR transporters which contribute to azole resistance. There are examples of compounds such as FK506, enniatins, milbemycins, synthetic D-octapeptides, cyclosporine A, isonitrile, disulfiram, ibuprofen and unnarmicins (Holmes *et al.*, 2008; Tanabe *et al.*, 2007), which inhibit fungal ABC transporters. Such inhibitors or chemo sensitizers probably directly act by affecting substrate binding and transport mediated by MDR efflux proteins.

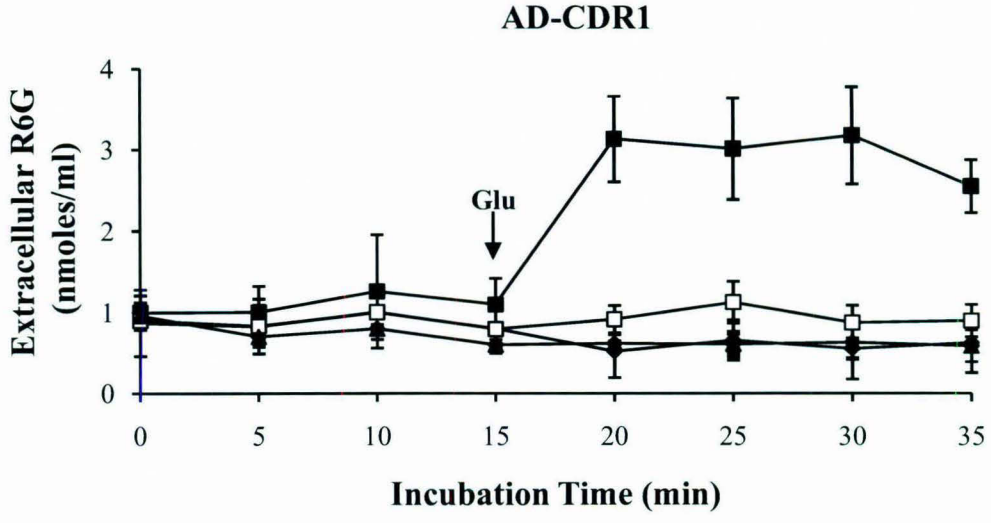
Notably, the effect of curcuminoids on fungal ABC transporters is not known. However, due to functional and structural similarities between *ABCB1* and ABC transporters in yeasts, it is very likely that the curcuminoids could act as "reversal agents" of drug resistance in yeast as well. In this study, we have examined the potency of CUR in modulating the efflux activity of CaCdr1p and have compared it with CaCdr2p and with ScPdr5p of *S. cerevisiae*. Our results demonstrate that CUR behaves as a specific modulator of Rhodamine 6 G (R6G) efflux mediated by CaCdr1p, CaCdr2p and ScPdr5p in *S. cerevisiae* cells over expressing these transporters. Notably, CUR did not have any impact on efflux activity mediated by MFS transporter CaMdr1p. Furthermore, CUR reversed drug resistance by displaying synergism with selected drugs.

3.1.2 Results

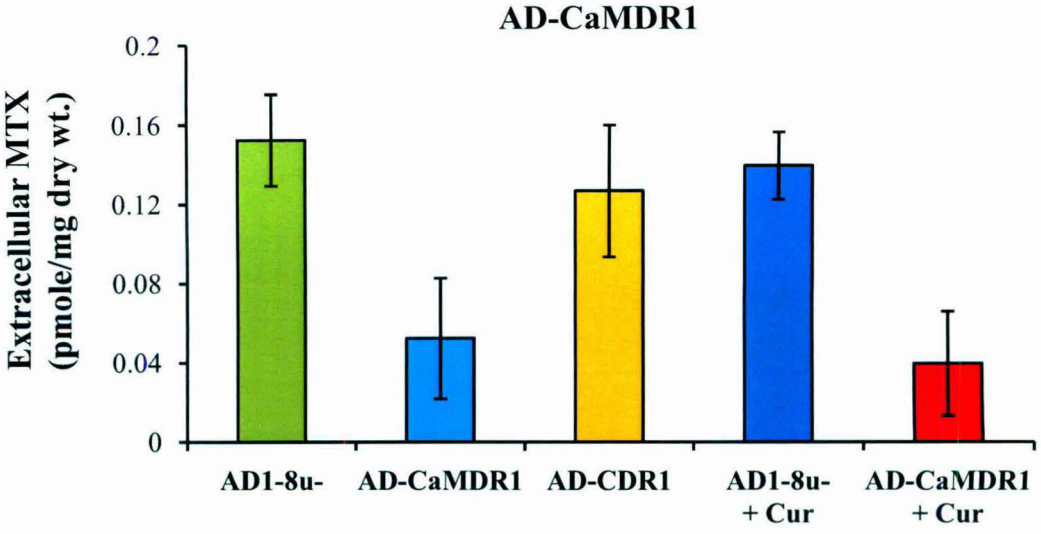
3.1.2.1 CUR inhibits R6G efflux

We used a commercial preparation of CUR to see its effect on MDR efflux proteins of pathogenic yeast *C. albicans*. For this, we monitored the efflux of R6G in cells where GFP tagged CaCdr1p (Cdr1p-GFP) was stably over expressed from a genomic *PDR5* locus in a *S. cerevisiae* mutant AD1-8u$^-$ (Nakamura *et al.*, 2001). The host AD1-8u$^-$ constructed by Goffeau's group (Decottignies *et al.*, 1998) was derived from a Pdr1-3 mutant strain with a gain of function mutation in the transcription factor Pdr1p, resulting in constitutive hyper induction of the *PDR5* promoter (Nakamura *et al.*, 2001). As depicted in Figure 10A, *S. cerevisiae* cells over-expressing CaCdr1p, showed energy dependent efflux of R6G, which was inhibited by CUR (100 μM). However, the addition of CUR had no effect on the leakage of preloaded R6G from deenergized *S. cerevisiae* cells. We tested if CUR could also affect a multidrug transporter belonging to the MFS, and we examined efflux mediated by CaMdr1p expressed in similar heterologous background. As shown in Figure 10B, the transport of well known substrate of CaMdr1p *viz* [^{3}H]-MTX remained unaffected by a fourfold excess of CUR. In Figure 10A, AD-CaMDR1 strain is used as a negative control for R6G transport and in Figure 10B AD-CDR1 strain is used as a negative control for MTX transport. The effect of CUR was also substrate specific because efflux of a well known substrate FLC remained unimpeded

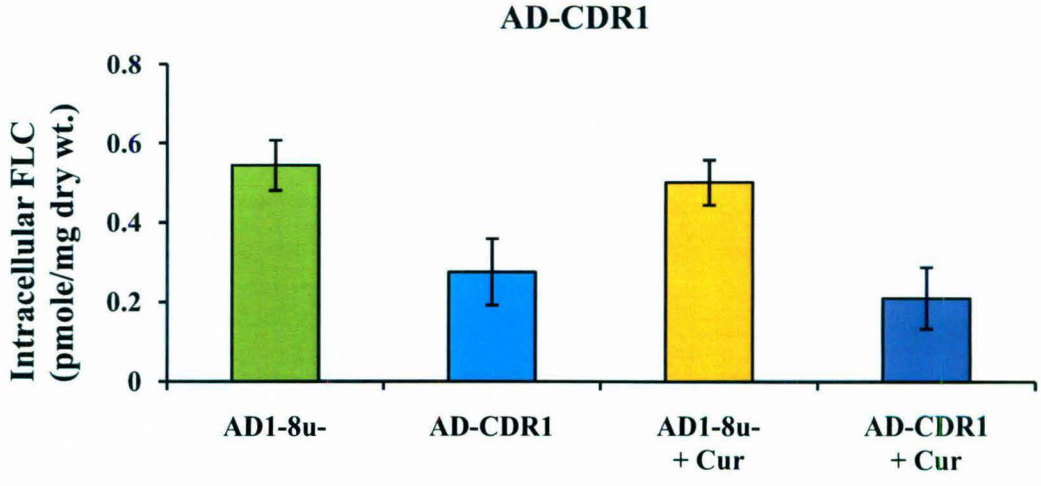
A.


AD-CDR1
Extracellular R6G (nmoles/ml)
Glu
Incubation Time (min)
0
1
2
3
4
0
5
10
15
20
25
30
35

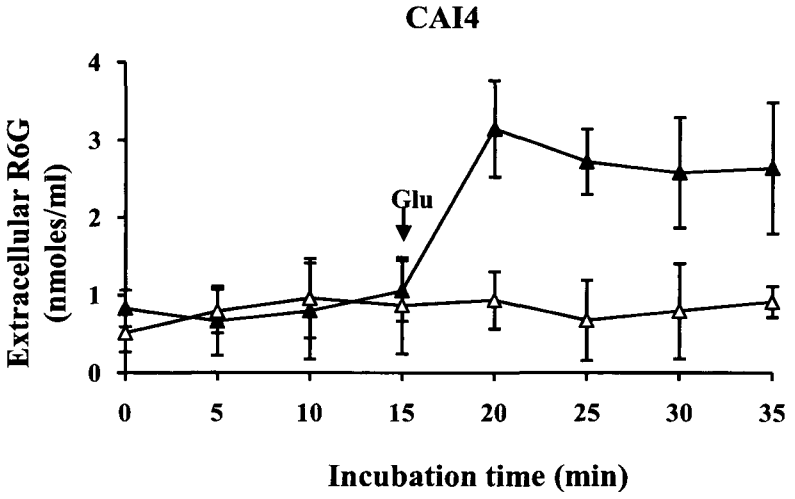
B.


AD-CaMDR1
Extracellular MTX (pmole/mg dry wt.)
0
0.04
0.08
0.12
0.16
0.2
AD1-8u-
AD-CaMDR1
AD-CDR1
AD1-8u- + Cur
AD-CaMDR1 + Cur

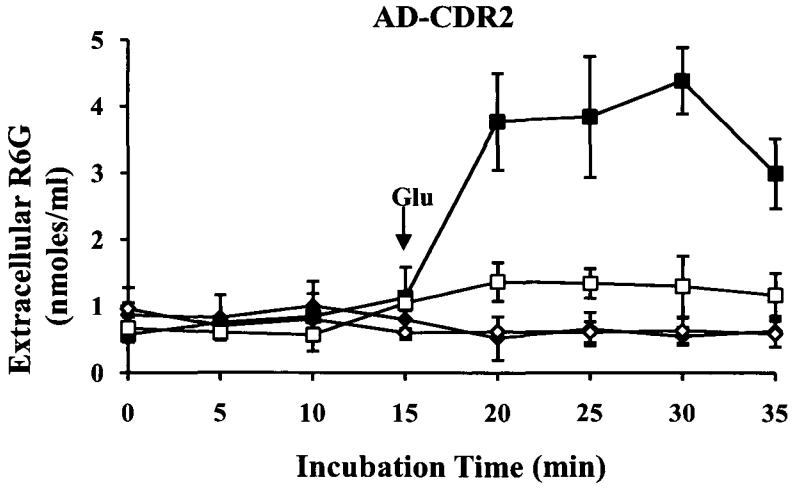
C.


AD-CDR1
Intracellular FLC (pmole/mg dry wt.)
0
0.2
0.4
0.6
0.8
AD1-8u-
AD-CDR1
AD1-8u- + Cur
AD-CDR1 + Cur

D.


CAI4
Extracellular R6G (nmoles/ml)
Glu
Incubation time (min)

E.


AD-CDR2
Extracellular R6G (nmoles/ml)
Glu
Incubation Time (min)

F.

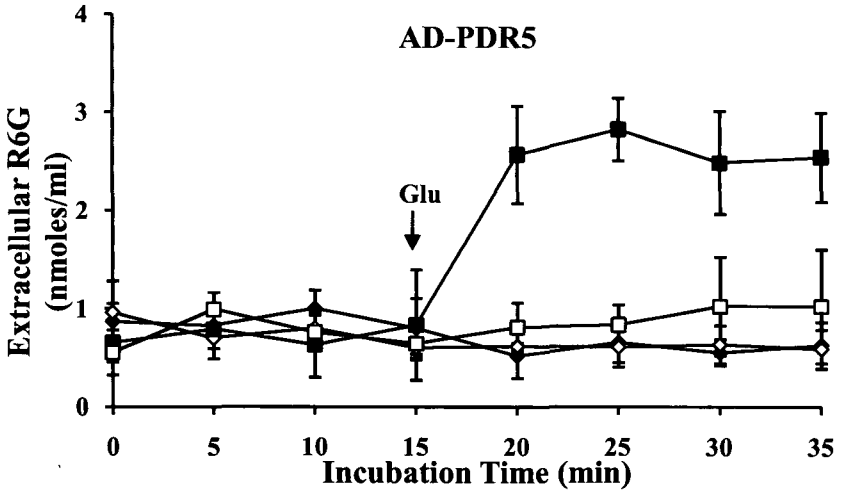

AD-PDR5
Extracellular R6G (nmoles/ml)
Glu
Incubation Time (min)

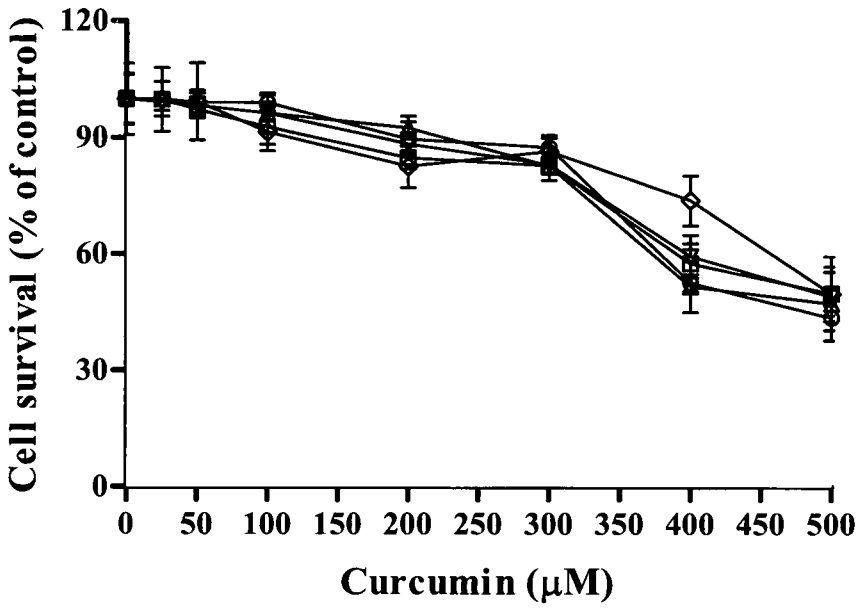
Figure 10. Effect of CUR on efflux of substrates in yeast cells.
(A) Extracellular R6G concentrations in *S. cerevisiae* control cells (AD1-8u$^-$) and in cells over expressing CaCdr1p (AD-CDR1), incubated with either R6G (10 μM) or R6G + CUR (100 μM) represented by filled/empty diamonds (AD1-8u$^-$) or filled/empty squares (AD-CDR1), respectively. Filled triangles represent AD-CaMDR1 cells. The energy dependent R6G efflux was initiated by adding glucose (2%, indicated by arrow) and quantified by measuring the absorbance of the supernatant at 527 nm. The values are the means and standard deviations (indicated by bars) of three independent experiments. **(B)** [^{3}H]-MTX accumulation in *S. cerevisiae* control cells (AD1-8u$^-$) and in cells overexpressing CaMdr1p (AD-CaMDR1). Cells were incubated with either [^{3}H]-MTX (25 μM, specific activity 8.60 Ci/mmol) or [^{3}H]-MTX + CUR (100 μM) represented by filled (grey)/empty bars, respectively. Filled bar (black) represents AD-CDR1 cells. The accumulated [^{3}H]-MTX was measured, after 40 min of initiation of efflux, using a liquid scintillation counter (Beckman, USA). The values indicated by the bars represent the means ± standard deviations (indicated by error bars) of three independent experiments **(C)** [^{3}H]-FLC accumulation in *S. cerevisiae* control cells and in cells over expressing CaCdr1p. Cells were incubated with either [^{3}H]-FLC (100 nM, specific activity 19 Ci/mmol) or [^{3}H]-FLC + CUR (100 μM) represented by filled/empty bars, respectively. The accumulated [^{3}H]-FLC was measured, after 40 min of addition of glucose (2%). The values indicated by the bars represent the means ± standard deviations (indicated by error bars) of three independent experiments. **(D)** Extracellular R6G concentrations in *C. albicans* strain, CAI4. Cells were incubated with either R6G (10 μM), or R6G + CUR (100 μM) represented by filled/empty triangles, respectively. The energy dependent R6G efflux was initiated by adding glucose (2%, indicated by arrow) and quantified by measuring the absorbance of the supernatant at 527 nm. The values are the means and standard deviations (indicated by bars) of three independent experiments. **(E)** Extracellular R6G concentrations in *S. cerevisiae* control cells (AD1-8u$^-$) and in cells over expressing CaCdr2p (AD-CDR2), incubated with either R6G (10 μM) or R6G + CUR (100 μM) represented by filled/empty diamonds (AD1-8u$^-$) or filled/empty squares (AD-CDR2), respectively. **(F)** Extracellular R6G concentrations in *S. cerevisiae* control cells (AD1-8u$^-$) and in cells over expressing ScPdr5p (AD-PDR5), incubated with either R6G (10 μM) or R6G + CUR (100 μM) represented by filled/empty diamonds (AD1-8u$^-$) or filled/empty squares (AD-PDR5), respectively. The energy dependent R6G efflux was initiated by the addition of glucose (2% indicated by arrows) and quantified by measuring the absorbance of the supernatant at 527 nm. The values are the means and standard deviations (indicated by bars) of three independent experiments.

in CaCdr1p expressing *S. cerevisiae* cells even though CUR was supplied in thousand fold excess(Figure 10C). Of note, CUR could also modulate R6G efflux in *C. albicans* cells (Figure 10D); however, for subsequent studies we used *S. cerevisiae* strain over expressing MDR transporters.

3.1.2.2 CUR selectively modulates ABC transporters

Before evaluating if CUR affects drug transporters, we examined whether it affected the viability of cells. For this, the control and the transporter over expressing cells were exposed to various concentrations of CUR for 48 hrs. and cytotoxicity was determined by MTT assay (Chearwae *et al.*, 2004; Chearwae *et al.*, 2006a). The percent of viable cells was calculated to determine the IC_{50} values (Figure 11A). As depicted in Figure 11B, the IC_{50} values for control (AD1-8u$^-$) as well as of cells expressing various transporters (AD-CDR1, AD-CDR2, AD-PDR5 and AD-CaMDR1) were not very different and ranged between 410.6 ± 9.4 μM and 498 ± 5.5 μM. Further, our transport assays confirmed that CUR is not a substrate of ABC or MFS proteins, since the extra cellular concentration of CUR remained the same even after initiation of efflux (Figure 12A and B). Our data suggests that CUR interacts with the yeast transporters but these multidrug transporters may not transport it since the IC_{50} values and relative resistance factor were similar whether cells were over expressing a transporter or not. We evaluated if CUR effect is specific to ABC transporters, and we examined R6G efflux mediated by CaCdr1p homologues such as CaCdr2p and ScPdr5p which were expressed in similar background. It was observed that CUR could inhibit the efflux of R6G mediated by both the proteins (Figure 10E and 10F). Our transport assays confirmed that CUR is not a substrate of ABC or MFS proteins, since the extracellular concentration of CUR remained the same even after initiation of efflux (Figure 12). This was further confirmed by a cytotoxicity assay (Chearwae *et al.*, 2004; Chearwae *et al.*, 2006a) where the IC_{50}s were similar for cells that did and did not over express transporters (Figure 11). It should be mentioned that the functionality of GFP tagged versions of ABC and MFS transporters was similar to that of untagged proteins (Pasrija *et al.*, 2007; Shukla *et al.*, 2003). To further examine the effect of CUR, the ABC transporter CaCdr1p was selected for detailed functional analyses.

A.



B.

Strain	IC_{50} (μM)	Relative resistance factor
AD1- 8u⁻	421.6 ± 2.2	1
AD-CDR1	492.8 ± 6.3	1.16
AD-CDR2	410.6 ± 9.4	0.99
AD-PDR5	498 ± 5.5	1.18
AD-CaMDR1	454.6 ± 7.4	1.07

Figure 11. Effect of CUR on the viability of *S. cerevisiae* cells as determined by MTT assay.

(A) % cell survival in control cells (AD1-8u⁻) and in cells over expressing ABC/MFS transporters represented by empty circle (AD1-8u⁻), empty inverted triangle (AD-CDR1), empty triangle (AD-CDR2) empty diamond (AD-PDR5) and empty square (AD-CaMDR1), respectively. The experiments were conducted in triplicate and the values represent mean ± S.D. of three independent experiments. **(B)** Table depicts the IC_{50} values and the relative resistance factor for AD1-8u⁻, AD-CDR1, AD-CDR2, AD-PDR5 and AD-CaMDR1 in presence of CUR.

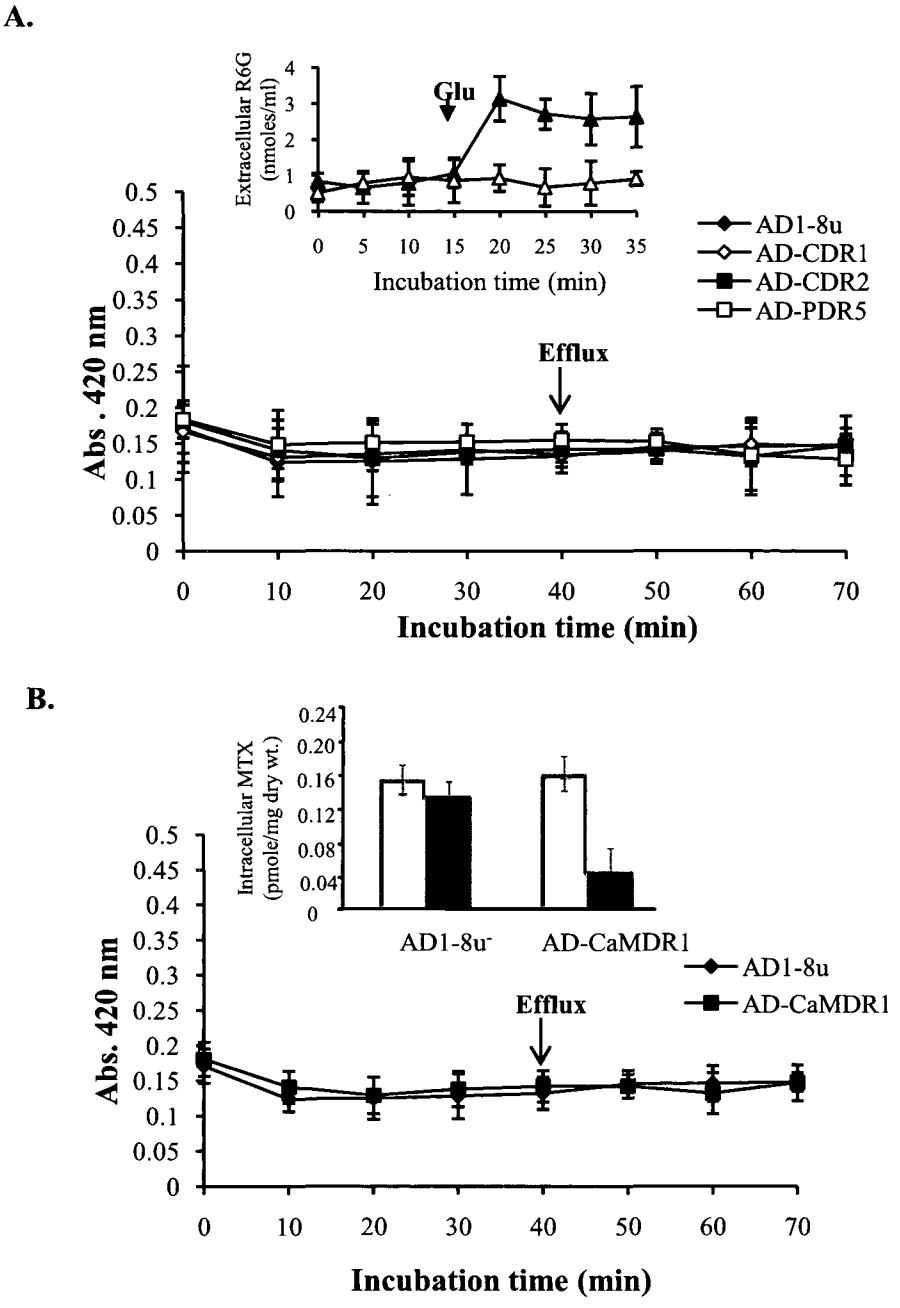


Figure 12. Transport of CUR by *S. cerevisiae* cells over expressing ABC or MFS transporters.

(A) Extracellular CUR concentrations in *S. cerevisiae* control cells (AD1-8u$^-$) and in cells over expressing CaCdr1p (AD-CDR1), CaCdr2p (AD-CDR2), ScPdr5p (AD-PDR5) represented by filled diamonds (AD1-8u$^-$)/ empty diamonds (AD-CDR1)/filled squares (AD-CDR2)/ empty squares (AD-PDR5), respectively. The values are the means and standard deviations (indicated by bars) of three independent experiments. Inset shows R6G transport (as control) in AD1-8u$^-$ (empty triangle), and AD-CDR1 cells (filled triangle). **(B)** Extracellular CUR concentrations in *S. cerevisiae* control cells (AD1-8u$^-$) and in cells over expressing CaMdr1p (AD-CaMDR1) represented by filled diamonds (AD1-8u$^-$)/ filled squares (AD-CaMDR1), respectively. The supernatant was collected and absorption was measured at 420 nm. Inset shows MTX transport (as control) in AD1-8u$^-$ and AD-CaMDR1 cells.

3.1.2.3 CUR competitively inhibits R6G efflux

Commercially available CUR is a mixture of three major curcuminoids as mentioned earlier and display a wide range of biological and pharmacological properties (Ammon and Wahl, 1991; Kuo *et al.*, 1996). We used purified curcuminoids (CUR-I, II and III) to see if these compounds show any selectivity as a modulator of R6G efflux. The efflux of R6G mediated by CaCdr1p was inhibited by all the pure forms of CUR in a concentration dependent manner with an IC_{50} value ranging between 40±5 and 45±5 μM (Figure 13A). The Lineweaver-Burk plot revealed that CUR competitively inhibits R6G efflux with an increase in apparent K_m (5.87 to 11.83 μM) but no effect on the V_{max} (Figure 14A).

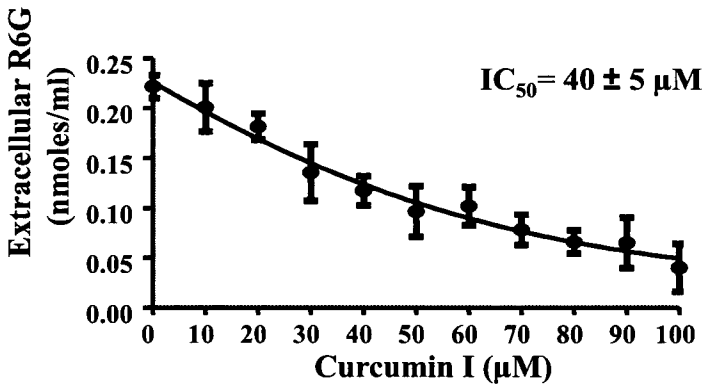
3.1.2.4 CUR inhibits drug binding and has no effect on ATPase activity and expression of CaCdr1p

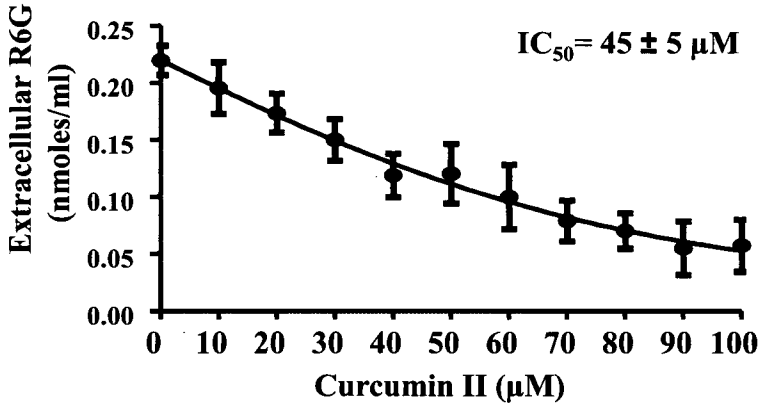
We had earlier shown that iodoarylazido prazosin (IAAP, a photoaffinity analogue of human P-gp substrate, prazosin) and azidopine (a dihydropyridine photoaffinity analogue of its modulator, verapamil) specifically bind to CaCdr1p (Shukla *et al.*, 2003). To monitor whether CUR affect drug binding, we labelled CaCdr1p with [^{125}I] IAAP as described in Materials and Methods. Figure 14B demonstrates that CUR effectively inhibited photo affinity labeling of CaCdr1p with [^{125}I] IAAP with IC_{50} value of 14.2 μM. We also monitored [^{125}I] IAAP labeling in presence of R6G as described in Materials and Methods. Interestingly, R6G could not inhibit [^{125}I] IAAP binding. In contrast to well known inhibitors of CaCdr1p ATPase activity such as vanadate, oligomycin, sodium azide and NEM (Krishnamurthy *et al.*, 1998; Shukla *et al.*, 2006; Shukla *et al.*, 2003) CUR at (100 μM) had no effect on the ATPase activity in presence of varying concentrations of ATP (Figure 14C). CUR also had no effect on the expression and localization of CaCdr1p and other ABC proteins (Figure 14D, E and F). Taken together, the data strongly indicate that although CUR is not transported, it acts as a competitive inhibitor at one of the transport sites used to transport clinically significant antifungal agents.

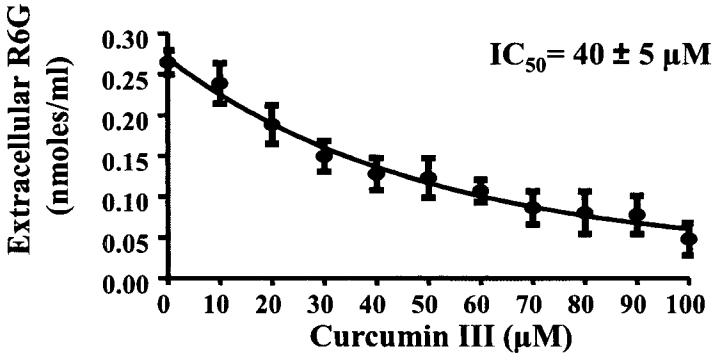
3.1.2.5 CUR displays synergism with selected azoles

When the control cells, AD1-8u$^-$, and the CaCdr1p expressing cells were grown either in the presence of drugs, FLC (6.52 μM), VRC (5.72 μM) MCZ (0.167

A.


Extracellular R6G
(nmoles/ml)
0.25
0.20
0.15
0.10
0.05
0.00
0 10 20 30 40 50 60 70 80 90 100
Curcumin I (μM)
IC50= 40 ± 5 μM


Extracellular R6G
(nmoles/ml)
0.25
0.20
0.15
0.10
0.05
0.00
0 10 20 30 40 50 60 70 80 90 100
Curcumin II (μM)
IC50= 45 ± 5 μM


Extracellular R6G
(nmoles/ml)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 10 20 30 40 50 60 70 80 90 100
Curcumin III (μM)
IC50= 40 ± 5 μM

B.

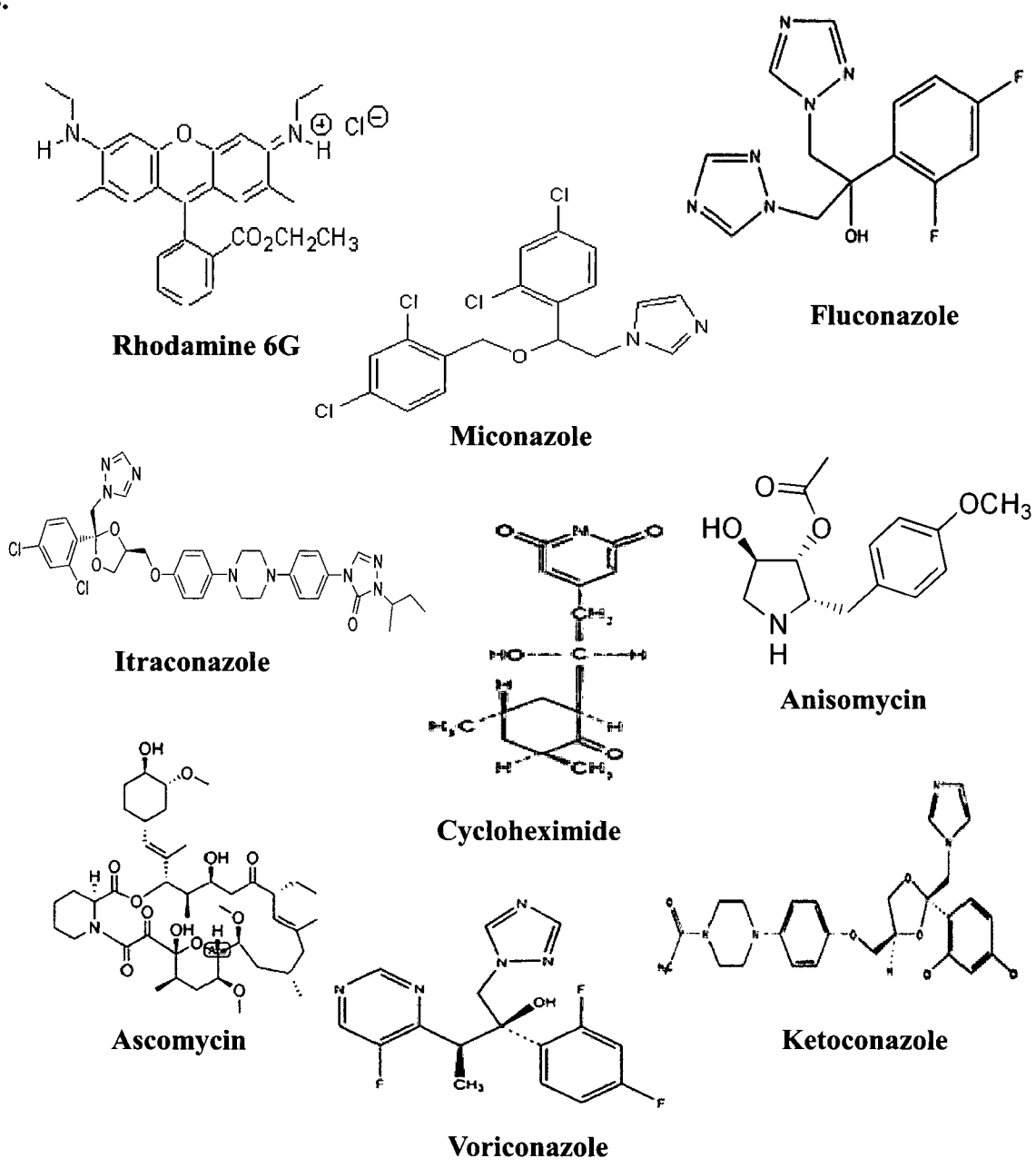
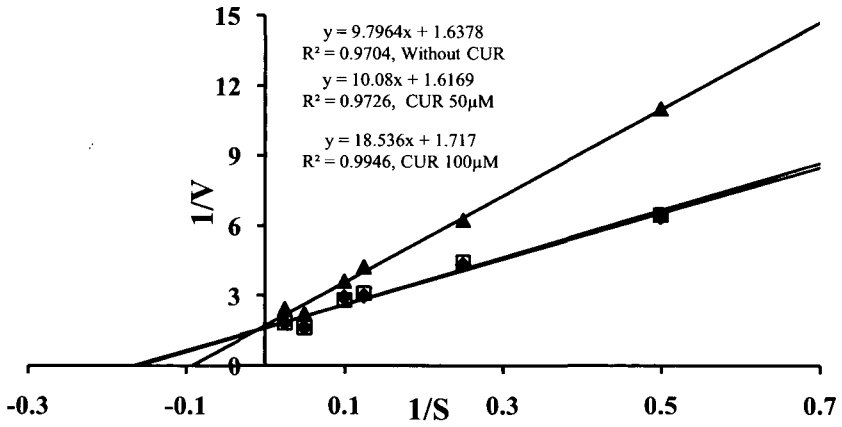


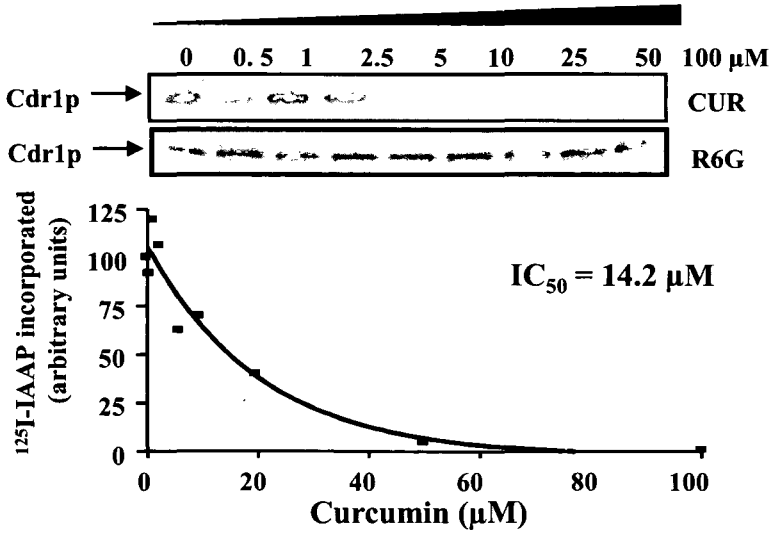
Figure 13. Effect of pure curcuminoids on R6G transport in *S. cerevisiae* cells over expressing CaCdr1p.

(A) Competition assay of R6G and CUR. CaCdr1p over expressing *S. cerevisiae* cells were incubated with either R6G (10 μM) or R6G (10 μM) + CUR (10-100 μM either I, II or III). R6G efflux was monitored after 40 min. of the addition of glucose (2%). The extra cellular R6G was quantified by measuring the absorbance at 527 nm. The data is plotted using Graph Pad Prism (http://www.graphpad.com/prism/Prism.htm). The values are the means and standard deviations (indicated by bars) of three independent experiments. **(B)** The structure of various substrates used.

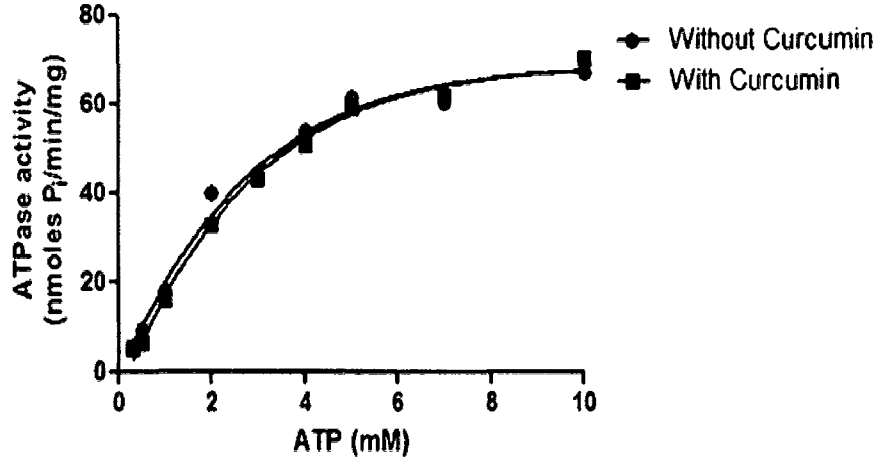
A.


y = 9.7964x + 1.6378
R² = 0.9704, Without CUR
y = 10.08x + 1.6169
R² = 0.9726, CUR 50μM
y = 18.536x + 1.717
R² = 0.9946, CUR 100μM
1/V
1/S
15
12
9
6
3
0
-0.3
-0.1
0.1
0.3
0.5
0.7

B.


0 0.5 1 2.5 5 10 25 50 100 μM
Cdr1p
CUR
Cdr1p
R6G
125I-IAAP incorporated (arbitrary units)
IC50 = 14.2 μM
Curcumin (μM)
125
100
75
50
25
0
0
20
40
60
80
100

C.


Without Curcumin
With Curcumin
ATPase activity (nmoles Pi/min/mg)
ATP (mM)
80
60
40
20
0
0
2
4
6
8
10

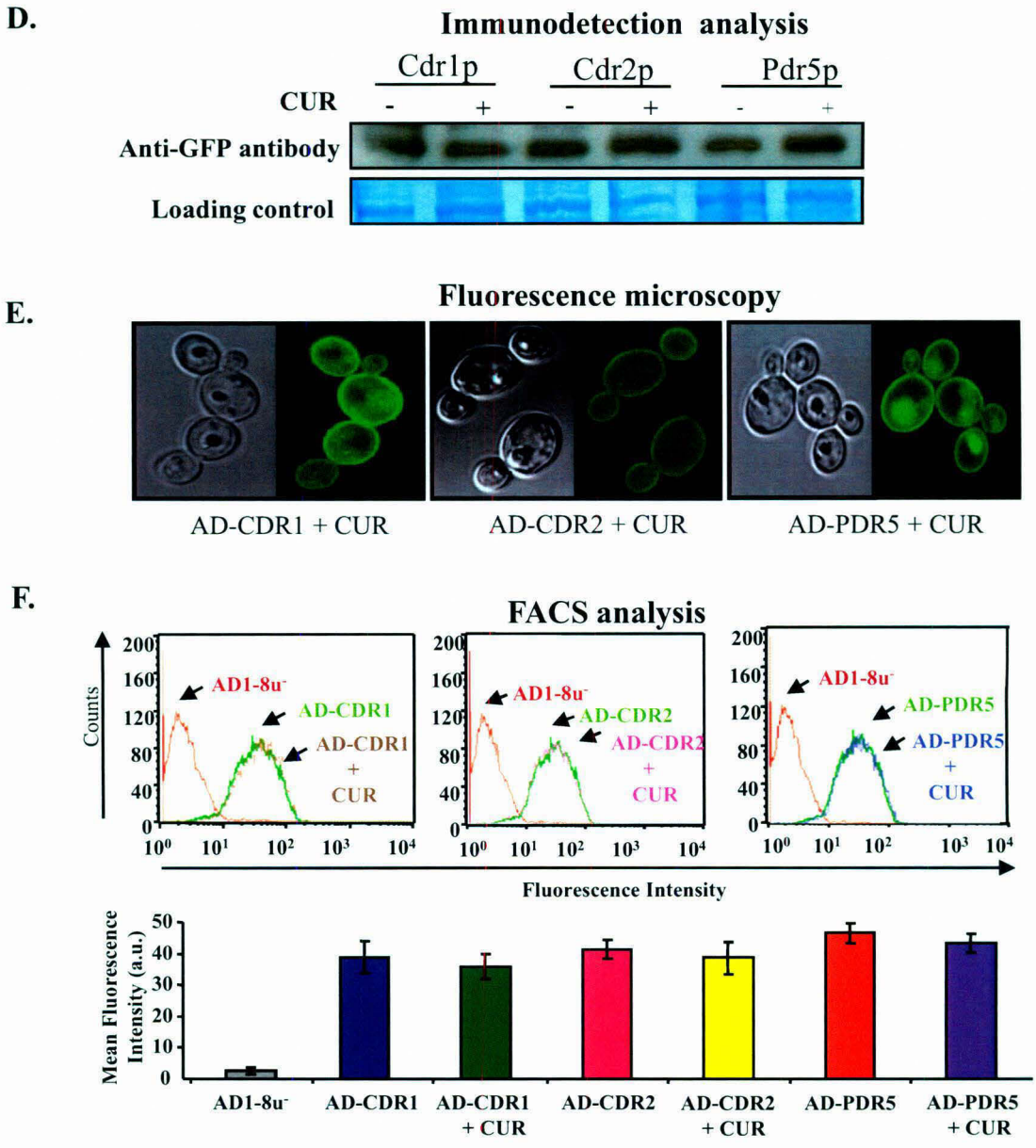


Figure 14. Biochemical analysis of CaCdr1p in presence of CUR.

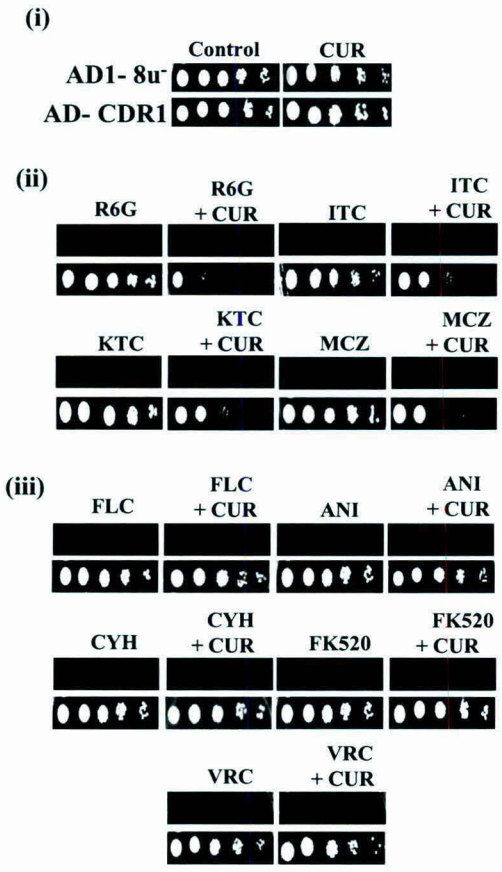
(A) Lineweaver-Burk plot of CaCdr1p mediated R6G efflux in presence of CUR after 5 min of addition of glucose (2%). Filled diamond/open square/filled triangle represents 0/50/100 μM of CUR, respectively. The rate of each reaction was calculated as nmoles of R6G released/min/5×10^6 cells. **(B)** Effect of CUR/ R6G on photoaffinity labeling of CaCdr1p with [^{125}I] IAAP. The autoradiogram represents the amounts of [^{125}I] IAAP incorporated into CaCdr1p in the presence of indicated concentrations of CUR (upper panel) and R6G (lower panel). The graph represents the amounts of [^{125}I] IAAP incorporated into CaCdr1p in the presence of indicated concentrations of CUR. **(C)** Effect of CUR on ATPase activity of CaCdr1p. Plasma membranes from cells over expressing CaCdr1p were incubated with and without 100 μM of CUR and varying concentrations of ATP (0.5 mM to 7 mM) in the ATPase buffer. The assay was performed essentially as described in the Materials and Methods. The data is plotted using Graph Pad Prism (http://www.graphpad.com/prism/Prism.htm). **(D)** Effect of CUR (100 μM) on expression of ABC proteins. Western blot analyses were done with an anti-GFP monoclonal antibody. Equal loading of protein was assessed by using a Coomassie stained gel. **(E)** Confocal microscopy and **(F)** Flow cytometry of *S. cerevisiae* cells expressing ABC proteins following CUR treatment.

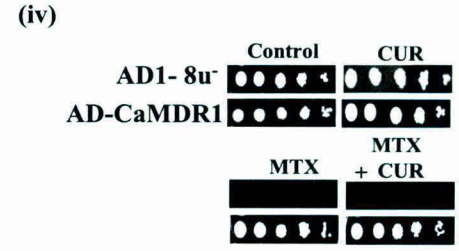
μM), KTC (0.037 μM), ITR (0.141 μM), ANI (2.97 μM), CYH (0.28 μM), FK520 (12.6 μM), R6G (0.209 μM), or in the presence of both CUR (75.6 μM) and the indicated drug, it was observed that CaCdr1p expressing cells displayed expected drug resistance and thus were able to grow in the presence of drug alone. Similar results were obtained with CaCdr2p and ScPdr5p expressing *S. cerevisiae* cells. However, the simultaneous presence of CUR with either R6G or azoles, *viz.*, KTC, ITR or MCZ, sensitized the cells, as evident by inhibition of growth of the cells (Figure 15A [ii]). Interestingly, the presence of CUR along with the non-competing drugs such as ANI, CYH, FLC, VRC and FK520 did not affect the level of resistance and the growth of cells expressing ABC proteins (Figure 15A [iii]). The observed inhibition in growth by CUR in presence of drugs was not due to loss of viability as determined by MTT assay (Figure 11). Notably, CUR (75.6 μM) alone did not inhibit growth of control cells (AD1-8u$^-$) and the cells over expressing CaCdr1p (Figure 15A [i]) or CaMdr1p (Figure 15A [iv]). The growth of CaMdr1p over expressing cells in the presence of MTX remained insensitive to CUR (Figure 15 A[iv]). We performed checkerboard assays in presence of CUR and various drugs. The FIC indices are below 0.5, for drugs such as KTC, ITR, MCZ and R6G in AD-CDR1 cells thus suggesting synergism with CUR (Figure 15B). Checkerboard analysis did not show any synergism for FLC, VRC, ANI, CYH and FK520 with CUR. Similar pattern of synergism between select drugs and CUR was observed with AD-CDR2 and AD-PDR5 cells.

3.1.2.6 CUR acts at the R6G transport site

We had recently subjected transmembrane segment 11 (TMS 11) of CaCdr1p to alanine scanning, replacing all 21 amino acids residues with alanine by site directed mutagenesis (Saini *et al.*, 2005). Out of 21 residues of TMS11, substitution of 5 residues, namely A1346G, A1347G, T1351A, F1360A and G1362A abrogated the efflux of R6G, while the rest of the mutant variants of CaCdr1p showed unaltered efflux (Figure 16A). Since CUR selectively modulates R6G efflux, we argued that if R6G and CUR share binding sites, abrogation of R6G efflux should affect its modulation by CUR. Interestingly, we observed that the ability of CUR to modulate R6G efflux was indeed considerably reduced in those mutant variants which showed abrogated efflux of R6G. Figure 16A depicts that in comparison to native CaCdr1p,

A.





B.

	FIC		FIC	FICI
KTC	0.250	CUR	0.005	0.255
MCZ	0.125	CUR	0.005	0.130
ITC	0.250	CUR	0.005	0.255
R6G	0.002	CUR	0.001	0.003

Figure 15. Synergistic effect of CUR on drug resistance.

(A) The control cells (AD1-8u$^-$) and the CaCdr1p expressing (AD-CDR1) *S. cerevisiae* cells were grown overnight on YEPD plates and then resuspended in normal saline to an OD_{600} of 0.1. The following stock solutions of drugs were used (the solvent used is given in parenthesis): R6G 1 mg/ml (DMSO), FLC 1 mg/ml (water), VRC 5 mg/ml (water), CYH 0.1 mg/ml (water), MCZ 1 mg/ml (methanol), KTC 1 mg/ml (methanol), ANI 1 mg/ml (DMSO), FK520 1 mg/ml (ethanol), MTX 1 mg/ml (10 mM Tris-Cl), CUR 11mg/ml (DMSO). Five microliters of fivefold serial dilution of each strain was spotted onto YEPD plates as described in material methods either in the (i) absence (control) or presence of (ii) R6G (0.209 µM), ITC (0.141 µM), KTC (0.037 µM), MCZ (0.167 µM), CUR (75.6 µM) and (iii) FLC (6.52 µM), ANI (2.97 µM), CYH (0.28 µM), FK520 (12.6 µM), VRC (5.72 µM) alone or in combination with CUR (75.6 µM) and (iv) MTX (11 µM), CUR (75.6 µM) alone or in combination. **(B)** Table depicts the interactions of CUR with KTC/MIC/ITC/R6G against AD-CDR1 cells, determined as FIC index. A FIC index ($\leq$ 0.5) interprets the synergistic interactions. The interaction of CUR with KTC/MCZ/ITC/R6G was evaluated by the checkerboard method recommended by the NCCLS and expressed as the sum of the fractional inhibitory concentration (FIC) index for each agent. The FIC of each agent is calculated as the MIC of this agent in combination divided by the MIC of this agent alone.

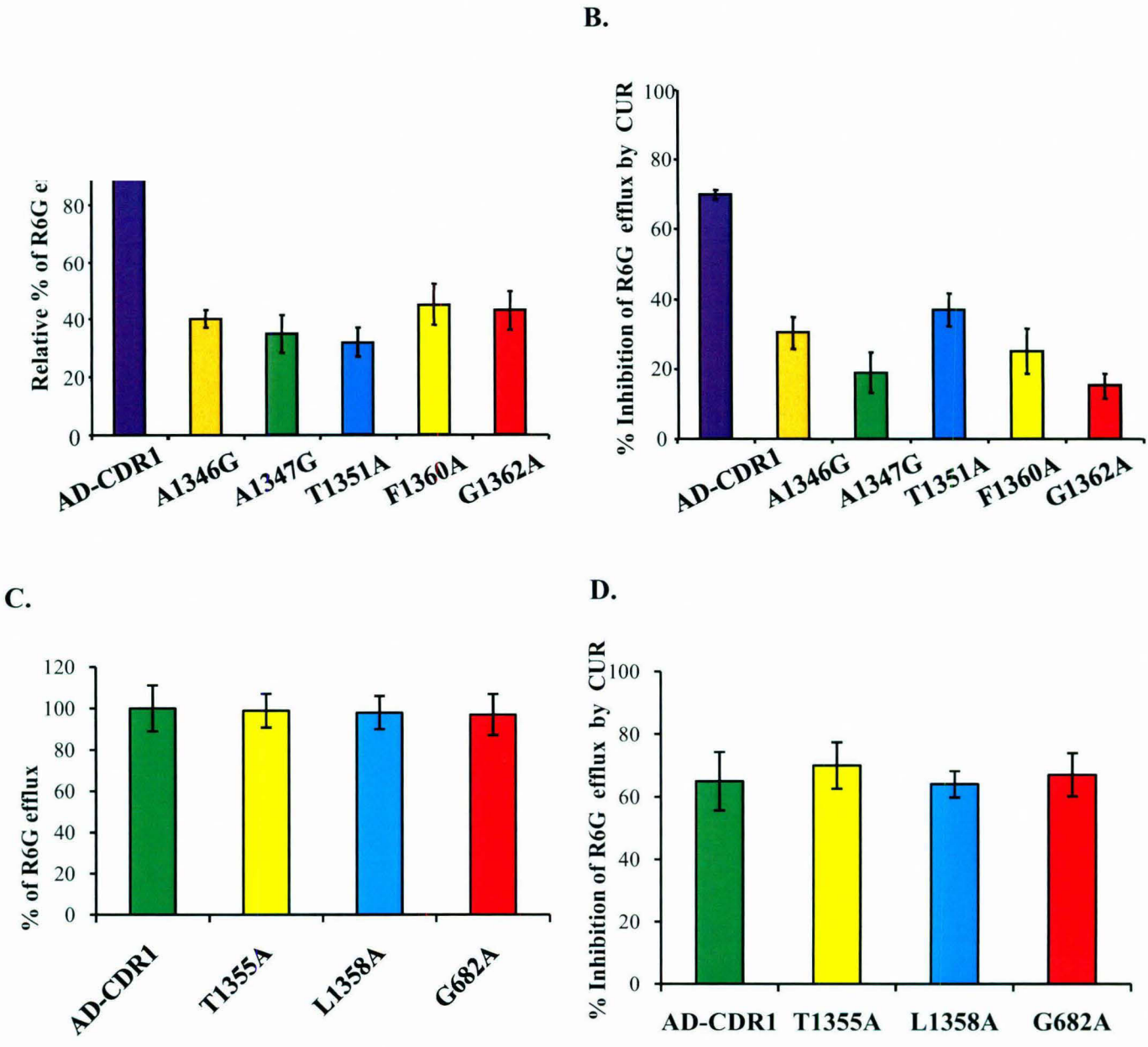


Figure 16. R6G transport in *S. cerevisiae* cells over expressing CaCdr1p or its mutant variants.

Extracellular R6G concentrations (expressed in %) in *S. cerevisiae* cells over expressing CaCdr1p (AD-CDR1) or mutant variants of CaCdr1p, incubated with either **(A)** and **(C)** R6G (10 μM) or **(B)** and **(D)** R6G (10 μM) + CUR (100 μM).The energy dependent R6G efflux was initiated by adding glucose (2%) and quantified by measuring the absorbance of the supernatant at 527 nm. The percentage inhibition by CUR was calculated taking each mutant R6G efflux value as 100%. The values are means ± standard deviations (indicated by bars) of three independent experiments.

the percentage inhibition of R6G efflux by CUR in mutant variants was considerably decreased. In addition, CUR inhibits R6G efflux even in those mutant variants which do not show abrogated efflux of the dye. This would suggest that in addition to common binding sites, CUR also has independent binding site (s) in CaCdr1p (Figure 16C and D). However, to resolve this issue, elaborate binding studies will be required.

3.1.3 Discussion

Among the 28 putative ABC and 95 MFS transporter genes identified in the *C. albicans* genome (Gaur *et al.*, 2005; Gaur *et al.*, 2008), there is overwhelming clinical and experimental evidence that only ABC transporters like CaCdr1p and CaCdr2p and MFS transporter, CaMdr1p are major determinants of azole resistance (Prasad *et al.*, 2006; Sanglard and Odds, 2002). The reversal of the functionality of these multidrug efflux pump proteins represents an attractive strategy to combat azole resistance. In this study, we explored whether CUR which inhibits the activity of the mammalian ABC multidrug transporters *ABCB1, ABCG2* and *ABCC1* (Chearwae *et al.*, 2004; Chearwae *et al.*, 2006a; Chearwae *et al.*, 2006b) could be exploited as a modulating agent of multidrug transporters of *C. albicans*. Our study reveals that CUR exclusively inhibits R6G transport in *S. cerevisiae* cells over expressing ABC drug transporters; CaCdr1p, CaCdr2p and ScPdr5p, and had no effect on efflux mediated by MFS transporter, CaMdr1p. All the three pure forms of CUR, i.e CUR I, II and III showed similar modulation of R6G efflux in *S. cerevisiae* cells expressing ABC transporters (Figure 13A). The modulatory effect of CUR was restricted to R6G while it had no effect on the efflux of another substrate FLC. We could observe a direct correlation between modulatory effect of CUR and the status of R6G efflux. For instance, those mutant variants of CaCdr1p which show abrogated efflux of R6G, also display decreased modulation by CUR.

Notably, R6G and FLC are both substrates of CaCdr1p but only the former gets competed with CUR (Figure 10A and C). If the structure of CUR is compared with the structures of R6G and FLC, it is apparent that electronic factors like number of π rings, extended π surface are some of the factors exist which could be important for CUR and other substrates such as ITR, KTC and MCZ which compete with R6G efflux (Figure 13B). In this context, it is noteworthy to mention that non-covalent π-π interactions have tremendous biological implications (Hunter *et al.*, 1990; Hunter *et*

al., 1991). On the other hand, if one considers FLC, VRC, ANI and CYH structures which do not compete with R6G, there are no such electronic factors but rather have a good number of tetrahedral sites. Therefore, these subtle differences in the properties between the structure of competing and non-competing substrates could explain as to why CUR is a selective modulator.

That the modulation of ABC transporter function would probably result in an increase in the intracellular concentration of the drugs to toxic levels became apparent from the growth studies. When CUR was used in combination, it was synergistic with drugs in cells over expressing ABC transporters. This synergism was restricted only to those drugs whose efflux was modulated by R6G (Saini *et al.*, 2005). Thus, the observed chemo-sensitization of cells by CUR was specific to competing drugs such as KTC, ITR and MCZ and was not the case with non-competitive drugs such as FLC, VRC, ANI, CYH and FK520. The fact that the presence of CUR along with some drugs did not inhibit the growth of cells not only points to its selectivity towards certain compounds but also suggests that R6G, KTC, ITR, MCZ and CUR may share overlapping binding sites of ABC multidrug transporter proteins. The modulatory and synergistic effect of CUR confirm our earlier result where we had observed that KTC, MCZ and ITR share CaCdr1p binding sites with R6G (Saini *et al.*, 2005).

A natural CUR mixture contains three major curcuminoids: CUR-I, CUR-II, and CUR-III. Various studies have tested these individual curcuminoids with mammalian ABC drug transporters P-gp, MRP1 and ABCG2 (Chearwae *et al.*, 2004; Chearwae *et al.*, 2006a; Chearwae *et al.*, 2006b). These individual curcuminoids inhibit the function of the above drug transporters with varying efficiency and CUR-I was most potent amongst them (Chearwae *et al.*, 2004; Chearwae *et al.*, 2006a; Chearwae *et al.*, 2006b). In addition, THC, a major metabolite of CUR also inhibits the above three mammalian ABC drug transporters (Limtrakul *et al.*, 2007). In this study, based on the above initial data with natural CUR mixture and its purified individual components on mammalian transporters, we evaluated CUR mixture alone for its activity to synergize the activity of antifungal agents. There are several CUR derivatives which are synthetic analogs and there may be a possibility that some of them may have better activity than the CUR mixture. Thus, these merit further study.

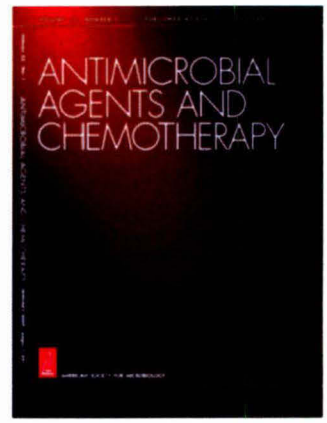
There are reports to suggest that CUR can down- regulate the expression of MDR linked transporter (ABCB1) and can even affect the function of several

transcription factors (Anuchapreeda *et al.*, 2002; Choi *et al.*, 2008). For this reason, we tested the effect of CUR on the expression of ABC transporter and observed that CUR did not affect the expression levels of CaCdr1p (Figure 14D), thus implying that the modulation of R6G efflux by CUR is restricted to its direct effect on the functionality of ABC transporter proteins. The direct effect of CUR on CaCdr1p was confirmed by its ability to compete photo affinity labelling of CaCdr1p with [^{125}I] IAAP (Figure 14B) and competitive inhibition of R6G efflux (Figure 14A). We excluded the possibility if CUR could be a preferred substrate of studied ABC transporters (Figure 12).

Our study shows that CUR, which is not a transport substrate of CaCdr1p, specifically modulates efflux of R6G mediated by the transporter. This is not surprising since it has been observed earlier that curcuminoids could modulate drug transport without being a substrate of mammalian *ABCG2* (Chearwae *et al.*, 2006a). Our cytotoxicity data (Figure 11) suggest that the presence or absence of efflux pump proteins did not affect the growth and viability of yeast cells which again points to the fact that CUR is not a substrate of these pumps. It is not clear, however, if CUR modulates R6G efflux by binding to substrate or allosteric site(s) of CaCdr1p. Considering the fact that the structure and substrate specificity of the fungal ABC transporters such as CaCdr1p, CaCdr2p and ScPdr5p are very different, our finding from this study that yeast transporters can be modulated by CUR is very significant.

It is reported that the poor bioavailability and low plasma concentrations of CUR decrease its effectiveness to modulate the function of ABC drug transporters in rodents and humans. However, recent studies indicate that the use of piperine to prevent CUR's glucuronidation as well as the use of encapsulation of CUR in liposomes can increase the absorption and plasma levels of the CUR (Shukla *et al.*, 2009). It is, however, not known whether CUR is metabolized via glucuronidation in yeast cells or whether the intracellular level of CUR is lower compared to the medium or plasma. These issues need to be resolved before CUR can be used as an effective *in vivo* or *in vitro* antifungal. In summary, the modulation of antifungal efflux by CUR is substrate and transporter specific. Nevertheless, curcuminoids are neither toxic to the cell nor are they transported by their target efflux pumps. Thus, their ability to sensitize cells to azoles opens up the possibility that it could be exploited in combination with conventional chemotherapy.

✓ The work presented in this chapter has been published as **"Curcumin modulates efflux mediated by yeast ABC multidrug transporters and is synergistic with antifungals"** in *Antimicrobial Agents and Chemotherapy* (2009) **53**: 3256-3265.



Section-II

3.2 Antifungal Curcumin Induces Reactive Oxygen Species And Triggers An Early Apoptosis But Prevents Hyphae Development By Targeting The Global Repressor *TUP1* In *Candida albicans*

3.2.1 Introduction

The dimorphic opportunistic pathogen, *Candida albicans* is normally a commensal organism in humans, but when the host is unable to mount an adequate immune response, as in AIDS, organ transplantation, diabetes, burn or in cancer patients, it results in mucosal, cutaneous or invasive mycoses (Odds *et al.*, 2003). Infections caused by *C. albicans* are commonly treated either by azoles or non-azole antifungal agents. Widespread and prolonged usage of antifungals, in recent years, has led to the emergence of azole-resistant strains of *Candida* which display MDR (White *et al.*, 1998). Various mechanisms, that contribute to the development of azole resistance have been reported which include over-expression of or point mutations in *ERG11*, the target enzyme of azoles, *i.e.* lanosterol 14α-demethylase (White *et al.*, 1998), and an over expression of the drug efflux pump encoding genes *viz CaCDR1*, *CaCDR2* and *CaMDR1* belonging to the ABC and MFS transporters, respectively (White *et al.*, 1998).

Although, mechanisms of antifungal resistance and major factors which contribute to it are fairly established, there is evidence to suggest that MDR is a multi-factorial phenomenon, which originates from yet unknown mechanisms. For example, morphological regulators such as Δ*efg1* (Prasad *et al.*, 2009), a homolog of bacterial two-component response regulators Δ*ssk1* and an iron deprivation display enhanced sensitivity to drugs in *C. albicans* (Chauhan *et al.*, 2007; Hameed *et al.*, 2008). There are azoles resistant clinical isolates of *C. albicans* where mechanisms of resistance appear to be different than the commonly known strategies adopted by *Candida* (White *et al.*, 2002).

The present study deals with the evaluation of antifungal activity of a natural plant polyphenol, CUR, produced by the rhizome of *Curcuma longa*. CUR, which is an important spice in Asian diet, has several important pharmacological properties, notably, antioxidant, antimutagenetic and antitumor activities (Anand *et al.*, 2007). It could block HIV-1 replication by inhibiting the activity of its long terminal repeat (LTR) and synergistically works with dideoxyinosine, a reverse transcriptase inhibitor

in HIV-1 cells (Li *et al.*, 1993). CUR alters cellular redox homeostasis, and disrupts mitochondrial function in cultured transformed cells (Fang *et al.*, 2005; Syng-Ai *et al.*, 2004).

In this study, we show that CUR can also be cidal to *C. albicans* as well as to non-*albicans* species, induces ROS levels and sets in early apoptosis which could be reversed by the addition of antioxidants in *C. albicans* cells.

3.2.2 Results

3.2.2.1 CUR inhibits growth of *Candida* cells

To investigate the antifungal effect of CUR on *C. albicans* cells, we used a commercial preparation of CUR which is a mixture of three major curcuminoids (Anand *et al.*, 2007). For this, we employed spot and broth microdilution drug susceptibility assays. CUR over a range of concentration was able to inhibit the growth of cells after concentrations ≥ 185 mg/L in a broth microdilution assay, while higher concentrations (296-370 mg/L) were needed to inhibit the growth in solid media (Figure 17A and B). The growth inhibitory effect of CUR was also evident with non-*albicans* species. For example, on solid media, CUR at ≥46.25 mg/L was able to inhibit the growth of *C. tropicalis, C. dubliniensis* and *C. utilis,* while ≥ 92.5 mg/L and ≥185 mg/L of it was needed to inhibit growth of *C. kefyr* and *C. krusei*, respectively. Both *C. parapsilosis* and *C. glabrata* required 370 mg/L CUR to show growth inhibition (Figure 17A and B). All the three purified curcuminoids from CUR did inhibit growth of *Candida* cells which was comparable to commercial preparation of CUR. For all the subsequent experiments, commercial CUR was used.

3.2.2.2 Co-administration of CUR and PIP reduces the *Candida* load *in vivo*

CUR has poor bioavailability due to its rapid metabolism in liver and intestinal walls. Co-administration of PIP, an inhibitor of hepatic and intestinal glucuronidation, is known to enhance the bioavailability of CUR (Shoba *et al.*, 1998). To determine the *in vivo* antifungal activity of CUR, against *C. albicans* (ATCC 36082), a systemic murine model of infection was employed (Wang *et al.*, 2009). As depicted in Figure 18, the effect of CUR was evaluated by comparing the live *Candida* load reduction in the kidneys of treated and untreated mice after administration of CUR or CUR+PIP. It

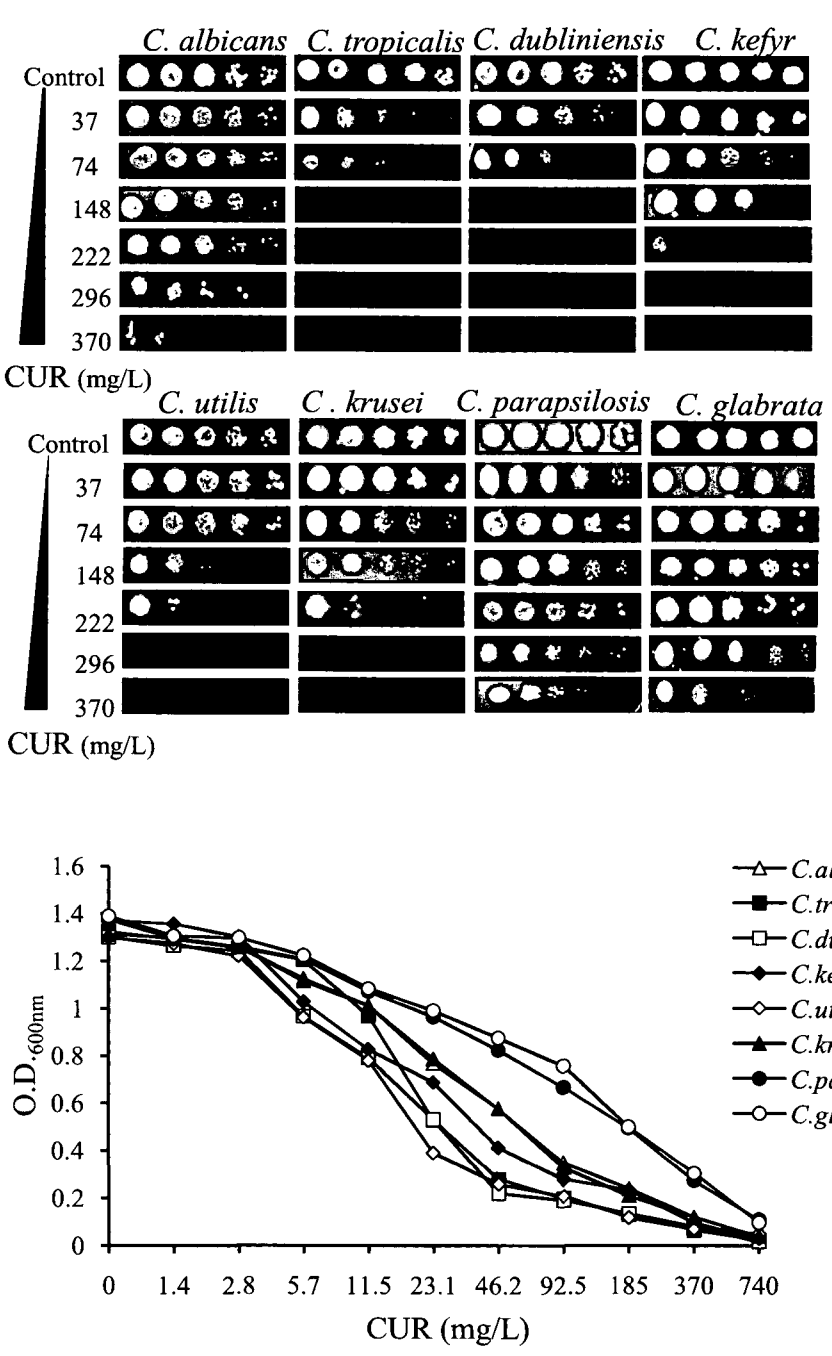
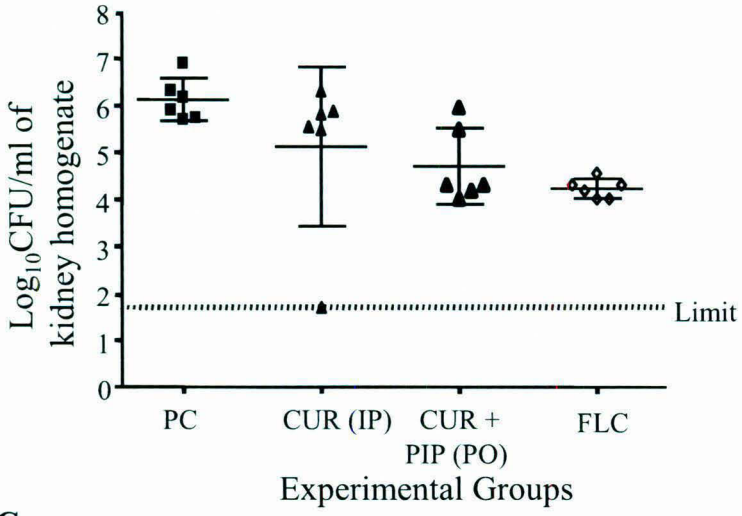


Figure 17. Effect of CUR on the growth of *Candida* cells.

(A) The cells were grown overnight on YEPD plates and then resuspended in normal saline to an OD_{600} of 0.1. Five microliters of fivefold serial dilution of each strain was spotted onto YEPD plates as described in material and methods, either in the absence or presence of varying concentrations of CUR (37-370 mg/L). **(B)** Determination of growth inhibition of the *Candida* cells by broth micro dilution assay in presence of CUR at concentrations ranging from 1.44-740 mg/L. Growth of the cells was evaluated both visually and by reading the absorbance (A_{600}) in a microtitre reader as described in material and methods. A stock solution of 11 mg/ml was used (DMSO). Growth was not affected by the presence of the solvent (data not shown). Filled/empty triangle, filled/empty square, filled/empty diamond and filled/empty circle represent *C. krusei, C. albicans, C. tropicalis, C. dubliniensis, C. kefyr, C. utilis, C. parapsilosis* and *C. glabrata*, respectively.

A.



B.

Groups (n=6)	Untreated (control)	CUR (IP)	CUR + PIP (PO)	FLC
Mean	6.12	5.12	4.71	4.22
S.D.	0.45	1.70	0.81	0.21
S.E.	0.18	0.69	0.33	0.09

C.

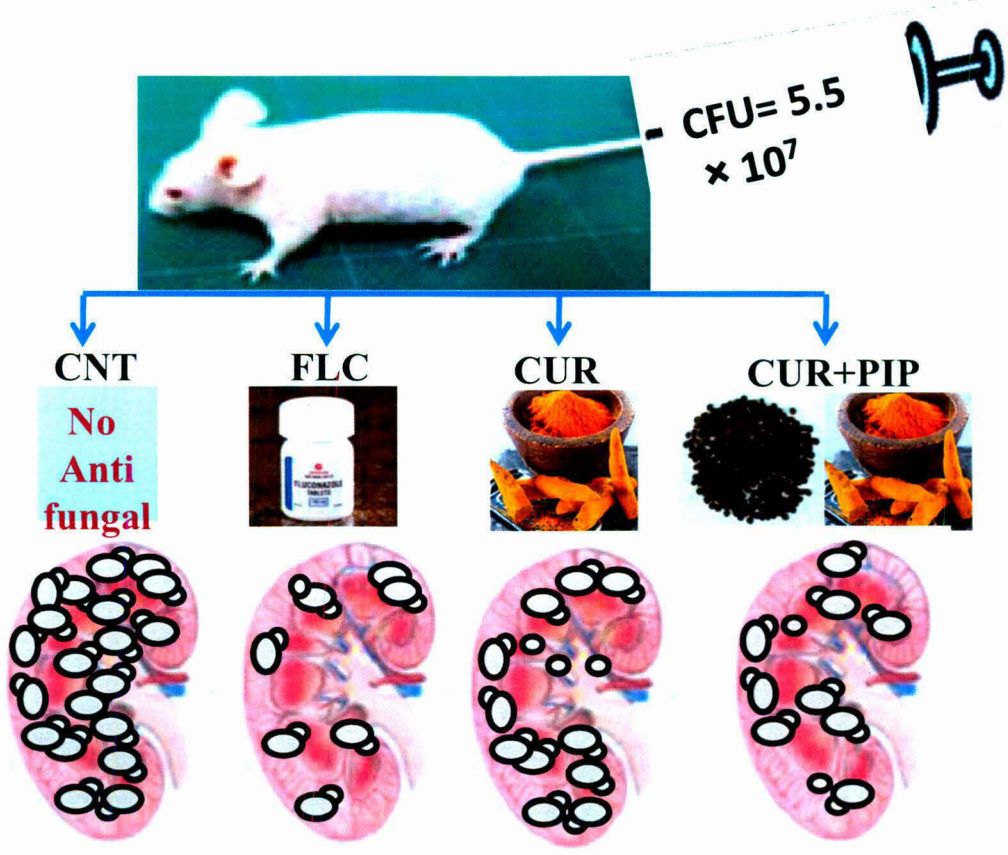


Figure 18. Kidney CFU assay of mice with systemic Candidiasis.
(A). *In vivo* efficacy of CUR against *C. albicans* (strain 36082) in murine systemic infection model. Swiss albino mice (n=6) of either sex weighing 20 ± 2g were used in four experimental groups. In one group, CUR was administered 100mg/kg body weight (IP, intra peritoneal), second group was dosed with CUR (100mg/kg) in combination with PIP (20mg/kg) administered concomitantly PO (oral administration), reference standard FLC (50mg/kg, PO) was given to the third group. Fourth group was kept as untreated control and was administered vehicle (20 % PEG and water) only. **(B)** Table represents the mean $\log_{10}$ CFU of *C. albicans* in the kidney of control (untreated) and treated groups. Statistical analysis was done using t test and results were considered significant when p values were less than 0.05. **(C)** Schematic representation of the *in vivo* experiment.

was observed that administration of CUR (IP) alone lead to an insignificant fungal load reduction of 1.0 $\log_{10}$ with a p value of 0.0887. However, the administration of CUR along with PIP (PO) causes a significant fungal load reduction of 1.4 $\log_{10}$ with a p value of 0.0199. In case of positive control FLC, there was a fungal load reduction of 1.9 $\log_{10}$, with a p value of 0.0029. Notably, MIC_{80} value of *C. albicans* (ATCC 36082) was similar to SC5314 (ATCC MYA2876) used in this study for the subsequent experiments.

3.2.2.3 Antifungal effect of CUR is independent of drug efflux pump

When the drug efflux pump (*CaCDR1*/*CaCDR2*/*CaMDR1*) (Sanglard *et al.*, 1996; Wirsching *et al.*, 2000) null mutants were grown in the presence of varying concentrations of CUR, the drug susceptibility pattern remained similar to the wild type strain (Figure 19A). The clinical matched pair of AS (azole susceptible) and AR (azole resistant) isolates which show increased resistance to azoles due to an over expression of either *CaCDR1*(Gu4,Gu5) (Franz *et al.*, 1999) or *CaMDR1* (F2,F5) (Franz *et al.*, 1998a) genes remained sensitive to CUR (Figure 19B). CUR also did not affect the expression of genes encoding MDR pump proteins (Figure 19C).

3.2.2.4 Oxidative stress null mutants are susceptible to CUR

To understand the molecular basis of antifungal activity of CUR, we evaluated its effect on various categories of mutants of *C. albicans*. These included morphological nulls Δ*tup1*, (Tup1p- a transcriptional co-repressor, represses filamentous growth (Braun *et al.*, 2001), Δ*efg1* (Efg1p- a transcriptional repressor required for hyphal growth (Stoldt *et al.*, 1997), Δ*cph1* (Cph1p - a transcription factor required for mating and hyphal growth on solid media (Stoldt *et al.*, 1997), Δ*nrg1* (Nrg1p- a transcription repressor regulates hyphal genes and virulence genes (Braun *et al.*, 2001), Δ*ras1* (Ras1p - a RAS signal transduction GTPase, regulates cAMP and MAP kinase pathways (Feng *et al.*, 1999), Δ*ssk1* (Ssk1p- a response regulator of two component system, role in cell wall biosynthesis and virulence (Calera *et al.*, 2000), oxidative stress nulls Δ*CaIpf7817*, (CaIpf7817p- putative NADH-dependent flavin oxidoreductase, involved in the regulation of redox homeostasis (Jia *et al.*, 2007) Δ*Cap1* (Cap1p- *C. albicans* AP-1, a transcription factor, that regulates the oxidative stress response (Alarco and Raymond, 1999; Wang *et al.*, 2006), Δ*tac1* (Tac1p- a transcription factor involved in the up regulation of *CDR1* and *CDR2* (Coste *et al.*,

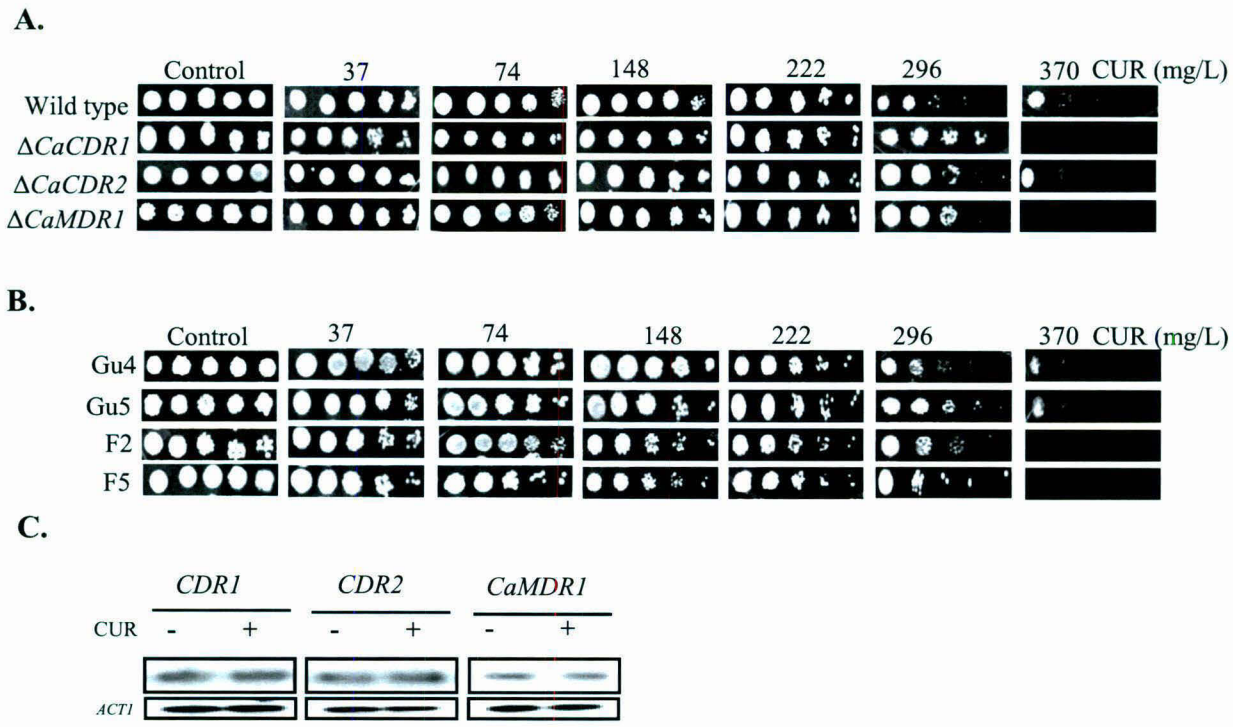


Figure 19. Antifungal effects of CUR against *C. albicans*, in cells either lacking or over expressing the drug efflux pumps.

The cells were grown overnight on YEPD plates and then resuspended in normal saline to an OD_{600} of 0.1. Five microliters of fivefold serial dilution of each strain was spotted onto YEPD plates as described in material and methods, either in the absence or presence of varying concentrations of CUR (37-370 mg/L). **(A)** Null mutants of *C. albicans* (Δ*CaCdr1*, Δ*CaCdr2*, Δ*CaMdr1*) lacking functional drug transporters. **(B)** Matched pair isolates of *C. albicans* (Gu4 and Gu5) over expressing the transporter *CaCDR1* or *CaMDR1* (F2 and F5). Growth differences were evaluated following 48 hrs. of incubation as mentioned in material and methods. **(C)** Transcript levels of *CDR1/CDR2/CaMDR1* in the wild type stain SC5314 in the absence and presence of CUR (185 mg/L, 16 hrs). *ACT1* mRNA levels were used as a loading control.

2004), Δ*ftr1* (Ftr1p- a high affinity iron permease (Ramanan and Wang, 2000) and Δ*ccc2* (Ccc2p- a copper transporting ATPase (Weissman *et al.*, 2002). Notably, only the oxidative stress nulls Δ*CaIpf7817* and Δ*Cap1* appeared to be highly susceptible to CUR than the wild type strain (Figure 20A). As depicted in Figure 20B there was a significant reduction in the MIC_{80} values of the oxidative stress mutants when grown in presence of CUR as compared to the wild type stain. None of the other tested mutants showed any difference in the MIC_{80} values when grown on CUR.

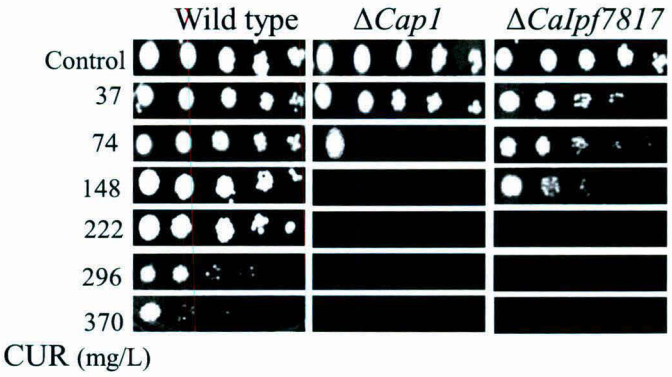
3.2.2.5 Antifungal effects of CUR could be reversed by antioxidants

We further confirmed the role of oxidative stress by performing spot assays in presence of CUR, and antioxidants such as PDTC or AA. For this, we used a range of antioxidant concentrations (5-100μM for PDTC and 25-100mM for AA) and observed that 10μM of PDTC or 25mM of AA could only partially restore CUR effect. A higher concentration of each antioxidant was toxic to cells. As depicted in Figure 21A, addition of PDTC (10μM) or AA (25mM) alone had no effect on the growth of cells but when added along with CUR (296 mg/L), the growth inhibition was reversed. This reversal of growth inhibition due to antioxidants was further confirmed by colony formation assay. As compared to CUR alone which killed ~96% of the cells, the percentage of viable cells in presence of CUR and antioxidants were considerably increased (Figure 21B).

3.2.2.6 CUR generates ROS

In the following experiments, we analyzed if ROS formation could be a key event in the CUR induced killing of *C. albicans.* Using a fluorimetric assay, we could demonstrate that in presence of CUR, there was an increase in fluorescence intensity (FI) which coincided with induced endogenous ROS in *Candida* Figure 22 (A and B). The net ROS production in cells raised by CUR could be reversed by the addition of antioxidant PDTC or AA. The ROS levels in non-*albicans* species were also raised to different levels when CUR was present in growth media which could be reversed by the addition of antioxidants (Figure 22C). It should be pointed out that PDTC and AA alone in the growth medium inhibited the basal level of ROS production between 10-20% (Figure 22B). Expectedly, the highly CUR susceptible oxidative stress mutants Δ*CaIpf7817* and Δ*Cap1* displayed 3-5 times higher basal ROS levels which was

A.



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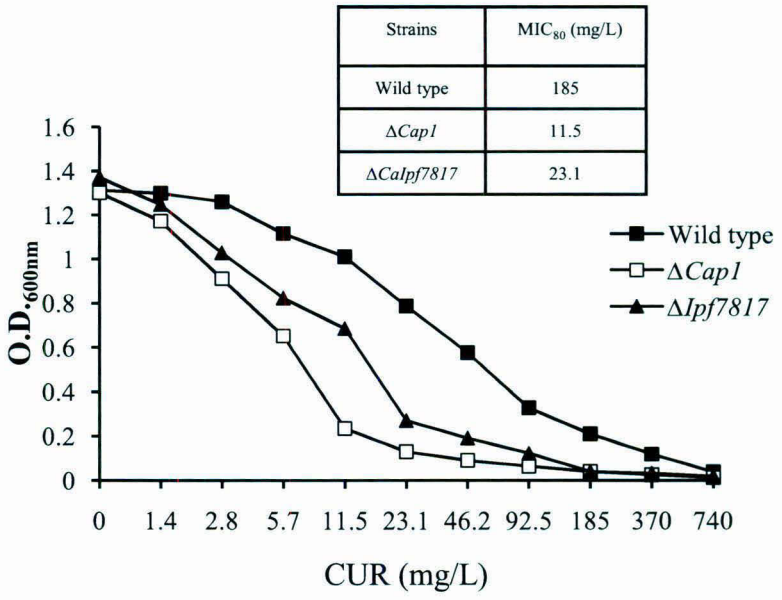
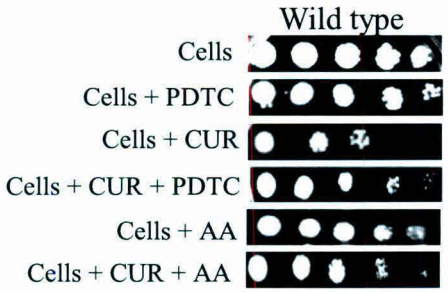


Figure 20. Susceptibility of oxidative stress mutants of *C. albicans* on CUR.
(A) The cells (SC5314/Δ*Cap1*/Δ*CaIpf7817*) were grown overnight on YEPD plates and then resuspended in normal saline to an OD_{600} of 0.1. Five microliters of fivefold serial dilution of each strain was spotted onto YEPD plates as described in material and methods, either in the absence or presence of varying concentrations of CUR (37-370 mg/L). **(B)** Determination of growth inhibition of the *Candida* cells by broth micro dilution assay in presence of CUR at concentrations ranging from 1.44-740 mg/L. Growth of the cells was evaluated both visually and by reading the A_{600} in a microtitre reader as described in material and methods. Filled/empty squares and filled triangles represent SC5314/Δ*Cap1* and Δ*CaIpf7817*, respectively. Inset shows the MIC_{80} values. A stock solution of 11 mg/ml was used (DMSO). Growth was not affected by the presence of the solvent.

A.



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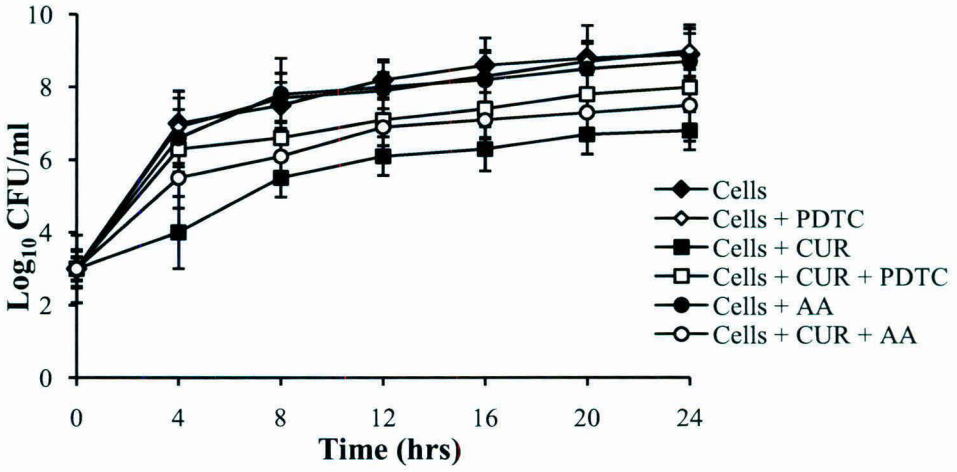


Figure 21. Spot assays in presence of antioxidants/CUR and the time kill assays. **(A)** The cells were grown overnight on YEPD plates and then resuspended in normal saline to an OD_{600} of 0.1. Five microliters of fivefold serial dilution of each strain was spotted onto YEPD plates as mentioned in material and methods, either in the absence or presence of CUR (296 mg/L) alone or CUR and antioxidants (10µM PDTC or 25mM AA). The antioxidants (10µM PDTC or 25mM AA) have no effect on the growth of cells. Growth differences were evaluated following 48 hrs. of incubation as mentioned in material and methods. **(B)** Time kill curves of wild type strain of *C. albicans* in presence of CUR/PDTC/AA alone or in combination were obtained by using initial inoculums of 10^3CFU/ml.. The filled / empty- diamond, square and circle represents cells alone, cells + PDTC, cells + CUR, cells + CUR + PDTC, cells + AA and cells + CUR + AA, respectively. The values are the means and standard deviations (indicated by bars) of three independent experiments.

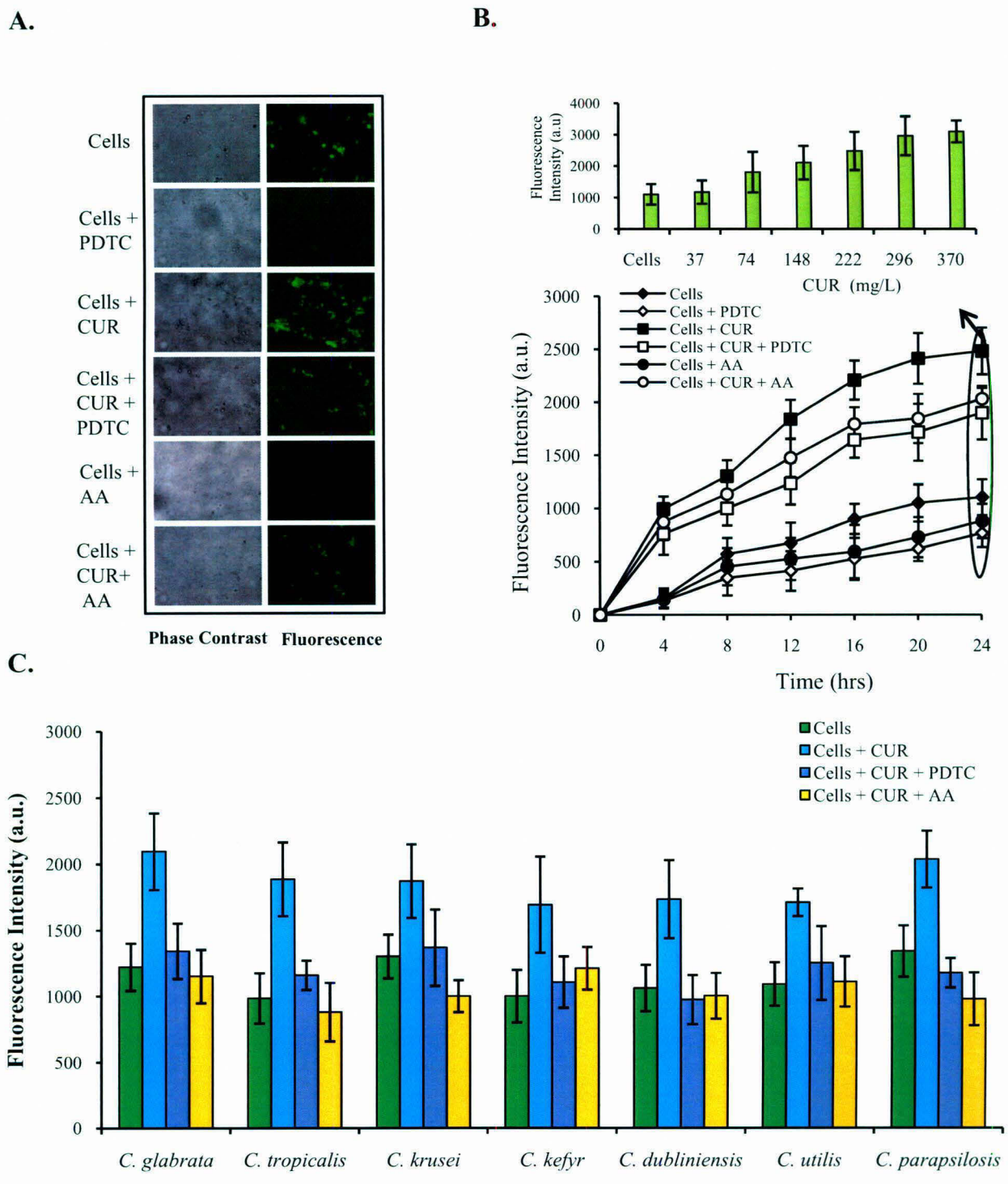


Figure 22. ROS levels in presence of CUR in *C. albicans* cells.

(A) Images of DCF Fluorescence due to CUR (185 mg/L) or CUR+antioxidants (10µM PDTC or 25mM AA) treatment were taken with a fluorescence microscope (Carl Zeiss, USA). **(B)** Amounts of ROS produced in CUR (185 mg/L) or CUR+antioxidants (10µM PDTC or 25mM AA) treated cells. The fluorescence emitted by the cells was measured using a spectrofluorometer (Varian, Cary Eclipse). An excitation and emission of 485 and 540 nm respectively, were used. The filled/ empty diamond filled/ empty square and filled/empty circle represent cells alone, cells + PDTC, cells + CUR, cells + CUR + PDTC, cells + AA and cells + CUR + AA, respectively. Inset shows the level of ROS produced at 24 hrs of administration of CUR. **(C)** ROS produced in non *albicans* species of *Candida* upon CUR (185 mg/L) or CUR+PDTC or CUR+AA (10µM PDTC, 25mM AA) treatment (16 hrs). The values are the means and standard deviations (indicated by bars) of three independent experiments.

further raised by CUR in a reversible manner (Figure 23A). As depicted in Figure 23 B, the presence of CUR increased the transcript levels of the genes (*CAP1, CaIPF7817, GRP2* [NADPH- dependent methyl glyoxal reductase], *CAT1* [Catalase] and *SOD2* [superoxide dismutase 2]) associated with maintenance of oxidative stress.

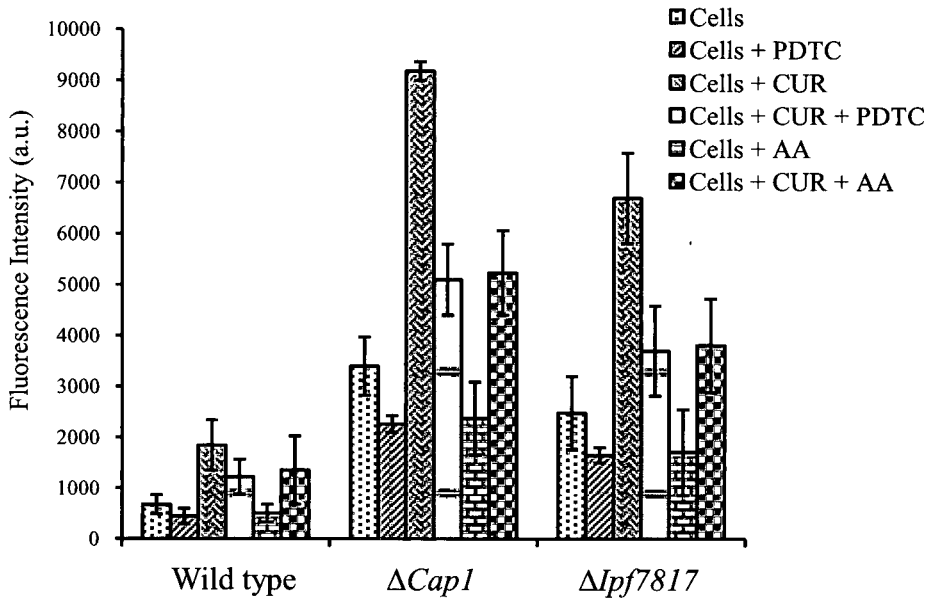
3.2.2.7 CUR induces apoptosis in *Candida* cells

Translocation of PS to the outer monolayer of the lipid bilayer of the PM is an early marker of apoptosis (Balzan *et al.*, 2004; Phillips *et al.*, 2003). We explored if CUR induces apoptosis in *C. albicans* by measuring PS externalization using Annexin V-FITC assay. As depicted in Figure 24A, after 4 hrs of incubation of cells with CUR, the population of cells had 22.88% exposed PS as compared to 3.17% in untreated cells (Figure 24A, panel II and I, respectively). Notably the CUR induced externalization of PS could be arrested to 2.28 or 3.77% if cells were pretreated with antioxidant PDTC or AA prior to the incubation with CUR (Figure 24A, panel III and panel IV, respectively).

Since following the loss of membrane integrity, Annexin V-FITC also labels necrotic cells, simultaneous addition of PI which does not permeate cells with an intact PM allows discrimination between apoptotic (Annexin V positive, PI negative), necrotic (both Annexin V and PI positive) and live (both Annexin V and PI negative) cells (Balzan *et al.*, 2004; Phillips *et al.*, 2003). Accordingly, we examined the effect of CUR on the overall population distribution between apoptotic and necrotic cells. For this, CUR (185 mg/L) treated cells were double stained with Annexin V-FITC and PI. As shown in Figure 24B, Panel II, a significant percentage (28.95%, lower right quadrants) of cells stained positive for Annexin V-FITC as compared to none (Figure 24, Panel I) in untreated cells. That the Annexin V labeled cells represents only apoptotic cells (Figure 24A panel II, lower right quadrants) was confirmed from the double staining where we could observe an almost equal number of apoptotic cells when compared with Annexin V-FITC and PI labeling (Figure 24B panel II, lower right quadrants). The late apoptotic/necrotic cells increased to 11.95% in 4 hrs (Figure 24B panel II, upper right quadrants) when treated with CUR which could be reversed by the antioxidants like PDTC or AA (Figure 24B panel III and IV, upper right quadrants).

MCA1 encodes a homologue of a mammalian caspase in *C. albicans* (Cao *et al.*, 2009). To determine the involvement of caspases in the CUR induced cytotoxicity

A.



B.

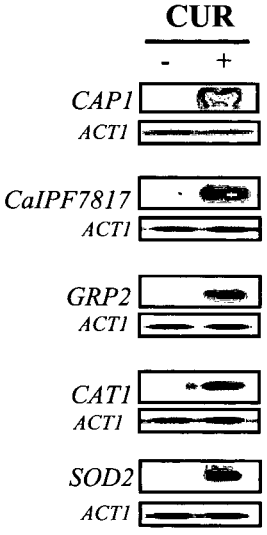


Figure 23. ROS levels in the oxidative stress mutants and mRNA transcript levels in C. *albicans* cells.

(A) Amounts of ROS produced in CUR (185 mg/L) or CUR + antioxidants (10μM PDTC or 25mM AA) treated cells *viz* wild type (SC5314), Δ*Cap1* and Δ*CaIpf7817*. The fluorescence emitted by the cells was measured using fluorescence spectrometer (at excitation and emission at 485 and 540 nm, respectively). Bars with dots, diagonal lines, weave, horizontal lines, bricks and checker board represent cells alone, cells + PDTC, cells + CUR, cells + CUR + PDTC, cells + AA and cells + CUR + AA, respectively. The values are the means and standard deviations (indicated by bars) of three independent experiments. **(B)** Transcript levels of *CAP1, CaIPF7817, SOD2, GRP2* and *CAT1* in the wild type stain SC5314 in the presence and in absence of CUR (185 mg/L, 4 hrs). *ACT1* mRNA levels were used as a loading control

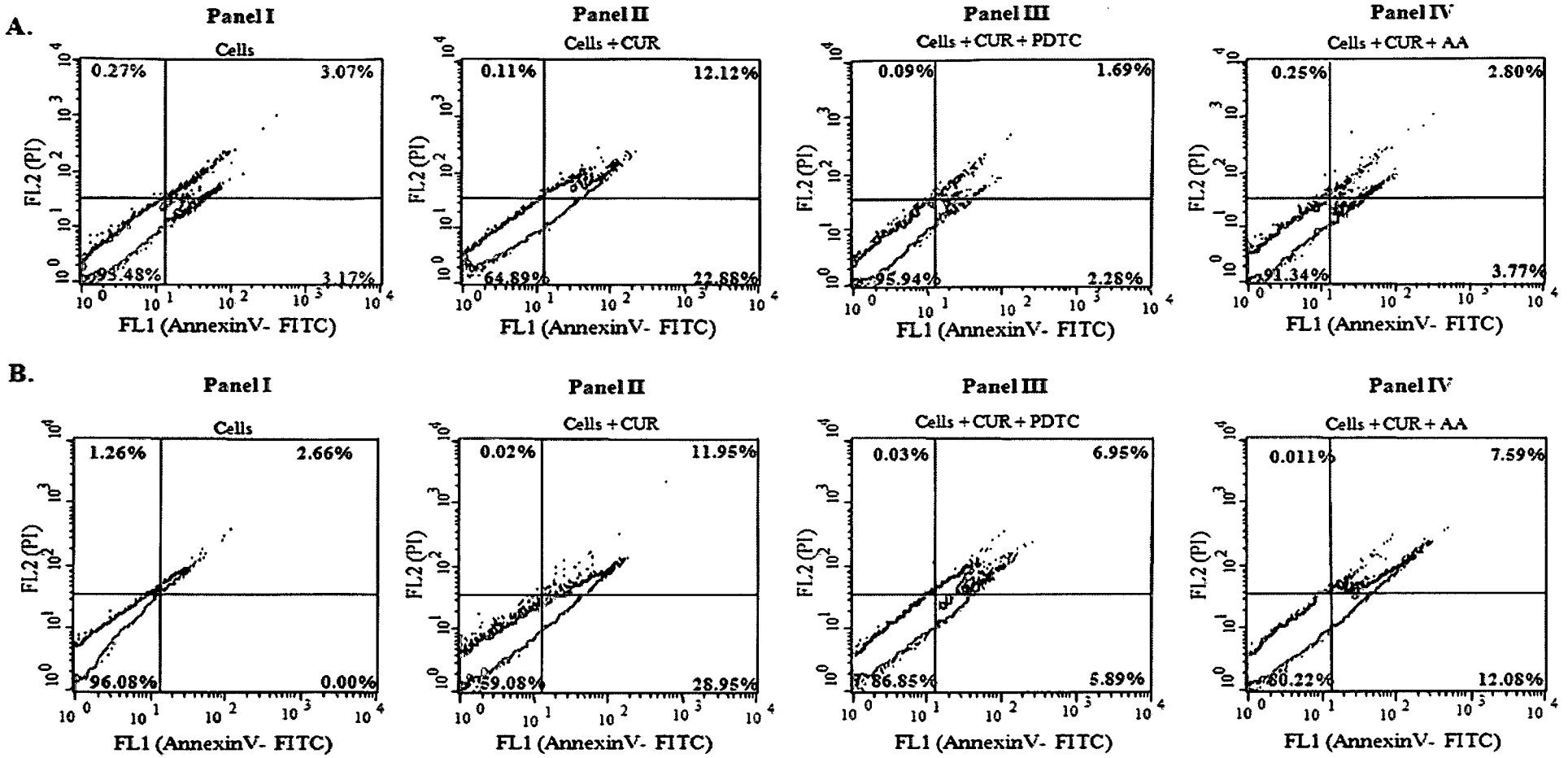


Figure 24. Externalization of phosphatidylserine in CUR treated cells.
Panel I represents untreated, stained *C. albicans* (SC5314) cells, Panel II shows CUR treated cells, Panel III and IV are the PDTC/AA pretreated cells in presence of CUR and stained with **(A)** Annexin V-FITC alone or **(B)** Co-stained with Annexin V-FITC and PI. For treatment, the cells were incubated with CUR (185 mg/L, 4hrs) and analyzed by flow cytometry as described in "Materials and Methods". The lower right quadrants of the various Panels represent early apoptotic cells, upper right quadrants represent late apoptotic or necrotic cells. **(C)** Transcript level of *CaMCA1* in the wild type strain SC5314 in the absence and presence of CUR (185 mg/L, 4hrs). *ACT1* mRNA levels were used as a loading control.

in *C. albicans*, the expression of *CaMCA1* was determined in log phase cells by Northern blot analysis. It was observed that following 4 hrs. exposure to CUR, *CaMCA1* transcript level was also increased when compared with untreated cells (Figure 24C).

3.2.2.8 CUR inhibits hyphae development by *Candida*

Mycelial development of *C. albicans* is influenced by many factors and is controlled by well known morphological regulators. In this study, we observed that CUR (37 mg/L) when added to hyphae inducing solid or liquid media prevented the development of hyphae of *C. albicans* cells. In contrast to the other effects of CUR, the inhibition of hyphae development could not be reversed by the addition of an antioxidant such as PDTC (10μM) or AA (25mM) (Figure 25).

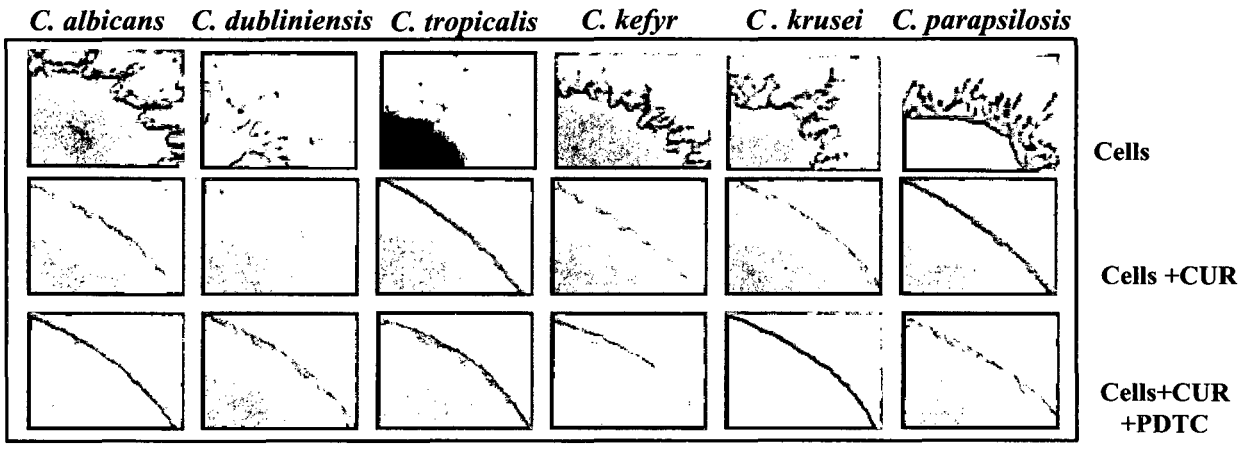
3.2.2.9 Hyphae development by *tup1* nulls could not be inhibited by CUR

We examined the effect of CUR (37 mg/L) in several null mutants lacking morphological transcription regulators and found that CUR could not prevent hyphae development in Δ*tup1* cells (Kebaara *et al.*, 2008) (Figure 26A). This coincided with a raised *TUP1* (Thymidine uptake 1) transcript levels by CUR in a wild type strain of *C. albicans* (Figure 26B).

3.2.2.10 CUR works independent of the quorum sensing molecule farnesol

It is known that the quorum sensing molecule farnesol raises ROS levels, inhibits hyphae development by targeting the repressor *TUP1* (Kebaara *et al.*, 2008). We observed that polyphenol CUR apparently mimics farnesol affect. To establish the link between CUR and farnseol pathways, we examined the various effects of CUR *viz* antifungal, ROS generation and the inhibitory effect on mycelial development in a *C. albicans* strain defective in farnesol production. *DPP3* (Diacylglycerol pyrophosphate phosphatase) encodes a phosphatase which converts farnesyl pyrophosphate to farnesol. It is reported that *DPP3* knock out (KWN2) produces six times less farnesol in comparison to the parent strain (SN152) (Navarathna *et al.*, 2007). Figure 27A shows that CUR could inhibit the growth of *DPP3* null strain similar to wild type strain. Additionally, the growth inhibition of Δ*dpp3* cells by CUR could be reversed by antioxidants (Figure 27B). Similarly, CUR effect to raise ROS

A.



B.

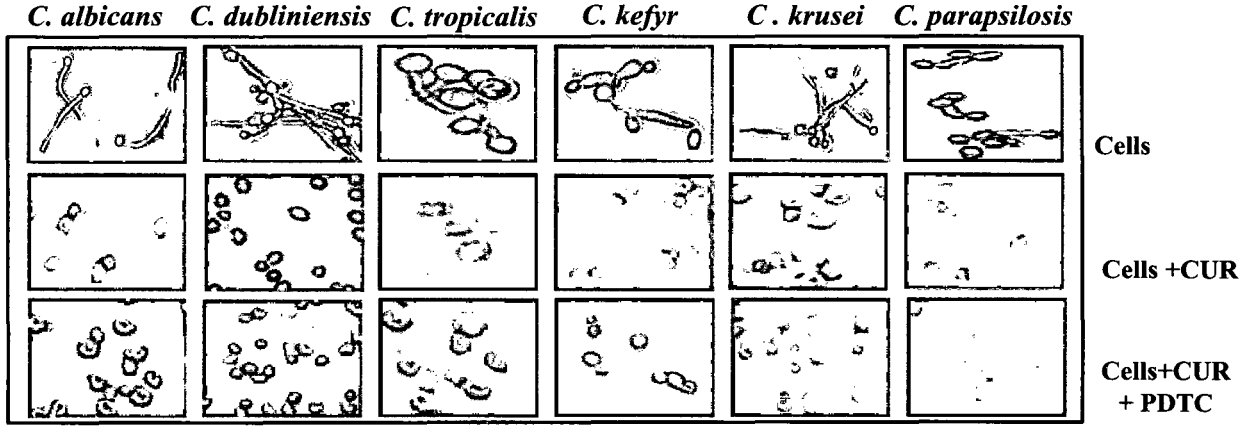


Figure 25. Hyphal development in presence and in absence of CUR/CUR+ PDTC in solid and liquid hyphae inducing media.

Response to CUR (37 mg/L) and CUR (37 mg/L) +PDTC (10μM) by *C. albicans* (SC5314) cells under conditions that promote hyphal growth. The cells were grown in the absence or in the presence of CUR alone or CUR+PDTC in **(A)** Solid YEPD (2.5% agar) with 10% fetal bovine serum (FBS) or in **(B)** Liquid spider media. Cells were incubated for 6 hrs in liquid media and for 3 days in case of solid media at 37°C. Colony morphologies on solid plates and filamentation in liquid media were examined microscopically (Carl Zeiss, USA) as mentioned in "Materials and Methods" section.

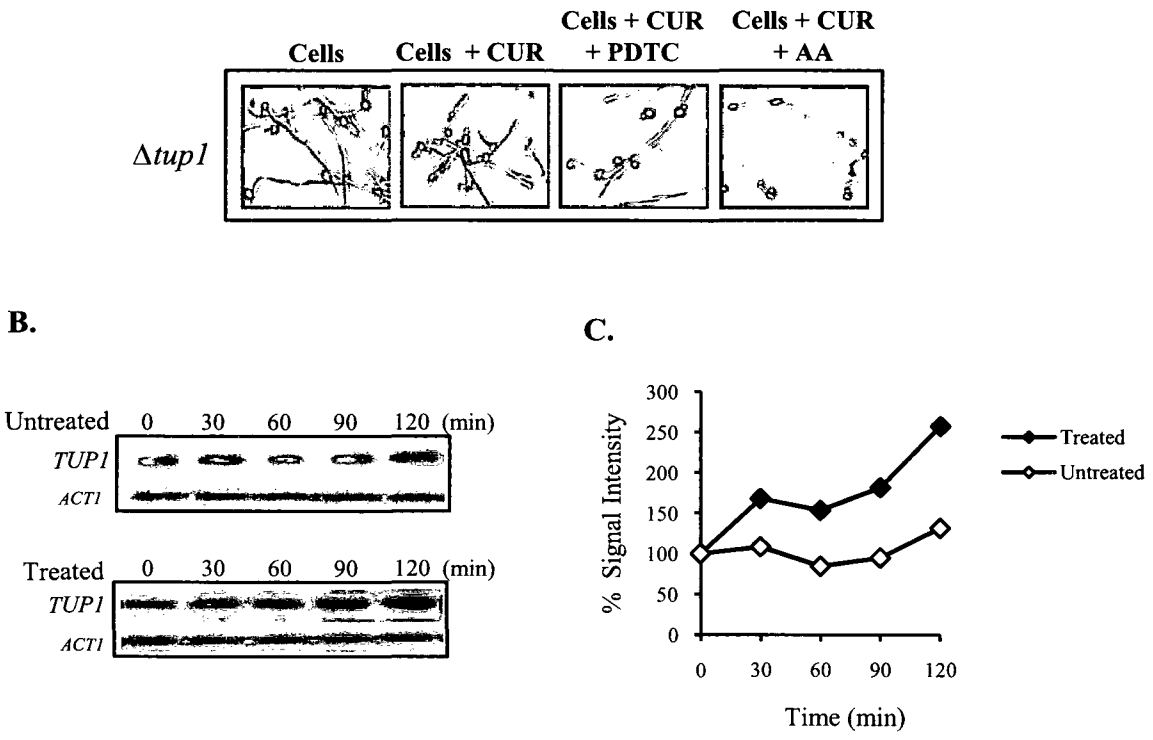


Figure 26. Effect of CUR on the hyphae development of Δ*tup1* cells.
(A) Response to CUR and CUR+PDTC by *C. albicans* under conditions that promote hyphal growth. The cells were grown in the presence or in the absence of CUR alone (37 mg/L) or CUR (37 mg/L) + PDTC (10μM) in YEPD with 10% fetal bovine serum (FBS) and incubated at 37°C for 6 hrs. The filamentation in liquid media was examined microscopically (Carl Zeiss, USA). **(B)** Transcript levels of *Tup1* in the wild type stain SC5314 in the presence and in absence of CUR (37 mg/L) at the indicated time points. **(C)** Quantitation of Northern blot hybridization. Constitutively expressing *ACT1* transcript was used as a loading control.

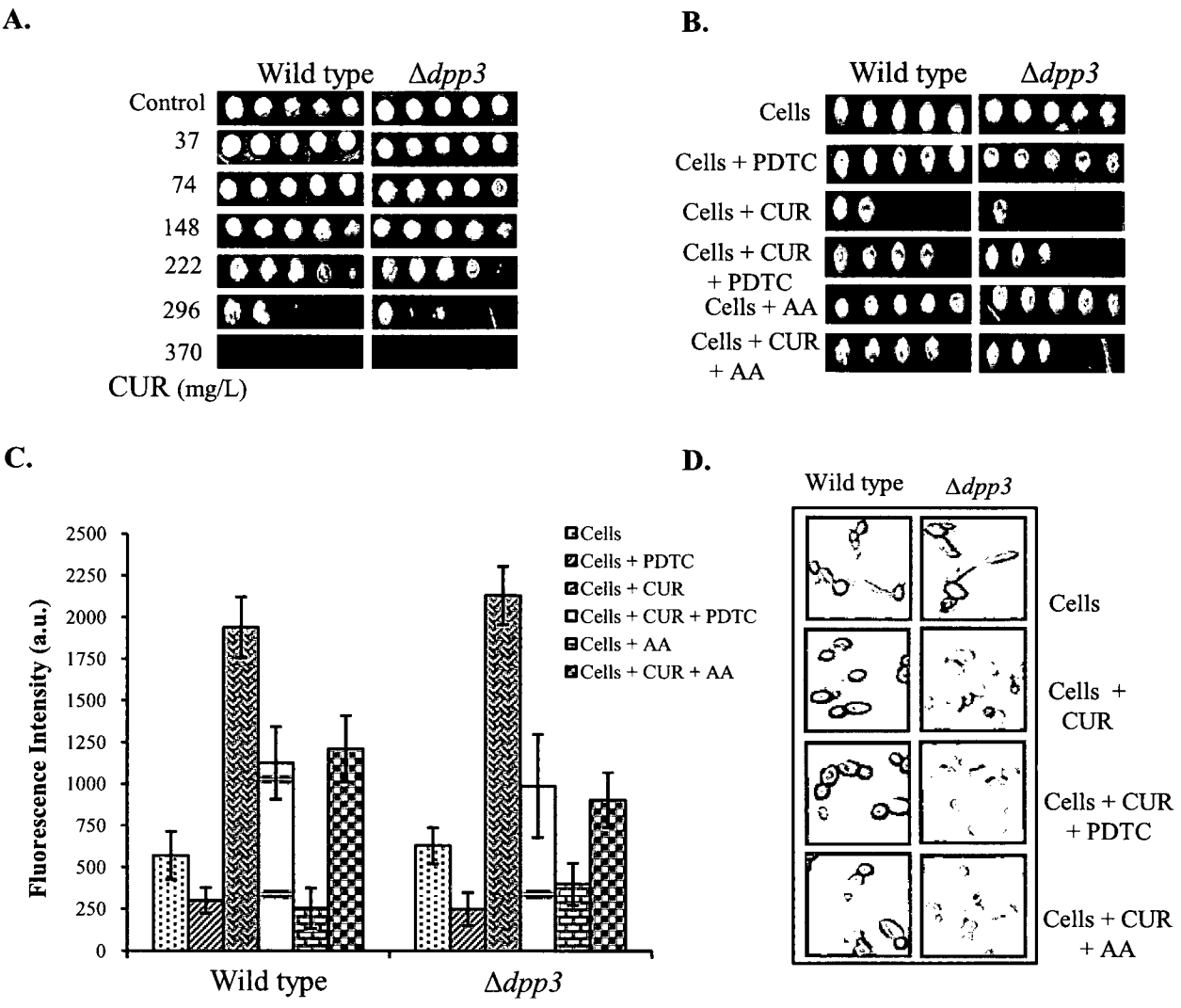


Figure 27. Effects of CUR on Δ*dpp3* cells.

(A) The wild type (SN152) and Δ*dpp3* (KWN2) cells were grown overnight on YEPD plates and then resuspended in normal saline to an OD_{600} of 0.1. Five microliters of fivefold serial dilution of each strain was spotted onto YEPD plates, as described in material and methods, either in the absence or presence of varying concentrations of CUR (37-370 mg/L). **(B)** Five microliters of fivefold serial dilution of each strain was spotted onto YEPD plates as mentioned in material and methods, either in the absence or presence of CUR (296 mg/L) alone or CUR and antioxidants (10μM PDTC or 25mM AA). The antioxidants (10μM PDTC or 25mM AA) have no effect on the growth of cells. Growth differences were evaluated following 48 hrs of incubation as mentioned in material and methods. **(C)** Amounts of ROS produced in CUR (185 mg/L) or CUR + antioxidants (10μM PDTC or 25mM AA) treated cells *viz* wild type (SN152) and Δ*dpp3* (KWN2) cells. The fluorescence emitted by the cells was measured by using fluorescence spectrometer (at excitation and emission at 485 and 540nm, respectively). Bars with dots, diagonal lines, weave, horizontal lines, bricks and checker board represent cells alone, cells + PDTC, cells + CUR, cells + CUR + PDTC, cells + AA and cells + CUR + AA, respectively. The values are the means and standard deviations (indicated by bars) of three independent experiments. **(D)** Response to CUR and CUR+PDTC by *C. albicans* under conditions that promote hyphal growth. The cells were grown in the presence or in the absence of CUR alone (37 mg/L) or CUR (37 mg/L)+PDTC (10μM) in YEPD with 10% fetal bovine serum (FBS) and incubated at 37°C for 6 hrs. The filamentation in liquid media was examined microscopically (Carl Zeiss, USA).

levels and to inhibit hyphae formation remained unaffected in Δ*dpp3* cells (Figure 27C and D).

3.2.3 Discussion

In the present study, we have investigated the antifungal effects of a natural polyphenol CUR against *albicans* and non *albicans* species of *Candida* and have shown that the growth of all the tested strains of *Candida* could be inhibited by CUR (Figure 17). While demonstrating anti-cancer effects of CUR it has been observed that the systemic exposure of CUR remains low to exhibit sufficient pharmacological activity. However, concomitant administration of PIP increases bioavailability of CUR both in humans and rats (Anand *et al.*, 2007; Shoba *et al.*, 1998a). Based on these findings, PIP, a known inhibitor of hepatic and intestinal glucuronidation, was combined with CUR and administered to check its *in vivo* efficacy. Indeed, a combination of CUR and PIP when administered together, resulted in a significant and higher reduction in fungal load ($1.4\log_{10}$) in kidneys of Swiss mice as compared to when CUR was administered alone ($1\log_{10}$) (Figure 18). Pharmacokinetic studies have indicated that following oral administration in rats and humans, CUR is transformed into metabolites like curcumin glucuronides (Anand *et al.*, 2007). Since PIP remarkably enhances the bioavailability of CUR in mice resulting in significant reduction in fungal load suggests that these metabolites may also contribute to antifungal effect of CUR. Elaborate *in vivo* studies are desired to find out the efficacy across the fungal species. Also, efforts are necessary to increase the medicinal value of CUR through structural modifications of the molecule and new formulations to increase the oral bioavailability.

To investigate the mechanism of CUR cidal activity against *Candida*, we checked the sensitivity of CUR on various morphological, iron transporters and oxidative stress mutants of *C. albicans* and observed that only the oxidative stress mutants (Δ*Cap1* and Δ*CaIpf7817*) were particularly susceptible to CUR (Figure 23) while other mutant strains behaved similar to the wild type strain. A striking feature was the growth inhibitory effects and elevated ROS levels due to CUR which could be reversed if the natural or synthetic antioxidants were also present in the growth medium (Figure 21 and 22).

ROS induction capacity of various antifungals has earlier been reported (Huang *et al.*, 2008). For example, azoles such as MCZ, as well as the polyene AMB and NYS and polyol macrolide such as niphimycin induce ROS levels in susceptible fungi (Kobayashi *et al.*, 2002; Phillips *et al.*, 2003; Nakayama *et al.*, 2002; Thevissen *et al.*, 2007). In addition, the benzo-naphthacenequinone antibiotic pradimicin A (Hiramoto *et al.*, 2003), natural perylenequinonoid pigments (Xing *et al.*, 2003), the isoprenoid alcohol farnesol (Machida *et al.*, 1998) and several antifungal peptides /proteins also induce ROS in yeast species. Some of the ROS inducing antifungals further trigger apoptosis in yeast cells (Phillips *et al.*, 2003; Nakayama *et al.*, 2002; Hiramoto *et al.*, 2003). An oxidative stress response in yeast is well documented (Wang *et al.*, 2006; Jia *et al.*, 2007; Alarco and Raymond, 1999; Kusch *et al.*, 2007). Yeast cells undergoing apoptosis display several characteristic markers, including the induction of endogenous ROS (Shirtliff *et al.*, 2009). With this background, our observation that CUR induced ROS stimulates the pro-apoptotic regulatory machinery in *Candida* cells is interesting. We could demonstrate that CUR increased the number of pre-apoptotic cells which could be prevented by the presence of an antioxidant (Figure 24). Raised *CaMCA1* levels in the presence of CUR points to caspase mediated apoptosis in *C. albicans* (Figure 24C). In our previous study, we have shown that CUR modulates the drug efflux of yeast ABC transporters without affecting the protein levels (Sharma *et al.*, 2009b) and here we could demonstrate that CUR exerts its growth inhibitory effects without affecting the transcript levels of genes encoding these transporters (Figure 19C). Hence the effect of CUR on *Candida* growth is independent of the levels of these transporters (Figure 19).

Interestingly, even at lower concentrations CUR (37 mg/L) could block the hyphae development in both *albicans* and non *albicans* species of *Candida* (Figure 25). However, unlike antifungal effect of CUR, the inhibition of hyphae development could not be reversed by the antioxidants. We found instead that CUR targeted global repressor *TUP1* since CUR could not inhibit hyphae development of Δ*tup1* which otherwise due to de-repression vigorously make hyphae. This was further confirmed by Northern experiments where CUR induced *TUP1* transcript levels in wild type cells (Figure 26). The signaling molecule farnesol, which also induces ROS levels, exerts its effect *via TUP1* (Navarathna *et al.*, 2007). In *C. albicans*, farnesol is endogenously generated by enzymatic dephosphorylation of farnesyl diphosphate, a precursor for the synthesis of sterols in the sterol biosynthesis pathway (Shirtliff *et al.*,

2009). Thus, CUR effect seems to mimic the effect of quorum sensing molecule farnesol. However, when we checked the effects of CUR on the growth, ROS generation and mycelial development in a knockout of *DPP3* which encodes a phosphatase for converting farnesyl pyrophosphate to farnesol, it was observed that CUR continued to inhibit the growth, which could be reversed by the addition of antioxidants in the mutant strain KWN2 (Δ*dpp3*) (Navarathna *et al.*, 2007). The raised ROS levels and the inhibition of hyphae development in *DPP3* knock out suggest that CUR only mimics the effects of farnesol phenotypically but is independent of farnesol (Figure 27). Taken together, CUR has dual affects on *Candida* cells. Its antifungal effect is mediated through the ROS signaling pathway which sets in an early apoptosis leading to cell death. Independent of the ROS pathway, CUR also inhibits hyphae development by regulating *TUP1* levels. Considering the success story of CUR as an anti-cancer and anti-inflammatory compound, our study opens up an opportunity wherein CUR can also be exploited as a potential natural antifungal.

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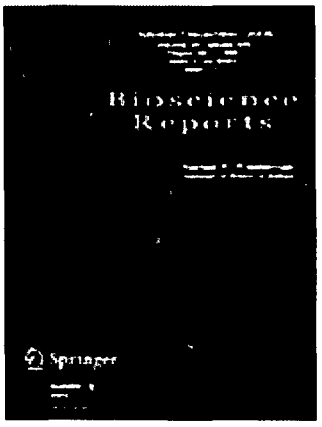
✓ The work presented in this chapter has been published as **"Antifungal curcumin induces reactive oxygen species and triggers an early apoptosis but prevents hyphae development by targeting the global repressor *TUP1* in *Candida albicans*"** *in Biosciences Reports* (2009) Epub. ahead of print.



Section-III

3.3 Synergistic anticandidal activity of pure polyphenol Curcumin I in combination with azoles and polyenes generates reactive oxygen species leading to apoptosis

3.3.1 Introduction

Combating *Candida* infections and particularly which are due to resistant strains remain a major challenge among clinicians (Sanglard *et al.,* 2002; White *et al.,* 2002). Coinciding with the occurrence of AR isolates, much efforts are directed to resolve this by: (i) searching for new antifungal agents (Andes *et al.,* 2006); (ii) developing new formulations (Pfaller *et al.,* 2005); and (iii) improving the efficacy of antifungal agents by using combination therapy. (Ghannoum *et al.,* 1995). For instance, a combination of voriconazole (VRC) and terbinafine (TRB) was found to be synergistic in *C. albicans* isolates obtained from HIV- infected patients (Weig *et al.,* 2001). It has been reported that there exists a synergy between micafungin (MCFG) and fluconazole (FLC), VRC, amphotericin B (AMB), flucytosine (5-FC) in clinical isolates of *Candida* (Nishi *et al.,* 2009). The anti-arrhythmic drug amiodarone is also reported to act synergistically with FLC, itraconazole (ITR) and VRC against drug resistant isolates of *C. albicans* (Guo *et al.,* 2008). In the clinical isolates of *C. glabrata,* a combination of posaconazole (POS) and caspofungin (CSP) was found to be effective (Oliveira *et al.,* 2005).

Many herbal products such as allicin (ALC), berberine (BER), grapefruit seed extract, tea tree oil, xanthorrhizol (isolated from *Curcuma xanthorrhiza*), retigeric acid B (RAB, isolated from the lichen, *Lobaria kurokawae*) are known to have antifungal properties either alone or in combination with known antifungals. For example, ALC has inhibitory activity against *C. albicans, Aspergillus fumigates, Scedosporium prolificand, Cryptococcus neoformans and Trichophyton* spp. (Guo *et al.,* 2009) and at the same time, ALC also enhances the antifungal activity of AMB against *S. cerevisiae*, *C. albicans*, and *A. fumigates* (Davis *et al.,* 2003). The combination of FLC and ALC exhibited good *in vivo* and *in vitro* synergistic antifungal effects, and FLC/ALC therapy was more efficacious than FLC monotherapy in clearing *Candida* from the kidneys (Guo *et al.,* 2009). The concomitant use of BER and FLC provided a synergistic action against AR *C. albicans* clinical strains *in vitro* (Quan *et al.,* 2006). A strong synergistic anti-yeast

activity of Garlic oil (GO) and allyl alcohol (AA) is known against *Candida utilis* (Chung *et al.,* 2007). Recently, Sun (Sun *et al.*, 2010) have reported synergistic mechanism of RAB and azoles against *C. albicans*.

We have earlier reported that the three pure curcuminoids isolated from natural Curcumin *viz.* diferuloylmethane (CUR-I), demethoxycurcumin (CUR-II) and bisdemethoxycurcumin (CUR-III) exhibit antifungal activity (Sharma *et al.,* 2009a). An antifungal activity of CUR is also reported against *Cryptococcus neoformans*, *Sporotix schenckii*, *Paracoccidioides brasiliensis* and *Aspergillus* spp. (Martins *et al.*, 2009). Our present study demonstrates that pure CUR-I which is the major component (~77%) of natural curcumin can be exploited even more successfully in combination with azoles or polyenes. We evaluated the combined effects of pure CUR-I with FLC, KTC, MCZ, ITR, VRC, AMB and NYS against WT and AR isolates of *C. albicans* by employing checkerboard microdilution method. We show that the use of pure CUR-I in combination with azoles and polyenes is synergistic which could be further validated by filter disc and time-kill assays.

3.3.2 Results

3.3.2.1 Pure CUR-I is synergistic to azoles and polyenes

In vitro antifungal effects of pure CUR-I alone as well as in combination with azoles and polyenes, were tested against WT and clinical AR isolates of *C. albicans*. In WT strain, the MIC_{80} (μg/ml) of FLC, KTC, MCZ, ITR, VRC, AMB and NYS alone was 0.5, 0.5, 0.2, 0.062, 0.062, 1.25 and 3.75, respectively, which in combination with CUR-I was reduced to 0.0155 (32 fold reduction), 0.0078 (64 fold reduction), 0.0062 (32 fold reduction), 0.0078 (7.9 fold reduction), 0.0078 (7.9 fold reduction), 0.078 (16 fold reduction) and 0.234 (16 fold reduction), respectively (Table 4). We explored, if the observed synergistic effects of CUR-I in WT strain can be extended to the clinical AR isolates. For this, we checked the interactions of azoles/polyenes and CUR-I against the AR clinical *Candida* isolates. Table (5-11) summarizes the checkerboard assay of pure CUR-I, azoles and polyenes against clinical AR isolates. In clinical AR isolates, MIC_{80} (μg/ml) varies from 64-128 (FLC), 0.156-10 (KTC), 0.312-5 (MCZ), 0.312-10 (ITR), 0.25-2 (VRC), 0.156-0.625 (AMB) and 0.312-10 (NYS) which in combination of CUR-I was reduced respectively to 4-

Table 4: Checkerboard assay of FLC, KTC, MCZ, ITR, VRC, AMB, NYS and CUR against WT strain of *C. albicans*

Antifungal agent	MIC_{80} of each agent (μg/ml)		FIC	FICI	Out Come
	Alone	Combination			
FLC	0.500	0.015	0.031		
CUR	185	11.5	0.062	0.093	SYN
KTC	0.500	0.007	0.015		
CUR	185	23.12	0.124	0.139	SYN
MCZ	0.200	0.006	0.031		
CUR	185	185	0.062	0.093	SYN
ITR	0.062	0.007	0.125		
CUR	185	23.12	0.124	0.244	SYN
VOR	0.062	0.007	0.125		
CUR	185	46.25	0.250	0.375	SYN
AMB	0.125	0.078	0.062		
CUR	185	23.12	0.124	0.187	SYN
NYS	3.750	0.234	0.062		
CUR	185	23.12	0.124	0.186	SYN

Table 5: Checkerboard assay of FLC and CUR against clinical AR isolates of *C. albicans*

Strain	MIC_{80} of FLC alone (μg/ml)	MIC_{80} of FLC in combination (μg/ml)	FIC (FLC)	MIC_{80} of CUR alone (μg/ml)	MIC_{80} of CUR in combination (μg/ml)	FIC (CUR)	FICI	Out Come
A1	128	8	0.060	370	23.12	0.060	0.124	SYN
A2	128	16	0.125	185	46.25	0.250	0.187	SYN
A3	64	8	0.124	185	46.25	0.250	0.374	SYN
D1	128	16	0.125	185	46.25	0.250	0.375	SYN
D2	64	4	0.062	185	46.25	0.250	0.312	SYN
D3	64	4	0.062	185	46.25	0.250	0.312	SYN
D4	64	8	0.125	185	46.25	0.250	0.375	SYN
D5	128	16	0.125	370	92.5	0.250	0.375	SYN
D6	128	16	0.125	185	23.12	0.125	0.249	SYN
D7	64	16	0.250	185	46.25	0.250	0.500	SYN
B1	128	32	0.250	185	46.25	0.250	0.500	SYN
B2	64	8	0.125	370	92.5	0.250	0.375	SYN
B3	64	16	0.250	185	46.25	0.250	0.500	SYN
B4	64	16	0.250	185	23.12	0 .125	0.375	SYN
B5	128	16	0.125	185	23.12	0.125	0.249	SYN
P1	64	8	0.125	185	23.12	0.125	0.249	SYN
P2	64	4	0.062	185	46.25	0.250	0.310	SYN
P3	128	16	0.125	185	46.25	0.250	0.250	SYN
P4	128	16	0.125	128	23.12	0.180	0.350	SYN
P5	64	16	0.250	185	46.25	0.250	0.500	SYN
P6	64	8	0.125	185	11.5	0.062	0.375	SYN

Table 6: Checkerboard assay of KTC and CUR against clinical AR isolates of *C. albicans*

Strain	MIC_{80} of KTC alone (µg/ml)	MIC_{80} of KTC in combination (µg/ml)	FIC (KTC)	MIC_{80} of CUR alone (µg/ml)	MIC_{80} of CUR in combination (µg/ml)	FIC (CUR)	FICI	Out Come
A1	2.500	0.312	0.124	370	23.12	0.060	0.184	SYN
A2	2.500	0.625	0.250	370	92.5	0.250	0.500	SYN
A3	0.156	0.039	0.250	185	46.25	0.250	0.500	SYN
D1	5.000	0.625	0.125	185	46.25	0.250	0.375	SYN
D2	2.500	0.312	0.124	185	23.12	0.124	0.248	SYN
D3	0.625	0.078	0.124	185	46.25	0.250	0.374	SYN
D4	0.312	0.078	0.250	185	23.12	0.124	0.374	SYN
D5	5.000	0.312	0.062	370	46.25	0.125	0.187	SYN
D6	2.500	0.156	0.062	185	23.12	0.124	0.186	SYN
D7	0.312	0.078	0.250	185	46.25	0.250	0.500	SYN
B1	5	0.625	0.125	185	23.12	0.124	0.249	SYN
B2	10	1.25	0.125	370	46.25	0.125	0.250	SYN
B3	2.500	0.625	0.250	370	46.25	0.125	0.375	SYN
B4	1.250	0.156	0.124	185	23.12	0.124	0.248	SYN
B5	10	0.312	0.031	185	23.12	0.124	0.155	SYN
P1	1.25	0.156	0.124	185	46.25	0.250	0.374	SYN
P2	2.500	0.156	0.062	185	46.25	0.250	0.312	SYN
P3	0.625	0.078	0.124	185	23.12	0.124	0.248	SYN
P4	0.625	0.078	0.124	185	23.12	0.124	0.248	SYN
P5	1.250	0.156	0.124	185	11.5	0.062	0.186	SYN
P6	1.250	0.312	0.249	185	11.5	0.062	0.311	SYN

Table 7: Checkerboard assay of MCZ and CUR against clinical AR isolates of *C. albicans*

Strain	MIC_{80} of MCZ alone (μg/ml)	MIC_{80} of MCZ in combination (μg/ml)	FIC (MCZ)	MIC_{80} of CUR alone (μg/ml)	MIC_{80} of CUR in combination (μg/ml)	FIC (CUR)	FICI	Out Come
A1	0.312	0.078	0.250	370	92.50	0.250	0.500	SYN
A2	0.312	0.039	0.125	370	92.50	0.250	0.375	SYN
A3	1.250	0.039	0.031	185	23.12	0.124	0.155	SYN
D1	2.500	0.078	0.032	185	46.25	0.250	0.281	SYN
D2	1.250	0.156	0.124	185	46.25	0.250	0.374	SYN
D3	2.500	0.156	0.060	185	46.25	0.250	0.310	SYN
D4	0.625	0.039	0.624	185	23.12	0.124	0.186	SYN
D5	1.250	0.039	0.031	370	46.25	0.125	0.156	SYN
D6	2.500	0.312	0.124	185	11.50	0.062	0.186	SYN
D7	1.250	0.078	0.062	185	23.12	0.124	0.187	SYN
B1	1.250	0.156	0.124	185	23.12	0.124	0.248	SYN
B2	2.500	0.312	0.124	370	92.50	0.250	0.374	SYN
B3	1.250	0.312	0.249	185	23.12	0.124	0.373	SYN
B4	1.250	0.156	0.124	185	23.12	0.124	0.248	SYN
B5	5	0.156	0.031	185	23.12	0.124	0.155	SYN
P1	2.500	0.312	0.124	185	46.25	0.240	0.374	SYN
P2	2.500	0.156	0.062	185	11.50	0.062	0.124	SYN
P3	0.620	0.156	0.249	185	23.12	0.124	0.373	SYN
P4	2.500	0.156	0.062	185	11.50	0.062	0.124	SYN
P5	2.500	0.625	0.250	185	46.25	0.250	0.500	SYN
P6	2.500	0.156	0.062	185	46.25	0.250	0.312	SYN

Table 8: Checkerboard assay of ITR and CUR against clinical AR isolates of *C. albicans*

Strain	MIC_{80} of ITR alone (μg/ml)	MIC_{80} of ITR in combination (μg/ml)	FIC (ITR)	MIC_{80} of CUR alone (μg/ml)	MIC_{80} of CUR in combination (μg/ml)	FIC (CUR)	FICI	Out Come
A1	10	0.625	0.124	370	23.12	0.060	0.122	SYN
A2	1.250	0.156	0.124	370	92.5	0.250	0.374	SYN
A3	0.312	0.078	0.25	185	46.25	0.250	0.500	SYN
D1	2.500	0.312	0.124	185	23.12	0.124	0.248	SYN
D2	1.250	0.156	0.124	185	11.5	0.062	0.186	SYN
D3	5.000	1.250	0.250	185	46.25	0.250	0.500	SYN
D4	0.625	0.078	0.124	185	23.12	0.124	0.248	SYN
D5	2.500	0.312	0.124	370	92.5	0.250	0.374	SYN
D6	10	1.250	0.125	185	46.25	0.250	0.375	SYN
D7	0.315	0.039	0.123	185	46.25	0.250	0.373	SYN
B1	5	0.625	0.125	185	23.12	0.124	0.249	SYN
B2	10	0.625	0.0625	370	46.25	0.125	0.187	SYN
B3	5	0.312	0.0624	370	46.25	0.125	0.187	SYN
B4	10	0.156	0.0156	185	23.12	0.124	0.140	SYN
B5	10	0.039	0.0039	185	23.12	0.124	0.127	SYN
P1	2.500	0.156	0.062	185	46.25	0.250	0.312	SYN
P2	0.625	0.078	0.124	185	46.25	0.250	0.374	SYN
P3	2.500	0.156	0.062	185	23.12	0.124	0.186	SYN
P4	1.250	0.156	0.124	185	11.5	0.062	0.186	SYN
P5	0.625	0.078	0.124	185	11.5	0.062	0.186	SYN
P6	0.625	0.039	0.062	185	11.5	0.062	0.124	SYN

Table 9: Checkerboard assay of VRC and CUR against clinical AR isolates of *C. albicans*

Strain	MIC_{80} of VRC alone (μg/ml)	MIC_{80} of VRC in combination (μg/ml)	FIC (VRC)	MIC_{80} of CUR alone (μg/ml)	MIC_{80} of CUR in combination (μg/ml)	FIC (CUR)	FICI	Out Come
A1	2	0.250	0.125	370	92.5	0.25	0.375	SYN
A2	2	0.125	0.062	370	46.25	0.125	0.150	SYN
A3	0.5	0.625	0.125	185	23.12	0.124	0.250	SYN
D1	0.25	0.031	0.124	185	11.5	0.062	0.186	SYN
D2	2	0.250	0.125	185	46.25	0.250	0.374	SYN
D3	2	0.125	0.062	185	23.12	0.124	0.186	SYN
D4	2	0.125	0.062	185	46.25	0.250	0.312	SYN
D5	2	0.500	0.250	370	11.5	0.038	0.281	SYN
D6	1	0.062	0.062	185	11.5	0.062	0.124	SYN
D7	2	0.125	0.062	185	11.5	0.062	0.124	SYN
B1	0.5	0.625	0.125	185	46.25	0.25	0.374	SYN
B2	2	0.125	0.062	370	46.25	0.125	0.187	SYN
B3	2	0.062	0.031	370	92.5	0.25	0.281	SYN
B4	1	0.125	0.125	185	11.5	0.062	0.187	SYN
B5	0.25	0.031	0.124	185	23.12	0.124	0.248	SYN
P1	2	0.062	0.031	185	11.5	0.062	0.093	SYN
P2	2	0.125	0.062	185	11.5	0.062	0.124	SYN
P3	0.5	0.625	0.125	185	11.5	0.062	0.187	SYN
P4	0.5	0.625	0.125	185	23.1	0.124	0.249	SYN
P5	1	0.125	0.125	185	11.5	0.062	0.187	SYN
P6	2	0.125	0.062	185	23.1	0.124	0.186	SYN

Table 10: Checkerboard assay of AMB and CUR against clinical AR isolates of *C. albicans*

Strain	MIC_{80} of AMB alone (µg/ml)	MIC_{80} of AMB in combination (µg/ml)	FIC (AMB)	MIC_{80} of CUR alone (µg/ml)	MIC_{80} of CUR in combination (µg/ml)	FIC (CUR)	FICI	Out Come
A1	0.156	0.019	0.121	370	46.25	0.125	0.246	SYN
A2	0.625	0.078	0.124	370	92.5	0.250	0.374	SYN
A3	0.312	0.039	0.125	185	23.12	0.124	0.249	SYN
D1	0.312	0.019	0.060	185	23.12	0.124	0.184	SYN
D2	0.625	0.019	0.030	185	11.5	0.062	0.092	SYN
D3	0.625	0.039	0.062	185	47.25	0.250	0.312	SYN
D4	0.312	0.039	0.125	185	11.5	0.062	0.187	SYN
D5	0.312	0.078	0.025	370	92.5	0.250	0.275	SYN
D6	1.250	0.039	0.031	185	23.12	0.124	0.155	SYN
D7	1.250	0.078	0.062	185	23.12	0.124	0.186	SYN
B1	0.625	0.039	0.062	185	46.25	0.250	0.312	SYN
B2	1.250	0.039	0.031	370	23.12	0.062	0.093	SYN
B3	0.312	0.019	0.060	370	46.25	0.125	0.185	SYN
B4	0.312	0.078	0.250	185	46.25	0.250	0.500	SYN
B5	0.625	0.039	0.062	185	46.25	0.250	0.312	SYN
P1	0.625	0.019	0.030	185	23.12	0.124	0.154	SYN
P2	0.312	0.078	0.124	185	23.12	0.124	0.248	SYN
P3	0.312	0.019	0.060	185	46.25	0.250	0.310	SYN
P4	1.250	0.078	0.062	185	46.25	0.250	0.312	SYN
P5	0.625	0.019	0.030	185	46.25	0.250	0.280	SYN
P6	0.312	0.039	0.125	185	23.12	0.124	0.249	SYN

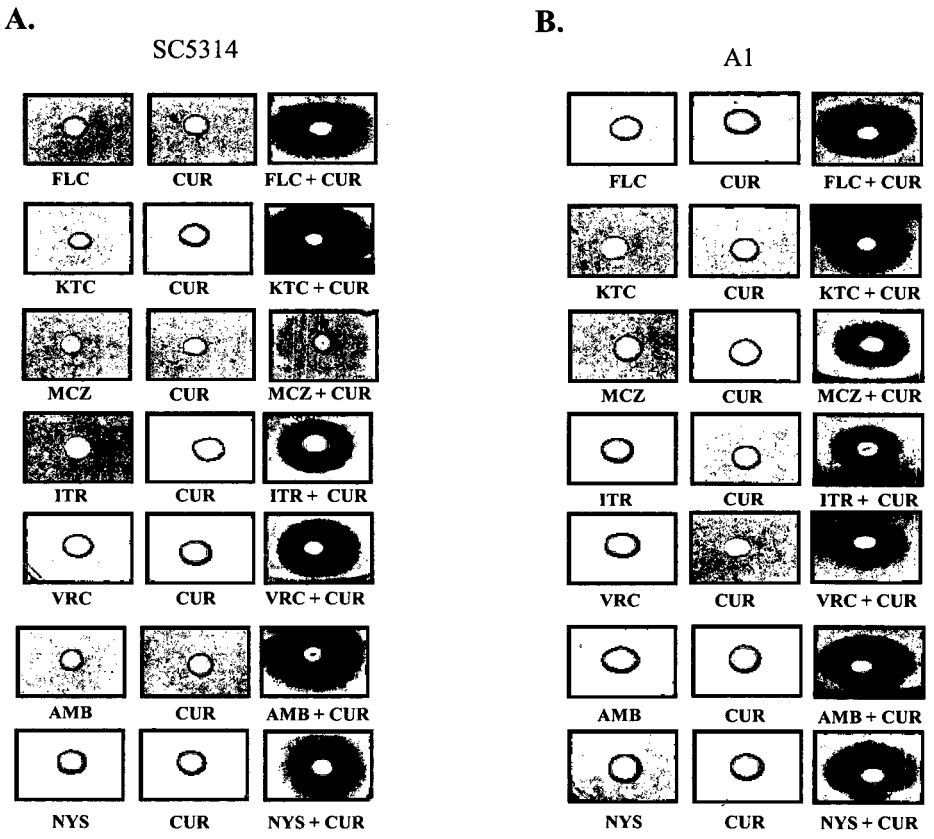
Table 11: Checkerboard assay of NYS and CUR against clinical AR isolates of *C. albicans*

Strain	MIC_{80} of NYS alone (µg/ml)	MIC_{80} of NYS in combination (µg/ml)	FIC (NYS)	MIC_{80} of CUR alone (µg/ml)	MIC_{80} of CUR in combination (µg/ml)	FIC (CUR)	FICI	Out Come
A1	3.75	0.234	0.062	370	92.5	0.250	0.312	SYN
A2	3.75	0.468	0.124	370	185	0.500	0.624	IND
A3	1.87	0.117	0.062	185	46.25	0.251	0.313	SYN
D1	7.50	0.93	0.124	185	46.25	0.251	0.375	SYN
D2	7.50	1.87	0.249	185	46.25	0.251	0.500	SYN
D3	3.75	0.468	0.124	185	47.25	0.250	0.374	SYN
D4	1.87	0.234	0.125	185	92.5	0.250	0.375	SYN
D5	7.50	0.93	0.124	370	185	0.500	0.624	IND
D6	3.75	0.234	0.062	185	11.5	0.062	0.124	SYN
D7	3.75	0.93	0.248	185	23.12	0.124	0.372	SYN
B1	7.50	0.93	0.124	185	46.25	0.250	0.374	SYN
B2	3.75	0.468	0.124	370	185	0.500	0.624	IND
B3	3.75	0.234	0.062	370	46.25	0.125	0.187	SYN
B4	1.87	0.117	0.062	185	11.5	0.062	0.124	SYN
B5	7.50	0.93	0.124	185	23.12	0.124	0.248	SYN
P1	7.50	1.87	0.249	185	23.12	0.120	0.369	SYN
P2	7.50	3.75	0.500	185	46.25	0.250	0.750	IND
P3	3.75	0.93	0.248	185	11.5	0.062	0.310	SYN
P4	3.75	0.468	0.124	185	46.2	0.250	0.374	SYN
P5	1.87	0.93	0.497	185	23.12	0.120	0.617	IND
P6	7.50	3.75	0.500	185	23.12	0.124	0.624	IND

32 (FLC), 0.039-0.625 (KTC), 0.039-0.312 (MCZ), 0.039-0.625 (ITR), 0.0312-0.25 (VRC), 0.019-0.078 (AMB) and 0.078-1.25 (NYS). As depicted from the FICI values, pure CUR-I was synergistic to azoles and polyenes in all of the tested strains with the exception of the clinical isolates A2, D5, B2, P2, P5 and P6, where the FICI values were between >0.5 and ≤4 which implied indifferent kind of interactions between CUR-I and NYS. Thus, a 100% synergy was recorded in case of CUR-I with tested azoles and AMB in WT and AR isolates. However, we did not find antagonistic interactions between CUR I and any of the drugs tested.

3.3.2.2 Filter-disc assay and time-kill curves confirm synergism

For validation of checkerboard assays in AR isolates, we selected one representative strain A1. Filter-disc assay visualized the synergistic interaction of CUR-I and tested drugs. Figure 28 shows the filter discs for the WT strain SC5314 (Figure 28A) and the representative strain A1 (Figure 28B). As depicted in Figure 28, at indicated concentrations there was no antifungal activity of pure CUR-I or tested drugs alone (absence of zones of inhibition). However, combination of pure CUR-I with tested drugs yielded significantly clear zones of inhibition, the diameter (mm) of various zones of inhibition are summarized in Figure 28C. The observed synergism between pure CUR-I and tested drugs was further confirmed by time-kill curves. Figure 29 shows the time-kill curves of the WT strain and the representative clinical AR isolate AI. CUR-I and either of the tested drugs at indicated concentration alone did not significantly affect the growth curve, however, combination of CUR-I with azoles or polyenes significantly affected the growth curve of the WT (Figure 29A) as well as of the representative clinical AR isolate A1 (Figure 29B). Given the initial inoculum of 10^3 CFU/ml for WT strain, combination therapy of CUR-I yielded a corresponding decline of 4, 6.6, 11, 6.3, 10, 7.8 and 8.5 $\log_{10}$ CFU/ml for FLC, KTC, MCZ, ITR, VRC, AMB and NYS when compared to respective drugs alone at 24 hrs. Similarly, for the representative clinical AR isolate AI the combination therapy yielded a sharp decline in the observed CFU/ml (Figure 29B).



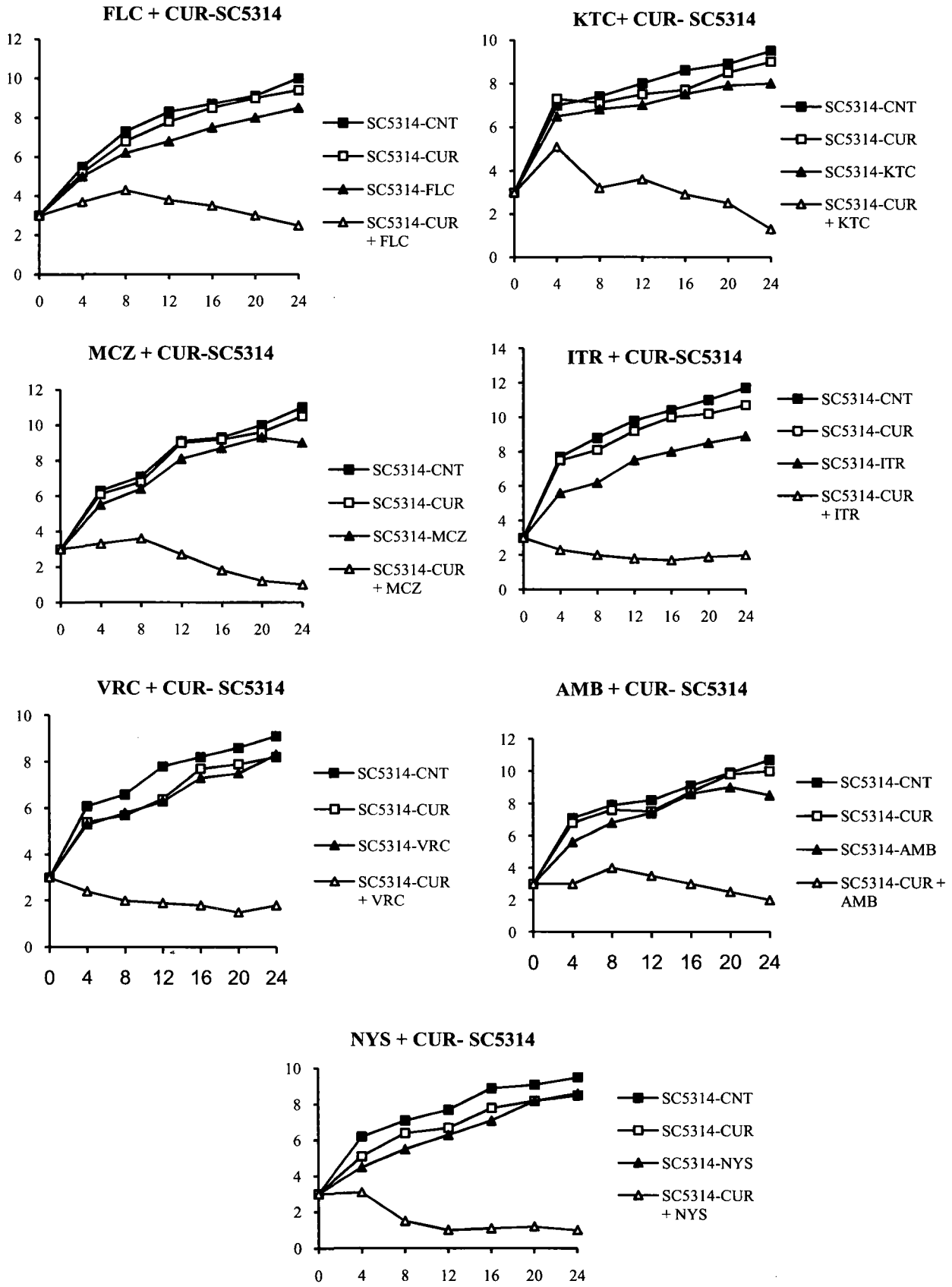
C.

Drug*	Strains SC5314	AI
	Diameter of zones of inhibition (mm)	
FLC + CUR	1.5	1.0
KTC + CUR	1.6	0.9
MCZ + CUR	1.3	0.6
ITR + CUR	1.0	0.5
VRC + CUR	1.2	0.8
AMB + CUR	1.3	0.9
NYS +CUR	1.4	1.0

*No zone of inhibition in drug alone

Figure 28. Filter disc assay of CUR-I in combination with azoles/polyenes.
(A) Synergistic interactions in WT strain SC5314, the various concentrations (μg/ml) spotted/ impregnated were 0.0155 FLC/23.12CUR-I, 0.0078 KTC/ 23.12 CUR-I, 0.0062 MCZ/11.5 CUR-I, 0.0078 ITR/ 23.12 CUR-I, 0.0078 VRC/ 46.25 CUR-I, 0.234 NYS/23.12 CUR-I, 0.078 AMB/23.12 CUR-I. **(B)** Shows the synergistic interactions in the clinical isolate A1, the various concentrations (μg/ml) spotted were 8 FLC/ 23.12 CUR-I, 0.312 KTC/ 23.12 CUR-I, 0.078 MCZ/ 92.5 CUR-I, 0.625 ITR/23.12 CUR-I, 0.25 VRC/92.5 CUR-I, 0.234 NYS/92.5 CUR-I, 0.019 AMB/ 46.25 CUR-I. **(C)** Table summarizes the diameter of zones of inhibition (in mm) in WT strain SC5314 and the AR isolate AI.

A.


FLC + CUR-SC5314
SC5314-CNT
SC5314-CUR
SC5314-FLC
SC5314-CUR + FLC
KTC+ CUR- SC5314
SC5314-CNT
SC5314-CUR
SC5314-KTC
SC5314-CUR + KTC
MCZ + CUR-SC5314
SC5314-CNT
SC5314-CUR
SC5314-MCZ
SC5314-CUR + MCZ
ITR + CUR-SC5314
SC5314-CNT
SC5314-CUR
SC5314-ITR
SC5314-CUR + ITR
VRC + CUR- SC5314
SC5314-CNT
SC5314-CUR
SC5314-VRC
SC5314-CUR + VRC
AMB + CUR- SC5314
SC5314-CNT
SC5314-CUR
SC5314-AMB
SC5314-CUR + AMB
NYS + CUR- SC5314
SC5314-CNT
SC5314-CUR
SC5314-NYS
SC5314-CUR + NYS

B.

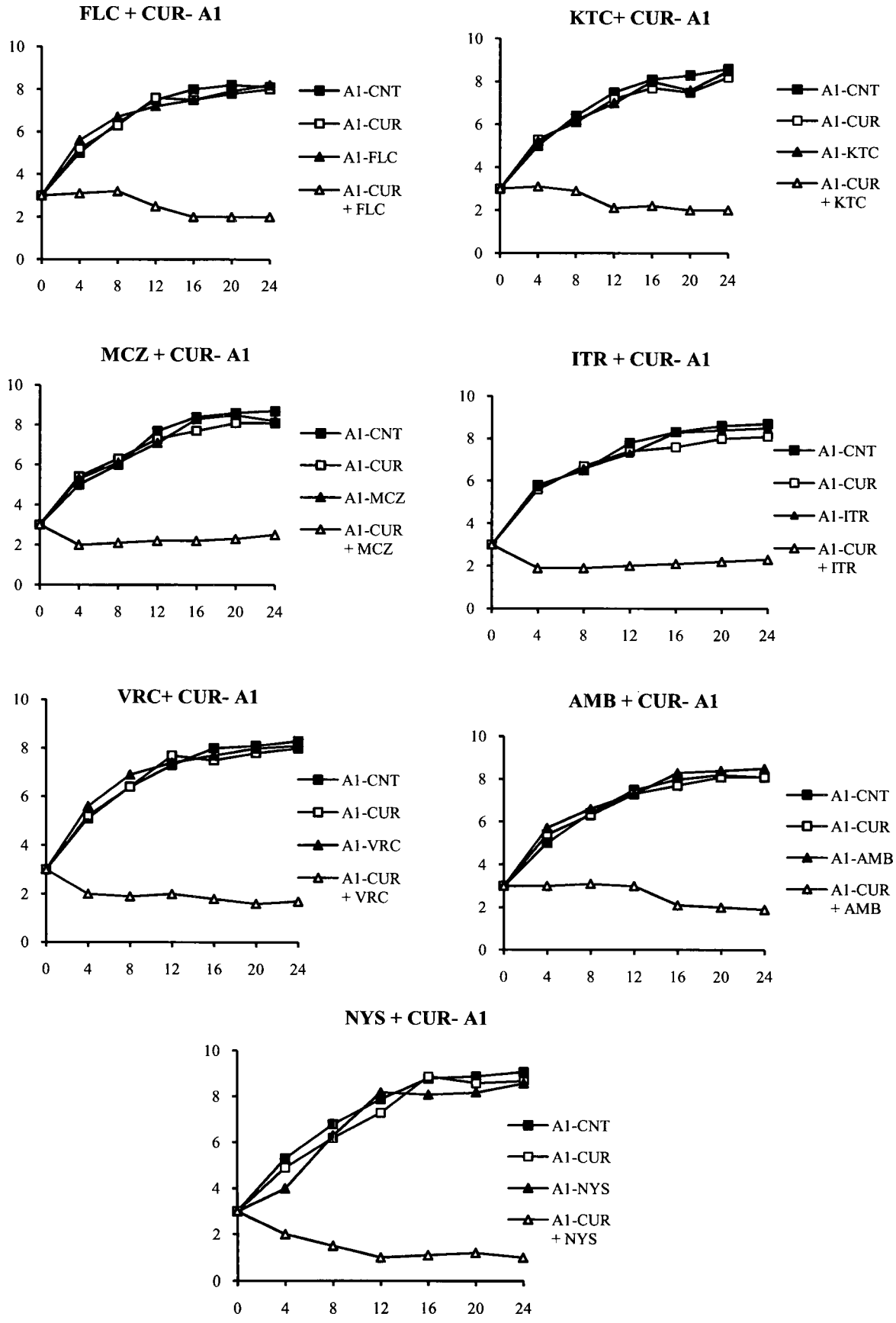

FLC + CUR- A1
A1-CNT
A1-CUR
A1-FLC
A1-CUR + FLC
KTC+ CUR- A1
A1-CNT
A1-CUR
A1-KTC
A1-CUR + KTC
MCZ + CUR- A1
A1-CNT
A1-CUR
A1-MCZ
A1-CUR + MCZ
ITR + CUR- A1
A1-CNT
A1-CUR
A1-ITR
A1-CUR + ITR
VRC+ CUR- A1
A1-CNT
A1-CUR
A1-VRC
A1-CUR + VRC
AMB + CUR- A1
A1-CNT
A1-CUR
A1-AMB
A1-CUR + AMB
NYS + CUR- A1
A1-CNT
A1-CUR
A1-NYS
A1-CUR + NYS

Figure 29. Time kill curves of CUR-I in combination with azoles/polyenes
(A) time kill curves of WT strain SC5314, the various concentrations (μg/ml) used were 0.0155, 0.0078, 0.0062, 0.0078, 0.0078, 0.234, 0.078 of FLC, KTC, MCZ, ITR, VRC, NYS, AMB and the concentration (μg/ml) of CUR-I combined was 23.12, 23.12, 11.5, 23.12, 46.25, 23.12 and 23.12, respectively **(B)** time kill curves of the clinical isolate A1, the concentrations (μg/ml) used were 8, 0.312, 0.078, 0.625, 0.25, 0.234, 0.019 of FLC, KTC, MCZ, ITR, VRC, NYS, AMB and the concentration (μg/ml) of CUR-I combined was 23.12, 23.12, 92.5, 23.12, 92.5, 92.5, and 46.25, respectively. The x-axis is time in hours and y- axis is log CFU/ml.

3.3.2.3 A combination of FLC/AMB and CUR-I raises ROS levels which could be reversed by an antioxidant

As depicted in Figure 30A, ROS levels were measured in cells (SC5314) treated with either of the drug alone *viz* FLC (0.0155μg/ml), AMB (0.078 μg/ml) and CUR-I (23.12μg/ml) or in combination, FLC (0.0155μg/ml)+CUR-I (23.12μg/ml) or AMB (0.078μg/ml)+CUR-I (23.12μg/ml). The treatment of FLC, AMB or CUR-I alone did not influence the concentration of endogenous ROS, however, FLC+CUR-I or AMB+CUR-I combination resulted in marked augmentation of endogenous ROS levels (16 and 26 fold in FLC+CUR-I and AMB+CUR-I, respectively). The increased ROS levels and the synergistic antifungal activity of FLC+CUR-I and AMB+CUR-I could be reversed in presence of an antioxidant as was evident from increased FICI values (Figure 30B). Of note, for ROS and subsequent determinations, we used FLC and AMB, as a representative drug from each category of azoles and polyenes.

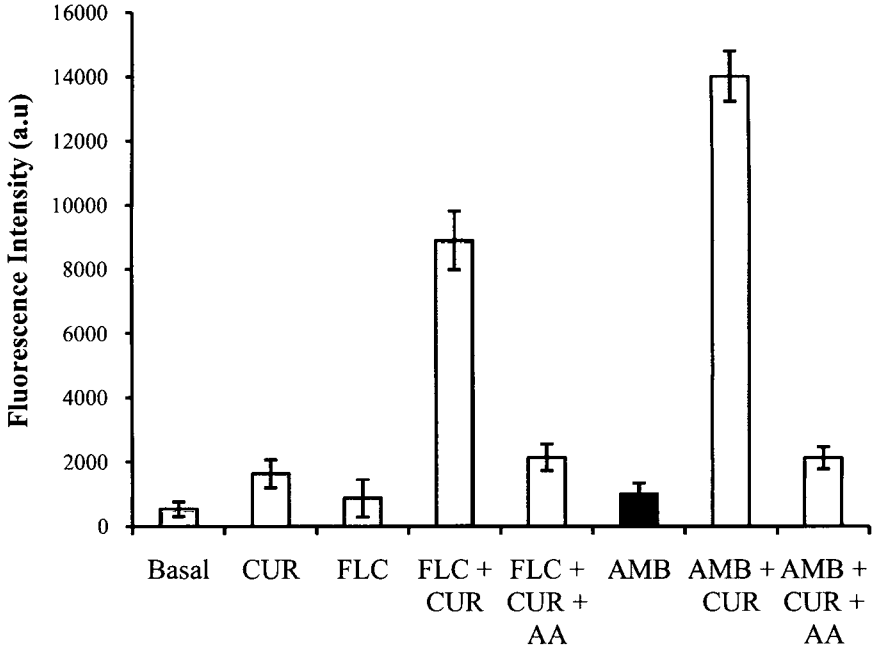
3.3.2.4 Increased ROS leads to apoptosis

As shown in Figure 31, there is almost no apoptotic population in untreated cells (0%, lower right quadrant, Panel I), CUR-I (0.01%, lower right quadrant, Panel II), FLC (1.54%, lower right quadrant, Panel III) and AMB treated (0.14%, lower right quadrant, Panel IV) cells. However, there was a significant population of apoptotic cells when CUR-I was used in combination with FLC (17.6%, lower right quadrant, Panel V) or AMB (18.46%, lower right quadrant, Panel VI). The treatment of cells with AA decreased the apoptotic population (2.05%, lower right quadrant, Panel VII and 4.18%, lower right quadrant, Panel VIII).

3.3.3 Discussion

In the present study, we demonstrate that pure polyphenol CUR-I is synergistic to antifungal drugs belonging to either azole or polyene categories. The synergism with CUR-I is independent of efflux pump proteins belonging to either ABC or MFS categories. For example, the synergistic effect of CUR-I was demonstrable in *CaCDR1, CaCDR2* and *CaMDR1* null mutants (Table 12). We also demonstrate that the synergism with CUR-I can be shown in AR clinical isolates with reduced antifungal sensitivity.

A.



B.

Drug	FICI	FICI in presence of antioxidant
FLC	0.093	0.374
AMB	0.187	0.498

Figure 30. ROS levels in presence of CUR-I and azoles/polyenes.
(A) Amounts of ROS produced due to CUR-I, FLC or AMB either alone or in combination with AA. The concentrations used are 23.12 μg/ml of CUR-I, 0.0155 μg/ml of FLC, 0.078 μg/ml of AMB and 25 mM AA. The fluorescence emitted by the cells was measured using a spectrofluorometer (Varian, Cary Eclipse). An excitation and emission of 485 and 540nm respectively, were used. The values are the means and standard deviations (indicated by bars) of three independent experiments. **(B)** Table summarises the FICI values of CUR-I+FLC and CUR-I+AMB in presence of AA (25 mM).

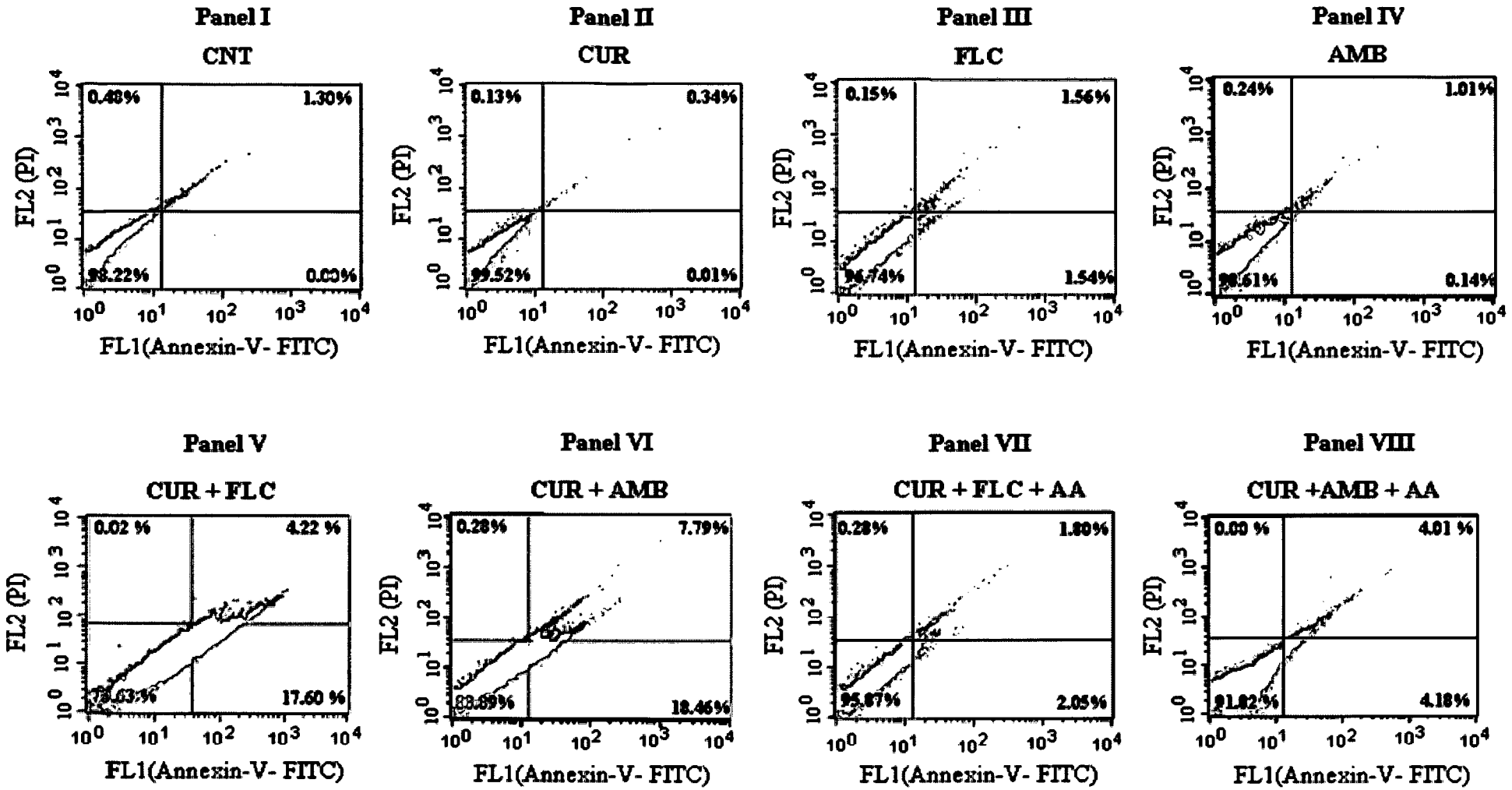


Figure 31. Externalization of phosphatidylserine in presence of CUR-I and azoles/polyenes.

Panel I represents untreated *C. albicans* (SC5314) cells, Panel II, III and IV shows CUR-I, FLC and AMB treated cells. Panel V and VI are CUR-I+FLC and CUR-I+AMB treated cells, respectively. Panel VII and VIII are AA pretreated cells, further treated with CUR-I+FLC and CUR-I+AMB, respectively. After treatment for 4 hrs, the cells were analyzed by flow cytometry as described in "Materials and Methods".

Table 12: Checkerboard assay of FLC and CUR against drug efflux pump and calcineurin null mutants of *C. albicans*

Strain/ Antifungal agent	MIC_{80} of each agent (μg/ml)		FIC	FICI	Out Come
	Alone	Combination			
ΔCdr1					
FLC	0.125	0.031	0.248	0.372	SYN
CUR	185	23.12	0.124		
ΔCdr2					
FLC	0.250	0.062	0.248	0.372	SYN
CUR	185	23.12	0.124		
ΔCaMDR1					
FLC	0.500	0.015	0.031	0.155	SYN
CUR	185	23.12	0.124		
Δcnb1					
FLC	0.500	0.015	0.031	0.155	SYN
CUR	185	23.12	0.124		

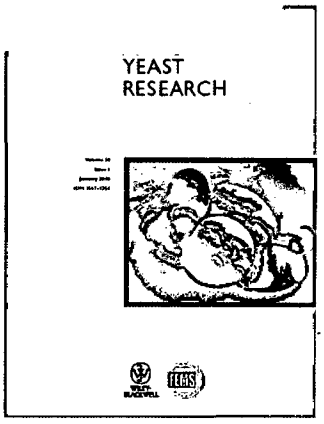
The synergistic effect of CUR-I was independent of well known calcineurin pathway. Cacineurin is a Ca^{2+}-calmodulin-activated phosphatase which is involved in fungicidal synergism of FLC with cyclosporine A (CsA) and tacrolimus (FK506) (Uppuluri *et al.*, 2008; Cruz *et al.*, 2002). The synergism with FLC/AMB and CUR-I combination remained unaffected in calcineurin nulls viz., Δ*cnb1*/*cnb1* (Table 12).

We had earlier shown that CUR-I (185μg/ml) acts as an antifungal *via* generation of ROS and triggers an early apoptosis in *C. albicans* cells (Sharma *et al.*, 2009a). Notably, a combination of CUR-I with azoles (FLC) or polyenes (AMB) generated ROS levels to several folds (16 and 26 folds, respectively) at a much lower concentration. Thus if used alone, neither FLC or AMB or CUR-I showed antifungal activity and increased ROS levels. Interestingly, the reversal of ROS levels by AA also reversed synergism which was evident from increased FICI values. The synergism of CUR-I with drugs also lead to an increase in apoptotic cell population which again could be reversed by the addition of an antioxidant (Figure 30).

Earlier, we observed that CUR modulated efflux mediated by ABC multidrug transporters of *C. albicans* hetrologously expressed in *S. cerevisiae* and was selectively synergistic with drug substrates such as Rhodamine 6G, KTC, ITR and MCZ but not with FLC and VRC (Sharma *et al.*, 2009b). However, in the present study, we could demonstrate synergism with FLC and VRC as well. This difference in the pattern of interaction of CUR with FLC/VRC in *S. cerevisiae* strain AD-CDR1 and *C. albicans* strain SC5314 may not be strictly comparable since the two strains represent different systems. In this study, we observed difference in drug interactions with CUR-I within *Candida* strains. For example, NYS was not synergistic with CUR-I in 6 of the clinical AR isolates tested but there was good synergism between CUR-I and NYS with all other isolates. *In vitro* assays like checkerboard method are preliminary steps to understand drug-drug interactions. However, detailed *in vivo* pharmacokinetic and pharmacodynamic studies are warranted to really comment upon these interactions.

We have demonstrated that the effect of CUR-I if given in combination with azoles and polyenes is synergistic; this synergy ir related to the generation of ROS and apoptosis. Considering the fact that MDR in *Candida* is associated with multiple signaling pathways, inhibition of signaling cascades with combination treatment of nontoxic natural products and antifungals represents a logical preventive/therapeutic approach for combating *Candida* infections.

✓ The work presented in this chapter has been published as **"Synergistic anticandidal activity of pure polyphenol Curcumin I in combination with azoles and polyenes generates reactive oxygen species leading to apoptosis"** in *FEMS Yeast Research* (2010), Epub. ahead of print.



Summary

SUMMARY

C. albicans is an opportunistic diploid fungus that causes infection in immunocompromised and debilitated patients (Odds, 1988). Wide spread and prolonged usage of azoles in recent years has led to the rapid development of the phenomenon of azole resistance which poses a major threat to antifungal therapy (Calderone, 2002; White *et al.*, 1998). Various mechanisms which contribute towards the development of azole resistance have been implicated in *Candida* such as overexpression of, or mutations in the target enzyme of azoles, lanosterol 14α-demethylase as well as overexpression of drug efflux pumps encoding genes belonging to either ABC or MFS category of proteins (Kohli *et al.*, 2002; Odds, 1988; White, 1998).

Coinciding with the occurrence of azole resistant isolates, many efforts are directed to resolve the challenging problem of MDR by **(i)** Development of inhibitors/modulators of these multidrug efflux pumps. **(ii)** Searching for new antifungal agents. **(iii)** Improving the efficacy of antifungal agents by using combination therapy. In light of the above mentioned goals, we have explored in detail the role of CUR in MDR of *C. albicans*. CUR, a natural product of turmeric, from rhizomes of *Curcuma longa* are known reversal agents of drug resistance phenotype in cancer cells over-expressing ABC transporters *viz. ABCB1, ABCG2* and *ABCC1*.

In the first section of the study, we evaluated whether CUR, could also modulate multidrug transporters of yeasts that belong to either the ABC or the MFS category. The effect of CUR on multidrug transporter proteins was demonstrated by examining Rhodamine 6 G (R6G) efflux in *S. cerevisiae* cells over expressing ABC transporters CaCdr1p and CaCdr2p, MFS CaMdr1p of *C. albicans* and ScPdr5p of *S. cerevisiae*. CUR decreased the extra cellular concentration of R6G in ABC transporter expressing cells while had no effect on MTX efflux mediated through MFS transporter CaMdr1p. CUR competitively inhibited R6G efflux and the photo labeling of CaCdr1p by a drug substrate prazosin analog [^{125}I]-iodoarylazidoprazosin (IC_{50}, 14.2 μM). Notably, the mutant variants of CaCdr1p which displayed abrogated efflux of R6G also showed reduced modulation by CUR. Drug susceptibility testing of ABC protein expressing cells by spot assays and checkerboard tests revealed that CUR was selectively synergistic with drug substrates such as R6G, KTC, ITR and MCZ, but not

with FLC, VRC, ANI, CYH and FK520. Taken together, our results provide the first evidence that CUR only modulates ABC multidrug transporters and could be exploited in combination with certain conventional antifungal drugs to reverse MDR in *Candida* cells.

In the second section of the study, we have investigated the antifungal effects of CUR, against *albicans* and non *albicans* species of *Candida* and shown its ability to inhibit the growth of all the tested strains. The inhibitory effects of CUR were independent of the status of the multi drug efflux pump proteins belonging to either ABC or MFS category of transporters. By using a systemic murine model of infection, we established that CUR and piperine when administered together caused a significant fungal load reduction ($1.4\log_{10}$) in kidneys of Swiss mice. Additionally, CUR raised the levels of ROS which as revealed by Annexin V-FITC labeling, triggered an early apoptosis in *Candida* cells. Coincident with the raised ROS levels, mRNAs of tested oxidative stress related genes (*CAP1, CaIPF7817, SOD2, GRP2* and *CAT1*) were also elevated. The growth inhibitory effects of CUR could be reversed by the addition of natural and synthetic antioxidants. Notably, independent of ROS status, polyphenol CUR prevented hyphae development in both liquid and solid hypha inducing media by targeting global suppressor *TUP1*. Taken together, our results provide the first evidence that CUR acts as an antifungal agent, *via* generation of oxidative stress, inhibits hyphae development by targeting *TUP1*.

In the last section of the present study, by employing checkerboard method, filter disc and time-kill assays, we have demonstrated that CUR-I at non antifungal concentration synergizes with azoles and polyenes. For this, pure polyphenol CUR-I was tested for synergy with five azole and two polyene drugs such as FLC, MCZ, KTC, ITR, VRC, NYS and AMB against 21 clinical isolates of *C. albicans*, with reduced antifungal sensitivity, as well as a drug-sensitive laboratory strain. Notably, there was a 10-35 fold drop in the MIC_{80} values of the drugs when CUR-I was used in combination, FICI values ranged between 0.09-0.5. Interestingly, the synergistic effect of CUR-I with FLC and AMB was associated with the accumulation of ROS which could be reversed by the addition of an antioxidant such as AA. Furthermore, the combination of CUR-I and FLC/AMB triggered apoptosis that too could be reversed by AA. We provide the first evidence that pure CUR-I in combination with azoles and polyenes represents a novel therapeutic strategy in improving activity of common antifungals.

Considering the success story of CUR as an anti-cancer and anti-inflammatory compound, the results presented in the thesis clearly opens up an opportunity wherein CUR can also be exploited as a potential natural antifungal.

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Appendices

APPENDIX-I

List of Strains used in the study

Strain	Genotype	Source
AD1-8u$^-$	*Mat a, pdr1-3, his1, ura3, Δyor1::hisG,Δsnq2::hisG , Δpdr5::hisG, Δpdr10::hisG,Δpdr11::hisG,Δycf1::hisG, pdr3::hisG,Δpdr15::hisG*	Decottignies *et al.* (1998)
AD-CDR1	AD1-8u$^-$ cells harboring CaCDR1-GFP ORF integrated at *PDR5* locus	Shukla *et al.* (2003)
A1346G	CDR1-GFP cells carrying A1346G mutation in *CDR1* ORF and integrated at *PDR5* locus	Saini *et al.* (2005)
A1347G	CDR1-GFP cells carrying A1347G mutation in *CDR1* ORF and integrated at *PDR5* locus	Saini *et al.* (2005)
T1351A	CDR1-GFP cells carrying T1351A mutation in *CDR1* ORF and integrated at *PDR5* locus	Saini *et al.* (2005)
F1360A	CDR1-GFP cells carrying F1360A mutation in *CDR1* ORF and integrated at *PDR5* locus	Saini *et al.* (2005)
G1362A	CDR1-GFP cells carrying G1362A mutation in *CDR1* ORF and integrated at *PDR5* locus	Saini *et al.* (2005)
L1358A	CDR1-GFP cells carrying L1358A mutation in *CDR1* ORF and integrated at *PDR5* locus	Saini *et al.* (2005)
T1355A	CDR1-GFP cells carrying T1355A mutation in *CDR1* ORF and integrated at *PDR5* locus	Saini *et al.* (2005)
G682A	CDR1-GFP cells carrying G682A mutation in *CDR1* ORF and integrated at *PDR5* locus	Puri *et al.* (unpublished data)
AD-CaMDR1	AD1-8u$^-$ cells harboring CaMDR1-GFP ORF integrated at *PDR5* locus	Pasrija *et al.* (2007)
AD-CDR2	AD1-8u$^-$ cells harboring CaCDR2-GFP ORF integrated at *PDR5* locus	Lamping *et al.* (2007)
AD-PDR5	AD1-8u$^-$ cells harboring ScPDR5-GFP ORF integrated at *PDR5* locus	Lamping *et al.* (2007)
CAI4	*Δura3::imm434/Δura3::imm434*	(Fonzi and Irwin, 1993)
ATCCMYA 2876	*Candida albicans*	(Fonzi and Irwin, 1993)
ATCC6258	*Candida krusei*	Ranbaxy Lab. India

ATCC750	*Candida tropicalis*	Ranbaxy Lab. India
ATCC22019	*Candida parapsilosis*	Ranbaxy Lab. India
ATCC2512	*Candida kefyr*	Ranbaxy Lab. India
ATCC33	*Candida dubliniensis*	Ranbaxy Lab. India
ATCC90030	*Candida glabrata*	Ranbaxy Lab. India
ATCC15239	*Candida utilis*	Ranbaxy Lab. India
Gu4	Fluconazole susceptible clinical isolate	Franz *et al.* (1999)
Gu5	Fluconazole resistant clinical isolate	Franz *et al.* (1999)
F2	Fluconazole susceptible clinical isolate	Franz *et al.* (1999)
F5	Fluconazole resistant clinical isolate	Franz *et al.* (1999)
DSY 449	Δ *cdr1*::hisG/Δ *cdr1*::hisG	Sanglard *et al.* (1996)
DSY 653	Δ*cdr2*::hisG-URA3-hisG/Δ*cdr2*::hisG	Sanglard *et al.* (1997)
F5M 432	*Δ mdr1*::FRT/*Δmdr1*::FRT	Wirsching *et al.* (2000)
HLC52	Δ *efg1*::hisG/Δ*efg1*::hisG-URA3-hisG	Stoldt *et al.* (1997)
DK9	Δ *tup1*::hisG/Δ *tup1*::p405-URA3 /Δ *ura3*	Braun *et al.* (2001)
CSSK22-1	*Δura3::imm434/Δura3::imm434Δ ssk1::hisG/Δssk1::hisG*	Calera *et al.* (2000)
DK152	Δ*nrg1*::hisG/Δ*nrg1*::hisG-URA3-hisG	Braun *et al.* (2001)
JKC19	Δ*cph1*::hisG/Δ*cph1::hisG-URA3-hisG*	Stoldt *et al.* (1997)
Δ *ccc2*	Δura3::imm434/Δura3::imm434Δ*ccc2* ::hisG/Δ*ccc2::his G*	Weissman *et al.* (2000)

Δ *ftr1*	Δura3::imm434/Δura3::imm434Δ *ftr1*::hisG/Δftr1::*hisG*	Ramanan *et al.*(2000)
DSY2906	Δ*tac1*::hisG/Δ*tac1*::hisG	Weissman *et al.* (2000)
CJD21	Δ*cap1*::hisG/Δ*cap1*::hisG	Alarco and Raymond, 1999
JH2	Δ *ipf7817*::hisG/Δ*ipf7817*::hisGΔ ura3::imm434/Δura3 ::imm434his1::hisG/his1::hisG	Jia, *et al.* (2007)
ras 1-2/ras1-3	Δ *ras1*::hisG/Δ*ras1*::hph-URA3-hph	Feng *et al.* (1999)
SN152	A wild type strain of *C.albicans*	Navarathna *et al.* (2007)
KWN2	Δ*dpp3*::hisG/Δ *dpp3*::hisG	Navarathna *et al.* (2007)
DAY364	Δ*cnb1*::hisG/ Δ*cnb1*::hisG	Cruz *et al.*(2002)
SC5314	Laboratory isolate of *C.albicans*	AIIMS, India
A1	Oropharyngeal lesions of HIV/AIDS patients	AIIMS, India
A2	Oropharyngeal lesions of HIV/AIDS patients	AIIMS, India
A3	Oropharyngeal lesions of HIV/AIDS patients	AIIMS, India
D1	Vaginal swabs of diabetic woman	AIIMS, India
D2	Vaginal swabs of diabetic woman	AIIMS, India
D3	Vaginal swabs of diabetic woman	AIIMS, India
D4	Vaginal swabs of diabetic woman	AIIMS, India
D5	Vaginal swabs of diabetic woman	AIIMS, India
D6	Vaginal swabs of diabetic woman	AIIMS, India
D7	Vaginal swabs of diabetic woman	AIIMS, India

B1	Wounds of burn patients	AIIMS, India
B2	Wounds of burn patients	AIIMS, India
B3	Wounds of burn patients	AIIMS, India
B4	Wounds of burn patients	AIIMS, India
B5	Wounds of burn patients	AIIMS, India
P1	Pancreatitis patients	AIIMS, India
P2	Pancreatitis patients	AIIMS, India
P3	Pancreatitis patients	AIIMS, India
P4	Pancreatitis patients	AIIMS, India
P5	Pancreatitis patients	AIIMS, India
P6	Pancreatitis patients	AIIMS, India

APPENDIX-II

YEPD (Yeast Extract Peptone Dextrose)

Component	Concentration (g/100ml)
Yeast extract	1
Bacto peptone	2
D-glucose	2
Bacto agar (when required)	2.5

LIQUID SPIDER MEDIA

Component	Concentration (g/100ml)
Nutrient broth	1
Mannitol	1
K_2HPO_4	0.2

APPENDIX-III

List of oligonucleotides used in the study

Primer	Sequence (5'-3')
MS-TUP –F	ATTCTTGCAAGATTTAGACATTGC
MS-TUP1- R	ATGACACCACGGTCCTTTGAGC
MS-CAP1-F	ACCGTGAACGTAAAGAACG
MS- CAP1-R	GCTACCACCAGTATATTTAGCC
MS-IPF7817-F	TGTTAAGGATTTTGGTGCTGC
MS-IPF7817-R	CCACCGTATTCATCTGTTCTC
MS-GRP2-F	ATGTCTTCATCTACTACAGTTTTCG
MS- GRP2-R	TAATTTCAAAAGCTTGTGGACC
MS- CAT1-F	CCCAGAAAGAGTTGTCCACGC
MS -CAT1-R	CCATGATGGGTAGTTACCAGCAGC
MS- SOD2-F	ATGTTTTCTATCAGATCATC
MS- SOD2-R	ACCACCACCTTGAGAGACAGGAGCC
KM-1 (CDR1-F)	CTTTTCCACTGGTAACTACT
KM-2 (CDR1-R)	ACTGTATCCTACGAAGTTACCATTGACCC
CDR2-F	GGTCCTTATACCGAAGCTGC
CDR2-R	TTTTTTCATCTTCTTTTCTCT
MDR-F	CACCGTTATGGAACCAGTTG
MDR-R	CAGCACCAAACAATGGACCAACCCAATGAG
ACT1-F-RM	GTTAGAAAAGAATTATACGG
ACT1-R-RM	GAAACATTTGTGGTGAACAATGG

APPENDIX- IV

8 % Polyacrylamide Gel (PAGE)

Components	Volume for 50 ml
30 % Polyacrylamide	13.3 ml
1.5M Tris (pH 8.8)	12.5 ml
10 % SDS	500 µl
10% APS	500 µl
TEMED	30 µl
M.Q. water	23.2 ml

ATPase assay buffer

Components	Concentration
Tris (pH 7.5)	59mM
$MgCl_2$	7mM
Ammonium molybdate	0.2mM
KNO_3	50mM
NaN_3	10mM

Zymolyase buffer

Components	Volume for 50ml
1M 50Mm Tris Cl (pH 7.5)	2.5ml
1M $MgCl_2$	0.5ml
1M DTT	1.5ml
2M Sorbitol	25ml
M.Q. water	20ml

Western Hybridization

Components	Amount
Transfer buffer (for 1000 ml)	
Tris	3.03g (25mM)
Glycine	14.4g (198mM)
Methanol	200ml (20%)
Blocking Buffer (for 10ml)	
Skimmed milk	500mg (5%)
Phosphate buffer saline-Tween 80(PBS-T)	10ml
Methanol	200ml (20%)
Monoclonal Anti-GFP antibody	1:5000

APPENDIX -V

Solvents for Drugs

Drugs	Solvent used
Rhodamine 6-G	DMSO
Fluconazole	Water
Itraconazole	DMSO
Ketoconazole	Methanol
Miconazole	Methanol
Voriconazole	Water
Nystatin	Methanol
Amphotericin B	DMSO
Cycloheximide	Water
Anisomycin	DMSO
FK520	Ethanol
Methotrexate	10 mM Tris-Cl
Curcumin	DMSO

PUBLICATIONS

- **Monika Sharma**, Raman Manoharlal, Suneet Shukla, Nidhi Puri, Tulika Prasad, Suresh V. Ambudkar and Rajendra Prasad (2009). Curcumin modulates efflux mediated by yeast ABC multidrug transporters and is synergistic with antifungals. (***Antimicrob. Agents Chemother.* 53, 3256-3265**).

- **Monika Sharma**, Raman Manoharlal, Nidhi Puri and Rajendra Prasad (2009). Antifungal curcumin induces reactive oxygen species and triggers an early apoptosis but prevents hyphae development by targeting the global repressor *TUP1* in *Candida albicans*. (***Biosciences Reports,* Epub. ahead of print**).

- **Monika Sharma,** Raman Manoharlal, Arvind Singh Negi and Rajendra Prasad (2010). Synergistic anticandidal activity of pure polyphenol Curcumin I in combination with azoles and polyenes generates reactive oxygen species leading to apoptosis. (***FEMS Yeast Research,* Epub. ahead of print**).

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Curcumin Modulates Efflux Mediated by Yeast ABC Multidrug Transporters and Is Synergistic with Antifungals▽

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Curcumin (CUR), a natural product of turmeric, from rhizomes of *Curcuma longa*, is a known agent of reversal of drug resistance phenotypes in cancer cells overexpressing ATP-binding cassette (ABC) transporters, viz., ABCB1, ABCG2, and ABCC1. In the present study, we evaluated whether CUR could also modulate multidrug transporters of yeasts that belong either to the ABC family or to the major facilitator superfamily (MFS). The effect of CUR on multidrug transporter proteins was demonstrated by examining rhodamine 6G (R6G) efflux in *Saccharomyces cerevisiae* cells overexpressing the *Candida albicans* ABC transporters Cdr1p and Cdr2p (CaCdr1p and CaCdr2p, respectively) and the MFS transporters CaMdr1p and *S. cerevisiae* Pdr5p. CUR decreased the extracellular concentration of R6G in ABC transporter-expressing cells but had no effect on methotrexate efflux mediated through the MFS transporter CaMdr1p. CUR competitively inhibited R6G efflux and the photolabeling of CaCdr1p by [^{125}I]iodoarylazidoprazosin, a drug analogue of the substrate prazosin (50% inhibitory concentration, 14.2 μM). Notably, the mutant variants of CaCdr1p that displayed abrogated efflux of R6G also showed reduced modulation by CUR. Drug susceptibility testing of ABC protein-expressing cells by spot assays and checkerboard tests revealed that CUR was selectively synergistic with drug substrates such as R6G, ketoconazole, itraconazole, and miconazole but not with fluconazole, voriconazole, anisomycin, cycloheximide, or FK520. Taken together, our results provide the first evidence that CUR modulates only ABC multidrug transporters and could be exploited in combination with certain conventional antifungal drugs to reverse multidrug resistance in *Candida* cells.

Overexpression of ATP-binding cassette (ABC) multidrug transporters, including P-glycoprotein (ABCB1), multidrug resistance protein (ABCC1), and mitoxantrone resistance protein (ABCG2), plays a major role in the development of multidrug resistance (MDR) in cancer cells (19). Among the various strategies to combat MDR, blocking the functioning of MDR transporters represents an attractive approach (11). Notably, several functional inhibitors of MDR proteins have been tested, but thus far none are clinically successful, due to the dose-limiting toxic effect of the modulators.

To circumvent this problem, extensive efforts have been under way in recent years to identify natural inhibitors of MDR exporters, since natural products have the potential to yield a large number of new drugs. Curcuminoids, from the rhizomes of *Curcuma longa*, have been reported to reverse the drug resistance phenotype in cancer cells overexpressing ABC transporters, viz., ABCB1, ABCG2, and ABCC1 (2, 4, 5). Curcuminoids blocked the efflux of fluorescent substrates calcein AM, rhodamine 123, and bodipy-FL-vinblastine in MDR cervical carcinoma cell lines overexpressing ABCB1 and the efflux of mitoxantrone and pheophorbide A, mediated by ABCG2, in HEK293 cells (3, 4).

In yeasts, including species of the pathogenic genus *Candida*, upregulation of multidrug transporter genes belonging either to the ABC family or to the major facilitator superfamily (MFS) is frequently observed in cells exposed to drugs and leads to the phenomenon of MDR (29, 31, 32). For clinical isolates of *Candida albicans*, it has been established that the ABC transporters *C. albicans* Cdr1p (CaCdr1p) and CaCdr2p and the MFS transporter CaMdr1p are major MDR transporters that contribute to azole resistance. There are compounds, such as FK506, enniatins, milbemycins, synthetic D-octapeptides, cyclosporine, isonitrile, disulfiram, ibuprofen, and unnarmicins (12, 30), that inhibit fungal ABC transporters. Such inhibitors or chemosensitizers probably act directly by affecting substrate binding and transport mediated by MDR efflux proteins.

Notably, the effect of curcuminoids on fungal ABC transporters is not known. However, due to functional and structural similarities between ABCB1 and ABC transporters in yeasts, it is very likely that the curcuminoids could act as "reversal agents" of drug resistance in yeasts as well. In this study, we have examined the potency of curcumin (CUR) in modulating the efflux activity of CaCdr1p and have compared it with those of CaCdr2p and *Saccharomyces cerevisiae* Pdr5p (ScPdr5p). Our results demonstrate that CUR behaves as a specific modulator of rhodamine 6G (R6G) efflux mediated by CaCdr1p, CaCdr2p, and ScPdr5p in *S. cerevisiae* cells overexpressing these transporters. Notably, CUR had no impact on efflux activity mediated by the MFS transporter CaMdr1p.

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TABLE 1. Strains used in this study

Strain no.	Strain name or mutation	Genotype or description[a]	Source
1	AD1-8u$^-$	MATα *pdr1-3 his1 ura* Δ*yor1::hisG* Δ*snq2::hisG* Δ*pdr5::hisG* Δ*pdr10::hisG* Δ*pdr11::hisG* Δ*ycf1::hisG pdr3::hisG* Δ*pdr15::hisG*	7
2	AD-CDR1	AD1-8u$^-$ cells harboring the CaCDR1-GFP ORF integrated at the *PDR5* locus	27
3	A1346G	CDR1-GFP cells carrying an A1346G mutation in the *CDR1* ORF and integrated at the *PDR5* locus	24
4	A1347G	CDR1-GFP cells carrying an A1347G mutation in the *CDR1* ORF and integrated at the *PDR5* locus	24
5	T1351A	CDR1-GFP cells carrying a T1351A mutation in the *CDR1* ORF and integrated at the *PDR5* locus	24
6	F1360A	CDR1-GFP cells carrying an F1360A mutation in the *CDR1* ORF and integrated at the *PDR5* locus	24
7	G1362A	CDR1-GFP cells carrying a G1362A mutation in the *CDR1* ORF and integrated at the *PDR5* locus	24
8	L1358A	CDR1-GFP cells carrying an L1358A mutation in the *CDR1* ORF and integrated at the *PDR5* locus	24
9	T1355A	CDR1-GFP cells carrying a T1355A mutation in the *CDR1* ORF and integrated at the *PDR5* locus	24
10	G682A	CDR1-GFP cells carrying a G682A mutation in the *CDR1* ORF and integrated at the *PDR5* locus	Puri et al., unpublished data
11	AD-CaMDR1	AD1-8u$^-$ cells harboring the CaMDR1-GFP ORF integrated at the *PDR5* locus	22
12	AD-CDR2	AD1-8u$^-$ cells harboring the CaCDR2-GFP ORF integrated at the *PDR5* locus	17
13	AD-PDR5	AD1-8u$^-$ cells harboring the ScPDR5-GFP ORF integrated at the *PDR5* locus	17
14	CAI4	Δ*ura3::imm434*/Δ*ura3::imm434*	8

[a] ORF, open reading frame.

Furthermore, CUR reversed drug resistance by displaying synergism with selected drugs.

MATERIALS AND METHODS

Materials. R6G, a commercial-grade mixture of curcuminoids (commonly known as CUR), protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, aprotinin, pepstatin A, *N*α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK], and *N*-tosyl-L-phenylalanine chloromethyl ketone [TPCK]), a bicinchoninic acid protein determination kit, miconazole (MCZ), ketoconazole (KTC), itraconazole (ITC), anisomycin (ANISO), cycloheximide (CYH), FK520, oligomycin, dinitrophenol, deoxyglucose, 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), and other molecular-grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-labeled fluconazole (FLC; specific activity, 19 Ci/mmol) was custom synthesized by Amersham Biosciences, United Kingdom, and [^{3}H]methotrexate (MTX; specific activity, 8.60 Ci/mmol) was procured from Amersham Biosciences, United Kingdom. Radiolabeled [^{125}I]iodoarylazidoprazosin (IAAP) (2,200 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA).

Yeast strains and growth media. The strains used in this study are listed in Table 1. The yeast strains were cultured in yeast extract-peptone-dextrose (YEPD) broth (Bio 101, Vista, CA). For agar plates, 2.5% (wt/vol) Bacto agar (Difco, BD Biosciences, NJ) was added to the medium. All strains were stored as frozen stocks with 15% glycerol at −80°C. Before each experiment, cells were freshly revived on YEPD plates from the stock.

Efflux of R6G. The efflux of R6G was determined essentially using a previously described protocol (27). Briefly, approximately 1×10^6 yeast cells from an overnight-grown culture were transferred to YEPD medium and allowed to grow for 5 h. Cells were pelleted, washed twice with phosphate-buffered saline (PBS) (without glucose), and resuspended as a 2% cell suspension, which corresponds to 10^8 cells (wt/vol) in PBS without glucose. The cells were then de-energized for 45 min in deoxyglucose (5 mM) and dinitrophenol (5 mM) in PBS (without glucose). The de-energized cells were pelleted, washed, and then resuspended as a 2% cell suspension (wt/vol) in PBS without glucose, to which R6G was added at a final concentration of 10 μM and incubated for 40 min at 30°C. The equilibrated cells with R6G were then washed and resuspended as a 2% cell suspension (wt/vol) in PBS without glucose. Samples with a volume of 1 ml were withdrawn at the indicated time and centrifuged at 9,000 × *g* for 2 min. The supernatant was collected, and absorption was measured at 527 nm. Energy-dependent efflux (at the indicated time) was measured after the addition of glucose (2%) to the cells resuspended in PBS (without glucose). Glucose-free controls were included in all the experiments. For competition assays, CUR (100 μM) was added to the de-energized cells 5 min before the addition of R6G and allowed to equilibrate.

Measurement of drug accumulation. The accumulation of [^{3}H]FLC (specific activity, 19 Ci/mmol) and [^{3}H]MTX (specific activity, 8.60 Ci/mmol) was determined essentially by the methods described previously (22). Briefly, cells from mid-log phase (5×10^6) were centrifuged at 3,000 × *g* for 3 min and resuspended in PBS as a 2% cell suspension. For accumulation studies, 100 nM FLC and 25 μM MTX were routinely used (22). CUR at 100 μM was added 5 min before the addition of drugs and was allowed to equilibrate. A 100-μl volume of the cell suspension containing drugs alone or drugs plus CUR was incubated at 30°C for 40 min, filtered rapidly, and washed twice with PBS (pH 7.4) on a Millipore manifold filter assembly using a 0.45-μm-pore size cellulose nitrate filter (Millipore). The filter discs were dried and put in cocktail "O," and the radioactivity was measured in a liquid scintillation counter (Beckman). Accumulation was expressed as picomoles per milligram (dry weight).

Photoaffinity labeling with IAAP. The crude membrane proteins (50 μg) prepared from AD-CDR1 cells (27) were incubated with CUR or with R6G for 10 min at 37°C in 0.1 ml of 50 mM Tris-HCl (pH 7.5). The samples were brought to room temperature, and 3 to 6 nM [^{125}I]IAAP (2,200 Ci/mmol) was added and incubated for an additional 5 min under subdued light. The samples were then illuminated with a UV lamp assembly (PGC Scientifics, Gaithersburg, MD) fitted with two black-light (self-filtering) UVA long-wavelength F15T8BLB tubes (365 nm) for 10 min at room temperature (21 to 23°C). One milliliter of radioimmunoprecipitation assay buffer was added to the samples, and CaCdr1p cross-linked with [^{125}I]IAAP was immunoprecipitated with 10 μg of a monoclonal antibody (BD Biosciences, Palo Alto, CA) against green fluorescent protein (GFP) (27). The samples were then separated on a 7% Tris-acetate gel at a constant voltage, and the gels were dried and exposed to Bio-Max MR film (Eastman Kodak, Rochester, NY) at −80°C for 12 to 24 h. The radioactivity incorporated into the CaCdr1p band was quantified using a Storm 860 PhosphorImager system (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software as described previously (27).

ATPase assay. The ATPase activity of the plasma membrane (PM) fractions was measured as oligomycin-sensitive release of inorganic phosphate either alone, as described previously (27), or in the presence of CUR (100 μM) and varying concentrations of ATP (0.5 mM to 7 mM).

Immunodetection of ABC proteins. PMs were prepared from *S. cerevisiae* cells overexpressing ABC transporters as described previously (27) or in the presence of CUR (100 μM). The PM protein concentration was determined by a bicinchoninic acid assay using bovine serum albumin as the standard. Western blot

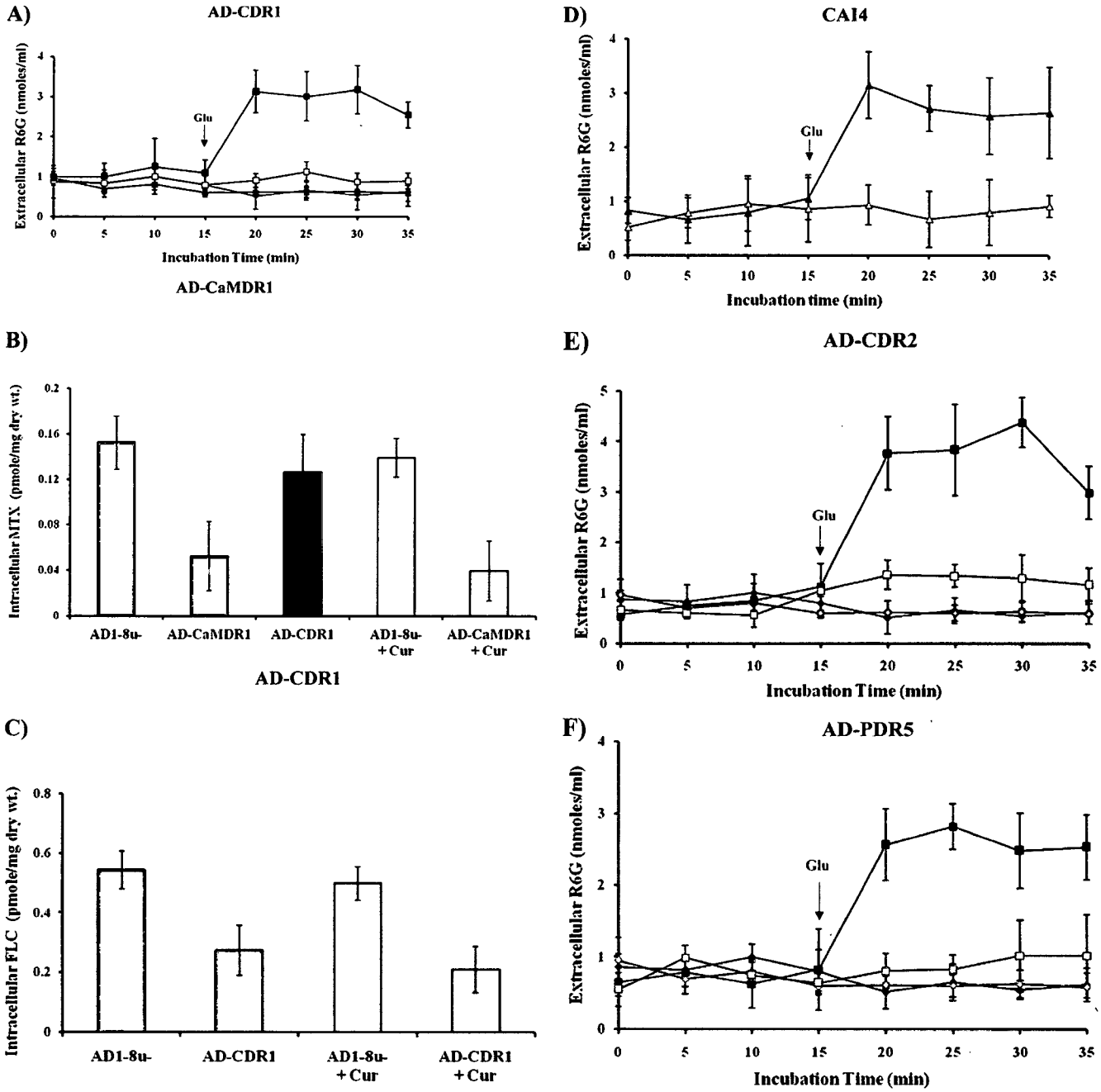


FIG. 1. Effects of CUR on efflux of substrates in yeast cells. (A) Extracellular R6G concentrations in *S. cerevisiae* control cells (AD1-8u$^-$) (diamonds) and in cells overexpressing CaCdr1p (AD-CDR1) (squares), incubated either with R6G (10 μM) alone (filled symbols) or with R6G plus CUR (100 μM) (open symbols). Filled triangles represent AD-CaMDR1 cells. Energy-dependent R6G efflux was initiated by adding 2% glucose (arrow) and was quantified by measuring the absorbance of the supernatant at 527 nm. Values are means and standard deviations (error bars) for three independent experiments. (B) [^{3}H]MTX accumulation in *S. cerevisiae* control cells (AD1-8u$^-$) and in cells overexpressing CaMdr1p (AD-CaMDR1). Cells were incubated either with [^{3}H]MTX (25 μM; specific activity, 8.60 Ci/mmol) alone (shaded bars) or with [^{3}H]MTX plus CUR (100 μM) (open bars). The solid black bar represents AD-CDR1 cells. The accumulated [^{3}H]MTX was measured, 40 min after the initiation of efflux, using a liquid scintillation counter (Beckman). Values are means ± standard deviations (error bars) for three independent experiments. (C) [^{3}H]FLC accumulation in *S. cerevisiae* control cells and in cells overexpressing CaCdr1p. Cells were incubated with either [^{3}H]FLC (100 nM; specific activity, 19 Ci/mmol) alone (filled bars) or [^{3}H]FLC plus CUR (100 μM) (open bars). The accumulated [^{3}H]FLC was measured 40 min after the addition of glucose (2%). Values are means ± standard deviations (error bars) for three independent experiments. (D) Extracellular R6G concentrations in *C. albicans* strain CAI4. Cells were incubated either with R6G (10 μM) alone (filled triangles) or with R6G plus CUR (100 μM) (open triangles). Energy-dependent R6G efflux was initiated by adding 2% glucose (arrow) and was quantified by measuring the absorbance of the supernatant at 527 nm. Values are means and standard deviations (error bars) for three independent experiments. (E) Extracellular R6G concentrations in *S. cerevisiae* control cells (AD1-8u$^-$) (diamonds) and in cells overexpressing CaCdr2p (AD-CDR2) (squares) incubated either with R6G (10 μM) alone (filled symbols) or with R6G plus CUR (100 μM) (open symbols). (F) Extracellular R6G concentrations in *S. cerevisiae* control cells (AD1-8u$^-$) (diamonds) and in cells overexpressing ScPdr5p (AD-PDR5) (squares), incubated either with R6G (10 μM) alone (filled symbols) or with R6G plus CUR (100 μM) (open symbols). Energy-dependent R6G efflux was initiated by the addition of 2% glucose (arrows) and was quantified by measuring the absorbance of the supernatant at 527 nm. Values are means and standard deviations (error bars) for three independent experiments.

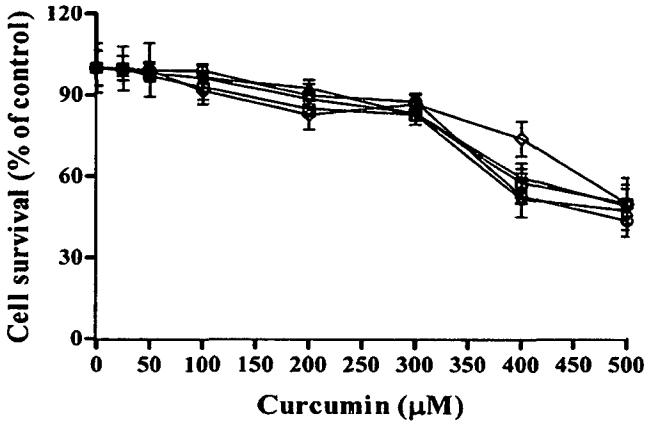


FIG. 2. Effect of CUR on the viability of *S. cerevisiae* cells as determined by an MTT assay. Shown is the percentage of survival among control cells (AD1-8u$^-$) (open circles) and among cells overexpressing ABC or MFS transporters: AD-CDR1 (open inverted triangles), AD-CDR2 (open triangles), AD-PDR5 (open diamonds), and AD-CaMDR1 (open squares) cells. The experiments were conducted in triplicate, and the values are means ± standard deviations for three independent experiments.

analysis was conducted using an anti-GFP monoclonal antibody (1:5,000) as described previously (27). Proteins on immunoblots were visualized using the enhanced chemiluminescence assay system (ECL kit; Amersham Biosciences, Arlington Heights, IL).

Drug susceptibility assay. The sensitivities of yeast cells to different drugs in the presence of CUR were determined by spot assays as described previously (22). The interaction of CUR with KTC, MCZ, ITC, R6G, FLC, ANISO, CYH, FK520, or MTX was evaluated by the checkerboard method recommended by the CLSI (formerly NCCLS) and was expressed as the fractional inhibitory concentration (FIC) index, the sum of the FICs for each agent. The FIC of each agent is calculated as the MIC of the agent in combination divided by the MIC of the agent alone (21). A range of concentrations were tried: 0.202 to 208 μM for FLC or voriconazole (VORI), 0.007 to 3.6 μM for KTC, 0.004 to 8.32 μM for MCZ, 0.002 to 5.6 μM for ITC, 0.200 to 103.5 μM for R6G, 0.177 to 92.5 μM for ANISO, 0.066 to 35 μM for CYH, 0.122 to 63 μM for FK520, and 1.05 to 540 μM for CUR. Each checkerboard test generates many different combinations, and by convention the FIC of the most effective combination is used in calculating the FIC index.

Cytotoxicity assay. The cytotoxic effect of CUR was determined by an MTT assay (3, 4). Yeast cells (10^4) were seeded into 96-well plates in the absence and the presence of varying concentrations of CUR (25 to 500 μM) and were grown for 48 h at 30°C. One hundred microliters of an MTT solution was added to each well and incubated for 3 to 4 h, and 200 μl of isopropanol was added to stop the reaction. Absorbance was measured using a microplate spectrophotometer at 570 nm with a reference wavelength of 650 nm. Cell survival (as a percentage of the survival of control cells) was calculated as (mean absorbance in test wells)/(mean absorbance in control wells) × 100.

RESULTS

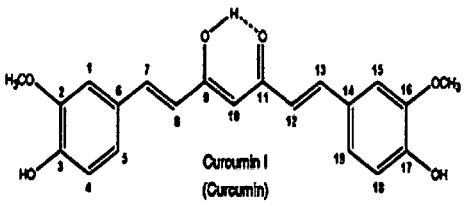
CUR inhibits R6G efflux. We used a commercial preparation of CUR in order to observe its effect on MDR efflux proteins of the pathogenic yeast *C. albicans*. For this purpose, we monitored the efflux of R6G in cells where GFP-tagged CaCdr1p (Cdr1p-GFP) was stably overexpressed from a genomic *PDR5* locus in the *S. cerevisiae* mutant strain AD1-8u$^-$ (20). The host AD1-8u$^-$, constructed by Goffeau's group (7), was derived from a Pdr1-3 mutant strain with a gain-of-function mutation in the transcription factor Pdr1p, resulting in constitutive hyperinduction of the *PDR5* promoter (20). As shown in Fig. 1A, *S. cerevisiae* cells overexpressing CaCdr1p showed energy-dependent efflux of R6G, which was inhibited by CUR (100 μM). However, the addition of CUR had no effect on the leakage of preloaded R6G from de-energized *S. cerevisiae* cells. We tested if CUR could also affect a multidrug transporter belonging to the MFS, and we examined efflux mediated by CaMdr1p expressed in a similar heterologous background. As shown in Fig. 1B, the transport of [^{3}H]MTX, a well-known substrate of CaMdr1p, remained unaffected by a fourfold excess of CUR. In the experiments for which results are shown in Fig. 1A, strain AD-CaMDR1 was used as a negative control for R6G transport, and in the experiments for Fig. 1B, strain AD-CDR1 was used as a negative control for MTX transport. The effect of CUR was also substrate specific, as evidenced by the fact that efflux of the well-known substrate FLC remained unimpeded in CaCdr1p-expressing *S. cerevisiae* cells, even though CUR was supplied in a 1,000-fold excess (Fig. 1C). Of note, CUR could also modulate R6G efflux in *C. albicans* cells (Fig. 1D); however, for subsequent studies, we used an *S. cerevisiae* strain overexpressing MDR transporters.

CUR selectively modulates ABC transporters. Before investigating whether CUR affects drug transporters, we examined whether it affected the viability of cells. For this purpose, control cells and transporter-overexpressing cells were exposed to various concentrations of CUR for 48 h, and cytotoxicity was determined by an MTT assay (3, 4). The percentage of viable cells was calculated in order to determine the 50% inhibitory concentrations (IC_{50}s) (Fig. 2). As shown in Table 2, the IC_{50}s for control cells (AD1-8u$^-$) and those for cells expressing various transporters (AD-CDR1, AD-CDR2, AD-PDR5, and AD-CaMDR1) were not very different, ranging from 410.6 ± 9.4 μM to 498 ± 5.5 μM. Further, our transport assays confirmed that CUR is not a substrate of ABC or MFS proteins, since the extracellular concentration of CUR remained the same even after the initiation of efflux (data not shown). Our data suggest that CUR interacts with the yeast transporters, but these multidrug transporters may not transport it, since the IC_{50}s and relative resistance factors were similar whether cells were overexpressing a transporter or not. We investigated whether the effect of CUR is specific to ABC transporters, and we examined R6G efflux mediated by CaCdr1p homologues, such as CaCdr2p and ScPdr5p, which were expressed in similar backgrounds. It was observed that CUR could inhibit the efflux of R6G mediated by both the proteins (Fig. 1E and F). Our transport assays confirmed that CUR is not a substrate of ABC or MFS proteins, since the extracellular concentration of CUR remained the same even after the initiation of efflux (data not shown). This was further confirmed by a cytotoxicity assay (3, 4), where the IC_{50}s were similar for cells that did and did not

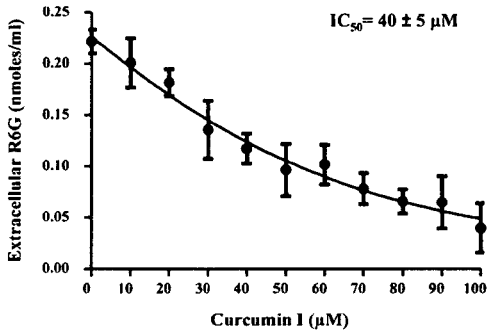
TABLE 2. IC_{50}s and relative resistance factors for fungal strains in the presence of CUR

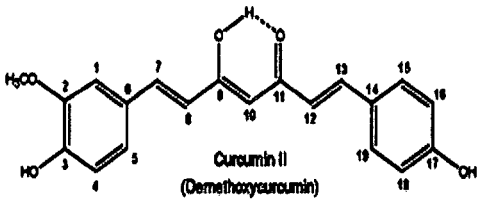
Strain	IC_{50} (μM)	Relative resistance factor
AD1-8u$^-$	421.6 ± 2.2	1
AD-CDR1	492.8 ± 6.3	1.16
AD-CDR2	410.6 ± 9.4	0.99
AD-PDR5	498 ± 5.5	1.18
AD-CaMDR1	454.6 ± 7.4	1.07

A)

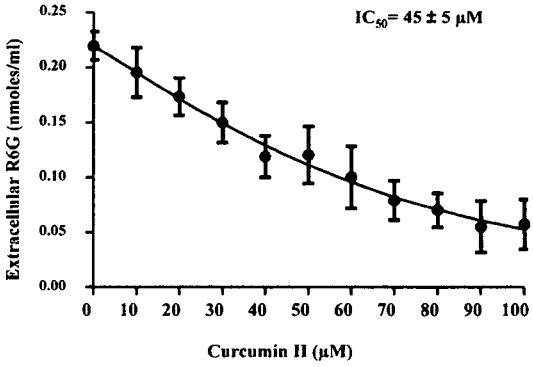


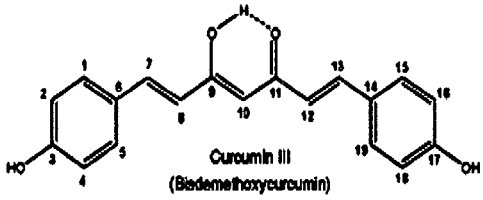
Curcumin I
(Curcumin)



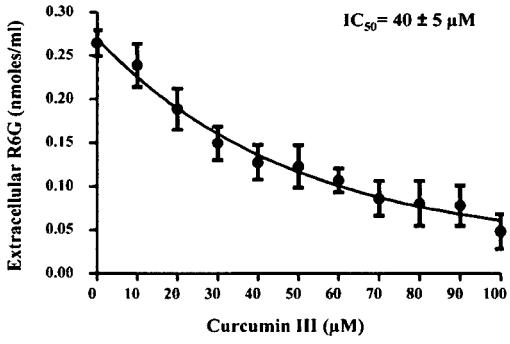


Curcumin II
(Demethoxycurcumin)

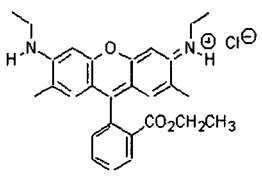




Curcumin III
(Bisdemethoxycurcumin)



B)



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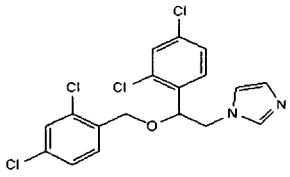
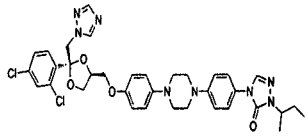


Miconazole



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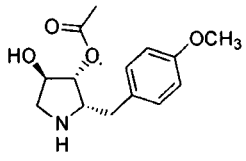

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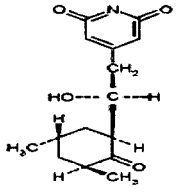
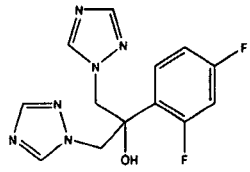
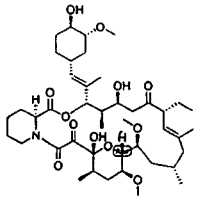


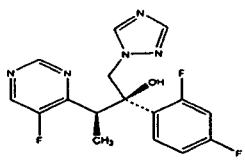
Cycloheximide



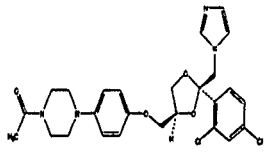
Fluconazole



Ascomycin



Voriconazole



Ketoconazole

overexpress transporters (Table 2). It should be mentioned that the functionality of GFP-tagged versions of ABC and MFS transporters was similar to that of untagged proteins (22, 27). To further examine the effect of CUR, the ABC transporter CaCdr1p was selected for detailed functional analyses.

CUR competitively inhibits R6G efflux. Commercially available CUR is a mixture of three major curcuminoids: curcumin, or diferuloylmethane (curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III). This mixture contains predominantly curcumin I (~77%), followed by curcumin II (17%) and curcumin III (3%), which display a wide range of biological and pharmacological properties (1, 16). We used purified curcuminoids (curcumins I, II, and III) to see if these compounds showed any selectivity as modulators of R6G efflux. The efflux of R6G mediated by CaCdr1p was inhibited by all the pure forms of CUR in a concentration-dependent manner, with IC_{50}s ranging from 40 ± 5 to 45 ± 5 μM (Fig. 3A). The Lineweaver-Burk plot revealed that CUR competitively inhibits R6G efflux, with an increase in apparent K_m (5.87 to 11.83 μM) but no effect on the V_{max} (Fig. 4A).

CUR inhibits drug binding and has no effect on ATPase activity or the expression of CaCdr1p. We had shown previously that IAAP (a photoaffinity analogue of the human P-glycoprotein substrate prazosin) and azidopine (a dihydropyridine photoaffinity analogue of its modulator, verapamil) specifically bind to CaCdr1p (27). To monitor whether CUR affects drug binding, we labeled CaCdr1p with [^{125}I]IAAP as described in Materials and Methods. Figure 4B demonstrates that CUR effectively inhibited the photoaffinity labeling of CaCdr1p with [^{125}I]IAAP, with an IC_{50} of 14.2 μM. We also monitored [^{125}I]IAAP labeling in the presence of R6G as described in Materials and Methods. Interestingly, R6G could not inhibit [^{125}I]IAAP binding. In contrast to well-known inhibitors of CaCdr1p ATPase activity, such as vanadate, oligomycin, sodium azide, and *N*-ethylmaleimide (15, 26, 27), CUR at 100 μM had no effect on ATPase activity in the presence of varying concentrations of ATP (Fig. 4C). CUR also had no effect on the expression of CaCdr1p (Fig. 4D). Taken together, the data strongly indicate that although CUR is not transported, it acts as a competitive inhibitor at one of the transport sites used to transport clinically significant antifungal agents.

CUR displays synergism with selected azoles. When the control (AD1-8u$^-$) cells and the CaCdr1p-expressing cells were grown either in the presence of drugs alone (FLC at 6.52 μM, VORI at 5.72 μM, MCZ at 0.167 μM, KTC at 0.037 μM, ITC at 0.141 μM, ANISO at 2.97 μM, CYH at 0.28 μM, FK520 at 12.6 μM, R6G at 0.209 μM) or in the presence of both CUR (75.6 μM) and the indicated drug, it was observed that CaCdr1p-expressing cells displayed the expected drug resistance and thus were able to grow in the presence of drug alone. Similar results were obtained with CaCdr2p- and ScPdr5p-expressing *S. cerevisiae* cells (data not shown). However, the simultaneous presence of CUR with either R6G or azoles, viz., KTC, ITC, or MCZ, sensitized the cells, as evidenced by inhibition of the growth of the cells (Fig. 5ii). Interestingly, the presence of CUR along with noncompeting drugs, such as ANISO, CYH, FLC, VORI, and FK520, did not affect the level of resistance or the growth of cells expressing ABC proteins (Fig. 5iii). The observed inhibition of growth by CUR in the presence of drugs was not due to loss of viability, as determined by an MTT assay (Fig. 2). Notably, CUR (75.6 μM) alone did not inhibit the growth of control cells (AD1-8u$^-$) or that of cells overexpressing CaCdr1p (Fig. 5i) or CaMdr1p (Fig. 5iv). The growth of CaMdr1p-overexpressing cells in the presence of MTX remained insensitive to CUR (Fig. 5iv). We performed checkerboard assays in the presence of CUR and various drugs. The FIC indices are below 0.5 for drugs such as KTC, ITC, MCZ, and R6G in AD-CDR1 cells, suggesting synergism with CUR (Table 3). Checkerboard analysis showed no synergism with CUR for FLC, VORI, ANISO, CYH, or FK520. Similar patterns of synergism between select drugs and CUR were observed with AD-CDR2 and AD-PDR5 cells (data not shown).

CUR acts at the R6G transport site. We had recently subjected transmembrane segment 11 of CaCdr1p to alanine scanning, replacing all 21 amino acid residues with alanine by site-directed mutagenesis (24). Out of 21 residues of transmembrane segment 11, substitution of 5 residues, namely, A1346G, A1347G, T1351A, F1360A, and G1362A, abrogated the efflux of R6G, while the rest of the mutant variants of CaCdr1p showed unaltered efflux (Fig. 6A). Since CUR selectively modulates R6G efflux, we argued that if R6G and CUR share binding sites, abrogation of R6G efflux should affect its modulation by CUR. Interestingly, we observed that the ability of CUR to modulate R6G efflux was indeed considerably reduced in those mutant variants that showed abrogated efflux of R6G. Figure 6B shows that the percentage of inhibition of R6G efflux by CUR was considerably lower in mutant variants than in native CaCdr1p-expressing cells. In addition, CUR inhibits R6G efflux even in those mutant variants that do not show abrogated efflux of the dye. This would suggest that in addition to common binding sites, CUR also has an independent binding site(s) in CaCdr1p (Fig. 6C and D). However, to resolve this issue, elaborate binding studies will be required.

DISCUSSION

Among the 28 putative ABC transporter genes and 95 putative MFS transporter genes identified in the *C. albicans* genome (9, 10), there is overwhelming clinical and experimental evidence that only ABC transporters, such as CaCdr1p and CaCdr2p, and the MFS transporter CaMdr1p are major determinants of azole resistance (23, 25). The reversal of the functionality of these multidrug efflux pump proteins represents an

FIG. 3. Effects of pure curcuminoids on R6G transport in *S. cerevisiae* cells overexpressing CaCdr1p. (A) Structures of curcumin I, curcumin II, and curcumin III and competition assays with R6G. CaCdr1p-overexpressing *S. cerevisiae* cells were incubated either with 10 μM R6G alone or with 10 μM R6G plus curcumin I, II, or III (10 to 100 μM). R6G efflux was monitored 40 min after the addition of glucose (2%). Extracellular R6G was quantified by measuring the absorbance at 527 nm. The data are plotted using GraphPad Prism. Values are means and standard deviations (error bars) for three independent experiments. (B) Structures of the various substrates used.

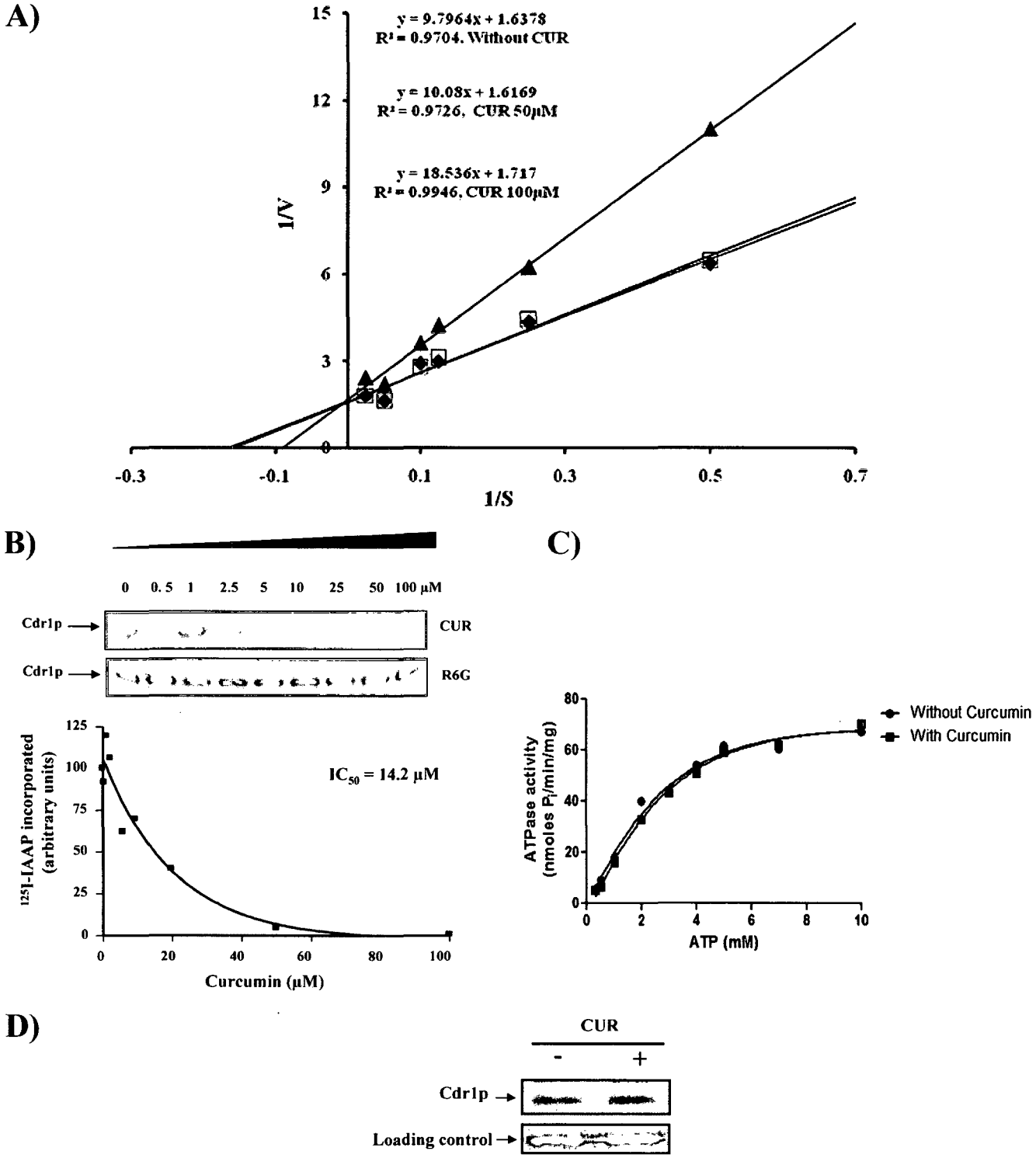


FIG. 4. Biochemical analysis of CaCdr1p in the presence of CUR. (A) Lineweaver-Burk plot of CaCdr1p-mediated R6G efflux in the presence of CUR 5 min after the addition of 2% glucose. Filled diamonds, open squares, and filled triangles represent 0, 50, and 100 μM CUR, respectively. The rate of each reaction was calculated as nanomoles of R6G released per minute per 5×10^6 cells. (B) Effect of CUR or R6G on the photoaffinity labeling of CaCdr1p with [^{125}I]IAAP. The autoradiogram represents the amounts of [^{125}I]IAAP incorporated into CaCdr1p in the presence of the indicated concentrations of CUR or R6G. The graph represents the amounts of [^{125}I]IAAP incorporated into CaCdr1p in the presence of the indicated concentrations of CUR. (C) Effect of CUR on the ATPase activity of CaCdr1p. PMs from cells overexpressing CaCdr1p were incubated with or without 100 μM CUR and varying concentrations of ATP (0.5 mM to 7 mM) in the ATPase buffer. The assay was performed essentially as described in Materials and Methods. The data are plotted using GraphPad Prism. (D) Effect of CUR (100 μM) on the expression of CaCdr1p. Western blot analyses were performed with an anti-GFP monoclonal antibody. Equal loading of protein was assessed by using a Coomassie-stained gel.

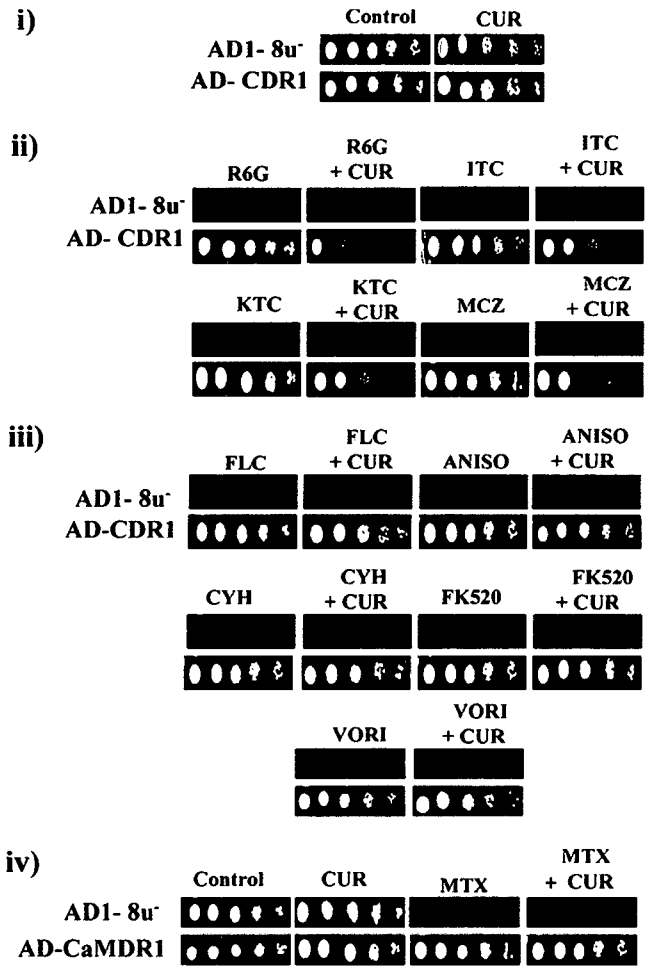


FIG. 5. Synergistic effects of CUR on drug resistance. Control (AD1-8u⁻) and CaCdr1p-expressing (AD-CDR1) *S. cerevisiae* cells were grown overnight on YEPD plates and then resuspended in normal saline to an optical density at 600 nm of 0.1. The following stock solutions of drugs were used: R6G at 1 mg/ml in dimethyl sulfoxide, FLC at 1 mg/ml in water, VORI at 5 mg/ml in water, CYH at 0.1 mg/ml in water, MCZ at 1 mg/ml in methanol, KTC at 1 mg/ml in methanol, ANISO at 1 mg/ml in dimethyl sulfoxide, FK520 at 1 mg/ml in ethanol, MTX at 1 mg/ml in 10 mM Tris-Cl, and CUR at 11 mg/ml in dimethyl sulfoxide. Five microliters of a fivefold serial dilution of each strain was spotted onto YEPD plates as described previously (27) either in the absence (control) (i) or in the presence of antifungals, alone or in combination with CUR (ii through iv). (ii) R6G (0.209 μM), ITC (0.141 μM), KTC (0.037 μM), or MCZ (0.167 μM), alone or in combination with CUR (75.6 μM). (iii) FLC (6.52 μM), ANISO (2.97 μM), CYH (0.28 μM), FK520 (12.6 μM), or VORI (5.72 μM), alone or in combination with CUR (75.6 μM). (iv) MTX (11 μM) or CUR (75.6 μM), alone or in combination.

attractive strategy for combating azole resistance. In this study, we explored whether CUR, which inhibits the activities of the mammalian ABC multidrug transporter genes *ABCB1*, *ABCG2*, and *ABCC1* (3, 4, 5), could be exploited as a modulating agent for multidrug transporters of *C. albicans*. Our study reveals that CUR inhibited R6G transport exclusively in *S. cerevisiae* cells overexpressing the ABC drug transporters CaCdr1p, CaCdr2p, and ScPdr5p and had no effect on efflux mediated by the MFS transporter CaMdr1p. All three pure forms of CUR—curcumins I, II, and III—showed similar levels of modulation of R6G efflux in *S. cerevisiae* cells expressing ABC transporters (Fig. 3A). The modulatory effect of CUR was restricted to R6G; it had no effect on the efflux of another substrate, FLC. We could observe a direct correlation between the modulatory effect of CUR and the status of R6G efflux. For instance, those mutant variants of CaCdr1p that show abrogated efflux of R6G also display decreased modulation by CUR.

Notably, R6G and FLC are both substrates of CaCdr1p, but only the former is competed with CUR (Fig. 1A and C). If the structure of CUR is compared with the structures of R6G and FLC, it is apparent that electronic factors, such as the number of π rings and an extended π surface, could be important for CUR and other substrates, such as ITC, KTC, and MCZ, which compete with R6G efflux (Fig. 3B). In this context, it is noteworthy that noncovalent π-π interactions have tremendous biological implications (13, 14). On the other hand, if one considers the structures of FLC, VORI, ANISO, and CYH, which do not compete with R6G, there are no such electronic factors but a good number of tetrahedral sites. Therefore, these subtle differences in properties between the structures of competing and noncompeting substrates could explain why CUR is a selective modulator.

The probability that the modulation of ABC transporter function would result in an increase in the intracellular concentrations of the drugs to toxic levels became apparent from the growth studies. When CUR was used in combination, it was synergistic with drugs in cells overexpressing ABC transporters. This synergism was restricted to those drugs whose efflux was modulated by R6G (24). Thus, the chemosensitization of cells by CUR was specific to competing drugs, such as KTC, ITC, and MCZ, and was not observed with noncompetitive drugs, such as FLC, VORI, ANISO, CYH, and FK520. The fact that the presence of CUR along with some drugs did not inhibit the growth of cells not only points to the selectivity of CUR for certain compounds but also suggests that R6G, KTC, ITC, MCZ, and CUR may share overlapping binding sites of ABC multidrug transporter proteins. The modulatory and synergistic effects of CUR confirm our previous observation that KTC, MCZ, and ITC share CaCdr1p binding sites with R6G (24).

A natural CUR mixture contains three major curcuminoids: curcumin I, curcumin II, and curcumin III. We tested these individual curcuminoids in our earlier studies with the mammalian ABC drug transporters P-glycoprotein, MRP1,

TABLE 3. Interaction of CUR with KTC, MCZ, ITC, or R6G against AD-CDR1 cells[a]

Antifungal or dye	FIC of the antifungal or dye	FIC of CUR	FICI
KTC	0.25	0.0054	0.255
MCZ	0.125	0.005	0.130
ITC	0.250	0.005	0.255
R6G	0.0026	0.0013	0.0039

[a] Evaluated by the checkerboard method recommended by the CLSI and expressed as the FIC index (FICI). A FICI of ≤0.5 indicates synergistic interaction. FICI is calculated as the sum of the FICs of each agent. The FIC of each agent is calculated as the MIC of the agent in combination divided by the MIC of the agent alone (21).

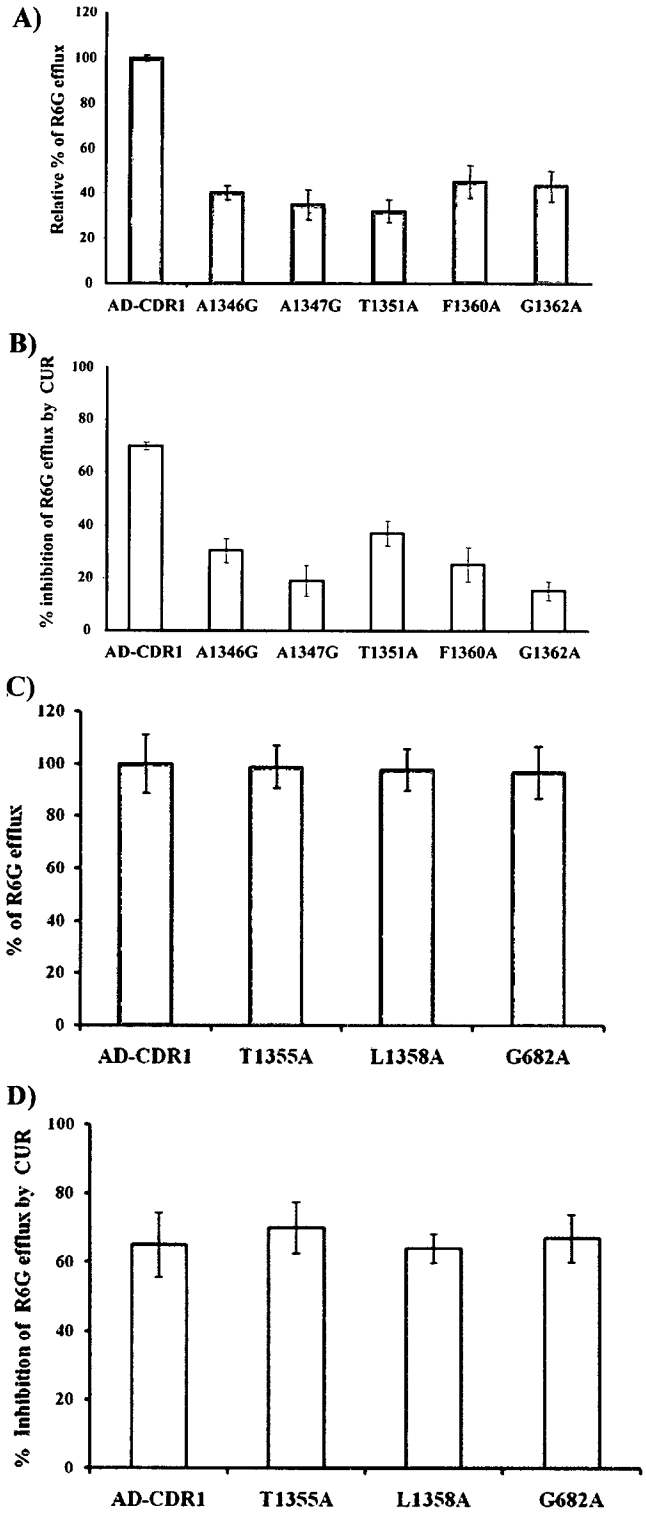


FIG. 6. R6G transport in *S. cerevisiae* cells overexpressing CaCdr1p or its mutant variants. (A and C) Extracellular R6G concentrations (expressed as percentages of those with wild-type CaCdr1p [AD-CDR1 cells]) in *S. cerevisiae* cells overexpressing CaCdr1p or mutant variants of CaCdr1p, incubated with 10 μM R6G alone. Energy-dependent R6G efflux was initiated by adding 2% glucose and was quantified by measuring the absorbance of the supernatant at 527 nm. (B and D) Percentages of inhibition of R6G efflux by CUR (100 μM), calculated by taking the level of R6G efflux with each mutant CaCdr1p variant in the absence of CUR as 100%. Values are means ± standard deviations (error bars) for three independent experiments.

and ABCG2 (3, 4, 5). We reported that these individual curcuminoids inhibited the function of these drug transporters with different efficiencies and that curcumin I was the most potent among them (3, 4, 5). In addition, we have also reported that tetrahydrocurcumin, a major metabolite of CUR, also inhibits these three mammalian ABC drug transporters (18). In this study, based on the initial data discussed above for the natural CUR mixture and its purified individual components with mammalian transporters, we evaluated the CUR mixture alone for its activity to synergize the activities of antifungal agents. There are several CUR derivatives that are synthetic analogues, and some of them may have better activity than the CUR mixture. Thus, these analogues merit further study.

There are reports to suggest that CUR can downregulate the expression of an MDR-linked transporter (ABCB1) and can even affect the function of several transcription factors (2, 6). For this reason, we tested the effect of CUR on the expression of an ABC transporter and observed that CUR did not affect the expression levels of CaCdr1p (Fig. 4D), implying that the modulation of R6G efflux by CUR is restricted to its direct effect on the functionality of ABC transporter proteins. The direct effect of CUR on CaCdr1p was confirmed by its ability to compete the photoaffinity labeling of CaCdr1p with [^{125}I]IAAP (Fig. 4B) and by its competitive inhibition of R6G efflux (Fig. 4A). We excluded the possibility that CUR could be a preferred substrate of the ABC transporters studied (Fig. 2).

Our study shows that CUR, which is not a transport substrate of CaCdr1p, specifically modulates the efflux of R6G mediated by the transporter. This is not surprising, since it has been observed previously that curcuminoids can modulate drug transport without being a substrate of mammalian ABCG2 (4). Our cytotoxicity data (Fig. 2; Table 2) suggest that the presence or absence of efflux pump proteins did not affect the growth and viability of yeast cells, again pointing to the fact that CUR is not a substrate of these pumps. It is not clear, however, whether CUR modulates R6G efflux by binding to the substrate or to an allosteric site(s) of CaCdr1p. Considering the fact that the structures and substrate specificities of fungal ABC transporters such as CaCdr1p, CaCdr2p, and ScPdr5p are very different, our finding from this study that yeast transporters can be modulated by CUR is very significant.

It is reported that the poor bioavailability of CUR and its low concentrations in plasma decrease its effectiveness in modulating the function of ABC drug transporters in rodents and humans. However, recent studies indicate that the use of piperine to prevent the glucuronidation of curcumin, as well as the encapsulation of CUR in liposomes, can increase the absorption of CUR and its levels in plasma (28). It is, however, not known whether CUR is metabolized via glucuronidation in yeast cells or whether the intracellular

level of CUR is lower than that in medium or plasma. These issues need to be resolved before CUR can be used as an effective in vivo or in vitro antifungal. In summary, the modulation of antifungal efflux by CUR is substrate and transporter specific. Nevertheless, curcuminoids are not toxic to the cell, nor are they transported by their target efflux pumps. Thus, their ability to sensitize cells to azoles opens up the possibility that they could be exploited in combination with conventional chemotherapy.

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Original Paper

Antifungal curcumin induces reactive oxygen species and triggers an early apoptosis but prevents hyphae development by targeting the global repressor *TUP1* in *Candida albicans*

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Synopsis

In the present study, we have investigated the antifungal effects of a natural polyphenol, CUR (curcumin), against *albicans* and non-*albicans* species of *Candida* and have shown its ability to inhibit the growth of all the tested strains. The inhibitory effects of CUR were independent of the status of the multidrug efflux pump proteins belonging to either ABC transporter (ATP-binding cassette transporter) or MFS (major facilitator) superfamilies of transporters. By using a systemic murine model of infection, we established that CUR and piperine, when administered together, caused a significant fungal load reduction ($1.4\log_{10}$) in kidneys of Swiss mice. Additionally, CUR raised the levels of ROS (reactive oxygen species), which, as revealed by annexin V–FITC labelling, triggered early apoptosis in *Candida* cells. Coincident with the raised ROS levels, mRNAs of tested oxidative stress-related genes [*CAP1* (Candida albicans AP-1), *CaIPF7817* (putative NADH-dependent flavin oxidoreductase), *SOD2* (superoxide dismutase 2), *GRP2* (NADPH-dependent methyl glyoxal reductase) and *CAT1* (catalase 1)] were also elevated. The growth inhibitory effects of CUR could be reversed by the addition of natural and synthetic antioxidants. Notably, independent of ROS status, polyphenol CUR prevented hyphae development in both liquid and solid hypha-inducing media by targeting the global suppressor *TUP1* (thymidine uptake 1). Taken together, our results provide the first evidence that CUR acts as an antifungal agent, via generation of oxidative stress, and inhibits hyphae development by targeting *TUP1*.

Key words: antioxidant, *Candida albicans*, curcumin, hypha, multidrug resistance, oxidative stress

INTRODUCTION

The dimorphic opportunistic pathogen *Candida albicans* is normally a commensal organism in humans, but when the host is unable to mount an adequate immune response, such as in AIDS, organ transplantation, diabetes, burn or cancer patients, it results in mucosal, cutaneous or invasive mycosis [1]. Infections caused by *C. albicans* are commonly treated either by azoles or by non-azole antifungal agents. Widespread and prolonged usage of antifungals, in recent years, has led to the emergence of azole-resistant strains of *Candida*, which display MDR (multidrug resistance) [2]. Various mechanisms that contribute to the development of azole resistance have been reported, which include overexpression of or point mutations in *ERG11* and the target enzyme of azoles, i.e. lanosterol 14α-demethylase [2], and an overexpression of the drug efflux pump encoding genes, namely *CaCDR1* (*C. albicans Candida* drug resistance 1), *CaCDR2* and *CaMDR1* (*C. albicans* MDR1) belonging to the ABC transporter (ATP-binding cassette transporter) and MFS (major facilitator) superfamilies of transporters respectively [2].

Although mechanisms of antifungal resistance and major factors that contribute to it are fairly established, there is evidence suggesting that MDR is a multifactorial phenomenon, which originates from as yet unknown mechanisms. For example, morphological regulators such as Δ*efg1* (Prasad, T., Hameed, S., Manoharlal, R., Biswas, S., Mukhopadhyay, C. K., Goswami, S. K. and Prasad, R., unpublished work), a homologue of the bacterial two-component response regulator Δ*ssk1*, and iron deprivation show enhanced sensitivity to drugs in *C. albicans* [4,5]. There are

Abbreviations used: AA, ascorbic acid; ABC transporter, ATP-binding cassette transporter; *CaCDR*, *Candida albicans Candida* drug resistance; *CAP1*, *Candida albicanse* A-P-1; *CAT1*, catalase 1; cfu, colony-forming units; CUR, curcumin; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; *DPP3*, diacylglycerol pyrophosphatae phosphatase; FBS, fetal bovine serum; FI, fluorescence intensity; FLC, fluconazole; *GRP2*, NADPH-dependent methyl glyoxal reductase; MCA, metacaspase; MFS, major facilitator superfamily; MDR, multidrug resistance; *CaMDR*, *Candida albicans* MDR; PDTC, pyrrolidinedithiocarbamate; PEG, poly(ethylene glycol); PIP, piperine; PM, plasma membrane; PS, phosphatidylserine; ROS, reactive oxygen species; *TUP1*, thymidine uptake 1; YEPD, yeast extract/peptone/dextrose.

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azole-resistant clinical isolates of *C. albicans* where mechanisms of resistance appear to be different from the commonly known strategies adopted by *Candida* [6].

The present study deals with the evaluation of antifungal activity of a natural plant polyphenol, CUR (curcumin), produced by the rhizome of *Curcuma longa*. CUR, which is an important spice in the Asian diet, has several important pharmacological properties, notably, antioxidant, antimutagenetic and antitumour activities [7]. It could block HIV-1 replication by inhibiting the activity of its LTR (long terminal repeat) and synergistically works with dideoxyinosine, a reverse transcriptase inhibitor in HIV-1 cells [8]. CUR alters cellular redox homoeostasis, and disrupts mitochondrial function in cultured, transformed cells [9,10].

In the present study, we show that CUR can be lethal to *C. albicans* as well as to non-*albicans* species, increases ROS (reactive oxygen species) levels and brings about early apoptosis, which could be reversed by the addition of antioxidants in *C. albicans* cells.

MATERIALS AND METHODS

Materials

Commercial-grade mixture of curcuminoids, commonly known as CUR, DCFH-DA (2′,7′-dichlorofluorescein diacetate), PDTC (pyrrolidinedithiocarbamate), AA (ascorbic acid), PEG [poly(ethylene glycol)], PIP (piperine) and other molecular grade chemicals were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). An annexin V–FITC apoptosis detection kit was obtained from BD Biosciences. Zymolyase 100T was purchased from Seikagaku Corporation. The oligonucleotides used in the present study were commercially synthesized by Sigma–Aldrich.

Yeast strains and growth media

Strains used in the present study are listed in Supplementary Table S1 (http://www.biosci.org/bsr/30/bsr30ppppadd.htm). The yeast strains were cultured in YEPD (yeast extract/peptone/dextrose) broth (BIO101; Vista). For agar plates, 2.5% (w/v) bacto agar (Difco, BD Biosciences) was added to the medium. All strains were stored as frozen stock with 15% (v/v) glycerol at −80°C. Before each experiment, cells were freshly revived on YEPD plates from the stock.

In vivo antifungal susceptibility testing

A *C. albicans* strain (ATCC 36082) was grown overnight on YEPD at 30°C and suspended in sterile normal saline to adjust the D_{600} (attenuance at 600 nm) to 1.0. The final inoculum was prepared by 1:20 dilution of the original suspension. The cfu (colony-forming units) per ml of inoculum was 5.5×10^7. CUR was dissolved in 20% (v/v) PEG, whereas FLC (fluconazole) and PIP were dissolved in sterile water. Swiss albino mice ($n = 6$) of either sex weighing 20 ± 2 g were procured from an in-house facility. Animals were taken 2–3 days before the start of experiments to acclimatize to the experimental environment. Feed and water were provided *ad libitum* during the entire study. We have complied with the ethical standards that were approved by the institutional ethics committee. All mice were infected with 200 μl of the cell suspension (1×10^7 cfu) by the intravenous route. Treatment started 1 h post-infection: in one group, CUR alone (100 mg/kg of body weight) was intraperitoneally administered; a second group was dosed with CUR plus PIP (100 and 20 mg/kg of body weight respectively) administered orally; whereas in the third group, reference standard FLC was given (50 mg/kg of body weight, orally). The fourth group was kept as untreated control and was administered vehicle (20% PEG, orally) only. In all the groups, a second dose was administered 6 h after the first dose and animals were treated for 2 days. The data were analysed by using GraphPad Prism, version 5.1. The limit of detection for live *C. albicans* was $1.7\log_{10}$.

Time kill assays

C. albicans cells at a concentration of 10^3 cfu/ml were inoculated in YEPD medium containing either CUR (185 mg/l) or antioxidants PDTC (10 μM)/AA (25 mM) alone or a combination of both CUR and antioxidants. At predetermined time points (0, 4, 8, 12, 16, 20 and 24 h, at 30°C incubation; agitation 200 rev./min), a 100 μl aliquot was removed, serially diluted (10-fold) in 0.9% saline and plated on to YEPD agar plates. Colony counts were determined after incubation at 30°C for 48 h [11].

Measurement of ROS production

Endogenous amounts of ROS were measured by a fluorimetric assay with DCFH-DA [12]. Briefly, the cells were adjusted to a D_{660} of 1 in 10 ml of PBS and centrifuged at 2500 *g* for 15 min. The cell pellet was then resuspended in PBS and treated with appropriately diluted PDTC or AA for 1 h or was left untreated at room temperature (25°C). After incubation with CUR at 37°C for different time intervals as indicated, 10 μM DCFH-DA in PBS was added. The FIs (fluorescence intensities) ($\lambda_{ex} = 485$ nm and $\lambda_{em} = 540$ nm) of the resuspended cells were measured with a spectrofluorimeter (Varian, Cary Eclipse) and the images of DCF fluorescence were taken by using a fluorescence microscope (Carl Zeiss).

Analysis of apoptotic markers

Protoplasts of *C. albicans* were stained with propidium iodide and FITC-labelled annexin V by using the annexin V–FITC apoptosis detection kit (BD Biosciences) to assess the cellular integrity and the externalization of PS (phosphatidylserine) as described earlier [13]. The cells were analysed by using an FACS® Caliber flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.) using $\lambda_{ex} = 488$ nm excitation and a 515 nm band pass filter for FITC detection and a filter >560 nm for propidium iodide detection. A total of 10000 events were counted at the flow rate. Data analysis was performed using Cell Quest software (Becton Dickinson Immunocytometry Systems) [13,14].

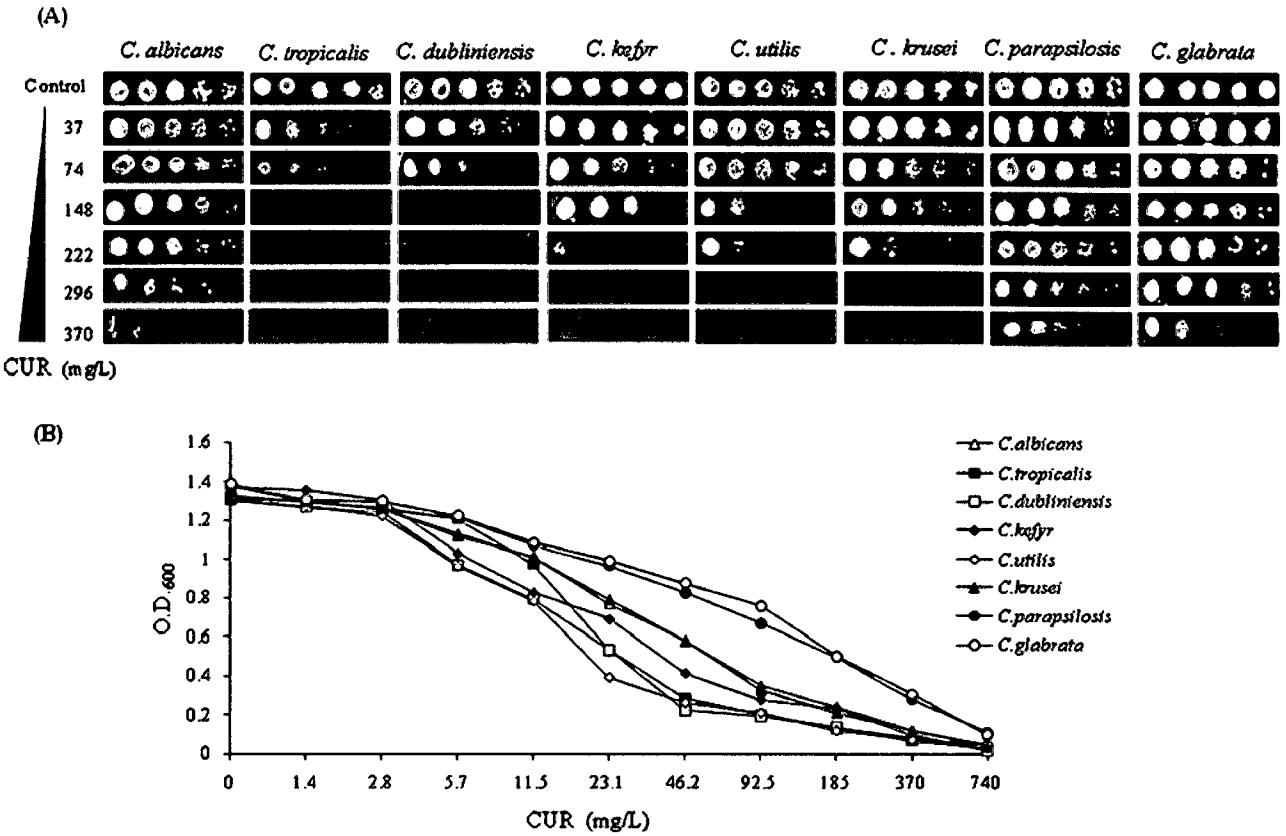
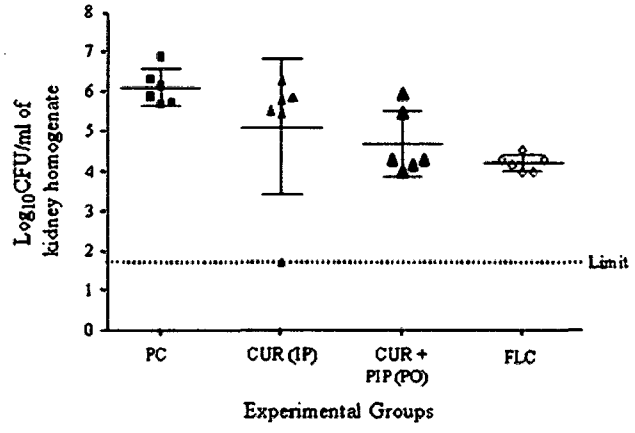


Figure 1 **Effect of CUR on the growth of *Candida* cells**

(A) The cells were grown overnight on YEPD plates and then resuspended in normal saline to a D_{600} of 0.1. A 5 μl portion of 5-fold serial dilution of each strain was spotted on to YEPD plates as described earlier [44], either in the absence or presence of various concentrations of CUR (37–370 mg/l). (B) Determination of growth inhibition of the *Candida* cells by the broth microdilution assay in the presence of CUR at concentrations ranging from 1.44 to 740 mg/l. Growth of the cells was evaluated both visually and by reading the attenuance (D_{600}) in a microtitre reader as described earlier [44]. A stock solution of 11 mg/ml was used (DMSO). Growth was not affected by the presence of the solvent (results not shown). Filled/empty triangle, filled/empty square, filled/empty diamond and filled/empty circle represent *C. krusei*, *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. kefyr*, *C. utilis*, *C. parapsilosis* and *C. glabrata* respectively.

(A)



(B)

Groups (n=6)	Untreated (control)	Curcumin (IP)	Curcumin + piperine (PO)	Fluconazole
Mean	6.12	5.12	4.71	4.22
S.D.	0.45	1.70	0.81	0.21
S.E.	0.18	0.69	0.33	0.09

Figure 2 Kidney cfu assay of mice with systemic candidiasis

(A) *In vivo* efficacy of CUR against *C. albicans* (strain 36082) in the murine systemic infection model. Swiss albino mice ($n=6$) of either sex weighing 20 ± 2 g were used in four experimental groups. In one group, CUR was administered 100 mg/kg of body weight (intraperitonealy, IP), the second group was dosed with CUR (100 mg/kg) in combination with PIP (20 mg/kg) administered concomitantly orally (oral administration, PO), and the reference standard FLC (50 mg/kg, orally) was given to the third group. The fourth group was kept as untreated control and was administered vehicle (20% PEG and water) only. 'Limit' indicates the minimum level of detection for live *C. albicans*. (B) The Table presents the mean $\log_{10}$ cfu of *C. albicans* in the kidney of control (untreated) and treated groups. Statistical analysis was performed using a *t* test and the results were considered significant when *P* values were less than 0.05.

Morphological studies

To check the hypha status, the cells were grown in the presence/absence of CUR alone or CUR+PDTC in liquid or solid (2.5% agar) YEPD with 10% (v/v) FBS (fetal bovine serum) or in liquid spider medium and incubated for 6 h (in liquid medium) or for 3 days (in the case of solid medium) at 37 °C. Colony morphologies on solid plates and filamentation in liquid medium were analysed microscopically (Carl Zeiss) [15].

RNA isolation and hybridization

Total RNA from the mid-exponentially grown *C. albicans* cells was prepared in the presence/absence of CUR [5]. Approx. 25 μg of total RNA from the above samples was hybridized with probes derived from gene-specific sequences as mentioned in Supplementary Table S2 (http://www.biosci.org/bsr/30/bsr30ppppadd.htm). Hybridization signal intensity was quantified with a phosphoimager scanner (FLA-5000; Fuji phosphoimager).

RESULTS

CUR inhibits the growth of *Candida* cells

To investigate the antifungal effect of CUR on *C. albicans* cells, we used a commercial preparation of CUR, which is a mixture of three major curcuminoids, namely diferuloylmethane (CUR I), demethoxycurcumin (CUR II) and bisdemethoxycurcumin (CUR III), and contains predominantly CUR I (~77%) followed by CUR II (17%) and CUR III (3%) [7]. For this, we employed spot and broth microdilution drug susceptibility assays. CUR over a range of concentrations was able to inhibit the growth of cells after concentrations $\geqslant 185$ mg/l in a broth microdilution assay, whereas higher concentrations (296–370 mg/l) were needed to inhibit the growth in solid medium (Figures 1A and 1B). The growth inhibitory effect of CUR was also evident with non-*albicans* species. For example, on solid medium, CUR at $\geqslant 46.25$ mg/l was able to inhibit the growth of *Candida tropicalis Candida dubliniensis* and *Candida utilis* whereas $\geqslant 92.5$ and $\geqslant 185$ mg/l of it was needed to inhibit the growth of *Candida kefyr* and

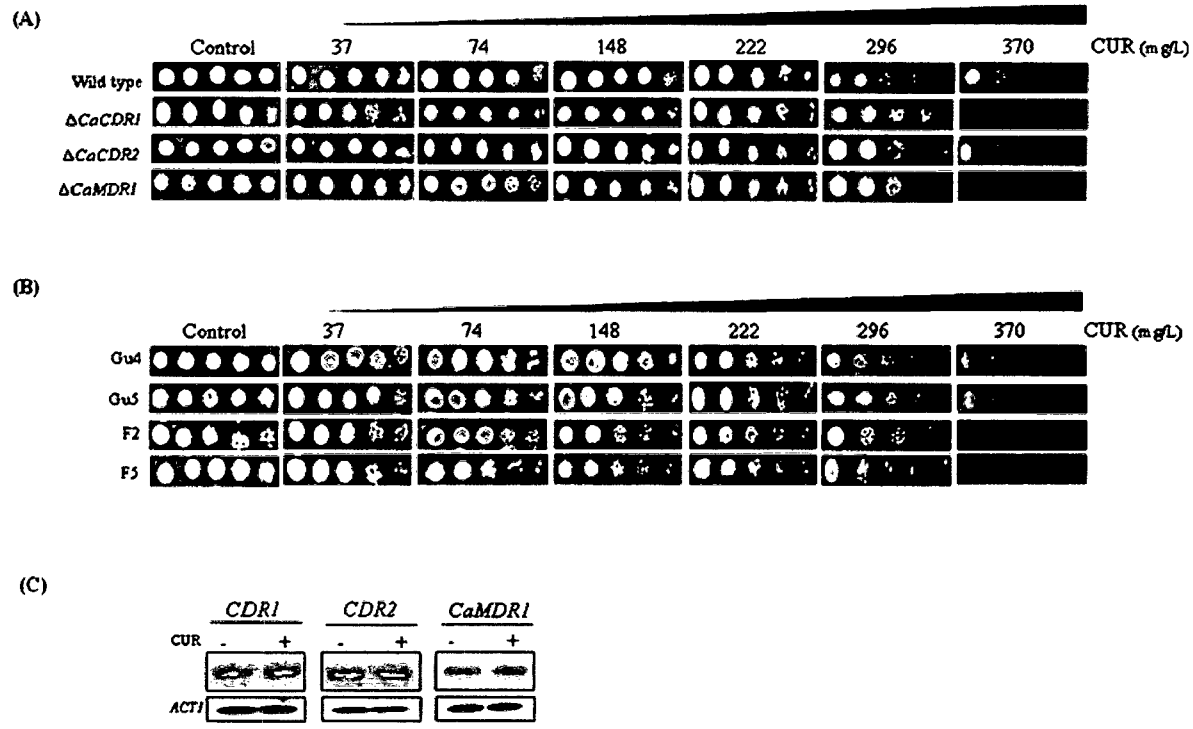


Figure 3 Antifungal effects of CUR against *C. albicans*, in cells either lacking or overexpressing the drug efflux pumps
The cells were grown overnight on YEPD plates and then resuspended in normal saline to a D_{600} of 0.1. A 5 μl portion of 5-fold serial dilution of each strain was spotted on to YEPD plates as described earlier [44], either in the absence or presence of various concentrations of CUR (37–370 mg/l). (A) Null mutants of *C. albicans* (Δ*CaCdr1*, Δ*CaCdr2*, Δ*CaMdr1*) lacking functional drug transporters. (B) Matched pair isolates of *C. albicans* (Gu4 and Gu5) overexpressing the transporter *CaCDR1* or *CaMDR1* (F2 and F5). Growth differences were evaluated after 48 h of incubation as mentioned earlier [44]. (C) Transcript levels of *CDR1/CDR2/CaMDR1* in the wild-type strain SC5314 in the absence and presence of CUR (185 mg/l, 16 h). *ACT1* mRNA levels were used as a loading control.

Candida krusei respectively. Both *Candida parapsilosis* and *Candida glabrata* required 370 mg/l CUR to show growth inhibition (Figures 1A and 1B). All the three purified curcuminoids from CUR did inhibit the growth of *Candida* cells, which was comparable with a commercial preparation of CUR (results not shown). For all the subsequent experiments, commercial CUR was used.

Co-administration of CUR and PIP reduces the *Candida* load *in vivo*

CUR has poor bioavailability owing to its rapid metabolism in liver and intestinal walls. Co-administration of PIP, an inhibitor of hepatic and intestinal glucuronidation, is known to enhance the bioavailability of CUR [16]. To determine the *in vivo* antifungal activity of CUR against *C. albicans* (ATCC 36082), a systemic murine model of infection was employed [17]. As depicted in Figure 2, the effect of CUR was evaluated by comparing the live *Candida* load reduction in the kidneys of treated and untreated mice after administration of CUR or CUR+PIP. It was observed that administration of CUR (intraperitoneally) alone led to an insignificant fungal load decrease of $1.0\log_{10}$ with a P value of 0.0887. However, the administration of CUR along with PIP (orally) causes a significant fungal load reduction of $1.4\log_{10}$ with a P value of 0.0199. In the case of positive control FLC, there was a fungal load reduction of $1.9\log_{10}$, with a P value of 0.0029. Notably, the MIC_{80} (lowest drug concentration that causes 80% inhibition of fungal growth) value of *C. albicans* (ATCC 36082) was similar to that of SC5314 (ATCC MYA2876) used in the present study for the subsequent experiments (results not shown).

Antifungal effect of CUR is independent of the drug efflux pump

When the drug efflux pump (*CaCDR1/CaCDR2/CaMDR1*) [18–20] null mutants were grown in the presence of various concentrations of CUR, the drug susceptibility pattern remained similar to the wild-type strain (Figure 3A). The clinical matched pair of AS (azole-susceptible) and AR (azole-resistant) isolates, which show increased resistance to azoles owing to an overexpression of either *CaCDR1*(Gu4, Gu5) [21] or *CaMDR1* (F2, F5) [22] genes, remained sensitive to CUR (Figure 3B). CUR did not affect the expression of genes encoding MDR pump proteins (Figure 3C).

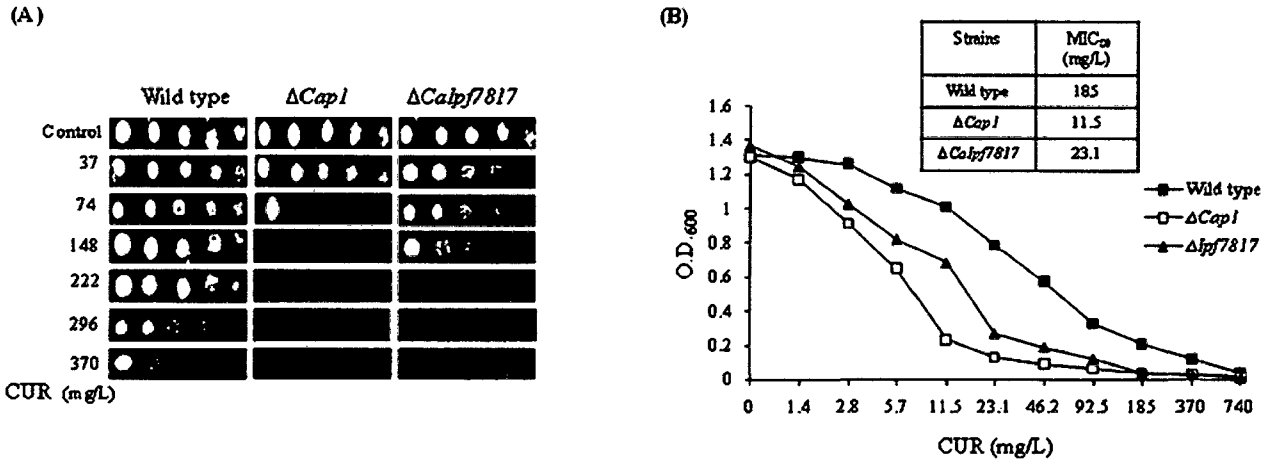


Figure 4 **Susceptibility of oxidative stress mutants of *C. albicans* to CUR**

(A) The cells (SC5314/Δ*Cap1*/Δ*Calpf7817*) were grown overnight on YEPD plates and then resuspended in normal saline to a D_{600} of 0.1. A 5 μl portion of 5-fold serial dilution of each strain was spotted on to YEPD plates as described earlier [44], either in the absence or presence of various concentrations of CUR (37–370 mg/l). (B) Determination of growth inhibition of the *Candida* cells by the broth microdilution assay in the presence of CUR at concentrations ranging from 1.44 to 740 mg/l. Growth of the cells was evaluated both visually and by reading the D_{600} in a microtitre reader as described earlier [44]. Filled/empty squares and filled triangles represent SC5314/Δ*Cap1* and Δ*Calpf7817* respectively. The inset shows the MIC_{80} values. A stock solution of 11 mg/ml was used (DMSO). Growth was not affected by the presence of the solvent (results not shown).

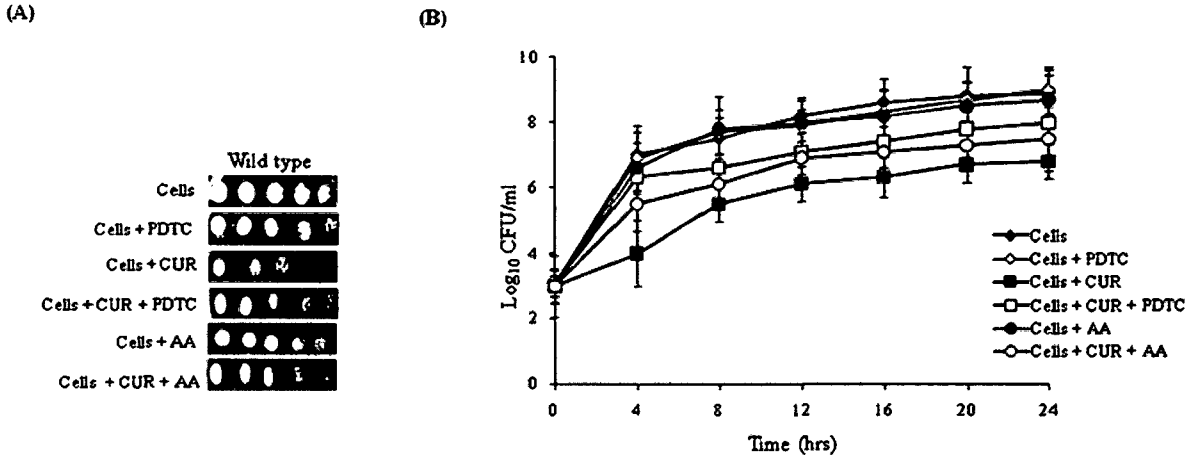


Figure 5 **Spot assays in the presence of antioxidants/CUR and the time kill assays**

(A) The cells were grown overnight on YEPD plates and then resuspended in normal saline to a D_{600} of 0.1. A 5 μl portion of 5-fold serial dilution of each strain was spotted on to YEPD plates as described earlier [44], either in the absence or presence of CUR (296 mg/l) alone or CUR and antioxidants (10 μM PDTC or 25 mM AA). The antioxidants have no effect on the growth of cells. Growth differences were evaluated after 48 h of incubation as mentioned earlier [44]. (B) Time kill curves of the wild-type strain of *C. albicans* in the presence of CUR/PDTC/AA alone or in combination were obtained by using initial inoculums of 10^3 cfu/ml. The filled/empty diamonds, squares and circles represent cells alone, cells+PDTC, cells+CUR, cells+CUR+PDTC, cells+AA and cells+CUR+AA respectively. The values shown are the means and S.D. (indicated by error bars) for three independent experiments.

Oxidative stress null mutants are susceptible to CUR

To understand the molecular basis of antifungal activity of CUR, we evaluated its effect on various categories of mutants of *C. albicans*. These included morphological nulls Δ*tup1* (Tup1p, which is a transcriptional co-repressor and represses filamentous growth [23]), Δ*efg1* (Efg1p, a transcriptional repressor required for hyphal growth [24]), Δ*cph1* (Cph1p, a transcription factor required for mating and hyphal growth on solid medium [24]), Δ*nrg1* (Nrg1p, a transcription repressor, regulates hyphal genes

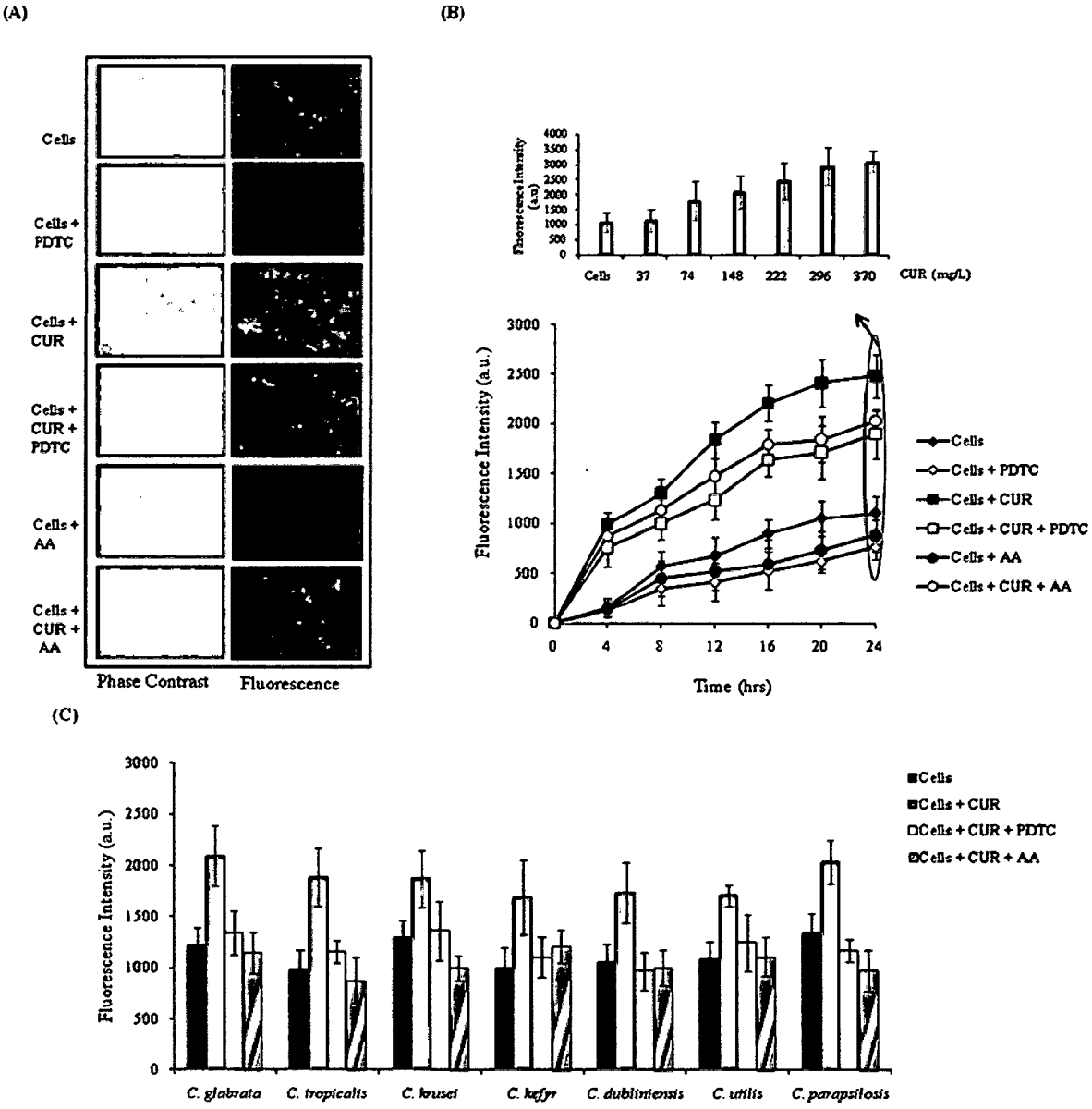


Figure 6 **ROS levels in the presence of CUR in *C. albicans* cells**

(A) Images of DCF fluorescence due to CUR (185 mg/l) or CUR+antioxidants (10 μM PDTC or 25 mM AA) treatment were taken with a fluorescence microscope (Carl Zeiss). (B) Amounts of ROS produced in CUR (185 mg/l) or CUR+antioxidants (10 μM PDTC or 25 mM AA)-treated cells. The fluorescence emitted by the cells was measured by using a spectrofluorimeter (Varian, Cary Eclipse; $\lambda_{ex} = 485$ and $\lambda_{em} = 540$ nm). The filled/empty diamond, filled/empty square and filled/empty circle represent cells alone, cells+PDTC, cells+CUR, cells+CUR+PDTC, cells+AA and cells+CUR+AA respectively. The bar graph shows the level of ROS produced at 24 h of administration of CUR. (C) ROS produced in non-*albicans* species of *Candida* on CUR (185 mg/l) or CUR+PDTC or CUR+AA (10 μM PDTC and 25 mM AA) treatment (16 h). The values are the means and S.D. (indicated by error bars) for three independent experiments.

and virulence genes [23]), Δ*ras1* {Ras1p, which is a RAS signal transduction GTPase and regulates cAMP and MAPK (mitogen-activated protein kinase) pathways [25]}, Δ*ssk1* (Ssk1p, which is a response regulator of the two-component system and plays a role in cell wall biosynthesis and virulence [26]), oxidative stress nulls Δ*Calpf7817* (Calpf7817p, which is involved in the regulation of redox homoeostasis [27]), Δ*Cap1* (Cap1p, which is a transcription factor and regulates the oxidative stress response [28,29]), Δ*tac1* (Tac1p, a transcription factor involved in the up-regulation of *CDR1* and *CDR2* [30]), Δ*ftr1* (Ftr1p, a

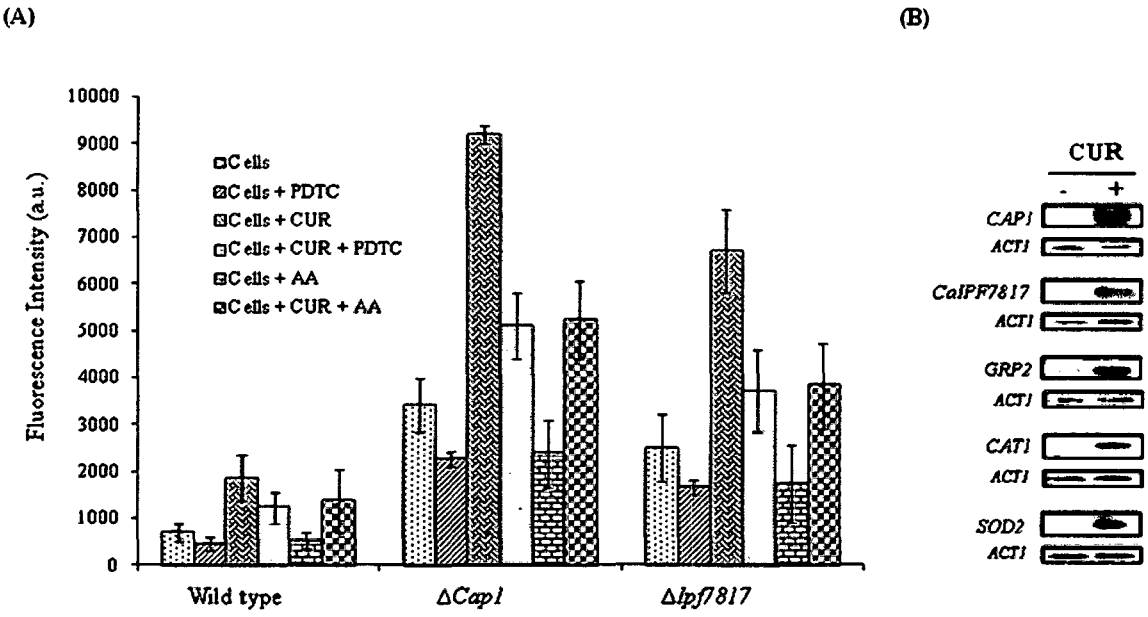


Figure 7 **ROS levels in the oxidative stress mutants and mRNA transcript levels in *C. albicans* cells**

(A) Amounts of ROS produced in CUR (185 mg/l) or CUR+antioxidants (10 μM PDTC or 25 mM AA)-treated cells, namely wild-type (SC5314), Δ*Cap1* and Δ*Calpf7817*. The fluorescence emitted by the cells was measured by using a fluorescence spectrometer ($\lambda_{ex} = 485$ nm and $\lambda_{ex} = 540$ nm). Bars with dots, diagonal lines, weave, horizontal lines, bricks and checker board represent cells alone, cells+PDTC, cells+CUR, cells+CUR+PDTC, cells+AA and cells+CUR+AA respectively. The values are the means and S.D. (indicated by error bars) for three independent experiments. (B) Transcript levels of *CAP1*, *CaIPF7817*, *SOD2*, *GRP2* and *CAT1* in the wild-type strain SC5314 in the presence and absence of CUR (185 mg/l, 4 h). *ACT1* mRNA levels were used as a loading control.

high-affinity iron permease [31]) and Δ*ccc2* (Ccc2p, a copper-transporting ATPase) [32]. Notably, only the oxidative stress nulls Δ*Calpf7817* and Δ*Cap1* appeared to be highly susceptible to CUR in comparison with the wild-type strain (Figure 4A). As depicted in Figure 4(B), there was a significant reduction in the MIC_{80} values of the oxidative stress mutants when grown in the presence of CUR as compared with the wild-type strain. None of the other tested mutants showed any difference in the MIC_{80} values when grown on CUR (see Supplementary Table S3 at http://www.biosci.org/bsr/30/bsr30ppppadd.htm).

Antifungal effects of CUR could be reversed by antioxidants

We further confirmed the role of oxidative stress by performing spot assays in the presence of CUR and antioxidants such as PDTC or AA. For this, we used a range of antioxidant concentrations (5–100 μM for PDTC and 25–100 mM for AA) and observed that 10 μM of PDTC or 25 mM of AA could only partially restore the CUR effect. A higher concentration of each antioxidant was toxic to cells (results not shown). As depicted in Figure 5(A), addition of PDTC (10 μM) or AA (25 mM) alone had no effect on the growth of cells but, when added along with CUR (296 mg/l), the growth inhibition was reversed. This reversal of growth inhibition due to antioxidants was further confirmed by the colony formation assay. As compared with CUR alone, which killed ~96% of the cells, the percentage of viable cells in the presence of CUR and antioxidants was considerably increased (Figure 5B).

CUR generates ROS

In the following experiments, we analysed whether ROS formation could be a key event in the CUR-induced killing of *C. albicans*. Using a fluorimetric assay, we could demonstrate that in the presence of CUR, there was an increase in FI, which coincided with induced endogenous ROS in *Candida* (Figures 6A and 6B). The net ROS production in cells raised by CUR could be reversed by the addition of antioxidant PDTC or AA. The ROS levels in non-*albicans* species were also raised to different levels when CUR was present in growth medium, which could be reversed by the addition of antioxidants (Figure 6C). It should be pointed out that PDTC and AA alone in the growth medium inhibited the basal level of ROS production between 10 and 20% (Figure 6B). Expectedly, the highly CUR-susceptible oxidative stress mutants Δ*Calpf7817* and Δ*Cap1* displayed 3–5 times higher basal ROS levels, which was further increased by CUR in a reversible manner (Figure 7A). As depicted in Figure 7(B), the presence of CUR increased the transcript levels of the genes [*CAP1* (*C. albicans* AP-1), *CaIPF7817* (putative NADH-dependent flavin oxidoreductase), *GRP2* (NADPH-dependent methyl glyoxal reductase), *CAT1* (catalase 1) and *SOD2* (superoxide dismutase 2)] associated with maintenance of oxidative stress.

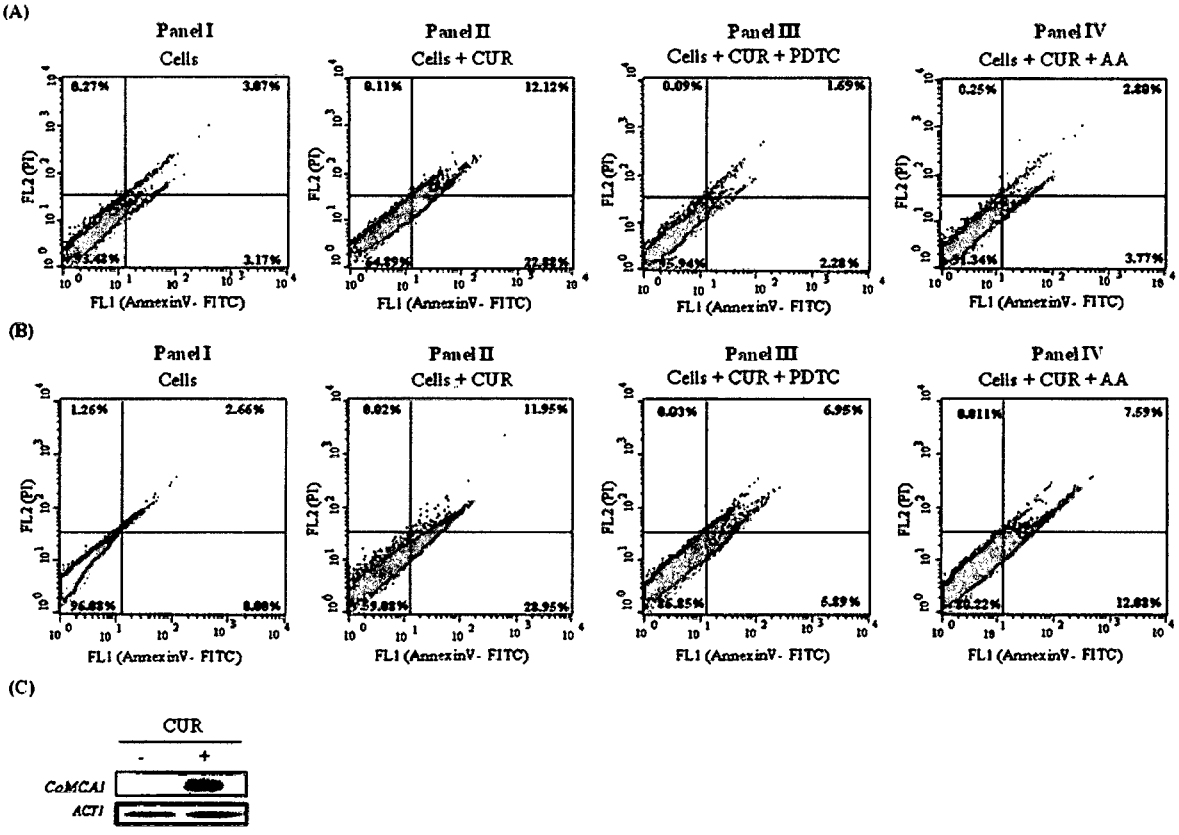


Figure 8 Externalization of PS in CUR-treated cells

Panel I represents untreated, stained *C. albicans* (SC5314) cells, panel II shows CUR-treated cells, panels III and IV are the PDTC/AA pretreated cells in the presence of CUR and stained with (A) annexin V–FITC alone or (B) co-stained with annexin V–FITC and propidium iodide. For treatment, the cells were incubated with CUR (185 mg/l, 4 h) and analysed by flow cytometry as described in the Materials and methods section. The lower right quadrants of the various panels represent early apoptotic cells, and the upper right quadrants represent late apoptotic or necrotic cells. (C) Transcript level of *CaMCA1* in the wild-type strain SC5314 in the absence and presence of CUR (185 mg/l, 4 h). *ACT1* mRNA levels were used as a loading control.

CUR induces apoptosis in *Candida* cells

Translocation of PS to the outer monolayer of the lipid bilayer of the PM (plasma membrane) is an early marker of apoptosis [13,14]. We explored whether CUR induces apoptosis in *C. albicans* by measuring PS externalization using the annexin V–FITC assay. As depicted in Figure 8(A), after 4 h of incubation of cells with CUR, the population of cells had 22.88% exposed PS as compared with 3.17% in untreated cells (Figure 8A, panels II and I respectively). Notably the CUR-induced externalization of PS could be arrested to 2.28 or 3.77% if cells were pretreated with antioxidant PDTC or AA before the incubation with CUR (Figure 8A, panels III and IV respectively).

Since after the loss of membrane integrity, annexin V–FITC also labels necrotic cells, simultaneous addition of propidium iodide, which does not permeate cells with an intact PM, allows discrimination between apoptotic (annexin V positive, propidium iodide negative), necrotic (both annexin V and propidium iodide positive) and live (both annexin V and propidium iodide negative) cells [13,14]. Accordingly, we examined the effect of CUR on the overall population distribution between apoptotic and necrotic cells. For this, CUR (185 mg/l)-treated cells were double stained with annexin V–FITC and propidium iodide. As shown in Figure 8(B) (panel II), a significant percentage (28.95%, lower right quadrants) of cells stained positive for annexin V–FITC as compared with none (Figure 8, panel I) in untreated cells. It was confirmed from the double staining (annexin V–FITC and propidium iodide, Figure 8B, panel II, lower right quadrants), that the single stained (annexin V–FITC) cells represents only apoptotic cells (Figure 8A, panel II, lower right quadrants). The late apoptotic/necrotic cells increased to 11.95% in 4 h (Figure 8B, panel II, upper right quadrant) when treated with CUR, which could be reversed by the antioxidants such as PDTC or AA (Figure 8B, panels III and IV, upper right quadrants).

MCA1 (metacaspase 1) encodes a homologue of a mammalian caspase in *C. albicans* [33]. To determine the involvement of caspases in CUR-induced cytotoxicity in *C. albicans*, the

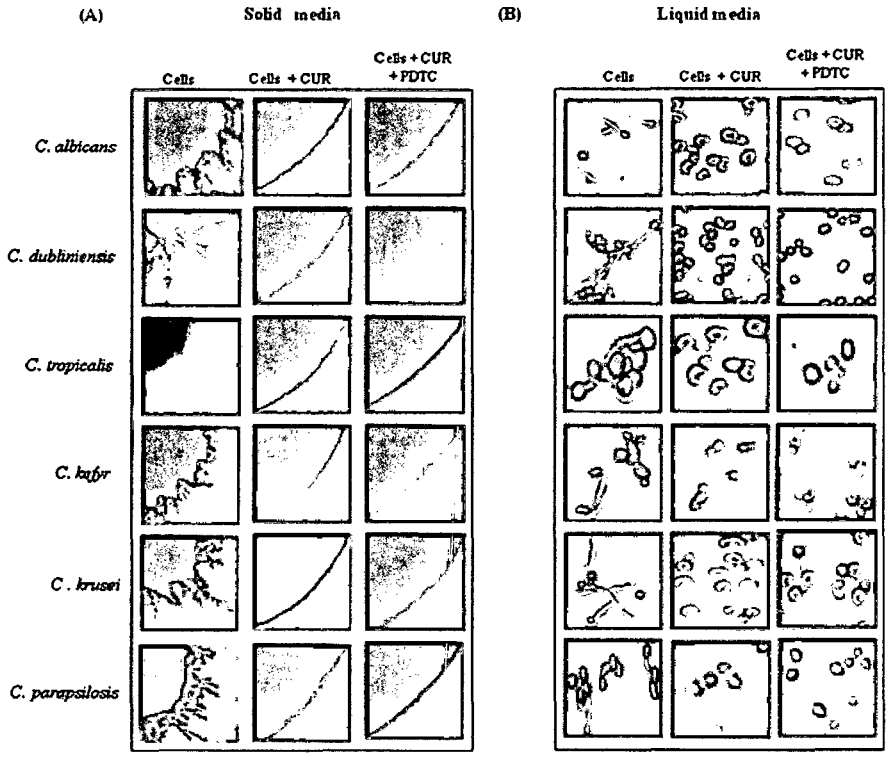


Figure 9 **Hyphae development in the presence and absence of CUR/CUR+ PDTC in solid and liquid hypha-inducing media**
Response to CUR (37 mg/l) and CUR (37 mg/l)+PDTC (10 μM) by *C. albicans* (SC5314) cells under conditions that promote hyphal growth. The cells were grown in the absence or presence of CUR alone or CUR+PDTC in (A) solid YEPD (2.5% agar) with 10% FBS or in (B) liquid spider medium. Cells were incubated for 6 h in liquid medium and for 3 days in solid medium at 37°C. Colony morphologies on solid plates and filamentation in liquid medium were examined microscopically (Carl Zeiss) as mentioned in the Materials and methods section.

expression of *CaMCA1* was determined in exponential phase cells by Northern-blot analysis. It was observed that after a 4 h exposure to CUR, the *CaMCA1* transcript level was also increased when compared with untreated cells (Figure 8C).

CUR inhibits hyphae development by *Candida*

Mycelial development of *C. albicans* is influenced by many factors and is controlled by well-known morphological regulators. In the present study, we observed that CUR (37 mg/l) when added to hypha-inducing solid or liquid medium prevented the development of hyphae of *C. albicans* cells. In contrast with the other effects of CUR, the inhibition of hyphae development could not be reversed by the addition of an antioxidant such as PDTC (10 μM) or AA (25 mM) (Figure 9).

Hyphae development by *tup1* nulls could not be inhibited by CUR

We examined the effect of CUR (37 mg/l) in several null mutants lacking morphological transcription regulators and found that CUR could not prevent hyphae development in Δ*tup1* cells [34] (Figure 10A). This coincided with a raised *TUP1* (thymidine uptake 1) transcript level due to CUR in a wild-type strain of *C. albicans* (Figure 10B).

CUR works independently of the quorum sensing molecule farnesol

It is known that the quorum sensing molecule farnesol raises ROS levels and inhibits hyphae development by targeting the repressor *TUP1* [34]. We observed that polyphenol CUR apparently mimics the farnesol affect. To establish the link between CUR and farnseol pathways, we examined the various effects of CUR, namely antifungal, ROS generation and the inhibitory effect on mycelial development in a *C. albicans* strain defective in farnesol production. *DPP3* (diacylglycerol pyrophosphate phosphatase) encodes a phosphatase that converts farnesyl pyrophosphate into farnesol. It is reported that *DPP3* knockout (KWN2) produces six times less farnesol in comparison with the parent strain (SN152) [35]. Figure 11(A) shows that CUR could inhibit the growth of the *DPP3* null strain similarly to the wild-type strain. Additionally, the growth inhibition of Δ*dpp3* cells by CUR could be reversed by antioxidants (Figure 11B). Similarly, the CUR effect to raise ROS levels and to inhibit hyphae formation remained unaffected in Δ*dpp3* cells (Figures 11C and 11D).

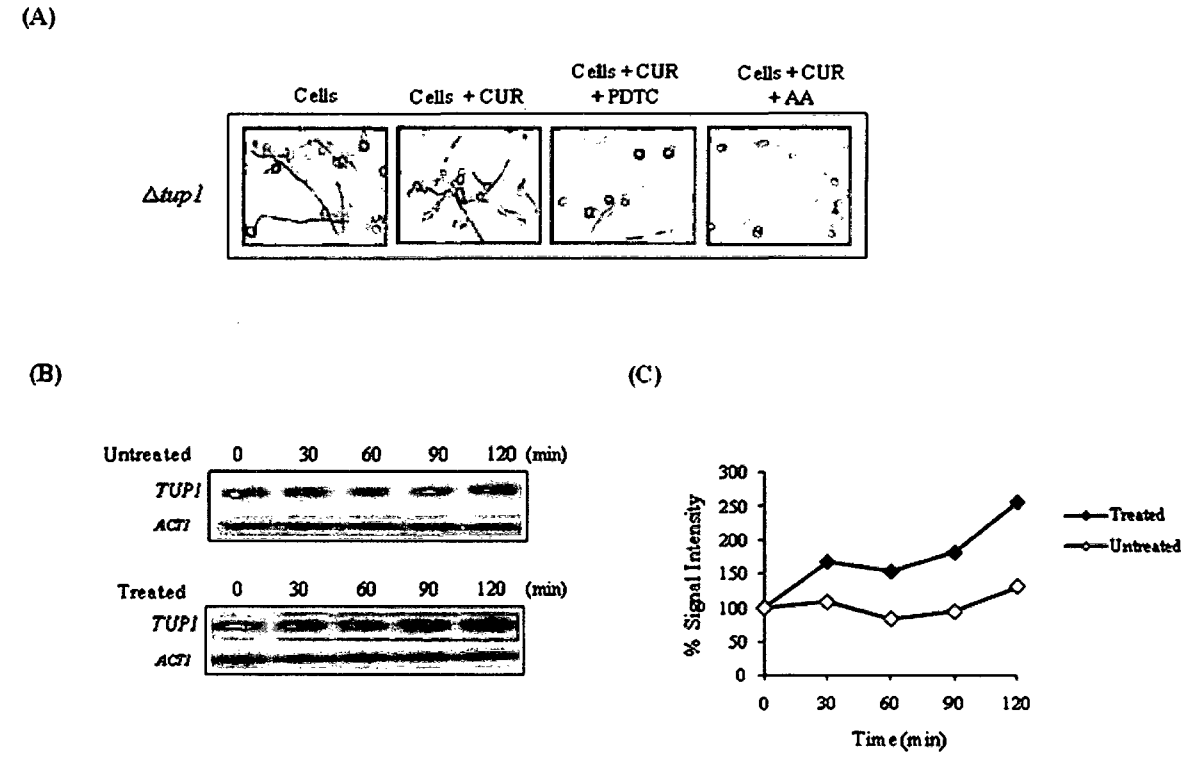


Figure 10 Effect of CUR on the hyphae development of *Δtup1* cells
(A) Response to CUR and CUR+PDTC by *C. albicans* under conditions that promote hyphal growth. The cells were grown in the presence or absence of CUR alone (37 mg/l) or CUR (37 mg/l)+PDTC (10 μM) in YEPD with 10% FBS and incubated at 37 °C for 6 h. The filamentation in liquid medium was examined microscopically (Carl Zeiss). (B) Transcript levels of *Tup1* in the wild-type strain SC5314 in the presence and absence of CUR (37 mg/l) at the indicated time points. Constitutively expressing *ACT1* transcript was used as a loading control. (C) Quantification of Northern-blot hybridization.

DISCUSSION

In the present study, we have investigated the antifungal effects of a natural polyphenol CUR against *albicans* and non-*albicans* species of *Candida* and have shown that the growth of all the tested strains of *Candida* could be inhibited by CUR (Figure 1). While demonstrating the anticancer effects of CUR, it has been observed that the systemic exposure to CUR remains too low to exhibit sufficient pharmacological activity. However, concomitant administration of PIP increases bioavailability of CUR both in humans and in rats [7,16]. On the basis of these findings, PIP, a known inhibitor of hepatic and intestinal glucuronidation, was combined with CUR and administered to check its *in vivo* efficacy. Indeed, when a combination of CUR and PIP was administered, there was a significant and higher reduction in fungal load ($1.4\log_{10}$) in kidneys of Swiss mice as compared with the case when CUR was administered alone ($1\log_{10}$) (Figure 2). Pharmacokinetic studies have indicated that after oral administration in rats and humans, CUR is transformed into metabolites like CUR glucuronides [7]. PIP remarkably enhances the bioavailability of CUR in mice, resulting in significant reduction in fungal load; this suggests that these metabolites may also contribute to the antifungal effect of CUR. Elaborate *in vivo* studies are required to find out the efficacy across the fungal species. Also, research efforts are necessary to increase the medicinal value of CUR through structural modifications of the molecule and new formulations to increase the oral bioavailability.

To investigate the mechanism of the antifungal effect of CUR against *Candida*, the sensitivity of CUR to various morphological, iron transporter and oxidative stress mutants of *C. albicans* was checked and it was observed that only the oxidative stress mutants (Δ*Cap1* and Δ*Calpf7817*) were particularly susceptible to CUR (Figure 4), whereas other mutant strains behaved similarly to the wild-type strain (Supplementary Table S3). A striking feature was the growth inhibitory effects and elevated ROS levels due to CUR, which could be reversed if the natural or synthetic antioxidants were also present in the growth medium (Figures 5 and 6).

The capacity to induce ROS possessed by various antifungals has been reported earlier [11]. For example, azoles such as miconazole, as well as the polyenes amphotericin B and nystatin and polyol macrolides such as niphimycin, induce ROS levels in susceptible fungi [12,14,36,37]. In addition, the benzo-naphthacenequinone antibiotic pradimicin A [38], natural perylenequinonoid pigments [39], the isoprenoid alcohol farnesol

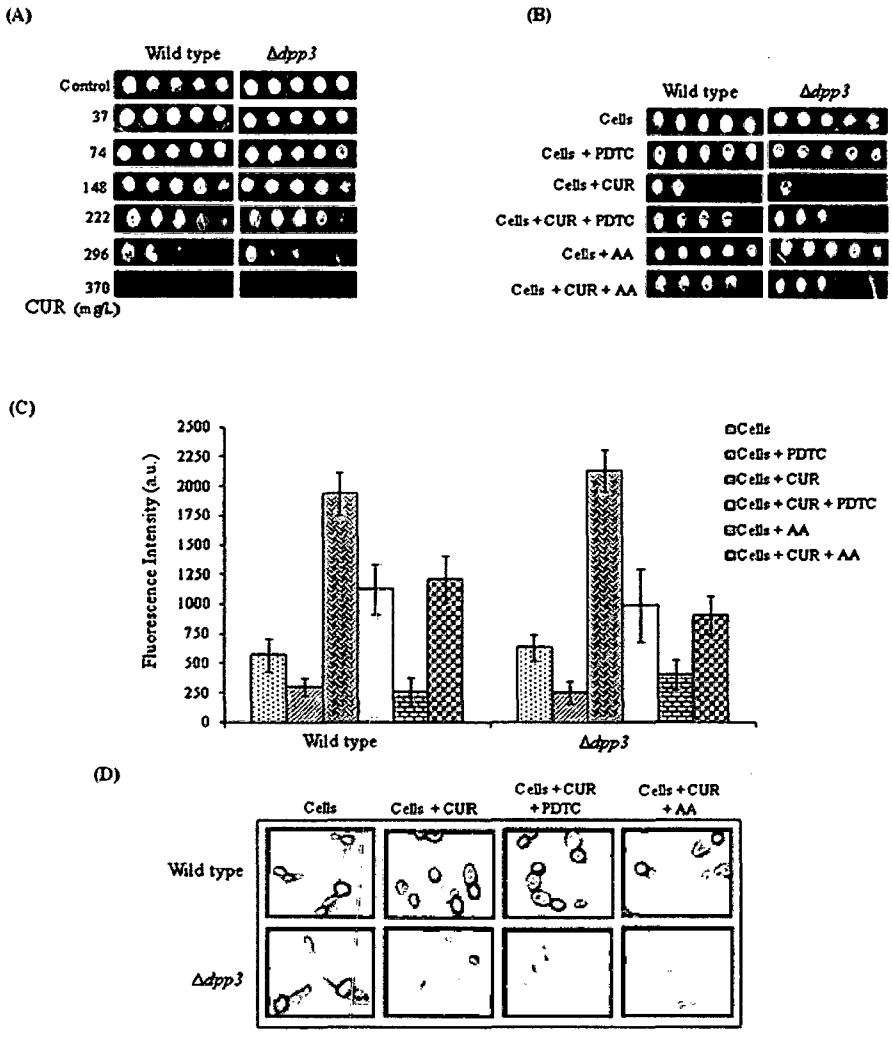


Figure 11 **Effects of CUR on Δ*dpp3* cells**

(A) The wild-type (SN152) and Δ*dpp3* (KWN2) cells were grown overnight on YEPD plates and then resuspended in normal saline to a D_{600} of 0.1. A 5 μl portion of 5-fold serial dilution of each strain was spotted on to YEPD plates as described earlier [44], either in the absence or presence of various concentrations of CUR (37–370 mg/l). (B) A 5 μl portion of 5-fold serial dilution of each strain was spotted on to YEPD plates as described earlier [44], either in the absence or presence of CUR (296 mg/l) alone or CUR and antioxidants (10 μM PDTC or 25 mM AA). The antioxidants (10 μM PDTC or 25 mM AA) have no effect on the growth of cells. Growth differences were evaluated after 48 h of incubation as mentioned earlier [44]. (C) Amounts of ROS produced in CUR (185 mg/l) or CUR+antioxidants (10 μM PDTC or 25 mM AA)-treated cells, namely wild-type (SN152) and Δ*dpp3* (KWN2) cells. The fluorescence emitted by the cells was measured by using a fluorescence spectrometer ($\lambda_{ex} = 485$ and $\lambda_{em} = 540$ nm). Bars with dots, diagonal lines, weave, horizontal lines, bricks and checker board represent cells alone, cells+PDTC, cells+CUR, cells+CUR+PDTC, cells+AA and cells+CUR+AA respectively. The values are the means and S.D. (indicated by error bars) for three independent experiments. (D) Response to CUR and CUR+PDTC by *C. albicans* under conditions that promote hyphal growth. The cells were grown in the presence or absence of CUR alone (37 mg/l) or CUR (37 mg/l)+PDTC (10 μM) in YEPD with 10% FBS and incubated at 37°C for 6 h. The filamentation in liquid medium was examined microscopically (Carl Zeiss).

[40] and several antifungal peptides/proteins also induce ROS in yeast species. Some of the ROS-inducing antifungals further trigger apoptosis in yeast cells [14,36,38]. An oxidative stress response in yeast is well documented [27–29,41]. Yeast cells undergoing apoptosis display several characteristic markers, including the induction of endogenous ROS [42]. With this background, our observation that CUR-induced ROS stimulates the pro-apoptotic regulatory machinery in *Candida* cells is interesting. We could demonstrate that CUR increased the number of pre-apoptotic cells that could be prevented by the presence of an antioxidant (Figure 8). Raised *CaMCA1* levels in the presence of CUR point to caspase-mediated apoptosis in *C. albicans* (Figure 8C). In our previous study, we have shown that CUR modulates the drug efflux of yeast ABC transporters without affecting the protein

levels [43], and here we could demonstrate that CUR exerts its growth inhibitory effects without affecting the transcript levels of genes encoding these transporters (Figure 3C). Hence the effect of CUR on *Candida* growth is independent of the levels of these transporters (Figure 3).

Interestingly, even at lower concentrations, CUR (37 mg/l) could block the hyphae development in both *albicans* and non-*albicans* species of *Candida* (Figure 9). However, unlike the antifungal effect of CUR, the inhibition of hyphae development could not be reversed by the antioxidants. We found instead that CUR targeted the global repressor *TUP1* since CUR could not inhibit hyphae development of Δ*tup1*, which otherwise due to de-repression vigorously makes hyphae. This was further confirmed by Northern-blot experiments where CUR induced an increase in *TUP1* transcript levels in wild-type cells (Figure 10). The signalling molecule farnesol, which also causes an increase in ROS levels, exerts its effect via *TUP1* [35]. In *C. albicans*, farnesol is endogenously generated by enzymatic dephosphorylation of farnesyl diphosphate, a precursor for the synthesis of sterols in the sterol biosynthesis pathway [42]. Thus the CUR effect seems to mimic the effect of the quorum sensing molecule farnesol. However, when we checked the effects of CUR on the growth, ROS generation and mycelial development in a knockout of *DPP3* that encodes a phosphatase for converting farnesyl pyrophosphate into farnesol, it was observed that CUR continued to inhibit the growth, which could be reversed by the addition of antioxidants in the mutant strain KWN2 (Δ*dpp3*) [35]. The raised ROS levels and the inhibition of hyphae development in *DPP3* knockout suggest that CUR only mimics the effects of farnesol phenotypically but is independent of farnesol (Figure 11). Taken together, CUR has dual affects on *Candida* cells. Its antifungal effect is mediated by the ROS signalling pathway, which brings about an early apoptosis leading to cell death. Independent of the ROS pathway, CUR also inhibits hyphae development by regulating *TUP1* levels. Considering the success story of CUR as an anticancer and anti-inflammatory compound, the present study opens up the possibility that CUR can also be exploited as a potential natural antifungal.

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online data

SUPPLEMENTARY ONLINE DATA

Antifungal curcumin induces reactive oxygen species and triggers an early apoptosis but prevents hyphae development by targeting the global repressor *TUP1* in *Candida albicans*

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Table S1 Strains used in the present study

Strains	Genotype	Source/reference
ATCC MYA2876	*C. albicans*	Fonzi and Irwin [1]
ATCC 6258	*C. krusei*	Ranbaxy Laboratories, India
ATCC 750	*C. tropicalis*	Ranbaxy Laboratories, India
ATCC 22019	*C. parapsilosis*	Ranbaxy Laboratories, India
ATCC 2512	*C. kefyr*	Ranbaxy Laboratories, India
ATCC 33	*C. dubliniensis*	Ranbaxy Laboratories, India
ATCC 90030	*C. glabrata*	Ranbaxy Laboratories, India
ATCC 15239	*C. utilis*	Ranbaxy Laboratories, India
Gu4	Fluconazole-susceptible clinical isolate	Franz et al. [2]
Gu5	Fluconazole-resistant clinical isolate	Franz et al. [2]
F2	Fluconazole-susceptible clinical isolate	Franz et al. [3]
F5	Fluconazole-resistant clinical isolate	Franz et al. [3]
DSY 449	Δ*cdr1*::hisG/Δ*cdr1*::hisG	Sanglard et al. [4]
DSY 653	Δ*cdr2*::hisG-URA3-hisG/Δ*cdr2*::hisG	Sanglard et al. [5]
F5M 432	Δ*mdr1*::FRT/Δ*mdr1*::FRT	Wirsching et al. [6]
HLC52	Δ*efg1*::hisG/Δ*efg1*::hisG-URA3-hisG	Stoldt et al. [7]
DK9	Δ*tup1*::hisG/Δ*tup1*::p405-URA3/Δ*ura3*	Braun et al. [8]
CSSK22-1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*Δ *ssk1*::*hisG*/Δ*ssk1*::*hisG*	Calera et al.[9]
DK152	Δ*nrg1*::hisG/Δ*nrg1*::hisG-URA3-hisG	Braun et al. [8]
JKC19	Δ*cph1*::hisG/Δ*cph1*::*hisG-URA3-hisG*	Stoldt et al. [7]
Δ*ccc2*	Δura3::imm434/Δura3::imm434Δ*ccc2*::hisG/Δ*ccc2*::*his G*	Weissman et al. [10]
Δ*ftr1*	Δura3::imm434/Δura3::imm434 Δftr1::hisG/Δftr1::*hisG*	Ramanan and Wang [11]
DSY 2906	Δtac1::hisG/Δtac1::hisG	Coste et al. [12]
CJD21	Δcap1::hisG/Δcap1::hisG	Alarco et al. [13]
JH2	Δipf7817::hisG/Δipf7817::hisGΔura3::imm434/Δura3 ::imm434his1::hisG/his1::hisG	Jia et al. [14]
ras1-2/ras1-3	Δras1::hisG/Δras1::hph-URA3-hph	Feng et al. [15]

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Table S2 Primers used in the present study

Primer	Sequence (5′–3′)
MS-TUP1-F	ATTCTTGCAAGATTTAGACATTGC
MS-TUP1-R	ATGACACCACGGTCCTTTGAGC
MS-CAP1-F	ACCGTGAACGTAAAGAACG
MS-CAP1-R	GCTACCACCAGTATATTTAGCC
MS-IPF7817-F	TGTTAAGGATTTTGGTGCTGC
MS-IPF7817-R	CCACCGTATTCATCTGTTCTC
MS-GRP2-F	ATGTCTTCATCTACTACAGTTTTCG
MS-GRP2-R	TAATTTCAAAAGCTTGTGGACC
MS-CAT1-F	CCCAGAAAGAGTTGTCCACGC
MS-CAT1-R	CCATGATGGGTAGTTACCAGCAGC
MS-SOD2-F	ATGTTTTCTATCAGATCATC
MS-SOD2-R	ACCACCACCTTGAGAGACAGGAGCC

Table S3 MIC_{80} values of the strains

Strain	MIC_{80} (mg/l)
SC5314	185
Δ*tup1*	185
Δ*efg1*	185
Δ*cph1*	185
Δ*nrg1*	185
Δ*ras1*	185
Δ*ssk1*	92.5
Δ*ftr1*	185
Δ*ccc2*	185

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RESEARCH ARTICLE

Synergistic anticandidal activity of pure polyphenol curcumin I in combination with azoles and polyenes generates reactive oxygen species leading to apoptosis

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Candida albicans; curcumin; fractional inhibitory concentration index; minimum inhibitory concentration; synergism.

Abstract

We have shown previously that pure polyphenol curcumin I (CUR-I) shows antifungal activity against *Candida* species. By employing the chequerboard method, filter disc and time–kill assays, in the present study we demonstrate that CUR-I at non-antifungal concentration interacts synergistically with azoles and polyenes. For this, pure polyphenol CUR-I was tested for synergy with five azole and two polyene drugs – fluconazole (FLC), miconazole, ketoconazole (KTC), itraconazole (ITR), voriconazole (VRC), nystatin (NYS) and amphotericin B (AMB) – against 21 clinical isolates of *Candida albicans* with reduced antifungal sensitivity, as well as a drug-sensitive laboratory strain. Notably, there was a 10–35-fold drop in the MIC_{80} values of the drugs when CUR-I was used in combination with azoles and polyenes, with fractional inhibitory concentration index (FICI) values ranging between 0.09 and 0.5. Interestingly, the synergistic effect of CUR-I with FLC and AMB was associated with the accumulation of reactive oxygen species, which could be reversed by the addition of an antioxidant such as ascorbic acid. Furthermore, the combination of CUR-I and FLC/AMB triggered apoptosis that could also be reversed by ascorbic acid. We provide the first evidence that pure CUR-I in combination with azoles and polyenes represents a novel therapeutic strategy to improve the activity of common antifungals.

Introduction

Combating *Candida* infections, particularly those that are due to resistant strains, remains a major challenge for clinicians (Sanglard & Odds, 2002; White *et al.*, 2002). Much effort has gone into devising a treatment for these azole-resistant (AR) isolates, including: (i) searching for new antifungal agents (Andes *et al.*, 2006); (ii) developing new formulations (Pfaller *et al.*, 2005); and (iii) improving the efficacy of antifungal agents using combination therapy (Ghannoum *et al.*, 1995). A combination of voriconazole (VRC) and terbinafine (TRB) was found to be synergistic against *Candida albicans* isolates obtained from HIV-infected patients (Weig & Muller, 2001). It has been reported that there exists a synergy between micafungin (MCFG) and fluconazole (FLC), VRC, amphotericin B (AMB) and flucytosine (5-FC) in clinical isolates of *Candida* (Nishi *et al.*, 2009). The antiarrhythmic drug amiodarone has also been reported to act synergistically with FLC, itraconazole (ITR), and VRC against drug-resistant isolates of *C. albicans* (Guo *et al.*, 2008). In the clinical isolates of *Candida glabrata*, a combination of posaconazole (POS) and caspofungin (CSP) was found to be effective (Oliveira *et al.*, 2005).

Many herbal products such as allicin (ALC), berberine (BER), grapefruit seed extract, tea tree oil, xanthorrhizol (isolated from *Curcuma xanthorrhiza*), and retigeric acid B (RAB, isolated from the lichen *Lobaria kurokawae*) are known to have antifungal properties either alone or in combination with known antifungals. For example, ALC has inhibitory activity against *C. albicans*, *Aspergillus fumigatus*, *Scedosporium prolificans*, *Cryptococcus neoformans* and *Trichophyton* spp. (Guo *et al.*, 2009). In addition, ALC enhances the antifungal activity of AMB against *Saccharomyces cerevisiae*, *C. albicans* and *A. fumigatus* (Davis *et al.*, 2003). The combination of FLC and ALC exhibited good *in vivo* and *in vitro* synergistic antifungal effects, and FLC/ALC

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therapy was more efficacious than FLC monotherapy in clearing *Candida* from the kidneys (Guo *et al.*, 2009). The concomitant use of BER and FLC provided a synergistic action against AR *C. albicans* clinical strains *in vitro* (Quan *et al.*, 2006). A strong synergistic antiyeast activity of garlic oil and allyl alcohol is known against *Candida utilis* (Chung *et al.*, 2007). Recently, Sun *et al.* (2010) have reported a synergistic mechanism of RAB and azoles against *C. albicans*.

We have reported previously that the three pure curcuminoids isolated from natural curcumin – diferuloylmethane (CUR-I), demethoxycurcumin (CUR-II), and *bis*demethoxycurcumin (CUR-III) – exhibit antifungal activity (Sharma *et al.*, 2009a). An antifungal activity of curcumin is also reported against *Cryptococcus neoformans*, *Sporothrix schenckii*, *Paracoccidioides brasiliensis* and *Aspergillus* spp. (Martins *et al.*, 2009). Our present study demonstrates that pure CUR-I, which is the major component (~77%) of natural curcumin, can be exploited even more successfully in combination with azoles or polyenes. We evaluated the combined effects of pure CUR-I with FLC, ketoconazole (KTC), miconazole (MCZ), ITR, VRC, AMB and nystatin (NYS) against wild type (WT) and AR isolates of *C. albicans* by employing a chequerboard microdilution method. We show that the effect of pure CUR-I in combination with azoles and polyenes is synergistic; this was further validated using filter disc and time–kill assays.

Materials and methods

MCZ, KTC, ITR, NYS, AMB, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), pyrrolidinedithiocarbamate (PDTC), ascorbic acid, and other molecular grade chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO). An Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from BD Biosciences (Franklin Lakes, NJ). Zymolyase 100 T was purchased from Seikagaku Corporation (Tokyo, Japan).

Extraction and isolation of CUR-I

The rhizomes of *Curcuma longa* were obtained from the Central Institute of Medicinal and Aromatic Plants (CIMAP) field GeneBank (Accession no. CIMAP-1554). The rhizomes were dried at room temperature (25–35 °C), pulverized and stored at 10–15 °C until extraction. The powdered rhizomes (145 g) were first defatted with hexane 1000 mL in a soxhlet apparatus for 4 h and then extracted with chloroform 1000 mL for 5 h. The chloroform solution was filtered and evaporated under vacuum (50 °C) to produce a curcuminoid-rich extract (5.221 g, 3.6%). The crude mass (5.1 g) was charged on a silica gel column (180 g, 60–120 mesh, 5 × 70 cm glass) and eluted successively with hexane 800 mL, chloroform: hexane (20–80%, 1000 mL each), chloroform 2000 mL and acetone–chloroform (1–5%, 2000 mL each). The fractions were collected and spotted on thin-layer chromatography (TLC) aluminium sheets coated with silica gel. Fractions that showed the same pattern on TLC (200 mL each) were pooled [CUR-I (fractions 39–63), CUR-II (fractions 67–73), and CUR-III (fractions 78–82) were obtained successively] and the organic solvent was removed to obtain the powder form. The purity of CUR I, II, and III was analysed by high-pressure liquid chromatography (HPLC) and was found to be 98.4%, 98.1%, and 97.6%, respectively (data not shown). CUR I was the major component (~77%) of the curcuminoid mixture used in the present study. The purity of CUR-I was further confirmed by MALDI-TOF analysis (Supporting Information, Fig. S1).

Yeast strains and growth media

Clinical *Candida* isolates were obtained from the All India Institute of Medical Sciences (AIIMS), New Delhi, India. The yeast strains were cultured in yeast extract peptone dextrose (YEPD) broth (BIO101, Vista, CA). For agar plates, 2.5% (w/v) Bacto agar (Difco, BD Biosciences) was added to the medium. All strains were stored as frozen stocks with 15% glycerol at − 80 °C. Before each experiment, cells were revived on YEPD plates from the stock. The clinical azole-resistant isolates were obtained from Delhi hospitals. The A1–A3 isolates were collected from oropharyngeal lesions of HIV/AIDS patients, D1–D7 isolates were collected from vaginal swabs of diabetic women with suspected vulvovaginal candidiasis, B1–B5 isolates were from wounds of burn patients, and P1–P5 isolates were from pancreatitis patients (Table S1).

Determination of minimum inhibitory concentration (MIC)

MICs of FLC, MCZ, KTC, ITR, VRC, NYS, AMB and CUR-I against the *Candida* strains mentioned above were determined by broth microdilution using twofold serial dilutions in RPMI 1640 medium, as described by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) method M27-A. The test was carried out in 96-well flat-bottomed microdilution plates according to previous reports (Guo *et al.*, 2009; Rukayadi *et al.*, 2009).

After agitation for 15 s, the plates were incubated at 30 °C without shaking and readings were performed after 48 h of incubation by visual reading and OD determination with a spectrophotometer set at 492 nm. Experiments were performed in triplicate, and the average MIC value was calculated. The susceptibility end point was defined as the lowest concentration of antifungal which resulted in 80% inhibition of growth compared with that of the drug-free control. The reference strain SC5314 (ATCC MYA2876) was included as a positive control in each batch of susceptibility tests.

Chequerboard titre tests

The interaction of CUR-I with FLC/KTC/MCZ/ITR/VRC/NYS/AMB was evaluated by the chequerboard method recommended by the NCCLS and expressed as the sum of the fractional inhibitory concentration (FIC) index for each agent. The FIC of each agent is calculated as the MIC of this agent in combination divided by the MIC of this agent alone (Guo *et al.*, 2009; Rukayadi *et al.*, 2009). In brief, serial double dilutions of the anticandidal compounds were prepared (in $\mu g\,mL^{-1}$): 0.25–128 (FLC), 0.019–10 (KTC and ITR), 0.019–10 (MCZ and AMB), 0.078–2 (VRC), 0.029–15 (NYS) and 1.4–740 (CUR). After drug dilution, a 100-μL suspension of *Candida* strains adjusted to 5×10^5 CFU mL^{-1} was added to each well and cultured at 30 °C for 48 h in RPMI 1640 medium. Visual readings of the MIC were then performed and the $OD_{492\,nm}$ values were measured. The background OD was subtracted from the OD of each well. Each isolate was tested in triplicate on different days. Each chequerboard test generates many different combinations and by convention the FIC value of the most effective combination is used in calculating the fractional inhibitory concentration index (FICI). FICI was calculated by adding both FICs:

$$FICI = FICA + FICB = C_A^{comb}/MIC_A^{alone} + C_B^{comb}/MIC_B^{alone}$$

where MIC_A^{alone} and MIC_B^{alone} are the MICs of drugs A and B when acting alone and C_A^{comb} and C_B^{comb} are concentrations of drugs A and B at the isoeffective combinations, respectively. Off-scale MICs were converted to the next highest or next lowest doubling concentration. The FICI was interpreted as synergistic when it was ≤ 0.5, antagonistic when it was > 4.0, and any value in between was interpreted as indifferent (Odds, 2003; Guo *et al.*, 2009; Rukayadi *et al.*, 2009).

Filter disc assays

Filter disc assays were done as described earlier (Shukla *et al.*, 2003) in RPMI 1640 medium. Aliquots of 5–10 μL of azole/polyene either alone or in combination with CUR were spotted on 6 mm paper discs. After incubation of the plates for 48 h at 30 °C, the respective zones of inhibition were recorded.

Time–kill assays

Candida albicans cells at a concentration of 10^3 CFU mL^{-1} were inoculated in RPMI 1640 medium. The concentrations used are the MIC_{80} values used alone and in combination (see Results). At various predetermined time points (0, 4, 8, 12, 16, 20 and 24 h) at 30 °C incubation, agitation 200 r.p.m., a 100-μL aliquot was removed, serially diluted (10-fold) in 0.9% saline and spread on Sabouraud dextrose agar plates. Colony counts were determined after incubation at 30 °C for 48 h. The experiment was performed in triplicate. Synergism was defined as a decrease of $\geq 2\log_{10}$ CFU mL^{-1} in antifungal activity produced by the combination compared with the more active agent alone after 24 h (Quan *et al.*, 2006).

Measurement of ROS production

Endogenous amounts of ROS were measured by a fluorometric assay with DCFH-DA (Kobayashi *et al.*, 2002). Briefly, the cells were adjusted to an $OD_{660\,nm}$ of 1 in 10 mL of phosphate-buffered saline (PBS) and centrifuged at 2500 ***g*** for 15 min. The cell pellet was then resuspended in PBS and treated with appropriately diluted ascorbic acid for 1 h or left untreated at room temperature. After incubation with CUR at 37 °C for different time intervals, as indicated, 10 μM DCFH-DA in PBS was added. The fluorescence intensities (excitation and emission of 485 and 540 nm, respectively) of the resuspended cells were measured with a Spectrofluorometer (Varian, Cary Eclipse) and the images of DCF fluorescence were taken using a fluorescence microscope (Carl Zeiss).

Analysis of apoptotic markers

Protoplasts of *C. albicans* were stained with propidium iodide and FITC-labelled Annexin V using the Annexin V-FITC apoptosis detection kit (BD Biosciences) to assess cellular integrity and the externalization of phosphatidylserine as described earlier (Balzan *et al.*, 2004). The cells were analysed using a fluorescence-activated cell sorter (FACS) calibre flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA) using 488 nm excitation and a 515 nm band-pass filter for FITC detection and a filter > 560 nm for propidium iodide detection. A total of 10 000 events were counted at the flow rate. Data analysis was performed using CELLQUEST software (Becton-Dickinson Immunocytometry Systems) (Phillips *et al.*, 2003; Balzan *et al.*, 2004).

Results

Pure CUR-I is synergistic with azoles and polyenes

In vitro antifungal effects of pure CUR-I alone as well as in combination with azoles and polyenes, were tested against WT and clinical AR isolates of *C. albicans*. In WT strain, the MIC_{80} ($\mu g\,mL^{-1}$) of FLC, KTC, MCZ, ITR, VRC, AMB and NYS alone was 0.5, 0.5, 0.2, 0.062, 0.062, 1.25 and 3.75, respectively; in combination with CUR-I this was reduced to 0.0155 (32-fold reduction), 0.0078 (64-fold reduction), 0.0062 (32-fold reduction), 0.0078 (7.9-fold reduction), 0.0078 (7.9-fold reduction), 0.078 (16-fold reduction) and

0.234 (16-fold reduction), respectively (Table 1). We explored whether the observed synergistic effects of CUR-I in WT strain can be extended to the clinical AR isolates. For this, we checked the interactions of azoles/polyenes and CUR-I with the clinical AR *Candida* isolates. Table 2 summarizes the chequerboard assay of pure CUR-I, azoles and polyenes against clinical AR isolates. In clinical AR isolates, MIC_{80} ($\mu g\,mL^{-1}$) were 64–128 (FLC), 0.156–10 (KTC), 0.312–5 (MCZ), 0.312–10 (ITR), 0.25–2 (VRC), 0.156–0.625 (AMB) and 0.312–10 (NYS); in combination with CUR-I this was reduced to 4–32 (FLC), 0.039–0.625 (KTC), 0.039–0.312 (MCZ), 0.039–0.625 (ITR), 0.0312–0.25 (VRC), 0.019–0.078 (AMB) and 0.078–1.25 (NYS). As shown from the FICI values, pure CUR-I was synergistic with azoles and polyenes in all of the tested strains with the exception of the clinical isolates A2, D5, B2, P2, P5 and P6, where the FICI values were between > 0.5 and 4, which implied indifferent interactions between CUR-I and NYS. Thus, a 100% synergy was recorded in the case of CUR-I with tested azoles and AMB in WT and AR isolates. We did not find antagonistic interactions between CUR I and any of the drugs tested.

Filter-disc assay and time–kill curves confirm synergism

For validation of chequerboard assays in AR isolates, we selected one representative strain, A1. Filter-disc assay visualized the synergistic interaction of CUR-I and tested drugs. Figure 1 shows the filter discs for the WT strain SC5314 (Fig. 1a) and the representative strain A1 (Fig. 1b). As depicted in Fig. 1, at the concentrations indicated, pure CUR-I or tested drugs alone showed no antifungal activity (absence of zones of inhibition). However, pure CUR-I

Table 1. Checkerboard assay of FLC, KTC, MCZ, ITR, VRC, AMB, NYS and CUR against WT strain of *Candida albicans*

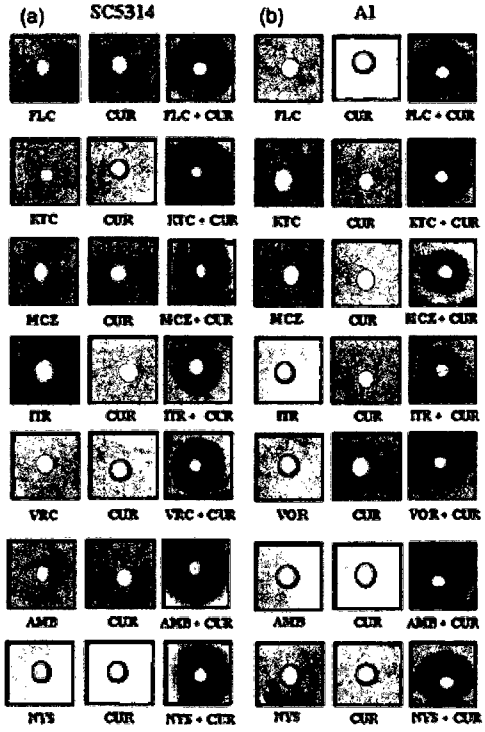
Antifungal agent	MIC_{80} of each agent ($\mu g\,mL^{-1}$)				
	Alone	Combination	FIC	FICI	Outcome
FLC	0.500	0.015	0.031		
CUR	185	11.5	0.062	0.093	SYN
KTC	0.500	0.007	0.015		
CUR	185	23.12	0.124	0.139	SYN
MCZ	0.200	0.006	0.031		
CUR	185	185	0.062	0.093	SYN
ITR	0.062	0.007	0.125		
CUR	185	23.12	0.124	0.244	SYN
VOR	0.062	0.007	0.125		
CUR	185	46.25	0.250	0.375	SYN
AMB	0.125	0.078	0.062		
CUR	185	23.12	0.124	0.187	SYN
NYS	3.750	0.234	0.062		
CUR	185	23.12	0.124	0.186	SYN

SYN, synergy.

Table 2. Interaction of azoles/polyenes and curcumin against 21 azole resistant clinical isolates of *Candida albicans* by checkerboard microdilution assay

Fluconazole and curcumin			Ketoconazole and curcumin			Miconazole and curcumin			Itraconazole and curcumin			Voriconazole and Curcumin			Amphotericin and Curcumin			Nystatin and Curcumin		
	MIC_{80} ($\mu g\,mL^{-1}$)			MIC_{80} ($\mu g\,mL^{-1}$)			MIC_{80} ($\mu g\,mL^{-1}$)			MIC_{80} ($\mu g\,mL^{-1}$)			MIC_{80} ($\mu g\,mL^{-1}$)			MIC_{80} ($\mu g\,mL^{-1}$)			MIC_{80} ($\mu g\,mL^{-1}$)	
Drug	Median	Range	Drug	Median	Range	Drug	Median	Range	Drug	Median	Range	Drug	Median	Range	Drug	Median	Range	Drug	Median	Range
FLC	64	64–128	KTC	2.5	0.312–10	MCZ	1.85	0.30–5.00	ITR	2.5	0.312–10	VOR	2	0.25–2	AMB	0.625	0.156–1.25	NYS	3.75	1.87–7.5
CUR	185	185–370	CUR	185	185–370	CUR	185	185–370	CUR	185	185–370	CUR	185	185–370	CUR	185	185–370	CUR	185	185–370
COM*	16	0.015–32	COM*	0.312	0.007–1.25	COM*	0.156	0.006–0.625	COM*	0.156	0.007–1.0	COM*	0.125	0.007–0.625	COM*	0.039	0.019–0.078	COM*	0.93	0.117–3.75
FICI	0.36	0.093–0.5	FICI	0.248	0.139–0.50	FICI	0.295	0.093–0.500	FICI	0.248	0.122–0.5	FICI	0.218	0.093–0.375	FICI	0.249	0.092–0.500	FICI	0.375	0.124–0.75

*COM, combination of curcumin with either azole or polyene.



(c)

Drug*	Strains SC5314	Strains AI
FLC + CUR	1.5	1.0
KTC + CUR	1.6	0.9
MCZ + CUR	1.3	0.6
ITR + CUR	1.0	0.5
VRC + CUR	1.2	0.8
AMB + CUR	1.3	0.9
NYS + CUR	1.4	1.0

*No zone of inhibition in drug alone

Fig. 1. Filter disc assay of CUR-I in combination with azoles/polyenes. (a) Synergistic interactions in WT strain SC5314, the various concentrations ($\mu g\,mL^{-1}$) spotted/impregnated were 0.0155 FLC/23.12 CUR-I, 0.0078 KTC/23.12 CUR-I, 0.0062 MCZ/11.5 CUR-I, 0.0078 ITR/23.12 CUR-I, 0.0078 VRC/46.25 CUR-I, 0.234 NYS/23.12 CUR-I, and 0.078 AMB/23.12 CUR-I. (b) The synergistic interactions in the clinical isolate A1. The various concentrations ($\mu g\,mL^{-1}$) spotted were 8 FLC/23.12 CUR-I, 0.312 KTC/23.12 CUR-I, 0.078 MCZ/92.5 CUR-I, 0.625 ITR/23.12 CUR-I, 0.25 VRC/92.5 CUR-I, 0.234 NYS/92.5 CUR-I, 0.019 AMB/46.25 CUR-I. (c) Summary of the diameter of zones of inhibition (in mm) in WT strain SC5314 and the AR isolate AI.

combined with tested drugs yielded significantly clear zones of inhibition; the diameters (mm) of various zones of inhibition are summarized in Fig. 1c. The observed synergism between pure CUR-I and tested drugs was further confirmed by time–kill curves. Figure 2 shows the time–kill curves of the WT strain and the representative clinical AR isolate AI. CUR-I and either of the tested drugs at the indicated concentrations alone did not significantly affect the growth curve; however, a combination of CUR-I with azoles or polyenes significantly affected the growth curve of the WT (Fig. 2a) as well as the representative clinical AR isolate A1 (Fig. 2b). Given the initial inoculum of $10^3\,CFU\,mL^{-1}$ for WT strain, combination therapy with CUR-I yielded a corresponding decline of 4 (FLC), 6.6 (KTC), 11 (MCZ), 6.3 (ITR), 10 (VRC), 7.8 (AMB) and 8.5 (NYS) $\log_{10}\,CFU\,mL^{-1}$ when compared to drugs alone at 24 h. Similarly, for the representative clinical AR isolate AI, the combination therapy resulted in a sharp decline in the observed $CFU\,mL^{-1}$ (Fig. 2b).

A combination of FLC/AMB and CUR-I raises ROS levels which could be reversed by an antioxidant

As depicted in Fig. 3a, ROS levels were measured in cells (SC5314) treated with either of the drugs alone: FLC ($0.0155\,\mu g\,mL^{-1}$), AMB ($0.078\,\mu g\,mL^{-1}$) and CUR-I ($23.12\,\mu g\,mL^{-1}$) or in combination, FLC ($0.0155\,\mu g\,mL^{-1}$)+CUR-I ($23.12\,\mu g\,mL^{-1}$) or AMB ($0.078\,\mu g\,mL^{-1}$)+CUR-I ($23.12\,\mu g\,mL^{-1}$). The treatment of FLC, AMB or CUR-I alone did not influence the concentration of endogenous ROS; however, FLC+CUR-I or AMB+CUR-I combination resulted in marked augmentation of endogenous ROS levels (16- and 26-fold in FLC+CUR-I and AMB+CUR-I, respectively). The increased ROS levels and the synergistic antifungal activity of FLC+CUR-I and AMB+CUR-I could be reversed in the presence of an antioxidant, as was evident from increased FICI values (Fig. 3b). Of note, for ROS and subsequent determinations, we used FLC and AMB as representative drugs from each category of azoles and polyenes.

Increased ROS leads to apoptosis

There was almost no apoptotic population in untreated cells (0%, lower right quadrant, panel I), CUR-I- (0.01%, lower right quadrant, panel II), FLC- (1.54%, lower right quadrant, panel III) or AMB-treated cells (0.14%, lower right quadrant, panel IV) cells (Fig. 4). However, there was a significant population of apoptotic cells when CUR-I was used in combination with FLC (17.6%, lower right quadrant, panel V) or AMB (18.46%, lower right quadrant, panel VI). The treatment of cells with ascorbic acid decreased the apoptotic population (2.05%, lower right quadrant, panel VII and 4.18%, lower right quadrant, panel VIII).

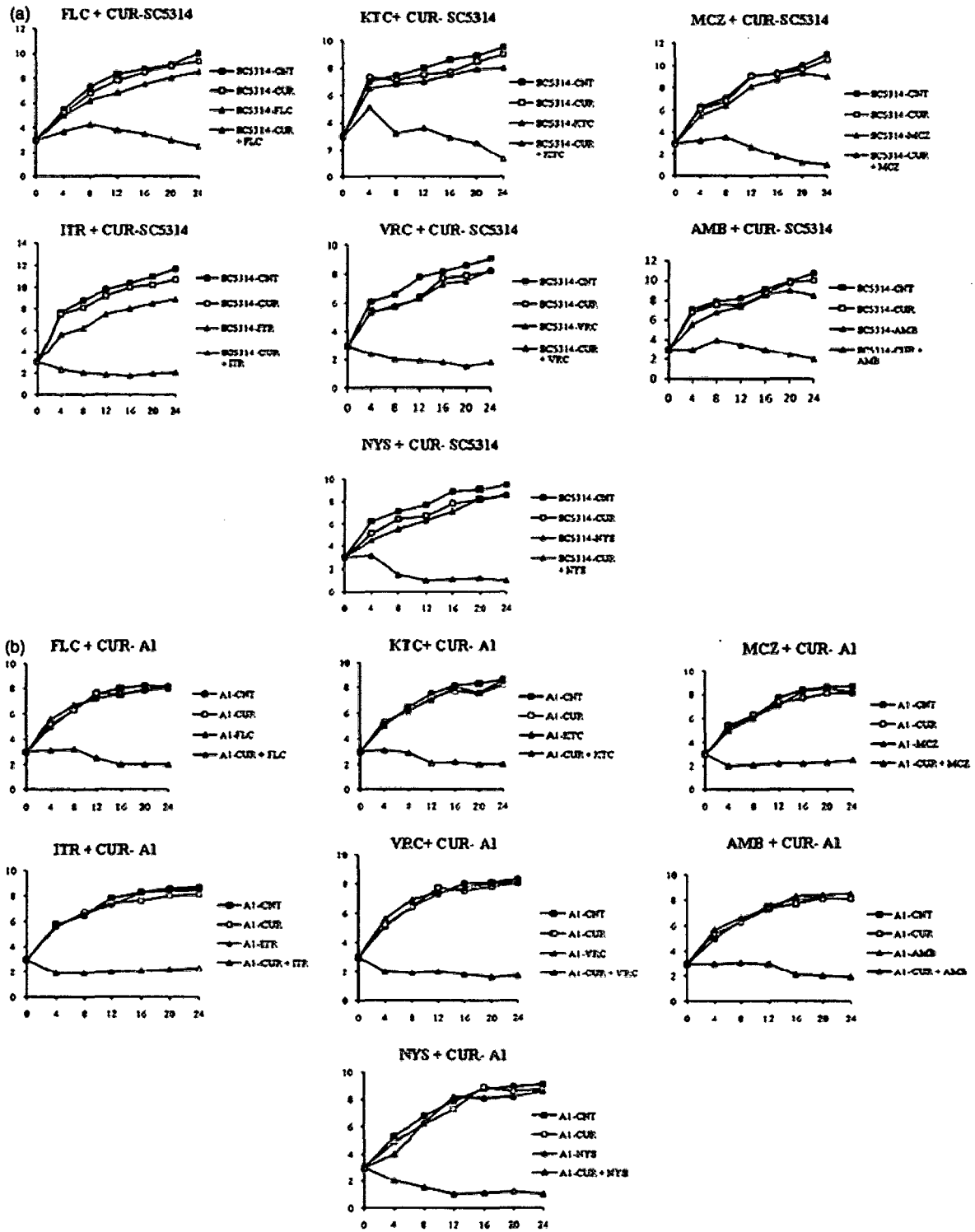
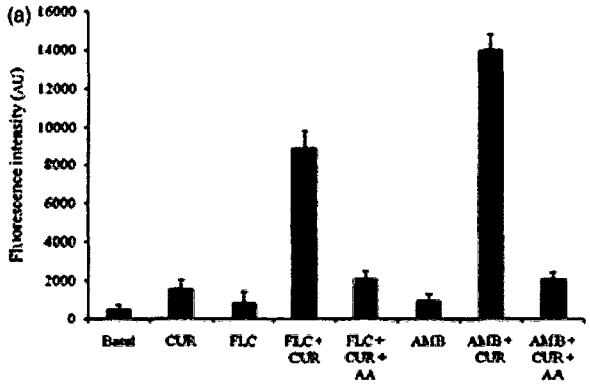


Fig. 2. Time–kill curves of CUR-I in combination with azoles/polyenes. (a) Time–kill curves of WT strain SC5314. The various concentrations (μg mL^{-1}) used were 0.0155, 0.0078, 0.0062, 0.0078, 0.0078, 0.234 and 0.078 of FLC, KTC, MCZ, ITR, VRC, NYS and AMB, respectively. The concentration (μg mL^{-1}) of CUR-I combined was 23.12, 23.12, 11.5, 23.12, 46.25, 23.12 and 23.12, respectively. (b) Time–kill curves of the clinical isolate A1. The concentrations (μg mL^{-1}) used were 8, 0.312, 0.078, 0.625, 0.25, 0.234 and 0.019 of FLC, KTC, MCZ, ITR, VRC, NYS and AMB, respectively. The concentration (μg mL^{-1}) of CUR-I combined was 23.12, 23.12, 92.5, 23.12, 92.5, 92.5 and 46.25, respectively. The *x*-axis is time in hours and the *y*-axis is log CFU mL^{-1}.



(b)

S. no.	Drug	FICI	FICI in presence of antioxidant
1.	FLC	0.093	0.374
2.	AMB	0.187	0.498

Fig. 3. ROS levels in the presence of CUR-I and azoles/polyenes. (a) Amounts of ROS produced due to CUR-I, FLC or AMB either alone or in combination with ascorbic acid. The concentrations used are 23.12 μg mL^{-1} of CUR-I, 0.0155 μg mL^{-1} of FLC, 0.078 μg mL^{-1} of AMB and 25 mM ascorbic acid. The fluorescence emitted by the cells was measured using a spectrofluorometer (Varian, Cary Eclipse). An excitation and an emission of 485 and 540 nm, respectively, were used. The values are the means and SDs (indicated by bars) of three independent experiments. (b) Summary of the FICI values of CUR-I+FLC and CUR-I+AMB in the presence of ascorbic acid (25 mM).

Discussion

In the present study, we demonstrate that pure polyphenol CUR-I interacts synergistically with antifungal drugs belonging to either azole or polyene categories. The synergism with CUR-I is independent of efflux pump proteins belonging to either ABC or MFS categories. For example, the synergistic effect of CUR-I was demonstrable in *CaCDR1*, *CaCDR2* and *CaMDR1* null mutants (data not shown). We also demonstrate that the synergism with CUR-I can be shown in AR clinical isolates with reduced antifungal sensitivity.

The synergistic effect of CUR-I was independent of the well-known calcineurin pathway. Calcineurin is a Ca^{2+}-calmodulin-activated phosphatase which is involved in the fungicidal synergism of FLC with cyclosporin A (CsA) and tacrolimus (FK506) (Cruz *et al.*, 2002; Uppuluri *et al.*, 2008). The synergism with the FLC/AMB and CUR-I combination remained unaffected in calcineurin nulls (Δ*cnb1*/*cnb1*) (data not shown).

We have shown previously that CUR-I (185 μg mL^{-1}) acts as an antifungal via generation of ROS and triggers an early apoptosis in *C. albicans* cells (Sharma *et al.*, 2009a). Notably, a combination of CUR-I with azoles (FLC) or polyenes (AMB) generated ROS levels several fold higher (16- and 26-folds, respectively) at a much lower concentration. Thus, if used alone, FLC, AMB and CUR-I showed no antifungal activity or increased ROS levels. Interestingly, reversal of ROS levels using ascorbic acid also reversed the synergism,

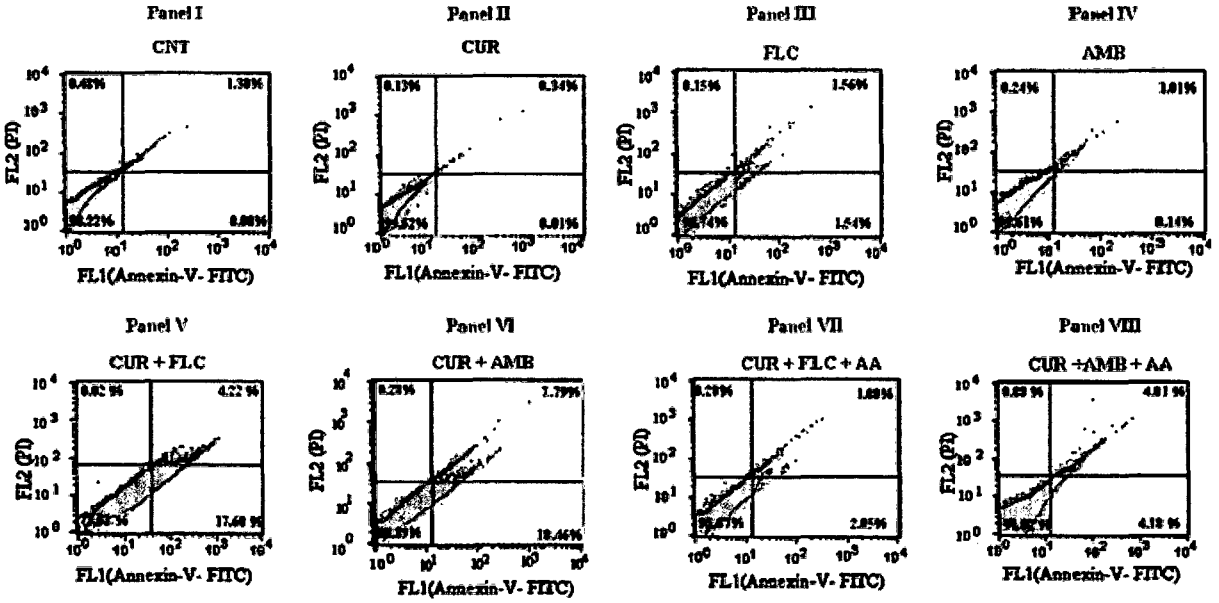


Fig. 4. Externalization of phosphatidylserine in the presence of CUR-I and azoles/polyenes. Panel I represents untreated *Candida albicans* (SC5314) cells, panels II, III and IV show CUR-I, FLC and AMB-treated cells, respectively. Panels V and VI are CUR-I+FLC and CUR-I+AMB-treated cells, respectively. Panels VII and VIII are ascorbic acid-pretreated cells, further treated with CUR-I+FLC and CUR-I+AMB, respectively. After treatment for 4 h, the cells were analysed by flow cytometry as described in Materials and methods.

as was evident from increased FICI values. The synergism of CUR-I with drugs also led to an increase in apoptotic cell population, which again could be reversed by the addition of an antioxidant (Fig. 3).

Earlier, we observed that CUR modulated efflux mediated by ABC multidrug transporters of *C. albicans* heterologously expressed in *S. cerevisiae* and was selectively synergistic with drug substrates such as rhodamine 6G, KTC, ITR and MCZ, but not with FLC or VRC (Sharma *et al.*, 2009b). However, in the present study, we demonstrated synergism with FLC and VRC as well. This difference in the pattern of interaction of CUR with FLC/VRC in *S. cerevisiae* strain AD-CDR1 and *C. albicans* strain SC5314 may not be strictly comparable, as the two strains represent different systems. In this study, we observed differences in drug interactions with CUR-I within *Candida* strains. For example, NYS was not synergistic with CUR-I in six of the clinical AR isolates tested but there was good synergism between CUR-I and NYS with all other isolates. *In vitro* assays like the chequerboard method are preliminary steps to understanding drug–drug interactions. However, detailed *in vivo* pharmacokinetic and pharmacodynamic studies are warranted to really comment upon these interactions.

We have demonstrated that the effect of CUR-I if given in combination with azoles and polyenes is synergistic; this synergy is related to the generation of ROS and apoptosis. Considering the fact that MDR in *Candida* is associated with multiple signalling pathways, inhibition of signalling cascades with combination treatment of nontoxic natural products and antifungals represents a logical preventive/therapeutic approach for combating *Candida* infections.

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Statement

Transparency declarations: none to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Determination of purity of curcuminoids.
Table S1. Description of the strains used in the study.

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