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Genetic prerequisites for additive or synergistic actions of 5-fluorocytosine and fluconazole in baker's yeast

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During applications of 5-fluorocytosine (5FC) and fluconazole (FLC), additive or synergistic action may even occur when primary resistance to 5FC is established. Here, we analysed conjoint drug action in *Saccharomyces cerevisiae* strains deficient in genes known to be essential for 5FC or FLC function. Despite clear primary resistance, residual 5FC activity and additive 5FC + FLC action in cells lacking cytosine permease (Fcy2p) or uracil phosphoribosyl transferase (Fur1p) were detected. In contrast, Δ*fcy1* mutants, lacking cytosine deaminase, became entirely resistant to 5FC, concomitantly losing 5FC + FLC additivity. Disruption of the orotate phosphoribosyltransferase gene (*URA5*) in the wild-type led to low-level 5FC tolerance, while an alternative orotate phosphoribosyltransferase, encoded by *URA10*, contributed to 5FC toxicity only in the Δ*ura5* background. Remarkably, combination of Δ*ura5* and Δ*fur1* resulted in complete 5FC resistance. Thus, yeast orotate phosphoribosyltransferases are involved in 5FC metabolism. Similarly, disruption of the ergosterol $\Delta^{5,6}$-desaturase-encoding gene *ERG3* resulted only in partial resistance to FLC, and concomitantly a synergistic effect with 5FC became evident. Full resistance to FLC occurred in Δ*erg3* Δ*erg11* double mutants and, simultaneously, synergism or even an additive effect with FLC and 5FC was no longer discernible. Since the majority of spontaneously occurring resistant yeast clones displayed residual sensitivity to either 5FC or FLC and those strains responded to combined drug treatment in a predictable manner, careful resistance profiling based on the findings reported here may help to address yeast infections by combined application of antimycotic compounds.

INTRODUCTION

The fluorinated pyrimidine 5-fluorocytosine (5FC) is one of the most widely used antimycotic agents, capable of disrupting both DNA and protein synthesis in fungal cells (Ghannoum & Rice, 1999). Fluconazole (FLC) on the other hand, exhibits a completely different mode of action, targeting Erg11, essential for the production of ergosterol in fungi (Kalb *et al.*, 1987; Vanden Bossche, 1985). These two agents are routinely administered in combination to combat a broad spectrum of fungal infections (Mukherjee *et al.*, 2005; Johnson *et al.*, 2004). The desired effect of such combination therapy is an additive or synergistic increase in antifungal activity when compared to singly applied compounds. In a previous study, *Saccharomyces cerevisiae* was used as a model organism to identify putative permeases which play a role in 5FC toxicity. In *S. cerevisiae*, 5FC uptake is brought about mainly by the cytosine permease Fcy2; however, in its absence, several other permeases ensure residual 5FC influx (Paluszynski *et al.*, 2006). However, the impact of disruptions in the 5FC metabolic pathway on synergy when combined with FLC has yet to be studied. Interestingly, in *Cryptococcus neoformans*, synergism was observed in cases in which primary resistance to one of the agents occurred (Schwarz *et al.*, 2003, 2006, 2007), but the reasons remained unknown. Depending on the agent and yeast species, however, adverse (antagonistic) effects of a combination therapy are also documented (Te Dorsthorst *et al.*, 2002). The outcome of a combined antimycotic treatment apparently varies within genera, species and even isolates, and is thus hardly predictable. Phylogenetic analyses of pathogenic yeast species indicated that *S. cerevisiae* is closely related to the major opportunistic fungal pathogen *Candida albicans* (Barns *et al.*, 1991; Hendricks *et al.*, 1989; Lupetti *et al.*, 2002). Thus, despite the above constraints, *S. cerevisiae* may serve as a valuable model to study antifungal drug action (Agarwal *et al.*, 2003).

5FC is a prodrug, which to exert its action requires uptake and metabolism to either 5-fluorouridine triphosphate (5FUTP, formed from 5FUDP; see Fig. 1) or 5-fluorodeoxyuridine monophosphate (5FdUMP), the former directly

Abbreviations: 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; FIC, fractional inhibitory concentration; FLC, fluconazole.

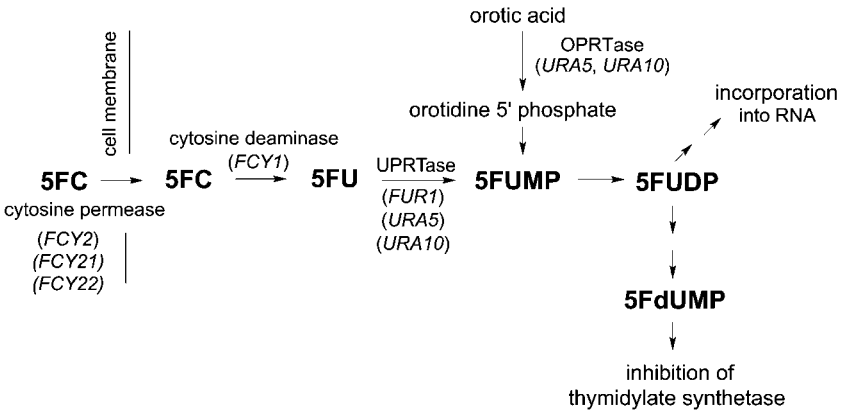

Fig. 1. Metabolism of 5-fluorocytosine in fungi. Enzyme and substrate names are abbreviated as follows: OPRTase, orotate phosphoribosyltransferase; 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; UPRTase, uridine phophoribosyltransferase; 5FUMP, 5-fluorouridine monophosphate; 5-FUDP, 5-fluorouridine diphosphate; 5-FdUMP, fluorodeoxyuridine monophosphate.

disturbing transcription and the latter – via inhibition of thymidylate synthetase and subsequent dTTP depletion – aborting DNA synthesis (Polak & Scholer, 1975; Hartmann & Heidelberger, 1961; Whelan, 1987; Wadler *et al.*, 1998). Essential steps in intracellular 5FC metabolism are the conversion to 5-fluorouracil (5FU) by the cytosine deaminase Fcy1 and subsequent processing to 5-fluorouridine monophosphate by the uracil phosphoribosyltransferase Fur1 (Chevallier *et al.*, 1975; Vanden Bossche *et al.*, 1994, 1987; Kern *et al.*, 1990; Kurtz *et al.*, 1999).

In *S. cerevisiae*, as for *C. albicans* and *Candida glabrata*, mutations in any of the corresponding genes involved in prodrug uptake and metabolism result in 5FC tolerance (Fasoli *et al.*, 1990), explaining the known rapid establishment of spontaneous resistance (Alexander & Perfect, 1997; Vanden Bossche *et al.*, 1987; Paluszynski *et al.*, 2006).

FLC and several other azole antimycotics interfere with the biosynthesis of ergosterol, a fungal-specific sterol that is important for membrane integrity (Smith *et al.*, 1996, Bammert & Fostel, 2000). The specific target of antimycotic azoles is the lanosterol 14α-demethylase, encoded by the *ERG11* gene (Kalb *et al.*, 1987; Vanden Bossche, 1985). By binding the iron atom of the haem moiety, activation of oxygen, necessary for demethylation of lanosterol, is prevented (Joseph-Horne & Hollomon, 1997), eventually resulting in the accumulation of toxic 14α-methylergosta-8,24(28)-dien-3β,6α-diol (14α-methyl-3,6-diol), which impairs membrane function (Kelly *et al.*, 1995).

Mechanisms of azole resistance in *S. cerevisiae* and *C. albicans* include overproduction or alteration of Erg11p, or modification of downstream enzymes (Bard *et al.*, 1993; Vanden Bossche *et al.*, 1992; Hitchcock, 1991). The loss of function of the sterol $\Delta^{5,6}$-desaturase encoded by *ERG3* results in accumulation of episterol, which permits fungal growth in the presence of azole drugs (Arthington *et al.*, 1991; Watson *et al.*, 1988; Kelly *et al.*, 1995). However, disruption of the aforementioned *ERG11* gene was shown to be lethal in *S. cerevisiae*, as accumulation of 14α-methyl-3,6-diols facilitates growth only under anaerobic conditions and in ergosterol-supplemented media (Watson *et al.*, 1989; Bard *et al.*, 1993). This growth arrest can be circumvented by the inactivation of *ERG3*, which results in the accumulation of methylfecosterol instead of 14α-methyl-3,6-diol. In contrast, *C. albicans ERG11* null mutants are capable of survival in the absence of a suppressor mutation in *ERG3*, albeit with a severe growth defect (Sanglard *et al.*, 2003). Hence, *C. albicans* either produces different levels of the diol and/or is less sensitive to its lethal effects as compared to *S. cerevisiae* (Watson *et al.*, 1989; Bard *et al.*, 1993).

To determine the prerequisites underlying the synergistic or additive increase in antifungal action of 5FC/FLC combinations, we set up a *S. cerevisiae* model system consisting of an isogenic strain collection with combinations of defined deletions in genes important for 5FC and/or FLC toxicity. Sensitivity profiling revealed residual drug responses and additive action of the two drugs in the majority of strains despite the establishment of primary resistance. The genetic basis of such residual activity was investigated and identified as being essential for increased drug action in combined applications of 5FC and FLC.

METHODS

Strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in Table 1. Cultivation was performed in complete YPD (1 %, w/v, Bacto-yeast extract, 2 %, w/v, Bacto-peptone, 2.2 %, w/v, glucose) or YNB (Yeast Nitrogen Base, Difco) media supplemented with (L-leucine 10 μg ml^{-1}, L-methionine 10 μg ml^{-1}, uracil 200 μg ml^{-1}, uridine 100 μg ml^{-1}) when required. Media were prepared according to Kaiser *et al.* (1994). Strains were cultivated at 30 °C in YPD liquid or on agar medium. *Escherichia coli* strains were grown in LB (1 %, w/v, peptone, 0.5 %, w/v, yeast extract, 0.5 %, w/v, NaCl, pH 7.3) at 37 °C (Sambrook *et al.*, 1989) with a working concentration of ampicillin of 100 μg ml^{-1}.

DNA techniques. Chromosomal yeast DNA was isolated from 50 ml YPD cultures essentially as described by Kaiser *et al.* (1994). Restriction and ligation of DNA was carried out according to the suppliers' recommendations (NEB and MBI-Fermentas). For Southern analyses (Southern, 1975) DNA fragments were labelled by applying the DIG-DNA-labelling and detection kit from Roche Biochemicals.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source
S. cerevisiae		
BY4741	*MATα his3 leu2 met15 ura3*	EUROSCARF
CG379	*MATα ade5-1 leu2-2 trp1-289 ura3-52 his7-2*	Bard *et al.* (1993)
BY Δ*fcy1*	As for BY4741, additionally *fcy1*	EUROSCARF
BY Δ*fcy2*	As for BY4741, additionally *fcy2*	EUROSCARF
BY Δ*ura5*	As for BY4741, additionally *ura5*::*SpHIS5*	This work
BY Δ*ura10*	As for BY4741, additionally *ura10*::*KlLEU2*	This work
BY Δ*ura5* Δ*ura10*	As for BY474,1 additionally *ura5*::*SpHIS5 ura10*::*KlLEU2*	This work
BY Δ*fur1*	As for BY4741, additionally *fur1*::*KlURA3*	This work
BY Δ*fur1* Δ*ura5*	As for BY4741, additionally *fur1*::*KlURA3 ura5*::*SpHIS5*	This work
BY Δ*erg3*	As for BY4741, additionally *erg3*::*KlLEU2*	This work
BY Δ*erg3* Δ*erg11*	As for CG379, additionally *erg3*::*KlLEU2 erg11*::*KlURA3*	Bard *et al.* (1993)
BY Δ*fcy1* Δ*erg3*	As for BY4741, additionally *fcy1 erg3*::*KlLEU2*	This work
BY Δ*fcy2* Δ*erg3*	As for BY4741, additionally *fcy2 erg3*::*KlLEU2*	This work
BY Δ*fur1* Δ*erg3*	As for BY4741, additionally *fur1*::*KlURA3 erg3*::*KlLEU2*	This work
E. coli		
JM109	F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)M15/Δ(*lac-proAB*) *glnV thi*	Yanisch-Perron *et al.* (1985)
Plasmids		
pSK−	ColE1 *ori*, Amp^R *lacZ*	Stratagene
pUG72 (AF298788)	*loxP-KlURA3-loxP*, Amp^R	EUROSCARF
pUG73 (AF298792)	*loxP-KlLEU2-loxP*, Amp^R	EUROSCARF

Transformation and gene disruptions. *E. coli* JM109 was transformed by the $CaCl_2$ method as described by Sambrook *et al.* (1989). Transformants of *S. cerevisiae* were obtained according to Gietz & Schiestl (1995), and selected on YNB agar.

The *ERG3* gene was disrupted using the *KlLEU2* marker gene cloned in pUG73. *ERG3* was amplified and blunt-end inserted into the *Hin*cII site of the pSK plasmid vector (see Table 2 for primers). Subsequently, the *KlLEU2* gene from pUG73 was excised (*Hin*cII and *Pvu*II) and inserted into the internal *ERG3 Hin*cII site. The disruption cassette *erg3*::*LEU2* was introduced into *S. cerevisiae* BY4741, *S. cerevisiae* Δ*fcy1* and *S. cerevisiae* Δ*fcy2* strains (Table 1). Mutants obtained from EUROSCARF (Frankfurt, Germany), Δ*fcy1* and Δ*fcy2*, were verified using primer pairs Fcy1F/R and Fcy2F/R, respectively. Homologous recombination with the *ERG3* disruption cassette was checked by PCR, in a Mini-Cycler (MJ Research Biozym) and Southern analysis.

The disruption of *URA5*, *URA10* and *FUR1* was carried out essentially as described previously (Wach *et al.*, 1994; Gueldener *et al.*, 2002). For *URA5* disruption, primers (URA5koF and URA5koR) flanking the *SpHIS5* marker gene from pUG27 and an additional 45 bp homologous to *URA5* were used. *URA10* was disrupted using the *KlLEU2* marker gene from pUG73, with primers URA10koF and URA10koR. *FUR1* was disrupted using Fur1koF and Fur1koR primers flanking the *KlURA3* marker gene. The *URA5*::*SpHIS5* knockout cassette was transformed into *S. cerevisiae* Δ*fur1* and Δ*ura10*, resulting in Δ*ura5* Δ*fur1* and Δ*ura5* Δ*ura10* double mutant strains. Disruption of *URA5* was PCR verified, employing primer pairs URA5outR/HIS5up and URA5outF/HIS5down, respectively. For verification of *URA10* and *FUR1* knockouts, primer pairs LEU2down/URA10outF, LEU2up/URA10outR and URA3down/Fur1outF, URA3up/Fur1outR were applied.

***In vitro* drug susceptibility tests.** Stock solutions of 5FC and FLC obtained from MP Bio-medicals, Frankfurt, Germany, were established with a final concentration of 5 mg ml^{-1}, and after sterile-filtration (cellulose acetate membrane, pore size 0.2 μm; Millipore), stored at −20 °C. Prior to use drugs were added to YPD agar at approximately 50 °C to establish appropriate concentrations. For testing resistance rapidly, cultures were diluted 10^{-1} to 10^4 and 8 μl of each dilution was spotted onto the agar plates. For proper quantification, 200 μl volumes of liquid YPD medium containing 5FC, FLC, and both in combination, were inoculated with 1×10^6 yeast cells and incubated at 30 °C in U-profile 96-well microtitre plates (Carl Roth) for 24 h; growth was monitored spectroscopically at 600 nm as previously described (Paluszynski *et al.*, 2006).

Where applicable, the minimal inhibitory concentration (MIC) for 5FC and FLC was read as the lowest drug concentration that gave 50 % or more growth inhibition, which is defined as MIC-2 as described by the National Committee for Clinical Laboratory Standards (NCCLS, 1998). The fractional inhibitory concentration (FIC) was calculated to quantify drug interaction (Elfopouios & Moellering, 1991), being defined as synergistic if the FIC was $\leqslant 0.5$, additive if FIC was >0.5 and $\leqslant 1$, indifferent if $1 <$FIC $\leqslant 4$, and antagonistic if FIC was >4.

The FIC index was determined as follows: FIC=[(MIC of drug A, in combination)/(MIC of drug A, tested alone)]+[(MIC of drug B, in combination)/(MIC of drug B, tested alone)].

Measurement of growth of Δ*erg3* and Δ*erg3* Δ*erg11* mutants. To measure the growth of Δ*erg3* and Δ*erg3* Δ*erg11* mutant strains, in comparison to the wild-type, growth curves were established by inoculating 1 % of overnight pre-cultures in 50 ml YPD medium. Cultivation was performed in the presence and absence of FLC at 30 °C, with the final concentration of FLC being 1 mg ml^{-1}. Growth was monitored spectroscopically at 600 nm until strains reached the stationary phase.

RESULTS AND DISCUSSION

Conjoint actions of 5FC and FLC in defined *S. cerevisiae* mutants

For systematic analysis of conjoint antifungal activities of 5FC and FLC in resistant yeast strains, we assembled a set

Table 2. Oligonucleotides used for PCR along with target sequences and positions according to the *Saccharomyces* genome database (http://www.yeastgenome.org/)

Primer	Sequence	Length (bp)	Target sequence (locus and nucleotide positions)
LEU2up	GGCGTATAGACCCAATTCC	19	*LEU2* nt 1041–1023
LEU2down	GGAATTGGGTCTATACGCC	19	*LEU2* nt 1041–1023
Erg3F	ATGGATTTGGTCTTAGAAGTCGC	23	*ERG3* Chrom. XII nt 5001–5023
Erg3R	TCAGTTGTTCTTCTTGGTATTTGGG	25	*ERG3* Chrom. XII nt 6074–6098
Erg3out5	GGTTGCAGAGGAGGTCAG	18	*ERG3* Chrom. XII nt 4928–4945
Erg3out3	GAGACAAACAAGGCAACCG	19	*ERG3* Chrom. XII nt 7002–7020
Fcy1F	GGGTATGGACATTGCCTATG	20	*FCY1* Chrom. XVI nt 677198–677217
Fcy1R	CCAATCCTGAGGTCTTTCATCG	22	*FCY1* Chrom. XVI nt 677595–677616
Fcy2F	CGTTGAATCATCAGAGGCCAC	21	*FCY2* Chrom. V nt 266655–266675
Fcy2R	GGTGCCCACAAAGCTTGAGCG	21	*FCY2* Chrom. V nt 267663–267683
URA3up	GACGCTGGCGTACTGGC	17	*URA3* nt 1067–1051
URA3down	GCCAGTACGCCAGCGTC	17	*URA3* nt 1067–1051
HIS5up	GACGCTGGCGTACTGGC	17	*HIS5* nt 1089–1073
HIS5down	GCCAGTACGCCAGCGTC	17	*HIS5* nt 1089–1073
Fur1koF	GAACGTCTACTTGCTACCTCAAACAAACCAATTGCTGGGTTTGT-AC/CAGCTGAAGCTTCGTACGC	65	*FUR1*/*URA3* nt 362139–362185/13–31
Fur1koR	GTATCTGTCACCAAAGTCACCCAACCCTGGAACTAGATACTTG-G/CATAGGCCACTAGTGGATCTG	65	*FUR1*/*URA3* nt 362714–362757/1604–1583
Fur1outF	GGCCGGTTTTTCTATAAGC	19	*FUR1* Chrom. VIII nt 361837–361819
Fur1outR	GGCTAGAGGACAGTACCCG	19	*FUR1* Chrom. VIII nt 363096–363077
URA5koF	CGAATGCCAGGCTCTAAGATTTGGTTCATTCAAGTTGAAATCA-G/CAGCTGAAGCTTCGTACGC	63	*URA5/HIS5* Chrom. XIII nt 56773–57453/1089–1073
URA5koR	CTGTTATTCTGCCTTCCAAATAGGTAATTATGTGAATTAAGGAG-AC/GCATAGGCCACTAGTGGATCTG	68	*URA5/HIS5* Chrom. XIII nt 57406–57342/1089–1073
URA5outF	GTAACTGCCCTACTTTCC	18	*URA5* Chrom. XIII nt 56272–56289
URA5outR	GGCAAGAGGTACCGAAAC	18	*URA5* Chrom. XIII nt 57491–57509
URA10koF	TGAACTGGGATTAGAATGCAAAGCACTAAGATTTGGGTCATTC-AAG/CAGCTGAAGCTTCGTACGC	65	*URA10*/*LEU2* Chrom. XIII nt 807595–807640/1051–1067
URA10koR	CATATACCATAGGCCTTACGGTAGTTTTCAATCGCTGATTTTTG-C/GCATAGGCCACTAGTGGATCTG	67	*URA10/LEU2* Chrom. XIII nt 808230–808185/1051–1067
URA10outF	GCTAAATTGCCTTCTGAG	18	*URA10* Chrom. XIII nt 807164–807182
URA10outR	GGCAAGAGGTACCGAAAC	17	*URA10* Chrom. XIII nt 808840–808824

of isogenic derivatives of *S. cerevisiae* BY4741 carrying mutations expected to provoke 5FC or FLC resistance. 5FC resistance was achieved by disruption of *FCY1*, *FCY2* and *FUR1*, encoding cytosine deaminase, purine-cytosine permease and uracil phosphoribosyltransferase, respectively (see also Fig. 1); FLC resistance was achieved by disruption of *ERG3*, encoding the C-5 sterol desaturase.

Resistance levels of respective mutants of the BY4741 background were initially monitored using a drop dilution plate assay employing various concentrations of 5FC and FLC. As to be expected, Δ*fcy1*, Δ*fcy2* and Δ*fur1* mutations clearly conferred 5FC resistance (Fig. 2a), whereas FLC resistance was solely observed in the Δ*erg3* strain (Fig. 2b). There was no cross-resistance to FLC in Δ*fcy1*, Δ*fcy2* or Δ*fur1* strains and vice versa; as for the wild-type 5FC sensitivity was seen in the Δ*erg3* mutant.

Growth capabilities in the presence of a broad concentration range of both 5FC and FLC, singly and in combination, were scored with the microtitre plate assay, which revealed differential effects of individual gene disruptions on the efficiency of combined drug treatments (Fig. 3). Neither in wild-type nor in any of the mutant strains were antagonistic effects of combined 5FC/FLC application observed. Rather, clear additive interactions of both drugs were detected in the wild-type (FIC=0.74) and the Δ*fcy2* mutant (FIC=0.576) or synergism in the case of the Δ*erg3* mutant (FIC=0.20), despite the fact that the latter two exhibit resistance to 5FC or FLC (Figs 3 and 4a). Such an increase in efficiency for 5FC+FLC was also seen in the Δ*fur1* mutant (FIC=0.63) (Fig. 3d). Significantly, there is a remarkable difference in the residual responses to the singly applied antimycotics that correlates with additive or synergistic interactions with the second drug. Despite the response being clearly distinct in strains deficient in either *FCY2* or *FUR1*, both show clear growth inhibition by 5FC, dose-dependent in Δ*fcy2* and dose-independent in the Δ*fur1* mutant (Fig. 3). Similarly, there is an apparent FLC-dependent growth retardation in the Δ*erg3* strain, which is, however, not aggravated with increasing FLC concentrations

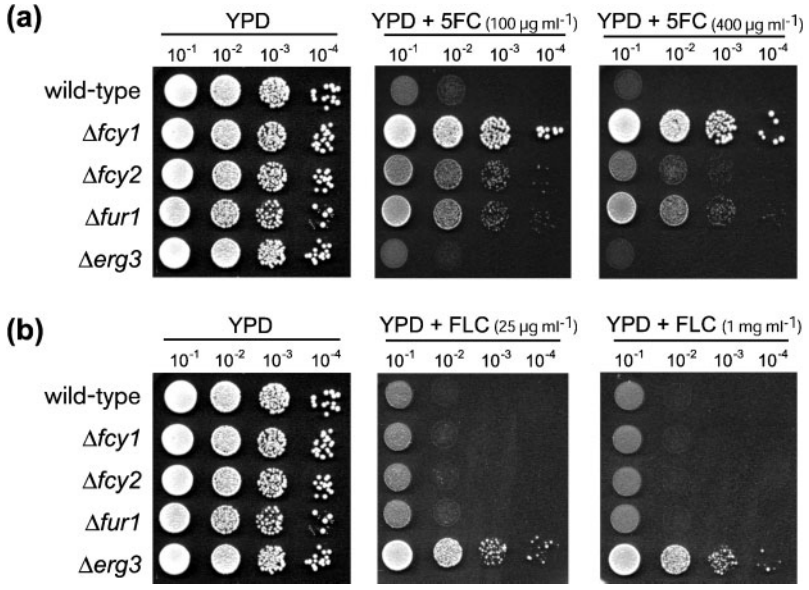

Fig. 2. Drop dilution assay for evaluating the sensitivity of various deletion mutants in *S. cerevisiae*: comparison of Δ*fcy1*, Δ*fcy2*, Δ*fur1* and Δ*erg3* mutant strains to the wild-type BY4741 when treated with (a) 5FC and (b) FLC. Each row represents a serial dilution from 10^{-1} to 10^{-4} from left to right; 8 μl of each dilution was spotted onto each series of YPD plates. The concentrations of 5FC and FLC are given above each panel. Without addition of antimycotics (YPD) all samples displayed the same growth capabilities and very similar cell numbers. Resistant phenotypes can be directly monitored by growth.

(Fig. 4a). By comparing growth of wild-type and Δ*erg3* cells, a general growth-affecting defect in Δ*erg3* became obvious; however with FLC, growth was additionally retarded (Fig. 4b). Thus, FLC inhibits growth even in cells carrying the FLC resistance mutation Δ*erg3*.

Mutation of *ERG3*, a late ergosterol biosynthesis gene, results in altered membrane sterol composition, with ergosterol being replaced by episterol (Arthington *et al.*, 1991). The Erg3 FLC-toxicity-promoting reaction is the generation of a toxic diol from 14α-methylfecosterol, the latter being formed from lanosterol by inhibition of the 14-α demethylase (Erg11) by FLC (Bard *et al.*, 1993; Watson *et al.*, 1989). Thus, Δ*erg3* mutants tolerate FLC, as no toxic diols are synthesized, but concomitantly exhibit an altered membrane sterol composition probably accounting for the observed slow growth of these mutants (see also Fig. 4).

Although the main target of FLC, Erg11, is normally essential, it is dispensable when Erg3 is additionally knocked out, as this abrogates formation of toxic diols (Bard *et al.*, 1993; Watson *et al.*, 1989). Δ*erg3* Δ*erg11* cells displayed full FLC resistance, but again, the strong FLC-independent growth defect became obvious (Fig. 4a, c). Interestingly, growth of the double mutant (Δ*erg3* Δ*erg11*), irrespective of the presence or absence of FLC, is comparable to that of the Δ*erg3* strain with FLC. In each of the latter, the ergosterol biosynthesis pathway yields 14α-methylfecosterol, evidently causing a more pronounced growth impairment than episterol but less than 14α-methyl-3,6-diol formed in wild-type cells when exposed to FLC (Fig. 4d). Importantly, synergistic actions of FLC and 5FC, as seen in Δ*erg3* cells, are cancelled by the additional Δ*erg11* mutation, indicating the requirement of at least residual FLC-mediated growth retardation for synergistic or additive drug action.

As for the Δ*erg3* Δ*erg11* mutant, the additive response was abolished when *FCY1* was lacking, with both displaying an FIC of 1 and, at least consistent with residual sensitivity being crucial for additive action, Δ*fcy1* mutants displayed no response to 5FC regardless of the concentration applied (Fig. 3b). Thus, synergistic or additive antifungal action of 5FC/FLC treatment is seen in Δ*erg3*, Δ*fcy2* and Δ*fur1* strains, whereas such an effect is absent in Δ*erg3* Δ*erg11* double and Δ*fcy1* single mutants, which do not respond to either FLC or 5FC, respectively.

5FC response in *fur1* mutants

We have recently shown that toxicity of 5FC in Δ*fcy2* mutants is due to the presence of several other permeases capable of low-level 5FC transport in *S. cerevisiae* (Paluszynski *et al.*, 2006). Growth inhibition of Δ*fur1* mutants by 5FC must, however, occur in a different manner, since the Fur1-catalysed conversion of 5FU to 5FUMP is crucial for downstream effects of 5FC (Fig. 1). Unlike mammals, *S. cerevisiae* lacks alternative enzymes capable of 5FU metabolism, such as thymidine or uridine phosphorylase (Jund & Lacroute, 1970). According to the *Candida* genome database (http://www.candidagenome.org/) and *Cryptococcus neoformans* genome project (http://www.tigr.org/tdb/e2k1/cna1/), there is also no evidence for the presence of thymidine or uridine phosphorylases in these genera. In mammals, 5FU can also be metabolized by orotate phosphoribosyltransferase, an enzyme involved in *de novo* pyrimidine biosynthesis (Peters *et al.*, 1984); the engagement of the respective yeast enzymes in 5FC antimycotic activity has, however, yet to be investigated. *S. cerevisiae* possesses two homologous orotate phosphoribosyltransferases, Ura5 and Ura10 (Fig. 5a), which are only distantly related to the functional analogues of mammals (de Montigny *et al.*, 1990). Hence, the involvement of such yeast orotate phosphoribosyltransferases in 5FU metabolism was checked by disrupting

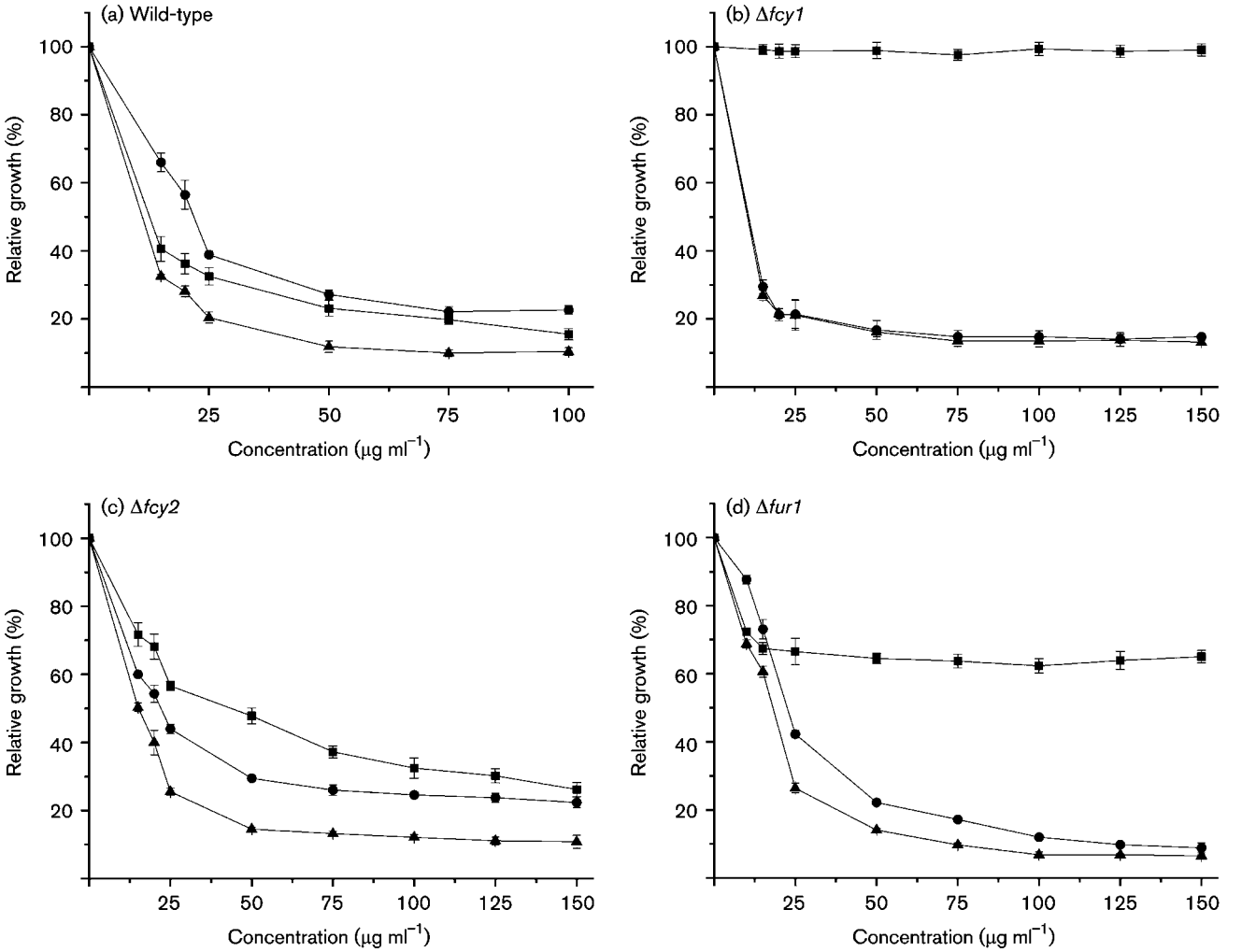

Fig. 3. Microtitre plate assay to monitor the response of yeast strains carrying single mutations in the cytosine metabolic pathway to 5FC (■), FLC (●) and 5FC+FLC (▲). (a) *S. cerevisiae* wild-type BY4741, (b) Δ*fcy1*, (c) Δ*fcy2*, (d) Δ*fur1*. Growth was measured as OD_{600} and is expressed as a percentage relative to the control. Values given represent the mean of at least three experiments, each carried out in triplicate; error bars represent SD (not plotted where smaller than symbols). There is a clear additive effect for 5FC and FLC for the wild-type (a), for Δ*fcy2* (c) and for Δ*fur1* (d), whereas Δ*fcy1* (b) behaves differently.

URA5 and *URA10* singly and in combination. Effects on 5FC tolerance were subsequently recorded by the microtitre plate assay (Fig. 5b). Indeed, the Δ*ura5* single mutant displayed moderate resistance at low 5FC concentrations (Fig. 5b). However, Δ*ura10* alone did not affect 5FC tolerance significantly, but slightly contributed to resistance in the Δ*ura5* background (not shown). Most remarkably, however, full dose-independent 5FC resistance was established when *URA5* was disrupted in the Δ*fur1* strain (Fig. 5b). Thus, both Ura5 and Ura10 are capable of 5FU metabolism and probably mediate residual 5FC toxicity in the absence of Fur1. As homologues of *S. cerevisiae* Ura5/10 have been identified in a variety of ascomycetous yeast species, including *Candida albicans* and *C. glabrata*, and also in the basidiomycetous yeast *Cryptococcus neoformans* (Fig. 5a), it appears probable that a requirement for uracil phosphoribosyltransferase in 5FC prodrug activation and antifungal activity can generally be bypassed to some extent by orotate phosphoribosyltransferases. Among three loci known to be involved in 5FC uptake and activation (*FCY1*, *FCY2* and *FUR1*) in *S. cerevisiae*, only one proved to be essential (*FCY1*). It has previously been shown that *S. cerevisiae* Δ*fcy1* mutants entirely lack cytosine deaminase activity, and Δ*fcy1* Δ*ura3* or Δ*ura2* double mutants, which are additionally defective in *de novo* pyrimidine synthesis, are unable to grow with exogenously supplied cytosine (Jund & Lacroute, 1970; Erbs *et al.*, 1997), clearly excluding an alternative enzyme for bypassing the Fcy1 deamination of cytosine or 5FC. In contrast, Fcy2 and Fur1 reactions can be catalysed by structurally or functionally related proteins, thereby explaining successful application of 5FC in instances where (partial) resistance was established by targeted gene disruption in the model system, or by spontaneous mutation in resistant clinical isolates.

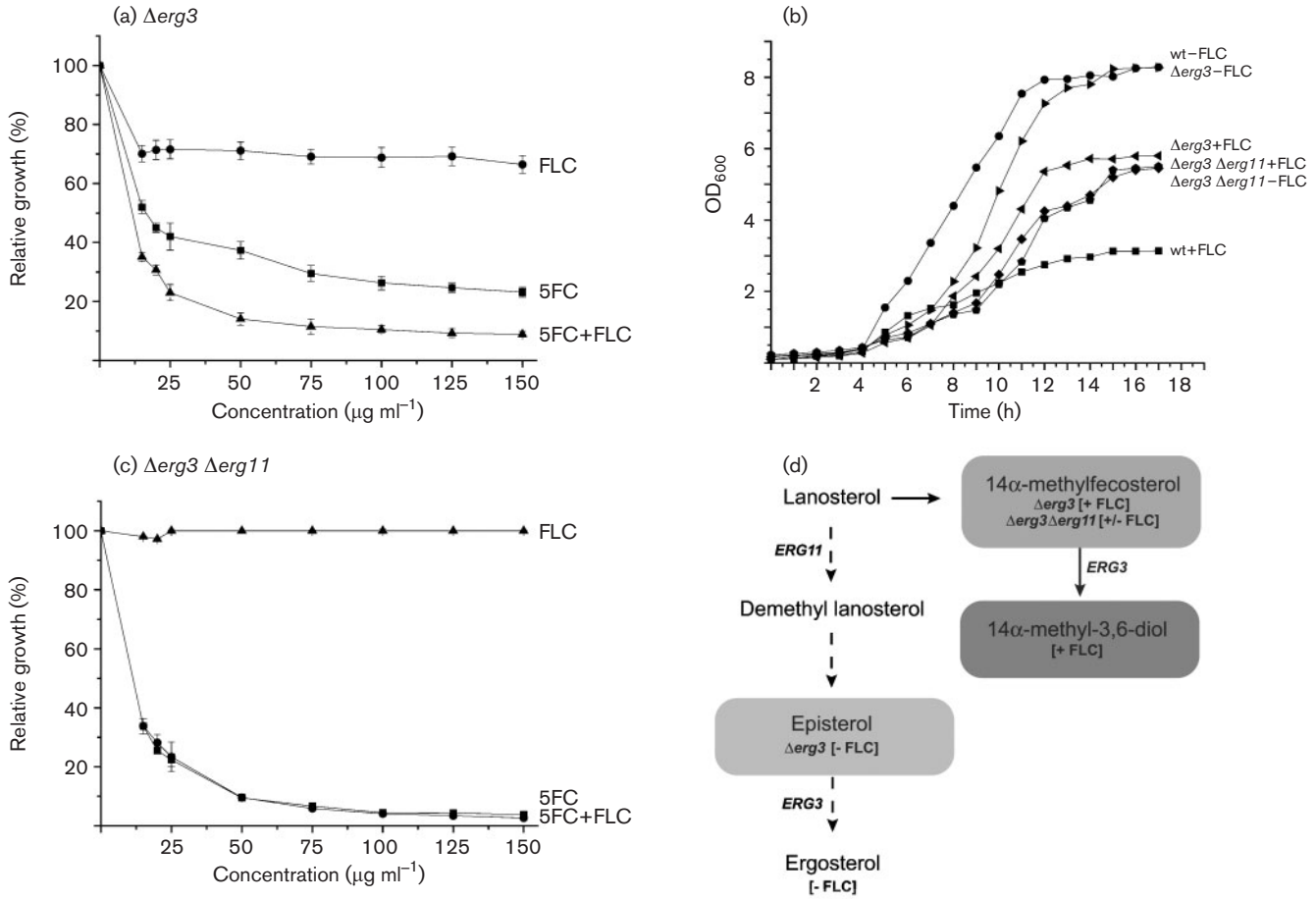

Fig. 4. Assessment of ergosterol mutant strains for FLC resistance using the microtitre plate test and evaluation of growth and synergism in combination with 5FC. (a) Response of Δ*erg3* to FLC (●), 5FC (■) and FLC+5FC (▲). (b) Growth of Δ*erg3*, and a Δ*erg3* Δ*erg11* double mutant, in comparison to wild-type in the presence and absence of FLC. (c) Response of an Δ*erg3* Δ*erg11* double mutant to FLC (▲), 5FC (■) and FLC+5FC (●). (d) Overview of the sterol composition in Δ*erg3* and Δ*erg3* Δ*erg11* mutant strains either treated or not treated with FLC. Error bars represent SD (not plotted where smaller than symbols).

Importantly, clinical isolates of *C. neofomans* displaying resistance to 5FC but increased susceptibility to 5FC plus amphotericin B (compared to amphotericin B alone) were shown by complementation to be defective in cytosine permease. It was speculated that addition of amphotericin B, which directly induces membrane damage (de Kruijff *et al.*, 1974; de Kruijff & Demel, 1974) might restore 5FC penetration, thus explaining synergistic antifungal activity in a cytosine-permease-defective strain (Schwarz *et al.*, 2007). However, synergism was also observed in 5FC-resistant isolates when this agent was combined with FLC, which does not directly affect membrane integrity (Allendoerfer *et al.*, 1991). As we detected additive action of 5FC/FLC not only in *fcy2* mutants of *S. cerevisiae* but also in a *fur1* strain, which is fully capable of 5FC import, we suggest that aided 5FC penetration by membrane-damaging antimycotics may increase synergism in 5FC-resistant strains, but is probably not generally required.

Effects of 5FC and FLC on spontaneously occurring resistant clones

To elucidate whether residual antimycotic response in spontaneous mutants is indeed detectable and functionally linked with susceptibility to combined 5FC/FLC application, naturally occurring drug-resistant mutants in *S. cerevisiae* were screened. Among 73 clones obtained in a screening for 5FC tolerance, 39 turned out to be stable. Almost all of the latter displayed additive effects when treated jointly with both drugs; only three of them eventually turned out to be fully resistant, and interestingly, for these isolates, the 5FC/FLC combination had the same effect as the singly applied 5FC (Table 3). All strains obtained in a screening for FLC tolerance (n=36) still displayed residual drug sensitivity. Remarkably, combined application of both antimycotic compounds increased their biological activities in either case, supporting the notion

(a)

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S.c. URA5        ----------MPIMLEDYQKNFLELAIECQALRFGSFKLKSGRE 34
S.c. URA10          ----------MSASTTSLEEYQKTFLELGLECKALRFGSFKLNSGRQ 37
C.g. XP_447993   ----------MP--LEDYQKNFLELAIESQALKFGSFTLKSGRQ 32
C.a. XP_718318   MLPFFSSHVEQHCYKHKQTTMASYKSSFLQLALDSQALKFGKFTLKSGRE 50
C.n. XP_572079   ----------MSSQALDSAKIAFIEAAIEHGVLLFGNFTLKSGRQ 35
                                                **.*.*:***:

S.c. URA5        SPYFFNLGLFNTGKLLSNLATAYAEAIIQSDL-KFDVIFGPAYKGIPLAA 83
S.c. URA10       SPYFFNLSLFNSGKLLANLATAYATAIIQSEL-KFDVIFGPAYKGIPLAA 86
C.g. XP_447993   SPYFFNLGLFNTGKLLSNLATAYAEAIIQSDL-KFDIIFGPAYKGIPLAA 81
C.a. XP_718318   SPYFFNLGLFNTGKLLSNLATSYAEAIIASGL-KFDILFGPAYKGIPLAA 99
C.n. XP_572079   SPYFFNAGLLYSSSLLSTTAQAYAKVLSSSRIPDFDVLFGPAYKGISLAA 85
                 ******. *: :..**:. * :** .: * : .**::********.***

S.c. URA5        IVCVKLAEIGGSKFQNIQYAFNRKEAKDHGEGGIIVGSALENKRILIIDD 133
S.c. URA10       IVCVKLAEIGGTKFQGIQYAFNRKKVKDHGEGGIIVGASLEDKRVLIIDD 136
C.g. XP_447993   IVCVKLAEIGGSKFQNVQYAFNRKEAKDHGEGGNIVGAALEGKKLIIIDD 131
C.a. XP_718318   ITVAKLAELDPINYGDIGYSFNRKEKKDHGEGGSIVGCSLKDKKILIIDD 149
C.n. XP_572079   VSAVSLYQQTG---KDIGYCYNRKEKKDHGEGGTMVGAPLKG-RIVIIDD 131
                 :  ..* :       .: *.:***: ******* :**..*:. :::****

S.c. URA5        VMTAGTAINEAFEIISNA-KGQVVGSIIALDRQEVVSTDD-KEGLSATQT 181
S.c. URA10       VMTAGTAINEAFEIISIA-QGRVVGCIVALDRQEVIHESD-PERTSATQS 184
C.g. XP_447993   VMTAGTAINEAFEIIAAN-QGQVVGSIIALDRQEVVGSPDSTDSMSATQA 180
C.a. XP_718318   VITAGTAINEAFEIIGNE-KGQVVGCIIALDRQETTATDP---TKSATQA 195
C.n. XP_572079   VLTSGKAIREAIDILKASPEAKLVGIVQLVDRQEKGQSGS---GKSTVQE 178
                 *:*:*.**.**::*:    :.::** :  :****          *:.*

S.c. URA5        VSKKYGIPVLSIVSLIHIITYLEG-RITAEEKSKIEQYLQTYGASA- 226
S.c. URA10       VSKRYNVPVLSIVSLTQVVQFMGN-RLSPEQKSAIENYRKAYGI--- 227
C.g. XP_447993   VSKRYNIPVLSIVSLSHVINFLDG-RITAEEKQKIEDYLQKYGA--- 223
C.a. XP_718318   VSERYQIPVLSIVNLGEVISILNG-KINDEDLKSIEQYRSKYGA--- 238
C.n. XP_572079   VEEEFGVPVEPIIGLDDIVKYLESSGKWEKELQEVRKYRAEYGVQRS 225
                 *.:.: :** .*:.* .::  : .    :: . :..*   **
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(b)

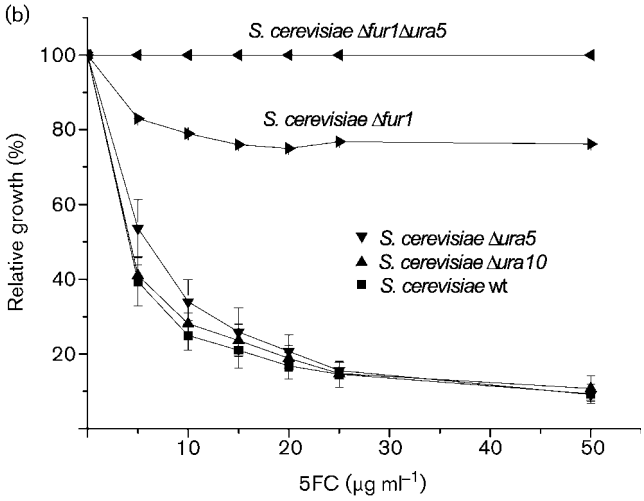

Fig. 5. (a) Amino acid alignment of orotate phosphoribosyl transferases (OPRTs), comparing *S. cerevisiae* Ura5 and Ura10 sequences with *Candida glabrata* (XP_447993), *Candida albicans* (XP_718318) and *Cryptococcus neoformans* (XP_572079); only one ORPT exists in the latter three species. All OPRT accession numbers refer to those deposited in the NCBI database. (b) Growth of Δ*ura5*, Δ*ura10* and Δ*fur1* single mutants, and a Δ*ura5* Δ*fur1* double mutant, with 5FC. 5FC concentrations ranging from 0 to 50 μg ml^{-1} were tested and relative growth was measured as for Fig. 3. Error bars represent SD (not plotted where smaller than symbols).

that residual drug response is required and sufficient for additive antifungal activity of the 5FC/FLC combination (Table 3). It is noteworthy that occurrence of full resistance to either drug was an exception rather than the rule in our experiments. In fact we have experienced it only for the three strains being fully resistant to 5FC. In all other instances susceptibility to combined drug treatment was prevalent.

Table 3. Screening for spontaneous mutants to 5FC and FLC resistance

A cell suspension of (*S. cerevisiae* BY4741) was plated out on YPD plates containing 5FC (100 μg ml^{-1}) or FLC (100 μg ml^{-1}) and incubated at 30 °C for approximately 5 days. Colony-forming units capable of growing in the presence of each agent were cultivated further for 1 day under the same conditions. Those that showed stable resistance were additionally tested by the micotitre plate assay at concentrations ranging from 10 to 100 μg ml^{-1} of each agent. Those strains classified as partially resistant displayed a relative growth of at least 25 % above the wild-type strain at concentrations up to 25 μg ml^{-1}, while those observed as resistant showed no response at all to the concentrations of 5FC and FLC applied.

Antimycotic compound	No. obtained	No. fully resistant/ additivity detected	No. partially resistant/ additivity detected
5FC	73	3/0	36/36
FLC	36	0/0	36/36

Since full FLC resistance requires a simultaneous loss of function of Erg3 and Erg11, whereas 5FC resistance is brought about by a mutation in a single gene (*FCY1*) our screening data fit with the drug sensitivity profiles for defined mutants (see above), as functional disturbances of 5FC uptake and metabolism can be bypassed by alternative permeases (Paluszynski *et al.*, 2006) or phosphoribosyltransferases (Fig. 5b), respectively. The finding that primary resistance does not generally abolish the effectiveness of 5FC, in particular when combined with FLC, may support the use of this agent in clinical applications despite the known rapid establishment of spontaneous resistance. Clearly, however, when 5FC resistance is due to loss of function of the cytosine deaminase, 5FC application is not appropriate.

Testing combined drug efficiency in defined mutants with primary resistance to 5FC and FLC

Double mutants carrying mutations conferring resistance to both agents were generated to check whether partial resistance to either 5FC or FLC still allows efficient combined drug action (Fig. 6). Indeed, combination of the Δ*erg3* mutation with either Δ*fcy2* or Δ*fur1* resulted in strains displaying robust resistance to both 5FC and FLC. However, as for the respective single mutants, residual response to both 5FC and FLC was observed in the Δ*erg3* Δ*fcy2* (FIC=0.57) and the Δ*erg3* Δ*fur1* strain, where a distinct FIC could not be determined (Fig. 6a, b). Combined application of both drugs led to increased antifungal activity; thus even a primary resistance against two antifungal agents does not a priori exclude additive

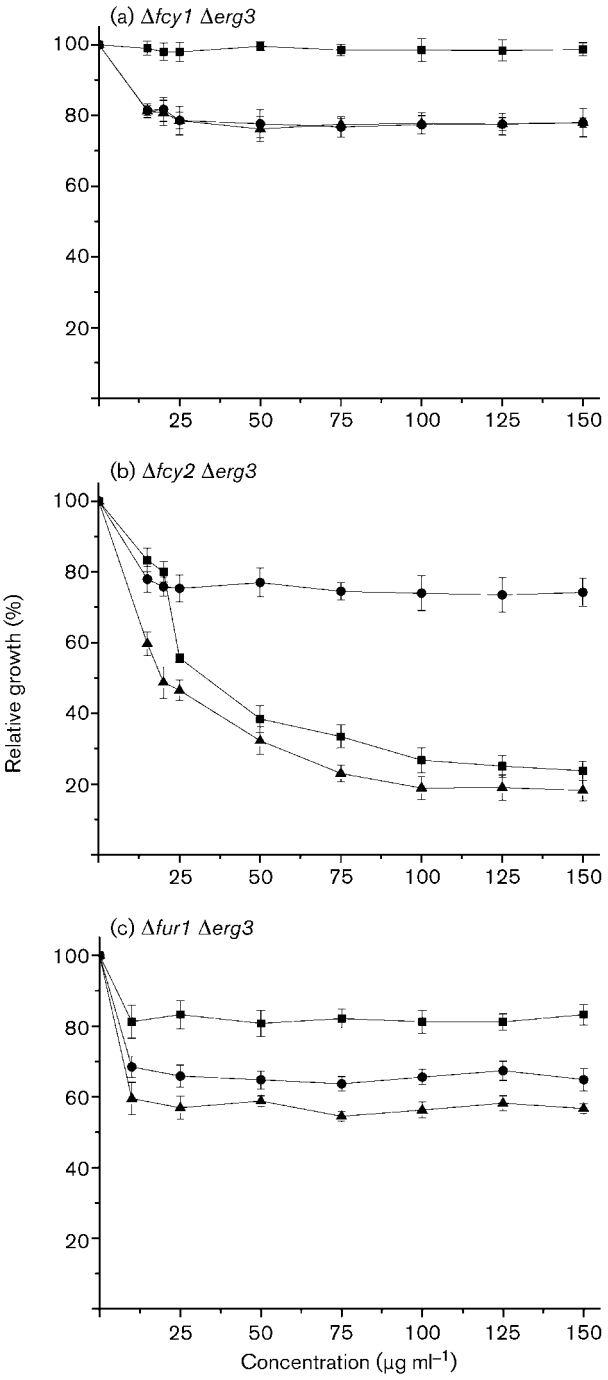

Fig. 6. Characterization of double mutant strains, carrying defects in both 5FC and ergosterol metabolic pathways, for 5FC/FLC additivity. (a) Δ*fcy1* Δ*erg3*, (b) Δ*fcy2* Δ*erg3*, and (c) Δ*fur1* Δ*erg3* treated with 5FC (■), FLC (●) and 5FC+FLC (▲). Microtitre plate tests were carried out and relative growth was calculated as for Fig. 3. Error bars represent SD (not plotted where smaller than symbols).

effects in combinational treatments. Combining full 5FC (Δ*fcy1*) with partial FLC (Δ*erg3*) resistance, however, completely abolished additivity, supporting the conclusion that at least a faint residual response to both of the drugs is required (Fig. 6a).

Conclusions

The majority of mutations leading to tolerance to 5FC and FLC affect genes that confer only partial resistance against those compounds. Only in rather rare cases is full resistance established and combined drug treatment not effective; in all other instances antifungal treatment by 5FC/FLC in combination is feasible. Careful examination of drug responses in clinical isolates is thus necessary, as resistance against one or even both of the agents does not a priori exclude an efficient therapy by combined application of 5FC and FLC.

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