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, Δfcy1 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 Δura5 background. Remarkably, combination of Δura5 and Δfur1 resulted in complete 5FC resistance. Thus, yeast orotate phosphoribosyltransferases are involved in 5FC metabolism. Similarly, disruption of the ergosterol Δ5,6-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 Δerg3 Δerg11 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 antifungal 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., 2003). The outcome of a combined antifungal 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
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 antifungics 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 antifungics 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 Δ24-desaturase encoded by ERG3 results in accumulation of episterol, which permits fungal growth in the presence ofazole 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, Bactopeptone, 2.2%, w/v, glucose) or YNB (Yeast Nitrogen Base, Difco) media supplemented with (1-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.
Transformation and gene disruptions. *E. coli* JM109 was transformed by the CaCl₂ 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 *KLEU2* marker gene cloned in pUG73. *ERG3* was amplified and blunt-end inserted into the *HincII* site of the pSK plasmid vector (see Table 2 for primers). Subsequently, the *KLEU2* gene from pUG73 was excised (*HincII* and *PvuII*) and inserted into the internal *ERG3* *HincII* site. The disruption cassette *erg3::LEU2* was introduced into *S. cerevisiae* BY4741, *S. cerevisiae* Δ*erg1* and *S. cerevisiae* Δ*erg2* 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 Bioczym) 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 pUG72 and an additional 45 bp homologous to *URA5* were used. URA10 was disrupted using the *KLEU2* marker gene from pUG73, with primers URA10koF and URA10koR. *FUR1* was disrupted using *Fur1koF* and *Fur1koR* primers flanking the *KIURA3* marker gene. The *UAR5::SpHIS* knockout cassette was transformed into *S. cerevisiae* Δ*fcy1* and Δ*ura10*, resulting in Δ*ura5 Δfur1* and Δ*ura5 Δura10* double mutant strains. Disruption of *URA10* was PCR verified, employing primer pairs *Ura5outR*/*His5up* and *URA10outF*/*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, Franklin, 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 10⁴ 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 × 10⁶ yeast cells and incubated at 30 °C in U-profile 96-well microtitre plates (Carl Roth) for 24 h; growth was monitored spectrophotometrically 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 (Efopoulos & Moellerling, 1991), being defined as synergistic if the FIC was <0.5, additive if FIC was >0.5 and ≤1, indifferent if 1 < FIC ≤4, and antagonistic if FIC >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 *Aerg3* and *Aerg3 Δerg11* mutants. To measure the growth of *Aerg3* and *Aerg3 Δerg11* 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 spectrophotometrically 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 of

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td><strong>BY4741</strong></td>
<td>MATa his3 leu2 met15 ura3</td>
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<tr>
<td><strong>CG379</strong></td>
<td>MATa ade1-1 leu2-2 trp1-289 ura3-52 his7-2</td>
<td>Bard et al. (1993)</td>
</tr>
<tr>
<td><strong>BY Δfcy1</strong></td>
<td>As for BY4741, additionally fcy1</td>
<td>EUROSCARF</td>
</tr>
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<td><strong>BY Δfcy2</strong></td>
<td>As for BY4741, additionally fcy2</td>
<td>EUROSCARF</td>
</tr>
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</tr>
<tr>
<td><strong>BY Aura10</strong></td>
<td>As for BY4741, additionally ura10::KLEU2</td>
<td>This work</td>
</tr>
<tr>
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<td>This work</td>
</tr>
<tr>
<td><strong>BY AΔfur1 Δura5</strong></td>
<td>As for BY4741, additionally fur1::KIURA3 ura5::SpHIS5</td>
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<tr>
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<tr>
<td><strong>E. coli</strong></td>
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</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td>Stratagene</td>
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<tr>
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<td>EUROSCARF</td>
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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 *Δerg3* 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 *Δerg3* 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 0.20), despite the fact that the latter two exhibit resistance to 5FC or FLC (Figs 3 and 4a). Such an increase in efficiency for 5FC of 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 *ERG3*, both show clear growth inhibition by 5FC.

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

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<th>Primer</th>
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<th>Length (bp)</th>
<th>Target sequence (locus and nucleotide positions)</th>
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et al. biosynthesis pathway yields 14-α-Demethylase (Erg11) by FLC (Bard et al., 1991). Thus, FLC inhibits growth even in cells carrying the FLC resistance mutation Δerg3.

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 14-α-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, Δerg3 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). Δerg3 Δerg11 cells displayed full FLC resistance, but again, the strong FLC-independent growth defect became obvious (Fig. 4a, c). Interestingly, growth of the double mutant (Δerg3 Δerg11), irrespective of the presence or absence of FLC, is comparable to that of the Δerg3 strain with FLC. In each of the latter, the ergosterol biosynthesis pathway yields 14-α-methylfecosterol, evidently causing a more pronounced growth impairment than episterol but less than 14-α-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 Δerg3 cells, are cancelled by the additional Δerg11 mutation, indicating the requirement of at least residual FLC-mediated growth retardation for synergistic or additive drug action.

As for the Δerg3 Δerg11 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, Δfcy1 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 Δerg3, Δfcy2 and Δfur1 strains, whereas such an effect is absent in Δerg3 Δerg11 double and Δfcy1 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 Δfcy2 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 Δfur1 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 (Jun & 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 antymycotic activity has, however, yet to be investigated. *S. cerevisiae* possesses two homologous orotate phosphoribosyltransferases, Ura5 and Ura10 (Fig. 5a), which are engaged in pyrimidine biosynthesis (Peters et al., 1984); the engagement of the respective yeast enzymes in 5FC antymycotic 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...
URA5 and URA10 singly and in combination. Effects on 5FC tolerance were subsequently recorded by the microtitre plate assay (Fig. 5b). Indeed, the Δura5 single mutant displayed moderate resistance at low 5FC concentrations (Fig. 5b). However, Δura10 alone did not affect 5FC tolerance significantly, but slightly contributed to resistance in the Δura5 background (not shown). Most remarkably, however, full dose-independent 5FC resistance was established when URA5 was disrupted in the Δfur1 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 Δfcy1 mutants entirely lack cytosine deaminase activity, and Δfcy1 Δura3 or Δura2 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. 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) Δfcy1, (c) Δfcy2, (d) Δfur1. Growth was measured as OD600 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 Δfcy2 (c) and for Δfur1 (d), whereas Δfcy1 (b) behaves differently.
Importantly, clinical isolates of *C. neoformans* 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 antifungal agents 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 antifungal 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 antifungal compounds increased their biological activities in either case, supporting the notion
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.

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 Δerg3 mutation with either Δfcy2 or Δfur1 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 Δerg3 Δfcy2 strain (FIC=0.57) and the Δerg3 Δfur1 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

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 microtitre 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.

<table>
<thead>
<tr>
<th>Antimycotic compound</th>
<th>No. obtained</th>
<th>No. fully resistant/ additivity detected</th>
<th>No. partially resistant/ additivity detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>5FC</td>
<td>73</td>
<td>3/0</td>
<td>36/36</td>
</tr>
<tr>
<td>FLC</td>
<td>36</td>
<td>0/0</td>
<td>36/36</td>
</tr>
</tbody>
</table>

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_781838) and Cryptococcus neoformans (XP_572079); only one ORPT exists in the latter three species. All ORPT accession numbers refer to those deposited in the NCBI database. (b) Growth of Δura5, Δura10 and Δfur1 single mutants, and a Δura5 Δfur1 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).
effects in combinational treatments. Combining full 5FC (Δfcy1) with partial FLC (Δerg3) 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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REFERENCES


Fig. 6. Characterization of double mutant strains, carrying defects in both 5FC and ergosterol metabolic pathways, for 5FC/FLC additivity. (a) Δfcy1 Δerg3, (b) Δfcy2 Δerg3, and (c) Δfur1 Δerg3 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).


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