Mechanisms of antifungal resistance

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Summary

The many drugs that are available at present to treat fungal infections can be divided into four broad groups on the basis of their mechanism of action. These antifungal agents either inhibit macromolecule synthesis (flucytosine), impair membrane barrier function (polyenes), inhibit ergosterol synthesis (allylamines, thiocarbamates, azole derivatives, morpholines), or interact with microtubules (griseofulvin).

Drug resistance has been identified as the major cause of treatment failure among patients treated with flucytosine. A lesion in the UMP-pyrophosphorylase is the most frequent clinical determinant of resistance to 5FC in Candida albicans. Despite extensive use of polyene antibiotics for more than 30 years, emergence of acquired resistance seems not to be a significant clinical problem. Polyene-resistant Candida isolates have a marked decrease in their ergosterol content. Acquired resistance to allylamines has not been reported from human pathogens, but, resistant phenotypes have been reported for variants of Saccharomyces cerevisiae and of Ustilago maydis. Tolerance to morpholines is seldom found. Intrinsic resistance to griseofulvin is due to the absence of a prolonged energy-dependent transport system for this antibiotic. Resistance to azole antifungal agents is known to be exceptional, although it does now appear to be increasing in importance in some groups of patients infected with e.g. Candida spp., Histoplasma capsulatum, or Cryptococcus neoformans. For example, resistance to fluconazole is emerging in C. albicans, the major agent of oropharyngeal candidosis in AIDS patients, after long-term suppressive therapy. In the majority of cases, primary and secondary resistance to fluconazole and cross-resistance to other azole antifungal agents seems to originate from decreased intracellular accumulation of the azoles, which may result from reduced uptake or increased efflux of the molecules. In most C. albicans isolates the decreased intracellular levels can be correlated with enhancedazole efflux, a phenomenon linked to an increase in the amounts of mRNA of a C. albicans ABC transporter gene CDR1 and of a gene (BEN r or CaMDR) coding for a transporter ke or increased efflux of the molecules. Not only fluconazole, ketoconazole and itraconazole are substrates for CDR1, terbinafine and amorolfine have also been established as substrates, BEN r overexpression only accounts for fluconazole resistance. Other sources of resistance: changes in membrane sterols and phospholipids, altered or overproduced target enzyme(s) and compensatory mutations in the A5,6-desaturase.

Key words

Resistance, Flucytosine, Polyenes, Allylamines, Morpholines, Azole antifungals, Griseofulvin

Mecanismos de resistencia a los antifúngicos

Los fármacos disponibles hoy en día para el tratamiento de las micosis pueden dividirse en cuatro grandes grupos según su mecanismo de acción. Los antifúngicos pueden inhibir la síntesis de macromoléculas (flucitosina), alterar la función de barrera de la membrana (políenos), inhibir la síntesis de ergosterol (allylaminas, thiocarbamatos, azoles, morfolinas) o interactuar con los microtúbulos (griseofulvina).

La resistencia farmacológica es la principal causa de fallo terapéutico entre los pacientes tratados con flucytosina (5FC). La causa más frecuente de la resistencia de Candida albicans a la 5FC es una alteración en la UMP-fosforilasa. A pesar del uso extenso de antibióticos poliénicos durante más de 30 años, la aparición de resistencia adquirida no parece ser un problema clínico importante. Los aislamientos de Candida resistentes a los políenos tienen una marcada reducción en su contenido en ergosterol. No se ha descrito resistencia adquirida a la alicafeminas en patógenos humanos aunque se han descrito fenotipos resistentes en Saccharomyces cerevisiae y en Ustilago maydis. La tolerancia a las morfolinas es rara. La resistencia intrínseca a la griseofulvina se debe a la ausencia de un sistema de transporte dependiente de energía para este antibiótico. La resistencia a antifúngicos azólicos es excepcional, aunque parece que está cobrando importancia en algunos grupos de pacientes infectados, por ejemplo, con Candida spp, Histoplasma capsulatum o Cryptococcus neoformans. Está apareciendo resistencia al fluconazol en C. albicans, el principal agente etiológico de la candidosis orofaringea en pacientes con sida, después de una terapia supresiva prolongada. En la mayoría de los casos, las resistencias primaria y secundaria al fluconazol y la resistencia cruzada a otros antifúngicos azólicos parece relacionarse con una menor acumulación intracelular de los azoles, que puede deberse a una entrada reducida o a un aumento de la eliminación de estas moléculas. En la mayoría de los aislamientos de C. albicans las bajas con-
centraciones intracelulares pueden relacionarse con el aumento de la eliminación de los azoles, fenómeno relacionado con un incremento en los niveles de mARN de un gen CDR1 transportador ABC de C. albicans y de un gen (BENr) o CaMDR que codifica para un transportador de la clase de los transportadores facilitadores de la eliminación de múltiples fármacos. El fluconazol, el ketoconazol y el itraconazol no son los únicos sustratos para el CDR1, ya que también lo son la terbinafina y la amorolfina, mientras que la expresión desmesurada de BENr sólo es responsable de la resistencia al fluconazol. Otras fuentes de resistencia son los cambios en los esteróles y fosfolípidos de la membrana, alteración o sobreproducción de enzimas diana y mutaciones compensatorias en la Δ5,6-desaturasa.

**Palabras clave** Resistencia, Flucitosina, Polienos, Alilaminas, Morfolinas, Azoles, Griseofulvina.

Although many antifungal agents are on the market, these agents are confined to relatively few chemical classes. They can be classified into four groups on the basis of their molecular mechanism of action (Table 1) [1].

Like other living organisms, fungal cells may become resistant to toxic compounds. Antifungal resistance may be defined as a stable, inheritable adjustment by a fungal cell to an antifungal agent, resulting in a less normal sensitivity to that antifungal. To express the level of resistance a resistance ratio may be used. This factor may be expressed as the ratio IC₅₀ resistant (post-treatment) strain/IC₅₀ wild-type (pre-treatment) fungus. According to Dekker [2] the term positive cross-resistance mediates resistance to one antifungal agent and at the same time increases sensitivity to a second antifungal, the term negative cross-resistance is used.

**Table 1. Molecular mechanisms of action of antifungal agents**

<table>
<thead>
<tr>
<th>Target</th>
<th>Chemical class</th>
<th>Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DNA &amp; RNA synthesis</td>
<td>Pyrimidine</td>
<td>Flucytosine (5FC)</td>
</tr>
<tr>
<td>2. Membrane barrier function (interaction with ergosterol)</td>
<td>Polyenes</td>
<td>Amphotericin B, Nystatin</td>
</tr>
<tr>
<td>3. Ergostane biosynthesis Squalene epoxidase 14α-Demethylase (cytochrome P450-14DM)</td>
<td>Allylamines, Thio compounds</td>
<td>Naftifine, Terbinfine, Tolnaftate</td>
</tr>
<tr>
<td></td>
<td>Azoles</td>
<td>Bifonazole, Clotrimazole, Econazole, Ketoconazole, Miconazole, Fluconazole, Itraconazole, Terconazole</td>
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<tr>
<td></td>
<td>Imidazoles</td>
<td>Amorolfine (see also Fenpropimorph)</td>
</tr>
<tr>
<td>4. Mitosis (sliding of microtubules)</td>
<td>Morfolines</td>
<td>Griseofulvin</td>
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*Partly taken from [1]

**5-FLUCYTOSINE**

The prevalence of resistance to 5-flucytosine among _Candida_ varies between countries (with a particularly high frequency in the USA), species and strains. Most experts would regard a fungus with a MIC for 5-flucytosine of ≥25 μg/ml as resistant. Among the _Candida_ species _Candida tropicalis_ and _Candida parapsilosis_ are generally the least susceptible to 5-flucytosine _in vitro_; almost 21% of the isolates are resistant prior to treatment (intrinsic resistance) compared with 8% of _Candida glabrata_ and ± 2% of _Cryptococcus neoformans_ isolates [3]. Next to intrinsic resistance, acquired drug resistance has been identified as a major cause of clinical failure in patients treated with flucytosine. Resistance to flucytosine can result from loss or mutation of any of the enzymes involved in its uptake (cytosine permease), metabolism (cytosine deaminase, uracil: phosphoribosyl transferase) and/or incorporation into RNA [1]. Acquired resistance in _Candida albicans_ usually results from a defect in uracil: phosphoribosyl transferase (UMP-pyrophosphorylase), an enzyme involved in the synthesis of uridine 5'-monophosphate (UMP), deoxyuridine monophosphate (dUMP), as well as 5-FdUMP and 5-FUTP [3].

In long-term therapy, flucytosine monotherapy has been replaced by a combination of amphotericin B and flucytosine which shows favourable interactions in tests with _C. albicans_ and _C. neoformans_. The combination significantly reduces the appearance of resistant isolates. For example, in cryptococcal meningitis 20 to 30% acquired resistance was observed under flucytosine monotherapy, whereas only 2 to 3% with the combination [4]. It is possible that amphotericin B enhances the uptake of flucytosine [5].

**POLYENES**

Mutant strains of _C. albicans_, _C. neoformans_ or _Aspergillus nidulans_, resistant to polyenes, can be readily obtained in the laboratory. Amphotericin B resistance, although rare in _Candida_ species other than _Candida lusitaniae_ is common in emerging pathogens such as _Trichosporon_ and _Fusarium_ species [6].

Treatment failure attributable to the development of amphotericin B resistance remained an uncommon problem. Resistant isolates that have been recovered during the treatment of patients with candidosis have so far belonged to species other than _C. albicans_, in particular _C. lusitaniae_ and _C. tropicalis_. But, in neutropenic patients, amphotericin B resistance may be a greater problem than has been supposed.

Powderly _et al._ [7] found that there was a higher mortality in neutropenic patients treated with amphotericin B when the MIC for the infecting _C. albicans_ was >0.8 μg/ml.

Polyenes complex with ergosterol in the membrane causing a cascade of cell disturbing events. Polyene-resistant _Candida_ isolates have a marked decrease in their ergosterol content compared with polynye-susceptible control isolates. For example, Dick _et al._ [8] recovered 27 polyene resistant _C. albicans_ isolates from three neutropenic patients. The polyene resistant _C. albicans_, _C. tropicalis_ and _C. glabrata_ demonstrated a marked decrease in ergosterol content as compared to polynye-susceptible control isolates of the same species. The interaction of
amphotericin B with the plasma membrane is complex, but, binding to ergosterol is required. Thus, the decreased ergosterol content should lead to decreased sensitivity to amphotericin B. Membrane alterations may also reduce virulence and this might explain why amphotericin B resistant strains are encountered mostly in immunocompromised patients.

ALLYLAMINES AND THIOCARBAMATES

At the moment, there are two allylamines, naftifine and terbinafine, and one thio carbamate antifungal, tolnafate in clinical use. All three are inhibitors of the squalene epoxidase. Resistance has not been reported from human pathogens, but, resistant phenotypes have been described in other reports of amorolfine-resistant human pathogens [6]. Amorolfine is a derivative of fenpropimorph, a morpholine fungicide used for several years against, for example, powdery mildew and rust fungi in cereals. The levels of fenpropimorph-resistance detected in the field are low [11].

AZOLE DERIVATIVES

The largest and most widely used class of antifungal agents is that of the azole-antifungals. All inhibit the 14α-demethylase, a cytochrome P450 in the ergosterol biosynthesis pathway [12]. Individual imidazole- and triazole antifungal agents differ in their effect on individual yeast or fungi because of different effects on the 14α-demethylase and/or other enzymes of the ergosterol biosynthesis pathway, and of differences in the extent of uptake and efflux of the antifungal agent in different species.

Candida spp.

Azole resistance is reported rather for nonimmunocompromised patients, thus, acquisition of azole resistance does not appear to be a major problem in clinical settings other than their long-term use in immunocompromised patients. There are few reports of resistance developing in C. albicans, C. tropicalis or C. glabrata during short term treatment of candidiasis. However, here again differences are seen between the different azole antifungal agents and different species. For example, Candida krusei isolates are natively resistant to fluconazole (MIC 50 µg/ml) [13]. Treatment with fluconazole suppressed relatively susceptible Candida species such as C. albicans and C. tropicalis while permitting the overgrowth of less sensitive Candida species such as C. krusei [14].
Several mechanisms describe how fungi try to escape from the effects of azoles (for a review see [1]). An overview of the mechanisms of resistance to 14α-demethylase inhibitors is given in Table 2. *C. krusei* is sensitive to ketoconazole and itraconazole [13], but, not to fluconazole, and the difference in sensitivity appeared to arise from differences in intracellular itraconazole, ketoconazole and fluconazole contents [15]. Depending on the experimental conditions, the *C. krusei* isolate accumulates 6-41 times more itraconazole than ketoconazole and the intracellular ketoconazole contents was 3.0-19.0 times higher than that of fluconazole [15]. A too low intracellular fluconazole content (unpublished results) may also be at the origin of its low if any activity (MIC > 100 µg/ml) against *Aspergillus fumigatus* [16].

The first ketoconazole resistant strains of *C. albicans* (AD & KB) were isolates from two American patients with chronic mucocutaneous candidiasis (CMC) [17]. These isolates are claimed to be impermeable to the azole antifungal ICI 153,066, and, thus are cross-resistant to this triazole derivative [18]. Resistance was thought to be due to changes in the properties of the cell membrane rather than internal enzymology. Further studies revealed that both resistant isolates contained increased amounts of non-esterified sterols which decreased their phospholipid/sterol ratio to half that of an azole-sensitive strain [19]. However, more recent studies showed that *C. albicans* AD and KB had much lower rates of fluconazole accumulation than the susceptible strains [20]. The net fluconazole uptake was increased by azide treatment; this provides evidence that the resistance mechanism is energy dependent and may be accounted for by fluconazole efflux. The fluconazole efflux may be associated with increased expression of *CDR1*, a member of the multidrug efflux systems. In the latter study, resistant organisms were defined as those for which the MICs of the following agents were as indicated: fluconazole > 2 µg/ml; itraconazole > 2 µg/ml; ketoconazole > 0.5 µg/ml [20]. A third resistant *C. albicans* strain (Darlington) was obtained from a British CMC patient [21-24]. Resistance in this isolate may be the result of multiple mechanisms [20]. The 14α-demethylase in this azole-resistant *C. albicans* isolate was less sensitive to ICI 153,066 than that of two clonally unrelated azole-resistant isolates [23]. Five *C. albicans* isolates from this patient contained larger amounts of fucosterol, relative to the amounts found in susceptible isolates [25]. This suggests decreased activity of the Δ5,6-desaturase. In the presence of ketoconazole or ICI 153,066, this isolate accumulates 14α-demethylase inhibitors. The 14α-methylsterol may partly satisfy the bulk sterol requirement for fungal viability. Kelly et al. [25, 26] have shown that *Saccharomyces cerevisiae* mutants, in which the *CYP51* gene (encoding the 14α-demethylase, also called CYP51A or P450 51) has been disrupted, are viable because the *CYP51* gene is up-regulated [27]. It should be noted that Albertson et al. [20] found *C. albicans* Darlington also resistant to fluconazole and amphotericin B, and that resistance may be associated with increased expression of *CDR1*. The fourth ketoconazole-resistant strain (B41628) was also isolated from a British patient with CMC. The MIC values for miconazole, ketoconazole, itraconazole and fluconazole were increased, and this strain appeared to be less or even non-pathogenic compared to other *C. albicans* isolates in several animal models of infection [28]. Resistance may be associated with a modification in cytochrome P450: microsomal P450 isolated from the resistant isolate had a reduced affinity for ketoconazole, itraconazole and fluconazole. When the isolate was subcultured in a drug free medium, this reduction in affinity decreased, indicating that the resistance to azole antifungals is reversible [29]. Alteration of the target CYP51A in the phytopathogen *U. maydis* has also been proposed as a possible route of resistance to theazole fungicide triadimefon [30,31].

Next to the four ketoconazole-resistant isolates a much greater number of fluconazole-resistant *C. albicans* isolates were found in HIV-infected patients, after long-term suppressive therapy (a few examples are given in Table 2). For these isolates distinct resistance mechanisms have been proposed. Some fluconazole-resistant clinical *C. albicans* isolates (MIC of fluconazole 32->128 µg/ml for the resistant isolates against 0.25 - 1 µg/ml for the sensitive isolates) exhibited up to a 10-fold increase of mRNA levels of the ABC (ATP binding cassette) transporter gene *CDR1*; in another azole-resistant isolate the gene for another efflux pump *BEN* (a gene conferring benomyl resistance) was massively overexpressed [32]. Ben (the product of *BEN*; also called *CaMDR1*) belongs to the superfamily of major facilitator multidrug efflux transporters [10,33,34]. Disruption of the multidrug resistance gene *CaMDR1* in *C. albicans* resulted in mutant strains that colonized mouse kidneys to very high levels, but, were markedly reduced in their virulence. These results suggest a physiological role in pathogenesis for this multidrug efflux transporter [35]. The gene of the major facilitator has been isolated by Fling et al. [36] that of the ABC-transporter by Prasad et al. [37]. Overexpression of drug efflux pumps is at the origin of the low intracellular fluconazole levels found in *C. albicans* isolates C39, C26, C56 and C40 [32]. Indeed, failure in accumulating fluconazole is related to a significant increase in the level of *CDR1* mRNA (isolates C39, C26, C56) or *BEN* mRNA [32]. As shown in Table 2 a number of the isolates are cross-resistant to ketoconazole (MICs = 2-4 µg/ml) and itraconazole (MICs = 1.0-> 2); one isolate (C39) was cross-resistant to all three azoles only. It should be noted that other azole resistance mechanisms not involving the multidrug transporters could also be identified in these isolates. This is so in a *C. glabrata* strain isolated from a patient after nine days of treatment with ciprofloxacin/fluconazole [38,39]. Phenotypic and restriction fragment length polymorphism (RFLP) analysis of genomic DNA from the pre- and post-treatment isolates gave similar patterns, indicating that these *C. glabrata* isolates may be clonally related [40]. The concentrations needed to get 50% (IC50) inhibition of growth of the susceptible pre-treatment *C. glabrata* isolate were 43 µM fluconazole, 0.7 µM ketoconazole and 1 µM itraconazole; growth of the post-treatment isolate was only slightly inhibited by 10 µM ketoconazole and was unaffected by 100 µM fluconazole or 10 µM itraconazole [39].

The cellular fluconazole content of the post-treatment isolate was 1.5- to 3-fold lower than that of the pretreatment isolate. This difference was smaller than the measured difference in susceptibility and therefore does not fully explain the fluconazole resistance of the post-treatment isolate [39]. Moreover, the intracellular content of ketoconazole and itraconazole in the two *C. glabrata* isolates were similar, indicating that uptake and/or efflux differences do not account for the azole cross-resistance of the post-treatment isolate [39]. All results obtained so far indicate that cross-resistance is partly due to a *CYP51*
gene (codes for the 14α-demethylase) amplification that results in an increased level of 14α-demethylase activity. The CYP51 gene was visualized by means of a 24-nucleotide fragment identical to all cytochrome P450 51 (P450 14DM) sequences so far reported. The blot was also probed with a S. cerevisiae actin (ACT1) reference probe. The ratio of CYP51/ACT1 in the post-treatment isolate was 0.26, that in the pre-treatment isolate was 0.07 indicating a 3.7-fold increase in copy number of the CYP51 gene [9]. Northern blots showed that the 14α-demethylase mRNA level was approximately 8-fold higher in the post-treatment isolate [9].

The observed gene amplification resulted in an increased synthesis of ergosterol from acetate, mevalonate, squalene and lanosterol [39,40]. Recent hybridization experiments on chromosomal blots indicate that the increase in copy number was due to amplification of the entire chromosome containing the CYP51 gene. The higher abundance of the amplified chromosome induced pronounced differences in the protein patterns between the susceptible versus the resistant isolates (unpublished results). Thus, the resistance in the C. glabrata isolate may be the result of multiple mechanisms. The amplification of the chromosome may also be associated with decreased sensitivity of the resistant isolate to terbinafine and amorolfine (unpublished results).

Another case of infection with C. glabrata in which the organism became resistant to fluconazole after a short course of treatment was reported by Hitchcock et al. [41]. The isolate was cross-resistant to ketoconazole and itraconazole. The MICs for the post-treatment isolate were 100 μg/ml, 3 μg/ml and 50 μg/ml respectively. Fluconazole resistance appeared to arise from a permeability barrier to this drug [42]. However, it is also possible that an overexpression of drug efflux pumps is involved. Indeed, this C. glabrata isolate was also shown to accumulate less rhodamine 123, a known substrate for a wide diversity of cells exhibiting the multidrug resistance phenotype [42].

Histoplasma capsulatum

Histoplasma capsulatum strains isolated from an HIV-infected male at week 8, 12 and 16 of therapy with fluconazole showed progressive increase in fluconazole minimum inhibitory concentration from 0.6 μg/ml to 20 μg/ml [43]. The pre- and post-treatment isolates are clonally related. Fluconazole is less potent against the post-treatment isolate, but, itraconazole is a 6-times more potent growth inhibitor of the relapse (post-treatment isolate). This is an example of negative cross-resistance. Negatively correlated cross-resistance has also been observed in laboratory isolates of Penicillium italicum. All isolates with a relatively high degree of resistance to 14α-demethylase inhibitors exhibited increased sensitivity to the morpholine fenpropimorph [44,45].

Reduced susceptibility of ergosterol synthesis to fluconazole appears to explain the inducible resistance noted in the Histoplasma relapse isolate. Induction of the accumulation of large amounts of obtusifolione (a 3-ketosteroid known to disturb membranes) by low concentrations of itraconazole in the relapse isolate suggests that the 3-ketosteroid reductase (an enzyme involved in the demethylation of, for example, obtusifoliol and 4,4-dimethylzymosterol) of the post-treatment isolate may be more susceptible to itraconazole than is that of the parent isolate. The increased amounts of obtusifolione formed in the relapse isolate incubated in the presence of itraconazole may explain the enhanced sensitivity to this triazole derivative. But, additional studies are certainly needed to elucidate both the mechanism(s) of fluconazole-resistance and of the enhanced sensitivity to itraconazole.

Cryptococcus neoformans

Lamb et al. [46] investigated the P450-mediated sterol 14α-demethylase of four Cryptococcus neoformans clinical isolates obtained from AIDS patients following failure of fluconazole therapy. Fluconazole tolerance was not associated with defective sterol biosynthesis. The isolates had slightly elevated P450-contents and slightly reduced fluconazole levels in the cells. A clear cause for resistance was the tenfold differences in sensitivity between the 14α-demethylase of the tolerant strains and that of wild-type strains.

GRISEOFULVIN

The last antifungal agent listed in Table 1 is griseofulvin. Griseofulvin, isolated from Penicillium griseofulvum, alters a process vital to the sliding of microtubules necessary for the separation of the chromosomes [47]. Griseofulvin's spectrum of activity is limited to dermatophytes. These fungi possess a prolonged energy-dependent transport system for the antibiotic. In contrast, in insensitive organisms, such as C. albicans, this system is replaced by a short-energy-independent transport system [48]. This is another example of intrinsic resistance with another mechanism.

CONCLUSION

The present available studies indicate that resistance to antifungal agents can originate from a too low intracellular drug content, the consequence of an impaired uptake or of overexpression of drug efflux pumps, resistance can also originate from amplification of the gene coding for the target enzyme, from changes at the target site(s), changes in the ergosterol level, from differences in the nature of the accumulating sterols or from a decreased activation of the antifungal agents. Thus, the fungus has several mechanisms to escape from the effects of antifungal agents.

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