Acta Biochimica Polonica

Vol. 42 No. 4/1995

509–516

QUARTERLY

Text of lecture presented at the 5th International Symposium on ***Molecular Aspects of Chemotherapy***

Mechanisms of resistance to azole antifungals

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Key words: fluconazole, ketoconazole, itraconazole, resistance, *Candida*, intracellular-content, cytochrome-P-450, gene, overexpression, Δ5-6-desaturase, review, *in vitro*

Until the late eighties, clinical resistance to azole antifungals was a rare phenomenon. Only a few cases of resistance to ketoconazole were found in patients with chronic mucocutaneous candidiasis (CMC). The spread of AIDS and the widespread prophylactic and therapeutic use of the hydrophilic azole compound fluconazole resulted both in the selection and induction of resistant strains and in a shift in the nature of the infecting organisms.

Most azole antifungals such as itraconazole, ketoconazole and fluconazole are active against a variety of fungal diseases. However, the concentration needed to inhibit growth is dependent on the nature of the infecting species. *Mucor* spp., e.g., are almost insensitive to present available azole compounds and can be regarded as intrinsically resistant to azole treatment.

Physicochemical features, such as the hydrophobicity and pK_a, of a given azole, define whether or not it will be active or cross-resistant against a given species. Fluconazole is almost inactive against *Candida krusei* and *Aspergillus fumigatus*, whereas the lipophilic itraconazole is active against these species.

A third type of resistance is acquired or induced resistance. This is the most controversial type because, even within a given species, organisms may differ in their response to the same azole. For these strains, convincing evidence can only be obtained when there is a genotypically related strain, which does not show resistance.

In a limited number of biochemical or molecular biological studies the mechanisms of resistance have been investigated at the molecular level. These studies show that resistance can occur when there is an insufficient intracellular content of the azole. This can be due to impermeability problems, inactivated uptake systems or, and more likely, the presence of active multidrug resistance gene products of the P-glycoprotein type. Alteration or overexpression of the target for azole antifungals, the cytochrome P450-dependent 14 α-demethylase, also induces resistance. The nature and amount of the accumulating sterols also are of great importance for azole-induced growth inhibition. This may explain why mutations in other enzymes of the ergosterol biosynthesis pathway, e.g. the Δ5–6 desaturase, can contribute to azole resistance.

Although many antifungal drugs have been developed, the clinically available antifungals interfere only with a limited number of targets (for a review see [1]). They impair membrane barrier function, inhibit macromolecule synthesis, and ergosterol synthesis, or interact with microtubules. Acquired resistance has been identified as a major cause of clinical failure and relapse in patients treated with the inhibitor of macromolecule synthesis, 5-fluorocytosine [2]. In contrast, treatment failure attributable to the development of amphotericin B

Abbreviations: CMC, chronic mucocutaneous candidiasis; MIC, minimal inhibitory concentration; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism.

resistance has remained an uncommon problem. The amphotericin B-resistant isolates that have been isolated during treatment of patients with candidiasis have so far belonged to species other than *Candida albicans*, in particular *Candida lusitaniae* and *Candida tropicalis* [3, 4]. Some isolates of *Candida guilliermondii* also have a high level of resistance to amphotericin B *in vitro* [5]. Until the late eighties, resistance to azole antifungals, the most important group of ergosterol biosynthesis inhibitors, was exceptional, although it does now appear to be increasing in importance in neutropenic and AIDS patients [4, 6].

From a clinician's point of view one could define strains resistant to an antifungal compound, when the compound given at the maximal possible concentration is unable to cure the fungal disease. However, the lack of activity could be due to host-related factors such as, e.g., immune supression, both reversible and irreversible; site and severity of infection; poor absorption; drug-drug interactions and patient compliance. Although important for the patient, these factors are irrelevant for the classification of the resistant nature of the infecting fungal organism.

Three different classes of resistance may be encountered in a patient: (1) Intrinsic resistance: the patient is colonized or infected with an organism that is not covered by the spectrum of azole antifungals. (2) Selective resistance: the patient is infected with multiple species or strains. During therapy the most sensitive organisms are eradicated, which favors the growth and selection of less sensitive or resistant ones. (3) Acquired resistance: the initially susceptible colonizing or infecting strain mutates and becomes resistant. The probability that a mutation is phenotypically visible depends on the number of copies of the gene: *Candida glabrata*, which is a haploid yeast, is much more prone to mutation than the diploid *Candida albicans*, which does not possess a haploid sexual stage in its life cycle [7]. In this last species both copies of a gene have to be mutated before the mutation will be phenotypically visible [8]. In order to classify a given case, reliable sensitivity measurements are needed as well as both pheno- and geno-typing of the organisms isolated before, during and after therapy. Reliable Minimal Inhibitory Concentration (MIC) determinations for azoles are difficult to obtain [9]. Despite substantial progress made by the National Committee for Clinical Laboratory Standards, no clearcut MIC breakpoints have yet been determined to define resistance for the different azoles. All azoles tend to have a Gaussian distribution of sensitivity within a given species. However, due to rapid development of DNA-based typing methods as, e.g., restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), karyotyping, hybridization with repetitive DNA base sequences; clonal relationships can be documented and azole sensitivity between these strains evaluated.

In 1978 Holt & Azmi [10] described a case of candidiasis in a neonate in which resistance to miconazole (and other azoles) developed following nine weeks of treatment. The MIC prior to treatment was 100-fold lower. No further tests were performed on these strains and this initial report was never confirmed.

The introduction of the first oral imidazole compound, ketoconazole, permitted a dramatic improvement in the management of patients with chronic mucocutaneous candidiasis. However, long-term treatment with ketoconazole was required if remission of infection was to be sustained. This form of management soon led to reports of treatment failure or relapse due apparent development of drug resistance. Rosenblatt *et al.* [11] were the first to mention such a case. Three more cases were reported in 1983 [12, 13] and a fifth in 1986 [14]. A reported characteristic of one of these azole -resistant *Candida albicans* strains is reduced virulence for animals [14].

In immuno-competent patients, mucosal and cutaneous forms of candidiasis can usually be eradicated with a short course of topical or oral treatment with an azole compound. Until 1986, no reports were published of emergent resistance to ketoconazole in *Candida albicans* associated with courses of treatment lasting for periods less than several months. However, in patients with AIDS, because of profound and sustained immunosuppression, persistent or recurrent oral infection with *Candida albicans* is common, and protracted or repeated courses of antifungal treatment are required if remission from infection is to be achieved and sustained.

Since 1990, there has been a substantial increase in the number of reports of HIV-infected patients with oral or esophageal candidiasis who have failed to respond to oral or parenteral

treatment with fluconazole, despite an earlier favorable response to this or other azole compounds [15–28]. In most cases, the patients affected have been in the advanced stages of AIDS with CD4-T cell counts of less than 50 per mm^3. Many had received several courses of treatment with fluconazole, or had been given protracted maintenance treatment with the drug at doses of 50 mg per day or every other day [4]. Dupont *et al.* [16] found that there is a clear correlation between persistent clinical signs of candidiasis and MICs to fluconazole > 12.5 mg × l^{-1} and between clearance of clinical signs and symptoms and MICs < 3 mg × l^{-1} (taken from [4]).

Bart-Delabesse *et al.* [20] studied serial *Candida albicans* isolates from four AIDS patients with recurrent oral candidiasis who developed clinical and mycological resistance to fluconazole. Three patients appeared to be infected by the same strains throughout. One patient appeared to acquire a new strain of *Candida albicans* at about the time of the rise in fluconazole MIC. In another investigation [29], no fewer than 61 different DNA sub-types were detected among 121 isolates of *Candida albicans* obtained from 29 AIDS patients with oral candidiasis who were being treated with fluconazole and/or clotrimazole. More than half the patients studied were infected or colonized with more than one strain and the introduction or selection of a different strain with a higher MIC was not uncommon. In contrast, few of the patients who were persistently infected with a single strain demonstrated an increase in fluconazole MIC over the course of treatment.

No instances of acquired resistance to the triazole antifungal compound, itraconazole, have so far been reported. However, at least one of the strains of *Candida albicans* recovered from patients with ketoconazole-resistant chronic mucocutaneous candidiasis showed cross-resistance to itraconazole (NCPF3363) [14].

A study by Ruhnke *et al.* [23] suggests that itraconazole may still serve as an effective antifungal agent in patients with HIV infection and oropharyngeal candidiasis nonresponsive to fluconazole. Barchiesi *et al.* [25] studied the *in vitro* activity of itraconazole against fluconazole-susceptible and -resistant *Candida albicans* isolates from the oral cavities of 100 HIV-infected patients. The data obtained demonstrated that itraconazole had good activity against most of the tested isolates; for 90% of all *Candida albicans* strains, MIC_{90}s were ≤ 1 µg/ml, and only one isolate was resistant to itraconazole (MIC_{90} > 16 µg/ml). It should be noted that the itraconazole MICs for the fluconazole-susceptible isolates were significantly lower than those for the fluconazole-resistant isolates. Thus HIV-infected patients who fail fluconazole therapy for oral and/or esophageal candidiasis may require higher doses than those used in standard therapy.

Another source of concern arising from the widespread use of azole drugs as prophylactic and therapeutic agents has been an apparent increase in the prevalence of colonization and infection with less common *Candida* species. Three studies in which prophylactic treatment with ketoconazole was given to immunosuppressed patients reported the emergence of *Candida glabrata* during the course of such treatment [30–32]. More recently, a number of case reports describe the failure of fluconazole to suppress infections with *Candida krusei* [33–38].

Most instances of treatment failure in patients with *Candida glabrata* or *Candida krusei* infection appear to be related to the intrinsic resistance of these organisms to fluconazole [39, 40]. However, there are reports [41, 42] of cases of *Candida glabrata* infection in which resistance developed during the course of short-term fluconazole treatment and in which pre- and post-treatment strains were available for comparison. In one reported case [42], a 30-fold rise in MIC of fluconazole was recorded following nine days of oral treatment. The pre- and post-treatment isolates were found to be similar by phenotypic and RFLP analysis, suggesting the development of drug-resistant mutants during treatment [43]. In a second case [41], comparison of the pre- and post-treatment *Candida glabrata* strains by RFLP analysis, showed that they were unrelated. This suggests that the resistant organism was selected from a mixed population of both strains as a result of the fluconazole treatment.

MECHANISMS OF ACTION

Imidazole and triazole antifungal compounds, such as miconazole, clotrimazole, econazole, ketoconazole, itraconazole and fluconazole inhibit the cytochrome P450-dependent

14α-demethylase (P450 14DM,P450 51), a key-enzyme of the ergosterol biosynthesis pathway. The interaction of these azole derivatives results in inhibition of ergosterol synthesis and accumulation of 14α-methylated sterols. Some of the latter sterols induce changes in membrane structure and function, including membrane leakiness and alterations in membrane-bound enzymes. These changes result in cessation of growth and, in a number of pathogenic fungi, cell death. In *Cryptococcus neoformans* [44] and *Histoplasma capsulatum* [45] itraconazole interacts directly or indirectly with a second target, the 3-ketosterol reductase. Due to inhibition of this enzyme, obtusifolione accumulates. This 3-keto-14-methylated sterol derivative has been shown to destabilize membranes. The accumulation of both 14-methylated sterols and obtusifolione may explain the potent effects of itraconazole on *Cr. neoformans* and *H. capsulatum*.

MECHANISMS OF RESISTANCE

Several mechanisms describe how fungi try to escape from the effects of azoles (for a review see [46]). The first ketoconazole-resistant strains of *Candida albicans* were isolated from two American patients (strains AD (NCPF3202) and KB (NCPF3303)) [12]. These isolates are impermeable to ICI 153,066, and are thus cross-resistant to this triazole derivative [47]. No intracellular ketoconazole contents were measured. Resistance is thought to be due to changes in the properties of the cell membrane rather than internal enzymology [47]. Subsequent studies revealed that both resistant isolates contained increased amounts of non-esterified sterol which decreased their phospholipid/sterol ratio to only half that of an azole-sensitive strain [48].

A third resistant *Candida albicans* strain (Darlington strain (NCPF 3310) was obtained from a British chronic mucocutaneous candidiasis (CMC) patient [13, 49]. Again the ketoconazole content was not measured, but this strain took up ICI 153,066 at a faster rate than sensitive strains [48]. The 14α-demethylase in this azole-resistant *Candida albicans* strain was less sensitive to ICI 153,066 than that of two clonally unrelated azole-sensitive strains. Since there was no direct correlation between the IC_{50} values for growth and ergosterol biosynthesis, the decreased sensitivity of 14α-demethylase may not be the only reason for the azole resistance observed [49]. More recent studies have shown that five *Candida albicans* isolates from this CMC patient contained larger amounts of fecosterol (ergosta-8,24(28)-dienol), relative to the amount found in susceptible isolates [50]. This suggests decreased activity of the $\Delta^{5,6}$-desaturase. In the presence of a 14α-demethylase inhibitor, such as ketoconazole or ICI 153,066, this isolate accumulates 14α-methylfecosterol, a sterol that can at least partly replace ergosterol. Indeed, Kelly *et al.* have shown that *Saccharomyces cerevisiae* mutants, in which the *CYP51* gene (encoding the 14α-demethylase) has been disrupted, are viable only when there is a concomittant defect in the $\Delta^{5,6}$-desaturase [51, 52]. A defective sterol $\Delta^{5,6}$-desaturase is also the cause of azole resistance in *Ustilago maydis* [53].

It should be noted that no pretreatment isolate from the British CMC patient was available for comparison. Therefore it cannot be excluded that a *Candida albicans* strain different from the pretreatment isolate was selected during treatment with ketoconazole.

This may also be the case with the fourth ketoconazole-resistant strain (strain B41628 (NCPF3363)) which was also isolated from a British patient with CMC [14]. 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 *Candida albicans* isolates in several animal models of infection [14]. Microsomal cytochrome P450 isolated from B 41628 had a 15-, 2.5- and 12-times reduced affinity for ketoconazole, itraconazole and fluconazole, respectively [54]. When the isolate was subcultured in a drug-free medium, this reduction in affinity decreased. The microsomal P450 from a new isolate (B44548), obtained from this patient after treatment with azole antifungals had been discontinued, also showed a much higher affinity for fluconazole, ketoconazole and itraconazole compared with isolate B41628 [54]. Both results suggest that the resistance to these azole antifungals is reversible.

The widespread use of fluconazole as a therapeutic, and especially as a prophylactic, at doses that are considered relatively low [55] has amplified the number of resistant *Candida albicans* and non-*Candida albicans* isolates (e.g. *Can-*

dida glabrata) available for investigation. For example, two fluconazole-resistant strains of *Candida albicans* (B67078 and B67081) were obtained from B. Dupont (France). Fifty percent inhibition of growth by fluconazole was reached at more than a 100-fold higher concentrations than those required with a sensitive *Candida albicans* isolate [56]. This decreased susceptibility may result, at least in part, from a reduced intracellular fluconazole content [56]. Indeed, the intracellular fluconazole content of a sensitive isolate (B2630) was 0.8 nmol per 10^9 cells, whereas that of strains B67078 and B67081 was 0.08 and 0.03 nmol per 10^9 cells, respectively. It should be noted that the intracellular itraconazole content of strains B2630, B67078 and B67081 was much greater: 14.4, 9.6 and 8.6 nmol per 10^9 cells, respectively. These higher contents correlated with itraconazole's greater inhibitory potency. For example, the IC_{50} values for growth inhibition of strain B2630 was 0.04 μM for itraconazole and 0.7 μM for fluconazole.

A case of infection with *Candida 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 susceptible pre-treatment *Candida glabrata* isolate were 12.5 mg × l^{-1} for fluconazole, 0.19 mg × l^{-1} for ketoconazole and 0.39 mg × $l^{-1}$for itraconazole. In the resistant isolate these MICs increased to 100 mg × l^{-1}, 3 mg × l^{-1} and 50 mg × l^{-1}, respectively. Fluconazole resistance appeared to arise from a permeability barrier to this drug [41]. The uptake of ketoconazole or itraconazole was not measured. However, the increase in MICs during treatment appeared to correlate with the increase in hydrophobicity. Itraconazole, the most hydrophobic compound, showed the greatest increase in MIC (128-fold). The 16-fold rise in MIC with the less hydrophobic compound, ketoconazole, is much closer to that found with the most water-soluble of the three compounds, fluconazole (8-fold). For this reason, it is possible that changes in the physical and biochemical properties of the membranes of this *Candida glabrata* isolate had a greater effect on the uptake and/or efflux of hydrophobic azole antifungals. It would certainly be of interest to compare the plasma membrane and cell wall composition of the pre- and post-treatment isolates. However, RFLP analysis of genomic DNA from both isolates gave different patterns, suggesting that these strains were clonally unrelated [41]. Therefore, it will be necessary to compare cell wall and plasma membrane composition of the resistant isolate with that of different sensitive isolates.

Warnock *et al.* [42] reported a case of infection with *Candida glabrata* in which the organism became resistant to fluconazole after nine days of treatment. Phenotypic and RFLP analysis of genomic DNA from the pre- and post-treatment isolates (B57148 (NCPF38XX) and B57149 (NCPF38XX)) gave similar patterns, indicating that these organisms may be clonally related [46, 56]. The IC_{50} values for growth inhibition of the pretreatment isolate (B57148) were 0.7 μM ketoconazole, 1 μM itraconazole, and 43 μM fluconazole. In contrast, growth of the post-treatment isolate (B57149) was only slightly inhibited by 10 μM ketoconazole and was unaffected by 10 μM itraconazole or 100 μM fluconazole [43]. The cellular fluconazole content of strain B57149 was 1.5–3-fold lower than that of strain B57148. Because the difference in susceptibility was greater than the difference in uptake, this reduced accumulation does not fully explain the fluconazole resistance of the post-treatment isolate. Moreover, the intracellular concentrations of ketoconazole and itraconazole in the two *Candida glabrata* isolates were similar, suggesting that uptake differences do not account for the azole cross-resistance observed [43]. Further studies of the mechanism of resistance (unpublished results) revealed major changes between both isolates. Southern-blot analysis revealed that the gene coding for the 14α-demethylase was amplified 3.7 fold. Northern blots showed that the 14α-demethylase mRNA level was approximately 8-fold higher in the post-treatment isolate. The observed gene amplification resulted in an increased synthesis of ergosterol from acetate, mevalonate, squalene and lanosterol [43]. In the presence of a 14α-demethylase inhibitor, lanosterol synthesis from squalene by a subcellular fraction of the post-treatment isolate was 3-fold that found for the pre-treatment isolate [43]. This suggests that next to the 14α-demethylase activities the squalene epoxidase and/or oxidosqualene cyclase activities are also enhanced. The increased ergosterol synthesis and content not only resulted in decreased sensitivity of the post-treatment isolate to azole anti-

fungals, but also amphotericin B, terbinafine and amorolfine are less active. After 159 subcultures of strain B57149 on fluconazole free-medium, the *CYP*51 gene levels, mRNA levels, 14α-demethylase activity, ergosterol synthesis and content, and sensitivity to itraconazole returned to pretreatment levels. However, the fluconazole activity was only partly restored by subculturing as compared with the pretreatment isolate ([43, 56], and unpublished data). This correlated with the still reduced intracellular fluconazole content in the subcultured strain.

In vitro testing has demonstrated that isolates of *Candida krusei* have much higher fluconazole MICs than most *Candida albicans* isolates [39, 40]. Therefore, it is not surprising that this species is frequently cited as emergent colonizing flora during the clinical use of fluconazole and as a significant problem in patients receiving treatment with this drug in specific settings such as neutropenic patients, in intensive therapy units and patients with AIDS [6]. The low susceptibility of *Candida krusei* to fluconazole appeared to arise from a low intracellular fluconazole accumulation. Exponentially growing cells retained after 1 h of incubation 0.06 nmol per 10^9 cells. Depending on the isolate used this is 42- to 125-times less than itraconazole [57].

In conclusion: the present available studies indicate that resistance to fluconazole can originate from a too low intracellular drug content, changes at the target site and from differences in the nature of the accumulating sterols.

The authors are grateful to F.C. Odds for help in preparing the manuscript.

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