Mechanisms of resistance to antifungal agents: Yeasts and filamentous fungi

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Summary Failure to respond to antifungal therapy could be due to in vitro resistance (intrinsic or developed during therapy) or clinical resistance; the latter is associated with numerous factors related to the host, the antifungal agent, or the infecting isolate. Recently, a susceptible MIC breakpoint (⩽ 2 µg/ml) was designed for *Candida* spp. to all three available echinocandins, anidulafungin (Pfizer), caspofungin (Merck) and micafungin (Astellas) and treatment failures have been associated with MICs > 2 µg/ml. In some of these cases, clinical failure was associated with the genetic mutations described below. Azole and flucytosine breakpoints, and the echinocandin susceptible breakpoint, are useful when isolates are tested by CLSI standardized methods; breakpoints are also available by the EUCAST method. More recently, in vitro resistant MIC breakpoints have been assigned for filamentous fungi (moulds) vs. five antifungal agents, but these categories are not based on correlations of in vitro with in vivo response to therapy. However, itraconazole (Janssen), amphotericin B (Bristol-Myers) and voriconazole (Pfizer) clinical failures in aspergillosis have been correlated with MICs > 2 µg/ml. This article provides a review of reported resistance molecular mechanisms to antifungal agents since 2005; previous related reviews are also listed.

Key words Antifungal resistance, Resistance mechanisms

Mecanismos de resistencia a los antifúngicos: levaduras y hongos filamentosos

Resumen La falta de éxito de la terapia antifúngica puede ser debida a la resistencia que se observa in vitro (resistencia que puede ser intrínseca a la cepa o adquirida durante el tratamiento) o a una resistencia clínica; esta última se asocia con numerosos factores relacionados con el huésped, el agente antifúngico o la cepa responsable de la infección. Recientemente se ha determinado un nuevo punto de corte CMI (⩽ 2 µg/ml) para los aislamientos de *Candida* sensibles a las tres equinocandinas comercializadas: anidulafungina (Pfizer), caspofungina (Merck) y micafungina (Astellas). Se ha asociado el fallo en el tratamiento antifúngico a valores de CMI > 2 µg/ml. En algunos de estos casos, el fallo clínico del tratamiento se relacionó con ciertas mutaciones genéticas que se describen en este artículo. Los puntos de corte establecidos para los azoles y la flucitosina, así como el de las equinocandinas, son de utilidad cuando se estudian los aislamientos con los métodos estandarizados propuestos por el CLSI; los puntos de corte también pueden establecerse mediante el método EUCAST. Más recientemente, se han establecido los puntos de corte para determinar la resistencia in vitro de los hongos filamentosos a cinco agentes antifúngicos, pero los intervalos determinados por estos puntos de corte no están basados en la comparación entre los resultados in vitro y la respuesta al tratamiento clínico. No obstante, se han relacionado tratamientos clínicos fallidos de aspergilosis con itraconazol (Janssen), anfotericina B (Bristol-Myers) y voriconazol (Pfizer) con valores CMI > 2 µg/ml. Este artículo es una revisión de los mecanismos de resistencia molecular a los antifúngicos descritos desde 2005; se mencionan, también, otras revisiones previas.

Palabras clave Resistencia a los antifúngicos, Mecanismos de resistencia

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The echinocandins

In vitro susceptibility. Anidulafungin, caspofungin and micafungin have excellent in vitro activity against most common *Candida* spp. (⩾ 99.6% susceptible); the exceptions are MICs for *Candida parapsilosis* and *Candida guilliermondii* (> 90% susceptible) [15,18,47]. Although in vitro cross resistance to echinocandins is rare, anidulafungin MICs have been lower than those of caspofungin for isolates of *Candida glabrata* [11] and *C. parapsilosis* [38]. The intrinsic reduced in vitro susceptibility reported for *C. parapsilosis* and *C. guilliermondii* [15,18,47] is unclear, since patients infected with some isolates of these two species have responded to echinocandin therapy. However, these higher MICs may predispose these strains to resistance development. On the other hand, treatment failures have been associated with MICs > 2 µg/ml [21,29,30,37,38]. Comparable amino acid mutations to those found in established caspofungin-resistant *Candida albicans* isolates have been reported in *C. parapsilosis* and *C. guilliermondii* [46]. It has been demonstrated that caspofungin inhibits ß-1,3-D-glucan synthesis and reduced in vitro growth not only of *Candida* spp. and *Aspergillus* spp. isolates, but also for some isolates of the following mould species: *Alternaria*, *Curvularia*, *Scedosporium*, *Acremonium*, *Bipolaris* and *Trichoderma* [25]. However, the determination of the minimum effective concentration (MEC) is a more practical alternative to assess the susceptibility of moulds to echinocandins in the clinical laboratory.

Molecular resistance mechanisms in* Candida *spp. The principal target of echinocandin activity is the protein Fks1; drug binding with this target produces a great deal of stress to the fungal cell due to glucan depletion by shutting down glucan synthase [46]. Specific mutations in *fks1* genes of the ß-1,3-D-glucan synthase complex of *Saccharomyces cerevisiae* (F639I, V641K, D646Y) and *C. albicans* (S645F, S645P, S645Y) are necessary for reduced susceptibility to caspofungin. These substitutions helped define the region, Ca*Fks1* Phe641-Pro649, as the "hot spot" 1 (HS1) [44,46]. The other "hot spot" has been termed HS2. Caspofungin MICs > 2 µg/ml for several clinical *C. albicans* isolates have also been associated with mutations in the *FKS1* gene (amino changes at ser 645F or 645P or 645Y Ca*Fks1p*) [5,44]. Progressive loss or reduced echinocandin susceptibility has been linked with HS1 and HS2 mutations in other *C. albicans* (F641Y), *C. glabrata* (analogous Fks2 residue) and *Candida krusei* (R1361G substitution in HS2 and T2080K in HS1) clinical isolates [24,27,44]. These *C. albicans* mutants also displayed 2 to 3 log shifts in the ED_{99} values in a murine model of disseminated candidiasis vs. caspofungin, and an equivalent shift in IC_{50} values in the inhibition of glucan synthase [44]. Although cross-resistance of caspofungin with micafungin and anidulafungin among the mutants has been reported [46], the MIC increase relative to a fully susceptible wild type strain has been consistently higher for micafungin and caspofungin than for anidulafungin. The clinical impact of these differences is yet to be fully understood. A relationship has been reported between *C. albicans* resistance to both fluconazole (increased expression of *MDR1* gene) and micafungin (*fks1* gene mutation) and its increased experimental pathogenicity (e.g., more rapid and extensive hyphae formation, an increased adherence to plastic and the propensity for biofilm formation), which could have an adverse effect during prolonged, dosage-increased treatment with both azole and echinocandin antifungal agents [4]. On the other hand, overexpression of *C. albicans CDR1, CDR2* or *MDR1* was not associated with significant reduction of either micafungin or caspofungin activity against this species [40], which indicates a class-specific resistance.

Molecular resistance mechanisms in other fungi. *Cryptococcus neoformans* is resistant to echinocandins. But this resistance is not related to the same target as that for *Candida* spp., since it was demonstrated that the *C. neoformans* target enzyme was sensitive to caspofungin despite the high echinocandin MICs usually obtained for this species [34]; this indicated a different resistance mechanism. However, substitutions in *FKS1* have resulted in resistance to anidulafungin, caspofungin and micafungin in *Aspergillus fumigatus* [20,51]. For more details regarding the clinical impact of resistance mechanism, see reference 26.

Paradoxical phenomenon. Some fungal yeast and mould isolates are able to grow at echinocandin high concentrations above the MIC, and this ability has been termed the paradoxical phenomenon or the "eagle effect". It is believed that this modest drug resistance or drug tolerance is the result of an adaptive cellular physiology [46,56]. Examination of the cell wall content of a *C. albicans* strain demonstrating this phenomenon in the presence of caspofungin showed an 898% chitin increase (to compensate for the decrease in ß-1,3-D-glucan) or a rapid shift or compensation in the key polymer, but this shift was not related to the Fks1 mutations described above [12,55]. An increased expression of the *C. albicans* and *C. glabrata* homolog *SLT2* gene, which encodes a mitogen–activated protein kinase of the cell wall integrity pathway, has been demonstrated during a paradoxical attenuation of caspofungin activity [62]. At elevated echinocandin concentrations, an incomplete growth inhibition and lack of fungicidal activity have been observed as compared to those obtained for the isogenic parent strain. The clinical impact of this phenomenon has not been fully determined, but it has not yet been reported in humans treated with high echinocandin doses [26]. However, the potential for other resistance mechanisms is underlined.

The azoles

In vitro susceptibility. The triazoles, fluconazole, itraconazole, posaconazole and voriconazole, are the most important azoles in the treatment of invasive fungal infec-

Table 1. Interpretive MIC breakpoints (µg/ml) and corresponding zone diameters (mm) for in vitro susceptibility testing of *Candida* species[a]

Antifungal Agent	Susceptible (S)	Susceptible-dose dependent (S-DD)	Intermediate (I)	Resistant (R)
Fluconazole	⩽ 8 ⩾ 19	16-32 15-18		⩾ 64 (µg/ml) ⩽ 14 (mm)
Voriconazole	⩽ 1 ⩾ 17	2 14-16		⩾ 4 (µg/ml) ⩽ 13 (mm)
Itraconazole	⩽ 0.125 NA	0.25-0.5 NA		⩾ 1 (µg/ml) NA
Flucytosine	⩽ 4 NA		8-16 NA	⩾ 32 (µg/ml) NA

[a] For fluconazole, these guidelines are based on mucosal candidiasis and limited data for invasive *Candida* infections. For itraconazole, the data were based on mucosal infections only. For fluconazole, susceptibility for S-DD MICs depends on achieving the maximum possible blood level: >400 mg/dose. For itraconazole, plasma concentration >0.5 µg/ml may be required for optimal response. For flucytosine, the clinical significance of intermediate MICs is still uncertain.

NA, not available

tions. *C. krusei* is intrinsically resistant to fluconazole, but susceptible to both posaconazole and voriconazole. Although itraconazole, voriconazole and posaconazole have in vitro activity against most moulds [14,53], fluconazole only has activity against some dermatophyte isolates and only posaconazole has activity against some zygomycetes. Interpretative breakpoints are available for fluconazole, itraconazole and voriconazole vs. *Candida* spp. (Table 1), but not yet for posaconazole. However, most posaconazole MICs for yeast and mould isolates are below 1 µg/ml [15,53]. Although resistance to the triazoles is not common among *Aspergillus* spp., it has been reported a few times, especially to itraconazole [14,18,53].

Molecular resistance mechanisms in* Candida *spp. The target of activity of the azoles is C14 lanosterol demethylase, an enzyme that is encoded by the *ERG11* gene (Erg11p). The result is ergosterol synthesis inhibition, accumulation of toxic products and growth inhibition. The most prevalent azole resistance mechanisms in *C. albicans* are (i) overexpression and mutations of the drug target enzyme (e.g., fluconazole resistance vs. decreased affinity of *ERG11p* to this agent) and (ii) overexpression of the efflux pump genes encoded by the *CDR* genes of the ATP-binding cassette (ABC) and Major Facilitator (MFS) class (e.g., overexpression of *CDR1*, *CDR*2, and *MDR1* and azole resistance association in *C. albicans*); the former mechanism impairs drug binding and the latter decreases intracellular drug concentration. Overexpression of *CgCDR1*, *PDH1*, *CdDR1* and *CdMDR1* transporters has been associated with resistance in *C. glabrata* and *Candida dubliniensis*, repectively [26,61]. In 2005, Pinjon et al. [48] reported the involvement of *CdCDR2* in *C. dubliniensis* azole resistance. Although the efflux pumps are localized on the plasma membrane and are quite numerous, only a few of these proteins are either drug transporters or have the ability to extrude drugs. For more details regarding *C. albicans,* see reference 1.

In vitro exposure of susceptible *C. glabrata* isolates (recovered before 1975) to azoles resulted in cross-resistance to these agents within four days of exposure to a single triazole (fluconazole) [6]. *C. glabrata* cross azole-resistance was associated with an increased expression of *CgCDR1*, *CgCDR2* [6] and, more recently, *CgSNQ2* genes [54]. PDR1 is known to encode a transcriptional regulator of multidrug transporters. It was reported in 2006 that *CgPDR1* contributed to azole resistance in both *C. glabrata* clinical isolates and petite mutants (mitochondrial dysfunction) [58]; until then, *PDR3* but not *PDR1* was known to be essential for the acquired high-frequency resistance that has been observed in *S. cerevisiae*

Vandeputte et al. [59] demonstrated that azole resistance in a *Candida tropicalis* isolate that was recovered from a patient treated with miconazole was due to overexpression of the Ct*ERG11* gene (missense mutation or tyrosine substitution). Only the *C. tropicalis MDR1* (*CtMDR1*) gene had been implicated in azole resistant in this species. However, this species has been the fourth most common species isolated from clinical specimens [46].

The membrane composition of the yeast cell has also been implicated in azole activity/resistance. Deletion of the gene *UPC2* (homolog of *UPC2*/*ECM22* genes in *S. cerevisiae*) in *C. albicans*, which is a regulator of the *ERG* genes involved in ergosterol synthesis, rendered cells hypersensitive to fluconazole and ketoconazole; overexpression of *UPC2* increased azole resistance. These results suggested that there was a link between changes in the ergosterol synthesis pathway and azole resistance [33]. The synthesis of sphingolipids (encoded by the *IPT1* gene), major constituents of membrane rafts, is essential to maintain the function of the plasma membrane in *S. cerevisiae*. The depletion or altered composition of sphingolipids impaired the function of the Cdr1p drug efflux pump, and as a consequence increased the susceptibility of *C. albicans* to azole activity [49]. The morphogenesis of the cell was also affected. The effect of altered membrane lipid composition on the localization of CaMdr1p and CaCdr1p multidrug transporters of *C. albicans* indicated that the latter transporter is selectively recruited over the former transporter for the proper localization as well as yeast function due to different lipid specificities [45]. Hypersensitivity of *Candida* spp. to antifungals has also been associated with either gene mutations or iron deprivation. It was reported that *C. albicans* isolates lacking either the response regulator Ssk1 or the Chk1p histidine kinase proteins (involved in adaptation to stress and the regulation of virulence) were hypersensitive (16-500 fold) to both fluconazole and voriconazole as compared to the wild type; the net result was an increased accumulation of these agents, especially of fluconazole, in the mutant strains [8]. These authors suggested that compounds which target these two proteins may be useful in synergistic therapy with triazoles by augmenting their activity.

In contrast to azole resistant isolates, susceptible *C. albicans* expressed *MDR1* at low levels; however, expression of *MDR1* has been induced by benomyl and other toxic compounds. Harry et al. [22] and Hiller et al. [23] have found that *cis*-activating regions of the *MDR1* promoter were responsible for the increased expression in response to azole agents. More recently, Cheng et al. [10] reported that in a *C. albicans* petite mutant (with uncoupled oxidative phosphorylation) the activity of both fluconazole and voriconazole was eightfold lower than that against the wild strain. The mutant overexpressed *MDR1*, which could have accounted for the decreased drug susceptibility. But other resistance mechanisms may be involved; further understanding is needed regarding the relationships between mitochondrial function, oxidative phosphorylation, sterol synthesis and azole resistance mechanisms [10].

Another less common mechanism is the inactivation of the sterol $\Delta^{5,6}$-desaturase (encoded by *ERG3*), an enzyme involved in the late stages of ergosterol synthesis [61]. Mutations in *EFG1* have been shown to affect hyphal formation and to reduce *C. albicans* virulence in a murine model. In addition, Efg1 was found to be involved in azole resistance by negatively regulating the expression of *ERG3* in this species [31].

***Molecular resistance mechanisms in* Aspergillus *spp*.** The mechanisms of resistance for *A. fumigatus* are different than those for *Candida* spp. Two related Cyp51 proteins (14-α-sterol demethylase, encoded by *cyp51A* and *cyp51B* genes) are present in *A. fumigatus*. Targeted disruption of the *cyp51A* in itraconazole-resistant and -susceptible isolates has yielded *A. fumigatus* strains with decreased azole-susceptibility (2-to 40-fold). These results confirmed that the drug target was Cyp51A. Two mechanisms of resistance to azoles have been described for *Aspergillus* spp.: point mutations of Cyp51A and reduced concentration of intracellular drug. The latter mechanism could be either the result of over expression of efflux pumps or due to reduced drug penetration [13,26]. However, although more than 40 ATP binding cassette transporters and more than 100 major facilitators transport genes have been identified [57], overexpression of efflux pumps as an azole resistance mechanism has been observed mostly in *A. fumigatus* mutants. The exception was the cli-

nical *A. fumigatus* isolate AF72, but it was only resistance to itraconazole. It has been reported that resistant mutant strains retained the ability to cause pulmonary disease in a neutropenic mouse model as compared to the wild-type isolate [36].

On the other hand, specific mutations in *cyp51A* have been associated with either cross-resistance to itraconazole and posaconazole (due to amino substitutions at glycine 54) or with itraconazole resistance and different susceptibility profiles to other azoles (due to substitutions at methionine 220) [9,35,36]. Mellado et al. [35] have recently suggested that substitutions of leucine 98 for histidine (L98H) in addition to the presence of two copies of a 34-bp sequence tandem in the promoter of the *cyp51A* gene was the mechanism responsible for azole cross-resistance and the increased levels of *cyp51A* expression. The synergistic activity of itraconazole and voriconazole was not present in gene-knockout *A. fumigatus* strains (*cyp51A*$^-$ and *cyp51B*$^-$); these results suggested that the inhibition of each of these two enzymes (Cyp51A and Cyp51B) had a similar effect and that they could be involved in the same ergosterol synthesis pathway. However, *cyp51A* and *cyp51B* could also act on either different substrata or they might aim at different steps in the ergosterol synthesis pathway [19]. The relevance of these in vitro results needs to be established.

It has also been demonstrated that although two genes (*erg3A* and *erg3B*) have been identified to be involved in the production of C-sterol desaturase enzymes, they did not have an apparent role in *A. fumigatus* viability. In contrast to findings in *Candida* spp., deletion of either one or both of these genes did not appear to alter the susceptibility to azoles or amphotericin B in *A. fumigatus* [2].

Amphotericin B

In vitro susceptibility. Few *Candida* clinical isolates have been reported to be resistant to amphotericin B, but high MICs (> 2 µg/ml) are frequently determined for a variety of filamentous fungi (e.g., *Paecilomyces lilacinus*, most *Scedosporium apiospermum* and *Scedosporium prolificans*, some *Aspergillus* spp. [especially *Aspergillus terreus* and *Aspergillus ustus*], *Alternaria* spp., *Fusarium* spp., *Penicillium marneffei*, *Phialophora* spp., and *Sporothrix schenckii*) [18]. In vitro breakpoints are available for filamentous fungi vs. amphotericin B (Table 2) and clinical resistance has been associated with MIC > 2 µg/ml [16,18]. Breakpoints with proven clinical relevance are not available for yeasts vs. amphotericin B due to methodology problems.

Table 2. In vitro interpretive MIC breakpoints (µg/ml) and corresponding zone diameters (mm) for in vitrosusceptibility testing of mould species[a]

Antifungal Agent	Susceptible (S)	Susceptible-dose dependent (S-DD)	Resistant (R)
Itraconazole	⩽ 1 ⩽ 17	2 14-16	⩾ 4 (µg/ml) ⩽ 13 (mm)
Posaconazole	⩽ 1 ⩾ 17	2 14-16	⩾ 4 (µg/ml) ⩽ 13 (mm)
Voriconazole	⩽ 1 ⩾ 17	2 14-16	⩾ 4 (µg/ml) ⩽ 13 (mm)
Caspofungin	⩽ 1 ⩾ 17	2 14-16	⩾ 4 (µg/ml) ⩽ 13 (mm)
Amphotericin B	⩽ 1 ⩾ 15	2 13-14	⩾ 4 (µg/ml) ⩽ 12 (mm)

[a] Adapted from reference [16]; categories are not based on correlation of in vitro vs. in vivo results.

Molecular resistance mechanisms in* Candida *spp. The polyenes (amphotericin B deoxycholate and its lipid-associated formulations) bind to ergosterol in the cell membrane. As a consequence, pores are formed which eventually lead to cell death. The mechanisms of resistance to amphotericin B are poorly understood, but decrease or lack of ergosterol content in the fungal cell membrane has been associated with resistance without affecting cell viability. These deficiencies could be the result of mutations in the genes that code some of the enzymes involved in the synthesis of ergosterol. Defects in the *ERG3* gene have been reported to lead to an accumulation of other sterols instead of ergosterol. In polyene-resistant *Candida* and *Cryptococcus* isolates, ergosterol content has been lower than in susceptible isolates [26]. Recently, Vandeputte et al. [60] studied the mechanisms responsible for the poor polyene susceptibility of a *C. glabrata* isolate that was associated with the production of pseudohyphal growth. These authors demonstrated that mutation in the *CgERG6* gene was the cause of both phenotypes. A possible association has been found between the high level of amphotericin B resistance and differential regulation of *ERG1*, *ERG25*, *SKN1*, and *KRE1* genes in *C. albicans* biofilms [28]. However, as it was previously hypothesized [3], the contribution of glucan changes to amphotericin B and fluconazole biofilm resistance in the same species has been recently reported [39]. References 32 and 50 reviewed this topic.

Flucytosine

In vitro susceptibility. Intrinsic resistance to flucytosine is relatively common among *C. albicans* (10%) and about 30% of this species isolates develop resistance during flucytosine treatment. Breakpoints are available for flucytosine vs. *Candida* spp. (Table 1) [18].

Molecular resistance mechanisms in* Candida *spp. After flucytosine is taken by the fungal cell and converts to 5-fluorouracil (the metabolically active form), it inhibits DNA replication and protein synthesis. Several enzymes are involved in the mode of action and resistance to flucytosine. This resistance is due to either (i) changes in the enzyme purine-cytosine permease (encoded by the *FCy2* gene), which is responsible for the uptake of the drug into the cell; or (ii) changes in the enzyme cytosine deaminase (encoded by the *FCy1* gene), which is responsible for the conversion to 5-fluorouracil; or (iii) changes in the enzyme uracil phosphoribosyltransferase, which is responsible for the transformation of 5-fluorouracil to 5-fluorouridine monophosphate (encoded by the gene *FUR1*). Most of these mechanisms have been specifically linked to resistance in *C. albicans* [61]. However, it has been shown that inactivation of the *C. lusitaniae FCY2* gene resulted in cross-resistance to flucytosine and fluconazole [7]. The contribution of *FCy1* and *FUR1*genes to this cross-resistance was more recently elucidated. Inactivation of the three genes (*FCY1*, *FCY2*, *FUR1*) in *Candida lusitaniae* produced two patterns of resistance to flucytosine, mutant *fur1* was resistant to 5-fluorouracil, while mutants *fcy1* and *fcy2* were resistant to fluconazole [43]. This is important information because flucitosine is mostly used in combination with amphotericin B or azoles such as fluconazole.

Terbinafine

Molecular resistance mechanisms. Although terbinafine is mostly used for the treatment of dermatophyte infections, it has been successfully administered in the combined therapy of systemic *S. prolificans* and other mould infections. Terbinafine belongs to the allylamines class that also inhibits the synthesis of ergosterol, but by the inhibition of the squalene epoxidase enzyme. The resulting fungal death is due to an increased membrane permeability (by high squalene concentration accumulation) and not to ergosterol deficiency. It has been reported that resistance to terbinafine was due to a single missense mutation in the squalene epoxidase gene leading to the amino acid substitutions L393F; this mutation was present in each of the six sequential terbinafine-resistant *Trichophyton rubrum* clinical isolates that were recovered from a single patient failing terbinafine treatment [41]. Amino acid substitution F397L in another single terbinafine-resistant clinical isolate was reported later [42]. In *Aspergillus* spp. mutants, resistance to terbinafine has been due to similar amino acid substitutions. The replacement of phenylalanine with leucine (Phe) at position 391 in the squalene epoxidase of *Aspergillus nidulans* and the equivalent mutation into the *ergA* gene of *A. fumigatus*, resulting in an F389L substitution, conferred terbinafine -resistance [52]. Since these isolates were not resistant to other classes of antifungal agents, this suggested that this mechanism was specific for terbinafine.

Conclusions

Much progress has been made toward a better understanding of the different molecular mechanisms of resistance; the clinical impact of this information is yet to be determined. In the meantime, the available standard methods and breakpoints could be helpful in monitoring the development of resistance during therapy as well as in identifying less susceptible or resistant isolates, because the probability of clinical response to therapy is much higher when the infecting isolate is susceptible to the therapeutic agent (60/90% rule).

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