Does hydrocortisone modify the in vitro susceptibility of *Aspergillus fumigatus* to itraconazole and amphotericin B?

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To analyse if hydrocortisone could modify the in vitro susceptibility of *Aspergillus fumigatus* to antifungal drugs, we developed a procedure to test the susceptibility of an *A. fumigatus* strain to amphotericin B and itraconazole, grown in the presence and in the absence of hydrocortisone. Conidia were germinated in the presence or the absence of hydrocortisone in Czapek medium without antifungal drug. A dilution of these cultures (5 × 10³ conidia ml⁻¹) was spread onto Czapek-agarose plates containing both antifungal drug and hydrocortisone. The cfu per plate were enumerated and compared. A therapeutic concentration of hydrocortisone induced a significant increase in the susceptibility to itraconazole. Conversely, the susceptibility to amphotericin B was not significantly modified when this antifungal drug was associated with hydrocortisone.

**Keywords** antifungal susceptibility, *Aspergillus fumigatus*, hydrocortisone

*Aspergillus fumigatus* is an opportunistic pathogen which often causes life-threatening diseases, namely invasive aspergillosis (IA) in immunocompromised patients. The incidence of this infection has increased greatly in recent years as a result of cancer therapy, organ transplantation and in AIDS patients [1–3]. Moreover, glucocorticoids are anti-inflammatory drugs that are sometimes used which constitute a risk factor in IA [4]. Prolonged treatment with high doses of these drugs severely impairs the macrophage and polymorphonuclear cell functions, which represent the most important process for the elimination of *A. fumigatus* [5–7].

Fungal–steroid interactions have long been studied but with different results. Progesterone, as well as deoxycorticosterone and dihydrotestosterone, has been shown to inhibit the growth of dermatophytic fungi although the biological significance of this phenomenon is not elucidated [8]. Oestrogens impaired mycelium-to-yeast-form transition of *Paracoccidioides brasiliensis* conidia [9]. A corticosteroid-binding protein and a possible endogenous ligand was found in the yeast *Candida albicans* [10]. More recently, two genes have been cloned and sequenced in *C. albicans*: one encoding an oestrogen-binding protein [11] and another encoding a corticosteroid-binding protein [12]. Furthermore, it has been shown that a therapeutic level of hydrocortisone directly enhances *A. fumigatus* growth in vitro [13]. This dual activity may potentiate the hormone action on *Aspergillus* allowing an easier dissemination in vivo [14]. As well as the difficulty of diagnosing invasive aspergillosis at an early stage [15], adapted treatment of this infectious disease involves antifungal drugs [16]. Cases of systemic mycoses are often treated with amphotericin B and itraconazole and they are sometimes used in combination for immunocompromised hosts, including AIDS patients [17]. As the same patient can be treated using both a glucocorticoid and an antifungal drug, it is important to study the simultaneous action of hydrocortisone and...
antifungal drugs (amphotericin B or itraconazole) on *A. fumigatus* development.

**Materials and methods**

**Amphotericin B and itraconazole**

Antifungal compounds were diluted in dimethyl sulphoxide (DMSO) to obtain a final concentration of 10%. Stock solutions were 1000 µg ml⁻¹ (amphotericin B) and 100 µg ml⁻¹ (itraconazole). They were stored at −20 °C (for the former) or at 4 °C (for the latter). Required amounts were added to Czapek-agarose medium which was then poured into Petri plates. Due to the light sensitivity of amphotericin B, the stock solution and the plates were poured and stored with an aluminium foil cover.

For each trial three antifungal drug concentrations were used: 0·005 µg ml⁻¹, 0·05 µg ml⁻¹ and 0·1 µg ml⁻¹ of itraconazole, and 0·005 µg ml⁻¹, 0·01 µg ml⁻¹ and 0·025 µg ml⁻¹ of amphotericin B.

**Hydrocortisone**

Hydrocortisone (Sigma, St Louis, USA) was dissolved in methanol at a concentration of 1 mM. This stock solution was diluted in the plate to a final concentration of 1 µM. Methanol at the same concentration was used as a control.

**Strain and culture**

An *A. fumigatus* strain (ALP⁺, CBS 144 89), kindly provided by M. Monod [18], was maintained on Sabouraud glucose agar–chloramphenicol slants at 4 °C. Conidia were harvested after 3 days at 41 °C on Sabouraud glucose agar. The conidial density was determined by haemocytometer count. A suspension of 10⁷ conidia ml⁻¹ in Czapek liquid medium supplemented with yeast extract (1 g l⁻¹) was allowed to germinate for 4–6 h both in the absence and in the presence of hydrocortisone 1 µM (Sigma, St Louis, USA) without antifungal drug. Culture conditions were 37 °C using an orbital shaker at 100 rpm. The germination rates obtained were consistently 80%. The germ tubes were from 2 µm to 15 µm in size without any ramifications. A 10 000-fold dilution of these suspensions (≈ 5 × 10⁶ germinated conidia ml⁻¹) were spread onto slants containing either amphotericin B or itraconazole (Janssen Pharmaceuticals, Beerse, Belgium). Hydrocortisone to a final concentration of 1 µM was allowed to diffuse for 1 h at 41 °C or 37 °C into plates. Controls were carried out allowing the corresponding amount of diluant to diffuse for the same time into control plates; cfu were counted after 12 and 24 h at 41 °C or 37 °C. The same procedure carried out in the absence of antifungal agents served as a control. Czapek-agarose (2%) slants were used and the significant results were checked using RPMI 1640-agar (Bio Medical Diagnostic, Marne La Vallée, France) medium as recommended by National Committee for Clinical Laboratory Standards for antifungal susceptibility testing of yeast [19]. Separate star-shaped colonies were obtained and counted as cfu after incubating overnight plus 24 h. (for the former) or at 4 °C (for the latter). Required amounts were added to Czapek-agarose medium which (five plates each) were compared using Student’s *t*-test.

Results

In the absence of antifungal drug, 233 (± 30·7) and 172 (± 23·9) cfu/plate were obtained with and without hydrocortisone, respectively. This difference was not significant at any temperature used. When the concentrations of amphotericin B and itraconazole were increased, the cell count was markedly decreased revealing the antifungal activity of the two drugs. Control experiments showed that DMSO (10% v/v) did not affect fungal growth. We used the 12-h mean cfu figure, as no discrepancies were observed between the cfu numbers at the two incubation times and for the two temperatures. The same result could be obtained at 37 °C after a 18 h incubation (data not shown).

**Itraconazole**

As shown in Fig. 1, the mean cfu number was significantly lower when using the medium supplemented with hydrocortisone for 0·05 and 0·1 µg ml⁻¹ of itraconazole (*P* < 0·001). Without hydrocortisone, the increase in itraconazole concentration resulted in a small significant decrease in the mean cfu/plate number: *P* < 0·01 for 0·05 µg ml⁻¹ over 0·1 µg ml⁻¹. This decrease became more significant in the presence of hydrocortisone: *P* = 0·001 for 0·005 µg ml⁻¹ over 0·05 µg ml⁻¹ on one hand and for 0·05 µg ml⁻¹ over 0·1 µg ml⁻¹ on the other hand. These results showed that hydrocortisone increased the susceptibility of this strain to increasing concentrations of itraconazole. The same result was obtained using RPMI 1640-agar medium (data not shown).

**Amphotericin B**

The mean cfu number/plate was greater when using media supplemented with hydrocortisone. This increase
Hydrocortisone and Aspergillus fumigatus

Fig. 2 Hydrocortisone incidence on amphotericin B action against A. fumigatus. Hydrocortisone decreased the susceptibility of A. fumigatus to increasing doses of amphotericin B. These results are the mean value of three determinations. § Statistically significant, \( P<0.05 \). Bars = ± SE.

Fig. 1 Hydrocortisone incidence on itraconazole action against A. fumigatus. Hydrocortisone increased the susceptibility of A. fumigatus to increasing doses of itraconazole. These results are the mean value of three determinations. * Statistically significant, \( P<0.001 \). Bars = ± SE.

was not significant for low amphotericin B levels (0.005 \( \mu \)g ml\(^{-1} \) and 0.01 \( \mu \)g ml\(^{-1} \)) and became slightly significant (\( P<0.05 \)) for the highest concentration of amphotericin B (0.025 \( \mu \)g ml\(^{-1} \)) tested, even though the cfu number is too low to be taken into account (Fig. 2).

Discussion

It has been shown previously that hormones such as progesterone can be hydroxylated by A. fumigatus [20]. In Cochliobolus lunatus a constitutive 17-beta-hydroxysteroid dehydrogenase activity was detected, the presence of androgen-binding proteins was found as well as the endogenous synthesis of testosterone, showing the existence of a complete signalling pathway which seemed to be related to the growth of the fungus [21]. Also, hydrocortisone was shown to increase the growth rate of A. fumigatus [13]. No effect of hydrocortisone on the germinative development was observed as no difference in the mean cfu/plate count was found. However, in vitro, hydrocortisone elicited some changes in the susceptibility of the strain of A. fumigatus tested to amphotericin B or itraconazole showing a non-significant decrease in the susceptibility for the former and an increased susceptibility for the latter antifungal drug. However, there may well be variation in the effect of hydrocortisone with different strains of A. fumigatus.

Amphotericin B is the ‘gold standard’ for invasive aspergillosis treatment. Part of its mechanism of action involves forming complexes with ergosterol in the fungal membrane causing membrane damage plus oxidative damage to the cell [22]. Our results showed a very weak impairment of amphotericin B action at the highest antifungal drug level, which may indicate an antagonistic effect of the two drugs enhancing the resistance of A. fumigatus.

Itraconazole has been used more recently for the treatment of invasive aspergillosis [23]. Numerous studies have shown that its most important target is the cytochrome P450-dependent enzyme lanosterol C\(_{14}\) \( \alpha \)-demethylase [22,24]. The blocking of this system creates an ergosterol depletion and a \( \Delta^{14} \) methyl sterol accumulation in the membrane which increases the susceptibility of the fungal cells to killing by phagocytes [25].

Microbial transformation of steroids such as progesterone or oestrogen are investigated for industrial purposes showing the involvement of constitutive cytochrome P450-dependent enzymes [26] allowing exogenous progesterone hydroxylation by A. fumigatus microsomes as well as whole cells. Progesterone hydroxylase activities can be found in multiple forms in this organism [27] and, among the cytochrome P450-related genes which have previously been cloned, some
are inducible such as the bphA gene of *A. niger* [28]. Conversely, glucocorticoids–*Aspergillus* interactions are poorly studied and until now no glucocorticoid receptor has been described in this fungus.

The decreased *A. fumigatus* cfu numbers in the presence of hydrocortisone corresponded to an increase in the fungus susceptibility to itraconazole. It could be hypothesized that the two drugs act synergistically on the same pathway. Moreover, in *C. albicans* imidazole antifungal drugs were found capable of displacing corticosterone from the corticosteroid-binding–protein-binding site [12]. Further investigations are needed to characterize more precisely the proteins of *A. fumigatus* involved in this process. As in the yeast, they could belong to the flavonoid-like protein superfamily possibly related to cytochrome P450 [12]. However, the mechanisms of action of both hydrocortisone and antifungal drugs on *A. fumigatus* may be more complex than suggested here. Even though hydrocortisone potentiates the itraconazole antifungal effect in *vitro* and at the two temperatures tested (37 °C and 41 °C), the glucocorticoid may compromise sufficiently the phagocytosis function to impair further treatment. Furthermore, although similar results were obtained in two different media, the transposition to the *in vivo* situation may be carefully carried out for at least two reasons.

The model developed here was elaborated to show the direct action of a glucocorticoid on *A. fumigatus*, so a therapeutic level of hydrocortisone was used. In contrast, the antifungal drug levels were too far below the MIC to allow us to compare a sufficient cfu number. In our experimental conditions the MIC were 0.05 μg ml⁻¹ and 0.25 μg ml⁻¹ for amphotericin B and itraconazole, respectively, although the E-test method (Bio Medical Diagnostic, Marne La Vallée, France), in current use in our laboratory, showed a MIC of 0.5 μg ml⁻¹ for itraconazole as well as amphotericin B (data not shown). This discrepancy results from the difference in the *in vitro* antifungal test used. Clinically, intravenous amphotericin B serum concentration can reach 1–2 μg ml⁻¹ and itraconazole 0.25 μg ml⁻¹ and no data are available on tissue concentration. However, the interaction that we found here with low antifungal drugs levels may not correlate with the situation *in vivo*.

Moreover, if glucocorticoids are impairing phagocytosis as well as macrophage development [29], then additional *in vitro* experiments including phagocytes or *in vivo* experiments should be used to extrapolate our findings to human disease and to assess if the direct action of a therapeutic concentration of hydrocortisone on the susceptibility of *A. fumigatus* to antifungal drugs may be of importance for antifungal therapy.

We are currently studying the effect of prednisolone and dexamethasone, glucocorticoids widely used in cancer and transplant therapy, on itraconazole and amphotericin B antifungal action using the same model. Thus we are also looking for a putative corticosteroid-binding protein in order to elucidate the mechanisms involved.

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**References**


Hydrocortisone and Aspergillus fumigatus


