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Interaction of local anaesthetics with other antifungal agents against pathogenic *Aspergillus*

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Abstract

Aspergillus spp. are responsible for an increasing number of fungal infections in immunocompromised and transplant patients. Local anaesthetics (LAs) are growth inhibitors of bacteria and yeasts. Subinhibitory concentrations of the LAs lidocaine and bupivacaine blocked the germination of *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger* whilst also showing a positive interaction in vitro with the antifungal activity of amphotericin B, itraconazole and caspofungin and a negative interaction with voriconazole. At higher concentrations, both LAs present fungicidal activity against resting conidia owing to cell membrane lesions. Verapamil, nifedipine and lanthanum produced a similar inhibitory effect on conidia germination. Calcium chloride reverted the inhibitory effect of verapamil and LAs. This study highlights that drug interactions may affect the clinical efficacy of antifungals, either promoting or limiting their action.
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Keywords: *Aspergillus*; Local anaesthetics; Calcium channel blockers; Conidial germination; Antifungal susceptibility

1. Introduction

Invasive aspergillosis is one of the most common and serious invasive fungal infections worldwide, usually associated with high mortality rates. Development of *Aspergillus* conidia to the hyphal form represents a crucial step in the progression to serious invasive disease [1–3]. *Aspergillus fumigatus* germinates significantly faster than *Aspergillus flavus* and *Aspergillus niger*, a fact probably related to increased virulence [4]. Thus, inhibitors of conidial germination may represent a new strategic target with significant therapeutic impact [1]. Combination therapy has recently been evaluated in vitro to validate its application in the clinical management of recalcitrant fungal infections [5]. However, drugs other than antifungals, usually administered simultaneously with antifungal therapy, may affect the development of fungal strains.

Several papers have described the antimicrobial activity of local anaesthetics (LAs) on different organisms. Lidocaine (LID) was the most active LA against pathogenic bacteria in vitro [6,7], an effect also confirmed in an in vivo model of surgical wound infection [8]. LID and bupivacaine (BUP) also showed fungicidal activity against *Candida* spp. by damaging the cytoplasmic membrane [9]. Additionally, both drugs are potent inhibitors of germ tube formation by *Candida albicans* as a result of calcium (Ca^{2+}) channel blockade [10].

Calcium and calmodulin are essential for the nuclear division cycle and growth of *Aspergillus nidulans*. Impairment of the expression of the unique gene for Ca^{2+}/calmodulin-dependent protein kinase prevents entry of conidia into the nuclear division cycle and germination, thus blocking development of the hyphal form [11]. LAs may show important antifungal activity against *Aspergillus*, helping to prevent the spread of infection. It would be of relevance to evaluate the effect of LID, BUP and Ca^{2+} channel blockers on the germination of pathogenic species of *Aspergillus*.

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2. Materials and methods

2.1. Organisms and growth conditions

Clinical isolates of *A. fumigatus* (six strains), *A. flavus* (five strains) and *A. niger* (five strains) belonging to the fungal collection of the Department of Microbiology, Faculty of Medicine, University of Porto were used. Organisms were grown in Sabouraud agar slants (Difco, Detroit, MI) at 25 °C for 5 days. Conidia were harvested by flooding the agar surface with phosphate-buffered saline (PBS) solution (Sigma–Aldrich Chemie GmbH, Steinheim, Germany).

2.2. Drugs and chemicals

Pure, preservative-free LAs (LID and BUP), the ion channel blockers verapamil (VER), nifedipine (NIF) and lanthanum oxide (LAN) as well as calcium chloride dihydrate were purchased from Sigma–Aldrich. LAN was dissolved in hydrochloric acid (Panreac Quimica SA, Barcelona, Spain). Amphotericin B (AMB) was purchased from Bristol-Myers Squibb Pharmaceuticals (Dublin, Ireland). Itraconazole (ITC) was a kind gift of Janssen-Cilag (Saunderton, UK), voriconazole (VRC) was donated by Pfizer Inc. (New York, NY) and caspofungin (CPF) by Merck Sharp & Dohme (Hoddesdon, UK). ITC and VRC were obtained as powder and treated according to Clinical Laboratory Standards Institute (CLSI) procedure M38-A [12]. CPF was diluted in sterile deionised water as recommended by the manufacturer.

2.3. Susceptibility testing

Determination of the minimal inhibitory concentration (MIC) of the antifungal agents AMB, ITC and VRC as well as of LID, BUP and the ion channel blockers was performed according to the CLSI M38-A protocol for susceptibility testing of moulds [12]. The concentration of antifungals ranged between 0.03 μg/mL and 16 μg/mL. Final LID concentrations ranged between 0.125 mg/mL and 4 mg/mL, BUP serial dilutions were 0.06–2 mg/mL, VER serial dilutions were 0.38–3 mg/mL, NIF serial dilutions were 1.25–10 mg/mL and LAN serial dilutions were 0.25–2 mg/mL. A reduction in growth of 90% defined the MIC, visually determined after incubation for 48 h at 37 °C. CPF activity was also determined according to CLSI M38-A protocol [12], except the minimal effective concentration (MEC) was evaluated [5]. The checkerboard dilution method was used for determination of the MIC or MEC whenever testing combinations of drugs, as previously described [5,13,14]. Interaction between antifungal agents and LAs was evaluated according to the following criteria: MIC or MEC four or more times lower than the control = synergistic effect; MIC or MEC two times lower than the control = additive effect; MIC or MEC two times higher than or equal to the control = indifferent effect; MIC or MEC four or more times higher than the control = antagonistic effect. Each evaluation was performed twice, on consecutive days.

2.4. Evaluation of conidia germination

Conidial germination was evaluated according to a previously described standard procedure [4] using a final inoculum of 5×10^5 conidia/mL. Conidia suspensions in RPMI-1640 with MOPS and serial dilutions of LID, BUP, VER, NIF or LAN (all at subinhibitory concentrations) were incubated at 37 °C for up to 12 h. The effect of calcium chloride on germination (concentration of Ca^{2+} ranging between 2.5 mM and 10 mM) was also studied. All controls were prepared in plain RPMI-1640 with MOPS. Germination (percentage) was checked hourly following the evaluation of 200 conidia under phase contrast microscopy (400×). Tests were performed in triplicate.

2.5. Evaluation of the mechanism of fungicidal activity

A final concentration of 10^6 conidia/mL was incubated both in RPMI-1640 with MOPS and in PBS, supplemented with serial dilutions of LID (concentration range, 1.25–10 mg/mL) and BUP (concentration range, 0.06–5 mg/mL). Following 7 h and 10 h incubation at 37 °C, conidia were washed and suspended in PBS with 2% glucose and stained with 1 μg/mL of propidium iodide (PI), which stains cells presenting serious damage to the cytoplasmic membrane [15]. A Beckman Coulter XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL) was used for detection and quantification of PI staining, as previously described [9]. Fluorescence was measured at FL3 (red, 620 nm).

2.6. Statistical analysis

Microsoft Excel 2000 and SPSS 11.5 (SPSS Inc., Chicago, IL) programs were used for data elaboration and analysis. The Wilcoxon signed rank test and Student's *t*-test for paired samples were used for statistical analysis. Data were considered significant at $P < 0.05$.

3. Results

All the isolates were susceptible to the antifungal agents AMB, ITC, VRC and CPF and showed high MIC values to LID, BUP and the ion channels blockers VER, NIF and LAN (Table 1). The activity of AMB, ITC and CPF on all *Aspergillus* strains was significantly enhanced in the presence of subinhibitory concentrations of both LAs (Table 2). No significant difference was found between *Aspergillus* species. Synergistic activity was found with the highest tested concentrations of both LAs: 0.5–2 mg/mL of LID and 0.25–1 mg/mL of BUP. LID activity corresponded to one-half of the activity of the same concentration of BUP. However, VRC showed a different pattern of effect compared with the other antifungals. In fact, in almost all isolates an indifferent effect was seen, whilst an antagonistic effect occurred in the presence of LID (five strains: three *A. flavus*, one *A.*

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