Antifungal Activity of Local Anesthetics Against Candida Species

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ABSTRACT

Objective: To evaluate the activity of benzydamine, lidocaine, and bupivacaine, three drugs with local anesthetic activity, against Candida albicans and non-albicans strains and to clarify their mechanism of activity.

Methods: The minimal inhibitory concentration (MIC) was determined for 20 Candida strains (18 clinical isolates and two American Type Culture Collection strains). The fungistatic activity was studied with the fluorescent probe FUN-1 and observation under epifluorescence microscopy and flow cytometry. The fungicidal activity of the three drugs was assayed by viability counts. Membrane alterations induced in the yeast cells were evaluated by staining with propidium iodide, by quantitation of intracellular K+ leakage and by transmission electron microscopy of intact yeast cells and prepared spheroplasts.

Results: The MIC ranged from 12.5-50.0 µg/mL, 5.0-40.0 mg/mL, and 2.5-10.0 mg/mL for benzydamine, lidocaine, and bupivacaine, respectively. The inhibitory activity of these concentrations could be detected with the fluorescent probe FUN-1 after incubation for 60 minutes. A very fast fungicidal activity was shown by 0.2, 50, and 30 mg/mL of benzydamine, lidocaine, and bupivacaine, respectively.

Conclusions: At lower concentrations, the tested drugs have a fungistatic activity, due to yeast metabolic impairment, while at higher concentrations they are fungicidal, due to direct damage to the cytoplasmic membrane. Infect. Dis. Obstet. Gynecol. 8:124–137, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS

lidocaine, bupivacaine, benzydamine, Candida, fungicidal

Candida albicans is a common causative agent of mucosal fungal infections,1,2 which are difficult to treat and tend to recur.1,3 There has been an increasing rate of candidosis, particularly due to a growing number of immunocompromised patients, e.g., due to iatrogenic measures and to persons infected by human immunodeficiency virus. The increasing use of antifungal drugs, both for prophylactic and therapeutic purposes, has been accompanied by the emergence of drug-resistant strains. The present study was undertaken to evaluate the activity of three local anesthetic drugs, benzydamine, lidocaine, and bupivacaine, against Candida albicans and non-albicans strains.
lactic and therapeutic purposes, has led to the emergence of resistant strains.\textsuperscript{4,5} This situation calls for the search for alternative antifungal drugs.

Many nonantibiotic drugs including antidiuretic, antidiabetic, \( \beta \)-blockers, psychotherapeutic, and nonsteroidal anti-inflammatory molecules possess an antimicrobial action, which has generally been regarded as a side effect\textsuperscript{6} and therefore neglected for potential clinical use. Benzylamine, lidocaine, and bupivacaine are known nonantibiotic antimicrobials. Topical use of these anesthetic drugs may be useful in the management of cutaneous and vaginal candidiasis. We studied the activity and mechanism of action of benzylamine, lidocaine, and bupivacaine against \textit{C. albicans} and non-\textit{albicans} strains.

**MATERIALS AND METHODS**

**Candida Strains**

Twenty \textit{Candida} strains were used: 18 clinical isolates and two American Type Culture Collection (ATCC) strains (Table 1). The yeasts were kept at -70°C in Brain-Heart broth (Difco Laboratories, Detroit, MI) with 5% glycerol until tested. For each experiment, the strains were subcultured twice on Sabouraud agar (Difco) for 24 hours at 35°C and either resuspended in saline (stationary growth phase cells) or subcultured in Sabouraud broth to the middle of the exponential growth phase.

**Antifungal Drugs**

Benzylamine was obtained from Lepori Angelini (Rome, Italy). Lidocaine and bupivacaine were purchased from Sigma (St. Louis, MO).

**Incubation of Yeast Cells With Drugs**

Yeast cells in stationary phase were resuspended in 10 mmol/L sodium N-2-hydroxyethylpiperazine-N-2-ethanesulfonic buffer (HEPES, pH 7.2), supplemented with 2% glucose (GH solution), at a density of \( 1 \times 10^6 \) to \( 5 \times 10^6 \) cells/mL, with or without serial concentrations of the drugs (see legends to figures). Incubations were carried out at 35°C, with shaking at 200 strokes/min. At the end of the incubation, the cells were centrifuged for 10 minutes at 1800g, and the antifungal activity of the drugs was assayed by viability counts and by staining with the fluorescent probes Propidium Iodide (PI) and FUN-I, as described below.

**Determination of Minimal Inhibitory Concentrations**

The minimal inhibitory concentrations (MICs) of the antifungals were determined by a macrodilution test, according to the reference method.
Yeast Cell Counts

The total number of yeasts in the suspensions was determined in a Neubauer hemocytometer (Agar Scientific Ltd, Stansted, UK). Enumeration of viable yeast cells in the untreated control suspensions and in those exposed to local anesthetics or sodium azide was carried out by counting colony-forming units (CFU) after plating serial dilutions (in saline) of the suspensions on Sabouraud agar plates. The number of colonies was counted after 48 hours of incubation at 35°C.

Studies of Membrane Damaging

Two independent procedures were used to assess the capacity of benzydamine, lidocaine, and bupivacaine to damage the fungal cytoplasmic membrane. One relied on the use of the membrane-impermeable fluorescent dye PI. Previous experiments have been carried out to optimize the flow cytometric conditions that were used in the current study (Pina-Vaz et al., in press). That is, optimal results were obtained when using 10⁶ yeast cells/mL, stained with 1µg/mL of PI, for 30 minutes in 0.05 mol/L sodium HEPES buffer, pH 7.2, at room temperature in the dark. Incubation with 1µg/mL of PI under the above-mentioned conditions had no toxicity to Candida cells (as determined by viability counts) and stained 100% of Candida cells killed by boiling for 30 minutes (Pina-Vaz et al., in press). For each sample, the percentage of PI-positive cells was determined by flow cytometry as described in detail below. From these values, the concentration of the assayed drugs resulting in 50% PI-positive cells was calculated according to a linear regression equation. The PI staining was found to be an adequate indicator of cell death (see “Results”). Therefore, we considered those drug concentrations causing half of the cells to be stained as representing median lethal concentration (LC₅₀).

The second method we used to study cytoplasmic membrane damage estimated the leakage of intracellular K⁺ from the yeast cells. Because the intracellular accumulation of K⁺ is higher in yeasts in the exponential growth phase, Candida cells grown at 35°C in Sabouraud broth supplemented with 0.5% K₂HPO₄ were harvested at the middle of the exponential growth phase. The yeasts were washed twice with saline and exposed at 35°C for 10 minutes to the assayed drugs dissolved in saline at indicated concentrations. After 5 and 10 minutes, treated and control suspensions were filtered through 0.45 µm Millipore filters (Millipore, MA). The filtrates were assayed for K⁺ using a K⁺-sensitive glass electrode connected to a Spotlyte analyzer (Menarini Diagnostics). The values are presented as the percentage of K⁺ leaked in comparison to that from cells boiled for 30 minutes. Viability counts and the percentage of cells stained by PI were also determined in the suspensions used for the K⁺ leakage assays. The leakage of K⁺ was also analyzed for Candida cells treated with 20 mM sodium azide, or with 2µg/mL of amphotericin B, for 10 minutes.

Assays of Metabolic Vitality

The processing of the fluorescent probe FUN-1 by the yeast cells was used to detect nonlethal metabolic alterations. Two methods to assess FUN-1
Fig. 2. Percentage of unviable (A, C, E) and PI-positive, (B, D, F) C. albicans, ATCC strain 10231 cells exposed for 30 min to increasing concentrations of benzylamine (benz.) (A, B), lidocaine (LID) (C, D) or bupivacaine (BUP) (E, F).

processing were used. Biochemically active cells, stained with this fluorescent membrane-permeable dye, exhibit under fluorescence microscopy orange/red cylindrical intravacuolar structures (CIVS), while nonviable cells or viable cells with severely impaired metabolism do not show these structures. Metabolically impaired yeast cells show an increased intracellular accumulation of the probe, which can be detected by flow cytometry. Untreated and treated yeast suspensions in GH solution were incubated with 0.5 µM of FUN-1 (Molecular Probes Europe BV, Leiden, Netherlands) for 30 minutes at 30°C in the dark. To evaluate CIVS formation, the FUN-1 stained cells were mounted on microscope glass slides with the anti-fading Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). The percentage of yeasts with CIVS was determined by observing 200 cells under epifluorescence microscopy in a Leitz Laborlux K (Leica, Buffalo, NY) microscope fitted
with a mercury 50-W lamp, a BP 450–490-nm excitation filter and a LP 515-nm emission filter. To quantify the intracellular concentration of FUN-1, flow cytometry was used as described below.

**Flow Cytometry**

The suspensions were analyzed at 620 nm (FL3) for PI and at 575 nm (FL2) for FUN-1, in a Beckman Coulter XL-MCL (Hialeah, FL) flow cytometer, equipped with a 15-mV argon laser with and without the fluorochrome (autofluorescence, as a control).

**Spheroplast Formation**

Spheroplasts of *C. albicans*, ATCC strain 10231, were obtained by enzymatic digestion of the cell wall with Lyticase (Boehringer Mannheim, Cat. No 1372464, Mannheim, Germany). Incubation was made at 35°C in YEPD medium (1% yeast extract, 2% bacto peptone, and 2% glucose), containing...
yeast cells grown to middle of exponential phase (about $1 \times 10^7$ cells/mL). The cells were collected by centrifugation at 1800g for 10 min, washed once with water and once with 1.4 mol/L sorbitol before the pellets were resuspended at a concentration of $1 \times 10^7$–5 $\times 10^7$ cells/mL in 0.04 M HEPES buffer (pH 7.4), 0.5 M MgCl$_2$, and 0.5% mercaptoethanol (Sigma) in 1.4 M sorbitol. Lyticase was added at a concentration of 10 units/10$^7$ cells, and the suspension was incubated at 30°C with gentle, occasional shaking. Spheroplast production was monitored by phase contrast microscopy, assessing the lysis of yeast cells exposed to 5% sodium dodecyl sulfate (SDS). Yeasts unexposed to Lyticase did not lyse with SDS. When most Candida cells were converted into spheroplasts, the suspension was cen-
Fig. 4. Exponential phase C. albicans, ATCC strain 10231 cells exposed for 10 min to 0.3 mg/mL of benzydamine (Benz), 50 mg/mL of lidocaine (LID), 30 mg/mL of bupivacaine (BUP) or 20 mM sodium azide (Na azide). In the same suspensions, three parameters were determined, i.e., the percentage of nonviable cells (determined by CFU counts) (A), the percentage of PI-positive cells (B), and K⁺ efflux (as percent of the total intracellular K⁺) (C).

Transmission Electron Microscopy
To analyze the ultrastructural alterations induced by the tested drugs, C. albicans blastoconidia and spheroplasts were studied. Blastoconidia of untreated and treated cells of C. albicans strain ATCC 10231, were prefixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, followed by washing in the same buffer. The cells were then fixed with 1.5% potassium permanganate in water for 1 hour, followed by washing with water and post-fixation with aqueous 1% uranyl acetate for 30 min. The samples were then centrifuged at 1800g for 10 minutes and the pellet resuspended in GH medium supplemented with 1.4 M sorbitol with indicated concentrations of the assayed antifungal drugs.
Samples of control and treated spheroplasts were prefixed with 2.5% glutaraldehyde (1 volume of 25% glutaraldehyde stock solution added to 9 volumes of spheroplast suspensions). After at least 4 hours at room temperature, the samples were washed with 0.1 M cacodylate buffer, pH 7.2, and fixed overnight at room temperature with 1% OsO₄ in 0.1 M acetate-veronal buffer, pH 7.0, supplemented with 10 mM calcium chloride. After washing with water, the cells were postfixed with aqueous 1% uranyl acetate for 30 minutes at room temperature. Fixed intact cells and spheroplasts were dehydrated in ethanol and embedded in Epon (TAAB Aldermaston, Berks, UK). Ultra-thin sections were cut with an LKB Ultratome III microtome (LKB-Produkter AB, Stockholm-Bromma, Sweden) and contrasted with uranyl acetate followed by lead citrate. To improve visualization of ribosomes in the spheroplasts, sections were treated with 3% hydrogen peroxide for 10 minutes before lead citrate staining. Observations and micrographs were done with a Zeiss EM 10C electron microscope (Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

Correlation coefficients (r) were calculated using the Anova program (program Statistica for Windows, StatSoft, CA).

RESULTS

The three drugs tested inhibited growth of all Candida strains studied, with MICs ranging from 6.25 to 50.0 μg/mL for benzydamine, from 1.25 to 40.0 mg/mL for lidocaine, and from 2.5 to 10 mg/mL for bupivacaine (Table 1). The correlation coefficients between the MIC of each drug for the 20 strains studied were good when comparing lidocaine with bupivacaine (r = 0.905), but not for benzydamine vs. lidocaine (r = 0.212) and not for benzydamine vs. bupivacaine (r = 0.328). Short exposure of Candida strains ATCC 10231 to the MIC of each of the three drugs did not result in any cell death and did not make the cells permeable to PI or induce any significant K⁺ leakage (not shown). However, under these conditions, the drugs significantly impaired the vitality of the Candida cells, as was shown by FUN-1 experiments. Thus, yeast cells exposed for 1 hour to the drugs at the MIC did not form CIVS (not shown), while an increase in the intracellular fluorescence was detected by flow cytometry (Fig. 1).

Treatment of C. albicans, ATCC strain 10231, with increasing concentrations of the anesthetics resulted in a dose-dependent fungicidal effect (Fig. 2A, C, and E). Staining with PI showed that the number of PI-positive cells correlated well with the number of nonviable cells (Fig. 2B, D and F). Figure 3 (A, B, and C) shows the effect of a 60-minute exposure of some of the Candida strains investigated to increasing concentrations of the drugs. The drug concentrations resulting in LC₅₀ strains were calculated for each strain (Table 1). The correlation coefficient between the LC₅₀ of each drug for the 12 strains tested was r = 0.073 when comparing benzydamine with lidocaine, r = 0.001 for benzydamine vs. bupivacaine, and r = 0.955 for lidocaine vs. bupivacaine.

The concentrations of the three drugs causing an extensive rate of killing (Fig. 4A) and permeability to PI (Fig. 4B) in C. albicans, ATCC 10231, cells induced a quick and extensive leakage of intracellular K⁺ (Fig. 4C). A similar pattern of K⁺ leakage was seen with the membrane-active antifungal amphotericin B, with 85.7% and 94.1% of intracellular K⁺ being lost after treatment of C. albicans ATCC 10231 cells with 2 μg/mL of the antibiotic for 5 and 10 minutes, respectively. Treatment of C. albicans, ATCC strain 10231 cells, with 20 mM sodium azide for 10 minutes resulted in extensive cell death (Fig. 4A) but did not make the cells permeable to PI (Fig. 4B), nor did it induce a significant K⁺ leakage (Fig. 4C). Candida glabrata strain H30 was the strain most resistant to the three drugs assayed. When it was treated with high concentrations of lidocaine, no K⁺ leakage was seen (not shown).

Transmission electron microscopy showed that exposure of C. albicans, ATCC strain 10231, to the described conditions—resulted in detergent-sensitive spheroplasts, which often had loose cell wall remnants (Fig. 6A). Only occasionally completely wall-free protoplasts were found. Spheroplasts not exposed to the drugs were intact and showed numerous ribosomes and normal intracellular organelles, i.e., the nucleus and the mitochondria (Fig. 6, A and B) and intact cell membranes with a continuous triple-layered profile (Fig. 7A). On the contrary, when...
Local anesthetic drugs possess antibacterial and antifungal properties. However, their antifungal mechanisms have not yet been elucidated.

Using a combination of complementary methodologies, we found the drugs to have a marked antifungal activity on C. albicans that ranged from growth inhibition to complete loss of viability. Growth inhibition can be detected by conventional protocols for MIC determination, as demonstrated in our study. We found that the fungistatic activity of the anesthetics could also be detected, with advantages, by use of FUN-1. Yeast cells exposed to MIC of the drugs showed both inhibition of conversion of the FUN-1 monomer into CIVS and an increase in diffuse intracellular fluorescence, as demonstrated by flow cytometry. These alterations were already detectable after an incubation period of 1 hour, thereby allowing for a more rapid assay as compared with the conventional protocol for MIC determination requiring 24 hours.

The following results point to the mechanism of the fungicidal action of the drugs being due to direct damage to the yeast cytoplasmic membrane. Thus, Candida cells exposed to fungicidal concentrations of the drugs quickly become permeable to the membrane-impermeable fluorochrome PI. This probe has previously been used to evaluate membrane permeability in yeasts and is considered a good marker for cell death associated with membrane alterations. Quantitation of K+ leakage from microorganisms, both from bacteria and yeasts, has also been used to evaluate membrane damage by various compounds. Yeast cells in the exponential growth phase are known to accumulate K+ intracellularly, with concentrations as high as 220 mM. This cation is quickly lost to the extracellular milieu when the selective membrane permeability is lost. A very quick and extensive K+ leakage was induced by fungicidal concentrations of the local anesthetics, with more than 90% of the intracellular cation being lost during the initial 10 minutes after exposure to 0.3, 50, or 30 mg/mL of benzydamine, lidocaine, or bupivacaine.
respectively. This indicates that the fungicidal effect results from a direct damage to the cell membrane, rather than from a metabolic impairment leading to secondary membrane damages. In support of this interpretation is our observation that exposure of the Candida cells to fungicidal concentrations of the metabolic inhibitor sodium azide induced K⁺ leakage at a much slower rate as compared to the three local anesthetics. As expected, the membrane-active antimicrobial agent amphotericin B also induced an extensive and rapid K⁺ leakage. The extensive loss of intracellular K⁺ may not be the sole factor responsible for the fungicidal activity of the drugs, as the membrane disorganization resulted in multiple perturbations that eventually could be lethal.

Additional support for a direct membrane damaging action of fungicidal concentrations of the anesthetics was evident from the quick lysis of spheroplasts in an osmotically protective medium, when exposed to fungicidal concentrations of the drugs. Bacterial protoplasts are quickly lysed by treatment with phenethyl alcohol, tetrazolium salts, or local anesthetics, molecules that are known to directly disorganize bacterial cell membranes. The ultrastructural studies revealed a second support for the assumption, which was the severe membrane alterations, with fracturing and solubilization, seen after 10 minutes of exposure to fungicidal concentrations of the drugs. The poor correlation found between the MIC or LC₅₀ of benzydamine and those of lidocaine and bupivacaine suggests that although the drugs kill Candida by acting on a common target (the cell membrane), the mechanisms for the membrane damage would differ in regards to benzydamine on the one hand and lidocaine and bupivacaine on the other.

Fig. 6. A. Untreated spheroplasts of C. albicans, ATCC strain 10231. Notice the loose partially digested cell walls (W), the intact protoplast with nucleus, mitochondria, and vacuoles. Section stained with uranyl-lead. (× 9600). B. Spheroplast from the same sample as in Fig. 6A. Section treated with 5% hydrogen peroxide for 10 minutes followed by lead staining. Notice the abundant ribosomes in the cytoplasmic matrix and 3 mitochondria (M). (× 50,600). C. Spheroplasts from the same preparation as in Fig. 6A but exposed to 0.3 mg/mL of benzydamine for 15 min. Notice several lysed spheroplasts (*) with collapsed cell walls. The nonlysed spheroplasts have a very altered ultrastructure. Section stained with uranyl-lead. (× 16,000).
The proposed mechanism for the fungicidal activity of the drugs is in conformity with the lipophilic properties of membrane-active molecules due to their lipophilic character. It is therefore expected that the antifungal activity of bupivacaine was higher than that of lidocaine because the former anesthetic is more lipophilic.\textsuperscript{30} The same difference in the activity between the two local anesthetics was also found in a previous study on the inhibition of germ tube formation by \textit{C. albicans}.\textsuperscript{19,22} A good correlation between the lipophilic and antibacterial activity of local anesthetics has also been described.\textsuperscript{9,18} These observations agree with the accepted interpretation that anesthetic-membrane interaction is of a hydrophobic character, whereby the anesthetic molecule ultimately penetrates the membrane bilayer and accommodates in its hydrophobic interior.\textsuperscript{31,32} Membrane splitting resulting from insertion of several lipophilic molecules, including the local anesthetic tetraacaine, into the hydrophobic core of bacterial membranes was described.\textsuperscript{33} This ultrastructural alteration was seen in the present study in lidocaine-treated \textit{Candida} plasma membranes (Fig. 7C).

The results showing that the three local anesthetics assayed possess an antifungal activity through a membrane-damaging action are in keeping with what has been reported for other lipophilic molecules. This is the case, among others, with amphotericin B,\textsuperscript{10} lipophilic azoles,\textsuperscript{34} butenafine,\textsuperscript{35} and phenothiazines.\textsuperscript{36,37}

The observed good correlation coefficients between the MIC (indicator of fungistatic activity) and LC\textsubscript{50} (indicating a fungicidal activity) of each of the drugs for the 12 \textit{Candida} strains tested would suggest that the mechanisms for the fungistatic and fungicidal activities of the drugs are similar, the plasma membrane being a common target. However, when exposing yeast cells to the fungistatic concentrations of the three drugs, no indication of membrane damage was found, since there was no permeability to PI nor production of K\textsuperscript{+} leakage. This may be explained by the short incubation time (10 minutes) used in the incubations prior to the above two membrane integrity tests.

The present results show that the three drugs now studied could be used topically to treat mucosal or cutaneous candidosis because concentrations with antifungal activity can be obtained with the commercial formulations available, mainly with benzydamine, the most active of the three drugs we tested. In fact, benzydamine is available in gels with 3% and 5% of the drug,\textsuperscript{38} and these concen-
Fig. 7.  **A.** High magnification of a spheroplast of control cells showing the continuous, triple-layered cytoplasmic membrane (arrow). W, partially digested cell wall. Section stained with uranyl-lead. (× 72,000).  
**B** and **C.** Same conditions as in Fig. 7A but the spheroplast had been exposed to 0.3 mg/mL of benzydamine (B) or 30 mg/mL of lidocaine (C) for 15 min. Notice the extensive solubilization of the cytoplasmic membrane, with only small remnants left (arrows). In C, the membrane remnants show zones with increased membrane thickness, indicating membrane splitting (arrow heads). W, partially digested cell wall. Sections stained with uranyl-lead. (× 72,000).
trations are 600 and 1000 times higher, respectively, than the MIC for the least susceptible Candida strain we studied. Benzydamine solutions for oral and vaginal applications contain 0.15%, which is 30 times higher than the MIC of the least susceptible Candida strain. Commercial formulations of lidocaine and bupivacaine contain drug concentrations that are up to 40 times above the MIC of most strains we studied. It should be emphasized, additionally, that the analgesic properties of the three drugs we studied represent an additional advantage for their topical use in the management of Candida infections.

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