In vitro antifungal activity and in vivo antibiofilm activity of cerium nitrate against Candida species

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Received 11 September 2014; returned 31 October 2014; revised 17 November 2014; accepted 23 November 2014

Objectives: The objective of this study was to clarify the antifungal properties of cerium, a lanthanide member, against Candida species. A comprehensive study with planktonic and sessile cells was performed. The ability of cerium nitrate (CN) to impair in vitro and in vivo biofilm formation was evaluated and its potential use in biofilm treatment was also evaluated.

Methods: Forty-eight clinical isolates of different Candida species and the type strain ATCC 90028 were tested according to the protocol M27-A3. The MICs and minimum lethal concentrations were determined. A time–kill assay was performed and a cytometric kinetic study was performed using live/dead markers. Biofilm inhibition and biofilm susceptibility in the presence of cerium was evaluated by quantification of the biofilm metabolic activity and total biomass with XTT and crystal violet assays, respectively. CN in vivo efficacy as a coating for medical indwelling devices was evaluated for the first time for Candida parapsilosis, using a mouse subcutaneous foreign body model using polyurethane catheter segments. Scanning electron microscopy was used to assess biofilm architecture after CN treatment.

Results: The MICs for planktonic cells correlated with severe cellular metabolic activity impairment and membrane damage after 3 h of incubation. Moreover, CN efficiently prevented biofilm formation both in vitro and in vivo in segments of polyurethane catheters. At higher concentrations, it was also able to disorganize and almost eradicate preformed biofilms.

Conclusions: Our results strongly suggest that CN application in the clinical setting might be effective in preventing the formation of biofilm-associated infections, namely through catheter coating and ultimately as an antimicrobial lock therapy.

Keywords: central venous catheters, healthcare-related infections, CVCs

Introduction

The dramatic increase in healthcare-related fungal infections involves a rise in the related mortality (30%–50%) and prolonged hospitalization, despite treatment with antifungal drugs.1–3 Candida albicans remains the most frequent fungal agent isolated in such settings. One of the several problems related to the treatment of Candida infections is therapeutic failure. Many of the most resistant infections are associated with biofilm formation.4,5 Biofilms are well-structured microbial communities where fungal cells are embedded within an extracellular matrix (ECM), becoming considerably more tolerant to antifungal therapy. That’s why biofilms are considered an important virulence factor.6–8 Biofilm infections usually involve medical indwelling devices, especially intravascular and urinary catheters, but also dental and laryngeal prostheses and other biomedical devices that are used in contact with skin and mucosal surfaces. Up to 40% of patients with Candida organisms isolated from intravascular catheters displayed concomitant fungaemia and the mortality rate of patients with catheter-related candidaemia is almost 40%.6,9–11 Despite C. albicans still being the most commonly isolated fungal species, biofilm-generating strains of
Candida parapsilosis, Candida tropicalis and Candida glabrata have been associated with considerably higher mortality rates.\textsuperscript{12,13}

Since classical antifungal drugs are not highly effective in eradicating sessile biofilm Candida cells, new drug alternatives are urgently needed.

Cerium is a member of the lanthanides or rare earth elements. It has diverse biological effects, due to its similarity to calcium, and despite its classification this element is not particularly rare. While the effects of cerium on calcium-dependent physiological processes are well characterized, its pharmacological properties remain to be fully explored.\textsuperscript{14} All applications addressed for cerium compounds remain controversial, with the exception of its use as a topical agent for the treatment of burn wounds. In fact, some studies describe a reduction in mortality of 20\%–50\% following treatment with cerium.\textsuperscript{14–19} This finding is mostly attributed to its immunomodulatory activity; the few studies addressing its antimicrobial effect produced contradictory results. The bacteriostatic or bactericidal effect of cerium nitrate has been reported by some studies, but refuted by others.\textsuperscript{14,15,18,20–22} Its synergism with silver (CN) or other cerium compounds has been reported by some studies addressing its antimicrobial effect produced contradictory results. The bacteriostatic or bactericidal effect of cerium nitrate has been associated with considerably higher mortality rates.\textsuperscript{12,13} Hence, the aim of this study was to clarify CN antimicrobial activity against Candida species and test its ability to either impair biofilm formation or to treat biofilms of the most clinically important Candida species.

Materials and methods

Strains

Forty-eight clinical isolates of Candida species corresponding to eight C. albicans, eight C. parapsilosis sensu stricto, eight C. glabrata, eight C. tropicalis, eight Candida krusei and eight Candida guilliermondii were used (see Table 1). C. guilliermondii strains were only characterized at the complex level. All strains had been previously isolated from patients admitted to Centro Hospitalar S. João (Porto, Portugal) and identified using the Vitek 2 system (bioMérieux, Vericieux, France). C. albicans type strain ATCC 90028 belonging to the ATCC was also used. Yeast strains were kept frozen in yeast peptone dextrose (YPD) medium (Formedium, Huntingston, UK) supplemented with 40\% glycerol at -70\°C until testing. For each experiment, microorganisms were subcultured twice on Sabouraud agar (Liofichem, Italy) at 35\°C for 24 h to assess the purity of the culture and its viability.

Chemicals

A 1 M stock solution of CN [cerium(III) nitrate hexahydrate, Sigma–Aldrich] was prepared in distilled water and filtered. Solutions of 0.5 \(\mu M\) FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-[benzo-1,3-thiazol-2-yl]-methylidene)-1-phenylquinolinium iodide] (Molecular Probes) and 1 \(\mu M\) propidium iodide (Sigma–Aldrich) were prepared in water supplemented with 2\% glucose. A 1 M stock solution of CN was prepared in distilled water and filtered. Solutions of 0.5 \(\mu M\) FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-[benzo-1,3-thiazol-2-yl]-methylidene)-1-phenylquinolinium iodide] (Molecular Probes) and 1 \(\mu M\) propidium iodide (Sigma–Aldrich) were prepared in water supplemented with 2\% glucose.

Antimicrobial activity

Antifungal susceptibility

The MIC of CN was evaluated according to the CLSI microdilution reference protocol M27-A3.\textsuperscript{23} For yeast strains. The tested concentrations ranged from 1000 to 1.9 \(\mu M\). The MIC endpoint was defined as the lowest drug concentration that inhibited 50\% of the microorganisms in microdilution wells when compared with the control.

To determine the minimum lethal concentration (MLC), 20 \(\mu L\) from each microdilution well was plated on Sabouraud agar and the plates were incubated at 35\°C for 48 h with subsequent counting of cfu. The MLC was defined as the lowest drug concentration that inhibited the growth of \(\geq 99.0\%\) of the inoculum.\textsuperscript{24}

Viability assays

C. albicans ATCC 90028 was grown overnight in 50 mL of Sabouraud broth at 37\°C and 180 rpm. Cells were harvested by centrifugation, washed with PBS (Sigma–Aldrich) and standardized to \(1\times10^6\) cells/mL in Sabouraud broth. Cells were allowed to grow in the presence of each of three concentrations of CN (0.03, 8 and 50 mM) for 24 h, at 37\°C and 180 rpm. At determined timepoints (0.5, 1.5, 3, 6 and 24 h), an aliquot from each culture was collected, spread in Sabouraud agar and incubated for 48 h at 37\°C. For each experimental condition, viable cells were counted in three different plates and the mean count was expressed as cfu/mL. The viability assays were performed three times on different days.

To unveil CN activity against Candida cells, flow cytometric studies were performed with two fluorescent markers: FUN-1, a probe that evaluates cell metabolic activity, and propidium iodide, a marker of cell death (only penetrates cells with a severe membrane lesion).\textsuperscript{25} At the same timepoints as the viability assays, a 1 mL aliquot from each treatment was collected, washed twice in distilled water and resuspended in 1 mL of FUN-1 or propidium iodide; the yeast suspensions were incubated for 30 min at 37\°C in the dark. Two controls were used for FUN-1 staining: untreated cells (CN free) stained with FUN-1 (negative control) and cells treated with 1 \(mM\) sodium azide (Sigma–Aldrich) for 1 h and stained with FUN-1 (positive control). Regarding propidium iodide, two staining controls were also used: untreated cells (CN free) stained with propidium iodide (negative control) and cells incubated at 90\°C for 30 min and stained with propidium iodide (positive control). The FACSCalibur flow cytometer (BD Biosciences, Sydney, NSW, Australia) analysed 20000 events in each acquisition. Results were expressed as: a staining index for FUN-1, defined as the ratio between the mean fluorescence of CN-treated cell suspensions and the corresponding value for the non-treated cells in the FL2 fluorescence channel (values \(\geq 1\) represent treatment effectiveness); for propidium iodide, as the percentage of cells showing fluorescence in the FL3 fluorescence channel.\textsuperscript{25}

Adhesion and biofilm formation

Medium and growth conditions

Candida strains were grown overnight in Sabouraud broth at 37\°C and 180 rpm. Afterwards, cells were harvested by centrifugation, washed with PBS and standardized to \(1\times10^6\) cells/mL. Yeast cell suspensions (with or without CN at subinhibitory concentrations) were mixed with \(2\times10^7\) microspheres/mL of 1 \(\mu m\) carboxylated highly green fluorescent polystyrene microspheres (Molecular Probes) and incubated at room temperature for 30 min with agitation (150 rpm). Following incubation, each suspension was vortexed and 50000 events were analysed in a FACSCalibur flow cytometer (BD Biosciences). Samples were analysed in the FL3 fluorescence channel (FL3: LP 670 nm) and the results were expressed according to two parameters: (i) percentage of cells with microspheres attached; and (ii) distribution pattern (Figure S1, available as Supplementary data at JAC Online). The CN concentrations were: C. albicans, 0.015 mM; C. glabrata, 0.001 mM; C. parapsilosis, 0.008 mM; C. tropicalis, 0.015 mM; C. krusei, 0.004 mM; and C. guilliermondii, 0.008 mM.
Biopfilm formation inhibition assays

To test the capacity of CN to inhibit the biopfilm formation of the six distinct Candida species studied, standardized yeast suspensions (1 × 10^6 cells/mL) were allowed to form biopfilm in 12-well polystyrene plates, for 24 and 48 h at 37°C in the presence or absence of CN. Four concentrations of CN were tested: 0.5× MIC, MIC, MLC and 50 mM. After incubation, biofilm metabolic activity was quantified using the semi-quantitative XTT reduction assay27 and biofilm total biomass was quantified with the crystal violet staining assay.28

Biofilm susceptibility assay

The biofilm susceptibility of the same Candida species to CN was evaluated as previously described.27 The 24 h biopfilms were challenged with CN concentrations ranging from 0.5 mM to 1 M and the minimum CN concentration that caused a 50% reduction in metabolic activity of the biofilm compared with the control (incubated in the absence of drug) was defined as the MIC for sessile cells (sMIC). Metabolic activity was measured by the XTT reduction assay. In order to evaluate the impact of this compound on biofilm biomass disorganization, the crystal violet staining assay was also performed.

In vivo biofilm formation

To test the ability of CN to prevent C. parapsilosis biofilm formation in vivo, a mouse subcutaneous foreign body infection model29 was used. C. parapsilosis OLO21 (clinical strain previously isolated from a central venous catheter (CVC)) was used in this study. Eight-week-old pathogen-free female BALB/c mice (Charles River), weighing 20 g, were used. Animals were housed in an environmentally controlled room with 12 h light/dark cycle and were maintained on a standard ad libitum diet. Animals were maintained in accordance with the Federation of European Laboratory Animal Science Associations criteria. Studies were approved by the Portuguese authority Direccão-Geral de Alimentacção e Veterena´ ria and by the Ethics Committee of the Faculty of Medicine, University of Porto.

Following anaesthesia, a 2 mm incision was made and six 1 cm segments polyurethane intravenous catheters (BD Vialon™ 16G, 1.7×45 mm) were implanted into the subcutaneous space. Before implantation, catheters were soaked for 24 h with CN at concentrations corresponding to 0.015, 8 and 50 mM. Catheters soaked with plain PBS were used as positive controls. Five animals were used for each experimental condition. Next, 1×10^6 cells/mL of C. parapsilosis was injected into the subcutaneous pockets. At day 7, animals were sacrificed, the catheter segments were aseptically removed and biofilm was quantified spectrophotometrically according to two different methodologies: XTT and crystal violet assays.27,28

Scanning electron microscopy (SEM) imaging

In order to evaluate the impact of CN on planktonic cells and biofilm architecture, SEM was performed. For imaging, planktonic cells were allowed to adhere to polystyrene coverslips for 24 h after each treatment.30 To visualize biofilms formed in the wells of polystyrene plates, the bottom of each well was extracted from the plate. To visualize biofilms formed in catheter segments, each segment was transected lengthwise. Sample preparation was carried out as described by Uppuluri et al.31 with minimum dehydration steps in an attempt to preserve the original structure of the cells and biofilm. Briefly, samples were placed overnight in a fixative solution of 4% (v/v) formaldehyde/1% (v/v) glutaraldehyde in PBS. After incubation, samples were washed in PBS and air dried in desiccators. For analysis, samples were coated with gold/palladium (40%/60%) and observed in a scanning electron microscope (JEOL JSM6301F/Oxford INCA Energy 350) in high-vacuum mode at 15 kV. Whenever justified, X-ray microanalysis (energy dispersive spectrometer) was performed in order to characterize sample elements.

CN resistance induction assays

A resistance induction assay32 was carried out to investigate whether CN is able to promote the development of Candida resistance following prolonged contact with the compound. C. albicans ATCC 90028 was used to perform this study. Yeast cells were incubated in YPD medium overnight at 150 rpm and 37°C; afterwards, an aliquot containing 10^6 cells/mL was transferred to different vials, each containing fresh culture medium with or without CN and incubated overnight as described above. Every following day, aliquots from each culture containing 10^6 cells were transferred into fresh medium containing the same concentration of CN and reincubated as described. Daily, during the 30 days of the assay, a 1 mL aliquot from each subculture was mixed with 0.5 mL of 40% glycerol and frozen at −70°C for later testing. In this experiment, four different concentrations of cerium(III) were used: one subinhibitory concentration (0.015 mM), one inhibitory concentration corresponding to the MIC and two concentrations above the MIC but under the MLC (3 and 6 mM).

Every 5 days of incubation, CN MIC values were redetermined according to the CLSI microdilution reference protocol M27-A3. The MLC endpoint was defined as the lowest drug concentration that inhibited 50% of the growth of microorganisms in microdilution wells when compared with the control.

Statistical analysis

Statistical analysis started with distribution normality assessment using the Kolmogorov–Smirnov test and histogram evaluation. Since the distribution of all variables was normal, only parametric tests were used. Adhesion in the presence of CN and CN biofilm inhibitory effect, at different concentrations, was evaluated with one-way ANOVA (with the Bonferroni correction). Student’s t-test (paired samples) was used to evaluate the activity of CN treatment in preformed biofilms. Statistical significance was considered as a P value <0.05. All statistical analysis was performed using SPSS software (v. 20.0).

Results

CN antmicrobial activity against planktonic cells

The susceptibility results and MLCs for planktonic Candida cells are shown in Table 1. The growth of all Candida species was inhibited by CN. The MLC corresponded to higher concentrations (8–13 dilutions above) of the MIC.

In order to understand CN antmicrobial kinetics, the viability of C. albicans cells treated with this compound was evaluated at determined timepoints (0.5, 1.5, 3.6 and 24 h). The tested concentrations were shown to be effective after 3 h of exposure, resulting in a decrease in viability of ~90% (P<0.05). Following 3 and 6 h of exposure, 50 mM promoted a significantly higher decrease in viability when compared with 8 mM; a total absence of viable cells was achieved following 24 h with both concentrations. Otherwise, cells treated with the MLC were able to recover after 3 h of incubation, showing no significant decrease of viability when compared with the control, even at 24 h (P=0.35) (Figure 1a). Under the same conditions, flow cytometric studies demonstrated that CN impairs cell metabolism; the MLC and 50 mM concentration caused a critical decrease in the cellular metabolism, starting after ~1.5 h of exposure and increasing over time. No differences were found between the two concentrations (MLC and 50 mM) for periods >3 h (including 6 and 24 h).
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The MIC endpoint for planktonic cells was based on visual determination of the lowest drug concentration that caused a 50% decrease in growth relative to the growth in the CN-free control well and the MLC was determined by cfu counting. The sMIC endpoint for biofilms corresponds to the lowest CN concentration resulting in a 50% reduction in metabolic activity relative to the metabolic activity of the untreated growth control, measured by the XTT reduction assay. Results are representative of at least three independent experiments performed in triplicate.
Figure 1. Antimicrobial activity of CN in planktonic cells. (a) A time-kill assay was performed to evaluate CN activity of MIC, MLC and 50 mM concentrations over 24 h. The percentage of viable cells, measured by cfu determination, was normalized to control (0 mM), which was taken as 100%. (b) Yeast cell metabolic activity measurement by flow cytometry after CN treatment. Metabolically active cells were able to convert FUN-1 into orange/red cylindrical intravacuolar structures; otherwise, cells with metabolism impairment were unable to convert FUN-1 and it remained dispersed in the cytoplasm, corresponding to the green fluorescence detected in the FL2 channel. Results are expressed as a staining index, i.e. the ratio between the mean fluorescence of treated cell suspensions and the corresponding value for the non-treated cells. (c) Flow cytometry measurement of the percentage of cells with membrane damage over time. Propidium iodide was used as a membrane injury marker, since its incorporation into nucleic acids only occurs following membrane disruption. Each result is representative of at least three independent experiments, performed in triplicate. (d–g) SEM representative images of *C. albicans* cells without CN treatment (d) and treated with 8 mM for 3 h (e), for 6 h (f) and for 24 h (g). White arrows highlight collapsed cells. Scale bars = 8 μm with 7000× magnification.
At 0.5 h, the MIC showed an effect (staining index = 1.04) that disappeared over time (Figure 1b). Propidium iodide was able to stain ~50% of the cells treated with 8 and 50 mM CN after 6 h of incubation, indicating cell death. After 24 h of treatment with these concentrations, almost 85% of cells presented membrane injury, with no statistically significant differences between the two concentrations ($P > 0.05$). The MIC was unable to induce membrane damage (Figure 1c).

SEM examination of cells treated with 8 mM CN revealed severe morphological alterations after 24 h of incubation. Cells were covered by a thin layer of cerium, exhibiting a wrinkled surface (Figure 1g). Cellular damage could be visualized, to a small extent, soon after 3 h of exposure, increasing progressively with time (Figure 1e and f).

**CN effect on adhesion and biofilm formation**

Since adhesion represents a crucial step in biofilm formation, we evaluated whether cerium has the ability to prevent *Candida* spp. adhesion. In fact, this compound was able to reduce significantly the adhesion of all *Candida* species tested, except *C. albicans* and *C. krusei*. Compared with the control for each species, which was assumed to be 100%, *Candida* species adhesion in the presence of CN was reduced to: *C. glabrata*, 39.2% ± 11.8%; *C. parapsilosis*, 42.2% ± 18.0%; *C. tropicalis*, 17.4% ± 8.2%; and *C. guilliermondii*, 61.0% ± 7.5%. CN antimicrobial activity together with the reduced adhesion observed for some species suggested the putative inhibition of biofilm formation by CN. In order to test this hypothesis, one subinhibitory and three inhibitory concentrations were tested for each species. Taking into account the tested concentrations, only the MLC and 50 mM efficiently reduced the biofilm metabolic activity (up to 90%) for all *Candida* species at 48 h (Figure 2a). For *C. glabrata* and *C. tropicalis*, the MIC significantly reduced biofilm metabolic activity. A significant reduction in biofilm total biomass was achieved with the MIC for *C. albicans*, *C. glabrata* and *C. krusei* and with the MLC for the other species (Figure 2b). No significant differences were found between the inhibition values of the MLC and 50 mM, both considering metabolic activity and total biomass. No differences were found in the biofilm metabolic activity from 24 to 48 h, but biofilm biomass was significantly reduced (data not shown).

Biofilm imaging of the bottom of polystyrene plates incubated with the CN MLC revealed an almost total absence of yeast cells (Figure 2g, h and i). Within strains of the different species used for SEM analysis, *C. tropicalis* were the only in which it was possible to visualize little spots of yeast cells, always surrounded by a relatively dense film (Figure 2j).

**Activity of CN against preformed biofilms**

CN was effective against planktonic cells with MICs ranging from 0.001 to 0.03 mM (Table 1). To understand whether CN exhibits antibiofilm activity, several concentrations were tested, as detailed in Figure 3. The sMICs, causing 50% reduction in metabolic activity, were much higher than the corresponding MICs for planktonic cells. Beyond its capacity to impair preformed biofilm metabolic activity, the effect of CN on biofilm biomass was also documented with the crystal violet assay. Notably, concentrations corresponding to the MLC for planktonic cells or just a few dilutions above promoted a dramatic decrease in biofilm biomass (reduction ranging between 63% and 92%) (Figure 3b).

SEM analysis supported the biofilm quantification results. As expected, the architectures of untreated biofilms belonging to different species were distinct. In the case of *C. albicans*, biofilms were composed of a network of hyphae and yeast cells that were well interconnected, while *C. glabrata* biofilms consisted of clusters of yeast cells adhered to each other by ECM. *C. parapsilosis* and *C. tropicalis* biofilms were essentially formed by yeast cell aggregates and pseudohyphae (Figure 3c–f). Treatment of preformed biofilms with 8 mM CN promoted a decrease in biofilm biomass accompanied by morphological changes (Figure 3g–j). Nevertheless, biofilms exposed to sMICs showed significantly less biomass and obvious morphological differences in comparison with control biofilms: yeast cells were smaller, displaying an irregular surface (Figure 3k–n). It was also possible to identify collapsed cells and other cells that were emptied of their contents (Figure 3g, h, j, l and n). Curiously, such findings were not found in *C. parapsilosis* strains. Additionally, yeast cells were surrounded with a film composed of cerium, carbon, oxygen, phosphorus and calcium as determined by X-ray microanalysis (Figure S2).

**CN activity against *C. parapsilosis* biofilms in an in vivo model**

The antimicrobial and antibiofilm activity of CN was assessed in an *in vivo* mouse subcutaneous foreign body infection model. To test the hypothesis that this compound could prevent catheter-related *Candida* infections, segments of polyurethane intravascular catheters soaked or not soaked with CN were challenged with *C. parapsilosis* yeast cells. Segments soaked with MLC and 50 mM cerium and challenged with *C. parapsilosis* exhibited a significant decrease in biofilm metabolic activity; up to 85.7% ($\pm 24.8\%$) reduction. The total biomass was reduced by 60% with MIC and MLC values.

SEM images show that both *C. parapsilosis* fully colonized the lumen of non-treated catheters; biofilms revealed a well-structured and dense network of yeast cells, pseudohyphae, matrix and possible components of the mouse immune system (Figure 4a and b). Catheters soaked with the CN MLC revealed a significant reduction in biofilm formation (Figure 4c). Moreover, the biofilm that still persisted displayed a very different architecture, being much thinner and unstructured (Figure 4d).

**CN failed to induce Candida resistance**

*C. albicans* yeast cells were challenged daily with different concentrations of CN for 30 days. All tested concentrations were unable to increase MIC values of this compound.

**Discussion**

The biological properties of the lanthanides have been the basis for research regarding their potential therapeutic application since the early part of the twentieth century. One of the most successful applications of this class of compounds involves the combination of cerium with nitrate. CN has been used as a topical antiseptic agent for the treatment of burn wounds with a
reduction in anticipated burn mortality. This decrease can be explained by the ability of CN to reduce alarm cytokine levels, to decrease leucocyte activation, to perform macromolecular leakage and finally to avoid burn oedema formation. On the other hand, its antimicrobial properties cannot be neglected. In the present study, MIC and MLC values were evaluated for all tested Candida species. MIC values differ slightly between the different species while the MLC is quite constant (8 mM for the majority of strains). The uniformity in Candida MLC values suggests a similar mechanism underlying cell death. Flow cytometric studies showed that metabolism impairment was significant after 3 h of exposure to the MLC, which ultimately resulted in membrane damage. Curiously, after 1.5 h of incubation with the MLC, a decrease in viability of 65% was observed. Even more puzzling were the results regarding the MIC, since it induced a decrease in cell viability over time, up to 3 h, similar to the decrease produced by the MLC. However, part of the population that maintained its membrane intact was able to overcome CN stress, displaying just a minimal impact on cell metabolism. Additionally, SEM analysis provided indications consistent with membrane and/or cell wall disruption.

Despite its described antibacterial activity, no serious attempts have been made to identify the mechanism underlying this phenomenon, except in one study involving Escherichia coli.

Figure 2. CN impairs biofilm formation by all tested Candida species. Quantification of biofilm metabolic activity (a) and biofilm biomass (b) after 48 h of incubation with four CN concentrations: a subinhibitory concentration (subMIC), the MIC and the MLC for each species and 50 mM. Results were normalized to control (0 mM), which was taken as 100%. Each result represents the mean of the eight strains tested for each species. Each strain was tested at least three times in triplicate. (c–f) Typical images of Candida biofilm grown for 48 h without CN treatment. (g–j) Images of Candida biofilm grown for 48 h in the presence of MLC. Scale bars = 60 µm with 1000x magnification. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
Figure 3. CN activity against preformed biofilms. Biofilms were grown for 24 h and then challenged with increasing concentrations of CN. (a) Biofilm susceptibility of preformed biofilms to CN was measured by an XTT reduction assay and (b) biofilm disaggregation was measured by a crystal violet assay.
which described cerium uptake into the cytoplasm with marked inhibition of cellular respiration, oxygen uptake and glucose metabolism. This study demonstrated that, contrary to what happens in human cells, cerium is taken up into the cytoplasm of bacteria and fungi, interfering with several metabolic functions.\textsuperscript{35} This pleiotropic effect is not surprising since the biological properties of cerium are strongly related to its similarity to calcium. This resemblance enables cerium to replace calcium in biological systems without necessarily replacing its functionality. Thus, it is plausible that cerium may act by blocking calcium channels or binding to calmodulin, inhibiting calcium-mediated processes and leading to an imbalance, disrupting cellular metabolism and the cell membrane.\textsuperscript{36,37}

Beyond its antimicrobial activity against planktonic cells, CN also demonstrated antiadhesion and antibiofilm activity. Nevertheless, concentrations below the MIC were not capable of impairing biofilm formation. Conversely, cells grown in the presence of CN lethal concentrations were unable to form biofilm or, in most cases, even replicate. Thus, the impairment of biofilm formation should be closely related to growth inhibition of planktonic cells rather than other events that inhibit adhesion, mostly due to cellular damage promoted by inhibitory concentrations. SEM visualization allowed confirmation that the bottoms of plates coincubated with the MLC were in general free of cells.

CN also exhibited a considerable effect on developed biofilms, resulting in decreases in biomass and metabolic activity of preformed biofilms. Biofilm cells showed some of the morphological alterations displayed by planktonic cells; nevertheless, the MICs for biofilms were higher than the planktonic MICs. The biofilm resistance appears to be a multifactorial complex event. In the present study, we can hypothesize that the ECM played an important role in the rise of MIC values. The ECM, beyond providing mechanical integrity, also has the ability to protect biofilm cells from environmental stresses as well as from the host immune system. It has also been described that one of the principal carbohydrate constituents of ECM, β-1,3-glucan, is involved in resistance to azoles, echinocandins, pyrimidines and polynides by restricting the antifungal drugs from reaching their targets.\textsuperscript{18–60} Previous studies by Al-Fattani and Douglas\textsuperscript{11,12} described a relationship between biofilm drug susceptibility and the matrix quantity visualized by microscopy. In our SEM images, it was possible to visualize that CN-treated biofilms exhibit a different matrix. Moreover, X-ray microanalysis revealed that cerium was one of the main elements present in ECM, suggesting that some CN was unable to reach the yeast cells. The differential composition of ECM found among the different species could also be involved in CN susceptibility. Although CN does not seem a better option for biofilm treatment, when compared with other available options such as echinocandins or amphotericin B lipid formulations, CN is very effective in biofilm disaggregation and could be a candidate for combined therapy.

Our results clearly show that CN disorganizes Candida preformed biofilms as well as impairing biofilm formation in vitro. One of the major problems related to biofilms is their ability to develop and thrive in medical indwelling devices. This colonization leads, in most cases, to catheter-related bloodstream infections (CRBSIs). CRBSIs result in longer hospital stay and considerably increased costs. It has been estimated that the global direct costs attributable to a hospital-acquired, laboratory-confirmed bloodstream infection correspond to €163.56 per case, while the costs are twice as high in the ICU setting compared with surgical wards.\textsuperscript{53–55} In order to combat CRBSIs, a number of new strategies and approaches have been developed. Early removal of the CVC, and thus the associated biofilm, has been advocated in some instances. To avoid catheter removal, which has risks for the patient, there has been a major focus on the implementation of clinical practice guidelines for the prevention of these infections and on the development of the ‘ideal catheter’. Different approaches such as antimicrobial lock therapy, catheter surface coating, material surface modification, novel drug delivery carrier systems, phage therapy and treatment with quarum-sensing molecules have been tried\textsuperscript{46–50} however, more effective alternatives would be desirable.

The ability of CN to reduce polyurethane colonization by C. albicans was already demonstrated in vitro.\textsuperscript{51} In vivo studies were also performed using catheter segments soaked with concentrations ranging from 1.5 to 6 mM, which resulted in inhibition of metabolic activity of ~40%–60%.\textsuperscript{59} Regarding C. parapsilosis, which is also a common fungal CVC colonizer, this is the first report describing CN activity both in vitro and in vivo. The inhibition results found for this species were similar to those for C. albicans, suggesting the 8 mM concentration for catheter coating.

Our in vivo results clearly demonstrated that, at relatively low concentrations, cerium efficiently prevented Candida biofilm formation on polyurethane catheters. Moreover, this effect seems to persist over time, which may be of great interest to the patient but also for economic reasons. Compared with other compounds used for CVC coating, CN exhibited the following advantages: it displays pleiotropic antimicrobial activity (bacteria and fungi); it is a chemical element abundant in nature, which makes it easily available and less expensive than present alternatives; and it is non-toxic since it cannot cross mammalian cell membranes and is used in clinical practice (at concentrations near 50 mM) to treat wound burns. Another important advantage described in this study is that cerium does not induce resistance in Candida, which is a problem with other available coatings.

This study undoubtedly demonstrated fungicidal activity of CN against planktonic Candida cells. However, additional efforts are required to completely elucidate its mechanism of action at a molecular and biochemical level. Most importantly, the study showed the ability of CN to prevent biofilm formation and to disorganize preformed biofilms. We strongly believe that the translation of this work to the clinical field would represent an important step towards the decrease in CRBSIs.

\textsuperscript{1091}
100 µm 100 µm

Figure 4. In vivo biofilm formation. C. parapsilosis were allowed to form biofilm for 7 days in segments of polyurethane catheters in a mouse subcutaneous pocket. The surface of untreated catheters (a and b) was completely covered by a complex, well-structured biofilm, while in catheters soaked with the MLC (c and d), CN almost completely prevented biofilm formation. Scale bars = 1 mm with 50x magnifications for catheter cross-sections and 100 µm with 500x magnifications for detailed biofilm structures.

Acknowledgements
We are grateful to Dr Daniela Silva from the Materials Centre of the University of Porto (CEMUP) for the helpful assistance with the SEM.

Funding
This work was supported by ‘Fundação para a Ciência e Tecnologia (FCT)’ in the scope of the project PTDC/CTP-EP/1660/2012. A. S.-D. was supported by a ‘Fundação para a Ciência e Tecnologia’ PhD grant (SFRH/BD/44896/2008) and J. M. M. was supported by FCT Ciência 2008 and cofinanced by the European Social Fund.

Transparency declarations
None to declare.

Supplementary data
Figure S1 and Figure S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


