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Investigation of the mechanism of action of 3-(4-bromophenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-one against *Candida albicans* by flow cytometry

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Abstract—The mechanism of action of the antifungal agent 3-(4-bromophenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-one against *Candida albicans* was investigated by flow cytometry, using propidium iodide, $DiBAC_4(3)$, and FUN-1 as the fluorescent dyes. A related but less active agent, together with amphotericin B and fluconazole, was tested in parallel for comparison of the results. The incrustoporine derivative was found to have a potent fungicidal activity on *C. albicans*, resulting in damage of cell membrane.

The incidence of invasive fungal infections caused by opportunistic pathogens, often characterized by high mortality rates, has been increasing over the past two decades, mainly due to the rise in the number of immunocompromised human hosts. Although some new drugs have recently been introduced to clinical practice, the number of available preparations to treat systemic fungal infections is still limited and more alternatives are needed, particularly with improved efficacy against emerging pathogens with limited susceptibility to the available preparations.[1,2]

3-(Halogenated phenyl)-5-acyloxymethyl-2,5-dihydrofuranones represent a novel class of butenolide antimycotics derived from (−)incrustoporine, a natural compound isolated from the extract of fermentation of the basidiomycete *Incrustoporia carneola* that was shown to have antifungal activity toward phytopathogenic molds.[3] Developments in the synthesis and tuning of antifungal activity of these incrustoporine derivatives have been reported over the last few years,[4–6] culminating in the recent publication of an extensive list of in vitro susceptibility testing results for three of these derivatives.[7] The compounds represent a promising group of wide-spectrum antifungal agents with high in vitro activities against a variety of relevant human pathogenic opportunistic yeasts and molds. From the clinical point of view, the significant activities found against fluconazole-resistant *Candida albicans* strains, *Candida krusei*, *Candida glabrata*, and *Aspergillus fumigatus*,[7] for example, are especially valuable.

As the next step in the study of incrustoporine derivatives, we have conducted preliminary investigations on the mechanism of action of 3-(4-bromophenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-one (LNO18–22) (the most potent derivative overall in the previous study)[7] in comparison with 3-(4-bromophenyl)-5-hydroxymethyl-2,5-dihydrofuran-2-one (LNO18),[5] plus amphotericin B and fluconazole as reference drugs with known

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mechanisms of action. We studied the influence of the compounds on a *C. albicans* strain by flow cytometry and simultaneous analysis of the kinetics of growth inhibition.

Flow cytometry has been previously recognized to have extremely valuable applications for such studies.[8] In this work, flow cytometric techniques involved staining with the fluorescent dyes PI (propidium iodide), $DiBAC_4(3)$ [bis-(1,3-dibutylbarbituric acid) trimethine oxonol], and FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1- phenylquinolinium iodide]. PI is an indicator of disturbance of plasmatic membrane integrity, a membrane-impermeant dye that only penetrates dead cells with porous membranes, increasing the fluorescence detected by flow cytometry after DNA intercalation.[9] $DiBAC_4(3)$ is an indicator of membrane potential that enters depolarized cells (but not mitochondria) where it binds to intracellular proteins or membranes and exhibits an enhancement of fluorescence.[10] Finally, FUN-1 is an indicator of metabolic vitality in yeast cells, a membrane-permeant fluorescent probe that is converted into orange/red cylindrical intra-vacuolar structures (CIVS) in metabolically active cells and remains in the cytoplasm as a bright diffuse green/yellow fluorescent stain in cells with impaired metabolism and/or disturbed plasma membrane integrity.[11]

The incrustoporine derivatives LNO18 and LNO18–22 (Fig. 1) were synthesized according to a previously described procedure.[5] Amphotericin B (Sigma) and fluconazole (Pfizer, Groton, United States) were used as standard antifungal drugs.[12]

The American Type Culture Collection *C. albicans* strain ATCC 90028 was used through the study and stored in skim milk medium (Becton Dickinson) at −40 °C before use. For each test the yeast strain was grown in Sabouraud's dextrose agar (Difco) for 24 h at 37 °C. Suspensions were prepared in RPMI 1640 medium, with L-glutamine, without sodium bicarbonate (Sevapharma, Prague, Czech Republic) and buffered to pH 7.0 ± 0.1 with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma), and determined microscopically using a Bürker's chamber in order to have $(2.5 \pm 0.1) \times 10^5$ CFU/mL. Solutions of the four compounds were added to the *C. albicans* suspensions in test tubes in order to obtain final concentrations of 0.1, 1, 10, and 100 μg/mL in a total volume of 5 mL. The final DMSO concentration in the test medium was 1% and all the solutions were prepared immediately before the tests. Growth control tubes containing drug-free cell suspensions in the test medium were included. Because of the expected formation of micelles by amphotericin B at high concentrations,[13] and the possibility of similar insolubility of the incrustoporine derivatives, drug control tubes with 100.0 μg/mL of each compound without cells were included. This allowed later flow cytometric confirmation of the discrimination between cells and the smaller compound micelles. All the tubes were later incubated for 22 h at 35 °C in humid atmosphere without agitation. The same samples were incubated in parallel for the Bioscreen broth test.[14] After the incubations, the cells were stained with the fluorescent dyes PI, $DiBAC_4(3)$, and FUN-1,[16] and analyzed in a flow cytometer.[17]

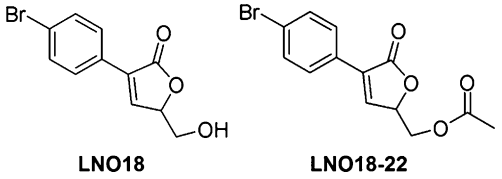

Figure 1. Structures of the incrustoporine derivatives LNO18 and LNO18–22.

In most cases, the kinetics of growth inhibition determined using the Bioscreen method (data not shown) did not reveal any additional information with the exception of the samples tested with a concentration of 1 μg/mL of LNO18–22. In this case, the compound caused a delay of about 8–10 h in the beginning of logarithmic phase, an effect that would not be detected by the isolated turbidimetric analysis of the growth inhibition at 24 h (Fig. 2).

Both the flow cytometric results (after staining with each of the three fluorescent dyes) and the values of the percentages of growth inhibition at the end of the incubation time are shown for each antifungal agent in Figure 3. Amphotericin B was the only agent to show influence on the cell growth with the lowest concentration (0.1 μg/mL). At higher concentrations complete growth inhibition was accompanied by cell death and damage of cell membranes evidenced by PI positivity, particularly at concentrations of 10 and 100 μg/mL (Fig. 3). With fluconazole, growth inhibition was never over 80% in comparison with the control and we observed a persistence of PI negativity through the whole range of concentrations, whereas the membrane potential and the metabolic activity were affected (Fig. 3).

Regarding the incrustoporine derivatives, the parental compound LNO18 only displayed total growth

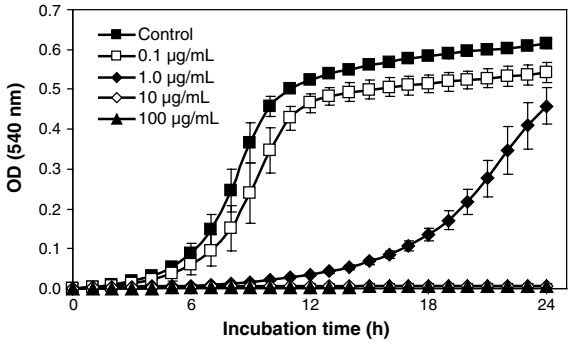

Figure 2. Growth curves of *Candida albicans* ATCC 90028 in control samples (closed squares) and samples treated with 0.1 (open squares), 1.0 (closed diamonds), 10 (open diamonds), and 100 μg/mL (closed triangles) of LNO18–22. *Notes.* Averages from a minimum of four values for PI and $DiBAC_4(3)$, and two values for FUN-1 ± standard deviations. In some cases the standard deviation bars are smaller than the symbols and thus not seen.

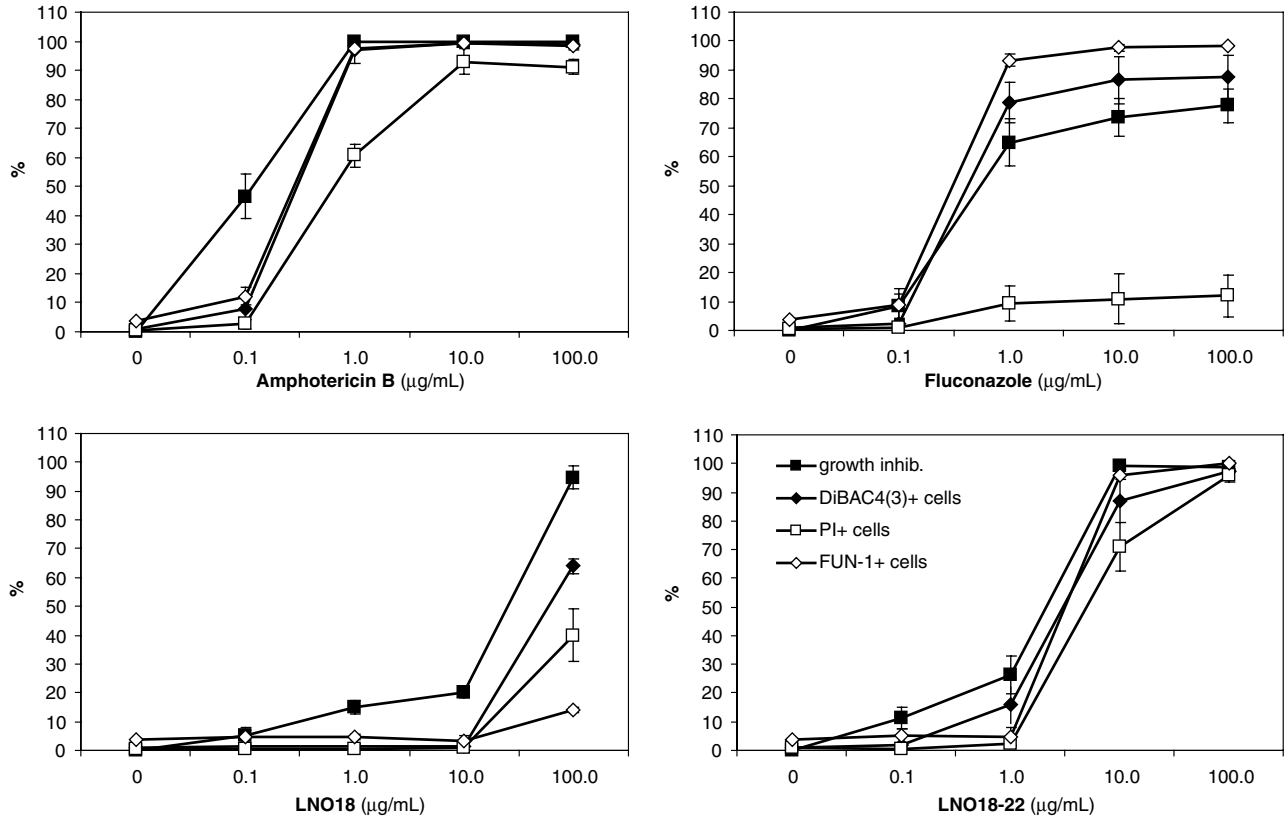

Figure 3. Percentages of growth inhibition in relation to the controls (closed squares) and $DiBAC_4(3)$ (closed diamonds), PI (open squares), and FUN-1 (open diamonds) positive cells after treatment of *Candida albicans* ATCC 90028 for 22 h with the logarithmic array of concentrations of amphotericin B, fluconazole, LNO18, and LNO18–22. *Notes.* Averages from a minimum of four values for PI and $DiBAC_4(3)$, and two values for FUN-1 ± standard deviations. In some cases the standard deviation bars are smaller than the symbols and thus not seen.

inhibition at the highest concentration (100 μg/mL) (Fig. 3). At the same concentration the flow cytometric results showed some degree of cell death (around 40% of the analyzed cells), while there was a somewhat higher incidence of plasmatic membrane depolarization, evidenced by over 60% of $DiBAC_4(3)$ positivity. On the other hand, LNO18–22 exhibited complete *C. albicans* ATCC 90028 growth inhibition and high cell death, cell membrane depolarization, and metabolic inactivity at 10 μg/mL. PI positivity closely correlated to the growth inhibition results through the range of LNO18–22 tested concentrations (Fig. 3).

The flow cytometric results obtained for amphotericin B and fluconazole were in perfect agreement with the expected findings according to the knowledge of their mechanisms of action.[18] The results obtained with amphotericin B confirmed its typical fungicidal mechanism of action through direct influence on the plasmatic membrane, producing high levels of cell death and impaired membrane integrity. On the contrary, PI negativity of fluconazole evidenced its fungistatic activity. Besides that, the fact that there was always at least 20% of growth in comparison to the controls, even for samples of cells treated with inhibitory concentrations of fluconazole, reflected its characteristic trailing effect.[19]

The closely related compounds LNO18 and LNO18–22 were previously shown to have significantly distinct antifungal activities.[5] LNO18–22 is obtained from the simple esterification of LNO18 with a consequent rise of lipophilicity, a characteristic that may have an important role in the ability of the compounds to penetrate fungal cells. LNO18–22 has a chiral center but there is no significant difference in antifungal activity between the enantiomers. The racemic mixture was even found to be more potent.[5] Previous studies of the effect of subinhibitory concentrations of incrustoporin derivatives (1/10 of the determined MICs) on the morphogenetic transition in *C. albicans* revealed that other compounds (particularly a 3,4-dichlorophenyl derivative which was shown to have an activity comparable to that of amphotericin B) could show more inhibition than LNO18–22, which had just a slightly higher influence than fluconazole.[7] Although the analysis of the kinetics of growth inhibition for LNO18–22 revealed the concentration of 1.0 μg/mL to cause the referred marked delay on the beginning of the exponential phase of *C. albicans* ATCC 90028 (Fig. 2), this effect was not accompanied by any physiological alterations detectable by our flow cytometric tests at the end of the incubation period. In fact, after 22 h of incubation the cells were already in full logarithmic phase and approaching the final cell density of control samples in stationary phase. No alterations of cell membrane potential and integrity or metabolic activity were detected at that concentration. At the concentrations of 10 and 100 μg/mL growth inhibition was accompanied by similarly high levels of cellular

membrane integrity disruption and consequent loss of viability as evidenced by PI positivity (Fig. 3). These high percentages of PI positivity, comparable to the ones found for amphotericin B, suggested a potent fungicidal activity resulting in damage of cell membranes, either by a direct interaction or by secondary results from metabolic impairment, leading to ion channel or pore formation and free diffusion of PI into the cytoplasm. On the contrary, a fungistatic mechanism would not be expected to produce significant alterations of cell viability, as seen with the results with fluconazole which maintained PI negativity through the whole range of test concentrations.

In conclusion, these flow cytometric results revealed a potent fungicidal activity for the incrustoporine derivative LNO18–22, resulting in disruption of plasmatic membrane integrity in *C. albicans*. Simultaneously, the known physiological consequences of the activities of the fungicidal amphotericin B and the fungistatic fluconazole were confirmed by our results, thus supporting the results for the experimental agent. More studies are required in the future to add further in-depth knowledge to these preliminary findings on the mechanism of action of this class of promising wide-spectrum antifungal agents.

Acknowledgments

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12. Stock solutions of 10 mg/mL were prepared for LNO18–22, LNO18, and amphotericin B in DMSO and of 5 mg/mL for fluconazole in sterile distilled water. The MICs of the drugs on *C. albicans* ATCC 90028 were available from previous work and were 0.50 μg/mL for LNO18–22, 1.00 μg/mL for amphotericin B, 0.25 μg/mL for fluconazole,[7] and 77.8 μg/mL for LNO18.[5]
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14. In every experiment, two aliquots (300 μL) of each incubation tube were distributed into wells of a flat-bottomed 100-well honeycomb microtiter plate and incubated in the Bioscreen reader.[15] The incubation program was set for 24 h at 35 °C with turbidimetric readings at intervals of 1 h. The reading wavelength was 540 nm and shaking of the plate was set for a period of 30 s at medium speed before each turbidimetric reading.
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16. Appropriate concentrations of the fluorescent dyes for our experimental conditions were determined through preliminary tests (data not shown). Stock solutions were prepared for PI (Sigma) in PBS at a concentration of 1 μg/mL, for $DiBAC_4(3)$ (Molecular Probes) in DMSO at 50 μg/mL, and for FUN-1 (Molecular Probes) in DMSO at 100 μM and stored at −40 °C before use. Each dye was used alone and experiments with PI and $DiBAC_4(3)$ simultaneously were also performed. After the described incubations with the drugs, the yeast cell suspensions were harvested by centrifugation for 10 min at 5000 rpm, washed, and resuspended in 3 mL of saline solution. Controls and other samples without growth inhibition during the incubation period were diluted to get the originally prepared cell densities. Aliquots (500 μL) of each sample were transferred to flow cytometry tubes and the dyes were added in volumes of 5 μL for PI, 2 μL for $DiBAC_4(3)$, and 2.5 μL for FUN-1, getting final dye concentrations of 0.01 μg/mL, 0.2 μg/mL, and 0.5 μM, respectively. Flow cytometric readings were started immediately after adding the dye to the last flow cytometry tube for PI and $DiBAC_4(3)$, corresponding to waiting times of around 10 min at room temperature. For FUN-1, the readings were done after a previous incubation of the suspensions with the dye for 20 min at 35 °C away from incident light.
17. Cytomix500 Flow Cytometer (Coulter Corporation, Hielah, FL), equipped with a 15-mW argon-ion laser with excitation at 488 nm. Forward scatter, side scatter, and fluorescence in FL3 channel (log red fluorescence, 620 nm) for PI, FL1 channel (log green fluorescence, 525 nm) for $DiBAC_4(3)$, and FL2 channel (log yellow/orange fluorescence, 575 nm) for FUN-1 were acquired and recorded using a logarithmic scale. For all samples a gate that excluded debris, cell clusters, and compound micelles was established in forward-scatter versus side-scatter dotplot cytograms and a minimum of 10,000 events (yeast cells) falling in the referred gate were acquired. The results were analyzed using WinMDI 2.8 software (Coulter Corporation, Hielah, FL). In order to obtain the percentage of positive cells regarding fluorescence in each sample, markers were adjusted in histogram representations of the number of events versus fluorescence of the controls to include a maximum of 5% of the events and then used in the analysis of all samples to define positive cells. In the experiments with PI and $DiBAC_4(3)$, dotplot cytograms of FL3 versus FL1 channel fluorescence were analyzed in addition to the referred separate histogram analysis.
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