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Imidazolium Salts as Antifungal Agents: Activity Against Emerging Yeast Pathogens, Without Human Leukocyte Toxicity

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Supplementary Information

Methods

Imidazolium Salts

The ILs C_4MImBF_4, $C_4MImNTf_2$, C_4MImPF_6, $C_6MImMeS$, $C_{10}MImMeS$, $C_{14}MImNTf_2$, $C_{16}MImBF_4$, $C_{16}MImPF_6$, $C_3OMImMeS$ and $C_7O_3MImMeS$ were synthesized through halide-free methodologies. The ILs $C_{10}MImCl$, $C_{16}MImCl$, $CH_2COOHMImCl$ and $C_3H_6COOHMImCl$ were synthesized through halide precursors. All the methods are described in the literature and the spectral data were in agreement with those reported previously[3,11-13].

Antifungal activity

A total of 32 clinical isolates of 4 opportunistic yeast species were tested for the antifungal susceptibility: *Trichosporonasahii* (TAH05, TAH09, TAH10, TAHA15, TAHA16, TAHARL40, TAHARL46, TBL23), *Candida parapsilosis* (RL11, RL01, RL05, RL07, RL13, RL20, RL27 e RL32), *C. glabrata* (RL 02, RL03, RL09, RL12, RL25, RL26, RL34 and RL35) and *C.tropicalis* (72A, 72P, 94P, 102A, 17P, RL18, RL17, RL16). The clinical isolates used in the study are all from infection cases including systemic, respiratory and urinary tract infections in HIV and non-HIV patients. All isolates are deposited in the Mycology Collection of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Minimal inhibitory concentrations (MIC) of the ILs against emergent yeasts were determined by the broth microdilution method according to M27-A3 documents of the Clinical Laboratory and Standards Institute (CLSI, 2008)[14], using RPMI-MOPS (RPMI 1640 medium containing L-glutamine, without sodium bicarbonate – Sigma-Aldrich – buffered to pH 7.0 with 0.165 $mol.L^{-1}$; MOPS buffer – Sigma). The concentrations of the ILs ranged from 1.9-500 $\mu g.mL^{-1}$ and 100 µL aliquots of the

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cultures were transferred to a flat-bottom 96-well microtiter plate. The MIC was defined as the lowest concentration of compounds at which the microorganism tested did not demonstrate visible growth. Fluconazole and ketoconazole were used for comparison and positive controls in the applied assay. All experiments were carried out in triplicate. Minimum fungicidal concentrations (MFCs) were determined by subculturing 10 µl from each well into 1 mL of sterile Sabouraud broth and incubation at 32 ºC for 3 days. The MFC was defined as the lowest test concentration without growth of the organism. Also the values of MIC50 were determined, representing the drug concentration that inhibits the growth of 50% of the isolates.

Ergosterol quantification method

In order to assess the ability of yeast sterol biosynthesis inhibition by the most effective IL (**C_{16}MImCl)**, the sterol present in the cell membrane was extracted as previously reported[16], determining the presence of ergosterol and the late sterol intermediate 24(28)-DHE [24(28)-dehydroergosterol]. The reduction of detectable ergosterol in extracts indicates action on the ergosterol biosynthesis.

For analysis, a 20 µL aliquot of sterol extract was diluted 5-fold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm. Theergosterol content was calculated as a percentage of the wet weight of the cell by the following equations: % ergosterol + % 24(28)DHE = [(A281.5/290) • F] / pellet weight, % 24(28)DHE = [(A230/518) • F]/pellet weight, and % ergosterol = [%ergosterol + % 24(28)DHE] – % 24(28)DHE, where F is the factor for dilution in ethanol and 290 and 518 are the E values (in percentages per cm) determined for crystalline ergosterol and 24(28)DHE, respectively.

Blood sample collection

The peripheral blood samples were collected by venipuncture, using syringe (BD Diagnostics, Plymouth, UK) and tubes with heparin. Subsequently, the leukocytes were isolated and a suspension was adjusted to obtain $8x10^3$ cell/mm^3. This leukocyte suspension was aliquoted and incubated with different ILs for one hour, namely: C_{10}MImCl (16 µg mL^{-1}), C_{16}MImCl (10 µg mL^{-1}), C_{16}MImPF_6 (35 µg mL^{-1}), C_{10}MImMeS (65 µg mL^{-1}), C_{16}MImBF_4 (16 µg mL^{-1}), and C_{14}MImNTf_2 (10 µg mL^{-1}). After that, one aliquot of each sample was used to perform the comet assay and the cell infeasibility analysis. The concentrations used in toxicity assays took into account the

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results obtained from the antifungal activity assay (MIC). The Research Ethics Committee approved the study protocol (No.23081) and informed consent was obtained from all individuals whose information was collected prospectively.

Single cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described in the literature in accordance with the general guidelines for use of the comet assay[20], which was performed in triplicate for each IL concentration tested. Each sample was analyzed in duplicate (two slides with one hundred cells per slide) and two different individuals evaluated each slide. Cells were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration). Therefore, the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration).

Cell infeasibility

The cell infeasibility assay was carried out using cells exposed to the same IL concentrations tested in the comet assay[23]. After the incubation, 100 mL of a leukocyte cell suspension was homogenized for 3 min. with 100 mL ofa 0.2% Trypan Blue solution in phosphate buffer. The cell infeasibility was determined microspically (400 × magnification) and two categories of cells were scored: (1) Living cells that appeared uncolored or light blue; and (2) dead cells that appeared blue colored. At least 300 cells were counted for each survival determination.

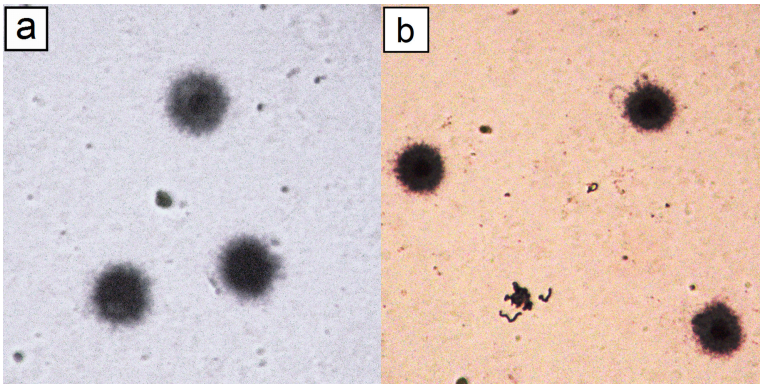

Fig. SI1 Microscopic images of leukocyte cells before (a) and after (b) treatment with IL C_{16}MImCl.

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REFERENCES

(1) N. Azie, D. Neofytos, M. Pfaller, H. U. Meier-Kriesche, S. P. Quan, and D. Horn, *Diagn. Microbiol. Infect. Dis.*, 2012, **73**, 293.

(2) Z. A. Kanafani and J. R. Perfect, *Clin. Infect. Dis*., 2008, **46**, 120.

(3) a) P. Wasserschied and T. Welton, *Ionic Liquids in Synthesis*, ed. VCH Wiley, Weinheim, 2nd edn, **2008**; b) J. Lu, F. Yan and J. Texter, *Prog. Polym. Sci.* 2009, **34**, 431.

(4) R. P. Swatloski, J. D. Holbrey, S. B. Memon, G. A. Caldwell, K. A. Caldwell, and R. D. Rogers, *Chem. Commun.*2004, 668.

(5) M. Petkovic, J. Ferguson, A. Bohn, J. Trindade, I. Martins, M. B. Carvalho, M. C. Leitão, C. Rodrigues, H. Garcia, R. Ferreira, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, *Green Chem.* 2009, **11**, 889.

(6) R. F. M. Frade and C. A. M. Afonso, *Hum. Exp. Toxicol.*, 2010, **29**, 1038.

(7) R. Ferraz, L. C. Branco, C. Prudêncio, J. P. Noronha and Z. Petrovski, *ChemMedChem.*, 2011, **6**, 975.

(8) D. Demberelnyamba, K.-S. Kim, S. Choi, S.-Y. Park, H. Lee, C.-J. Kimb and I.-D. Yoo, *Bioorg. Med. Chem.*, 2004, **12**, 853..

(9) W. L. Hough-Troutman, M. Smiglak, S. Griffin, W. M. Reichert, I. Mirska, J. Jodynis-Liebert, T. Adamska, J. Nawrot, M. Stasiewicz, R. D. Rogers and J. Pernak, *New J. Chem.* 2009, **33**, 26.

(10) G. R. Thompson III, J. Cadena and T. F. Patterson, *Clin. Chest. Med.* 2009, **30**, 203.

(11) H. S. Schrekker, D. O. Silva, M. A. Gelesky, M. P. Stracke, C. M. L. Schrekker, R. S. Gonçalves and J. Dupont, *J. Braz. Chem. Soc.*, 2008, **19**, 426.

(12) C. C. Cassol, G. Ebeling, B. Ferrera and J. Dupont, *Adv. Synth. Catal.*, 2006, **348**, 243.

(13) Z. Fei, D. Zhao, T. J. Geldbach, R. Scopelliti and P. J. Dyson, *Chem. Eur. J.* 2004, **10**, 4886.

(14) Clinical laboratory standards institute (CLSI): Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard – Third Edition. CLSI document M27-A3. Wayne: Clinical Laboratory Standards Institute, 2008.

(15) M. Cuéllar-Cruz, E. López-Romero, J. C. Villagómez-Castro and E. Ruiz-Baca, *Future Microbiol*. 2012, **7**, 755.

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(16) B. A. Arthington-Skaggs, H. Jradi, T. Desai and C. J. Morrison, *J. Clin. Microbiol.* 1999, **37**, 3332.

(17) J. R. A. Santos, L. F. Gouveia, E. L. S. Taylor, M. A. Resende-Stoianoff, G. A. Pianetti, I. C. César and D. A. Santos, *Antimicrob. Agents Chemother.* **56**, 2553 (2012).

(18) R. Musiol and W. Kowalczyk, *Curr. Med. Chem.*, 2012, **19**, 1378.

(19) N. Hirayama, T. Higo and H. Imura, *Anal. Sci.*, 2012, **28**, 541.

(20) a) G. F. F. S. Montagner, M. Sagrillo, M. M. Machado, R. C. Almeida, C. P. Mostardeiro, M. M. M. F. Duarte, I. B. M. Cruz, *Toxicol. in Vitro*, 2010, **24**, 1410; b) R. R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.-C. Ryu and Y. F. Sasaki, *Environ. Mol. Mutagen.*, 2000, **35**, 206; c) A. Hartmann, E. Agurell, C. Beevers, S. Brendler-Schwaab, B. Burlinson, P. Clay, A. Collins, A. Smith, G. Speit, V. Thybaud and R. R. Tice, *Mutagen.*, 2003, **18**, 45; d) S. Nadin, L. Vargas-Roig and D. Ciocca, *J. Histochem. Cytochem.*, 2001, **49**, 1183.

(21) E. I. Cortés-Gutiérrez, M. I. Dávila-Rodríguez, J. L. Fernández, C. López-Fernández, A. Gosálbez and J. Gosálvez, *J. Histochem. & Cytochem.*, 2011, **59**, 655.

(22) P. Moller, *Basic Clin. Pharmacol. Toxicol.*, 2006, **98**, 336.

(23) B. B. Mischell and S. M. Siingi, *Selected methods in cellular immunology* (W. H. Freeman Company, San Francisco, 1980).

(24) A. R. Collins, A. A. Oscoz, G. Brunborg, I. Gaivao, L. Giovannelli, M. Kruszewski, C. C. Smith, R. Stetina, *Mutag*.2008, **23**, 143.

(25) M. A. Ghannoum and L. B. Rice, *Clin. Microbiol. Rev.*1999, **12**, 501.