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FULL PAPER

Nitrate esters of heteroaromatic compounds as novel *Candida albicans* CYP51 enzyme inhibitors

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Abstract: Four heteroaromatic compounds bearing nitrate esters were selected using a virtual screening procedure as putative sterol 14α-demethylase (CYP51) *Candida albicans* inhibitors. Compounds were examined for their inhibition on *C. albicans* growth and biofilm formation as well as for their toxicity. NMR studies*, in silico* docking and molecular dynamics simulations were used to further investigate selectivity of compounds to fungal CYP51. All compounds exhibited good antimicrobial properties, indicated with low minimal inhibitory concentrations and ability to inhibit formation of fungal biofilm. Moreover, all the compounds had the ability to inhibit growth of *C. albicans* cells. *N*-(2-nitrooxyethyl)-1*H*-indol-2-carboxamide was the only compound with selectivity on *C. albicans* CYP51 which did not exhibit cytotoxic effect on cells isolated from liver and should be further investigated for selective application in new leads for the treatment of candidiasis.

Introduction

Dimorphic yeast *Candida albicans* is among the most common fungal pathogen for humans causing different health problems from vaginal and oral infections to systemic and lethal candidiasis.[1] Fungal infection caused by *Candida* species is increasing during the last years due to immunosuppressed patients from HIV, organ transplantation and cancer.[2] More than 17 different *Candida* species are known to be the cause of infections, among them *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida crusei* are the main cause of more than 90% of invasive infections.[3]

Azole drugs, which usually constitute the first choice for antifungal therapy, act by inhibiting sterol 14α-demethylase (CYP51) enzyme that is involved in ergosterol biosynthesis. They bind to and inhibit the heme moiety of enzyme and lead to depletion of ergosterol and accumulation of toxic intermediates of ergosterol biosynthesis.[4] Azoles are in clinical use since 1969,[5] and since then numerous different compounds have been investigated and new compounds have been introduced. Increased number of immunocompromised patients as well as increasing resistance to azole drugs are the main reasons for further investigation of potential new antifungals.

During *in silico* screening of a small in-house chemo-library, nitrate esters of heteroaromatic compounds were predicted to have the potential to inhibit CYP51 *Candida albicans* enzyme. Organic nitrate esters have previously shown antianginal activity, while their conjugates with NSAIDs are not ulcerogenic. However, the antimicrobial activity of this class of bioactive compounds was not investigated. There is only one recent report by Kutty *et al.*,[6] on nitrate esters possessing strong biofilm inhibition activity. To this extent, in the present study, four nitrate esters have been selected and evaluated *in vitro* for their binding and selectivity on candidal CYP51 and their consequent anticandidal activity. To a

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step further, NMR studies, *in silico* docking and molecular dynamics simulations were implemented to shed light on the binding mode of the proposed structures and provide information regarding the observed differences in activity.

Results and Discussion

Selection of compounds

Virtual screening was performed using a High throughput docking algorithm (Glide HTVS) on the homology model of *Candida albicans* CYP51 enzyme. The four compounds were selected on the basis of their ability to interact with (a) the heme iron, (b) crucial aminoacids of the active site and (c) scoring values. The docked poses of each of the selected compounds are presented in Figure S1 (Supporting Information) and the corresponding docking scores are shown in Table S1 (Supporting Information).

Anti-candidal activity of selected compounds

The anti-candidal activity of selected compounds is presented in Table 1. All of the *C. albicans* clinical isolates were susceptible to tested compounds with the similar susceptibility. Good antifungal activity of compounds (average minimal inhibitory concentration of 0.0028 mg/mL and minimal fungicidal concentration of 0.0056 mg/mL) was recorded. Compound MK94 had strongest inhibitory activity towards *C. albicans* followed by compound MK56. Commercial drug, ketoconazole, was used as positive control and it showed lower inhibitory and fungicidal concentrations compared to tested compounds.

Table 1. Anti-candidal activity of tested compounds.

Fungal strains	*Candida albicans* 475/15		*Candida albicans* 527/14		*Candida albicans 10/15*		*Candida albicans* 13/15	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
MK55	0.056±0.001^{c} 224.692±4.01	0.112±0.002^{c} 449.384±8.02	0.028±0.001^{b} 112.346±4.01	0.056±0.002^{b} 224.692±8.02	0.056±0.001^{c} 224.692±4.01	0.112±0.01^{c} 449.384±40.12	0.028±0.001^{b} 112.346±4.01	0.056±0.0[illegible]1^{b} 224.692[illegible]
MK56	0.028±0.001^{b} 107.181±3.83	0.056±0.001^{b} 214.362±3.83	0.028±0.001^{b} 107.181±3.83	0.056±0.001^{b} 214.362±3.83	0.056±0.001^{c} 214.362±3.83	0.112±0.01^{c} 428.725±38.28	0.028±0.001^{b} 107.181±3.83	0.056±0.002^{b} 214.362±7.66
MK129	0.056±0.001^{c} 179.349±3.2	0.122±0.002^{c} 390.725±6.41	0.028±0.001^{b} 89.675±3.2	0.056±0.001^{b} 179.349±3.2	0.056±0.002^{c} 179.349±6.41	0.112±0.01^{c} 358.698±32.03	0.028±0.001^{b} 89.675±32.03	0.056±[illegible]^{b} 179.349±6.[illegible]
MK94	0.028±0.002^{b} 80.149±5.72	0.056±0.001^{b} 160.298±2.86	0.028±0.002^{b} 80.149±5.72	0.056±0.002^{b} 160.298±5.72	0.028±0.001^{b} 80.149±2.86	0.056±0.002^{b} 160.298±5.72	0.028±0.002^{b} 80.149±5.72	0.056±0.0[illegible]2^{b} 160.298±5.72
Ketoconazole	0.0031±0.0001^{a} 5.833±0.19	0.0062±0.0001^{a} 11.667±0.19	0.00312±0.0001^{a} 5.871±0.19	0.00625±0.0001^{a} 11.761±0.19	0.0031±0.0001^{a} 5.833±0.19	0.05±0.0001^{a} 94.086±0.19	0.0016±0.0002^{a} 3.011±0.38	0.05±0.000[illegible] 94.086±0.[illegible]

MIC – minimum inhibitory concentration.
MFC – minimum fungicidal concentration.
The results are expressed in mg/mL (upper raw) and in μM.(lower raw) for each compound and strain.
Values are expressed as means ± SD. In each row, different letters mean significant differences between samples ($p<0.05$)

Antibiofilm activity of selected compounds

Impact on biofilm formation was anti-quorum sensing trait detected for all of the tested compounds, with MK56 showing the strongest effect with 80% inhibition at MIC and 87% at ½ MIC. Ketoconazole, together with MK55 also had strong activity on biofilm formation and at MIC concentration inhibited it for 80% and 73% and at ½ MIC for 73% and 61%, respectively (Table 2).

Table 2. Percentage of inhibition of biofilm formation after treatment with minimal inhibitory and sub inhibitory concentrations of the compounds.

	MIC	**½ MIC**	**¼ MIC**	**1/8 MIC**
MK 55	73	61	27	-
MK 56	80	87	74	54
MK 94	45	15	-	-
MK129	56	48	14	11
Ketoconazole	80	73	71	52

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Binding affinities on CYP51 enzyme

Different concentrations of compounds were incubated with CYP51 enzyme isolated from *C. albicans* but also human and bovine. Recorded binding spectra showed that compound MK55 binds to CYP51 enzyme isolated from *C. albicans* with dissociation constant (K_D) 4.19μM (Figure 1a), while the other tested compounds MK56, MK94 and MK129 didn't exhibit any binding affinity towards CYP51 enzyme, indicating a different mode of action. Since MK55 binds to candidal enzyme, it was tested for selectivity on human and bovine CYP51 enzymes showing good selectivity only to candidal CYP51 (Figure 1b). Commercial antifungal drug ketoconazole was also tested and showed dissociation constant CaCYP51 (<0.05μM); hCYP51 (<0.05μM).

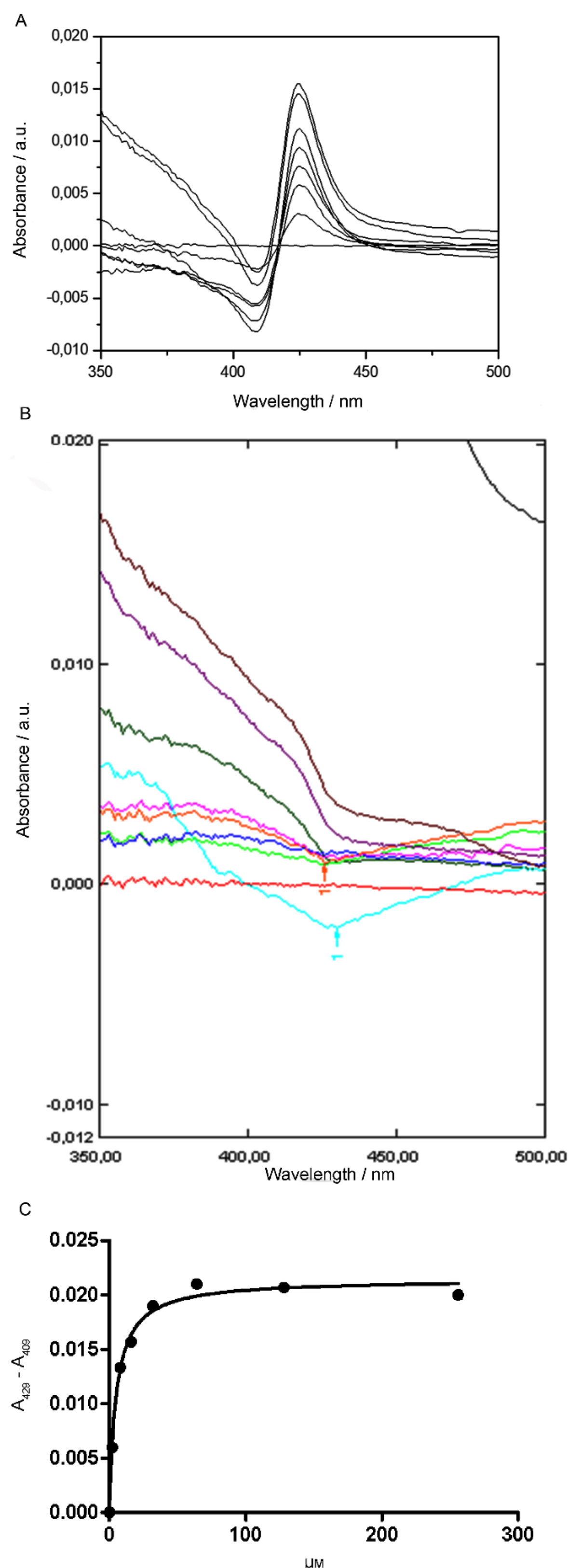

Figure 1. Binding spectra 350-500 nm recorded after titrating (A) *Candida albicans* and (B) human enzyme with increasing concentrations of MK55; (C) saturation curve of MK55.

NMR studies

The binding of compound MK55 to CYP51 isolated from *C. albicans* was further investigated at atomic level by the application of 1D STD and 2D transferred NOESY experiments in the D_2O buffer. The STD effects were observed for all carbon bound protons indicating that MK55 is buried inside the enzyme providing contacts of all proton rich moieties with the protein. The sign change of the ligand NOE cross peaks was observed upon addition of the enzyme in the NOESY spectra of MK55, which is typically observed at ligand-enzyme binding. In the NOESY spectra of MK55 recorded in the absence of the enzyme only trivial positive NOE cross-peaks were observed. In the transferred NOESY spectra of MK55 in the presence of the enzyme besides the strong negative trivial NOE cross-peaks, weak negative NOE cross-peaks between the H3 proton of indole ring and the methylene protons of the ethyl spacer were observed. This is the only difference in NOE pattern between the free and bound ligand states, which indicate a different relative orientation of the nitooxyethyl moiety with respect to the indole ring in the two ligand states. In the ligand bound state the methylene protons of the nitooxyethyl moiety are in close spatial proximity (< 5Å) to H3 proton of the indole ring (Figure 2).

Molecular docking studies

A structural model of *C. albicans* CYP51 was prepared based on the crystal structure of Saccharomyces cerevisiae CYP51 (Figure 3), in order to perform docking of MK55 and evaluate its binding affinity in respect to the experimental one. MK55 was the only compound which was experimentally found to bind selectively to *C. albicans* CYP51. For comparison reasons MK56 was also docked, since it shares common structural features with MK55 but fails to bind *C. albicans* CYP51 as presented above. Commercial antifungals voriconazole and ketoconazole, with already known affinity to CYP51 were also tested *in silico* to compare and validate the MK55 and MK56 docking results. Voriconazole showed a docking pose identical to the previously determined voriconazole crystal structures to other CYP51 cytochromes (pdb codes 5HS1, 4ZE0 and 4UYM). Similarly, the highest scored pose of ketoconazole was in agreement with previous ketoconazole-CYP51 crystal structures (pdb codes 3LD6 and 2JJP). Compounds MK55 and MK56 showed similar docking poses, in the heme prosthetic group region, surrounded by Y118, L121, T122, F126, Y132, L139, F228, G307, L376 and M508.

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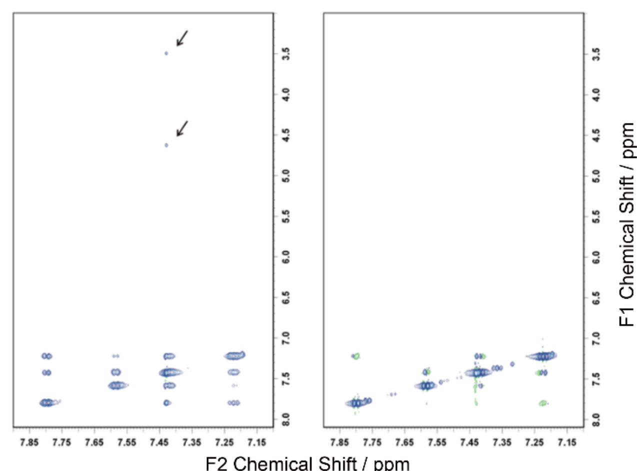

Figure 2. Expanded regions of the transferred NOESY spectrum (left) of compound MK55 in the presence of CYP51 isolated from *C. albicans* and of the NOESY spectrum (right) of MK55 recorded in the absence of the enzyme. The observed weak negative NOE cross-peaks between the H3 proton (7.43 ppm) of indole ring and the methylene protons (4.63 ppm and 3.49 ppm) of the ethyl spacer are indicated with arrows.

```
5EAF/30-529     30 RISLIIIIPFIYNIVWQLLYSLRKDRPPLVFYWIPWVGSAVVYGMKPYEFF 80
P10613/22-528   22 QISILLGVPFVYNLVWQYLYSLRKDRAPLVFYWIPWFGSAASYGQQPYEFF 72

5EAF/30-529     81 EECQKKYGDIFSFVLLGRVMTVYLGPKGHEFVFNAKLADVSAEAAYAHLTT 131
P10613/22-528   73 ESCRQKYGDVFSFMLLGKIMTVYLGPKGHEFVFNAKLSDVSAEDAYKHLTT 123

5EAF/30-529    132 PVFGKGVIYDCPNSRLMEQKKFVKGALTKEAFKSYVPLIAEEVYKYFRDSK 182
P10613/22-528  124 PVFGKGVIYDCPNSRLMEQKKFAKFALTTDSFKRYVPKIREEILNYFVTDE 174

5EAF/30-529    183 NFRLNERTTGTIDVMVTQPEMTIFTASRSLLGKEMRAKLDTDFAYLYSDLD 233
P10613/22-528  175 SFKLKEKTHGVANVMKTQPEITIFTASRSLFGDEMRRIFDRSFAQLYSDLD 225

5EAF/30-529    234 KGFTPINFVFPNLPLEHYRKRDHAQKAISGTYMSLIKERRKNNDI-QDRDL 283
P10613/22-528  226 KGFTPINFVFPNLPLPHYWRRDAAQKKISATYMKEIKSRRERGDIDPNRDL 276

5EAF/30-529    284 IDSLMKNSTYKDGVKMTDQEIANLLIGVLMGGQHTSAATSAWILLHLAERP 334
P10613/22-528  277 IDSLLIHSTYKDGVKMTDQEIANLLIGILMGGQHTSASTSAWFLLHLGEKP 327

5EAF/30-529    335 DVQQELYEEQMRVLD--GGKK-ELTYDLLQEMPLLNQTIKETLRMHHPLHS 382
P10613/22-528  328 HLQDVIYQEVVELLKEKGGDLNDLTYEDLQKLPSVNNTIKETLRMHMPLHS 378

5EAF/30-529    383 LFRKVMKDMHVPNTSYAIPAGYHVLVSPGYTHLRDEYFPNAHQFNIHRWNN 433
P10613/22-528  379 IFRKVTNPLRIPETNYIVPKGHYVLVSPGYAHTSERYFDNPEDFDPTRWDT 429

5EAF/30-529    434 D-----SASSYSVGEEVDYGFGAISKGVSSPYLPFGGGRHRCIGEHFAYCQ 479
P10613/22-528  430 AAAKANSVSFNS-SDEVDYGFGKVSKGVSSPYLPFGGGRHRCIGEQFAYVQ 479

5EAF/30-529    480 LGVLMSIFIRTLKWHYPEGKTVPPPDFTSMVTLPTGPAKIIWEKRNPEQK 529
P10613/22-528  480 LGTILTTFVYNLRWTI-DGYKVPDPDYSSMVVLPTEPAEIIWEKRETCMF 528
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Figure 3. Sequence alignment between Saccharomyces cerevisiae CYP51 and *C. albicans* CYP51. Blue colour intensity corresponds to amino acid sequence similarity.

Molecular dynamics simulations

In order to further evaluate the binding mode and stability, voriconazole, ketoconazole, MK55 and MK56 complexes with CYP51 were subjected to unconstrained molecular dynamics simulations for 200 ns each. In three of the compounds (voriconazole, ketoconazole and MK55), the docking pose was stable through the molecular dynamics simulations, depicting a rather consistent topology, except for MK56. The distance of the imidazole nitrogen atom and nitro group oxygens (center of mass) to the iron atom was monitored to evaluate the consistence of the interaction with the Fe atom (Figure 4A). The results indicate that the ketoconazole indole nitrogen has a constant interaction with the heme iron at ~2 Å, whereas, the voriconazole indole nitrogen does not, at least after the first ~50 ns, in which however, is in constant proximity of ~3 Å. For the case of MK55, the nitro group is kept stable most of the simulation time in a distance between 2 and 3 Å with the iron atom, whereas this is not observed for MK56 where the nitro group does not maintain its interaction with Fe.

Ketoconazole had a higher binding affinity for CYP51 than MK55. The docking and subsequent MD simulations showed a very stable topology for the compound, which forms several hydrophobic interactions with the cytochrome protein. Specifically, the imidazole group interacts with L376, the dichlorophenyl group interacts with I131, F126 and Y132, the dioxolan ring with Y118 and F228, the phenyl group with F235, F380 and M508 and the piperazine group with the Y64, H377, and F380 hydrophobic side chains (Figure 4B). Likewise, MK55 has a stable interaction with the heme iron and forms several interactions, especially with F126, Y118, M508 and V509 (Figure 4C). Important interactions include the indole ring, which is stabilized by the aromatic side chain ring of F228 and the aliphatic chain of L121. The methylene protons of the nitrooxyethyl moiety were also monitored for their proximity to the MK55 H3 proton as observed by the NMR NOE cross-peaks (Figure 2) and were found to be consistent with the NMR data (Figure 4D). The set of interactions that govern this position of MK55 with the *C. albicans* CYP51 pocket is highlighted in figure 4E. The phenyl ring from the indole group, interacting with L376, L121, T311, M508 and V509 seems to play the most significant role in the binding of MK55. When superimposed, MK55 and ketoconazole have a similar topology in the interaction site and relative to the heme cofactor (Figure 4F)

Cytotoxicity of N- (2-nitooxyethyl)-1H-indol-2-carboxamide MK55

Compound MK55 was tested in cytotoxicity assay on PLP2 cells, since it was the only one with proposed mode of action on *C. albicans*. MK55, with GI_{50} values (μg/mL) >400, didn't present cytotoxic activity compared to ellipticine which was used as a positive control (GI_{50} = 3.22 μg/mL).

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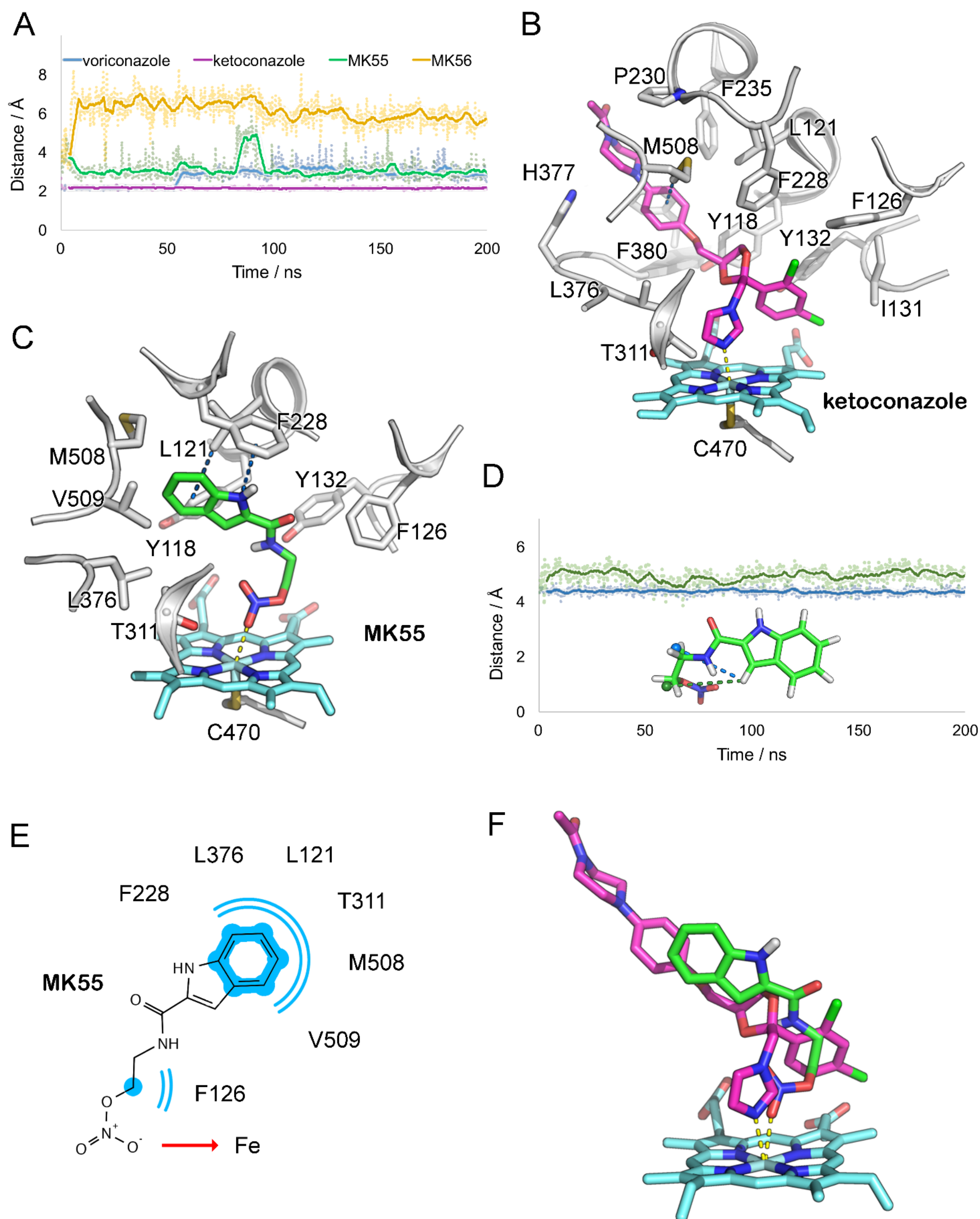

Figure 4. A) Distances in Å, monitored during the molecular dynamics simulations between the imidazole nitrogen atom (vericonazole and ketoconazole) or nitro group oxygens center of mass (MK55 and MK56) to the heme iron. B) Representative binding pose of ketoconazole (in magenta stick representation) to CYP51 (gray). C) Representative binding pose of MK55 (in green stick representation) to CYP51 (gray). Electrostatic interactions are shown in yellow and certain hydrophobic interactions in blue. D) Distances in Å, during the molecular dynamics simulations between H3 of MK55 and the center of mass of the ethyl spacer methylene hydrogens E) MK55 pharmacophoric groups and their proximal interacting residues in a 2D representation. Hydrophobic interactions are highlighted in blue. F) Superimposition of ketoconazole (magenta) and MK55 (green) in respect to the heme cofactor.

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Conclusions

Four nitrate esters were selected from an in-house chemical library and tested for their activity towards four different strains of *C. albicans*. All compounds exhibited moderate to good antifungal activities although still lower compared to the commercial antifungal drug ketoconazole. Compounds also presented identical susceptibility. Two of the tested nitrate esters, namely MK56 and to a lesser extent MK55 exhibited good inhibition effects on biofilm formation and can be considered as novel anti-quorum sensing agents.

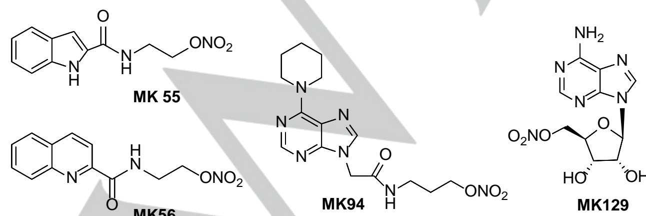
Although all four compounds exhibited moderate to good anti-candidal activities *in vitro*, their mechanism of action is different. Among the four tested compounds only MK55 bound to CaCYP51 via interaction with heme Fe, indicating that its mode of action relates to inhibition of ergosterol biosynthesis. MK55 dissociation constant (KD) for candidal enzyme was higher than the corresponding one of ketoconazole indicating lower binding affinities of MK55. To a step further, NMR studies, *in silico* docking and molecular dynamic simulations revealed a putative binding pose for MK55 proposing a metal coordination complex which was not present for the case of the other nitrate esters.

Most importantly, this study indicates that compound MK55 selectively binds the *C. albicans* with no activity to human CYP51 while ketoconazole is not selective towards fungal enzyme having a low KD for hCYP51. This is in accordance to previous investigation where it was concluded that ketoconazole has hepatotoxic side effect,[7] probably as a consequence of non-specific binding. To a step further, and since one of the most common side effects of azole drugs is liver damage,[8] MK55 toxicity was tested on PLP2 cell line from pig's liver and presented no cytotoxic effects. From the above findings, it is concluded that MK55 (N- (2-nitrooxyethyl)-1H-indol-2-carboxamide) could be further investigated as a potential antifungal lead for future development of novel antifungal compounds.

Experimental Section

Synthesis of nitrogen heterocyclic compounds

The synthesis of the nitrate esters of heteroaromatic compounds (MK55, MK56, MK94 and MK129) was previously described.[9],[10] Compounds N-(2-nitrooxyethyl)-1H-indol-2-carboxamide (MK55), N-(2-nitrooxyethyl)-quinoline-2-carboxamide (MK56), N-(2-nitrooxyethyl)-6-(piperidin-1-yl)-9H-purine acetamide (MK94), 5´-O-nitroadenosine (MK129) were subject of investigation (Scheme 1).



Scheme 1. Compounds tested in this study

Microbial culture conditions

Four strains of *C. albicans* were used in this study. Isolates were isolated from oral cavities of patients at ENT Clinic, Clinical Hospital Centre Zvezdara, Belgrade, Serbia by rubbing sterile cotton swab upon obtaining informed written consent. Strains were determined on CHROMagar plates (Biomerieux, France). *Candida albicans* strains were maintained on Sabourand Dextrose Agar (Merck, Germany) at 4˚C and subcultured once a month.

Anticandidal activity of selected compounds

Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) were established by the use of microdilution standardized technique[11] with modification. In brief, with the use of sterile saline, fresh overnight yeast cultures were adjusted to a concentration 1.0 x 105 CFU/per well. The microplates were incubated at 37 °C for 24 h after which MIC and MFC were determined.

The MIC values were considered as the lowest concentrations without microscopically observed growth. Following the serial sub-cultivations of 10 µl into microtiter plates containing 100 µl of broth/well, as well as subsequent 24 h incubation at 37 °C, the lowest concentrations with no visible growth were defined as the MFC values, indicating 99.5% killing of the original inoculum[12]. Ketoconazole was used as a positive control (Sigma Aldrich, Germany).

Antibiofilm activity of selected compounds

Impact of azole compounds on biofilm formation was determined as described[13] with some modifications. *C. albicans* cells were incubated for 24 hours in 96 well microtiter plates with adhesive bottom (Sarstedt, Germany) at 37ºC with MIC and subMIC concentrations of the compounds. After 24 hours each well was washed twice with sterile PBS (Phosphate buffered saline, pH 7.4). Fixation of adhered cells was done with methanol, after which plate was air dried and stained with 0.1 % crystal violet (Bio-Merieux, France) for 30min. Wells were washed with water to remove any unbound stain, air dried and to suspend all the bound stain 100 µl of 96% ethanol was added (Zorka, Serbia). Absorbance was read at 620nm on Multiskan™ FC Microplate Photometer, Thermo Scientific™. Percentage of inhibition of biofilm formation was calculated by formula:

$$[(A_{620control} - A_{620sample}) / A_{620control}] \times 100.$$

UV-visible spectra and ligand binding

Sterol 14α-demethylase (CYP51) from *Candida albicans*, human and bovine was previously isolated at Laboratory of Biomolecular Structure at National Institute of Chemistry, Ljubljana, Slovenia.[14] Spectra were taken at room temperature using a double beam Shimadzu UV-1800 spectrophotometer. For the ligand binding, 1 µM of human, bovine or *Candida albicans* CYP51 in 50 mM phosphate buffer, pH 7.4, 100 mM NaCl and 10% glycerol were progressively titrated with investigated ligands at concentrations 0, 2, 8, 16, 32, 64, 128, 256, 300 µM. After each incremental addition of the ligand, an equivalent volume of solvent was added to the reference cuvette. Spectra were recorded from 350-500 nm and ligand-induced spectral changes were monitored as difference type II spectral responses. Each binding assay was repeated three times. Plots of absorbance changes against ligand concentration were constructed and the apparent dissociation constants (KD) were determined by nonlinear

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regression in GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA) using the equation:

$$\Delta A = \Delta Amax \times \frac{[L]}{K_D + [L]}$$

where ΔA is the peak to trough absorbance change in the difference spectra, ΔAmax is the maximum absorbance change, [L] are the concentrations of the ligand used for the titration, respectively, and KD is the dissociation constant for the ligand-enzyme complex.

Nuclear Magnetic Resonance

The high-resolution NMR spectra were recorded on Varian DirectDrive 800 MHz spectrometers at 25 °C. All data were collected using pulse sequences and phase-cycling routines provided in Varian libraries of pulse programs. The cryogenic triple-resonance NMR probe was used. NMR samples for the STD and the transferred NOESY experiments were prepared in a buffer containing 20 mM Tris-D11, 10% glycerol-D8, and 100 mM NaCl in D_2O, pD 7.2. DSS (0.1 mM) was used as an internal standard. All the spectra were recorded at a protein/ligand ratio of 1:100, the protein concentration was 8 μM and the ligand concentration was 0.8 mM.

The transferred NOESY[15],[16] spectra were acquired with an 8802.8 Hz spectral width, 4096 data points in t2, 16 scans, 180-256 complex points in t1, a relaxation delay of 1.5 s, and a mixing time of 200 and 300 ms. The residual water signal was suppressed using excitation sculpting[17],[18] and adiabatic pulses[19] were applied for the suppression of zero quantum artefacts during the mixing time. A T1ρ filter of 30 ms was used to eliminate the background protein resonance. The spectrum was processed and analyzed with the FELIX 2007 software package from Felix NMR Inc. The spectrum was zero-filled twice and apodized with a squared sine bell function shifted by π/2 in both dimensions.

The STD experiment[20] was performed with an 8802.8 Hz spectral width, 16384 data points, a saturation time of 300 ms, a relaxation delay of 6.3 s, and 6000 scans. Saturation time and relaxation delay were selected according to the shortest and the longest 1H T1 relaxation time of the ligand to attain the experimental conditions for quantitative STD measurements[21]. Selective saturation was achieved by a train of 50 ms long Gauss-shaped pulses separated by 1 ms delay. Water was suppressed via excitation sculpting. The on-resonance selective saturation of CYP51 was applied at -0.38 ppm. The off-resonance irradiation was applied at 30 ppm for the reference spectrum. Subtraction of the on and off-resonance spectra was performed internally via phase cycling. The spectrum was zero-filled twice and apodized by an exponential line-broadening function of 1 Hz.

Preparation of CYP51, Virtual Screening and Molecular docking

Production of the *Candida albicans* CYP51 protein structural model (Uniprot code P10613) was made by homology modeling with MODELLER 9.17[22] using the crystal structure of Saccharomyces cerevisiae CYP51 complexed with fluquinconazole (pdb code 5EAF) as a template. The two sequences share a 65% identity. Gaps and insertions between residues in the alignment were only introduced in loop regions. Twenty models were produced and their overall stereochemical quality was evaluated by means of visual inspection and the discrete optimized energy (DOPE)[23] to conclude to the final model.

For the virtual screening process, all compounds from the in-house library were primarily sketched and prepared at pH 7.5 ± 0.5 with LigPrep of Maestro (LigPrep, Schrödinger, LLC, New York, NY, 2017). For the CYP51 modeled receptor, the Fe (II) ion formal charge was set to +2. Subsequently, the examined library was screened using Glide High Throughput Virtual Screening (Glide HTVS) - Precision mode, Maestro, version 11.2.013 (Glide, Schrödinger, LLC, New York, NY, 2017). [24-26].

For the explanatory docking, selected compounds were flexibly docked into the binding sites of receptor using AutoDock Vina.[27] The AutoDockTools program was used to prepare the corresponding PDBQT-protein file. The receptor was held rigid during the docking process, while the ligand was allowed to be flexible. Docking simulations in both cases were performed using a grid box with dimensions of 25 25 25 Å, a search space of 20 binding modes, and the search parameter was set to 5. The best docking pose for each compound was selected on the basis of the lowest energy docked conformation.

Molecular dynamics simulations

All MD simulations were performed using the GROMACS software v5.1.[28] Following the structural model, a minimization of the receptor topology was performed in order to remove steric clashes between the residues. The minimized topology was then inserted in a pre-equilibrated box containing water and a 0.15M concentration of Na and Cl ions. The latest AMBER99SB-ILDN[29] force field was used for all the dynamics simulations along with the TIP3P water model. Force field parameters for the ligands were generated using the general Amber force field (GAFF) and HF/6-31G*-derived RESP atomic charges.[230] AMBER-compatible heme parameters were derived from elsewhere[31]. Each system consisted of the protein, the ligand, ~15.000 water molecules and ~160 ions in a 10x10x10nm simulation box. The model systems were energy minimized and subsequently subjected to a 10 ns MD equilibration, with positional restraints on protein coordinates. These restraints were released, and 200 ns MD trajectories were produced in constant temperature of 300K using separate v-rescale thermostats for the protein, the peptide and solvent molecules. A time step of 2 fs was used and all bonds were constrained using the LINCS algorithm. Lennard-Jones interactions were computed using a cutoff of 10 Å, and the electrostatic interactions were treated using PME with the same real-space cutoff.

Cytotoxicity of compounds in a porcine liver primary cell culture

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin, 100 μg/mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 ºC with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Sulforhodamine B assay was performed according to a procedure previously described.[32] The results were expressed in GI_{50} values (compound concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

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Keywords: antimicrobial activity • biofilm • nitrate esters • selective inhibitors • CYP51

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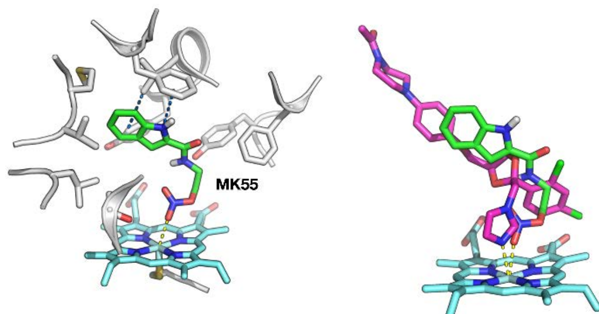

Virtual screening revealed some nitrate esters as compounds with antimicrobial activity. From them, N- (2-nitooxyethyl)-1H-indol-2-carboxamide has exhibited good inhibitory potential and selectivity on C. *albicans* growth and no cytotoxic effects.

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