Synthesis, Cytotoxicity Evaluation, and Antifungal Activity of Novel Nitroglycerin Derivatives against Clinical Candida albicans Isolates

Niloofar Rashidi¹, Sassan Rezaie¹, *Sayed Jamal Hashemi¹, Aziziollah Habibi², Mohammad Hadi Baghersad³, Roshanak Daie¹, Sadegh Khodavaisy¹, Heidar Bakhshi¹, Ali Salimi⁴, Mohammad Ebrahim Getso¹, Zahra Rafat¹

1. Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
2. Department of Chemistry, Faculty of Chemistry, Kharazmi University, Tehran, Iran
3. Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
4. Nanobiotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

*Corresponding Author: Email: sjhashemi@tums.ac.ir

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Abstract

**Background:** Candida albicans remains the main cause of candidiasis in most clinical settings. Available drugs for candidiasis treatment have many side effects. In this work, novel nitroglycerin derivatives were synthesized and their cytotoxic and antifungal effects evaluated against fluconazole susceptible and resistant clinical C. albicans isolates.

**Methods:** This experimental study was performed in Tehran University of Medical Sciences and Baqiatallah University of Medical Sciences, Tehran, Iran between Feb to Dec 2019. The in vitro activities of two novel nitroglycerin derivatives (1b and 2b) against 25 clinical fluconazole-susceptible and resistant C. albicans isolates and four standard C. albicans strains were determined according to CLSI reference M27-A3 documents. The cytotoxicity of chemical compounds was investigated near the SNL76/7 cells by colorimetric assay. Real-time PCRs were performed to evaluate the alterations in the regulation of ERG11 and CDR1 genes under nitroglycerin derivatives-treated and untreated conditions.

**Results:** The derivatives 1b and 2b exhibited potent antifungal activity against C. albicans isolates; MICs and MFCs varied from 18 μg/ml to 72 μg/ml and 36 μg/ml to 144 μg/ml, respectively. The cell viability evaluation demonstrated that both chemical compounds are safe within 24h. The nitroglycerin derivatives were able to reduce the transcription level of CDR1 and ERG11 genes in all susceptible and resistant C. albicans isolates.

**Conclusion:** Considering the potential and efficacy of these compounds against clinical C. albicans isolates, the complementary in vivo and clinical trials should be investigated.

**Keywords:** Nitroglycerin; Candida albicans; Antifungal activities

Introduction

Candida species are the fourth most common agents causing of hospital-acquired infections (1). Candida albicans remains the main cause of candidiasis in most clinical settings (2). The epidemi-
ology of Candida infections has changed in recent years. Invasive candidiasis (IC) is a major problem around the world, especially in immunocompromised patients (3). The high incidence of IC presents many challenges to clinicians due to the associated difficulties in the diagnosis and treatment of these infections. Candida spp. exhibit varying degrees of susceptibility to common antifungal agents available for therapy.

In recent years, the invasive infections caused by drug-resistant Candida species have resulted in increased morbidity and mortality (2). Additionally, the available antifungal agents have undesirable toxicities such as hepatotoxicity caused by azoles, cardiomyopathy caused by echinocandins, and nephrotoxicity caused by amphotericin B (4-7). Thus, there is an urgent need for the development and evaluation of new antifungal compounds that can complement the management of Candida infections. In view of this need, nitroglycerin has been researched as a novel potential antifungal drug. Nitroglycerin is a non-antibiotic drug that received prominence in the field of medicinal chemistry and was shown to have significant antifungal characteristics (8). Nitroglycerin derivatives were designed based on the structure that nitroglycerin core contains nitrate groups as the only functional group which can be responsible for the antifungal activity. We theorized that increasing the number of nitrate groups might increase the antifungal activity of this molecule. We aimed to introduce, synthesize, characterize, and evaluate the potential antifungal activities of two novel organic nitrate-esters derivatives of nitroglycerin against C. albicans.

Materials and Methods

Synthesis and characterization

This study was performed at Tehran University of Medical Sciences and Baqiatallah University of Medical Sciences, Tehran, Iran, from Feb to Dec 2019. All materials used in this study were obtained from commercial suppliers (Fluka and Merck). The synthesized compounds were dissolved in CDCl₃ and the nuclear magnetic resonance (NMR) spectrum was prepared using a (300 MHz, Bruker AMX) spectrometer with tetramethylsilane as the internal standard. We used an infrared (IR) spectrophotometer (Shimadzu IR-460 DR-11) to prepare the infrared spectra in the range of 400-4000 cm⁻¹ wavelength for the identification of functional groups.

Synthesis of compounds 1a and 2a

Sodium (4.8 g, 0.21 g-atoms) was added to 20 ml of allyl alcohol. While stirring, the desired selected dihalide compound (0.1 mol) was added over a 30 min period. The mixture was refluxed for 3h and then taken up in ether; the ether layer was washed and evaporation of the solvent was induced by the heat generated by a rotary evaporator. The residue was dried over anhydrous sodium sulfate, and then distilled twice under reduced pressure to obtain the desired product. All synthesized compounds were completely identified by infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR) and elemental analysis.

Synthesis of compounds 1b and 2b

Twenty mmol of iodine was added to a mixture of solution of silver nitrate (61 mmol) and compounds 1a or 2a (10 mmol) in acetonitrile (150 ml). The resultant mixture was refluxed for 12 hours. The mixture was then filtered using a filter paper to separate the formed silver iodide crystals. A solution of saturated brine (150 ml) was added to the filtered solution to precipitate the excess silver nitrate as silver chloride. The solution was washed twice with distilled water and dried with anhydrous sodium sulfate. The solvent was evaporated under vacuum to afford the compounds 1b or 2b as a yellow liquid.

C. albicans isolates

Twenty-five C. albicans isolates were obtained from the reference culture collection of the Tehran Medical Mycology Laboratory strain collection (TMML). The sixteen fluconazole-susceptible and nine fluconazole-resistant C. albicans isolates were recovered from patients with
various forms of clinical candidiasis. Four standard strains including a fluconazole-resistant strain (ATCC76615), a fluconazole-susceptible strain (ATCC10231), an amphotericin B-resistant strain (ATCC200955), and an amphotericin B-susceptible strain (ATCC90028) were used as control strains. All the isolates were cultured on Sabouraud dextrose agar (SDA) and incubated at 4 °C.

**Antifungal activity assay**

Antifungal susceptibility testing was performed according to CLSI guidelines document M27-A3 (9, 10). The antifungal agents were prepared to the final concentrations of 0.016 to 16 µg/ml for amphotericin B (AMB, Bristol-Myers-Squib, Woerden, The Netherlands) and 0.063 to 64 µg/ml for fluconazole (FLU, Pfizer, Groton, CT, USA). The drugs were diluted in standard RPMI-1640 medium (Sigma, st. Louis, MO, USA) and buffered to pH 7.0 with 0.165M morpholinepropanesulfonic acid (MOPS, Sigma-Aldrich). The sensitivity and resistance to fluconazole (≤ 2 μg/ml as susceptible, 4 μg/ml as susceptible dose-dependent, and ≥ 8µg/ml as resistant) were classified according to the CLSI clinical breakpoints values (10). The minimum fungicidal concentration (MFC) of the drug was determined as the lowest concentration that had more than 99.9% of cell-killing activity (11).

**RNA Extraction and Quantitative Real-Time PCR Assay**

The changes in the expression of ERG11 and CDR1 genes were studied for the two fluconazole-resistant *C. albicans* isolates (TMML1291 and TMML1292) and the two fluconazole-susceptible *C. albicans* strains (TMML 213 and TMML 229) under nitroglycerin derivatives-treated and untreated conditions. Total RNA extraction was performed using the RNAX plus kit (Sinaclon, Karaj, Iran) following the instructions of the manufacturer. RNA concentration and purity were determined spectrophotometrically (BiochromWPABiowave II, UK). The complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit (Vivantis, Malaysia). Primers were designed using the PRIMER 3 web-based software (http://bioinfo.ut.ee/primer3-0.4.0) (Table 1).

**Table 1:** Primers used for real-time polymerase chain reaction

| Name  | Nt | GC% | Tm  | Sequence (5′-3′)                  |
|-------|----|-----|-----|----------------------------------|
| CDR1-F | 23 | 43.5 | 58.87 | 5′-AGGGTAGTACTGGAAAGTGTTGTAG-3′ |
| CDR1-R | 21 | 42.9 | 55.92 | 5′-AGAATCCAAACGCTGACCAT-3′      |
| ERG11-F | 20 | 50  | 57.30 | 5′-ACTCATGGGGTTGCCAATGT-3′      |
| ERG11-R | 21 | 50  | 57.30 | 5′-AGCAGCATCAGCTCGGAAT-3′       |
| βactin-F | 20 | 55  | 60.39 | 5′-TGAGAGGGAATCGTGCGTG-3′       |
| βactin-R | 21 | 52.38 | 61.29 | 5′-TGGCTTGTGATTCACATCTG-3′      |

The β-actin gene was used as the endogenous reference gene. The Specificity of primers was checked with the BLAST search available on the NCBI website (http://blast.ncbi.nlm.nih.gov/blast.cgi). Standard curves for each gene were established from serially diluted cDNA that was obtained from the cells grown to mid-logarithmic phase using specific primers. Real-time RT-PCR was performed using the Corbett rotor-gene 2.1.0.9 (Qiagen, Iran) based on 3 step cycling protocol; with power SYBR ® Green Master Mix Kit (Qiagen, Iran) that was specifically designed for intercalator-based real-time PCR. Duration of operation program for amplification cycles was 15 min of heating/incubation at 95 °C as initial denaturation; followed by a further heating/incubation 20 sec at 95 °C, 20 sec at 58 °C; and 30 sec at 72 °C according to 2X real-time PCR master mix kit (Biofact, Korea). Each test was performed in tripli-
cate and the mean values of the relative expression were determined for ERG11 and CDR1 gene. The expression of all the genes was normalized to the housekeeping gene and analyzed using REST (2009) software.

**Cytocompatibility assessment and cell viability**

The cytotoxicity of chemical compounds (1b, 2b) were investigated in the vicinity of the SNL76/7 cells using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay at 24 and 72 hours. The samples were divided into two groups: the highest concentration of MIC (72 μg/ml) and the Tissue Culture Polystyrene (TCPS) as a control (placed in a 24-well culture plate). The SNL76/7 cells were seeded with a density of 1×10^4 cells/well and incubated at 37 °C and 5% CO_2. In order to assess the number of viable cells at each point in-time, 50µl MTT solution (5 mg/ml in DMEM) was added to each well, followed by another incubation period of 3 hours. Next, the supernatant was removed and 100 µl of DMSO solvent was added to dissolve the formazan precipitates. The optical absorbance was measured at 570 nm using a microplate reader (ELISA reader, ELX808, Biotech, USA). The optical absorption of scaffolds was reduced from each well’s absorbance of each well. Cell proliferation test was performed in triplicates and the results were reported as the average. Cell attachment and proliferation of SNL76/7 cells in 1b and 2b were qualitatively assessed using Acridine Orange/Ethidium Bromide (AO/EB) staining. The dual fluorescent staining solution containing 100μg/ml AO and 100 μg/ml EB (AO /EB, Sigma) was added to wells. Then, the wells each well was rinsed with PBS and examined using a fluorescent microscope (Leica Inc., Foster City, CA).

**Statistical analysis**

Statistical analysis of the data was carried out using the t-test for two dependent and Fisher’s exact tests, with the SPSS statistical package, version 7.0 (IBM Corp. Armonk, NY). A P-value of <0.01 was considered statistically significant.

**Results**

**Synthesized compounds (1b and 2b)**

The synthesis of the new nitrate ester compounds was carried out in two synthetic steps; in step 1, formation of two concerted ether bonds using allylalcohol and an appropriate alkyl dihalide and in step 2, concerted nitration of double bonds using iodine-nitration reaction. Allyl alcohol was first de-protonated by sodium and then the desired alkyl dihalide was added to prepare allyloxide salt (Fig. 1). Iodine-nitration reaction was used for direct and concerted nitration of both double bonds in compounds 1a and 2a. The comparison of the infrared spectrum of the 1a and 2a compounds with the nitrate esters 1b and 2b clearly shows the removal of the stretching vibration related to the carbon-carbon double bond at about 3100 and 1640 cm\(^{-1}\) and the observation of a stretching vibration related to nitrate groups in the 1260 and 1630 cm\(^{-1}\). The absorption peak at 1440 and 2890 cm\(^{-1}\) was related to the hydrocarbon skeleton in the molecular structure of these compounds (Figs. 2 and 3). In the \(^1\)H-NMR spectrum of compound 1b, the presence of a quintuplet peak with an integration factor of 2 at 1.82 ppm shows a methylene proton group in the middle of the molecule. A triplet peak with the integration factor of 4 at 3.54 ppm showed methylene protons connected to oxygen. In compound 2b, all protons of methylene groups, except the methylene connected to nitrate group at 3.69 to 3.79 ppm, are observed as two peak sets with integration factor of 4 and 8. \(^1\)H-NMR spectrum of both compounds, shows similar and clear peaks at 4.6-4.85 ppm region that are related to two protons of methylene group connected to nitrate group.
Fig. 1: Synthesis of novel nitrate ester compounds 1b and 2b

Fig. 2: IR spectrum of the compounds 1a and 1b
The synthesized compounds all have two chiral centers, the two protons of each methylene group are chemically nonequivalent, but in the $^1$H-NMR spectrum of these compounds, this non-equivalence was only clearly observed for methylene groups close to these centers. Methine protons connected to nitrate group in both compounds were observed as multiple peaks with an integration factor of 2 at about 5.4 ppm. In 13C-NMR of both compounds of 1a and 2b according to molecule symmetry, five kinds types of peaks are identifiable, all of them located in 25 to 80 ppm.

**Antifungal activity assay**

1b and 2b exhibited antifungal activity against *C. albicans* isolates with MICs and MFCs varied from 18 μg/ml to 72 μg/ml and 36 μg/ml to 144 μg/ml, respectively. The MICs and MFCs of AMB, FLU, 1b and 2b against *C. albicans* isolates were shown in Tables 2 and 3. In all investigated isolates, fluconazole and amphotericin B had the lowest MIC$_{50}$ (0.5 μg/ml), while 1b and 2b had the highest MIC$_{50}$ (36 μg/ml). Geometric mean (GM) was the lowest for amphotericin B (0.3731 μg/ml), followed by fluconazole (2.3937 μg/ml), 2b (27.02 μg/ml), and 1b (36 μg/ml).

**Real-time RT-PCR**

The *CDR1* and *ERG11* genes expression rates were evaluated in two fluconazole-resistant (TMML1291 and TMML1292) and two fluconazole-susceptible (TMML 213 and TMML 229) *C. albicans* isolates under 1b-treated (9 μg/mL) and 2b-treated (18 μg/mL) conditions. The *CDR1* gene was downregulated (0.051-0.468 fold) in two resistant and (0.037-0.337 fold) in two susceptible *C. albicans* isolates. The *ERG11* gene was downregulated (0.018-0.258 fold) in two resistant and (0.048-0.314 fold) in two susceptible *C. albicans* isolates.
Table 2: Antifungal activity of 1b and 2b against *C. albicans* isolates

| Isolates       | MIC (μg/ml) | MFC (μg/ml) |
|----------------|-------------|-------------|
|                | AMB         | FLU         | 1b | 2b | 1b | 2b |
| ATCC90028      | 0.5         | 0.125       | 72 | 36 | 72 | 36 |
| ATCC200955     | 4           | -           | 36 | 18 | 72 | 36 |
| ATCC10231      | -           | 0.5         | 36 | 36 | 72 | 72 |
| ATCC76615      | -           | >64         | 36 | 36 | 72 | 36 |
| TMML205        | 0.25        | 0.5         | 36 | 36 | 72 | 72 |
| TMML210        | 0.5         | 0.125       | 36 | 36 | 72 | 72 |
| TMML213        | 1           | 0.5         | 18 | 18 | 36 | 36 |
| TMML214        | 0.5         | 0.5         | 72 | 36 | 72 | 72 |
| TMML215        | 1           | 1           | 36 | 36 | 72 | 72 |
| TMML218        | 0.25        | 0.125       | 36 | 18 | 72 | 36 |
| TMML219        | 0.5         | 0.062       | 36 | 36 | 72 | 72 |
| TMML222        | 0.5         | 0.5         | 18 | 18 | 36 | 36 |
| TMML225        | 0.25        | 0.062       | 72 | 36 | 144| 72 |
| TMML226        | 0.5         | 0.5         | 18 | 18 | 36 | 36 |
| TMML227        | 0.25        | 0.125       | 72 | 72 | 144| 144|
| TMML228        | 1           | 0.5         | 36 | 18 | 36 | 36 |
| TMML229        | 0.25        | 0.5         | 36 | 36 | 72 | 72 |
| TMML231        | 0.5         | 0.5         | 72 | 36 | 144| 72 |
| TMML247        | 0.5         | 0.5         | 18 | 36 | 36 | 36 |
| TMML249        | 0.25        | 0.125       | 18 | 18 | 36 | 36 |
| TMML246        | -           | >64         | 18 | 18 | 36 | 36 |
| TMML277        | -           | >64         | 36 | 18 | 72 | 36 |
| TMML258        | -           | >64         | 36 | 72 | 72 | 72 |
| TMML202        | -           | >64         | 18 | 18 | 36 | 36 |
| TMML1290       | -           | >64         | 36 | 18 | 36 | 36 |
| TMML1291       | -           | >64         | 36 | 18 | 72 | 36 |
| TMML1292       | -           | >64         | 36 | 18 | 36 | 36 |
| TMML1293       | -           | >64         | 36 | 18 | 36 | 36 |
| TMML1294       | -           | 16          | 72 | 36 | 144| 36 |

Abbreviations: AMB; amphotericin B; FLU; fluconazole; MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration

Table 3: Antifungal activity profile of 1b and 2b against fluconazole susceptible and resistant *Calbicans* strains

| Isolates         | 1b MICs (μg/ml) | 1b MFCs (μg/ml) | 2b MICs (μg/ml) | 2b MFCs (μg/ml) |
|------------------|----------------|----------------|----------------|----------------|
| Fluconazole      | 18 3 6        | 36 72 144      | 36 72 36       | 72 144         |
| susceptible      | isolates      |                |                |                |
| (N=16)           |                |                |                |                |
| Fluconazole      | 5 7 8         | 7 8 1          | 1              | 1              |
| resistant        | isolates      |                |                |                |
| (N=9)            |                |                |                |                |

**Cytocompatibility and cell viability**

On the first day, the cells showed a decrease of about 10% and 20% in the vicinity of the 1b (72 μg/ml) and 2b (72 μg/ml), respectively. Moreover, cells showed about a 50% growth reduction in the vicinity of 1b and 2b compared to the control on the third day (Fig. 4). The acridine orange results also confirm the results of MTT.
Discussion

Invasive fungal infections are major causes of morbidity and mortality in critically ill patients (12, 13). The increasing frequency of IC is not only associated with a higher mortality rate but also a higher clinical treatment failure due to either acquired and/or intrinsic resistance to a number of antifungal drugs (14, 15). On the other hand, most antifungal drugs are relatively toxic and have are associated with significant side effects. With the increased potency of antifungals, the toxicity of chemical compound increases as well (16). The wide range of non-antibiotic drugs, such as antiepileptics and anesthetics, have significant antibacterial and antifungal properties (17-19). Nitroglycerin is a cardiovascular drug with significant antifungal effects alone individually or in combination with other compounds especially on *Candida* strains (20, 21).

We prepared nitroglycerin compounds (1b and 2b) using Williamson’s method - a well-known, easy, and classical method for synthesis of ethers from alcohols and alkylhalides (22, 23). In this method, the alcohol was initially de-protonated by sodium, sodium hydride, or any other strong base. Then, the corresponding alkylhalide is added to the prepared alkoxide salt. The product obtained by this method was purified using a saturated sodium chloride solution that removed the excess silver ions (24). The results of our study showed that both nitroglycerin compounds (1b and 2b) have relatively similar and noticeable antifungal activity but 2b was slightly more potent and effective ($P<0.05$). The increased potency of 2b is due to its solubility. The molecule intermediate chain structure contains more oxygen molecules and as a result it is more polar and more soluble. The MIC results of clinical fluconazole-resistant and susceptible isolates were similar (range 18-72 μg/ml). In line with the present results, the nitroglycerin has antifungal activity upon *Candida* spp. (MIC from 0.15mg/mL to 0.30 mg/mL) and *C. albicans* was the most susceptible species tested (0.15 mg/mL).

Some previous studies demonstrated the in vitro synergy of glyceryl trinitrate in combinations with citrate, caprylic acid, and ethanol in rapidly and completely eradicating resistant Gram-positive, Gram-negative, and *C. albicans* biofilms (25).
use of 1b and 2b in combination with other new chemical compounds requires further testing for treating and eradicating pathogenic biofilms. In our study, cell viability evaluation demonstrated that both chemical compounds are safe within 24h, but 2b showed more toxicity than 1b. Generally, the chemically synthesized compounds were safe. In previous studies, the mechanisms of azole resistance in different *Candida* isolates, including decreased intracellular concentrations of the target enzyme, changes in the drug target, and increased production of lanosterol 14α-demethylase have been identified (26). *CDR1* and *ERG11* genes are azoles-resistant genes in *C. albicans* and play important roles in fluconazole resistance. Their reduced expression could be a useful and effective mechanism of reducing the pathogenicity of *C. albicans* strains (27-29). In the current study, the new chemical compounds (1a and 2b) demonstrated the ability of reducing the transcription level of *CDR1* and *ERG11* genes in both the susceptible and the resistant strains.

**Conclusion**

We synthesized and evaluated the nitroglycerin-derivatives (1b and 2b) compounds that exhibited potent *in vitro* antifungal activities against clinical fluconazole-resistant and susceptible *C. albicans* isolates. The chemically synthesized compounds demonstrated a reduction in the the expression of genes involved in azole resistance mechanisms. The effectiveness of these nitroglycerin derivatives, as a monotherapy or in combination with fluconazole for the treatment of *Candida* infections needs to be determined further in vivo and clinical trials should be investigated.

**Ethical considerations**

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

**Financial disclosure**

No financial interests related to the materials of this manuscript have been declared.

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**Conflict of interest**

The authors declare that there is no conflict of interests.

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